Brian Austin · Dawn A. Austin

Bacterial Fish Pathogens

Disease of Farmed and Wild Fish

Sixth Edition

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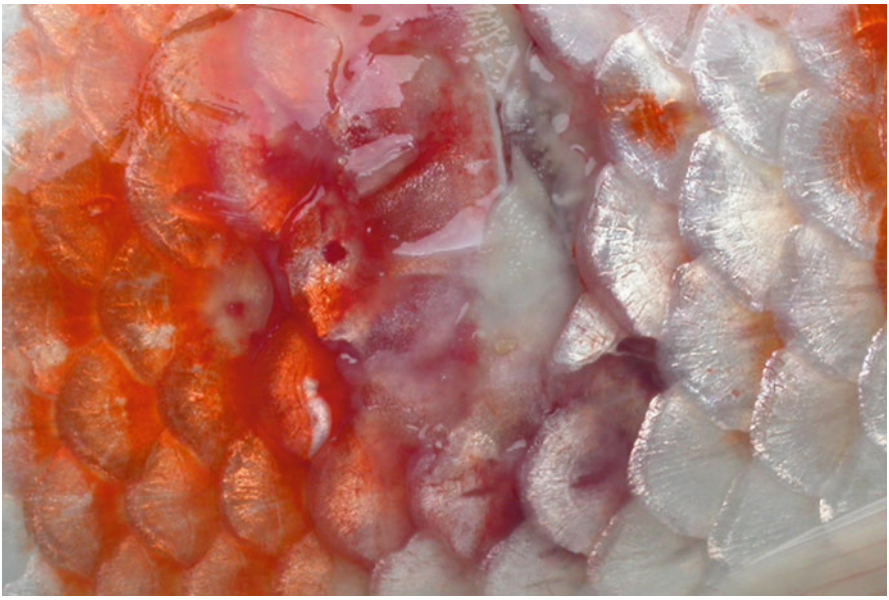

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To Aurelia Jean

Preface

This sixth edition of *Bacterial Fish Pathogens* is the successor to the original version, first published by Ellis Horwood Limited in 1987, which was planned to fill the need for an up-to-date comprehensive text on the biological aspects of the bacterial taxa which cause disease in fish. In those days, interest focused largely on bacterial kidney disease, the *Cytophaga*-like bacteria (CLBs), furunculosis and vibriosis (caused by *Vibrio anguillarum).* Over the intervening years, we have witnessed changes in impetus as new methodologies, notably involving molecular biology, have been developed. Interest in vaccines has been overtaken by probiotics and plant products. In terms of the number of publications, *Aeromonas hydrophila* is the pathogen of the moment. The impetus to prepare a sixth edition stemmed initially from discussion with the publisher when it became apparent that the book was particularly well used (there have been >19,000 downloads since the publication of the fifth edition in 2012). Since publishing the fifth edition in 2012, there has been only a comparatively few new bacterial fish pathogens described, but numerous publications deal with diagnosis, pathogenicity and especially control. Consequently with all the new publications, we considered that it is timely to prepare a new edition. Thinking back to the preparation of the first edition, it is mind numbing how the mechanics of publication have changed. For the first edition, we spent ages in libraries, using interlibrary loans; pouring over paper copies of journals, books and monographs; making handwritten notes; and preparing text for typing [on a typewriter – corrections led to use of white-out or the typing of replacement pages]. Line drawings were done using a drawing board, Rotring pens, set squares/French curves and stencils. Photographs were developed in a dark room and could be hit or miss in terms of quality. Now, we have access to an even wider range of journals electronically, which have been accessed from the laptop computer via a wireless Internet connection. Weeks of waiting for interlibrary loans from around the globe did not even feature during the initial research phase of the fifth or sixth editions. Because of the extensive new information published since the start of the millennium, we have been selective and, in particular, have once again condensed details of the pathology of the diseases, because there are excellent texts already available, which cover detailed aspects of the pathological conditions. For some diseases that have

occurred only occasionally and mostly in the distant past, we have condensed the text. Nevertheless, this sixth edition will hopefully meet the needs of the readership. As with all the preceding editions, it is emphasised that most of the information still appertains to diseases of farmed, rather than wild, fish.

The scope of the book covers all of the bacterial taxa, which have at one time or another been reported as fish pathogens. Of course, it is realised that some taxa are merely secondary invaders of already damaged tissues, whereas others comprise serious, primary pathogens. Shortcomings in the literature or gaps in the overall understanding of the subject have been highlighted, sometimes with subtlety. As a general comment, it is still apparent that little is known about anaerobic or microaerophilic pathogens – does this mean that they do not exist, or are scientists ignoring them? Also, it is mostly unclear whether organisms act by themselves or with others to cause a disease manifestation. Uncultured pathogens have been recognised in the form of *Candidatus* whereby the organisms may be visualised in pathological material, but not grown in the laboratory. The cause of some diseases is uncertain, although in the case of red mark syndrome/strawberry disease of rainbow trout, there is serological and molecular evidence for the involvement of rickettsial-like organisms, but which are still yet to be cultured. One wonders how many times the presence of uncultured or difficult-to-culture organisms is masked by culturable contaminants or secondary invaders. A final comment is that the overwhelming bulk of publications deal with diseases caused by single species of pathogen. Is this really true in that disease is a pure culture phenomenon, or is science missing microbial consortia that could work synergistically, simultaneously or sequentially to cause disease?

In preparing the text, we have sought both advice and material from colleagues. We are especially grateful to the following for the supply of photographs:

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Contents

1 Introduction 1
Conclusions 19
References 19

2 Gram-Positive Bacteria (Anaerobes and 'Lactic Acid' Bacteria) 21
Anaerobes 21
Clostridiaceae Representative 22
Clostridium botulinum 22
Eubacteriaceae Representative 25
Eubacterium tarantellae 25
Lactic Acid Bacteria 27
Carnobacteriaceae Representative 27
Carnobacterium piscicola (and the Lactobacilli) 28
Leuconostocacceae Representative 31
Weissella sp. 31
Weissella ceti 33
Gram-Positive Cocci in Chains: The Early Literature 34
General Comments 34
Isolation 36
Epizootiology 36
Development of Vaccines 37
Use of Inhibitory Compounds 38
Enterococcaceae Representatives 38
Enterococcus (Streptococcus) faecalis subsp. *liquefaciens* 38
Vagococcus salmoninarum 39
Streptococcaceae Representatives 41
Lactococcus garvieae (= Enterococcus seriolicida) 41
Lactococcus lactis subsp. *lactis* 46
Lactococcus piscium 47
Streptococcus agalactiae (= Str. difficilis) 48
Streptococcus dysgalactiae subsp. *dysgalactiae* 53
Streptococcus ictaluri 55

Streptococcus iniae 56
Streptococcus milleri 63
Streptococcus parauberis 64
Streptococcus phocae 66
References 68

3 Aerobic Gram-Positive Rods and Cocci 83
Erysipelothrix rhusiopathiae 83
Characteristics of the Disease 83
Isolation 83
Characteristics of the Pathogen 84
Pathogenicity 84
Disease Control 84
Renibacterium salmoninarum 84
Characteristics of the Disease 84
Isolation 86
Characteristics of the Pathogen 89
Diagnosis 93
Epizootiology 98
Pathogenicity 101
Disease Control 103
Aerococcaceae Representative 106
Bacillaceae Representatives 107
Corynebacteriaceae Representatives 110
Coryneform Bacteria 112
Microbacteriaceae Representative 113
Micrococcaceae Representative 113
Mycobacteriaceae Representatives 115
Nocardiaceae Representatives 125
Planococcaceae Representative 136
Staphylococcaceae Representatives 138
References 141

4 Aeromonadaceae Representatives (Motile Aeromonads) 161
Aeromonas allosaccharophila 162
Characteristics of the Disease 162
Isolation 162
Characteristics of the Pathogen 162
Diagnosis 163
Pathogenicity 164
Aeromonas bestiarum 164
Aeromonas caviae 165
Characteristics of the Disease 165
Isolation 165
Characteristics of the Pathogen 165

Aeromonas dhakensis 165
Characteristics of the Disease 166
Isolation. 166
Characteristics of the Pathogen. 166
Diagnosis. 166
Epizootiology 167
Pathogenicity. 167
Disease Control. 167
Aeromonas hydrophila 167
Characteristics of the Diseases 167
Isolation. 170
Characteristics of the Pathogen. 170
Diagnosis. 172
Pathogenicity. 173
Outer Membrane Proteins (OMPs) 176
Extracellular Products (ECPs) 176
Quorum Sensing 179
Disease Control. 179
Immunostimulants/Dietary Supplements 183
Aeromonas jandaei 193
Characteristics of the Disease 193
Characteristics of the Pathogen. 193
Pathogenicity. 194
Aeromonas piscicola 194
Characteristics of the Pathogen. 194
Aeromonas schubertii 194
Characteristics of the Disease 194
Isolation. 194
Characteristics of the Pathogen. 195
Pathogenicity. 195
Disease Control. 196
Aeromonas sobria 196
Characteristics of the Disease 196
Isolation. 196
Characteristics of the Pathogen. 196
Epizootiology 197
Pathogenicity. 197
Disease Control. 198
Aeromonas veronii biovar sobria. 198
Characteristics of the Disease 198
Isolation. 198
Characteristics of the Pathogen. 198
Diagnosis. 199
Pathogenicity. 199
Control 199

Aeromonas veronii biovar veronii 200
References 200

5 Aeromonadaceae Representative (*Aeromonas salmonicida*) 215
Aeromonas salmonicida 215
Characteristics of the Diseases 215
Isolation 220
Characteristics of the Pathogen 223
Atypical Isolates of *Aeromonas salmonicida* 230
Plasmid Profiles of *Aeromonas salmonicida* 233
The Taxonomic Dilemma 234
Serology 236
Bacteriophage Typing 238
Haemophilus piscium 239
Diagnosis 239
Epizootiology 242
Pathogenicity 262
Disease Control 281
References 298

6 Enterobacteriaceae Representatives 323
Citrobacter freundii 323
Characteristics of the Disease 323
Isolation 324
Characteristics of the Pathogen 324
Epizootiology 324
Pathogenicity 324
Disease Control 325
Edwardsiella anguillarum 325
Characteristics of the Pathogen 325
Pathogenicity 325
Edwardsiella ictaluri 326
Characteristics of the Disease 326
Isolation 327
Characteristics of the Pathogen 327
Diagnosis 328
Epizootiology 328
Pathogenicity 329
Disease Control 331
Edwardsiella piscicida 333
Characteristics of the Disease 333
Isolation 333
Characteristics of the Pathogen 333
Diagnosis 334
Pathogenicity 334
Edwardsiella tarda (=Paracolobactrum anguillimortiferum, Edw.anguillimortifera) 334

Characteristics of the Disease . . . 334
Isolation . . . 335
Characteristics of the Pathogen . . . 336
Diagnosis . . . 338
Epizootiology . . . 338
Pathogenicity . . . 339
Disease Control . . . 342
Enterobacter cloacae . . . 347
Characteristics of the Disease . . . 347
Isolation . . . 347
Characteristics of the Pathogen . . . 348
Pathogenicity . . . 348
Escherichia vulneris . . . 348
Characteristics of the Disease . . . 348
Isolation . . . 348
Characteristics of the Pathogen . . . 349
Pathogenicity . . . 349
Hafnia alvei . . . 349
Characteristics of the Disease . . . 349
Isolation . . . 350
Characteristics of the Pathogen . . . 350
Pathogenicity . . . 351
Klebsiella pneumoniae . . . 351
Characteristics of the Disease . . . 351
Isolation . . . 351
Characteristics of the Pathogen . . . 351
Pathogenicity . . . 352
Pantoea agglomerans . . . 352
Characteristics of the Disease . . . 352
Isolation . . . 352
Characteristics of the Pathogen . . . 353
Epizootiology . . . 353
Disease Control . . . 353
Plesiomonas shigelloides . . . 354
Characteristics of the Disease . . . 354
Isolation . . . 354
Characteristics of the Pathogen . . . 354
Pathogenicity . . . 355
Epizootiology . . . 355
Disease Control . . . 355
Providencia rettgeri . . . 355
Characteristics of the Disease . . . 355
Isolation . . . 356
Characteristics of the Pathogen . . . 356
Epizootiology . . . 356
Pathogenicity . . . 356

Providencia vermicola 357
Characteristics of the Disease 357
Isolation. 357
Characteristics of the Pathogen. 357
Epizootiology 357
Pathogenicity. 357
Salmonella enterica subsp. arizonae 358
Characteristics of the Disease 358
Isolation. 358
Characteristics of the Pathogen. 358
Epizootiology 359
Pathogenicity. 359
Disease Control. 359
Serratia liquefaciens 359
Characteristics of the Disease 359
Isolation. 360
Characteristics of the Pathogen. 360
Epizootiology 361
Pathogenicity. 361
Disease Control. 361
Serratia marcescens 361
Characteristics of the Disease 361
Isolation. 362
Characteristics of the Pathogen. 362
Epizootiology 362
Pathogenicity. 363
Disease Control. 363
Serratia plymuthica. 363
Characteristics of the Disease 363
Isolation. 363
Characteristics of the Pathogen. 364
Epizootiology 364
Pathogenicity. 364
Disease Control. 364
Yersinia intermedia 365
Characteristics of the Disease 365
Isolation. 365
Characteristics of the Pathogen. 365
Epizootiology 366
Pathogenicity. 366
Yersinia ruckeri 366
Characteristics of the Diseases 366
Isolation. 368
Characteristics of the Pathogen. 368
Diagnosis. 370
Epizootiology 371

Pathogenicity . . . 373
Disease Control . . . 375
References . . . 379

7 Flavobacteria and Cytophagas . . . 397
Chryseobacterium spp. . . . 401
Chryseobacterium aahli . . . 401
Characteristics of the Disease . . . 401
Isolation . . . 401
Characteristics of the Pathogen . . . 402
Pathogenicity . . . 402
Chryseobacterium balustinum (= Flavobacterium balustinum) . . . 402
Isolation . . . 402
Characteristics of the Pathogen . . . 403
Chryseobacterium indologenes . . . 403
Characteristics of the Disease . . . 403
Isolation . . . 403
Characteristics of the Pathogen . . . 404
Pathogenicity . . . 404
Chryseobacterium piscicola . . . 404
Characteristics of the Disease . . . 404
Characteristics of the Pathogen . . . 404
Pathogenicity . . . 405
Chryseobacterium scophthalmum (= Flavobacterium scophthalmum) . . . 405
Characteristics of the Disease . . . 405
Isolation . . . 405
Characteristics of the Pathogen . . . 406
Epizootiology . . . 406
Pathogenicity . . . 406
Disease Control . . . 406
Flavobacterium sp. . . . 407
Characteristics of the Diseases . . . 407
Isolation . . . 407
Characteristics of the Pathogen . . . 407
Diagnosis . . . 407
Disease Control . . . 408
Flavobacterium branchiophilum . . . 408
Isolation . . . 408
Characteristics of the Pathogen . . . 408
Diagnosis . . . 409
Epizootiology . . . 409
Pathogenicity . . . 409
Flavobacterium columnare (=Flexibacter/Cytophaga columnaris) . . . 410
Characteristics of the Disease . . . 410
Isolation . . . 410

Characteristics of the Pathogen . . . 411
Diagnosis . . . 412
Epizootiology . . . 413
Pathogenicity . . . 414
Disease Control . . . 416
Flavobacterium hydatis (= Cytophaga aquatilis) . . . 419
Isolation . . . 419
Characteristics of the Pathogen . . . 419
Pathogenicity . . . 420
Disease Control . . . 420
Flavobacterium johnsoniae (= Cytophaga johnsonae) . . . 420
Characteristics of the Disease . . . 420
Isolation . . . 420
Characteristics of the Pathogen . . . 421
Disease Control . . . 421
Flavobacterium oncorhynchi . . . 421
Isolation . . . 422
Characteristics of the Pathogen . . . 422
Flavobacterium psychrophilum (=Cytophaga psychrophila = Flexibacter psychrophilum) . . . 422
Characteristics of the Diseases . . . 422
Isolation . . . 423
Characteristics of the Pathogen . . . 423
Diagnosis . . . 426
Epizootiology . . . 428
Pathogenicity . . . 429
Disease Control . . . 431
Flavobacterium piscicida . . . 434
Flavobacterium spartansii . . . 434
Characteristics of the Disease . . . 434
Isolation . . . 435
Characteristics of the Pathogen . . . 435
Flavobacterium succinicans . . . 436
Flectobacillus roseus . . . 436
Characteristics of the Disease . . . 436
Isolation . . . 436
Characteristics of the Pathogen . . . 436
Pathogenicity . . . 437
Flexibacter spp. . . . 437
Myroides odoratimimus . . . 437
Characteristics of the Disease . . . 437
Isolation . . . 437
Characteristics of the Pathogen . . . 438
Epizootiology . . . 438
Pathogenicity . . . 438

Tenacibaculum spp. 438
Characteristics of the Disease 438
Characteristics of the Pathogen 438
Tenacibaculum dicentrarchi 439
Characteristics of the Disease 439
Isolation 439
Characteristics of the Pathogen 439
Tenacibaculum discolor 440
Characteristics of the Disease 440
Isolation 440
Characteristics of the Pathogen 440
Pathogenicity 441
Tenacibaculum gallaicum 441
Characteristics of the Disease 441
Isolation 441
Characteristics of the Pathogen 441
Pathogenicity 442
Tenacibaculum maritimum (= Flexibacter maritimus = Cytophaga marina) 442
Characteristics of the Diseases 442
Isolation 442
Characteristics of the Pathogen 443
Diagnosis 444
Epizootiology 444
Pathogenicity 444
Disease Control 445
Tenacibaculum ovolyticum (=Flexibacter ovolyticus) 446
Characteristics of the Disease 446
Isolation 446
Characteristics of the Pathogen 446
Tenacibaculum soleae 447
Characteristics of the Disease 447
Characteristics of the Pathogen 447
Identification/Diagnosis 448
Pathogenicity 448
Sporocytophaga spp. 448
References 448

8 Francisellaceae Representatives 467
Isolation 467
Francisella noatunensis 467
Characteristics of the Disease 467
Characteristics of the Pathogen 468
Diagnosis 469
Epizootiology 469

Pathogenicity . . . 469
Control . . . 470
Francisella noatunensis subsp. *orientalis* . . . 470
Characteristics of the Disease . . . 470
Characteristics of the Pathogen . . . 470
Pathogenicity . . . 471
Disease Control . . . 471
Francisella spp. . . . 471
References . . . 471

9 Pseudomonads . . . 475
Pseudomonas aeruginosa . . . 475
Pseudomonas alcaligenes . . . 476
Characteristics of the Disease . . . 476
Isolation . . . 476
Characteristics of the Pathogen . . . 476
Pathogenicity . . . 477
Pseudomonas anguilliseptica . . . 477
Characteristics of the Disease . . . 477
Isolation . . . 478
Characteristics of the Pathogen . . . 478
Diagnosis . . . 479
Epizootiology . . . 480
Pathogenicity . . . 481
Disease Control . . . 481
Pseudomonas baetica . . . 482
Characteristics of the Disease . . . 482
Isolation . . . 482
Characteristics of the Pathogen . . . 482
Pathogenicity . . . 483
Pseudomonas chlororaphis . . . 483
Characteristics of the Disease . . . 483
Isolation . . . 483
Characteristics of the Pathogen . . . 484
Epizootiology . . . 484
Pathogenicity . . . 484
Pseudomonas fluorescens . . . 484
Characteristics of the Diseases . . . 484
Isolation . . . 485
Characteristics of the Pathogen . . . 486
Epizootiology . . . 486
Pathogenicity . . . 486
Disease Control . . . 487
Pseudomonas koreensis . . . 487
Characteristics of the Pathogen . . . 487

Pseudomonas luteola 488
Characteristics of the Disease 488
Isolation 488
Characteristics of the Pathogen 488
Pathogenicity 488
Pseudomonas mosselii 489
Pseudomonas plecoglossicida 489
Characteristics of the Disease 489
Characteristics of the Pathogen 489
Epizootiology 490
Pathogenicity 490
Disease Control 491
Pseudomonas pseudoalcaligenes 491
Characteristics of the Disease 491
Isolation 491
Characteristics of the Pathogen 492
Epizootiology 492
Pathogenicity 492
Disease Control 492
Pseudomonas putida 493
Characteristics of the Disease 493
Characteristics of the Pathogen 493
Stenotrophomonas maltophilia 493
Characteristics of the Disease 494
Isolation 494
Characteristics of the Pathogen 494
Pathogenicity 494
References 495

10 Vibrios 499
Photobacterium damselae subsp. *damselae* 501
Characteristics of the Disease 501
Isolation 501
Characteristics of the Pathogen 502
Diagnosis 502
Epizootiology 503
Pathogenicity 503
Photobacterium damselae subsp. *piscicida (= Pasteurella piscicida)* . . . 504
Characteristics of the Disease 504
Isolation 505
Characteristics of the Pathogen 506
Diagnosis 507
Epizootiology 508
Pathogenicity 509
Disease Control 511

Aliivibrio (= Vibrio) fischeri 513
Aliivibrio logei 513
Characteristics of the Disease 513
Isolation. 513
Characteristics of the Pathogen. 514
Epizootiology 514
Aliivibrio salmonicida. 514
Characteristics of the Disease 514
Isolation. 515
Characteristics of the Pathogen. 515
Epizootiology 516
Pathogenicity. 516
Disease Control. 517
Aliivibrio wodanis. 518
Characteristics of the Disease 518
Characteristics of the Pathogen. 518
Vibrio aestuarianus. 519
Characteristics of the Disease 519
Characteristics of the Pathogen. 519
Pathogenicity. 520
Vibrio alginolyticus. 520
Characteristics of the Disease 520
Isolation. 521
Characteristics of the Pathogen. 521
Diagnosis. 521
Epizootiology 522
Pathogenicity. 522
Disease Control. 523
Vibrio anguillarum 524
Characteristics of the Disease 524
Isolation and Detection 525
Characteristics of the Pathogen. 526
Diagnosis. 529
Epizootiology 530
Pathogenicity. 532
Disease Control. 537
Vibrio cholerae (non-O1) 544
Characteristics of the Disease 544
Isolation. 545
Characteristics of the Pathogen. 545
Epizootiology 546
Pathogenicity. 546
Vibrio furnissii 546
Vibrio harveyi (= V. carchariae = V. trachuri). 547
Characteristics of the Diseases 547
Isolation. 548

Characteristics of the Pathogen . . . 548
Diagnosis . . . 549
Pathogenicity . . . 550
Disease Control . . . 551
Probiotics . . . 553
Dietary Supplements . . . 553
Inhibitors of Quorum Sensing . . . 554
Antimicrobial Compounds . . . 554
Vibrio ichthyoenteri . . . 554
Characteristics of the Disease . . . 554
Characteristics of the Pathogen . . . 555
Control . . . 555
Vibrio mimicus . . . 555
Characteristics of the Disease . . . 555
Isolation . . . 556
Characteristics of the Pathogen . . . 556
Pathogenicity . . . 556
Disease Control . . . 556
Vibrio ordalii . . . 556
Characteristics of the Disease . . . 556
Isolation . . . 557
Characteristics of the Pathogen . . . 557
Diagnosis . . . 558
Epizootiology . . . 558
Pathogenicity . . . 559
Disease Control . . . 559
Vibrio parahaemolyticus . . . 559
Disease Control . . . 559
Vibrio pelagius . . . 560
Characteristics of the Disease . . . 560
Isolation . . . 560
Characteristics of the Pathogen . . . 560
Epizootiology . . . 561
Pathogenicity . . . 561
Disease Control . . . 561
Vibrio ponticus . . . 561
Characteristics of the Disease . . . 561
Isolation . . . 562
Characteristics of the Pathogen . . . 562
Pathogenicity . . . 562
Vibrio scophthlalmi . . . 562
Characteristics of the Pathogen . . . 563
Pathogenicity . . . 563
Vibrio splendidus . . . 563
Characteristics of the Disease . . . 563
Isolation . . . 564

Characteristics of the Pathogen . . . 564
Epizootiology . . . 565
Pathogenicity . . . 565
Disease control . . . 565
Vibrio tapetis . . . 566
Characteristics of the Disease . . . 566
Isolation . . . 566
Characteristics of the Pathogen . . . 566
Disease Control . . . 567
Vibrio vulnificus . . . 567
Characteristics of the Disease . . . 567
Isolation . . . 567
Characteristics of the Pathogen . . . 568
Diagnosis . . . 569
Epizootiology . . . 569
Pathogenicity . . . 570
Disease Control . . . 570
References . . . 572

11 Miscellaneous Pathogens . . . 603
Alteromonadaceae Representatives . . . 603
Pseudoalteromonas piscicida . . . 603
Pseudoalteromonas undina . . . 604
Shewanella putrefaciens . . . 605
Campylobacteriaceae Representative . . . 607
Arcobacter cryaerophilus . . . 607
Comamonadaceae Representative . . . 608
Delftia acidivorans . . . 608
Hahellaceae Representative . . . 608
Hahella chejuensis . . . 608
Halomonadaceae Representative . . . 610
Halomonas (=Deleya) cupida . . . 610
Moraxellaceae Representatives . . . 611
Acinetobacter sp. . . . 611
Acinetobacter johnsonii and *Acinetobacter lwoffii* . . . 613
Moraxella sp. . . . 613
Moritellaceae Representatives . . . 615
Moritella marina (= V. marinus) . . . 615
Moritella viscosa (= V. viscosus) . . . 616
Mycoplasmataceae Representative . . . 618
Mycoplasma mobile . . . 618
Myxococcaceae Representative . . . 619
Myxococcus piscicola . . . 619
Neisseriaceae Representative . . . 620
Aquaspirillum sp. . . . 620
Oxalobacteraceae Representative . . . 620
Janthinobacterium lividum . . . 620

Pasteurellaceae Representative 622
Pasteurella skyensis 622
Piscirickettsiaceae Representative 624
Piscirickettsia salmonis. 624
Rickettsia-Like Organisms 628
Characteristics of the Disease 628
Streptobacillus 629
'*Candidatus*' 630
'*Candidatus* Actinochlamydia clariae' 630
'*Candidatus* Arthromitus' 630
'*Candidatus* Branchiomonas cysticola' 631
'*Candidatus* Clavochlamydia salmonicola' 631
'*Candidatus* Piscichlamydia salmonis' 632
'*Candidatus* Renichlamydia lutjani' 632
'*Candidatus* Similichlamydia latridicola' 632
'*Candidatus* Syngnamydia venezia' 632
Chlamydiales Representative 632
Unidentified Gram-Negative Bacteria. 633
References. 635

12 Isolation/Detection 643
Appendix – Media Used for the Isolation and Growth of Bacterial Fish Pathogens 647
Anderson and Conroy's Medium for *Sporocytophaga* – Like Organisms 647
Blood and Serum Free Medium for *Piscirickettsia salmonis* (Henriquez et al. 2013) 647
Bootsma and Clerx's Medium for *Flavobacterium columnare* 648
Brewer's Thioglycollate Medium 648
Charcoal Agar; for the Growth of *Renibacterium salmoninarum*. 648
Columbia Agar 649
Coomassie Brilliant Blue Agar (CBB) 649
Cysteine Blood Agar. 649
Cytophaga Agar (Anacker and Ordal 1959) 649
Improved Growth Medium for *Fla. psychrophilum* (Daskalov et al. 1999). 650
LG Agar for *Lactococcus garvieae* (Chang et al. 2014a). 650
Nutrient Agar Supplemented with Activated Charcoal for *Fla. psychrophilum* (Álvarez and Guijarro 2007). 650
Dorset-Egg Medium 650
Edwardsiella tarda (ET) Medium (Lindquist 1991; Castro et al. 2011). 651
Emerson Agar 651
Flavobacterium columnare Selective Medium (Fijan 1969) 651
FLP Medium (for *Fla. psychrophilum*; Cepeda et al. 2004). 652
Kidney Disease Medium 2 (KDM2). 652
L-F Medium 652

Loeffler (Serum) Medium 653
Löwenstein-Jensen Medium 653
MacConkey Agar 653
Medium K (Mudarris and Austin 1988) 653
Middlebrook 7H10 Agar 654
Myxobacterium Selective Medium (Hsu et al. 1983) 654
Peptone Beef Extract Glycogen Agar (PBG) 655
Petragnani Medium 655
Photobacterium damselae subsp. *piscicida* Medium (Hashimoto et al. 1989) 655
Pseudomonas F Agar 656
Ribose Ornithine Deoxycholate Medium for the Isolation of *Yersinia ruckeri* (Rodgers 1992) 656
Rimler-Shotts Medium (Shotts and Rimler 1973) 656
Robertsons Meat Broth (= Cooked Meat Medium) 657
Selective Flexibacter Medium (Bullock et al. 1986) 657
Selective Kidney Disease Medium (SKDM) 657
Semi Defined Medium for *Renibacterium salmoninarum* 658
Shotts and Waltman Medium for the Isolation of *Edwardsiella ictaluri* (Shotts and Waltman 1990) 658
Skimmed Milk Agar 659
TCY Medium (Hikida et al. 1979) 659
Thioglycollate Broth 659
Thiosulphate Citrate Bile Salt Sucrose Agar (TCBS) 659
Todd-Hewitt Broth 660
Vibrio alginolyticus Agar (VAL) 660
Vibrio anguillarum Medium (VAM) 660
Waltman-Shotts Medium for the Isolation of *Yersinia ruckeri* (Waltman and Shotts 1984) 661
Yeast Extract Glucose Agar (Michel et al. 1997) 661
Yersinia ruckeri Selective Medium (Furones et al. 1993) 661
References 661

13 Diagnosis 663
Gross Clinical Signs of Disease 664
Histopathological Examination of Diseased Tissues 666
Bacteriological Examination 667
Tissues to be Sampled 667
Identification of Bacterial Isolates 667
Serology 667
Immunohistochemistry 671
Molecular Techniques 671
Phenotypic Tests 673
References 689

14 Control 693
Wild Fish Stocks 693
Farmed Fish 694
Husbandry/Management 695
Disinfection/Water Treatments 696
Genetically Resistant Stock 696
Adequate Diets/Dietary Supplements/Non-Specific Immunostimulants 696
Vaccines 698
Composition of Bacterial Fish Vaccines 698
Methods of Vaccine Inactivation 699
Methods of Administering Vaccines to Fish 699
Antimicrobial Compounds 701
Preventing the Movement and/or Slaughtering of Infected Stock 704
Probiotics/Biological Control 705
References 706

15 Conclusions 711
The Recognition of New and Emerging Conditions 712
Co-Infections 712
Taxonomy and Diagnosis 715
Isolation and Selective Isolation of Pathogens 715
Ecology (Epizootiology) 715
Pathogenicity 716
Control Measures 716
The Effects of Environmental Stress Including Physical and Chemical Pollution 716
Zoonoses 718
References 718

Index 723

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Abbreviations

ABC	Adenosine triphosphate-binding-cassette
Aer.	*Aeromonas*
AFLP	Amplified fragment length polymorphism
AHL	Acylated homoserine lactone
A-layer	The additional surface layer of *Aer. salmonicida*
Ali.	*Aliivibrio*
Arc.	*Arcobacter*
ARISA	Automated ribosome intergenic spacer analysis
ATCC	American Type Culture Collection, Rockville, Maryland, USA
ATP	Adenosine triphosphate
Bac.	*Bacillus*
BCG	Bacillus Calmette Guérin
BHI	Brain-heart infusion
BHIA	Brain-heart infusion agar
BKD	Bacterial kidney disease
BLIS	Bacteriocin-like substance
BMA	Basal marine agar
Bp	Base pair
Car.	*Carnobacterium*
CBB	Coomassie brilliant blue agar
CDC	Centers for Disease Control and Prevention, Atlanta, USA
CE	Carp erythrodermatitis
CFU	Colony-forming unit
CgP	Cytidine-phosphate-guanosine
Chrys.	*Chryseobacterium*
CHSE-214	Chinook salmon embryo 214 cell line
Cit.	*Citrobacter*
Cl.	*Clostridium*
CLB	*Cytophaga*-like bacteria

CLED	Cystine lactose electrolyte-deficient agar
Cor.	*Corynebacterium*
Cyt.	*Cytophaga*
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
ECP	Extracellular product
EDTA	Ethylenediaminetetraacetic acid
Edw.	*Edwardsiella*
ELISA	Enzyme-linked immunosorbent assay
Ent.	*Enterobacter*
En.	*Enterococcus*
EPC	Epithelioma papulosum cyprini (cell line)
ERIC	Repetitive intergenic consensus
ERM	Enteric redmouth
Esch.	*Escherichia*
ET	*Edw. tarda* medium
Eu.	*Eubacterium*
EUS	Epizootic ulcerative syndrome
FAME	Fatty acid methyl ester
FAT	Fluorescent antibody test
Fc	Crystallisable fragment
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
Fla.	*Flavobacterium*
Fle.	*Flexibacter*
Fr.	*Francisella*
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCAT	Glycerophospholipid:cholesterol acyltransferase
GFP	Green fluorescent protein
GMO	Genetically manipulated organism
GRP	Glucose-regulated protein
G+C	Guanine plus cytosine
H.	*Haemophilus*
Haf.	*Hafnia*
HG	Hybridisation group
h.i.	Hyperosmotic infiltration
HSP	Heat shock protein
iFAT	Indirect fluorescent antibody test
IgM	Immunoglobulin M
i.m.	Intramuscular
i.p.	Intraperitoneal
IROMP	Iron-regulated outer membrane protein
ISR	Intergenic spacer region
IU	International unit
J.	*Janthinobacterium*
HG	Hybridisation group

kb	Kilobase
kDa	Kilodalton
KDM2	Kidney disease medium 2
LAMP	Loop-mediated isothermal amplification
LD_{50}	Lethal dose 50 %, i.e., the dose needed to kill 50 % of the population
LD_{100}	Lethal dose 100 %
Lis	*Listeria*
LPS	Lipopolysaccharide
MARTX	Multifunctional, autoprocessing repeats-in-toxin
mDa	Megadalton
MEM	Minimal essential medium
MHC	Mueller-Hinton agar supplemented with 0.1 % (w/v) L-cysteine hydrochloride
MIC	Minimum inhibitory concentration
MLD	Median lethal dose
MLSA	Multilocus sequence analysis
Mor.	*Moraxella*
mRNA	Messenger RNA
MRVP	Methyl red Voges Proskauer
msa	Major soluble antigen (gene)
MSS	Marine salts solution
Myc.	*Mycobacterium*
NCBV	Non-culturable but viable
NCFB	National Collection of Food Bacteria, Reading, England
NCIMB	National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland
NCTC	National Collection of Type Cultures, Colindale, London, England
Nec.	*Necromonas*
Noc.	*Nocardia*
ODN	Oligodeoxynucleotide
OMP	Outer membrane protein
ORF	Open reading frame
p57	57 kDa protein (of *Ren. salmoninarum)*
Pa.	*Pasteurella*
PAGE	Polyacrylamide gel electrophoresis
PAP	Peroxidase-antiperoxidase enzyme immunoassay
PBG	Peptone beef extract glycogen medium
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PFU	Plaque-forming unit
Ph.	*Photobacterium*
PMSF	Phenylmethyl-sulphonyl fluoride
Pr.	*Providencia*
Ps.	*Pseudomonas*
PFGE	Pulsed-field gel electrophoresis

qPCR	Quantitative polymerase chain reaction
RAPD	Randomly amplified polymorphic DNA
Ren.	*Renibacterium*
REP	Repetitive extragenic palindromic
RFLP	Restriction fragment length polymorphism
RLO	*Rickettsia*-like organism
ROS	Reactive oxygen species
RPS	Relative percent survival
rRNA	Ribosomal ribonucleic acid
RTFS	Rainbow trout fry syndrome
RTG-2	Rainbow trout gonad-2 cell line
RT-PCR	Reverse transcriptase polymerase chain reaction
SBL	Striped bass larvae
S_D	Dice coefficient
SEM	Scanning electron microscopy
S-layer	Surface layer
Sal.	*Salmonella*
SDS	Sodium dodecyl sulphate
Ser.	*Serratia*
SKDM	Selective kidney disease medium
SNP	Single-nucleotide polymorphism
SSCP	Single-strand conformation polymorphism
SSH	Suppression subtractive hybridisation
Sta.	*Staphylococcus*
Str.	*Streptococcus*
T.	*Tenacibaculum*
T3SS	Type III secretion system
T6SS	Type VI secretion system
TCBS	Thiosulphate citrate bile salts sucrose agar
TCID	Tissue culture infectivity dose
TEM	Transmission electron microscopy
TLR	Toll-like receptor
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TYES	Tryptone yeast extract salt medium
UV	Ultraviolet
V.	*Vibrio*
Vag.	*Vagococcus*
VAL	*Vibrio alginolyticus* medium
VAM	*Vibrio anguillarum* medium
vapA	Virulence array protein gene A
VHH	*Vibrio harveyi* haemolysin
VHML	*Vibrio harveyi* myovirus-like (bacteriophage)
VNTR	Variable number tandem repeats
Y.	*Yersinia*

List of Figures

Fig. 2.1 A rainbow trout displaying haemorrhaging in the eye caused by infection with *Lactococcus garvieae* 41

Fig. 3.1 The rainbow trout on the left has bilateral exophthalmia caused by *Ren. salmoninarum.* The second fish is a healthy specimen..... 85

Fig. 3.2 A distended abdomen on a rainbow trout with BKD 85

Fig. 3.3 A blood blister on the surface of a rainbow trout with BKD..... 86

Fig. 3.4 Swollen kidneys associated with BKD..... 86

Fig. 3.5 A virtual pure culture of *Ren. salmoninarum* on SKDM (right). On KDM2 (KDM2), the pathogen has been outcompeted by contaminants..... 88

Fig. 3.6 An API ZYM strip after inoculation, incubation, and the addition of reagents. The organism is the type strain of *Ren. salmoninarum* 93

Fig. 3.7 Mycobacteriosis in yellowtail. Extensive granulomas are present on the liver and kidney 115

Fig. 3.8 Nocardiosis in yellowtail. Extensive granulomas are present on the liver and kidney 126

Fig. 4.1 Generalised liquefaction of rainbow trout tissues resulting from infection with *Aer. aquariorum*..... 162

Fig. 4.2 Surface haemorrhaging and mouth erosion on a carp which was infected with *Aer. bestiarum*..... 164

Fig. 4.3 Erosion and haemorrhaging of the mouth of a ghost carp. The aetiological causal agent was *Aer. bestiarum* 164

Fig. 4.4 Extensive surface haemorrhaging on tilapia infected with *Aeromonas* sp., possibly *Aer. hydrophila* 168

Fig. 4.5 A crucian carp displaying extensive surface haemorrhaging attributed to infection with *Aer. hydrophila*..... 168

Fig. 4.6 An extensive abscess with associated muscle liquefaction in the musculature of rainbow trout. The aetiological agent was *Aer. hydrophila* 169
Fig. 4.7 A dissected abscess on a rainbow trout revealing liquefaction of the muscle and haemorrhaging. The aetiological agent was *Aer. hydrophila* 169
Fig. 4.8 *Aeromonas* infection in goldfish, which is also displaying abdominal swelling (dropsy) and some surface haemorrhaging 170
Fig. 4.9 Generalised liquefaction of a rainbow trout associated with infection by *Aeromonas* 170
Fig. 4.10 Extensive erosion of the tail and fins on a rainbow trout. Also, there is some evidence for the presence of gill disease. The aetiological agent was *Aer. hydrophila* 171

Fig. 5.1 A furuncle, which is attributable to *Aer. salmonicida* subsp. *salmonicida,* on the surface of a rainbow trout 216
Fig. 5.2 A dissected furuncle on a rainbow trout revealing liquefaction of the muscle 216
Fig. 5.3 Carp erythrodermatitis. The aetiological agent is likely to be atypical *Aer. salmonicida* 218
Fig. 5.4 Extensive skin and muscle haemorrhaging in black rockfish caused by *Aer. salmonicida* subsp. *masoucida* 219
Fig. 5.5 A well developed ulcer on a koi carp. The aetiological agent was atypical *Aer. salmonicida* 219
Fig. 5.6 An ulcerated goldfish on which the lesion has extended across the body wall, exposing the underlying organs. The aetiological agent was atypical *Aer. salmonicida* 220
Fig. 5.7 *Aer. salmonicida* subsp. *salmonicida* producing brown diffusible pigment around the colonies on TSA. The smaller (= rough) and larger (= smooth) colonies are readily observable 221
Fig. 5.8 The dark blue colonies of *Aer. salmonicida* subsp. *salmonicida* on CBB 222
Fig. 5.9 An intensively brown pigmented L-form colony (~0.5 mm in diameter) of *Aer. salmonicida* subsp. *salmonicida* growing within L-F agar 224
Fig. 5.10 An L-form colony (~0.5 mm in diameter) of *Aer. salmonicida* subsp. *salmonicida* growing within L-F agar and stained by Dienes method 224

Fig. 6.1 *Edw. ictaluri* infection in *Pangasianodon hypophthalmus.* Pale areas of cellular necrosis are evident in the anterior (AK) and posterior (PK) kidney 326

Fig. 6.2 A rainbow trout displaying extensive haemorrhaging in the mouth caused by ERM 367
Fig. 6.3 A blind rainbow trout that survived infection with ERM..... 367

Fig. 7.1 Hyperplasia, hypertrophy and fusion of the gill lamellae in rainbow trout 398

Fig. 9.1 Petechial haemorrhages on the surface of an eel with Sekiten-byo..... 477
Fig. 9.2 Clown fish with systemic *Pseudomonas* infection 485

Fig. 10.1 Extensive surface haemorrhaging on a turbot with vibriosis..... 500
Fig. 10.2 An ulcer, caused by *Vibrio* sp., on the surface of olive flounder 500
Fig. 10.3 Surface haemorrhaging on a tongue sole (*Cynoglossus semilaevis*) infected with *Ph. damselae* subsp. *piscicida* 505
Fig. 10.4 Haemorrhaging on the fins and around the opercula of a sea bass. The aetiological agent was *V. anguillarum*..... 525

Fig. 11.1 The *reddened* area associated with *red mark* disease syndrome (= winter strawberry disease) in >500 g rainbow trout 629
Fig. 11.2 Epitheliocystis (*see arrow*) in *Sparus aurata* 630
Fig. 11.3 Limited tail erosion and an ulcer on the flank of rainbow trout. The casual agent was considered to be linked to ultramicrobacteria..... 635

Fig. 13.1 Exophthalmia and surface haemorrhaging on a carp that was suspected to have been infected with *Aer. hydrophila*..... 665
Fig. 13.2 Pronounced bilateral exophthalmia on a trout..... 665
Fig. 13.3 Distended abdomen/dropsy on a fathead minnow for which the causal agent was considered to be *Aeromonas*..... 666
Fig. 13.4 Surface haemorrhaging and ulceration on an eel..... 666

Fig. 15.1 Pronounced bloat in goldfish, which revealed the presence of multiple bacterial pathogens 713

Chapter 1
Introduction

Abstract There has been a progressive increase in the number of new bacterial taxa associated with fish diseases over the last 20 years, with examples including *Pasteurella skyensis* and *Francisella noatunensis* and the emergence of so-called unculturables, e.g. *Candidatus*, intact cells of which have been observed in diseased tissue but culture has not yet been achieved.

Keywords Introduction • Cultures • Molecular diagnosis • Unculturables • Disease definition

The traditional view of microbiology was that bacteria should be culturable in the laboratory, although it is now appreciated the many micro-organisms are unable to grow on artificial media. Although most of the recognised bacterial fish pathogens are capable of growth in laboratory media, the value of the cultures to understanding the biology of the organisms may be questioned. Introductory microbiology courses emphasise that bacteria multiply by binary fission with single cells being cloned to form visible colonies. Furthermore, there is an assumption that the colony contains a homogeneous population of cells, i.e. a clone, although this may be challenged because cells may mutate, and swap and/or share genes (e.g. Pennisi 2002). Then, there is the issue about the relationship of cultured cells to those in the natural environment [= diseased fish] from which they were obtained. Cultured cells may be bigger (Torrella and Morita 1981) and have reduced phenotypic abilities (e.g. Koskiniemi et al. 2012), which may reflect the loss of DNA by gene deletion or from plasmids and bacteriophages, or the switching off of specific genes. Let us not forget that cells more suited for the laboratory environment may well outcompete those more closely associated with the disease pathology. The result will be laboratory cultures with limited relationship to the cells in diseased fish. Thus, ensuing studies to include pathogenicity determination would be of limited value in explaining the true role of the organism in fish.

Within the realm of fish diseases, it is all too apparent that the names of bacterial species are often used with little supporting evidence to justify the use of those names. Over the last two decades, there has been a trend away from the conventional phenotypic approach of characterising fish pathogens to molecular methods; and the description of new taxa is often based on minimal phenotypic data, which poses

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problems for determining reliable diagnostic traits. In many laboratories, identification is now routinely accomplished by means of sequencing of the 16S rRNA gene; a move that has led to greater confidence in the outputs although this will reflect the accuracy of the data in the databases. However, whereas the use of new technologies is to be encouraged, an on-going dilemma remains about the authenticity and value of isolates. Also, many studies are based on the examination of single isolates the relevance of which to fish pathology or science in general is perhaps doubtful. Certainly, too many conclusions result from the examination of too few isolates. Nevertheless, the study of pathogenicity mechanisms, diagnostics and disease control by means of vaccines have all benefited from molecular approaches.

It is apparent that there has been a progressive increase in the number of new bacterial taxa associated with fish diseases, with examples including *Pasteurella skyensis* and *Francisella noatunensis.* However, some elementary questions/concerns about bacterial fish diseases remain to be addressed:

- Why are so few anaerobes associated with fish diseases? Could this reflect a lack of interest/expertise/suitable methods as opposed to a lack of occurrence?
- Are the majority of diseases really caused by single bacterial taxa or could there be many more incidences of infections caused by two or more taxa either acting simultaneously or sequentially? [Would diagnosticians recognise infections caused by more than one pathogen?]. Does the same organism instigate and develop the disease situation or could there be a microbial succession with one organism initiating an infection, and a second or third developing the disease. Could conventional diagnoses differentiate primary pathogens from secondary invaders/contaminants of diseased tissue?
- Unculturables, e.g. *Candidatus,* are becoming associated with fish diseases, i.e. situations where pathogens may be detected microscopically or serologically but not cultured. The question to be resolved is whether such organisms are incapable of growing outwith a host or if suitable media have not been developed. It is speculative how many more of these unculturable organisms remain to be recognised. Moreover, how many times has *Candidatus* been missed, and secondary invaders/contaminants labelled as the actual pathogen? Then, there is the situation, such as with red mark syndrome, whereby an organism may be detected by serology or molecular methods, but not readily observed.
- There is the issue regarding the value of the cultures obtained during diagnoses. The conventional dogma is that where isolation from an active disease situation is attempted the result on laboratory media will be dense virtually pure cultures and not a comparatively few diverse colony types; the latter being interpreted as indicative of the presence of contaminants. This approach would miss infections caused by two or more different organisms.
- The value of cultures is a major concern insofar as the assumption is that the pure culture is representative of the organism in its natural habitat, and thus may be used for meaningful studies of pathogenicity, ecology and disease control.
- Certainly, an initial weakening process to the host may be possible in the absence of pathogens, and involve pollution or a natural physiological state (e.g. during

the reproductive phase) in the life cycle of the fish. A weakened host is then prone to infection.

Notwithstanding these concerns, representatives of many bacterial taxa have, at one time or another, been associated with fish diseases. There remains doubt about whether some of these bacteria should really be considered as true fish pathogens. In some cases, the supportive evidence is either weak or non-existent – scientists may have failed to prove that the isolate from diseased fish is actually pathogenic -, or there have been only single reports of disease without any repeat cases over many years. Possibly, such organisms constitute contaminants or even innocuous saprophytes. However, it is readily apparent that there is great confusion about the precise meaning of disease. A definition, from the medical literature, states that:

> "… a disease is the sum of the abnormal phenomena displayed by a group of living organisms in association with a specified common characteristic or set of characteristics by which they differ from the norm of their species in such a way as to place them at a biological disadvantage…"
>
> (Campbell et al. 1979)

This definition is certainly complex, and the average reader may be excused for being only a little wiser about its actual meaning. Dictionary definitions of disease are more concise, and include "an unhealthy condition" and "infection with a pathogen [= something that causes a disease]". One conclusion is that disease is a complex phenomenon, leading to some form of measurable damage to the host. Yet, it is anticipated that there might be profound differences among scientists about just what constitutes a disease. Fortunately, infection by micro-organisms is one aspect of disease that finds ready acceptance within the general category of disease.

For his detailed treatise on diseases of marine animals, Kinne (1980) considered that disease might be caused by

- genetic disorders
- physical injury
- nutritional imbalance
- pathogens
- pollution.

This list of possible causes illustrates the complexity of disease. An initial conclusion is that disease may result from biological (= *biotic)* factors, such as pathogens, and *abiotic* causes, e.g. the emotive issue of pollution. Disease may also be categorised in terms of epizootiology (Kinne 1980), as:

> *Sporadic* diseases, which occur sporadically in comparatively small members of a fish population;
>
> *Epizootics*, which are large-scale outbreaks of communicable disease occurring temporarily in limited geographical areas;
>
> *Panzootics,* which are large-scale outbreaks of communicable disease occurring over large geographical areas;
>
> *Enzootics,* which are diseases persisting or re-occurring as low level outbreaks in certain defined areas.

The study of fish diseases has concentrated on problems in fish farms (= aquaculture), where outbreaks either begin suddenly, progress rapidly often with high mortalities, and disappear with equal rapidity (= *acute* disease) or develop more slowly with less severity, but persist for greater periods (= *chronic* disease). As we move further into the 21st century, issues about global warming/climate change are discussed – could this impact on the emergence and spread of fish diseases? A situation could easily arise in which the host becomes stressed by increasing temperature, and more prone to disease. Clearly, the deteriorating situation in the natural environment is of increasing concern. Indeed, there is already concern about the health of corals, worldwide, and the initial evidence that some coral pathogens may also infect fish. In another example, it is curious why mycobacteria appear to have increased in significance in fish within confined areas, notably the Chesapeake Bay, USA.

This text will deal with all the diseases caused by bacteria. Cases will be discussed where infectious disease is suspected but not proven. An example includes red mark syndrome/disease (also known as winter strawberry disease) of rainbow trout in the UK where the causal agent is suspected to be bacterial of which rickettsia is the possible aetiological agent.

Disease is usually the outcome of an interaction between the host (= fish), the disease causing situation (= pathogen) and external stressor(s) (= unsuitable changes in the environment; poor hygiene; stress). Before the occurrence of clinical signs of disease, there may be demonstrable damage to/weakening of the host. Yet all too often, the isolation of bacteria from an obviously diseased fish is taken as evidence of infection. Koch's Postulates may be conveniently forgotten.

So, what are the bacterial fish pathogens? A comprehensive list of all the bacteria, which have been considered to represent fish pathogens, has been included in Table 1.1. Some genera, e.g. *Vibrio*, include many species that are acknowledged to be pathogens of freshwater and/or marine fish species. Taxa (highlighted by quotation marks), namely '*Catenabacterium*', '*H. piscium*' and '*Myxobacterium*' are of doubtful taxonomic validity. Others, such as *Pr. rettgeri* and *Sta. epidermidis*, are of questionable significance in fish pathology insofar as their recovery from diseased animals has been sporadic. A heretical view would be that enteric bacteria, e.g. *Providencia,* comprise contaminants from water or from the gastro-intestinal tract of aquatic or terrestrial animals. Certainly, many of the bacterial pathogens are members of the normal microflora of water and/or fish. Others have been associated only with clinically diseased or covertly infected (asymptomatic) fish. Examples of these 'obligate' pathogens include *Aer. salmonicida* and *Ren. salmoninarum*, the causal agents of furunculosis and bacterial kidney disease (BKD), respectively. It will be questioned whether or not bacteria should be considered as obligate pathogens of fish, at all. It is a personal view that the inability to isolate an organism from the aquatic environment may well reflect inadequate recovery procedures. Could the organism be dormant/damaged/senescent in the aquatic ecosystem; a concept which has been put forward for other water-borne organisms (Stevenson 1978)?

It is undesirable that any commercially important species should suffer the problems of disease. Unfortunately, the aetiology of bacterial diseases in the wild is

Table 1.1 Bacterial pathogens of freshwater and marine fish

Pathogen	Disease	Host Range	Geographical distribution
Anaerobes			
'*Catenabacterium*' sp	–	grey mullet (*Mugil auratus*),	USA
Clostridiaceae representative		redfish (*Sebastes* sp.)	
Clostridium botulinum	botulism, visceral toxicosis	Salmonids, channel catfish *(Ictalurus punctatus)*	Denmark, England, USA
Eubacteriaceae representative			
Eubacterium tarantellae	eubacterial meningitis	striped mullet (*Mugil cephalus*)	USA
Gram-positive bacteria – The 'Lactic Acid' bacteria			
Carnobacteriaceae representative			
Carnobacterium maltaromaticum	pseudokidney disease	Lake whitefish *(Coregonus clupeaformis)*	USA
	meningoencephalitis	trout and salmon (*Oncorhynchus* sp.)	
		salmon shark *(Lemna*	
Carnobacterium piscicola	lactobacillosis, pseudokidney disease	salmonids	North America, UK
Enterococcaceae representatives			
Enterococcus (Streptococcus) faecalis subsp. *liquefaciens*	–	bullhead (*Amiurus bebulosus*)	Croatia
Vagococcus salmoninarum	lactobacillosis, pseudokidney disease, peritonitis, septicaemia	Atlantic salmon *(Salmo salar)*, brown trout *(Salmo trutta)*, rainbow trout	Australia, France, North America, Turkey
Lactobacillaceae representative			
Lactobacillus spp.	lactobacillosis, pseudokidney disease	salmonids	North America, UK

(continued)

Table 1.1 (continued)

Pathogen	Disease	Host Range	Geographical distribution
Streptococcaceae representatives			
Lactococcus garvieae (= Enterococcus seriolicida)	streptococcicosis/streptococcosis	many fish species	Australia, Brazil, Europe, Israel, Japan, Saudi Arabia, Red Sea, South Africa, Taiwan, USA
Lactococcus lactis subsp. *lactis*	lactococcosis	hybrid sturgeon, silver carp	Taiwan, USA
Lactococcus piscium	lactobacillosis, pseudokidney disease	rainbow trout	North America
Streptococcus dysgalactiae	streptococcosis	Amur sturgeon (*Acipenser schrenckii*), amberjack *(Seriola dumerili)*, Nile tilapia *(Oreochromis niloticus),* yellowtail *(Seriola quinqueradiata)*	Brazil, China, Japan
Streptococcus agalactiae (= Str. difficilis)	meningoencephalitis	carp *(Cyprinus carpio),* grouper *(Epinephelus lanceolatus),* rainbow trout, silver pomfret *(Pampus argenteus),* tilapia *(Oreochromis* spp.)	Australia, Columbia, Israel, Kuwait, USA
Streptococcus ictaluri	streptococcosis	channel catfish	USA
Streptococcus iniae (Str. shiloi)	acute septicaemia, meningoencephalitis, streptococcicosis/streptococcosis	various freshwater and marine fish species	Australia, Bahrain, China, Europe, Israel, Japan, Saudi Arabia, South Africa, USA
Streptococcus milleri	–	koi carp *(Cyprinus carpio)*	UK
Streptococcus parauberis	streptococcicosis/streptococcosis	olive flounder, turbot *(Scophthalmus maximus)*	Portugal, Spain, USA
Streptococcus phocae	streptococcosis	Atlantic salmon	Chile

Aerobic gram-positive rods and cocci			
Renibacterium salmoninarum	bacterial kidney disease (BKD; Dee disease; corynebacterial kidney disease)	salmonids	Europe, Japan, North and South America
Erysipelothrix rhusiopathiae	haemorrhagic septicaemia	eels	Australia
Aerococcaceae representative			
Aerococcus viridans	–	tilapia	China
Bacillaceae representatives			
Bacillus spp.	septicaemia; bacillary necrosis	various freshwater fish species including catfish *(Pangasius hypophthalmus)*	Nigeria, Vietnam
Bacillus cereus	branchio-necrosis	carp *(Cyprinus* sp.), striped bass *(Morone saxatilis)*	USA
Bacillus mycoides	ulceration	channel catfish *(Ictalurus punctatus)*	Poland, USA
Bacillus subtilis	branchio-necrosis	carp	Poland
Corynebacteriaceae representatives			
Corynebacterium aquaticum	exophthalmia	striped bass	USA
Coryneform bacteria	'corynebacteriosis'	salmonids	England
Leuconostocaccaceae representative			
Weissella spp.	weissellosis	rainbow trout	Brazil, China, USA
Weissella ceti	weissellosis	rainbow trout	Brazil, China
Microbacteriaceae representative			
Microbacterium paraoxydans	?	Nile tilapia	Mexico
Micrococcaceae representative			
Micrococcus luteus	micrococcosis	rainbow trout	England

(continued)

Table 1.1 (continued)

Pathogen	Disease	Host Range	Geographical distribution
Mycobacteriaceae representatives			
Mycobacterium spp. *(Myc. abscessus, Myc. anabanti, Myc. avium, Myc. chelonei* subsp. *piscarium, Myc. fortuitum, Myc. gordonae, Myc. marinum, Myc. montefiorense, Myc. neoaurum, 'Myc. piscium', 'Myc. platypoecilus', Myc. poriferae. Myc. pseudoshottsii, 'Myc. ranae', Myc. salmoniphilum, Myc. shottsii, Myc. scrofulaceum, Myc. simiae, Myc. smegmatis, Myc. ulcerans*	mycobacteriosis (fish tuberculosis)	most fish species	worldwide
Nocardiaceae representatives			
Nocardia spp. *(Noc. asteroides, Noc. salmonicida; Noc. seriolae)*	nocardiosis	most fish species	worldwide
Rhodococcus sp.	ocular oedema	chinook salmon *(O. tshawytscha)*	Canada
Rhodococcus erythropolis	?	Atlantic salmon	Norway, Scotland
Rhodococcus qingshengii	?	Atlantic salmon	Chile
Planococcaceae representative			
Planococcus sp.	–	salmonids	England
Staphylococcaceae representatives			
Staphylococcus aureus	eye disease, jaundice	silver carp *(Hypophthalmichthys molitrix),* African sharp-tooth catfish *(Clarias gariepinus)*	India, Africa
Staphylococcus epidermidis	–	gilthead sea bream *(Sparus aurata),* red sea bream *(Chrysophrus major),* yellowtail *(Seriola quinqueradiata*)	Japan, Turkey

Staphylococcus warneri	ulcerations	rainbow trout	Spain, Turkey
Gram-negative bacteria			
Aeromonadaceae representatives			
Aeromonas allosaccharophila	–	elvers	Spain
Aeromonas bestiarum	–		USA
Aeromonas caviae	septicaemia	Atlantic salmon *(Salmo salar)*	Turkey
Aeromonas dhakensis	generalised septicaemia	Nile tilapia	Mexico
Aeromonas hydrophila (= Aer. liquefaciens, Aer. punctata)	haemorrhagic septicaemia, motile aeromonas septicaemia, redsore disease, fin rot	many freshwater fish species	worldwide
Aeromonas jandaei	–	eel *(Anguilla* sp.)	Spain
Aeromonas piscicola	–	?	Spain
Aeromonas salmonicida (subsp. *achromogenes, masoucida, salmonicida* and *smithia) {= Haemophilus piscium}*	furunculosis, carp erythrodermatitis, ulcer disease	salmonids, cyprinids, and marine species (dabs, cod)	worldwide
Aeromonas sobria	–	Garra rufa (*Garra rufa*), perch *(Perca fluvialitis),* gizzard shad *Dorosoma cepedianum),* stone loach (*Triplophya siluroides*) tilapia *(Oreochromis niloticus)*	China, Slovakia, Switzerland, USA
Aeromonas schubertii	tuberculous lesions	Snakehead *(Ophiocephalus argus)*	China
Aeromonas veronii biovar sobria	epizootic ulcerative syndrome, infectious dropsy	African catfish *Clarias gariepinus)*, rajputi *(Puntius gonionotus),* rui *(Labeo rohita),* catla (*Catla catla*), shole *(Channa striatus),* oscar *(Astronotus ocellatus)*	Bangladesh, India

(continued)

Table 1.1 (continued)

Pathogen	Disease	Host Range	Geographical distribution
Aeromonas veronii biovar veronii	–	Chinese longsnout (*Leiocassis longirostris*)	China
Alteromonadaceae representatives			
Pseudoalteromonas piscicida	egg disease	damselfish	USA
Pseudoalteromonas undina		sea bass, sea bream	Spain
Shewanella putrefaciens	septicaemia	rabbit fish *(Siganus rivulatus)*	Saudi Arabia
Campylobacteriaceae representative			
Arcobacter cryaerophilus	–	rainbow trout	Turkey
Comamonadaceae representative			
Delftia acidovorans	–	glass eels	Spain
Enterobacteriaceae representatives			
Citrobacter freundii	–	salmonids, sunfish *(Mola mola),* carp *(Cyprinus carpio)*	Europe, India, USA
Edwardsiella anguillarum	–	eels, sea bream, red sea bream	China
Edwardsiella ictaluri	enteric septicaemia of catfish	Ayu, bagrid catfish (*Pelteobagrus nudiceps*), brown bullhead (*Amieurus nebulosus),* channel catfish, freshwater catfish *(Pangasius hypophthalmus),* danio *(Danio devario),* striped catfish *(Pangasius hypophthalmus),* yellow catfish (*Pelteobagrus fulvidraco*)	China, Indonesia, Japan, USA, Vietnam
Edwarsiella piscicida	–	Catfish,white fish (*Coregulus lavaretus*)	Asia, Europe, USA

Edwardsiella tarda (Paracolobactrum anguillimortiferum, Edw. anguillimortifera)	redpest, edwardsiellosis, emphysematous putrefactive disease of catfish	freshwater and some fish species	Japan, Spain, USA
Enterobacter cloacae	–	mullet (*Mugil cephalus*)	India
Escherichia vulneris	septicaemia	various freshwater fish species	Turkey
Hafnia alvei	haemorrhagic septicaemia	cherry salmon *(O. masou),* rainbow trout	Bulgaria, England, Japan
Klebsiella pneumoniae	fin and tail disease	rainbow trout	Scotland
Plesiomonas shigelloides	–	African catfish *(Heterobranchus bidorsalis),* eel, gourami *(Osphyronemus gourami),* rainbow trout, sturgeon *(Acipenser sturio)*	Germany, Portugal, Spain
Pantoea (= Enterobacter) agglomerans	–	dolphin fish *(Coryphaena hippurus)*	USA
Providencia (Proteus) rettgeri	–	silver carp	Israel
Providencia vermicola	–	Indian major carp	India
Salmonella enterica subsp. *arizonae (= Sal. choleraesuis* subsp. *arizonae = Sal. arizonae)*	septicaemia	pirarucu *(Arapaima gigas)*	Japan
Serratia liquefaciens	septicaemia	Arctic charr *(Salvelinus alpinus),* Atlantic salmon, turbot	France, Scotland, USA
Serratia marcescens	–	white perch *(Morone americanus)*	USA
Serratia plymuthica	–	rainbow trout	Poland, Scotland, Spain
Yersinia intermedia	–	Atlantic salmon	Australia

(continued)

Table 1.1 (continued)

Pathogen	Disease	Host Range	Geographical distribution
Yersinia ruckeri	enteric redmouth (ERM), salmonid blood spot	salmonids	Australia, Europe, North and South America
Flavobacteriaceae representatives			
Chryseobacterium balustinum (= *Flavobacterium balustinum*	flavobacteriosis	marine fish	USA
Chryseobacterium aahli	–	Great Lakes salmonids	USA
Chryseobacterium indologenes	–	Yellow perch (*Perca flavescens*)	USA
Chryseobacterium piscicola	skin and muscle ulceration	Atlantic salmon, rainbow trout	Chile, Finland
Chryseobacterium scophthalmum (= *Flavobacterium scophthalmum)*	gill disease; generalised septicaemia	turbot	Scotland
Flavobacterium branchiophilum	gill disease	salmonids	Europe, Korea, Japan, USA
Flavobacterium columnare (=*Flexibacter/Cytophaga columnaris*	columnaris, saddleback disease	many freshwater fish species	worldwide
Flavobacterium hydatis (= *Cytophaga aquatilis)*	gill disease	salmonids	Europe, USA
Flavobacterium johnsoniae (= *Cytophaga johnsonae)*	gill disease, skin disease	barramundi *(Lates calcarifer),* koi carp, rainbow trout, longfin eel (*Anguilla mossambica*)	Australia, France, South Africa
Flavobacterium oncorhynchi	–	rainbow trout	Spain
	bacterial gill disease	salmonids	Europe, USA
Flavobacterium psychrophilum (= *Cytophaga psychrophila)*	coldwater disease, rainbow trout fry syndrome, necrotic myositis	perch (*Perca fluviatilis*), salmonids, sea lamprey *(Petromyzon marinus)*	Australia, Europe, Japan, North America
Flavobacterium spartensis	–	Chinook salmon	USA
Flavobacterium succinicans	bacterial gill disease	rainbow trout	USA
Flectobacillus roseus	flectobacillosis	rohu	India

Myroides odoratimimus	–	grey mullet	India
Tenacibaculum dicentrarchi	–	sea bass	Spain
Tenacibaculum discolor	–	sole (*Solea senegalensis*)	Spain
Tenacibaculum gallaicum	–	turbot (*Psetta maxima*)	Spain
Tenacibaculum maritimum (=*Flexibacter maritimus*)	bacterial stomatitis, gill disease, black patch necrosis	many marine fish species	Europe, Japan, North America
Tenacibaculum ovolyticum (= *Flexibacter ovolyticus*)	larval and egg mortalities	halibut (*Hippoglossus hippoglossus*)	Norway
Tenacibaculum soleae	tenacibaculosis	sole (*Solea senegalensis*), wedge sole (*Dicologoglossa cuneata*), brill (*Scophthalmus rhombus*)	Spain
'Cytophaga rosea')	gill disease	salmonids	Europe, USA
Sporocytophaga sp.	saltwater columnaris	salmonids	Scotland, USA
Francisellaceae representatives			
Francisella sp.	granulomatous inflammatory disease	Atlantic cod (*Gadus morhua*), hybrid striped bass (*Morone chrysops x M. saxatilis*), three-line grunt (*Parapristipoma trilineatum*), tilapia	Costa Rica, Japan, Norway, USA
Francisella noatunensis subsp. *orientalis*	francisellosis	tilapia, French grunt, Caesar Grunt,	Austria,Brazil, Costa Rica, England, Japan, USA
Francisella noatunensis (= *Fr. philomiragia* subsp. *noatunensis* = *Fr. piscicida*)	francisellosis, visceral granulomatosis	Atlantic cod, Atlantic salmon, three-line grunt, striped bass, tilapia	Chile, Costa Rica, Japan, Norway

(continued)

Table 1.1 (continued)

Pathogen	Disease	Host Range	Geographical distribution
Hahellaceae representative			
Hahella chejuensis	red egg disease	tilapia	Thailand
Halomonadaceae representative			
Halomonas (=Deleya) cupida	–	black sea bream *(Acanthopagrus schlegeli)*	Japan
Moraxellaceae representatives			
Acinetobacter sp.	acinetobacter disease	Atlantic salmon, channel catfish	Norway, USA
Acinetobacter johnsonii	–	common carp, rainbow trout	Poland
Acinetobacter lwoffii	–	common carp, rainbow trout	Poland
Moraxella sp.	–	striped bass	USA
Moritellaceae representatives			
Moritella marina (V. marinus)	skin lesions	Atlantic salmon	Iceland
Moritella viscosa	winter ulcer disease/syndrome	Atlantic salmon	Iceland, Norway, Scotland
Mycoplasmataceae representative			
Mycoplasma mobile	red disease	tench *(Tinca tinca)*	USA
Myxococcaceae representative			
Myxococcus piscicola	gill disease	green carp *(Ctenopharyngodon idelluls)*	China
Neisseriaceae representative			
Aquaspirillum sp.	epizootic ulcerative syndrome	snakeheads *(Ophicephalus striatus)* and catfish *(Clarias batrachus)*	Thailand
Oxalobacteraceae			
Janthinobacterium lividum	anaemia	rainbow trout	Scotland

Pasteurellaceae representative			
Pasteurella skyensis	?	Atlantic salmon	Scotland
Piscirickettsiaceae representative			
Piscirickettsia salmonis	coho salmon syndrome, salmonid rickettsial septicaemia	salmon, sea bass *(Atractoscion nobilis)*	Canada, Chile, Greece, Norway, Scotland, USA
Rickettsia-like organism	red mark syndrome/strawberry disease	rainbow trout	UK, USA
Pseudomonadaceae representatives			
Pseudomonas aeruginosa	–	Nile tilapia; Mozambique tilapia	India, Tanzania
Pseudomonas alcaligenes	–	Chinese sturgeon	China
Pseudomonas anguilliseptica	red spot (Sekiten-byo), winter disease	rainbow trout, marine fish species, and particularly cod, eels *(Anguilla anguilla, A. japonica)*, black spot sea bream (*Pagellus bogaraveo*), gilthead sea bream *(Sparus aurata)*	Finland, France, Japan, Portugal, Scotland, Spain
Pseudomonas baetica	–	wedge sole (*Dicologoglossa cuneata*)	Spain
Pseudomonas chlororaphis	–	amago trout *(Oncorhynchus rhodurus)*	Japan
Pseudomonas fluorescens	generalised septicaemia	most fish species	Worldwide
Pseudomonas koreensis	eye disease	golden mahseer (*Tor putitora*)	India
Pseudomonas luteola	generalised septicaemia	rainbow trout	Turkey
Pseudomonas mosselii	–	Nile tilapia	Mexico
Pseudomonas plecoglossicida	bacterial haemorrhagic ascites	ayu *(Plecoglossus altivelis)*, pejerrey *(Odonthestes bonariensis)*,olive flounder	China, Japan

(continued)

Table 1.1 (continued)

Pathogen	Disease	Host Range	Geographical distribution
Pseudomonas pseudoalcaligenes	skin ulceration	rainbow trout	Scotland
Pseudomonas putida	haemorrhagic ascites, ulceration	ayu, rainbow trout	Japan, Turkey
Stenotrophomonas maltophilia	–	catfish, African catfish	China, India
Vibrionaceae representatives			
Photobacterium damselae subsp. *damselae (= Photobacterium histaminum)*	vibriosis	damsel fish *(Chromis punctipinnis),* redbanded sea bream *(Pagrus auriga)* rainbow trout, sea bass (*Lates calcarifer*), sharks, turbot, yellowtail	Asia, Europe, USA
Photobacterium damselae subsp. *piscicida (= Pasteurella piscicida)*	pasteurellosis, pseudotuberculosis	bluefin tuna *(Thunnus thynnus),* gilthead sea bream *(Sparus aurata),* sole *(Solea senegalensis),* striped bass *(Morone saxatilis*), white perch *(Roccus americanus*), yellowtail	Europe, Japan, USA
Aliivibrio fischeri	–	gilt-head sea bream , turbot	Spain
Aliivibrio logei	skin lesions	Atlantic salmon	Iceland
Aliivibrio salmonicida	coldwater vibriosis, Hitra disease	Atlantic salmon	Canada, Norway, Scotland
Aliivibrio wodanis	winter ulcer disease/syndrome	Atlantic salmon	Iceland, Norway, Scotland
Vibrio aestuarianus	–	tongue sole (*Cynoglossus semilaevis*)	China
V. alginolyticus	eye disease, septicaemia	cobia *(Rachycentron canadum),* gilt-head sea bream, grouper *(Epinephelus malabanicus),* sea bream *(Sparus aurata)*	Asia, Europe, Israel

V. anguillarum (= *Listonella anguillarum)*	vibriosis	most marine fish species	worldwide
V. cholerae (non-01)	ascites disease, septicaemia	ayu, goldfish *(Carassius aurata),* Mandarin fish, Cardinal tetra, Raphael catfish	Australia, Czech Republic Japan
V. furnissii	–	eel	Spain
V. harveyi (= *V. carchariae* and *V. trachuri)*	eye disease (blindness), necrotising enteritis, vasculitis, granuloma	gilt-head sea bream, sea bass, common snook *(Centropomus undecimalis),* horse mackerel (*Trachurus japonicus*), milkfish, red drum *(Sciaenops ocellatus),* sharks *(Carcharhinus plumbeus, Negaprion breviorstris)*, sole *(Solea senegalensis)*, summer flounder *Paralichthys dentatus),* tiger puffer *(Takifugu rubripes)*	Europe (notably Spain), Japan, Taiwan, USA
V. ichthyoenteri	intestinal necrosis/enteritis	Japanese flounder *(Paralichthys olivaceus),* summer flounder, olive flounder	Japan, Korea, USA
V. mimicus	ascites disease	grass carp, yellow catfish	China
V. ordalii	vibriosis	most marine fish species	Worldwide
V. parahaemolyticus	–	kelp grouper	China, India
V. pelagius	–	turbot	Spain
V. ponticus	ulcerative disease	Japanese sea bass (*Lateolabrax japonicus*)	China
V. scophthalmi	–	olive flounder	Korea

(continued)

Table 1.1 (continued)

Pathogen	Disease	Host Range	Geographical distribution
V. splendidus	septicaemia, vibriosis	corkwing wrasse *(Symphodus melops),* gilt-head sea bream, turbot	Norway, Spain
V. tapetis	vibriosis	corkwing wrasse, ovate pompano *(Trachinotus ovatus),* Dover sole	Norway
V. vulnificus	septicaemia	eel	Europe, Japan, P.R.C., USA
Miscellaneous pathogens			
'*Candidatus* Actinochlamydia clariae'	epitheliocystis	African sharptooth catfish	Uganda
'*Candidatus* Arthromitus'	summer enteritic syndrome, rainbow trout gastroenteritis	rainbow trout	Croatia, France, Italy, Spain, UK
'*Candidatus* Branchiomonas cysticola'	epitheliocystis	Atlantic salmon	Ireland, Norway
'*Candidatus* Clavochlamydia salmonicola'	epitheliocystis	freshwater salmonids	North America, Norway
'*Candidatus* Piscichlamydia salmonis'	epitheliocystis	Atlantic salmon	Ireland, Norway
'*Candidatus* Renichlamydia lutjani'	"epitheliocystis"-like	blue-striped snapper *(Lutjanus kasmira)*	Hawaii, USA
'*Candidatus* Similichlamydia latridicola'	epitheliocystis	striped trumpeter	Tasmania
'*Candidatus* Syngnamydia venezia	epitheliocystis	broad nosed pipefish	–
Chlamydiales representative	epitheliocystis	leopard sharp (*Triakis semifasciata*)	Swiss aquarium
Streptobacillus	–	Atlantic salmon	Ireland
unidentified	gill lesions	rockfish	Japan
unidentified	*Varracalbmi*	Atlantic salmon	Norway
unidentified	ulceration	rainbow trout	Scotland

Names in quotation marks are not included in the Approved Lists of Bacterial Names (Skerman et al. 1980) or their supplements

often improperly understood. Moreover, it seems that little if anything may be done to aid wild fish stocks, except, perhaps, by controlling pollution of the rivers and seas, assuming that when environmental quality deteriorates this influences disease cycles. In contrast, much effort has been devoted to controlling diseases of farmed fish.

Conclusions

- The list of fish pathogens has extended substantially since 1980. Current interest focuses on the motile aeromonads, enterics, vibrios, flavobacteria, francisellas and streptococci-lactococci.
- A question mark hangs over the significance of some organisms to fish pathology – are they truly pathogens or chance contaminants?
- There has been considerable improvement in the taxonomy of some groups, for example vibrios, particularly with the widespread use of sequencing of the 16S rRNA gene.
- There have been substantive advances in the understanding of pathogenicity mechanisms as a result of molecular approaches.
- The advent of molecular methods has revolutionised diagnostics, particularly in terms of accuracy.
- There has been a shift from emphasis on culture-dependent to culture-independent techniques as molecular methods have become commonplace in laboratories.
- The value of pure laboratory cultures to their counterparts in pathological material may be questioned.

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Chapter 2
Gram-Positive Bacteria (Anaerobes and 'Lactic Acid' Bacteria)

Abstract A wide range of aerobic and anaerobic Gram-positive taxa have been associated with fish diseases with new pathogens including *Lactococcus lactis* subsp. *lactis.* Lactococcosis and streptoccoccosis have developed into significant constraints of many fish species over a wide geographical area. Recent research has aimed to improve diagnostics by use of sensitive and specific molecular methods and disease control especially by vaccination, probiotics and plant products.

Keywords Anaerobes • Lactococcosis • Streptococcosis • Pseudokidney disease • Weisellosis

Anaerobes

Although only two species of anaerobic bacteria, namely *Clostridium botulinum* and *Eubacterium tarantellae*, have been implicated as fish pathogens, it is likely that detailed bacteriological investigations, including the use of suitable methods, may reveal that anaerobes cause more widespread problems than has been hitherto realised. In the first place, fish disease diagnostic laboratories do not normally use anaerobic methods. Therefore, it is unlikely that isolation of an offending anaerobic pathogen would ever be achieved. Consequently, the true cause of disease may not be recognised, or may be attributed to an aerobic secondary invader. Although there is no evidence that anaerobic pathogens have been missed, there are puzzling cases of mortalities among fish populations for which the aetiological agent has never been determined. It is recognised that anaerobes occur widely in the natural environment, and are commonplace in aquatic sediments (Davies 1969; Rouhbakhsh-Khaleghdoust 1975) and in the gastro-intestinal tract of fish (Sakata et al. 1978, 1980; Trust et al. 1979; Austin 2006), where they would be readily available for initiation of a disease cycle. Of relevance, Trust and colleagues recovered *Actinomyces, Bacteroides, Fusobacterium* and *Peptostreptococcus* from grass carp, goldfish and rainbow trout. Subsequently, Sakata et al. (1980) described two groups of anaerobes from the intestines of freshwater fish, i.e. ayu, goldfish and tilapia, in Japan. These Gram-negative, non-motile, asporogenous rods were considered to be

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representatives of the family Bacteroidaceae. It is of course questionable whether or not these bacteria will be recognised as fish pathogens in the future.

Clostridiaceae Representative

Clostridium botulinum

Characteristics of the Disease

The first report of botulism as a disease of fish was from Denmark, stemming from the work of Huss and Eskildsen (1974) when it was shown that the disease was chronic, termed 'bankruptcy disease', and occurred in farmed trout, with the causal agent recognised as *Cl. botulinum* type E. Later, the disease was found on one farm of rainbow trout in Great Britain (Cann and Taylor 1982, 1984) and was similarly identified among farmed coho salmon in the USA (Eklund et al. 1982), and subsequently associated with channel catfish in the USA (Chatla et al. 2012). Characteristic disease symptoms were very vague, but fish have been observed to exhibit abnormal sluggish, erratic swimming, appeared to be listless, and alternately floated and sank, before showing temporary rejuvenation. This pattern was repeated until death eventually ensued (Cann and Taylor 1982; Khoo et al. 2011; Beecham et al. 2014). Internally, there may be ascites, the brain, spleen and liver may be congested, vascular dilation, oedema (fluid retention) in the kidneys and gastro-intestinal tract, and the blood supply interrupted to the intestines which appeared to be blanched (Khoo et al. 2011). When the swimming speed is adversely affected, the fish are likely to become more prone to predation (Beecham et al. 2014).

Isolation/Diagnosis of the Pathogen

Isolation is achieved by use of straightforward anaerobic techniques. Samples of intestinal contents, which probably support a resident anaerobic microflora, and internal organs should be homogenised in 1 % (w/v) peptone phosphate buffer at pH 7.0, and diluted five-fold. These diluted samples should be inoculated into 100 ml or 200 ml aliquots of Robertson's meat broth (see Appendix in Chap. 12), with subsequent anaerobic incubation at 30 °C for up to 6 days. Thereupon, the presence of *Cl. botulinum* and its toxicity may be assessed (Cann et al. 1965a, b; Cann and Taylor 1982). In addition, sterile culture filtrates may be injected into mice and/or fish to assess the presence of toxic factors (Cann and Taylor 1982). However, the recovery of *Cl. botulinum* from intestinal samples should not be used

as the sole reason for diagnosing botulism, in view of its possible widespread presence in this habitat (see Trust et al. 1979). Alternatively, diagnosis may be achieved using bioassays to detect the presence of the neurotoxin in serum, which are especially useful if culturing is not achieved (Gaunt et al. 2007; Khoo et al 2011).

Characteristics of the Pathogen

Descriptions of fish pathogenic clostridia have tended towards extreme brevity. However, it is clear that the outbreaks of botulism in fish have been caused by predominantly *Cl. botulinum* type E.

Box 2.1: *Clostridium botulinum*
Cultures comprise anaerobic, chemo-organotrophic, Gram-positive, non-acid-fast rods, of 3.4–7.5 × 0.3–0.7 μm in size, which are motile by means of peritrichous flagella. It is important to note that care should be taken in interpreting Gram-stained smears, because cells may appear Gram-negative with age. Oval, sub-terminally positioned endospores are formed, which have characteristics appendages and exosporia. The cell wall contains diaminopimelic acid. Surface colonies are 1–3 mm in diameter, slightly irregular with lobate margins and raised centres, and translucent to semi-opaque with a matt appearance. Poor to moderate growth occurs in cooked meat broth, but abundant growth occurs in broth containing fermentable carbohydrates at the optimum temperature of 25–30 °C. Lecithinase, lipase, neurotoxins and haemolysins, but not catalase, caseinase, H_2S, indole or urease, are produced. Gelatinase is not usually produced by *Cl. botulinum* type E. Nitrates are not reduced, nor is the Voges Proskauer reaction positive. Some carbohydrates, such as fructose and glucose, but not aesculin, cellobiose, dulcitol, glycogen, inulin, mannitol, melezitose, melibiose, raffinose, rhamnose, salicin, sorbose, starch, sucrose and xylose, are fermented to acetic and butyric acids. The G + C ratio of the DNA is in the range of 26–28 moles % (Cato et al. 1986).

Diagnosis

Diagnosis of botulism has been accomplished by isolation of *Cl. botulinum* from diseased tissues, and more importantly, by demonstrating the presence of circulating toxin (particularly to *Cl. botulinum* type E) in the blood of moribund fish (Cann and Taylor 1982).

Epizootiology

Cl. botulinum is widespread in soil, marine and freshwater sediments and in the gastro-intestinal tract of man and other animals, including fish (Bott et al. 1968; Cato et al. 1986). In one study of 530 trout from Danish earthern ponds, *Cl. botulinum* type E was discovered to occur in 5–100 % of the fish in winter, increasing to 85–100 % of the population in late summer (Huss et al. 1974a). It was supposed that the principal source of contamination with this organism was from minced trash fish used as feed, although soil and water could also be involved (Huss et al. 1974a). Moreover, it was considered likely that clostridia become established in the mud and bottom-living invertebrates in trout ponds (Huss et al. 1974b). In Britain, it has been determined from an examination of 1400 trout collected from 17 fish farms, that the incidence of *Cl. botulinum* in whole fish and viscera was 9.4 % and 11.0 % respectively. Nevertheless, *Cl. botulinum* lingers in the fish farm environment for considerable periods following outbreaks of disease. Thus, at the English trout farm, which experienced botulism, the organism (possibly as endospores) was recovered for a year after the outbreak of disease. The numbers ranged from 1 to 800 organisms/g of sediment, compared to <1/g at an unaffected control site (Cann and Taylor 1982). There are no data available to assess the level of contamination in wild fish stocks (Cann et al. 1975). Similarly, there is no evidence to suggest that trout contaminated with *Cl. botulinum* could comprise a human health hazard (Bach et al. 1971).

Pathogenicity

Botulinum toxin has been introduced into fish to determine the level of toxicity. Thus using channel catfish fingerlings, Chatla et al. (2012) established that the MLD of serotype E toxin as 13.7 pg of toxin/fish of 5.3 g weight. This was determined after 96 h of intracoelomical injection of the toxin. Initially, the fish displayed hyperactivity, which led to erratic swimming leading to death. The mortalities revealed exophthalmia, ascites, congestion of the spleen, and the cutting off (intussusception) of the blood supply and thus blanching of the intestine, i.e. visceral toxicosis (Chatla et al. 2012).

Disease Control

Disinfection As there is no effective chemotherapy for botulism in fish, disinfection of the ponds has been advocated. Success has resulted from moving the stock to clean areas, draining the contaminated ponds, and removing surface mud and detritus before disinfecting with slaked lime at 1.6 kg/m^2. The disinfectant should be worked well into the layers at the bottom of the ponds, left for a period of not less

than 7 days, and the pond then returned to use (Huss et al. 1974b; Cann and Taylor 1982). To remove clostridia from the gastro-intestinal tract of trout, it has been recommended that the fish should be starved for 5 days (Wenzel et al. 1971). However, Huss et al. (1974b) showed that whereas starvation does indeed reduce contamination with *Cl. botulinum*, the overall rate of success depends on other factors, including the nature of the water supply. It should be emphasised that clostridia may be normal inhabitants of the fish intestine.

Eubacteriaceae Representative

Eubacterium tarantellae

Characteristics of the Disease

The term 'eubacterial meningitis' was coined for this disease (Winton et al. 1983), which is a neurological condition whereby infected fish twirl in the water until death results (Udey et al. 1976). There was little, if any, external pathology observed, but cells of the pathogen may be readily observed in sections of brain tissue. It is interesting to note from Udey's work that some fish were also infected with other organisms, namely, trematodes (*Bucephalus* sp.) and *Vibrio* spp.; whereas ~20% possessed low numbers of *Myxosoma cephalus* spores in the brain cavity. Therefore, the question must be asked whether this anaerobe represented a primary or secondary pathogen during the original outbreak of disease in Biscayne Bay, Florida, USA.

Isolation of the Pathogen

Samples of tissues should be plated on BHIA, whereupon bacterial cultures will develop after 7 days anaerobic incubation at room temperature. Additionally, tissues may be inoculated into Brewer's thioglycollate medium (Appendix in Chap. 12; Udey et al. 1977). The organism has been recovered from diseased larval gut flora of common dentex (*Dentex dentex*) in Turkey (Akalyi et al. 2015).

Characteristics of the Pathogen

It was reported initially by Udey et al. (1976) that a novel anaerobic organism was capable of producing a neurological disease in striped mullet (*Mugil cephalus*). This conclusion resulted from an investigation into major fish mortalities in Biscayne Bay. The anaerobe was subsequently elevated to species status, as *Eubacterium tarantellus* (Udey et al. 1977), and then corrected to *Eu. tarantellae* (Trüper and de'Clari 1997). It seems likely that *Catenabacterium*, previously described as an

anaerobic pathogen of fish (Henley and Lewis 1976), may well be synonymous with *Eu. tarantellae* (Udey et al. 1977).

Box 2.2: *Eubacterium tarantellae*
On BHIA, the organism produces flat, translucent colonies, approximately 2–5 mm in diameter, which are colourless, rhizoidal and slight mucoid. These contain long, unbranched, filamentous, Gram-positive, asporogenous rods, which fragment into smaller bacilli of 1.3–1.6 × 1.0–17.0 µm. Good growth occurs at 25–37 °C. All isolates degrade blood (ß-haemolysis) and lecithin, but not aesculin, gelatin or starch. Catalase, H_2S and indole are not produced; nitrates are not reduced, and carbohydrates are generally not fermented. However, there is some evidence for the production of acid from fructose, glucose and lactose, but not from aesculin, amygdalin, arabinose, cellobiose, maltose, mannitol, mannose, melezitose, raffinose, rhamnose, salicin, starch, sucrose, trehalose or xylose.

Unfortunately, the G+C ratio of the DNA was not determined. From these phenotypic characteristics, Udey et al. (1977) proposed that the organism should be classified in a new species, as *Eubacterium tarantellus*. Clearly, the fish isolates possess the general characteristics of *Eubacterium*, i.e. Gram-positive anaerobic, asporogenous, chemo-organotrophic, non-motile, catalase-negative rods, which grow well at 37 °C (Moore and Holeman-Moore 1986). A comparison between the descriptions of *Eu. limosum* and *Eu. tarantellae* reveals that, among comparative tests, there are similarities. In fact, the only major differences concern hydrolysis of aesculin and acid production from lactose and mannitol.

Epizootiology

So far, the organism has only been recovered from the brain of mullet and ten other unnamed species of estuarine fish caught in Biscayne Bay and Florida Bay. It has not been found outside this area. Moreover, the inability to grow in 2% (w/v) sodium chloride implies that the organism is likely to be restricted to estuarine environments (Udey et al. 1977). These authors considered that isolates recovered from moribund fish, caught off the Texas coast and tentatively identified as *Catenabacterium* (Henley and Lewis 1976), also belong in *Eubacterium*, as *Eu. tarantellae*. Therefore, the range would appear to be restricted to the warmer waters of the southern part of the USA. It is uncertain whether or not the organism occurs in water, or indeed as part of the resident microflora of fish, although Trust et al. (1979) isolated eubacteria from the intestinal tract of three fish species. Therefore, it is conceivable that *Eu. tarantellae* could comprise part of the anaerobic microflora of the digestive tract, although it will be necessary for further study to clarify this point.

Pathogenicity

Invasion of the body may occur through wounds or as a result of damage inflicted through parasites, weak pathogens or stress. Once inside the body tissues, further damage may be inflicted as a result of exo- or endotoxins. The organism produces haemolysins and lecithinase, which may well harm fish. Nevertheless, it should be emphasised that the precise pathogenicity mechanisms have yet to be elucidated (Udey et al. 1976).

Disease Control

Use of Antimicrobial Compounds Udey et al. (1977) reported that isolates were sensitive to chloramphenicol, erythromycin, novobiocin, penicillin and tetracycline when examined by in vitro methods. It is possible that one or more of these compounds may be useful for chemoprophylaxis, but the value of any of these antimicrobial agents for chemotherapy is unproven. However, it seems likely that once the pathogen has entered brain tissue, antimicrobial compounds would probably not be able to reach the site of infection.

Lactic Acid Bacteria

Carnobacteriaceae Representative

Carnobacterium maltaromaticum and *Car. maltoromaticum*-Like Organisms

A total of 1286 whitefish *(Coregonus cupeaformis)* were recovered from the Great Lakes, USA between 2003 and 2006 and subjected to microbiological examination during which 23 carnobacterial isolates were recovered but pathogenicity not confirmed in laboratory challenge experiments. These organisms were equated with *Car. maltoaromaticum*-like organisms (Loch et al. 2008). Subsequent publications have reported *Car. maltoaromaticum*-like organisms from stranded juvenile salmon sharks *Lamna ditropis)* (Schaffer et al. 2013) and *Car. maltoaromaticum* from feral salmon and trout in the USA (Loch et al. 2011).

Characteristics of the Disease

The organisms were associated with thickening [hyperplasia] of the air bladder wall, splenomegaly, renal and splenic congestion, and accumulation of mucus exudate (Loch et al. 2008). The terms pseudokidney disease (Loch et al. 2011) and meningoencephalitis (Schaffer et al. 2013) have been used to describe the disease. Nephrocalcinosis was observed in some fish (Loch et al. 2011).

Isolation

Kidney and samples from external and internal lesions were inoculated onto TSA and cresol red thallium acetate sucrose inulin agar, which is selective for carnobacteria, with incubation at 22 °C for up to 72 h (Loch et al. 2008).

Characteristics of the Pathogen

Box 2.3: *Carnobacterium maltaromaticum*-Like Organisms
Whitish colonies on TSA comprise nonmotile, asporogenous, facultatively anaerobic Gram-positive rods of 1.0–1.5 × 0.5 μm in size arranged in pallisades, which produce arginine dihydrolase and ß-galactosidase but not catalase, H_2S or indole, lysine or ornithine decarboxylase, oxidase or phenylalanine deaminase, or reduce nitrates. Aesculin is degraded. The methyl red test is positive. Citrate and malonate are not utilised. Acid is produced from cellobiose, galactose, glucose, lactose, maltose, mannose, salicin, sucrose and trehalose, but not arabinose, adonitol, inositol, raffinose, rhamnose or xylose. Growth does not occur on acetate agar or MacConkey agar. Partial sequencing of the 16S rRNA gene revealed a 97 % homology with *Carnobacterium maltaromaticum* (Loch et al. 2008), which is below the threshold for a confirmed identification.

Scrutiny of the characteristics reveal phenotypic similarities to the organisms reported by Hiu et al. (1984), Baya et al. (1991), Starliper et al. (1992) and Toranzo et al. (1993). The taxonomic status of these putative carnobacteria remains to be firmly established. Subsequently, Loch et al. (2011) recovered 57 carnobacterial isolates from the kidney, spleen, swimbladder, and/or external ulcers on 51 trout and salmon broodstock in Michigan, USA. The isolates were phenotyped and the partial 16S rDNA gene sequenced with the result that the majority (29/30 isolates) were identified as *Car. maltoaromaticum,* and a solitary culture as *Car. divergens,* albeit with evidence of phenotypic and genotypic heterogeneity. In another study, Schaffer et al. (2013) sequenced the 16S rDNA gene of 7 isolates from 7 juvenile salmon sharks from the Californian coast, and recorded a 99 % homology to *Car. maltoaromaticum.* A 97 % homology value was scored using the large ribosomal DNA intergeneric spacer region (ISR).

Carnobacterium piscicola (and the Lactobacilli)

Lactic acid bacteria may be beneficial to fish as probiotics. Other representatives are pathogenic to fish. Certainly, there is confusion over the role of carnobacteria as fish pathogens.

Characteristics of the Disease

According to Ross and Toth (1974), the abdomen of moribund fish were distended because of the presence of ascitic fluid. However, it was readily admitted that mortalities could not be directly attributed to the lactobacillus "pathogen". The subsequent report of Cone (1982) indicated that the condition was stress mediated, insofar as it was recognised mostly in post-spawning fish. In these specimens, there was an accumulation of ascitic fluid in the peritoneal cavity, and extensive damage in the liver, kidney and spleen. Fin rot and other external signs of disease were absent. However, petechial haemorrhages in the muscle and hyperaemic air bladder were observed in some fish. The heart and gills appeared normal. According to Hiu et al. (1984), the disease occurred in fish >1 year old, which may have undergone stress, namely handling and spawning. Disease symptoms were varied, including septicaemia, the abdomen distended with ascitic fluid, muscle abscesses, blood blisters just beneath the skin, and internal haemorrhaging.

Isolation

The use of TSA or BHIA with incubation at 22–24 °C for 48 h has been advocated (Ross and Toth 1974; Cone 1982; Hiu et al. 1984).

Characteristics of the Pathogen

There have been only a few reports citing *Lactobacillus* spp. as fish pathogens. The initial work was by Ross and Toth (1974), who described mortalities among 3-year old rainbow trout in a hatchery in California. The term pseudokidney disease was coined by these workers to distinguish the condition from BKD. However, it is not clear what pathological significance, if any, could be attributed to the lactobacilli, because pure cultures were incapable of reproducing infection. Nevertheless, lactobacilli were again implicated in an infection of female 2–3 year old rainbow trout from a hatchery in Newfoundland, Canada (Cone 1982), but this was a mixed bacterial infection involving primarily lactobacilli and also *Aer. hydrophila, Ps. fluorescens* and Enterobacteriaceae representatives. There is no report about any pathogenicity experiments using the Canadian *Lactobacillus* strain; therefore, its precise role as a fish pathogen is open to question. Additional lactic-acid producing organisms have been isolated from the kidneys of moribund rainbow trout (B. Austin, unpublished data). Moreover, these isolates produced clinical disease upon intraperitoneal injection into salmonids. Similar isolates have been studied by Professor Stemke (personal communication).

Hiu et al. (1984)) proposed a new species, namely *Lactobacillus piscicola,* to accommodate a group of 17 isolates recovered from diseased chinook salmon, cutthroat trout and rainbow trout. Similar isolates were recovered from juvenile salmonids and carp in Belgium and France (Michel et al. 1986) and from striped bass and catfish in the U.S.A. (Baya et al. 1991). Then, *Lactobacillus piscicola* was reclassified as *Carnobacterium piscicola* (Collins et al. 1987). In addition, fish-

pathogenic lactobacilli, with similarities to *Lactobacillus alimentarius* and *Lactobacillus homohiochi,* have been recovered from post-spawning rainbow trout in the USA (Starliper et al. 1992).

Box 2.4: *Carnobacterium piscicola*
On TSA, *Car. piscicola* produces small round, entire, shiny, opaque colonies that develop within 48 h. These contain non-motile, non-acid-fast, Gram-positive fermentative cocco-bacilli or rods of approximately 1.1–1.4 × 0.5–0.6 μm in size. Other phenotypic traits of the lactobacilli include the inability to produce catalase (catalase-positive isolates were described by Starliper et al. 1992), H_2S, indole, gelatinase, urease, the Voges Proskauer reaction, arginine hydrolysis, or lysine or ornithine decarboxylases or reduce nitrates. However, the isolates produce acid from fructose, galactose, glucose, glycerol, inulin, lactose, maltose, mannitol, melibiose, salicin, starch, sucrose and trehalose, but not raffinose, sorbitol or xylose (Schmidtke and Carson 1994). There are a few differences between the Californian and Canadian isolates, namely hydrolysis of aesculin, and acid production from lactose. Growth occurs at 10 °C but not 40 °C, in 6.5 % (w/v) sodium chloride, and at pH 9.6.

In most respects, all the isolates bore similarities, and by comparison of the phenotypic traits with conventional identification schemes, it is apparent that an identification of *Lactobacillus* or *Streptococcus* could result. Furthermore, the photomicrographs of cells, published by Ross and Toth (1974), could be interpreted as chains of cocci rather than short rods, which would be more in keeping with an identification of *Streptococcus.* The isolates recovered from England and Canada, by Austin and Stemke, respectively, possessed more of the characteristics attributable to *Lactobacillus* or the related genera. Cultures comprised Gram-positive, non-motile, fermentative rods of approximately 3.0 × 1.0 μm in size, forming round, raised entire white colonies on TSA after incubation for 48 h at 20 °C. Growth occurred at 4–26 °C but not 37 °C, and in 2.5 % but not 7.5 % (w/v) sodium chloride. A resemblance to *Car. piscicola* is readily apparent. Indeed, the only differences reflect growth at 37 °C and acid production from lactose. Both the Canadian isolates of Stemke and the US isolates of Hiu et al. (1984) and Starliper et al. (1992) produced lactic acid from the fermentation reactions. It is possible that these lactobacilli may all belong in the same taxon, i.e. *Car. piscicola*, but it will be necessary for further study to determine the true relationship of the English and Canadian isolates to those of Hiu et al. (1984).

The cell wall peptidoglycan of *Car. piscicola* was found to comprise diaminopimelic acid, alanine and glutamic acid, but no lysine. DNA:DNA hybridisation revealed negligible, i.e. 10 %, homology with the reference cultures of *Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus jensenii, Lactobacillus salivarius* and *Lactobacillus yamanashiensis,* compared to 70 % re-association between isolates of *Car. piscicola* (Hiu et al. 1984. Thus in their publication, it was concluded that the fish pathogens were most closely related to *Lactobacillus yamanashiensis* subsp. *yamanashiensis* in terms of G + C ratio of the DNA and

fermentation profile. Subsequently, Collins et al. (1987) demonstrated closer relationships with other carnobacteria.

Epizootiology

It would appear that the disease is confined to Europe and North America. However, it is unclear whether fish are the natural hosts for *Car. piscicola* and other lactobacilli, or if they comprise part of the natural aquatic microflora.

Pathogenicity

Small-scale experiments with rainbow trout maintained in fresh water at 18 °C have shown that death may result within 14 days of i.p. injection of 10^5 cells/fish. Dead and moribund fish had swollen kidneys, and ascitic fluid accumulated in the abdominal cavity. However, adverse effects were not recorded following injection of cell-free extracts. This suggests that exotoxins did not exert a significant role in pathogenicity. It remains for further work to elucidate the effect, if any, of endotoxins (Ross and Toth 1974; Cone 1982; Hiu et al. 1984.

Disease Control

Antimicrobial Compounds In vitro methods have shown that isolates are sensitive to ampicillin, cephaloridine, chloramphenicol, furazolidone and tetracycline, but not to erythromycin, novobiocin, streptomycin, sulphamerazine or sulphamethoxazole (Michel et al. 1986; B. Austin, unpublished data). Unfortunately, these antimicrobial compounds have not been evaluated in fish.

Leuconostocacceae Representative

Weissella sp.

Characteristics of the Disease

Liu et al. (2009) reported *Weissella* as the causative agent of a disease in rainbow trout culture in China. Subsequently, a similar organism has been associated with haemorrhagic septicaemia of rainbow trout farmed in Brazil during 2008 and 2009 when there was increased water temperature (≥17 °C). Disease signs included anorexia, lethargy, ascites, exophthalmia and hemorrhages in/on the eyes, mouth, oral cavity and tongue (Figueiredo et al. 2012). Weissellosis was recognised during 2011 in rainbow trout in the USA with disease signs including darkening of the skin, lethargy, bilateral exophthalmia, corneal opacity, ocular haemorrhaging and occasional corneal rupture, and possibly cerebral haemorrhaging (Welch and Good 2013).

Isolation

Swabbed material of ascites, brain, kidney and liver were inoculated onto sheep blood agar with incubation at 25 °C for 72 h (Figueiredo et al. 2012).

Characteristics of the Pathogen

Seventy seven isolates were obtained and equated with *Weissella* by phenotyping and by sequencing of the 16S rRNA gene. The US isolates were 99 % homologous with those from China and Brazil (Welch and Good 2013).

Box 2.5: *Weissella* sp.
Colonies contain Gram-positive cocci, that do not produce catalase or oxidase, and are variable for α-haemolysis. Growth occurs over a wide range of temperatures (20–45 °C), but not on MacConkey agar. Acid is produced from D-maltose, pullulan, D-ribose and D-trehalose, but not from D-arabitol, α-cyclodextrin, glycogen, D-lactose, D-mannitol, D-melibiose, D-melezitose, D-saccharose, D-sorbitol or D-tagatose. The Voges Proskauer reaction is positive. Aesculin and sodium hippurate (variable result) are attacked. L-arginine is hydrolysed by some isolates. Pyruvate, 2-naphthyl-ß-D-galactopyranoside, L-leucyl-2-naphthylamide, naphthol α-D-glucuronate, 2-naphthylphosphate and pyrrolidonyl-2-naphthylamide were not metabolised. There is a >98 % 16S rRNA sequence homology with *Weissella* (Figueiredo et al. 2012).

Epizootiology

Figueiredo et al. (2012) considered that transmission occurred through water.

Pathogenicity

Isolates infected fish in laboratory-based challenge experiments of rainbow trout involving use of i.m. and i.p. injection and immersion (Figueiredo et al. 2012).

Control

Vaccine Formalised whole cells were protective when administered by injection; the RPS was reported 87.5 % and 85 % at 38 and 72 days after vaccination, respectively (Welch and Good 2013). Moreover, the preparation could be combined successfully as a bivalent product with ERM antigens (Welch and Good 2013).

Weissella ceti

Characteristics of the Disease

Weissellosis has been described in rainbow trout, and between 2010 and 2012 was reported to occur on five farms in Brazil, with disease signs of haemorrhagic septicaemia (Costa et al. 2015). In addition, the disease has been reported in China and the USA, and caused substantive mortalities in market-sized fish (Snyder et al. 2015).

Isolation

Diseased tissues were inoculated onto Columbia blood agar with incubation at 37 °C for 24 h (Vela et al. 2011). Costa et al. *(*2015) inoculated brain, eye, kidney and liver tissue were inoculated onto 5 % (v/v) sheep blood agar and MRS agar with incubation at 25 °C for 48 h

Characteristics of the Pathogen

Box 2.6: *Weissella ceti*
Cultures on blood agar are 0.75–1.0 mm in diameter, nonpigmented and α -haemolytic. The cultures contain Gram-positive facultatively anaerobic, nonmotile asporogenous coccoid rods occurring singly or in pairs. Growth occurs at 22–37 °C, but not at 15 or 42 °C, and in 3–6.5 % (w/v) sodium chloride, and not at pH 3.9. Alkaline and acid phosphatases, naphthol-AS-BI-phosphohydrolase and pyrazinamidase, but not N-acetyl-ß-glucosaminidase, catalase, α-chymotrypsin, cysteine arylamidase, esterase (C4), ester lipase (C8), α-fucosidase, α- or ß-galactosidase, α- or ß-glucosidase, ß-glucuronidase, lipase (C14), leucine arylamidase, α- or ß-mannosidase, trypsin or valine arylamidase is produced. Acid is produced from N-acetylglucosamine, D-glucose, maltose, D ribose trehalose, but not from D-adonitol, aesculin, amygdalin, L- or D-arabinose, L- or D-arabitol, arbutin, cellobioise, dulcitol, erythritol, D-fructose, D-galactose, gentiobiose, glycerol, glycogen, inositol, inulin, lactose, D-mannitol, D-mannose, melezitose, melibiose, methyl α-D-glucopyranoside, 2- or 5-ketogluconate, raffinose, L-rhamnose, salicin, D-sorbitol, starch, sucrose or L- or D-xylose. Nitrates are not reduced. The Voges Proskauer reaction is positive. Aesculin is degraded, but not gelatin, sodium hippurate or urea. The G+C ratio of the DNA is 39.2 mol% (Vela et al. 2011). Thirty-four isolates from Brazil were studied genotypically, and determined to be homogeneous by REP-PCR, enterobacterial repetitive intergenic consensus sequences PCR and pulsed field gel electrophoresis. Sequencing of the 16S rRNA gene revealed 99 % homology with *Weissella ceti* in the GenBank data base (Costa et al. 2015).

Diagnosis

A duplex PCR identified the pathogen in culture and diseased brain and spleen tissues (Snyder et al. 2015).

Disease Control

Vaccine Whole-cell formalin-inactivated vaccine was formulated with and without Montanide ISA 763 adjuvant, and 0.1 ml volumes were injected i.p. into rainbow trout, with challenge at 30 and 60 days after vaccination. The oil adjuvanted vaccine achieved the better RPS of 92 % at 30 and 60 days after vaccination. The corresponding non-adjuvanted vaccine achieved RPS values of 67 % and 58 % after 30 and 60 days, respectively (Costa et al. 2015).

Gram-Positive Cocci in Chains: The Early Literature

General Comments

Streptococcicosis (= streptococcosis) was initially described among populations of rainbow trout farmed in Japan (Hoshina et al. 1958). Since then, the disease has increased in importance, with outbreaks occurring in numerous fish species including yellowtails (Kusuda et al. 1976; Kitao et al. 1979), coho salmon (Atsuta et al. 1990), Jacopever *(Sebastes schlegeli)* (Sakai et al. 1986), Japanese eels (Kusuda et al. 1978), ayu and tilapia (Kitao et al. 1981). The disease, also known as 'popeye', has assumed importance in rainbow trout farms in Australia, Israel, Italy and South Africa (Barham et al. 1979; Boomker et al. 1979; Carson and Munday 1990; Ceschia et al. 1992; B. Austin, unpublished data) and in Atlantic croaker *(Micropogon undulatus),* blue fish *(Pomatomus saltatrix),* channel catfish, golden shiner *(Notemigonous chrysoleuca),* hardhead (sea) catfish *(Arius felis),* menhaden *(Brevoortia patronus),* pinfish (*Lagodon rhomboides),* sea trout *(Cynoscion regalis),* silver trout *(Cynoscion nothus),* spot *(Leiostomus xanthurus),* stingray *(Dasyatis* sp.), striped bass *(Morone saxatilis)* and striped mullet *(Mugil cephalus)* in the USA (Robinson and Meyer 1966; Plumb et al. 1974; Cook and Lofton 1975; Baya et al. 1990). There is good evidence that streptococcicosis is problematical in both farmed and wild fish stocks.

Since the first publication in 1958, there has been considerable confusion about the number of and the nature of the bacterial species involved in the disease. Thus at various times, the fish pathogenic streptococci have been linked with *Str. agalactiae, Str. difficilis, Str. dysgalactiae, Str. equi, Str. equisimilis, Str. (= En.) faecium, Str. ictaluri, Str. iniae, Str. milleri, Str. parauberis, Str. phocae, Str. pyogenes* and *Str. zooepidemicus.* In addition, we have found that *Enterococcus faecalis* NCTC 775^{T}, *En. faecium* NCTC 7171^{T}, *Lactococcus lactis* NCFB 604, *Str. mutans* NCFB 2062

will cause similar diseases in Atlantic salmon and rainbow trout. Also, *En. faecalis* and *En. faecium* have been recovered from diseased rainbow trout in South Africa (Bekker et al. 2011), and *En. faecalis* from farmed fish in Taiwan (Young et al. 2012a). Certain traits of the causal agent(s) have been repeatedly emphasised as having supposedly taxonomic significance. In particular, the ability to attack blood has been highlighted. Thus fish pathogenic strains have been described, at one time or another, as either α- (Kusuda et al. 1976; Al-Harbi 1994) or ß-haemolytic (Robinson and Meyer 1966; Minami et al. 1979; Kitao et al. 1981; Ugajin 1981; Iida et al. 1986) or as non-haemolytic (Plumb et al. 1974; Cook and Lofton 1975; Iida et al. 1986). Superficially, this information could infer heterogeneity among the pathogens, although some well-established taxa, e.g. *Str. agalactiae,* contain both α - and ß-haemolytic strains). Nevertheless, many characteristics are shared by many of the fish pathogens. Yet, there is also some variance in the overall descriptions reported by different groups of workers. For example, Boomker et al. (1979) reported that isolates, recovered from the Transvaal in South Africa, grew on MacConkey agar and at 45 °C, hydrolysed sodium hippurate, and produced acid from a range of carbohydrates, including galactose, glucose, lactose, maltose, salicin, starch and trehalose, but not from arabinose, glycerol, inulin, mannitol, raffinose, sorbitol, sucrose or xylose. In contrast, Japanese isolates did not grow at 45 °C or hydrolyse sodium hippurate (Minami et al. 1979; Kitao et al. 1981; Ugajin 1981). Of course, such differences may reflect the lack of standardisation in the testing regimes or, indeed, point to heterogeneity in the species composition of the organisms.

A comparison of the characteristics of fish pathogenic streptococci and lactobacilli with the results of the comprehensive taxonomy study by Bridge and Sneath (1983) revealed that the isolates, described by Hoshina et al. (1958), Robinson and Meyer (1966), Boomker et al. (1979), Minami et al. (1979), Cone (1982) and Kitao (1982a), approximated to *En. faecalis, Str. equinus, Str. lactis, Str. casseliflavus,* pediococci and the 'aerococcal' group, respectively. Subsequently, some of these taxa have been re-classified in the genus *Enterococcus*. However, the organisms recovered by Kusuda et al. (1976), Onishi and Shiro (1978), Minami (1979), Kitao et al. (1981) and Ugajin (1981) did not match the descriptions of any of the 28 phena defined by Bridge and Sneath (1983).

With such information, it could readily be assumed from the early literature that streptococcicosis is a syndrome caused by more than one species. To some extent, geographical differences have been implied. For example, the South African isolates of Boomker et al. (1979) have been described as comprising unidentified Lancefield Group D *Streptococcus*; Japanese isolates linked with, but not identical to, *En. faecalis* and *En. faecium* [note: the type strain of *En. faecium* has been determined to be pathogenic to salmonids in laboratory-based infectivity experiments; Austin, unpublished data]*;* whereas American strains approximate to the description of *Str. agalactiae* (Kusuda and Komatsu 1978). It is interesting that isolates from cases of streptococcicosis in rainbow trout farmed in Italy were originally linked with *En. faecalis* and *En. faecium* (Ghittino and Pearo 1992; 1993) before taxonomic re-appraisal, as indicated below.

Serology, although indicating a multiplicity of serotypes, has confirmed that the fish pathogens are indeed *bona fide* representatives of *Streptococcus/Enterococcus*. Thereafter, serological techniques have not improved the understanding of the precise taxonomic status of the strains. The organisms described by Cook and Lofton (1975) and considered as identical to those of Plumb et al. (1974) were identified as Group B type 1_b *Streptococcus* by the CDC, Atlanta. Also, Baya et al. (1991) identified their isolates as Group B. However, Boomker et al. (1979) regarded the isolates as Group D. To further complicate the issue, Kitao et al. (1981) reported a new serotype in Japan, which did not react with specific antisera to Lancefield groups A, B, C, D, E, F, G, H, K, L, N, O and MG; this conclusion was also reached by Kitao (1982a) and Kusuda et al. (1982). The confusion was only resolved when a proper speciation of fish pathogenic streptococci began to be developed and has continued until now with the recognition of some new species, e.g. *Str. phocae.*

Isolation

Recovery is usually straightforward, involving use of bovine blood tryptose agar (Naudé 1975; Roode 1977; Boomker et al. 1979), Columbia agar (Appendix in Chap. 12), 5 % (v/v) defibrinated sheep blood agar (Doménech et al. 1996), Todd-Hewitt broth (Appendix in Chap. 12), Todd-Hewitt agar (Nomoto et al. 2004), nutrient agar supplemented with rabbit blood (Kitao et al. 1981), TSA (Teskeredzic et al. 1993; Michel et al. 1997), 10 % (v/v) horse blood in Columbia agar (Oxoid), 10 % (v/v) horse serum in Columbia agar (Austin and Robertson 1993), yeast extract glucose agar (Appendix in Chap. 12; Michel et al. 1997) or BHIA (Minami et al. 1979; Ugajin 1981; Kusuda et al. 1991; Eldar et al. 1994). Media may be supplemented with 1 % (w/v) sodium chloride (Austin and Robertson 1993). Media should be inoculated with diseased tissue, notably from the kidney, and incubated at 15–37 °C for up to 7 days (usually 48 or 72 h) when 'dull grey' colonies approximately 1–2 mm in diameter develop. These colonies contain cocci in chains. It must be emphasised that an incubation temperature of 37 °C is in excess of the normal growth temperature of many fish species, notably salmonids. This indicates that the organisms may well have been derived from warm-blooded animals, and may, therefore, constitute a public health risk.

Epizootiology

By scrutiny of the early literature, there is evidence that the pathogens abound throughout the year in the aquatic environment, occurring in water, mud and in the vicinity of fish pens (Kitao et al. 1979). Some seasonality has been recorded, with higher numbers present in seawater during summer. In contrast, greatest numbers were isolated in mud during autumn and winter (Kitao et al. 1979). This is interesting, but unfortunately the authors did not comment further about the reasons for the

presence of streptococci in the aquatic environment. Conceivably, the organisms may have been released from infected fish and were being merely retained in the water and underlying sediment. Alternatively with the inconclusive taxonomic status of the fish pathogenic streptococci at the time, it would be difficult to conclude that any environmental isolates correspond precisely to the description of the fish pathogens. Therefore, any environmental isolates could be merely indicators of an unsanitary condition and not necessarily imply the presence of fish pathogenic strains. However, this evades the question about the precise source of infection. Minami (1979) determined that streptococci, with similarities to the fish pathogens, were present in fresh and frozen fish used for yellowtail diets. This worker reported that the isolates were pathogenic, and could survive for over 6 months in the frozen state. The suggestion was made, therefore, that the contaminated diets served as an important source of infection. The importance of food-borne infection was further highlighted by Taniguchi (1982a, b, 1983).

It is recognised that streptococcicosis may be transmitted by contact with infected fish. In this context, Robinson and Meyer (1966) transmitted the disease by cohabiting an infected golden shiner with healthy specimens of the same species. The healthy fish succumbed to streptococcicosis, and died within 5 days. Some host specificity to Gram-positive cocci in chains exists, insofar as trout suffer heavy mortalities whereas Mozambique bream (*Sarotherodon mossambicus),* banded bream (*Tilapia sparramanii),* carp (*Cyprinus carpio)* and largemouth bass (*Micropterus salmoides)* do not (Boomker et al. 1979). It has been established that challenge with low-virulence isolates or low doses of high-virulence isolates together with cell-free culture supernatants are sufficient to establish infection (Kimura and Kusuda 1979). The toxic activity of supernatants was further researched, and two fractions were demonstrated to have a significant effect on pathogenicity (Kimura and Kusuda 1982). These were recovered in Todd-Hewitt broth after incubation at 30 °C for 48 h. The fraction, although not toxic by oral administration (presumably the compounds were digested), produced damage, i.e. exophthalmia and petechial haemorrhages, following percutaneous injection of yellowtails.

Development of Vaccines

A formalised suspension of ß-haemolytic *Streptococcus* was successful when applied to rainbow trout by immersion or by injection with or without FCA (Sakai et al. 1987; 1989). A RPS of 70 % was achieved, which was superior to the results of Iida et al. (1982). Yet, only low titres of agglutinating antibody occurred in fish vaccinated by injection. Conversely, antibodies were not detected in trout, which were vaccinated by immersion (Sakai et al. 1987; 1989). A toxoid enriched whole cell *Enterococcus* vaccine, administered to turbot by i.p injection and immersion, gave long term protection, with RPS of 89–100 % and 67–86 % recorded for 45 g and 150 g fish, respectively (Toranzo et al. 1995).

Use of Inhibitory Compounds

Erythromycin, dosed at 25 mg/kg body weight of fish/day for 4–7 days, controlled streptococcicosis in yellowtail (Kitao 1982b), and worked better than oxytetracycline or ampicillin (Shiomitsu et al. 1980). Doxycycline, at 20 mg/kg body weight of fish/day for an unspecified duration (Nakamura 1982), and josamycin, dosed at 30 mg/kg body weight of fish/day for 3 days (or dosed at 20 mg/kg body weight of fish/day for 5 days) (Kusuda and Takemaru 1987; Takemaru and Kusuda 1988) have also been advocated. It is of particular interest that a novel fisheries therapeutant, namely sodium nifurstyrenate dosed at 50 mg/kg body weight of fish/day for 3–5 days (Kashiwagi et al. 1977a,b), has found use for streptococcicosis. This drug appears to be particularly effective, and should not have problems with plasmid-mediated resistance. The benefits of ionophores, namely lasalocid, monensin, narasin and salinomycin, were reported for an *Enterococcus*-like pathogen of rainbow trout in Australia. Following a comparison of 40 isolates, it was noted that the MIC of the ionophores was markedly less than erythromycin (0.1–0.8 μg/ml). Thus, the MIC for lasalocid, monensin, narasin and salinomycin were 0.8 μg/ml, 0.4–1.5 μg/ml, 0.2–04 μg/ml and 0.4–0.8 μg/ml, respectively. Perhaps, there are future opportunities for the use of some of these compounds in aquaculture.

The allocation of species names has sought to clarify the understanding of the disease, and the following narrative will deal with the individual species.

Enterococcaceae Representatives

Enterococcus (Streptococcus) faecalis subsp. *liquefaciens*

Characteristics of the Disease

Although a question mark remains over the accuracy of the identification of this pathogen, its inclusion is justified for the sake of completion. Also, this is the first indication of streptococci as fish pathogens in Croatia. Farmed brown bullhead *(Amiurus nebulosus)* developed deep ulcers predominantly between the dorsal and caudal fins, and there was haemorrhaging at the anus. Internal organs displayed unspecified changes, and fluid was present in the digestive tract. Gram-positive cocci were observed in the kidney and liver (Teskeredzic et al. 1993).

Characteristics of the Pathogen

We are not satisfied with the identification of these isolates. However, in the absence of an alternative, details from the original publication are included here (Teskeredzic et al. 1993):

Box 2.7: *Enterococcus faecalis* subsp. *liquefaciens*
Yellow colonies of 1–2 mm in diameter are obtained from kidney and liver on TSA. Colonies comprise Gram-positive cocci, which do not produce catalase, H_2S or indole and are negative for the methyl red test and the Voges Proskauer reaction. Nitrates are reduced. DNA and gelatin are attacked. Citrate is utilised.

Although there are insufficient data for a meaningful comparison with other taxa, it is interesting to note that streptococci are normally associated with the production of white colonies.

Vagococcus salmoninarum

Characteristics of the Disease

In France, the organism was attributed to significant losses, i.e. up to 50 % in a year, in rainbow trout farmed at low water temperature (Michel et al. 1997). Disease signs included listless behaviour, impaired swimming, unilateral exophthalmia, external haemorrhages, petechial haemorrhages on the gills, and enlarged liver and spleen (Michel et al. 1997) and peritonitis (Schmidtke and Carson 1994). Subsequently in rainbow trout farmed in Turkey, the organism was associated with ~55 % mortalities, and disease signs including anorexia, lethargy, darkened pigmentation, exophthalmia, disruption of the eyeball, boils, erosion on the side of the body, haemorrhaging in the jaw, mouth, abdomen and anus, and prolapse of the anus Didinen et al. 2011). The pathogen was detected in the heart and kidney of diseased rainbow trout Cagatay and Gumus 2014)).

Isolation

Schmidtke and Carson (1994) used Oxoid blood agar base supplemented with 7 % (v/v) defibrinated sheep blood with incubation at 25 °C for 48 h to recover cultures from brain, kidney, peritoneum, spleen and testes.

Characteristics of the Pathogen

In 1968, a so-called lactobacillus was recovered from diseased adult rainbow trout in Oregon, USA and later subjected to detailed taxonomic examination. This isolate, designated OS1-68^T, has become the type strain of *Vagococcus salmoninarum*

(Wallbanks et al. 1990). Further isolates have been studied by Schmidtke and Carson (1994).

Box 2.8: *Vagococcus salmoninarum*
Cultures comprise short or oval non-motile, facultatively anaerobic Gram-positive rods, which produce H_2S but not arginine dihydrolase or catalase. Aesculin and blood (α -haemolysis), but not sodium hippurate or urea, are degraded. Nitrates are not reduced. The Voges Proskauer reaction is negative. Acid is produced from amygdalin, arbutin, N-acetylglucosamine, cellobiose, fructose, ß-gentiobiose, glucose (gas is not produced), maltose, mannose, α-methyl-D-glucoside, ribose, salicin, starch, sucrose, D-tagatose and trehalose, but not from D or L-arabinose, D or L-arabitol, adonitol, dulcitol, erythritol, D or L fucose, galactose, gluconate, glycogen, glycerol, 2 or 5-keto-gluconate, inulin, inositol, lactose, D-lyxose, melibiose, melezitose, methyl-xyloside, methyl-D-mannoside, mannitol, rhamnose, raffinose, sorbose, sorbitol, D-turanose, D or L-xylose or xylitol. Growth occurs at 5–30 °C but not 40 °C, and at pH 9.6. The major cellular fatty acids are of the straight-chain saturated and mono-unsaturated types. The G+C ratio of the DNA is 36.0–36.5 mol %.

Based on an examination of only one culture of *Vag. salmoninarum*, it was established that there was 96.3 % homology with *Vag. salmoninarum* in the 1,340-nucleotide region of the 16S rRNA. Slightly lower homology values of 94.5 %, 94.1 %, 94.0 %, 93.8 % and 93.7 % were obtained with *En. durans, Car. divergens, En. avium, Car. piscicola* and *Car. mobile,* respectively. Despite the very high similarity to *Vag. fluvialis*, strain 051–68^T was described in a new species, as *Vagococcus salmoninarum.* It remains for the examination of further isolates to determine the level of genetic variability within the taxon.

Pathogenicity

Laboratory infections with *Vag. salmoninarum* were achieved using a comparatively high dose of 1.8×10^6 cells/rainbow trout (Michel et al. 1997).

Streptococcaceae Representatives

Lactococcus garvieae (= Enterococcus seriolicida)

Characteristics of the Disease

Infection of ayu with this pathogen may be exacerbated by prior infection/infestation with other organisms, such as blood flukes, that may weaken the host (Kumon et al. 2002). Internal signs of disease were absent in golden shiners, although raised lesions were apparent on the body surface (Robinson and Meyer 1966). Yellowtails were damaged in the liver, kidney, spleen and intestine, and there was a concomitant accumulation of ascitic fluid in the peritoneal cavity (Kusuda et al. 1976; 1991; Ugajin 1981). In rainbow trout, the disease was of sudden onset, and was described as a hyperacute systemic disease (Eldar and Ghittino 1999); haemorrhaging in the eye may be observed (Fig. 2.1). Marine fish showed pronounced enteritis, pale livers and blood in the peritoneal cavity, although the kidneys were apparently unaffected (Plumb et al. 1974). The pathogen appears to be spreading, and has been detected in wild Red Sea wrasse *Coris aygula* (Colorni et al. 2003) and in Brazilian Nile tilapia and pintado (*Pseudoplathystoma corruscans*) (Evans et al. 2009). Sequencing the 16S rDNA gene confirmed the presence of *Lactococcus garvieae* in Taiwan (Chen et al. 2002). *Lactococcus garvieae* (*Vibrio* spp. were also present in diseased fish) has been associated with green liver syndrome in cage-reared red lip mullet (*Chelon haemotocheilus*) in Korea (Han et al. 2015).

Fig. 2.1 A rainbow trout displaying haemorrhaging in the eye caused by infection with *Lactococcus garvieae* (Photograph courtesy of Dr. J.W. Brunt)

Isolation

LG agar was developed as a selective medium for *Lactococcus garvieae* that differentiated the virulent capsulated from the non-virulent uncapsulated cultures. The medium exploited the ability of the pathogen to grow in high concentrations of bile salts and potassium tellurite, and to reduce tetrazolium dye to coloured formazans. Thus, noncapsulated cultured produced white colonies without red halos whereas capsulated *Lactococcus garvieae* cultures comprised black colonies with red halos (Chang et al. 2014).

Characteristics of the Pathogen

The first attempt at clarifying the taxonomic status of the causal agents of streptococcicosis/streptococcosis was the landmark publication of Kusuda et al. (1991), who described a new species, i.e. *Enterococcus seriolicida,* to accommodate 12 isolates recovered from eels and yellowtail in Japan.

Box 2.9: *Lactococcus garvieae*
Cultures comprise non-motile facultatively anaerobic Gram-positive cocci in short chains, which do not produce catalase, H_2S, indole or oxidase. Blood is degraded (α-haemolysis). Aesculin and arginine are hydrolysed, but not so casein, gelatin or sodium hippurate. Acid is produced from a wide range of carbohydrates, namely aesculin, cellobiose, D-fructose, galactose, D-glucose, maltose, mannitol, D-mannose, salicin, sorbitol and trehalose, but not from adonitol, D-arabinose, glycerol, glycogen, inositol, lactose, melezitose, melibiose, raffinose, L-rhamnose, starch, sucrose or D-xylose. The methyl red and tetrazolium reduction tests and the Voges Proskauer reaction are positive, but not nitrate reduction. Growth occurs at 10–45 °C but not 50 °C, in 0–6.5 % (w/v) sodium chloride, and at pH 4.5–9.6. The G+C ratio of the DNA is 44 mols % [G+C ratio was quoted as 38 % for strain 8831 in a publication describing the genome sequence; Aguado-Urda et al. 2011]. The organisms do not belong to Lancefield groups, A, B, C, D, E, F, G, H, K, L, M, N or O.

Interestingly in the original publication describing *En. seriolicida*, only low DNA homology values were obtained with reference species of *Enterococcus.* Indeed, the greatest DNA homology, i.e. 24 %, was with *En. hirae* (Kusuda et al. 1991). Perhaps, it was inevitable that the association of these fish pathogens with *Enterococcus* would be challenged. Thus, it was determined that *En. seriolicida* was really identical (77 % DNA:DNA homology) with a previously described lactococcus, namely *Lactococcus garvieae* (Tiexeira et al. 1996). This view has been reinforced by others. For example, Pot et al. (1996) detailed research by SDS-PAGE of

whole cell proteins, concluding that *En. seriolicida* was closely related to *Lactococcus garvieae.* Also, Eldar et al. (1996) reached the same conclusion after studying the type strains phenotypically and by DNA:DNA hybridisation. The taxon is certainly homogeneous (Kawanishi et al. 2006), as verified by RFLP (Eyngor et al. 2004), although three groupings along geographical lines were recognised by RAPD, the outcome of which should have value for epizootiology (Ravelo et al. 2003). However, *Lactococcus garvieae* appears to be similar in terms of phenetic data, to *Lactococcus lactis* (Zlotkin et al. 1998). This similarity could result in mis-identification of fresh isolates.

Cultures were recovered from diseased yellowtail in Japan and although regarded as *Lactococcus garvieae* did not agglutinate with antiserum raised against capsulated cells (Oinaka et al. 2015). Such isolates could confuse diagnosticians, and lead to mis-diagnosis.

Diagnosis

Phenotypic Methods Presumptive identification of *Lactococcus garvieae*-like organisms has been made following growth on bile (40%)-aesculin agar (see Facklam and Moody 1970), with hydrolysis of aesculin and by the characteristic growth on eosin-methylene blue agar (lactose is not fermented).

Molecular Methods A PCR using a 1100 bp fragment has distinguished *Lactococcus garvieae* from *Lactococcus lactis* (Zlotkin et al. 1998). In terms of sensitivity, the PCR detected *Lactococcus garvieae* in 1 μl of fish plasma. Another publication reported a PCR based on a 709 bp fragment that was specific for *Lactococcus garvieae,* enabling a positive result to be obtained in 4 h (Aoki et al. 2000). Two primers, namely ITSLg30F and ITSLg319R, from the sequence in the 16S-23S ITS region and incorporated into a PCR reportedly gave improved specificity (Dang et al. 2012).

Multiplex PCR A multiplex PCR has been developed, and successfully recognised the fish pathogenic lactococci-streptococci, i.e. *Lactococcus garvieae, Str. difficilis (= Str. agalactiae), Str. iniae* and *Str. parauberis* from culture and fish tissues with a sensitivity for the purified DNA of 30 pg, 12.5 pg, 25 pg and 50 pg, respectively (Mata et al. 2004). The value of multiplex PCR was reinforced by Itsaro et al. (2013), who distinguished *Lactococcus garvieae* (sensitivity = 19.53 pg of DNA) from *Str. galactiae* and *Str. iniae.*

RT-PCR RT-PCR proved successful for the recognition of *Lactococcus garvieae* with a detection limited of 32 fg (Jung et al. 2010).

LAMP-PCR A LAMP PCR was specific and sensitive, detecting 300 CFU, which was considered to be 100-fold more sensitive than conventional PCR (Tsai et al. 2013a).

Epizootiology

Bacteriophages of *Lactococcus garvieae* have been found in seawater and sediment, but after defining 14 phage types (among 111 isolates), it was concluded that there was not any correlation between phage type and geographical source of the isolates (Park et al. 1998).

Pathogenicity

Experimental infections with organisms likely to correspond with *Lactococcus garvieae* have been achieved by injection of 10^4 to 10^5 cells (Cook and Lofton 1975), and by exposure of fish for 10 min to 10^6 bacteria (Robinson and Meyer 1966). Cells have been found in the spleen within 2-hours of infection (Young et al. 2012a). Thereafter, disease becomes established, and death ensues. Adherence of cells of *Lactococcus garvieae* to intestinal and brain gangliosides has been documented in yellowtail (Shima et al. 2006). Then, the pathogen becomes internalised and survives within the phagosomes of macrophages (Young et al. 2012a). Questions have been asked about the genes of *Lactococcus garvieae* that are needed for survival in fish, and as a result of signature-tagged mutagenesis, mutants were found which could not be recovered from rainbow trout after challenge. Sequence analysis pointed to roles including the pathogenesis of transcriptional regulatory proteins homologous to GidA and MerR, metabolic enzymes asparagine synthetase A and α–acetolactate synthase, the ABC transport system of glutamine, the calcium-transporting ATPase, and the *dltA* locus involved in alanylation of teichoic acid (Menéndez et al. 2007).

Capsules have been reported, with encapsulated cultures being more virulent (Barnes et al. 2002) and less efficient at fixing complement compared to non-encapsulated isolates (Barnes and Ellis 2004). Non-encapsulated cultures were more susceptible to normal rainbow trout serum than capsulated isolates (Barnes et al. 2002). Two capsular types have been found among *Lactococcus garvieae,* one of which produces a well developed capsule, whereas the second demonstrates a micro-capsule which contains fimbrial-type components projecting from the cell surface (Ooyama et al. 2002).

Disease Control

Vaccines Using formalin-inactivated capsulated and uncapsulated cells of *Lactococcus garvieae,* which were applied to yellowtail by i.p. injection, long-term protection resulted with challenge with a capsulated culture (Ooyama et al. 1999). Because of differences in antibody response (to uncapsulated but not capsulated cultures), these workers concluded that the capsule affected immunogenicity, and the protective antigens were most likely to be on the surface of uncapsulated cells

and not in the capsule. It was noted that capsulated cells became phagocytosed, and fimbrial-like appendages were seen in the cells after treatment with immune serum (Ooyama et al. 1999).

Bivalent formalin-inactivated whole cell preparations containing *Lactococcus garvieae* and *Aer. hydrophila* with and without Montanide ISA-763 as adjuvant were administered by intraperitoneal injection (0.1 ml amounts containing 1×10^8 cells/fish) to rainbow trout, and challenged after 30 days with a RPS for *Lactococcus garvieae* of 100 % and 95.3 % for non adjuvanted and adjuvanted preparations, respectively. At 90 days after vaccination, challenge resulted in decreased protection doe the non-adjuvanted preparation against *Lactococcus garvieae* (RPS = 76.2 %) but not so for the adjuvanted product (RPS = 90 %) (Bastardo et al. 2012).

The gene for the 40 kDa GAPDH was overexpressed in *Esch. coli* adjuvanted with Montanide ISA 763A, and 50 µg/fish volumes injected i.p. into tilapia. Another group of fish received formalin-inactivated whole cells adjuvanted with Montanide ISA 763A, or a mixture of with bacteria, formalized bacteria and adjuvant. Challenge was after 4-weeks when the combination vaccine [whole cells, protein and adjuvant] produced the highest RPS of 100 %. The whole cells and protein [with adjuvant] administered separately resulted in RPS values of 87.5 % and 50 %, respectively (Tsai et al. 2013b).

The efficacy of a formalin-inactive commercial vaccine for *Str. iniae*, a second for *Lactococcus garvieae*, and a combination of both was evaluated in thread-sail filefish, and led to significantly lower mortalities in each of the three vaccinated groups after challenge for the respective pathogen (Minami et al. 2013) with protection lasting for a year after the vaccination (Minami et al. 2014).

Immunostimulants/Dietary Supplements

Clove Oil

Natural plant products, namely clove oil at 3 % (w/v) supplemented diets, protected tilapia against challenge with *Lactococcus garvieae,* although the authors included 0.5 % (w/v) oxytetracyline as well (Rattanachaikunsopon and Phumkhachorn 2009).

Shirazi Thyme Shirazi thyme (*Zataria multiflora*) oil inhibited lactococcosis by suppressing the expression of the virulence genes, *PavA* and *Hly*, which encode fibronectin and haemolysin proteins, respectively (Soltani et al. 2015).

Mushroom The mushroom *(Lentinula edodes)* extract fed at 2 % for 6-weeks enhanced the immune response (immunoglobulin) of rainbow trout, and provided protection against challenge with *Lactococcus garvieae* (Baba et al. 2015).

Chinese Traditional Medicine Modified Huanglian Jiedu decoction (comprising *Cortex phellodendri, Flos lonicerae japonicae, Fructus forsythia, Fructus gardenia, Radix scutellaria* and *Rhizoma coptidis*) has been proposed as a feed additive when

dosed at 1 % for 28 days for the control of lactococcosis in grey mullet (*Mugil cephalus*). Resistance to experimental challenge was reported to be significantly enhanced (Choi et al. 2014).

Probiotics Lactococcosis, which is often difficult to control by other means, has been receptive to the use of probiotics, with *Aer. sobria* GC2 (recovered from the digestive tract of ghost carp) dosed at 5×10^7 cells/g of feed and fed over 14 days, stimulating the innate immune response (increase in leucocytes, phagocytosis, and respiratory burst activity) and conferring excellent protection against challenge (Brunt and Austin 2005). *Leuconostoc mesenteroides* and *Lactobacillus plantarum,* which were isolated from salmonids, were also effective at controlling lactococcosis when dosed at 10^7 cells/g of feed for 30 days with mortalities reduced from 78 % in the controls to 46 and 54 % in the experimental groups (Vendrell et al. 2008). When a bacteriocin (Nisin Z) producing *Lactococcus lactis* subsp. *cremoris* was administered orally to rainbow trout at 10^6 CFU/g for 21 days, only 20 % of the fish died (compared to 50 % of a non-bacteriocinogenic knockout isogenic mutant, and 72.5 % of the controls) following challenge with *Lactococcus garvieae* (Araujo et al. 2015).

Biological Control There are promising signs that bacteriophage may be able to moderate infections by *Lactococcus garvieae* in yellowtail (Nakai et al. 1999) and rainbow trout (Ghasemi et al. 2014).

Antimicrobial Compounds Resistance to erythromycin, lincomycin and oxytetracycline has been reported among Japanese isolates of *Lactococcus garvieae*, with the problem recognised for well over a decade (Kawanishi et al. 2005).

Lactococcus lactis subsp. *lactis*

Characteristics of the Disease

In hybrid sturgeon (*Huso huso x Acipenser ruthenus*) in Taiwan, diseased fish were anorexic, generally pale, with haemorrhagic ulcers on the tail and abdomen, which was enlarged [with ascites], enteritis, liquefied spleen, and rapid respiration, with 100 % mortality (Chen et al. 2012). Extensive skin lesions were observed near the caudal peduncle of an adult silver carp (*Hypophthalmichthys molitrix*) in the USA. Also, there was a musculoskeletal lesion. The disease was coined lactococcosis (Khoo et al. 2014).

Isolation

Material from diseased tissues was inoculated onto TSA, TSA plus 5 % (v/v) goat blood, and BHIA with incubation at 25 °C for 3 days (Chen et al. 2012). In a subsequent study, brain, kidney and material from a musculoskeletal lesion was inoculated onto Mueller Hinton agar supplemented with 5 % (v/v) sheep blood with incubation at 26 °C for 48 h when the predominant white-tan colony type was subcultured (Khoo et al. 2014).

Box 2.10: *Lactococcus lactis* subsp. *lactis*
Cultures comprise Gram-positive oval nonmotile cocci that grow at 45 °C and in 6.5 % (w/v) sodium chloride. Arginine dihydrolase, leucine arylamidase and pyrrolidonyl arylamidase are produced, but not alkaline phosphatase, ß-glucuronidase, H_2S, catalase or oxidase. Nitrates are not reduced. The Voges Proskauer reaction is positive. Acid is produced from amygdalin, mannitol, ribose and sucrose, but not from L-arabinose, glycogen, lactose, raffinose or sorbitol. Citrate is not utilized. Aesculin and blood (α-haemolysis) are attacked, but not sodium hippurate or urea (Chen et al. 2012; Khoo et al. 2014). Sequence homology of the 16S rRNA/16S SSU rDNA gene revealed 100 % similarity with *Lactococcus lactis* subsp. *lactis* in the GenBank data base (Chen et al. 2012; Khoo et al. 2014).

Pathogenicity

Chen et al. (2012) examined pathogenicity in tilapia, and reported LD_{50} values following i.p. injection the isolates of 10^2–10^5 CFU/ml. Pathogenicity was not confirmed in laboratory based infectivity experiments by Khoo et al. (2014).

Lactococcus piscium

Characteristics of the Disease

The disease condition encompasses lactobacillosis or pseudokidney disease of rainbow trout.

Characteristics of the Pathogen

The taxonomy of the Group N streptococci has undergone extensive revision. On the basis of 23S rRNA:DNA hybridization and superoxide dismutase studies, the genus *Lactococcus* was defined to accommodate these organisms. A group of fish

pathogenic lactococci/Group N streptococci have been studied, and named as a new species, i.e. *Lactococcus piscium* (Williams et al. 1990).

Box 2.11: *Lactococcus piscium*
From the available information, it is apparent that cultures comprise Gram-positive non-motile, facultatively anaerobic short (ovoid) rods, which are catalase negative, grow at 5–30 °C, produce acid from amygdalin, L-arabinose, arbutin, N-acetylglucosamine, cellobiose, D-fructose, galactose, ß-gentiobiose, gluconate, glucose, lactose, maltose, D-mannose, mannitol, melibiose, melezitose, D-raffinose, ribose, salicin, sucrose, trehalose, D-turanose and D-xylose, but not adonitol, D-arabinose, D or L-arabitol, dulcitol, erythritol, D and L-fucose, glycogen, glycerol, inositol, inulin, 2 and 5-ketogluconate, D-lyxose, α-methyl-D-glucoside, α -methyl-D-mannoside, ß-methyl-xyloside, rhamnose, L-sorbose, sorbitol, D-tagatose, xylitol and L-xylose. Aesculin and starch (slow, weak reaction) but not arginine is degraded. H_2S is not produced. The long chain cellular fatty acids are considered to be of the straight chain saturated, mono-unsaturated and cyclopropane-ring types. The major acids correspond to hexadecanoic acid, Δ 11-octadecanoic acid and Δ 11-methylenoctadecanoic acid. The G+C ratio of the DNA is calculated as 38.5 moles %.

Streptococcus agalactiae (= Str. difficilis)

Characteristics of the Disease

Str. difficilis was named as a result of an outbreak of disease in St. Peter's fish (tilapia) and rainbow trout within Israel during 1986. The disease spread rapidly, and caused severe economic losses in the farmed fish (Eldar et al. 1994). Diseased tilapia were lethargic, swam erratically, and showed signs of dorsal rigidity. In rainbow trout, the disease signs were consistent with a septicaemia, with brain damage (Eldar et al. 1994). An organism, identified as *Str. agalactiae*, was recovered from diseased cultured silver pomfret in Kuwait (Duremdez et al. 2004; Azad et al. 2012). Duremdez et al. (2004) described disease signs including inappetance, lethargy, swollen abdomen, the stomach and intestine filled with a gelatinous or yellowish fluid, and in some fish slight haemorrhaging in the eye, exophthalmia and corneal opacity. Also, the liver was enlarged, there was evidence of congestion of the kidney and spleen, and fluid occurred in the peritoneal cavity (Duremdez et al. 2004). Azad et al. (2012) reported anorexia and gradual darkening of larvae. Bacteria were recovered from brain tissue. Exophthalmia, haemorrhagic septicaemia, meningoencephalitis and multiple necrotic foci in tissues was reported by others (Suanyuk et al. 2008). An outbreak in wild fish including the giant Queensland grouper

(Epinephelus lanceolatus), was described, and which effectively extended the geographical range of the pathogen to Australia (Bowater et al. 2012).

Characteristics of the Pathogen

Str. difficilis was described to accommodate what was perceived to be a new species of fish pathogen causing meningo-encephalitis in cultured fish, which was first recognised in Israel during 1984 (Eldar et al. 1994, 1995b). The initial work with diseased fish resulted in the recognition of two groups of streptococci; the separation being achieved by use of API 50 CH and API 20 STREP, and by growth and haemolysis characteristics (Eldar et al. 1994). A fairly unreactive non-haemolytic mannitol negative group was labelled as *Str. difficile* (Eldar et al. 1994), and the specific epithet corrected to *difficilis,* i.e. *Str. difficilis* (Euzéby 1998), whereas a second more reactive α-haemolytic, mannitol positive group became known as *Str. shiloi.* On the basis of a comparative study including use of DNA:DNA hybridisation, *Str. difficilis* recognised to be a synonym of *Str. agalactiae,* which has precedence in nomenclature (Kawamura et al. 2005).

Box 2.12: *Streptococcus agalactiae*
Colonies on BHIA are 1 mm in diameter, and non-pigmented after incubation aerobically for 24 h at 30 °C. Cultures comprise encapsulated fermentative, catalase-negative Gram-positive cocci of varying diameters in small chains, which do not grow at 10, 37 or 45 °C, or in 40 % bile or 6.5 % (w/v) sodium chloride, but do grow at pH 9.6. The isolates attack (produced acid from) N-acetyl-glucosamine, D-fructose, D-glucose, maltose, D-mannose, ribose and saccharose, but not adonitol, aesculin, amygdalin, L- or D-arabinose, L- or D-arabitol, arbutin, cellobiose, dulcitol, erythritol, L- or D-fucose, galactose, gentiobiose, M-D-glucoside, glycerol, glycogen, inositol, inulin, lactose, melibiose, D-raffinose, rhamnose, salicin, sorbitol, L-sorbose, starch, turanose, xylitol, L- or D-xylose or M-xyloside. Alkaline phosphatase, arginine dihydrolase and leucine arylamidase are produced, but not α- nor **ß**-galactosidase, pyrrolidonylarylamidase nor ß-glucuronidase. The Voges Proskauer reaction is positive. Bovine blood is not attacked (Eldar et al. 1994).

These isolates were considered to belong to a separate and distinct DNA homology group, with DNA relatedness between members of 89–100 % (Eldar et al. 1994). The level of DNA relatedness with *Str. shiloi (= Str. iniae)* was 17 % (Eldar et al. 1994). Then, whole-cell protein electrophoresis revealed that the type strain of *Str. difficilis* was indistinguishable to *Str. agalactiae* (Vandamme et al. 1997), which had also been named as a fish pathogen (e.g. Evans et al. 2002). Moreover, it was determined that *Str. difficilis,* which was originally regarded as serologically untypeable (Eldar et al. 1994), cross reacted with group B *Streptococcus,* namely capsular

polysaccharide antigen type Ib (Vandamme et al. 1997). By single stranded conformation polymorphism analysis of the interspacer region, 46 isolates from different hosts and geographical origins were recovered in 5 genotypes, with genotype 1 accommodating cultures from Kuwait. AFLP profiling of the same cultures led to the definition of 13 genotypes, with Kuwaiti cultures recovered in two clusters (Olivares-Fuster et al. 2008). The genetic diversity of isolates was studied leading to the recognition of two capsular types (Ia and Ib) and four sequence types (103, 260, 552 and 553). The combined evaluation of serotype, sequence type and pattern of the presence or absence of *cylE* and *hylB* enabled the recognition of nine genetic profiles, which reportedly enabled better discrimination than previous methods (Godoy et al. 2013). Fifty-one isolates from 27 tilapia farms in China were collected, and analyzed for mobile genetic elements, genetic markers, and virulence-related genes, with results revealing only one genotype. This points to a similar origin for all the cultures included in the study. Bacteriophage typing differentiated all isolates into two distinct molecular types, with the second prevalent in tilapia culture in China (Zhang et al. 2013). Cultures (no = 181) from red hybrid tilapia (*Oreochromis* sp.) and golden pompano (*Trachinotus blochii*) in Malaysia were studied by RAPDs and REP-PCR leading to the delineation of 13 and 9 groups, respectively. Three cultures from golden pompano were genetically dissimilar to those from tilapia (Amal et al. 2013).

Detection

Nested PCR A nested PCR has been described for the detection of *Str. agalactiae* in naturally infected frozen and paraffin-embedded tissues of tilapia within positivity recorded from adult but not larvae or fry (Jiménez et al. 2011). The detection limit in the absence of fish DNA was given as 1.58 fg (Jiménez et al. 2011).

LAMP A LAMP detected 2.8×10^3 CFU/ml (=2.8 CFU/reaction), and was regarded as specific insofar as it did not recognise other related taxa (Wang et al. 2012). Similarly, a specific and sensitive (~20 cells/reaction) LAMP involved four primers that were designed to target the pathogen's *cfb* gene. The assay, which could be used with diseased tissues, was carried out at 65 °C for 60 min, and stopped at 80 °C for 10 min in a water bath, leading to results that could be observed visually (Ke et al. 2014). An accelerated and specific colorimetric LAMP with the pre-addition of calcein was developed for the detection of *Str. agalactiae* and *Str. iniae* in tilapia, with positivity reflecting a clearly observable colour change from orange to green. The LAMP was reported as 10 times more sensitivity than nested PCRs (Suebsing et al 2013).

Pathogenicity

Intraperitoneal injection of 0.5 ml amounts (10^7 CFU/ml) of *Str. agalactiae* into Nile tilapia significantly induced T-cell receptor ß chain expression more so than a higher dose (10^9 CFU/ml). This may have importance in the host response to attack

by pathogens (Nithikulworawong et al. 2012). The presence of a polysaccharide capsule is important for virulence, and the CpsA protein, which is a likely transcriptional regulator, has a role in capsule synthesis and unspecified cell wall-associated factors (Hanson et al. 2012). The duodenase-1 gene, which is involved in the immune response, appears to have an important role in conferring disease resistance in hybrid tilapia (Shen et al. 2015).

The water temperature has a direct relationship to the outcome of infection, and in one study 0, 50 and 70 % mortalities were recorded among populations of tilapia challenged with *Str. agalactiae,* and maintained at 20, 25 and 30 °C, elevating water temperature increases the severity of disease (Kayansamruaj et al. 2014; Zhao et al. 2015). Conversely, rainbow trout remained asymptomatic at 12 °C (Sepahi et al. 2013). Haemolytic activity was 5-fold higher when the pathogen was grown at 35 °C compared to 28 °C. Similarly, expression of *cylE* ß-haemolysin cytolysin), *cfb* (CAMP [Christie Atkins Munch-Petersen] factor) and *PI-2b* (pili backbone) was higher at 35 °C than 28 °C. Between 6 and 96 h after infection, at the higher temperature, tilapia showed profound inflammation, which was attributed to a 30–40 fold increase in upregulation of the inflammatory-related genes *cyclooxygenase-2*, *IL-1 ß* and *TNF-* α. In short, massive inflammatory type responses lead to acute mortalities at higher water temperatures (Kayansamruaj et al. 2014). Metabolomics revealed difference in the metabolic response of the tilapia according to the water temperature at which the fish were maintained. Thus, there was an increase in metabolites in the liver with increase in water temperature – 36 to 45 metabolites with less amino acids – particularly of L-proline – but more carbohydrates at 25 and 30 °C, respectively. Thus, the application of L-proline by injection or orally led to an increase in amino acid metabolism, and a concomitant reduction in mortalities (Zhao et al. 2015).

Disease Control

Vaccine Development Formalin-killed cells and a culture extract containing 50 % protein conjugated to alum, administered intraperitoneally, protected tilapia against challenge with a virulent strain, with protection correlated by the presence of humoral antibodies (Eldar et al. 1995c). Of relevance for vaccine development, western blots indicated that only a few proteins were actually protective (Eldar et al. 1995c). Using ECPs and encapsulated formalin-inactivated cells, which were administered to Nile tilapia by i.p. injection, Pasnik et al. (2005a) reported good protection even after 6 months when challenged. Also, there was demonstrable antibodies produced in the vaccinates, with a 55 kDa ECP antigen being implicated in vaccine efficacy (Pasnik et al. 2005b). Bath vaccination was less successful, with an RPS of 34 % compared to 80 % after administration intraperitoneally (Evans et al. 2004).

A live attenuated vaccine was developed by continuous passage of a culture in vitro, and the non-virulent variant evaluated in tilapia. Following administration

of 1.0×10^8 CFU/fish via injection, immersion or orally with challenge after 15-days, the RPS values obtained were 96.88, 67.22, and 71.81 %, respectively. Challenge after 30-days led to a reduction in protection with corresponding RPS values of 93.61, 60.56, and 53.16 %, respectively. Vaccination with less vaccine (1×10^6 and 1×10^7 CFU/fish) led to inferior protection. The antibody levels peaked at 14–21 days after vaccination, but decreased significantly after 28 days. The bacteria were recovered from the brain, liver, kidney, and spleen of fish after oral vaccination, and persisted in the spleen for up to 15 days (Li et al. 2015).

Two surface proteins, i.e. phosphoglycerate kinase and ornithine carbamoyltransferase were over-expressed in *Esch. coli*, purified and used as subunit vaccines and as components in whole cell vaccines in tilapia. The results revealed the the compound vaccine with purified protein – particularly phosphoglycerate kinase - led gave superior survival after challenge (Wang et al. 2014b). In a further development, surface immunogenic protein of *Str. agalactiae* was expressed in *Esch. coli,* and the purified recombinant protein was administered to Nile tilapia before challenge in which the RPS was 90.62 % (Wang et al. 2014b). Two genes encoding cell surface proteins, i.e. fibrinogen-binding protein A and α-enolase were expressed in *Esch. coli,* and the purified recombinant proteins examined as potential subunit vaccines to protect Nile tilapia leading to RPS values of 40.63 +/– 17.21 % and 62.50 +/– 18.75 %, respectively. In comparison, an inactivated whole cell vaccine led to an RPS of 93.75 +/– 5.41 % (Yi et al. 2014). An inactivated recombinant vaccine expressing the cell wall surface anchor family protein was administered orally with an equivalent dose of 10^6 CFU/ml to tilapia leading to an RPS of 70 % after challenge (Nur-Nazifah et al. 2014).

A recombinant plasmid pVAX1-sip, which contained a 1.02 kb DNA fragment encoding for a portion of the surface immunogenic protein (Sip) was inserted into an attenuated *Salmonella enteritidis* serovar Typhimurium and as a DNA vaccine used orally at 10^8 CFU/fish to vaccinate tilapia. The *Salmonella* localized in the digestive tract, liver and spleen for up to 4-weeks without harmful effects, and protected against lethal challenge (Huang et al. 2014a)

Immunostimulants/Dietary Supplements

Rosemary Leaves Feeding dried rosemary leaves to tilapia led to reduced mortalities following challenge (Zilberg et al. 2010).

Saccharomyces cerevisiae *Saccharomyces cerevisiae* cell wall with or without intraperitoneal vaccination [0.5 ml of a vaccine containing 10^8 CFU/ml] on day 60 and challenge 15 days later. All the fish receiving the dietary supplement survived, whereas 28.5 % of those that were not fed with yeast cell wall died compared to 38.09 % mortality among the controls. The mode of action reflected stimulation of innate and cellular immunity, specifically related to lymphocyte, macrophage and thrombocyte activity (Salvador et al. 2012).

Cinnamaldehyde Liposome-encapsulated cinnamaldehyde, which is from cinnamon, improved survival of zebra fish against challenge with *Str. agalactiae* (Faikoh et al. 2014).

Soybean Corn, Linseed, Fish and Olive Oils Soybean, corn, linseed, fish and olive oils were fed to Nile tilapia leading to evidence of immunostimulation, and in the case of soybean oil protection against experimental challenge (Ferreira et al. 2015).

Traditional Chinese Medicine Plant *Sophora flavescens* was fed at 0.025–0.4 % to Nile tilapia leading to immunostimulation (enhanced anti-protease, complement, myeloperoxidase and serum lysozyme activities, and increased production of reactive oxygen species and reactive nitrogen intermediates by peripheral blood leucocytes) and protection against experimental challenge (RPS = 73.3 %) (Wu et al. 2013).

Probiotics *Lactobacillus rhamnosus* was microencapsulated in alginate and skimmed milk-alginate, and fed to tilapia for 14 days leading to improved growth and protection after challenge (Pirirat et al. 2015).

Biological Control The ability of filter-feeding bivalve mussels (*Pilsbryoconcha exilis*) to control populations of *Str. agalactiae* (~3.5×10^5 CFU/ml in 40 l volumes of water) around juvenile Nile tilapia was examined in the laboratory. The results revealed that in the presence of mussels, the numbers of streptococci were reduced by 83.6–87.1 % over 3-weeks. Conversely, in the absence of mussels, the bacterial numbers increased by 31.5 % over the same period. The presence of the mussels led to higher growth and less mortalities than in the absence of the filter-feeders (Othman et al. 2015).

Antimicrobial Compounds *Str. difficilis* was susceptible to ampicillin, cefuroxime, cephalothin, chloramphenicol, ciprofloxacin, erythromycin, fusidic acid, methicillin, mezlocillin, nitrofurantoin, penicillin, potentiated sulphonamide, tetracycline and vancomycin, but resistant to amikacin, colistin, gentamycin and nalidixic acid.

Streptococcus dysgalactiae subsp. *dysgalactiae*

Characteristics of the Disease

This Lancefield group C streptococcus was recovered in Japan from amberjack and yellowtail that had been previously vaccinated with a commercial vaccine against lactococcosis caused by *Lactococcus garvieae* and were displaying necrotic lesions of the caudal peduncle. Some fish revealed splenic hypertrophy (Nomoto et al. 2004). The organism has been associated with severe necrosis of the caudal peduncle and mortalities of amberjack and yellowtail in Japan (Nomoto et al. 2006), and

with septicaemia and subcutaneous abscesses in the tail region of Nile tilapia in Brazil (Netto et al. 2011). The organism has also been recovered from diseased Amur sturgeon (*Acipenser schrenckii*) in China (Yang and Li 2009).

Isolation

Str. dysgalactiae has been recovered as orange colonies on Todd-Hewitt agar supplemented with 30 µg/ml of Congo red with incubation at 37 °C for 30 h (Abdelsalam et al. 2009). These could be readily distinguished from *Lactococcus garvieae* that produced white or pale orange colonies on the media (Abdelsalam et al. 2009).

Characteristics of the Pathogen

Box 2.13: *Streptococcus dysgalactiae* subsp. *dysgalactiae*
White colonies develop in 48–72 h at 25 °C. Cultures comprise Lancefield serological group C, catalase-negative, α-haemolytic on sheep blood (ß-haemolytic after prolonged incubation), Gram-positive cocci, which form long chains. Electron microscopy reveals fimbriae-like structures surrounding the cell wall. Growth does not occur at 10 or 45 °C, or at pH 9.6. Resistance is not recorded to 40 % bile salts. The Voges Proskauer reaction is negative. Aesculin and sodium hippurate are not hydrolysed. ß-glucuronidase, α-glucosidase, acid and alkaline phosphatase and leucine arylamidase are produced, but not pyrolidonylamidase, α-or ß-galactosidase (Yang and Li 2009). Acid is produced at 37 °C from trehalose and amygdalin, but not from arabinose, inulin, lactose, mannitol, raffinose or sorbitol (Nomoto et al. 2004; 2006).

Identification was confirmed by the results of 16S rDNA sequencing (Nomoto et al. 2004; 2006; Yang and Li 2009), with Nomoto and colleagues concluding that fish isolates differed by sequencing and phylogenetic analysis of the *sodA* manganese-dependent superoxide dismutase gene from pigs (Nomoto et al. 2008).

Diagnosis

Latex beads were coated with recombinant surface immunogenic protein and used in slide agglutination when positive responses were obtained with sera from infected farmed amberjack (*Seriola dumerili).* Negative responses were recorded with sera from non-infected fish and from healthy vaccinates, i.e. fish that received *Lactococcus garvieae* and *Str. iniae* vaccine (Nishiki et al. 2014).

Pathogenicity

When administered i.m. at doses of just over 10^6 cells/fish, *Str. dysgalactiae* led to clinical disease resembling those of naturally infected fish (Nomoto et al. 2004). The pyrogenic, exotoxin G, a superantigen, and streptolysin S genes are regarded as the most importance virulence traits, with cultures recovered from moribund fish harbouring the streptolysin S structural gene, *sagA* (Abdelsalam et al. 2010).

Streptococcus ictaluri

Characteristics of the Disease

The pathogen has been associated with a range of conditions, including arthritis, emaciation, myositis, osteolysis and spinal meningitis leading to low level mortalities in channel catfish (Camus et al. 2008).

Characteristics of the Pathogen

Sequencing of the 16S rRNA gene revealed that the three isolates from diseased channel catfish were most related phylogenetically to *Str. iniae, Str. parauberis* and *Str. uberis* (Shewmaker et al. 2007).

Box 2.14: *Streptococcus ictaluri*
Colonies on TSA supplemented with 5 % (v/v) defibrinated sheep blood comprise α-haemolytic catalase-negative Gram-positive cocci in pairs and short chains, which grows at optimally at 30 °C, but not at 45 °C or in 6.5 % (w/v) sodium chloride. Produces alanine-phenylalanine proline arylamidase, alkaline phosphatase, leucine aminopeptidase, pyroglutamic acid arylamidase and pyroglutamylaminopeptidase, but not arginine dihydrolase, ß-glucosidase, ß-galactosidase, *N*-acetyl-ß-glucosaminidase, glycine-L-tryptophan arylamidase or ß-mannosidase. Does not degrade aesculin, arginine, hippurate or urea. Produces acid from maltose, but not L-arabinose, D-arabitol, cyclodextrin, glycogen, lactose, mannitol, melibiose, melezitose, methyl-ß-D-glucopyranoside, pullulan, raffinose, sorbitol, sucrose, tagatose or trehalose. Does not utilise pyruvate. The Voges Proskauer reaction is negative. Tellurite is not tolerated. Susceptible to vancomycin. The G+C ratio of the DNA is 38.5 mol%. DNA:DNA hybridisation revealed a DNA relatedness of ≤22 % with other streptococci, namely *Str. canis, Str. dysgalactiae* subsp. *dysgalactiae, Str. iniae, Str. parauberis, Str. pyogenes, Str. uberis* and *Str. urinalis* (Shewmaker et al. 2007).

By use of the Rapid ID32 Strep system, the isolates were equated with *Gemella haemolysin* (good identification – 96 % confidence).

Pathogenicity

Str. ictaluri infected juvenile channel catfish by immersion ($\geq 10^7$ CFU/fish) and injection ($\geq 10^7$ CFU/fish) methods causing low level mortalities within 21 days, but only at high doses (Pasnik et al. 2009).

Disease Control

Passive immunisation of channel catfish with fish antiserum followed by challenge led to some protection and an indication of the involvement of antibodies in the process (Pasnik et al 2011).

Streptococcus iniae

Characteristics of the Disease

Str. iniae was initially recovered from an Amazon freshwater dolphin, *Inia geoffrensis* (Pier and Madin 1976). The association with fish diseases came when it was described as a cause of mortality in tilapia hybrids *(Tilapia nilotica x T. aurea)* (Perera et al. 1994) and later in dusky spinefoot *(Siganus fuscenscens)* (Sugita 1996) and hybrid striped bass (Stoffregen et al. 1996). *Str. shiloi* was named as a result of an outbreak of disease in St. Peter's fish and rainbow trout within Israel during 1986 when the disease spread rapidly, and caused severe economic losses in farmed fish (Eldar et al. 1994). Diseased tilapia were lethargic, swam erratically, and showed signs of dorsal rigidity. In rainbow trout, the disease signs were consistent with a septicaemia, with brain damage including meningitis (Eldar et al. 1994; Eldar and Ghittino 1999). *Str. iniae,* was diagnosed as causing disease in two tanks of hybrid striped bass in a commercial farm, using recirculating freshwater, in the USA (Stoffregen et al. 1996), in white spotted rabbitfish *(Siganus canaliculatus)* in Bahrain (Yuasa et al. 2003), in barramundi *(Lates calcarifer)* in Australia (Bromage et al. 1999) and in caged and wild fish from the Red Sea (Colorni et al. 2002). The pathogen appears to be spreading, and has now been recognized in red porgy (*Pagrus pagrus*) and gilthead sea bream (*Sparus aurata*) in Spain (El Aamri et al. 2010). In the case of Nile tilapia, there is evidence that enhanced mortalities may well ensue where co-infection, namely with ichthyophthiriasis, occurs (Xu et al. 2009). Channel catfish, which were farmed in China, have been found with acute septicaemia, and linked to infection with *Str. iniae.* Here, bacterial cells were observed in the macrophages (Chen et al. 2011). *Str. iniae* serotype II infection led to rainbow trout displaying lethargy, discoloration, loss of orientation, bilateral exophthalmia, corneal

opacity and haemorrhaging in the eye, and surface and internal (mostly in the spleen and fat around the intestine) haemorrhaging leading to death (Lahav et al. 2004).

Isolation

Heart infusion agar supplemented with thallium acetate and oxolinic acid or with colistin sulphate and oxolinic acid was evaluated for the selective recovery of *Str. iniae* from Japanese flounder and the fish farm environment. Defibrinated horse blood was also added to determine haemolysin pattern. The result was recovery of *Str. iniae* from brain, intestine and kidney of diseased fish (Nguyen and Kanai 1999).

Characteristics of the Pathogen

Box 2.15: ***Streptococcus iniae (= Str. shiloi)***
Colonies on BHIA are 1 mm in diameter, and non-pigmented after incubation aerobically for 24 h at 30 °C. Cultures comprise fermentative, catalase and oxidase-negative [virulent cultures are encapsulated; Barnes et al. 2003a] Gram-positive cocci in pairs and chains (some degree of pleomorphism has been observed), which grow at 37 °C but not at 10 or 45 °C, or in 40 % bile or 6.5 % (w/v) sodium chloride, but do grow at pH 9.6. Isolates attack (produced acid from) N-acetyl-glucosamine, aesculin, arbutin, cellobiose, D-fructose, gentiobiose, D-glucose, glycogen, maltose, mannitol, D-mannose, melezitose, ribose, salicin, starch and trehalose, but not adonitol, amygdalin, L- or D-arabinose, L- or D-arabitol, dulcitol, erythritol, L- or D-fucose, galactose, gluconate, glycerol, inositol, inulin, lactose, melibiose, D-raffinose, rhamnose, sorbitol, L-sorbose, tagatose, turanose, xylitol, or L- or D-xylose. Alkaline phosphatase, arginine dihydrolase, ß-glucuronidase, leucine arylamidase and pyrrolidonylarylamidase are produced, but not α- or ß-galactosidase. The Voges Proskauer reaction is negative. Nitrates are not reduced. α-haemolysis is recorded for bovine blood. Aesculin but not gelatin or sodium hippurate is degraded (Eldar et al. 1994; Zhou et al. 2008).

On the basis of DNA:DNA hybridisation, i.e. 77–100 % DNA homology, *Str. iniae* was realised to be synonymous with *Str. shiloi,* which had been previously named as the causal agent of a septicaemic condition in cultured fish, which occurred in Israel in 1984 (Eldar et al. 1994, 1995a, b). This change in the taxonomy was confirmed by others (e.g. Tiexeira et al. 1996). The organism was recovered from rainbow trout, which had been previously vaccinated with a streptococcus vaccine, with the conclusion that a new serotype had emerged (Bachrach et al. 2001). Indeed, a study of 26 Israeli and 9 other isolates using phenotypic, RAPD, and AFLP and 16S rDNA sequencing revealed a new variant among the Israeli cultures (Kvitt and

Colorni 2004). *Str. iniae* serotype II, which differed in arginine hydrolase activity, was described as the cause of disease in rainbow trout initially within Israel and then the USA (Bachrach et al. 2001; Barnes et al. 2003a; Lahav et al. 2004). The pathogen is now recognised in Saudi Arabia in tilapia culture (Al-Harbi 2011). Two phenotypes were recognised serologically among cultures from Japan (mostly from flounder), with differences reflecting the presence or absence of polysaccharide capsule (Kanai et al. 2006). Genetic variability as a result of PFGE has been recorded among Australian isolates (Nawawi et al. 2008). Korean isolates recovered during 2000–2005 from diseased olive flounder were examined by REP-PCR and RAPDs enabling division into two genotypes reflective of pathogenicity of which genotype 1 was more highly virulent than genotype 2 (Kim et al. 2014).

By serology using streptococcal specific antisera, the original isolates equated with *Str. shiloi* were untypeable. Moreover, these isolates were considered to belong to a separate and distinct DNA homology group, with DNA relatedness between members of 89–100 % (Eldar et al. 1994). Some phenotypic differences were noted between fish and human isolates, however molecular techniques did not discriminate the two sets of cultures (Dodson et al. 1999).

Diagnosis

Serology iFAT incorporating monoclonal antibodies has successfully recognised *Str. iniae* in tissues (Klesius et al. 2006).

Molecular Methods A multiplex PCR has been developed, and successfully recognised from cultures and fish tissues a range of fish pathogenic lactococci-streptococci, i.e. *Lactococcus garvieae, Str. difficilis, Str. iniae* and *Str. parauberis* with a sensitivity for the purified DNA of 30 pg, 12.5 pg, 25 pg and 50 pg, respectively (Mata et al. 2004). Specifically for *Str. iniae,* a PCR amplifying a 377-bp DNA fragment detected only 10 CFU (Zhou et al. 2011). Yet other studies, advocated LAMP for *Str. iniae* with a stated detection limit of 100 fg of purified genomic DNA, which corresponds to ~4.63×10^1 genomic copies/reaction, and is ~10 times more sensitive that conventional PCR (Han et al. 2011b). Cai et al. (2011; 2012) reported a sensitivity of only 12.4 cells/reaction, and commented on the high degree of specificity. An accelerated and specific colorimetric LAMP with the pre-addition of calcein was developed for the detection of *Str. agalactiae* and *Str. iniae* in tilapia, with positivity reflecting a clearly observable colour change from orange to green. The LAMP was reported as 10 times more sensitivity than nested PCRs (Suebsing et al 2013).

Pathogenicity

Environmental factors, notably water temperature, have profound effect on the progression of infection caused by *Str. iniae.* Thus, water temperatures of 25–28 °C led to the highest mortalities in barramundi; pH and salinity could not be linked with

mortalities in terms of statistical significance (Bromage and Owens 2009). Also, co-infection of *Str. iniae* with aquabirnavirus has led to higher mortalities in Japanese flounder (Pakingking et al. 2003).

Polysaccharide capsules have been found on *Str. iniae* (Barnes et al. 2003a), with evidence that the capsule may be involved with the resistance to opsonophagocytosis in yellowtail (Yoshida et al. 1997). This view by was reinforced by Miller and Neely (2005), who when using capsular mutants showed that the polysaccharide capsule was indeed important for the virulence of *Str. iniae.* Again, an effect on avoiding phagocytosis by avoiding phagocytic clearance was reported (Lowe et al. 2007; Locke et al. 2007). Eyngor et al. 2008) described the emergence of an extracellular polysaccharide producing strain of *Str. iniae* and thus the (re-)occurrence of disease outbreaks following vaccination.

A surface-located α-enolase, which is a plasmin and plasminogen binding and cell wall associating protein and may be associated with tissue invasion, has been found in *Str. iniae*. This enzyme may help *Str. iniae* to cross tissue barriers (Kim et al. 2007). Moreover, there is sound evidence for an extracellular polysaccharide leading to death of rainbow trout (Eyngor et al. 2010).

The pathogen produces a cytolysin with haemolytic traits, which is a functional homologue of streptolysin S. Expression of this cytolysin is necessary for local tissue necrosis but not to bacteraemia (Fuller et al. 2002). When grown in serum, this streptococcus expresses surface factors that are capable of binding to trout immunoglobulin by the Fc region [= crystallisable fragment of the immunoglobulin] (Barnes et al. 2003b). A range of isolates from fish, a dolphin and humans produced apoptosis and/or necrosis in tilapia nonspecific cytotoxic cells and tilapia continuous cell line (Taylor et al. 2001). Only serotype II strains entered, multiplied and survived in pronephros phagocytes (leading to apoptosis) for >48 h. This is relevant because it was estimated that ~70 % of the bacteria contained in blood during sepsis were located within phagocytes, which suggests a preferred intracellular existence (Zlotkin et al. 2003).

Scavenging for iron is necessary for the survival of pathogens within host tissues. Siderophores have been found in *Str. iniae.* Instead, the pathogen has been determined to require iron-containing proteins for growth under conditions of iron-restriction. Indeed, the ABC transporter system was recognized to be responsible for haem utilization. A putative lipoprotein exerts a role in haem utilization and is produced in vivo during infection with the pathogen (Wang et al. 2013).

A putative bacteriocin, Sil, which is composed of 101 amino acid residues, is secreted by *Str. iniae,* interacted with turbot head kidney monocytes inhibiting the innate immune response of the host cells and enhancing the cellular infection of the pathogen. The data suggested that Sil promotes infection by impairing the immune system of the fish (Li et al 2014).

Examination by GC-MS of the metabolites important to the defence of tilapia against infection highlighted the importance of elevated N-acetylglucosamine levels in survivors. Thus, supplying the chemical to fish enhanced survival (Cheng et al. 2014).

Disease Control

Vaccine Development With the spread of *Str. iniae,* it became an obvious candidate for vaccine development with commercial products now available. A *Str. iniae* vaccine was applied orally to Nile tilapia for 5 days, and following challenge 23 days after the conclusion of vaccination achieved an RPS of 63 % thus demonstrating the feasibility and usefulness of the oral approach (Shoemaker et al. 2006). Subsequently, a formalin-inactivated whole cell preparation has been used successfully in Nile tilapia to protect against a wide range of *Str. iniae* isolates with RPS values of 79–100 % (Shoemaker et al. 2010). Similar success (RPS = 100 %) has been achieved in farmed grouper 1 month following intraperitoneal application in adjuvant (Huang et al. 2014b). A trivalent formalin-inactivated vaccine comprising *Edw. tarda, Str. iniae* and *Str. parauberis,* protected olive flounder when administered intraperitoneally in 0.1 ml amounts (Han et al. 2011a). An inactivated bivalent vaccine containing cells of *Str. iniae* and *V. vulnificus* was administered intraperitoneally to sex reversed hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) leading to protection against both pathogens after challenge with RPS values of 69–100 % and 79–89 %, respectively (Shoemaker et al. 2012). The efficacy of a formalin-inactive commercial vaccine for *Str. iniae*, a second for *Lactococcus garvieae*, and a combination of both was evaluated in thread-sail filefish, and led to significantly lower mortalities in each of the three vaccinated groups after challenge for the respective pathogen (Minami et al. 2013) with protection lasting for a year after the vaccination (Minami et al. 2014). A commercial ß-haemolytic *Streptococcus* vaccine for Japanese flounder was administered by i.p. injection in threadsail filefish (*Stephanolepis cirrhifer*) leading to an RPS of >85 %, and protection that lasted >7-months (Ishii et al. 2013).

A recombinant subunit vaccine, i.e. a putative iron-binding protein, Sip11, of serotype 1 expressed in *Esch. coli* was protective (RPS = 69.7 %) particularly when linked to an inert carrier protein and administered as a live vaccine by i.p. injection in Japanese flounder (Cheng et al. 2010). Similarly, the putative hydrophobic cytoplasmic membrane protein, MtsB, of the ATP-binding cassette transporter system, was protective (RPS = 69.9 %) following i.p. injection of 28 μg quantities in FCA into tilapia. A booster dose was administered 14 days later (Zou et al. 2011).

A DNA vaccine involving a putative secretory antigen, Sia10, was identified, and used in the form of a plasmid, pSia10, which led to an RPS of 73–92 % in turbot (Sun et al. 2010).

A formalin inactivated whole cell vaccine was compared with live attenuated products in hybrid striped bass by bath and i.p. injection with the outcome that the live vaccine lacking M-like protein gave complete protection (RPS = 100 %) by both methods of administration albeit with some (12–16 %) pre-challenge mortalities (Locke et al. 2010). An attenuated novobiocin-resistant strain has been proposed as a vaccine candidate for use in Nile tilapia. Administration was by i.p. injection of 2×10^7 CFU and following challenge the RPS was up to 100 % (Pridgeon and Klesius 2011b).

The 759 bp *srtA* gene, which encodes sortase A - influences the correct positioning of surface proteins in bacterial cells, was cloned, and a mutant constructed via allelic exchange mutagenesis. This mutant was used as a live vaccine, administered to Nile tilapia intraperitoneally, which after challenge resulted in a RPS of 95.5 % (Wang et al. 2014b).

On a less positive note, the health of the fish at the initiation of vaccination is of paramount importance insofar as parasitism of Nile tilapia with *Gyrodactylus cichlidarum*, *Ichthyophthirius multifiliis* and *Trichodina heterodentata* led to reduced protection (Martins et al. 2011).

Immunostimulants/Dietary Supplements

Yeasts Yeasts have furnished nucleotides, which have shown promise with controlling infections caused by *Str. iniae.* Using a commercial product, Ascogen, which comprises oligonucleotides from brewer's yeast, feeding trials were carried out for 7–8 weeks with hybrid striped bass followed by bath challenge with *Str. iniae,* with the result that experimental groups showed a higher level of protection when compared to the controls (Li et al. 2004).

Garlic Garlic *(Allium sativum)* powder administered at 1.3 % [4 % was less effective] in feed for 14 days to orange-spotted grouper (*Epinephelus coioides*) gave better weight gain, and protection to challenge with *Str. iniae* (Guo et al. 2012). Garlic powder was fed at 1.2 g/kg body weight to cobia *(Rachycenron canadum)* for 21-days, leading to lower mortalities after challenge with *Str. iniae* (Guo et al. 2012).

Rosemary Rosemary (*Rosmarinus offiinalis)* was used as dried and powdered leaves and as ethyl acetate extracts in feeds in a ratio of 1:17 and 1:24, respectively, to tilapia for 5 days followed by infection with *Str. iniae* and feeding with the rosemary and its extract for a further 10 days with a resulting marked reduction in mortalities (Abutbul et al. 2004). This was substantiated in a later study when feeding dried leaves to tilapia led to reduced mortalities following challenge with *Str. iniae* (Zilberg et al. 2010).

Indian Lettuce Indian lettuce (*Lactuca indica*) fed to kelp grouper (*Epinephelus bruneus*) at 1 and 2 % enhanced immune function (lysozyme, phagocytic and respiratory burst activities) at 2 weeks, and led to lower mortalities after challenge with *Str. iniae* (Harikrishnan et al. 2011).

Sweet Orange Essential oil extracted from sweet orange (*Citrus sinensis*) peel was fed at 0.1 %, 0.3 % and 0.5 % for 90-days to Mossambique tilapia leading to protection from challenge (Acar et al. 2015).

Puncture Vine An extract of puncture vine *(Tribulus terrestris)* was fed at 400 mg/kg to first-feeding Mossambique tilapia fry for 45 days before challenge with results indicating enhanced growth and resistance to *Str. iniae* (Yilmaz et al. 2014).

Cumin Cumin (*Cuminum cyminum*) was fed at 1.14 % to Mozambique tilapia fry for 45 days leading to enhanced growth and protection after challenge (Yilmaz et al. 2013).

Allspice Allspice (*Pimenta dioica*) dosed 10 g of allspice/kg of fish feed and fed for 50 days to Mossambique tilapia fry led to improved growth and protection against challenge (Yilmaz and Ergun 2014).

Other Examples of Beneficial Plants Other examples that led to recipient fish resisting challenge were thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*) or fenugreek (*Trigonella foenum graecum*) (Yilmaz et al. 2013; Gultepe et al. 2014).

Barodon The anionic alkaline mineral, Barodon, was fed at 0.1–0.3 % to juvenile olive flounder for 10-weeks leading to improvements in growth, immunostmulation (enhanced anti-protease, serum lysozyme and superoxide dismutase activities) and resistance to experimental infection (Shin et al. 2014).

Probiotics Streptococcicosis, which is difficult to control by other means, has been receptive to probiotics, with *Aer. sobria* GC2 (recovered from the digestive tract of ghost carp) dosed at 5×10^7 cells/g of feed and fed over 14 days, stimulating the innate immune response (increase in leucocytes, phagocytosis, and respiratory burst activity) and conferring excellent protection against challenge (Brunt and Austin 2005). *Lactococcus lactis* BFE920 was fed at 1×10^6, 5×10^6, 2.5×10^7 and 1.25×10^8 CFU/g of feed for 2-weeks to olive flounder (*Paralichthys olivaceus*), demonstating 68–77 % survival after challenge. Consequently, a field trial was carried out using 12000 fish for 3-months with reports revealing improved growth, immunostimulation (increased lysozyme activity, and production of IL-12 and IFN-gamma) and survival (=66 %) after subsequent challenge with *Str. iniae* (Kim et al. 2013). A mixture of *Aspergillus oryzae* (2.0×10^9 CFU/g), *Bac. subtilis* (1.5×10^9 CFU/g) and *Saccharomyces cerevisiae* (10^9 CFU/g) were administered orally at 5 g/kg and 10 g/kg for 6-weeks to Nile tilapia leading to immunostimulation (enhanced leucocyte numbers, and respiratory burst activity) and protection against experimental challenge (Iwashita et al. 2015).

Bacteriocins *Lactococcus lactis* subsp. *lactis,* which produced a bacteriocin termed nisin Z, was recovered from the intestine of olive flounder, and inactivated *Str. iniae* within 3 h at a dose of 3,200 arbitrary units. As a consequence, nisin Z was suggested as an alternative to chemotherapy for the control of streptococcosis (Heo et al. 2012).

Use of Antimicrobial Compounds Success has been reported with the fluoroquinolone compound, enrofloxacin, dosed at 5 or 10 mg/kg body weight of fish/day for 10 days (Stoffregen et al. 1996). Mortalities declined rapidly upon initiating treatment, such that by the end of treatment, a total of 11 % and 17 % of the treated fish has died compared to 55 % and 40 % of the untreated controls, respectively. Also, laboratory studies have indicated the value of oxytetracycline (Darwish et al. 2003) and amoxicillin (Darwish and Ismaiel 2003) for the control of infection in blue tilapia and sunshine bass, respectively. Experimental evidence has pointed to the efficacy of using amoxicillin for controlling *Str. iniae* infection especially when dosed at 80 mg/kg of fish/day for 12 days when mortalities dropped from 96 % in the controls to 6 % in the treated blue tilapia (Darwish and Hobbs 2005). Moreover, the treated survivors did not carry the pathogen

Streptococcus milleri

Characteristics of the Disease

During 1992, Gram-positive chaining cocci and atypical *Aer. salmonicida* were recovered from newly imported koi carp, which displayed pronounced surface ulcers of 4–20 mm in diameter on the flank or tail. Internal damage was not recorded (Austin and Robertson 1993).

Characteristics of the Pathogen

Two cultures were obtained from kidney samples in ulcerated koi carp (Austin and Robertson 1993). The following characteristics were displayed:

Box 2.16: *Streptococcus milleri*
Cultures contain catalase and oxidase-negative fermentative cocci in chains, that produce acid and alkaline phosphatase, arginine dihydrolase, chemotrypsin, esterase (caprylate and lipase), ß-galactosidase, leucine and valine arylamidase and pyrrolidonylarylamidase but not cystine arylamidase, α-fucosidase, α -galactosidase, α – and ß-glucosidase, ß-glucuronidase, H_2S, indole, α -mannosidase, nitrate reductase, trypsin or tryptophan deaminase. The methyl red and bile aesculin tests are negative. Aesculin, arginine, casein and horse blood (weakly ß-haemolytic) are degraded, but not DNA, gelatin, sodium hippurate, starch or urea. Growth occurs in 0–1.5 % but not 8 % (w/v) sodium chloride and on MacConkey agar. Citrate is utilised. Acid is produced from N-acetyl-glucosamine, amygdalin, D-fructose, D-glucose, inositol, maltose, D-mannose, α-methyl-D-glucoside, ribose, saccharose, sucrose, D-trehalose, xylitol and xylose, but not from L-arabinose, glycogen, inulin, lactose, D-mannitol, D-melibiose, raffinose, rhamnose or sorbitol.

An identification to *Str. milleri* (probability of a correct identification = 98 %) resulted from use of the Bacterial Identifier Program (Bryant and Smith 1991). By means of the API 20 STREP system, an identification of *Lactococcus lactis* resulted (probability of a correct identification = 96.7 %). Yet from the published description, there was a closer fit with *Str. milleri.* The only discrepancies concerned utilisation of citrate, and acid production from inositol, xylitol and xylose. The isolates from koi carp were positive for these tests.

Pathogenicity

An isolate of *Str. milleri* (G3K) injected at 5×10^6 cells/fish caused 20 % mortalities in Atlantic salmon. Interestingly, all the fish darkened, albeit with negligible signs of internal or external abnormalities. With rainbow trout, there was evidence of kidney liquefaction (Austin and Robertson 1993).

Disease Control

Use of Inhibitory Compounds The two isolates were sensitive to tetracycline, which might be of value for chemotherapy (Austin and Robertson 1993).

Streptococcus parauberis

Characteristics of the Disease

This form of streptococcosis was originally recognised in farmed turbot (weight: 0.8–2 kg) from 5 sites in northern Spain during 1993 and 1994 (Doménech et al. 1996), and in northern Portugal in 2004 (Ramos et al. 2012). Overall in Spain, the farms, which used the same fish food, reported losses of 0.1–5 %. Disease signs, which were more severe during summer, included weight loss, haemorrhaging on the anal and pectoral fins, petechial haemorrhages on the abdomen, bilateral exophthalmia, haemorrhaging and pus in the eyes, pale liver, congested kidney and spleen, ascites, and mucohaemorrhagic enteritis (Doménech et al. 1996). The organism has developed into a major cause of streptococcosis in olive flounder *(Paralichthys olivaceus)* in Asia resulting in heavy mortalities and severe economic loss (Nho et al. 2011). Also, there is evidence for its occurrence in wild striped bass in Chesapeake Bay, USA (Haines et al. 2013).

Characteristics of the Pathogen

Isolates were identified by phenotypic (Rapid ID32 and API 50CH systems) and genotypic data (16S rRNA sequencing) as *Str. parauberis;* an organism known previously as *Str. uberis* genotype II. Eighteen isolates recovered from diseased turbot in Northwest Spain (Galicia) were subjected to ribotyping and RAPD analyses with the data demonstrating marked homogeneity among the cultures (Romalde et al. 1999). Two serotypes are recognised (Kanai et al. 2009; Han et al. 2011a), and the complete genome sequence of serotype I has been determined revealing the presence of a single chromosome of 2,143,887 bp containing 1,868 predicted coding sequences (Nho et al. 2011). Serotype I (may be subdivided into Ia, Ib and Ic; Kanai et al. 2015) is the more pathogenic of the two in experiments with olive flounder (Han et al. 2011a).

Box 2.17: *Streptococcus parauberis*
After overnight incubation, pure cultures produce whitish slightly α -haemolytic colonies of 1.5–2 mm in diameter. These contain non-motile encapsulated Gram-positive short rods/cocco-bacilli in pairs or short chains, which produce alkaline phosphatase, α -galactosidase, ß-glucuronidase and pyrrolidonyl arylamidase, but not catalase, indole or catalase, grow at 10–37 °C but not at 4 or 45 °C, in 4.5 but not 6.5 % (w/v) sodium chloride or at pH 9.6 or on MacConkey agar, and degrade arginine and hippurate (some strains).

There was a 100 % sequence homology between the fish isolates and *Str. parauberis*. The only reliable difference between the turbot isolates and the type strain concerned the action on D-raffinose which was negative for the latter (Doménech et al. 1996).

Diagnosis

RT-PCR, using the *Taq*Man probe assay targeting the *gyrB* gene sequences, detected only 10 fg of *Str. parauberis* genomic DNA/reaction specifically and reproducibly (Nguyen et al. 2016).

Epizootiology

Work has demonstrated that *Str. parauberis* has the potential to survive in the marine environment in a dormant, i.e. non-cultured form, after an initial culturable phase that lasted for approximately 1 and 6-months in water and sediment, respectively (Currás et al. 2002). The addition of nutrients to the experimental microcosms led to a return to a culturable state (Currás et al. 2002). Also, *Str. parauberis* has been associated with raw milk and bovine mastitis (Doménech et al. 1996).

Pathogenicity

Str. parauberis was examined for the presence of putative surface-associated virulence factors relevant to turbot for which the data indicated haemagglutination activity (against turbot erythrocytes), variable hydrophobicity due possibly to the presence of capsular material, and the ability to adhere to and invade cultured cells, e.g. CHSE-214 (Chinook salmon embryo) and SBL (striped bass larvae) cell lines (Romalde et al. 2000). Mutation of the phosphoglucomutase gene impaired capsule production, and attenuated virulence, thus indicating that expression of the enzyme is important for virulence by influencing bacterial survival against the fish defence mechanisms (Woo and Park 2014).

Disease Control

Vaccines A trivalent formalin-inactivated vaccine comprising *Edw. tarda, Str. iniae* and *Str. parauberis,* protected olive flounder when administered intraperitoneally in 0.1 ml amounts (Han et al. 2011a). Subsequently, formalised cells of *Str. parauberis* serotype I and II gave complete protection (RPS = 100 %) after 3-weeks when administered intraperitoneally to Japanese flounder and challenged with the homologous serotype; less protection was recorded when challenge was with the heterologous serotype (Mori and Fukuda 2012). These data pointed to issues with cross-protection when heterologous serotypes were present.

Dietary Supplements Material derived from the deciduous tree, kozo *(Broussonetia kazinoki)* was administered at 1.0 and 2.0 % as a feed additive to olive flounder leading to enhanced immune function (complement, lysozyme, phagocytic and respiratory burst activities) (Kim et al. 2012).

Antimicrobial Compounds It was reported that isolates were resistant to flumequine, oxolinic acid and streptomycin, moderately susceptible to oxytetracycline, tetracycline and sulphamethoxazole-trimethoprim, and highly sensitive to ampicillin, chloramphenicol, erythromycin, nitrofurantoin and penicillin (Doménech et al. 1996).

Streptococcus phocae

Characteristics of the Pathogen

This is a ß-haemolytic streptococcus, which was originally recovered from seals, but which has been associated with disease of Atlantic salmon in Chile since 1999 (Gibello et al. 2005; Romalde et al. 2008; Yañez et al. 2013). Chilean isolates are

genetically, serologically and phenotypically homogeneous (Romalde et al. 2008; Valdés et al. 2009).

Box 2.18: *Streptococcus phocae*
Cultures comprise ß-haemolytic facultatively anaerobic, non-motile, cocci in pairs or chains, that do not produce catalase. Growth occurs at 37 °C but not at 10 or 45 °C, or in 6.5 % (w/v) sodium chloride or in 40 % (v/v) bile. Alkaline phosphatase is produced, but not ß-glucuronidase, hyaluronidase or pyrrolidonyl arylamidase. The Voges Proskauer reaction is negative. Neither aesculin, hippurate nor starch is degraded. Acid is produced from D-fructose, maltose, D-mannose, *N*-acetyl-glucosamine and ribose, but not from galactose, glycerol, inulin, lactose, mannitol, melezitose, D-raffinose, salicin, sorbitol, or trehalose. The G+C ratio of the DNA is 38.6 mol% (Skaar et al. 1994).

Diagnosis

Molecular Techniques Detection/diagnosis of *Str. phocae* has quickly been the subject of molecular-based approaches, with detection levels of 10^2 and 10^4 cells/per PCR tube achieved for primer pair PX1-PXVQ2 (Avendaño-Herrera 2008). Using seeded Atlantic salmon tissues, this primer pair enabled the detection of $5.1 \times 10^5 - 6.4 \times 10^7$ CFU/g of kidney, liver and spleen, with nested-PCR being most sensitive (Avendaño-Herrera 2008). A multiplex PCR was developed for the simultaneous detection *Aer. salmonicida, Pis. salmonis, Str. phocae* and *V. anguillarum*. The detection limit using purified total bacterial DNA was 5 pg/µl ($=1.26 \times 10^4$ CFU/ml). The limits of detection using spiked tissues, i.e. kidney, liver, muscle or spleen, were, $9.03 \pm 1.84 \times 10^5$ CFU/g (Tapia-Cammas et al. 2011).

Pathogenicity

Injection of Atlantic salmon with *Str. phocae* cells but not ECPs led to mortalities with cultures resisting the killing ability of mucus and serum and multiplying within them (González-Contreras et al. 2011). The latter may be the result of capsular material. Haemagglutination was not reported. The pathogen was capable of adhering to but not of entering cells [this was determined by use of the CHSE cell line] (González-Contreras et al. 2011). Iron uptake mechanisms involved siderophores and constitutive iron binding components. At least two iron-regulated membrane proteins increased when the pathogen was cultured in iron-restricted medium (Retamales et al. 2012).

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Chapter 3
Aerobic Gram-Positive Rods and Cocci

Abstract Aerobic heterotrophic Gram-positive rods and cocci have received great attention from fisheries microbiologists, largely because of the severity of the diseases caused by pathogenic representatives. The group includes *Ren. salmoninarum, Mycobacterium* and *Nocardia,* the causal agents of BKD mycobacteriosis and nocardiosis, respectively. These organisms have been associated with slowly developing diseases that occur both in the wild and in aquaculture.

Keywords Mycobacteriosis • Nocardiosis • BKD • Bacillary necrosis • Micrococcosis

Erysipelothrix rhusiopathiae

Characteristics of the Disease

The organism was associated with haemorrhagic septicaemia in Australian eels, and may have been triggered by high temperatures, i.e. >40 °C. Haemorrhages were apparent in the gill arches and opercula, and in the pale liver. The intestinal mucosa was haemorrhagic (Chong et al. 2015).

Isolation

Kidney and gill tissue was inoculated onto blood agar, MacConkey agar and TCBS with incubation at 25 and 37 °C for 48–120 h whereupon pinprick colonies on blood agar (but not MacConkey agar or TCBS) developed (Chong et al. 2015).

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Characteristics of the Pathogen

Box 3.1: *Erysipelothrix rhusiopathiae*
The isolates from kidney were Gram-positive pleomorphic to curved rods that produced H_2S, but not did not produce catalase or oxidase. Glucose, lactose and maltose were fermented, blood (α-and ß-haemolysis) was attacked, but not gelatin or urea. Sequencing of the 16S rRNA gene was used to link the organism with *Erysipelothrix rhusiopathiae* (Chong et al. 2015).

Pathogenicity

Pathogenicity was not confirmed in laboratory-based infectivity experiments.

Disease Control

Erythromycin was used successfully to control disease (Chong et al. 2015).

Renibacterium salmoninarum

Characteristics of the Disease

Bacterial kidney disease (BKD, Dee disease, corynebacterial kidney disease, salmonid kidney disease) was described initially in 1930 for a condition in Atlantic salmon (*Salmo salar)* found in the Rivers Dee and Spey in Scotland (Mackie et al. 1930; Smith 1964). Histological examination of fixed material revealed the presence of large numbers of Gram-positive rods in lesions. In 1935, a disease was reported in the USA, where it occurred in hatchery reared brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis)* (Belding and Merrill 1935). Additional evidence indicated the presence of BKD in chinook salmon (*Oncorhynchus tshawytscha),* coho salmon (*O. kisutch)* and sockeye salmon (*O. nerka)* (Rucker et al. 1951). Since then, the disease has been reported to occur in 13 species of salmonids in Canada, Chile (especially during transfer of chinook salmon from fresh to seawater; Uribe et al. 1995), England, France, Germany, Iceland, Italy, Spain, U.S.A. and Yugoslavia (see Fryer and Sanders 1981; Hoffman et al. 1984; Uribe et al. 1995; Loch et al. 2012). During 1997, BKD was demonstrated for the first time in Denmark (Lorenzen et al. 1997). Workers have highlighted the presence of the disease in farmed salmonid stocks but less often has it been found in wild fish populations (Rucker et al. 1951, 1953; Smith 1964; Pippy

1969; Evelyn et al. 1973; Wood 1974; Ellis et al. 1978; Paterson et al. 1979, 1981; Mitchum and Sherman 1981; Banner et al. 1986; Loch et al. 2012), and it was cultured in two specimens of wild caught grayling (*Thymallus thymallus*) and Atlantic salmon in the UK; additional PCR positive wild fish were also identified mostly from rivers with rainbow trout farms known to be positive for BKD (Chambers et al. 2008). The presence of BKD in Japanese farmed ayu from April to July 2001, possibly reflecting horizontal transmission from masu salmon, has been documented (Nagai and Iida 2002). BKD may be exacerbated by or exacerbate other conditions, for example, Weiland et al. (1999) documented that Chinook salmon that were traumatized with gas (= gas bubble trauma) died more rapidly than those fish that were only exposed to renibacterium alone. Wire-tagging appears to influence horizontal transmission of the disease insofar as the wounds in the snout caused by tagging can become infected with renibacteria, and lead to the spread to internal organs. Possibly renibacteria are spread with the tagging devises (Elliott and Pascho 2001).

External signs include exophthalmia (Fig. 3.1), lesions in the eyes, swollen abdomen (full of ascitic fluid; Fig. 3.2), blood filled blisters on the flank (Fig. 3.3), and the presence of ulcers/abscesses (Fryer and Sanders 1981; Hoffman et al. 1984). Internally, lesions may develop in the kidney (this may become swollen; Fig. 3.4), brain (= meningoencephalitis; Speare 1997), liver, heart and spleen. The lesions contain a fluid mass of leucocytes, bacteria and cellular debris (Fryer and Sanders 1981). A false membrane, covering some internal organs, has been described (Snieszko and Griffin 1955; Bell 1961). This membrane, the presence of which may be influenced by water temperature (Smith 1964), consists of layers of fibroblasts

Fig. 3.1 The rainbow trout on the left has bilateral exophthalmia caused by *Ren. salmoninarum.* The second fish is a healthy specimen

Fig. 3.2 A distended abdomen on a rainbow trout with BKD

Fig. 3.3 A blood blister on the surface of a rainbow trout with BKD

Fig. 3.4 Swollen kidneys associated with BKD

and histiocytes, degenerating leucocytes with macrophages, and fibrin (Wolke 1975). In Atlantic salmon, petechial haemorrhages have been described on the muscle lining the peritoneum (Smith 1964). However, *Ren. salmoninarum* has been found in salmonids, e.g. char and grayling in Alaska, without any evidence of clinical disease (Meyers et al. 1993). Renibacterial cells have been found in intra-abdominal adhesions in Atlantic salmon and coho salmon that may have previously or were considered to have received adjuvant (= adjuvanted vaccine) intraperitoneally (Bruno and Brown 1999). Evidence suggests an impact of the disease on appetite with infected fish consuming less than their less diseased counterparts (Pirhonen et al. 2000). A reduction in growth of infect Chinook salmon has been observed (Sandell et al. 2015). One point that needs to be emphasised is that BKD does not always manifest itself in a clinical form, sub-clinical disease poses a real risk of transfer of the pathogen with fish movements, especially if the animals are perceived to be healthy (Murray et al. 2011).

Isolation

Cultivation of the causal agent of BKD *in vitro* from chinook salmon was not achieved until Earp (1950) used a nutrient-rich medium, containing fish extract, glucose, yeast extract and bovine serum/meat infusion, with incubation at 15 or 20 °C. An improvement resulted from use of minced chick embryos in 1 % (w/v) agar or on Dorset egg medium (Earp et al. 1953). Nevertheless, growth was generally poor, even after prolonged incubation periods of ≥14 days. To prove that the growth was of the pathogen, Earp successfully inoculated the bacterial culture into

healthy chinook salmon, and eventually recovered it again from the resultant kidney lesion. Continuing this pioneering work, Ordal and Earp (1956) supplemented Dorset egg medium with 0.05–1.0 % (w/v) L-cysteine, tryptone and yeast extract, and succeeded in isolating the pathogen in 3–4 weeks following incubation at 17 °C. The outcome of this work was the formulation of cysteine blood agar with which Koch's postulates were fulfilled (Appendix in Chap. 12; Ordal and Earp 1956). Foetal calf serum was substituted for human blood in a modification proposed by Evelyn et al. (1973). This was developed further by removing sodium chloride and substituting peptone for tryptone and beef extract (KDM2; Appendix in Chap. 12; Evelyn 1977); this medium is now used commonly for growth of the BKD organism. In a parallel development, Wolf and Dunbar (1959) used Mueller-Hinton agar supplemented with 0.1 % (w/v) L-cysteine hydrochloride (MHC) to culture the pathogen, although success with this medium did not occur with Smith (1964). However, Bullock et al. (1974) confirmed the value of MHC, although this has been subsequently contended by Evelyn (1977). Serum-rich KDM2 was considered to be superior to serum-deficient MHC, indicating the benefit of serum for the cultivation of the BKD organism. This was further supported by Paterson et al. (1979), who supplemented MHC with 10 % (v/v) foetal calf serum, and successfully used the medium for isolating the pathogen from Atlantic salmon. Daly and Stevenson (1985) proposed replacing serum with charcoal, which serves as a detoxicant (Appendix in Chap. 12 – charcoal agar). However, these media, being extremely rich in composition, are generally suitable for the growth of many aerobic, heterotrophic bacteria. Moreover, fast-growing organisms may rapidly outcompete and overgrow the slower-growing BKD organism. A solution was proposed by Evelyn (1977), who advocated the use of a drop-plating technique (essentially, this is analogous to dilution plates, which dilute out potential interference by fast-growing heterotrophs). In a later report, Evelyn (1978) recommended the use of peptone (0.1 % w/v)-saline (0.85 % w/v) as a diluent to remove any inhibiting factors against the pathogen, which may be present in kidney tissue (Evelyn et al. 1981; Austin 1986). Some inconsistencies in the performance of KDM2 were attributed to variation in the composition of the commercial peptone (Evelyn and Prosperi-Porta 1989). To overcome this inconsistency when single lots of peptone were not available, two possible modifications were suggested. Firstly, a "nurse" culture technique was reported. This technique accelerated the growth of the BKD organism, and increased the sensitivity at which the pathogen could be detected. The technique, which was based on satellitism or cross feeding, involved inoculating the nutritionally fastidious pathogen next to a non-fastidious feeder – the nurse organism. Evelyn et al. (1989) placed drops of a dense suspension of a stock culture of the BKD organism (= the nurse organism) onto the centre of KDM2 plates. Samples, suspected of containing the pathogen, were placed as 25 µl drops around the periphery of the nurse culture. With incubation, the nurse culture grew rapidly, presumably modified the conditions in the KDM2, and thus enhanced the growth of the pathogen in the periphery. For example, colonies of the BKD organism were observable after incubation for 19 days, compared to 25 days for the conventional approach. The second modification involved supplementing KDM2 agar with a small amount

of spent (KDM2) broth that was previously used for growing the pathogen. In both cases, it seems that an unknown metabolite serves as a growth stimulant (Evelyn et al. 1989, 1990). This was substantiated by Matsui et al. (2009), who substituted the serum component of KDM2 with spent medium, and found better recovery of *Ren. salmoninarum* from dilute suspensions, i.e. ≤300 CFU.

Until the advent of selective isolation techniques, initial isolation of the pathogen from fish tissues was an uncertain affair, prone to contamination by fast-growing aerobic heterotrophs. With this in mind, a selective isolation medium, SKDM (Appendix in Chap. 12; Austin et al. 1983) was devised, which proved to be effective for isolation of the pathogen from dilute samples. SKDM permitted the recovery of the pathogen from seeded river water, and from the kidney and faeces of experimentally infected fish (Fig. 3.5; Embley 1983; Austin and Rayment 1985). In contrast, the pathogen was not recovered on corresponding KDM2 plates, which were completely overgrown by other bacteria. Clearly, selective media, such as SKDM, should prove useful in further ecological studies on the causal agent of BKD. In a comparison of KDM2, SKDM and the charcoal-containing derivative, it was determined that the selective medium (SKDM) was most effective for the primary isolation of the pathogen from Atlantic salmon (Gudmundsdóttir et al. 1991). In this comparison of positive samples, 91 %, 60 % and 35 % were positive on SKDM, the charcoal containing derivative and KDM2, respectively. Clearly, the selective medium enhanced significantly the ability to recover the pathogen. Moreover, serum was more advantageous than charcoal as a medium supplement. However, long incubation periods of 12–19 weeks were necessary to recover colonies on the media from dilute samples (Benediktsdóttir et al. 1991). In a subsequent comparison of media for the recovery of *Ren. salmoninarum* from head kidney of rainbow trout, the best recovery was on KDM2 supplemented with 10 % (v/v) spent medium [used previously for the growth of the pathogen followed by SKDM, and then KDM2 with charcoal (Chambers and Barker 2006).

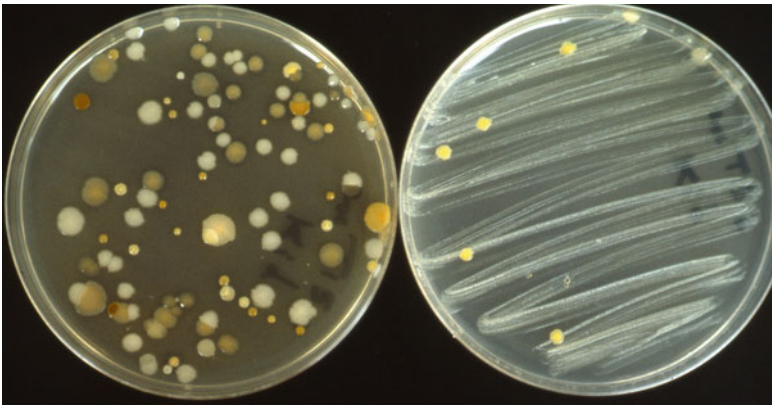

Fig. 3.5 A virtual pure culture of *Ren. salmoninarum* on SKDM (right). On KDM2 (left), the pathogen has been outcompeted by contaminants

The question concerning the necessary growth requirements for the pathogen was addressed in a detailed study by Embley et al. (1982). These workers formulated a rich semi-defined medium devoid of serum (Appendix in Chap. 12 – semi defined medium), which was suitable for the cultivation of cells, but not for the initial isolation of cells from infected fish tissues. The semi-defined medium was used, however, to obtain biomass destined for lipid analyses (Embley et al. 1983), and inocula for nutritional and physiological studies. Subsequently, Shieh (1989a) described a complex blood free medium, which permits the growth of the pathogen.

Characteristics of the Pathogen

At various times, the causal agent of BKD has been linked with *Corynebacterium* (Ordal and Earp 1956; Smith 1964; Sanders and Fryer 1978; Austin and Rodgers 1980), *Brevibacterium* (Smith 1964), *Listeria* (Bullock et al. 1975), *Lactobacillus* (Vladik et al. 1974) and *Rickettsia* (Snieszko and Griffin 1955). Subsequently, it was appreciated that the organisms were sufficiently unique to warrant separate species status, so, *Corynebacterium salmoninus* was described (Sanders and Fryer 1978). With further information, these authors realised that the pathogen belonged in a new, as yet undescribed genus and, therefore, proposed *Renibacterium*. Thus, the causal agent of BKD became classified as *Renibacterium salmoninarum* (Sanders and Fryer 1980).

The initial difficulties experienced in culturing the pathogen contributed significantly to the uncertainty over its precise taxonomic status. Early work emphasised a few morphological features, namely the presence of small (0.3–1.5 × 0.1–1.0 μm), Gram-positive, asporogenous, non-motile, non-acid-fast rods, which frequently occurred in pairs. Evidence of pleomorphism, metachromatic granules and a 'coryneform' appearance (Ordal and Earp 1956; Smith 1964) led to the initial, tenuous association with the coryneform group of bacteria, namely *Corynebacterium*, It is interesting to note that the later investigation of Young and Chapman (1978) did not substantiate the 'coryneform' morphology. However, transmission electron microscopy of negatively stained cells, obtained from 28-day-old cultures on growth medium, i.e. KDM2, revealed the presence of pleomorphism and intracellular vacuoles/granules (B. Austin, unpublished data). By using FAT on kidney smears from coho salmon, Cvitanich (2004) observed small short rods, termed bar forms because of their staining reaction in FAT, which could not be cultured and were not virulent.

Earp (1950) and Ordal and Earp (1956) demonstrated catalase and proteolytic activity, and realised that there was a growth requirement for cysteine. Additional attributes of the organism were slowly realised; in particular, Smith (1964) indicated the temperature range of growth, i.e. most rapid at 15 °C, slow at 5 and 22 °C, and not at all at 37 °C, and determined an inability to degrade gelatin. During the period of the late 1970s to early 1980s, a wealth of knowledge was accumulated on *Renibacterium*. A low genetic diversity among North American isolates has been indicated from multilocus enzyme electrophoresis using 44 enzymes (Starliper 1996). Thus, from 40 isolates, 21 electrophoretic types were recognised. Grayson

et al. (1999) highlighted the inability of conventional systems to differentiate among *Renibacterium* isolates, and investigated molecular methods that might be useful to identify intraspecific variation. The outcome was the differentiation of isolates by RAPD according to host and geographical location.

Box 3.2: *Renibacterium salmoninarum*

Characteristically, *Ren. salmoninarum* produces cream (non-pigmented), shiny, smooth, round, raised, entire, 2-mm diameter colonies on KDM2 after incubation at 15 °C for 20 days. Subclinical infections may lead to two colony types, the smooth colonies described above and a thin film of growth, the latter of which does not develop on SKDM (Hirvelä-Koski et al. 2006). Old cultures, i.e. 12 weeks, may become extremely granular or crystalline in appearance. Indeed, a transverse section through such colonies will reveal the presence of a few Gram-positive rods embedded in a crystalline matrix. Subculturing at this stage often leads to the development of more crystalline 'colonies'. It is thought that the material is principally cystine, which has been precipitated from the medium. For some strains, a uniformly turbid growth occurs in broth, but for others, a sediment may develop. The cell wall peptidoglycan of renibacteria contains D-alanine, D-glutamic acid, glycine and lysine as the diamino acids (Fiedler and Draxl 1986). The principal cell wall sugar is glucose, but arabinose, mannose and rhamnose are also present (Sanders and Fryer 1980). Here, there is a discrepancy with the more recent work of Kusser and Fiedler (1983). These authors reported that the principal cell wall sugar is galactose, with lesser amounts of N-acetyl-glucosamine, rhamnose and N-acetyl-fucosamine. This is a curious anomaly insofar as the same strain, i.e. the type strain (ATCC 33209), is common to both studies. Mycolic acids are absent. Methyl-branched fatty acids form over 92 % of the total fatty acid component of the cells, with 12-methyltetradecanoic (anteiso-C_{15}), 13-methyldecanoic (iso-C_{15}) and 14-methylhexadecanoic (anteiso-C_{17}) as the major components. Straight chain fatty acids generally account for 1 % of the total fatty acids, and unsaturated fatty acids are not detected at all. Over 81 % of the total fatty acids are composed of the lower melting point anteiso acids, which may contribute to membrane fluidity at low temperatures. Unsaturated menaquinones with nine isoprene units are present. All strains contain diphosphatidylglycerol, two major and six or seven minor glycolipids and two unidentified minor phospholipids (Embley et al. 1983). Although renibacteria were considered to be serologically homogeneous (Bullock et al. 1974; Getchell et al. 1985), two antigenic groups have been described (Bandín et al. 1992). These groups have been defined after analyses of membrane proteins, which determined the presence of 57 kDa and 30 kDa molecules in the respective groups. The G+C ratio of the DNA has been calculated as 53.0 +/− 0.46 moles % by Sanders and Fryer (1980) and as 55.5 moles % by Banner et al. (1991). Additional characteristics of *Ren. salmoninarum* have been included in Table 3.1.

(continued)

Box 3.2 (continued)

Table 3.1 Characteristics of *Renibacterium salmoninarum*[a]

Character	Response
Production of:	
Acid and alkaline phosphatase	+
Butyrate esterase	–
Caprylate esterase	+
Catalase	
Chymotrypsin	–
Cystine arylamidase	–
α-fucosidase	–
α- and ß-galactosidase	–
ß-glucosaminidase	–
α-glucosidase	+
ß-glucosidase	–
ß-glucuronidase	–
Leucine arylamidase	+
α-mannosidase	+
Myristate esterase	–
Oxidase	–
Trypsin	+
Valine arylamidase	–
Nitrate reduction	–
Degradation of:	
Adenine, aesculin, arbutin, chitin, chondroitin, DNA	–
Casein, Tributyrin, Tween 40 and 60	+
Elastin, gelatin, guanine, hyaluronic acid, hypoxanthine	–
Lecithin, RNA, starch, testosterone Tween 80, tyrosine	–
Xanthine	–
Acid production from sugars	–
Growth on/at:	
pH 7.8	+
0.025 % (w/v) bile salts, 0.001 % (w/v) methylene blue	–
0.0001 % (w/v) crystal violet, 0.00001 % (w/v) nile blue	+
0.005 % (w/v) phenol, 1 % (w/v) potassium thiocyanate	–
1 % (w/v) sodium chloride	+ (poor)
0.01 % (w/v) sodium selenite, 0.001 % (w/v) thallous acetate	–
Utilisation of:	
4-umbelliferyl – acetate, 4-umbelliferyl – butyrate	+
4-umbelliferyl – ßD-cellobiopyranoside monohydrate	–
4-umbelliferyl – elaidate, 4-umbelliferyl – α-L-arabinopyranoside	–

(continued)

Box 3.2 (continued)

Table 3.1 (continued)

Character	Response
4-umbelliferyl – 2-acetamido-2-deoxy-ß-D-galactopyranoside	–
4-umbelliferyl – ß-L-fucopyranoside	–
4-umbelliferyl – heptanoate, 4-umbelliferyl – laurate	+
4-umbelliferyl – nonanoate, 4-umbelliferyl – oleate	+
4-umbelliferyl – palmitate	–
4-umbelliferyl – propionate	+

[a]From Embley (1983) and Goodfellow et al. (1985)

The exact taxonomic position of *Ren. salmoninarum* is uncertain. However, the numerical phenetic study of Goodfellow et al. (1985) confirmed the homogeneity of the taxon, and demonstrated its dissimilarity to *Lactobacillus* and *Listeria (Lis. denitrificans)*. The results of the chemotaxonomy study discussed above, also indicated the unique position of renibacteria. On the basis of the fatty acid data, *Renibacterium* is distinguishable from *Corynebacterium sensu stricto* and other representatives of mycolic-acid-containing taxa, which have predominantly straight chain and monounsaturated fatty acids. In short, the data indicate that *Renibacterium* is distinct from other Gram-positive organisms (Embley 1983; Embley et al. 1983; Goodfellow et al. 1985), although its relationship to *Cor. (Actinomyces) pyogenes* needs clarification. On the basis of 16S rRNA cataloguing, *Ren. salmoninarum* was considered to comprise a member of the actinomycete subdivision, being related to *Arthrobacter, Brevibacterium, Cellulomonas, Jonesia, Micrococcus, Promicromonospora, Stomatococcus* and *Terrabacter* (Stackebrandt et al. 1988; Gutenberger et al. 1991). The complete genome of the pathogen was determined, and revealed a circular chromosome of 3155250 bp that was predicted to contain 3507 ORFs. The genome is 1.9 Mb smaller than *Arthrobacter* sp. FB24 and *Arthrobacter aurescens* TC1 genomes, and has a lower G+C content. The suggestion is that *Ren. salmoninarum* underwent significant reductive evolution from an ancestral *Arthrobacter* with the acquisition of putative virulence genes by horizontal gene transfer (Wiens et al. 2008).

Multilocus VNTRs have been used to investigate inter-strain variation, and distinguished 17 different haplotypes amongst 41 isolates from Atlantic salmon and rainbow trout in Scotland, Norway and the USA. The first group of two haplotypes featured isolates from Atlantic salmon and rainbow trout in Norwegian and Scottish aquaculture, and the type strain. The second group comprised isolates from only Atlantic salmon, of mostly wild origin, and including the original Dee disease isolates from Scotland (Matejusova et al. 2013). The data suggested a possible exchange of pathogens between Atlantic salmon and rainbow trout in Norwegian and Scottish aquaculture over the last two decades. Moreover, it would appear that isolates from European aquaculture are genetically distant to to those of the original Dee disease (Matejusova et al. 2013).

Next-generation sequencing technology was used to generate genome-wide SNP data from 68 isolates representing a wide range of hosts and geographical and temporal origin, with the result that two lineages were delineated. Lineage 1 was

regarded as having spread over the last century with the developments of aquaculture and stocking of fisheries; lineage 2 was endemic in wild salmonids from the eastern Atlantic Ocean (Brynildsrud et al. 2014).

Diagnosis

Histology Historically, diagnosis of BKD was achieved histologically, by the presence of Gram-positive cocco-bacilli in kidney tissue. However, the reliability was impaired by the presence of melanin granules and other morphologically similar bacteria (Chen et al. 1974). A subsequent derivative has been a histochemical technique using Lillie's allochrome to stain glycogen in the bacteria (Bruno and Munro 1992). Again, interference could result from the presence of other morphologically similar glycogen-containing bacteria.

Culturing The early spate of interest in culturing techniques improved diagnosis but the effectiveness was marred by the apparently slow growth of the organisms, i.e. up to 6 weeks at 15 °C. In fact, recent evidence suggests that 19 weeks may be necessary for the initial incubation period. Therefore, there was widespread attention focused on serological procedures as being the saviour of diagnosticians.

Certainly, the selective medium (SKDM; Austin et al. 1983) has proved useful in isolating *Renibacterium* from mixed cultures. In addition, during a comparative exercise with KDM2, SKDM consistently enabled a greater recovery of cells from infected fish. In some cases, scant growth of only one or two colonies were recovered from kidney tissue on SKDM, although the parallel KDM2 plates were devoid of any growth (Fig. 3.5; Austin et al. 1983). Suspect cultures of renibacteria were subsequently confirmed by the characteristic profile on API-ZYM and other phenotypic traits, namely catalase production and inability to produce oxidase. A characteristic profile is obtained on API-ZYM (Fig. 3.6):

−+−+−+−−+−++−−−+−−+/−−

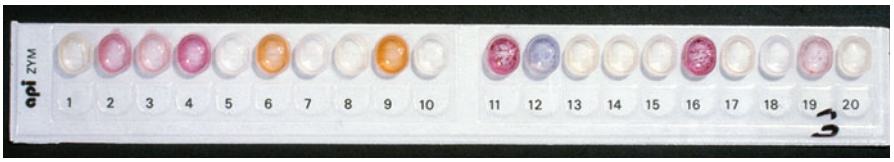

Fig. 3.6 An API ZYM strip after inoculation, incubation, and the addition of reagents. The organism is the type strain of *Ren. salmoninarum*

Serology An immunodiffusion test for BKD, based on the detection of soluble antigens in infected tissues, was developed by Chen et al. (1974) and discussed further by Kimura et al. (1978). Use of this method together with more classical agglutination reactions on ten isolates led to the conclusion by Bullock et al. (1974) that the causal agent of BKD was antigenically homogeneous. Following examination of over 50 isolates, we concur with this conclusion. Immunodiffusion was, of course, much quicker than cultivation, with diagnosis, achieved on the basis of specific precipitin lines, taking no more than 24 h.

The co-agglutination test of Kimura and Yoshimizu (1981) showed considerable promise for rapid detection of BKD, i.e. within 2 h. The anti-*Renibacterium* antibody coated staphylococcal cells are reacted with the supernatant from heated (i.e. 100 °C for 30 min) kidney tissues. Unlike iFAT/FAT, it does not require an expensive fluorescence microscope, and would, therefore, be more suited to field conditions.

iFAT (Bullock and Stuckey 1975; Mitchum et al. 1979; Paterson et al. 1979; Laidler 1980) and FAT (Bullock et al. 1980) have been developed for the diagnosis of BKD. Improvements in the FAT included a 60 min staining time with the fluorescent antibody (Cvitanich 1994). A further refinement involved concentrating samples - in this case coelomic fluid from spawning chinook salmon - on membrane filters, which were used with FAT (Elliott and McKubben 1997). This modification was regarded as more sensitive than FAT on smears (Elliott and McKubben 1997). iFAT has found use for detecting asymptomatic or overt cases of BKD (Bullock and Stuckey 1975; Lee and Gordon 1987), although the technique is not always as sensitive and reliable as culturing (Armstrong et al. 1989). Indeed, Paterson et al. (1979) pointed to the enzootic nature of BKD in one Canadian river. These workers reported asymptomatic infections in 33.4 % of 456 Atlantic salmon parr and 35.1 % of 37 adult salmon in the Margaree River. Seemingly, iFAT was more sensitive than the examination of Gram-stained kidney tissue or cultivation on Mueller-Hinton agar supplemented with 10 % foetal calf serum and 0.1 % L-cysteine hydrochloride (Paterson et al. 1979). A view has been expressed about the value of western blots (e.g. Lovely et al. 1994). But which is more efficient at detecting renibacterium, culturing or serology? Hsu et al. (1991) described a monoclonal antibody based ELISA which appears to be effective for the diagnosis of BKD. This system detected 0.05–0.1 μg of antigen/ml within a few hours.

The developments of serological methods for the detection and/or diagnosis of BKD must be examined sceptically because the reports preceded detailed taxonomic study of the organisms. It is unclear how workers knew that the aetiological agent possessed a unique antigenic profile, distinguishing it from other Gram-positive organisms. Unfortunately, the reliability of serological methods may now be questioned insofar as cross-reactions with apparently unrelated organisms have been recognised. Bullock et al. (1980) observed large bacteria, in faecal samples of brook trout, which fluoresced with antiserum to *Renibacterium*. After studying authentic representatives of 44 Gram-positive bacterial taxa and 101 cultures from fish and water, Austin and Rayment (1985) reported false positive reactions with

coryneform bacteria obtained from fish, a fish pathogenic *Mycobacterium* spp., and *Rothia dentocariosa*. Yoshimizu et al. (1987) noted a cross reaction between a *Pseudomonas* and *Ren. salmoninarum* in iFAT. A 60 kDa heat-shock protein (HSP60) of *Chlamydia psittaci* migrated with the 57 kDa protein of *Ren. salmoninarum,* and may explain the cross-reactivity of polyclonal renibacterium antiserum (Wood et al. 1995). Of course, antisera can be made more specific by cross-absorbing with these organisms. Reinforcing a separate article (Toranzo et al. 1993) by using western blots, Bandín et al. (1993) reported a common antigen, i.e. the 57 kDa protein, between *Cor. aquaticum, Car. piscicola* and *Ren. salmoninarum.* Also, it was noted that some isolates of *Ren. salmoninarum* did not produce the 57 kDa protein (Bandín et al. 1993). This is interesting because using the same strains, McIntosh et al. (1996) could not find the 57 kDa protein in *Cor. aquaticum* or *Car. piscicola.* Also in contrast to Bandín and co-workers, *Ren. salmoninarum* strain K57 was found to produce the 57 kDa protein. Brown et al. (1995) produced evidence that bacteria other than renibacterium could cross react with antiserum to *Ren. salmoninarum.* Moreover, these workers used PCR and confirmed the conclusion of McIntosh et al. (1996) that *Cor. aquaticum* and *Car. piscicola* lacked the p57 antigen.

Investment may be placed in the development of monoclonal antibodies, which should be totally specific for *Renibacterium* (Arakawa et al. 1987; Wiens and Kaattari 1991). Evelyn (1978) contradicted the Utopian opinion of serology, by reporting that culturing was more sensitive than fluorescent antibody techniques for the detection of renibacteria in kidney tissue by a factor of 10:1. This theme was continued in a later study (Evelyn et al. 1981) when experiments were undertaken to determine whether or not there was correlation between culturing and fluorescent antibody based diagnoses of the BKD carrier state. Again, culturing was reported as more sensitive then fluorescent antibody methods (Evelyn et al. 1981). Nevertheless, from the work of Paterson and colleagues, it could not be explained what was present in the fish which gave a positive fluorescence test but which could not be cultured. Explanations include the presence of dead cells which retain the ability to fluoresce, anaerobes which would require specialized isolation procedures, fastidious aerobes, damaged, dormant or inhibited cells of renibacteria, or even inanimate particles which microscopically could be mistaken for bacteria. Obviously, caution is needed in interpreting serological diagnoses. Whenever possible, culturing should be used for confirmation.

Molecular Techniques Since the early studies addressing the development of molecular methods for the detection/diagnosis of BKD, emphasis has moved to a comparison of the efficacy of different methods (Sandell and Jacobson 2011). León et al. (1994a, b) published details of a PCR assay using a 149 base pair DNA sequence, which was sensitive enough to detect 22 renibacterial cells even in tissue, and of sufficient specificity to recognise *Ren. salmoninarum* but not *Aer. hydrophila, Aer. salmonicida, Car. piscicola, Fla. columnare, V. anguillarum, V. ordalii* or *Y. ruckeri.* Then, this group detailed a 2282 base pair DNA fragment that appeared to be responsible for internalisation of renibacteria, at least into CHSE-tissue culture cells (Maulén et al. 1996). A nested RT-PCR has shown promise for the detection of

mRNA from viable cells of *Ren. salmoninarum* from fish tissues (kidney and ovarian fluid) with detection limits stated to be 1–10 bacterial cells (Cook and Lynch 1999). Subsequently, a nested reverse transcription PCR of 16S rRNA sequences successfully detected 1–10 renibacterial cells in ovarian fluid, but was unreliable with kidney (Magnússen et al. 1994). Although sensitive, this system took 1–2 days to carry out. In a further development, McIntosh et al. (1996) devised a simplified PCR invoking the 376-base pair region of the gene encoding the 57 kDa surface antigen. This system had a minimum detection limit of 5×10^3 renibacterial cells/ml in rainbow trout lymphocytes. Two 24-base oligonucleotide primers used to amplify a 501 base-pair region of the gene encoding the 57 kDa soluble protein (p57) formed the basis of a PCR, which was capable of detecting 2 renibacterial cells within individual salmonid eggs (Brown et al. 1994). This PCR was considered to have value for the screening of broodstock for the presence of BKD (Brown et al. 1994). A nested PCR amplifying a 320 bp fragment of the p57 antigen was suitable for detecting *Ren. salmoninarum* in ovarian samples (Pascho et al. 1998). Terminal-RFLP permitted the detection of ~30 CFU/mg of artificially inoculated kidney tissue (Nilsson and Strom 2002). A qPCR was developed to detect the pathogen in chinook salmon, and correlated well with ELISA at high levels of infection (Powell et al. 2005).

A clone, pRS47, of 5.1 kb, was used to develop a specific DNA probe (Hariharan et al. 1995). In a dot blot assay, the biotinylated pRS47/*Bam*HI insert probe hybridised with only DNA from three strains of *Ren. salmoninarum* but not with *Arthrobacter protophormiae, Aer. salmonicida, Cor. aquaticum, Car. piscicola, Micrococcus luteus, Ps. fluorescens, V. anguillarum, V. ordalii* or *Y. ruckeri.* With kidney tissue from fish challenged with *Ren. salmoninarum,* the dot blot assay was regarded as sensitive as culture and FAT. With the latter technique, samples negative by dot blot assay and culturing revealed the presence of ≤1 fluorescing object, presumed to be a bacterium/50 microscope fields. Consequently, this probe was regarded as having potential for the diagnosis of BKD (Hariharan et al. 1995).

A specific, sensitive real-time PCR was developed to recognise BKD in kidney tissue, and was capable of detecting 1–10 *Ren. salmoninarum* genomes/reaction (Jansson et al. 2008). The method was deemed to be more sensitive than ELISA insofar as BKD was detected in 39.9 % of fish by real-time PCR, but only 28 % by ELISA incorporating polyclonal antisera (Jansson et al. 2008).

LAMP methods have been proposed as a sensitive [more sensitive than quantitative real time PCR] and specific method for the detection of *Ren. salmoninarum* (Saleh et al. 2008; Gahlawat et al. 2009). Saleh et al. (2008) developed a rapid, i.e. 1 h, system which amplified a fragment of the p57 gene, and was specific to *Ren. salmoninarum,* and detected 1 pg of genomic DNA. Notwithstanding all the excellent publications, the dilemma remains about which method is best. Sandell and Jacobson (2011) compared different qPCRs and compared the value and sensitivity of *msa*/non-fluorescent quencher qPCR and *abc*/non-fluorescent quencher qPCR to ELISA.

Which Method Is Best? Confusion surrounds which of the available methods is most suitable for detecting and thus diagnosing the presence of BKD. Essentially, the opposing opinions include those who favour serology or molecular methods and those who recommend cultivation. Certainly molecular methods are now becoming accepted for their reliability (Etchegaray et al. 1991). However, it is generally difficult for most microbiologists to determine which of the alternative approaches is best, but comparative work on the various methods has been conducted (Bruno et al. 2007).

Comparing kidney and ovarian fluid from broodstock Atlantic salmon, the selective medium, SKDM (see Austin et al. 1983) detected a higher number of positive BKD samples than iFAT, which in turn was more sensitive than ELISA or western blots (Griffiths et al. 1996). Interestingly, renibacterium were found in either kidney or ovarian fluid, but not both. However, the use of culturing in SKDM broth followed by western blotting increased the sensitivity beyond the maximum level recorded by SKDM alone (Griffiths et al. 1996). This is interesting because the benefit of this broth stage in increasing the detection rate for renibacterium paralleled an observation with peptone water during the 1970s. Here, we found that preincubation of kidney, spleen and more importantly heart tissue in peptone water enhanced the level of resulting colonies, and therefore positivity, on solid medium. Another study concluded that ELISA, using a polyclonal rather than a monoclonal [this was less sensitive] antiserum, was more sensitive than SKDM. Here, SKDM detected *Ren. salmoninarum* in 45 % of kidney samples, compared to ELISA, which found that 50 % of the kidneys were positive (Jansson et al. 1996).

Pascho et al. (1987) compared five techniques for the detection of *Ren. salmoninarum* in coho salmon. The conclusion was that the ELISA (Dixon 1987) was most sensitive, followed by FAT, filtration-FAT, culturing, counterimmuno-electrophoresis and immunodiffusion. This view was echoed by results from Meyers et al. (1993), who regarded ELISA as more sensitive than FAT. Specifically, FAT did not detect *Ren. salmoninarum* in 80 % of the samples positive by ELISA. However, a complication was that in the same study FAT detected *Ren. salmoninarum* in 28 % of the samples negative by ELISA (Meyers et al. 1993). The comparative benefit of a membrane-filtration FAT over ELISA was illustrated when the former detected *Ren. salmoninarum* in 66/103 (= 64 %) ovarian samples compared to 40/103 (= 39 %) of positives with the latter (Pascho et al. 1998). The membrane-filtration FAT was capable of detecting ≥25 renibacterial cells/ml of ovarian fluid, whereas the ELISA was not consistent in detection at levels of $\leq 1.3 \times 10^4$ cells/ml (Pascho et al. 1998). Yet both methods were inferior to a nested PCR amplifying a 320 bp fragment of the p57 antigen. This PCR detected *Ren. salmoninarum* in all of the ovarian samples (Pascho et al. 1998). The method of McIntosh et al. (1996) for the detection of the p57 antigen by PCR was modified by using an improved DNA isolation procedure and by redesigning the forward primers and the conditions for carrying out the procedure to prevent false positives (Chambers et al. 2009). The modifications led to the minimum detection limit of only 5–72 CFU/mg of head kidney. Okuda et al. (2008) opined that there was not any difference in the detection levels between iFAT and PCR.

Following an examination of 1239 kidney samples, Gudmundsdóttir et al. (1993) considered that a double sandwich ELISA was more sensitive than culturing on SKDM. Yet, Bandín et al. (1996) reported the comparatively high cut-off for ELISA of ~10^6 bacteria/g of tissue. An amount of 0.3 μg of antigen/ml was noted by Olea et al. (1993). Sakai et al. (1989a, b) favoured the indirect dot blot assay (Sakai et al. 1989a, b; Sakai and Kobayashi 1992) [involving peroxidase and diaminobenzidine tetrahydrochloride as enzyme and substrate, respectively], which detected 10^2 cells/g of kidney tissue, over iFAT, co-agglutination, microscopy by Gram-stain, immunodiffusion and latex agglutination. Confirmatory diagnoses were made using dot blot and western blot assays (Sakai et al. 1990; see also Olivier et al. 1992). Certainly, Griffiths et al. (1991) highlighted the value of western blots over FAT and culturing for the detection of renibacteria. Immunohistochemistry is another approach, which is gaining popularity for the efficient detection of BKD. In particular, the indirect peroxidase technique, as applied to tissue sections, was deemed to be more sensitive than iFAT or Gram-staining (Hoffman et al. 1989). Further work has also highlighted the value of the peroxidase-antiperoxidase immunohistochemical technique for the detection of *Renibacterium* (Jansson et al. 1991). Mucus quantitative RT-PCR was regarded as the only non-lethal system with the necessary sensitivity and specificity, as an alternative to the lethal approach of examining kidney (Elliott et al. 2015).

Whereas debate has centred over the most effective means of detecting BKD, it may be concluded that effective diagnosis should encompass a multiplicity of methods. These include isolation and characterisation, and serology on infected tissue. It is proposed that clinical cases of disease should be examined by FAT and culturing methods. Asymptomatic cases should be the subject of full bacteriological examination.

Epizootiology

To date, there has been no evidence to suggest that *Renibacterium* is a component of the normal aquatic microflora. Indeed in one study, water and sediment from 56 fish farms were examined for the presence of renibacteria, but to no avail (Austin and Rayment 1985). Twelve days after experimentally infecting Chinook salmon with a high challenge dose, which led to infections with high numbers of renibacterial cells as determined by ELISA and FAT, the pathogen could be detected in the water (McKibben and Pascho 1999). Survival experiments confirmed that *Renibacterium* could survive in fish tank sediment/faecal material for up to 21 days in the absence of any fish. However, the organism was not at any time recovered from the overlying water, suggesting that renibacteria have an affinity with organic matter. Longer survival times of 13 weeks in river but not ground water were reported by Hirvelä-Koski (2004). The question regarding survival of the pathogen in water was the topic of detailed experimentation. This confirmed earlier work that laboratory-grown cultures were short-lived in river water. In the absence of

indigenous water-borne organisms, i.e. using filter-sterilised river water, renibacterial cells survived for 28 days, after which there was a rapid decline in numbers. Essentially, these data show that renibacteria have the potential to survive outside of fish for limited periods, although in water it is probably unable to compete with members of the normal aquatic microflora (Austin and Rayment 1985). In one study, the pathogen was found only in association with asymptomatic and clinically diseased fish (Austin and Rayment 1985). In another investigation, it was determined that the blue mussel *(Mytilus edulis)* cleared and killed most *Ren. salmoninarum* cells from seawater (Paclibare et al. 1994). However, some renibacterial cells could be found in mussel faeces during settling. Yet, it was conceded that mussels were unlikely to pose a realistic threat to fish farms regarding the survival and spread of renibacterium. But what if infected mussels are transferred to clean seawater? The answer according to Paclibare et al. (1994) was that the mussel cleared *Ren. salmoninarum* from within them upon transfer to clean sites. Clearly, early studies may have been hampered by lack of a suitable selective medium. Nevertheless, the use of SKDM has not, as yet, produced any definite evidence to suggest a non-fish reservoir for the organism (Austin et al. 1983; Embley 1983; Austin and Rayment 1985). The precise source of infection is unclear, but may include clinically or asymptomatically diseased fish (Wood and Wallis 1955; Wolf 1966; Bucke 1978; Mitchum et al. 1979; Paterson et al. 1979; Fryer and Sanders 1981). The organism has been recovered from faeces of both cultured and wild salmonid stocks. According to Balfry et al. (1996), renibacterium is shed from faeces, and may survive in seawater for a week. Attention has also been focused on the role of eggs in the transmission of BKD ('vertical' transmission) (Allison 1958; Wolf 1966; MacLean and Yoder 1970; Mitchum et al. 1979; Lee and Evelyn 1989). Allison (1958) indicated the involvement of eggs when BKD occurred following transfer of ova from an infected site. Similarly, Bullock et al. (1978)) implicated disinfected eggs of chinook salmon in the spread of the disease. Moreover, the preliminary data of Paterson et al. (1981) pointed to the presence of *Renibacterium* within fertilised eggs. Evelyn et al. (1984) demonstrated the presence of renibacteria in 11.6–15.1 % of eggs from a coho salmon which was infected with BKD, such that the coelomic fluid was cloudy because of high numbers of the organism. These authors suggested that *Ren. salmoninarum* was present in the yolk of the eggs, even after treatment with erythromycin (Evelyn et al. 1986a). Artificial contamination experiments led to the observation that infection occurred by entry of renibacterial cells from the egg surface to the perivitelline space through the micropyle during water-hardening (Kohara et al. 2012). Of greater significance was the finding that iodophors were ineffective at preventing intra-ovum infections. However, using rainbow trout and amago salmon (*Oncorhynchus masou*), the pathogen was not isolated from egg contents suggesting that there was minimal risk of intra-ovum infection of salmonid eggs in the coelomic cavity (Kohara et al. 2013).

The manifestation of the disease is complicated by certain environmental factors, including water hardness (Warren 1963), temperature, salinity and diet. Belding and Merrill (1935) were the first workers to describe the seasonal nature of BKD, with a correlation between water temperature and level of mortality. Earp et al. (1953)

found that BKD occurred over a wide range of water temperatures from 8 to 18 °C. Most epizootics occurred in the autumn and winter, i.e. during periods of declining water temperatures. However, most mortalities occurred at higher temperatures, a conclusion which has been echoed by Austin (1985), although the reverse has also been reported (Sanders et al. 1978). At low temperatures, the effect was the continual loss of small numbers of fish (Snieszko and Griffin 1955). This is quite a feat for a supposedly unreactive organism!

BKD has been diagnosed in fish following movement from fresh to seawater (Earp et al. 1953; Bell 1961). Indeed, the disease may be of paramount importance in the ability to acclimatize to seawater (Frantsi et al. 1975) and the survival of salmonids in the oceanic environment (Fryer and Sanders 1981).

Data obtained with ELISA have shown that *Ren. salmoninarum* occurs commonly, in the absence of pathological signs of BKD, in wild fish, i.e. Arctic charr and brown trout in Iceland (Jónsdóttir et al. 1998). Thus, the route of transmission to aquaculture may be from wild fish.

The effect of nutritional (dietary) status on the development of BKD is only partially understood. Some diets, notably those containing corn gluten (Wedemeyer and Ross 1973) or lipid (Austin 1985), enhanced the disease. Nutritional studies with Atlantic salmon have shown that levels of vitamin A, iron and zinc are lowered in BKD infected fish (Paterson et al. 1981). Subsequent experiments in which fish were administered diets rich in trace elements resulted in reduced incidences of BKD. This theme should be exploited further for control purposes.

In fish culture, *Ren. salmoninarum* appears to be a most unaggressive organism, generally devoid of much production of exoenzymes (exotoxins). Yet, it causes such a severe problem in salmonids. With lack of evidence to the contrary, it is our hypothesis that the organism is a normal resident of some fish, in (or on) which it exists, probably in fairly low numbers. Conceivably, it may be a normal resident of kidney tissue, forming a synergistic or controlled parasitic relationship with the host, possibly in the macrophages. Alternatively, it may be a normal resident of the digestive tract (Austin 1986). To continue the scenario, we postulate that at times of stress to the host, such as sub-clinical infections, damage to the digestive tract, starvation, kidney damage or temperature shock, the organism is able to migrate to the kidney (if not already there) and multiply. This would lead ultimately to the condition known as BKD. The problems with recovery of the organism, particularly from asymptomatic fish, may be explained within the realms of this concept. Evelyn and co-workers (Evelyn 1978; Evelyn et al. 1981) have considered the presence of inhibitors in the kidney that suppress the development of *Renibacterium* on solid medium. Could these unnamed compounds control the growth and development of the organism in healthy fish? This remains a possibility. However, there are other equally plausible explanations, namely dormancy, damage, or the presence of altered - osmotically fragile - cells. The renibacteria may normally be in a dormant or altered phase within the fish and, thus, would require to be triggered back into activity in order to produce colonies. Alternatively, renibacteria may be in some way damaged in the fish, and require repair before being able to produce colonies. This parallels the problem of damaged coliform bacteria in the aquatic environment

(Olson 1978). Of course, it is also possible that the media are deficient in certain essential nutrients, necessary for the replication of *Renibacterium.* A long lag phase, which has been suggested by Embley (1983), would be necessary for the organism to adjust to the new environment of the laboratory medium, prior to replication. Any or all these possibilities could apply to *Renibacterium.* Careful thought is necessary to unravel many of the mysteries still surrounding the biology of this pathogen.

Pathogenicity

Pathogenicity experiments have met with varying degrees of success. Mackie et al. (1933, 1935) succeeded in transmitting 'Dee disease' to brown trout by subcutaneous and i.m. injections of emulsified spleen from Atlantic salmon. In these experiments, death followed in 5 weeks, although typical lesions, as found in field situations, did not occur. A similar observation was made by Belding and Merrill (1935), injected, intramuscularly, brook trout with purulent material collected from kidney abscesses in the same species. Death followed in 18–25 days, but characteristic BKD lesions did not occur. This was, however, achieved by Earp (1950) following the injection of chinook salmon with a pure culture of the BKD organism. Koch's postulates were finally satisfied by Ordal and Earp (1956) following the establishment of BKD in chinook salmon after i.p. injection of an organism obtained from sockeye salmon. Mortalities started after 12 days, and continued until day 23, when all the fish were dead. At this point, the organism was re-isolated. Sakai et al. (1989b) found mortalities began 17 days after rainbow trout were injected with 4×10^8 cells. In comparison, carp (*Cyprinus carpio)* were markedly resistant. Failure greeted the attempt by Snieszko and Griffin (1955) to transmit BKD to brook trout by co-habiting with diseased fish for 21 days, followed by feeding with infected viscera. However using feeding, success was achieved by Wood and Wallis (1955) with 100 % infection of 993 chinook salmon fingerlings. Later, Wolf and Dunbar (1959) achieved success by immersing experimentally wounded brook trout into a suspension of the pathogen. Murray et al. (1992) succeeded in inducing BKD in chinook salmon by immersion (10^4–10^6 cells/ml for 15–30 min) and co-habitation with other experimentally infected fish. However, the time to death was much longer than in most experimental models. By co-habitation and immersion, the average periods leading to mortalities were 145 and 203 days, respectively. Transmission from wild to cultured fish has been reported (Mitchum and Sherman 1981) and *vice versa* (Frantsi et al. 1975). Prior infection with *Ren. salmoninarum* may well contribute to the poor survival of coho salmon upon transfer from fresh to sea water (Moles 1997).

Evidence has pointed to the ability of *Ren. salmoninarum* becoming internalised within non-phagocytic cells (González et al. 1999) and macrophages in which putative virulence factors are produced (McIntosh et al. 1997). Fish cell lines coupled with iFAT were used to study the internalisation of the pathogen with results revealing that *Ren. salmoninarum* became localised in the vacuoles of CHSE-214 and

RTG-2 cells with some escape into the cytoplasm (González et al. 1999). Within the phagocytic cells, renibacterium exhibits a slow rate of division, and survives certainly for 10 or more days (Gutenberger et al. 1997). Conversely, the macrophages may well inhibit the growth of and kill renibacterium by the live bacterial cells generating respiratory burst products (Hardie et al. 1996; Campos-Pérez et al. 1997). With this scenario, exposure to *Ren. salmoninarum* would enhance the killing activity of the macrophages (Hardie et al. 1996).

The hydrophobic, soluble cell surface p57 protein is common to all isolates (Wiens and Dale 2009), and is released in large quantities as a monomer into the external environment from broth cultures and in infected fish (Wiens et al. 1999). It is responsible for cell agglutination e.g. of salmonid leucocytes (Senson and Stevenson 1999; Wiens et al. 1999), and is encoded by *msa* [= major soluble antigen] genes – *msa1* and *msa2* and *msa3* [this is a duplicate of *msa1* but is not present in all isolates of *Ren. salmoninarum;* Rhodes et al. 2002, 2004a], both *msa1* and *msa2* are needed for complete virulence (Coady et al. 2006). It is produced in comparatively large amounts and consequently has been a target for vaccine development. The role of p57 protein in the pathogenicity process has prompted some excellent research. Incubation of *Ren. salmoninarum* at 37 °C for >4 h decreased cell surface hydrophobicity (this decrease was negated by pre-incubation in PMSF), as measured by salt aggregation, and decreased the quantity of cell-associated p57 protein (Piganelli et al. 1999). Cell surface hydrophobicity was re-instigated following incubation in ECP; reflecting re-association of the p57 protein onto the bacterial cell surface (Piganelli et al. 1999). An attenuated culture, MT 239, differs from virulent isolates in expressing less p57 protein (O'Farrell and Strom 1999). It has been demonstrated that a Norwegian isolate, strain 684, lacked a specific epitope [designated 4C11] and contained single alanine to glutamine substitution in the amino terminal region resulted in enhanced binding to leucocytes from Chinook salmon (Wiens et al. 2002).

There is a divergent opinion as to the presence of biological activity in ECP of *Ren. salmoninarum*. One view is that the ECP is generally devoid of extracellular enzymes; haemolytic and cytolytic activity being absent (Bandín et al. 1991). Yet in other investigations, proteases (Sakai et al. 1989b) and haemolysins (Grayson et al. 1995a, 2001) have been detected. ECP at 0.1 mg/ml and 1.0 ml/ml inhibited respiratory burst but not phagocytic activity in brook trout splenic phagocytes (Densmore et al. 1998). Hydrophobicity, haemagglutination and haemolysin activity to rabbit and trout erythrocytes have been recorded from water soluble extracts (proteins) (Bandin et al. 1989; Daly and Stevenson 1987; 1990; Evenden et al. 1990). In particular, hydrophobicity and auto-aggregation have been linked with virulence (Bruno 1988). *Ren. salmoninarum* has agglutinated spermatozoa from salmonids and goldfish (Daly and Stevenson 1989). Shieh (1989b) reported an unidentified toxin from *Renibacterium,* which was lethal to fingerling Atlantic salmon. Also, an iron acquisition mechanism has been found (Grayson et al. 1995b).

There is some evidence that fish respond to infection with renibacterium by the production of stress factors, including plasma cortisol and lactate, and reduced levels of plasma glucose (Mesa et al. 1999). Thus, a 70 kDa stress protein (HSP70) was recognised in coho salmon with BKD (Forsyth et al. 1997).

Disease Control

Slaughter of Infected Stock Evidence from Iceland has revealed that the culling of infected Atlantic salmon brood stock led to a reduction in the incidence of BKD (Gudmundsdóttir et al. 2000). At the start of the programme the incidence of infection was reported as ~35 % of the broodstock on two ranch sites, but after a few years of adopting the programme of culling, the incidence fell to <2 % (Gudmundsdóttir et al. 2000).

Disease Resistant Fish Work has pointed to some strains of fish which are resistant to BKD (Suzumoto et al. 1977; Winter et al. 1979; Withler and Evelyn 1990) and selective breeding enhance resistance to ERM and RTFS (Henryon et al. 2005). Studying comparative resistance to BKD in three juvenile coho salmon and steelhead trout strains (transferrin genotypes AA, AC and CC), it was found that the AA genotype was the most susceptible to BKD, whereas the CC genotype was the most resistant. Withler and Evelyn (1990) found a variation in resistance to BKD in two strains of coho salmon from British Columbia, Canada. In particular, survival was greater and the time to death was longer in juvenile animals from the Kitimat River strain than from the Robertson Creek strain.

Vaccine Development There is evidence that under some conditions, renibacteria elicit a humoral and innate immune response in fish (Sanders et al. 1978; Young and Chapman 1978; Bruno 1987; Jansson and Ljungberg 1998; Jansson et al. 2003), for example directed to metalloprotease and haemolysin (Grayson et al. 2001). However, it is apparent that early exposure to the p57 antigen can lead to long term immunosuppression (Brown et al. 1996). Conversely, removal of the p57 antigen from the surface of renibacterial cells has led to enhanced immunogenicity (Wood and Kaattari 1996). Administration of experimental vaccines prepared in FCA resulted in the development of humoral antibody (Evelyn 1971; Baudin-Laurençin et al. 1977). Evelyn (1971) detected antibodies in immature sockeye salmon at least 16 months after an i.p. injection with a heat-killed suspension in adjuvant. A second injection after 13 months resulted in a sharp increase in antibody titre from 1:2560 (after the first injection) to 1:10247. The protective ability of vaccines is, however, questionable (Sakai et al. 1989c, 1993). Sakai et al. (1993) compared formalised (RPS = 10–23.8 %), heat-killed, pH lysed (RPS = 35–36 %) and UV killed (RPS = 25 %) cells of *Ren. salmoninarum* and streptococci, and concluded that protection of rainbow trout did not develop. Also, Baudin-Laurençin et al. (1977) found no protective effect after injection of coho salmon with cells contained in FCA. Paterson et al. (1981), using a similar vaccine in Atlantic salmon, reported high agglutination titres and a reduced incidence of BKD lesions after 1 year, but FAT revealed the same number of bacteria in both vaccinated and unvaccinated (control) fish (Paterson et al. 1981). Although McCarthy et al. (1984) reported optimistically that their vaccine worked in fish, close scrutiny of the data suggests success comparable to that of Paterson et al. (1981). McCarthy and co-workers used a

number of vaccine formulations without adjuvants, including a formalised (0.3 % v/v formaldehyde) suspension of cells grown in KDM2, a lysed cell suspension (this was lysed at pH 9.5 by the addition of 10 N sodium hydroxide for 1 h, after which the pH was re-adjusted to 7.2 with 10 N hydrochloric acid), and 50 % concentrates of the vaccine. Juvenile rainbow trout were vaccinated by i.p. injection, hyperosmotic infiltration, and by 2 min immersion. Vaccinated fish were maintained for 6 weeks at 11 °C and then challenged by i.p. injection with living cells of the homologous organism. Best success occurred with the lysed preparation, administered by i.p. injection, although failure greeted attempts to vaccinate fish by immersion or hyperosmotic infiltration. When ≥80 % of the unvaccinated controls were infected, ≤10 % of the vaccinated fish were affected. This seems encouraging until it is realised that the workers measured the presence of infection by the presence of macroscopic lesions and the occurrence of Gram-positive bacteria in the anterior part of the kidney. The occurrence of carriers could not be assessed, because the Gram staining method is not the most sensitive technique for ascertaining the presence of renibacteria. Attenuated cells of *Ren. salmoninarum* or *Arthrobacter davidanieli* (Salonius et al. 2005) (a commercially available live vaccine named Renogen) gave limited protection but addition of purified *Ren. salmoninarum* genomic DNA or synthetic oligodeoxynucleotides did not improve protection of chinook salmon following i.p. challenge with a virulent culture (Rhodes et al. 2004b). More recently, a comparison was made between inactivated whole cells of two cultures including the type strain without or without prior heating at 37 °C for 48 h that destroys the p57 antigen, a recombinant product based on the p57 antigen in FIA, Renogen and PBS with or without FIA. Following i.p. injection vaccination, the chinook salmon were cohabited with mortalities recorded up to 285 days with the result that protective immunity was not demonstrated in any group (Alcorn et al. 2005).

Although renibacterium is normally regarded as being nutritionally fastidious, two "strains" were isolated from colonies on KDM2 that could grow on regular laboratory media, i.e. TSA and BHIA., and were non-pathogenic when injected i.p. into Atlantic salmon at a dose of 5×10^6 (Daly et al. 2001). When evaluated as live vaccines, the culture which grew on TSA (= Rs TSA1) led to an RPS of 50 and 74 % at 74 and 60 days after challenge (Daly et al. 2001).

Dietary Supplements Paterson et al. (1981) discussed the importance of nutrition in the manifestation of BKD in Atlantic salmon. These workers noted that infected fish had lower serum levels of vitamin A, zinc and iron than uninfected animals. Subsequent experimentation showed that the level of BKD could be reduced by feeding with high levels of trace elements, notably cobalt, copper, iodine, iron, fluorine and manganese, and reducing the quantity of calcium. In further experiments, Lall et al. (1985) concluded that high levels of iodine and fluorine, each dosed at 45 mg/kg of food, reduced the occurrence of natural infections of BKD to 3 % and 5 %, respectively, as compared to 95 % and 38 % infection in Atlantic salmon fed with commercial diets. Earlier, Woodall and Laroche (1964) demonstrated a reduction in BKD infections by feeding chinook salmon with high levels

of iodine (i.e. 10.1 µg/g). This theme was continued by Bell et al. (1984), who investigated the effects of sodium-L-ascorbate, zinc, iron and manganese as dietary supplements on the manifestation of BKD. They noted that survival time was inversely related to dietary ascorbate levels when the food was otherwise low in zinc and manganese.

Disinfection Disinfection of egg surfaces has also been utilised to control BKD. Iodophors, at 25–100 mg/l for 5 min, have proved beneficial at reducing transmission of the disease (Amend and Pietsch 1972; Ross and Smith 1972; Bullock et al. 1978), although they will not eliminate the pathogen from inside eggs (Evelyn et al. 1984). The use of erythromycin phosphate, at 1–2 mg/l for 30 min, has been advocated as an additive for water-hardening of eggs (Klontz 1978). However, it is debatable whether or not it is wise to use antibiotics in this way.

Another approach has been to disinfect the water in fish farms. In particular, a level of only 0.05 mg of free chlorine/l was sufficient to inactivate cells of the pathogen in 18 s (Pascho et al. 1995). With such rapid inactivation, there must surely be a use for the technique in hatcheries.

Use of Antimicrobial Compounds Chemotherapy offers some promise of success (Bandín et al. 1991). Although BKD has become regarded as one of the most difficult bacterial fish diseases to treat (Bullock et al. 1975; Fryer and Sanders 1981), some success at chemotherapy has been reported with erythromycin (Wolf and Dunbar 1959), sulphonamides (Rucker et al. 1951), chloramphenicol (Rucker et al. 1953; Wood and Wallis 1955; Millan 1977), penicillin (Decew 1972), clindamycin, kitasamycin and spiramycin (Austin 1985) and enrofloxacin [Baytril] (Hsu et al. 1994). A MIC of 0.25–05 µg of enrofloxacin/ml was calculated (Hsu et al. (1994). Furthermore, some beneficial effects have been indicated from trials using a dose of 20 mg of enrofloxacin/kg body weight/day for 10 days when there was a reduction in mortalities compared to controls. Over two trials, the deaths in the treated groups and the controls were 43 % and 72 %, and 93 % and 100 %, respectively (Hsu et al. 1994). In addition, cephradine, lincomycin and rifampicin were found to be effective for prophylaxis of BKD, although they were of no use for therapeutic purposes (Austin 1985). Undoubtedly, many of the problems with control measures revolve around the intracellular nature of the organism (Young and Chapman 1978). Quite simply, many of the drugs probably do not reach the actual foci of infection. Nevertheless, experiments with liposomes, which target drugs to given organs, proved to be disastrous, insofar as BKD was exacerbated (Austin 1985). Perhaps, the value of micro-encapsulation techniques should be assessed.

The pioneering work with drugs for the control of BKD was undertaken by Rucker et al. (1951). They reported a decrease in the level of mortalities following the administration of sulphadiazine, via the oral route, at 250 mg of drug/kg body weight of fish/day for 15 days. This was confirmed by Earp et al. (1953) and Allison (1958). However, the drug failed to eliminate the pathogen from the fish. Subsequently, Wolf and Dunbar (1959) in a comparison of 34 compounds concluded

that erythromycin, dosed at 100 mg of drug/kg body weight of fish/day for 21 days, gave the best result. The value of erythromycin at this concentration was confirmed by Austin (1985), although it was suggested that treatment need only be continued for 10 days. Erythromycin has also been reported to prevent vertical transmission of renibacteria (Evelyn et al. 1986b; Brown et al. 1990). Also, an injection of 20 mg of erythromycin/kg of broodstock fish is useful in preventing vertical transmission (Lee and Evelyn 1994). However, there is evidence for the development of a reduction in susceptibility to macrolide antibiotics (Rhodes et al. 2008).

Aerococcaceae Representative

Aerococcus viridans

Characteristics of the Disease

A disease, which led to losses of 30–40 %, was reported among farmed tilapia in Southwest China during 2010, with signs including congestion of the gills and abdomen, swollen gall bladder, and diffuse liver. Some fish displayed exophthalmia and spiral swimming behaviour (Ke et al. 2012).

Isolation

Isolation was achieved from brain and liver samples from moribund tilapia, using BHIA and sheep blood agar with incubation at 28 °C for up to 48 h.

Characteristics of the Pathogen

Box 3.3: ***Aerococcus viridans***
Off-white colonies are 1–2 mm in diameter after incubation for 48 h at 28 °C, and contain non-motile, α-haemolytic Gram-positive cocci of 0.6–2.0 μm in diameter in pairs, tetrads or small clusters. Neither arginine dihydrolase, catalase, ß-N-acetyl-glucosamine, Ala-Phe-Pro-arylamidase, alkaline phosphatase, glycyl tryptophan arylamidase, α-or ß-galactosidase, ß-glucuronidase, leucine aminopeptidase, pyroglutamic acid arylamidase, oxidase nor urease is produced. The Voges Prokauer reaction is negative. Growth occurs in 6.5 % (w/v) NaCl (Ke et al. 2012).

By use of the Rapid ID 32 Strep system, identification of *Aerococcus viridans* was achieved (99 % certainty of a correct identification. This was confirmed by

sequencing of the 16S rRNA gene (homology = >99.9 % to *Aerococcus viridans)* (Ke et al. 2012).

Pathogenicity

Two isolates were injected intraperitoneally (3.0×10^6 CFU/fish) and administered by bathing (2 h in 1.5×10^7 CFU/ml) to tilapia leading to mortalities (85 % and 45 %, respectively) with signs similar to those observed on the affected fish farm (Ke et al. 2012).

Bacillaceae Representatives

Bacillus spp.

Characteristics of the Disease

The initial outbreak of disease during 1989–1991 led to mortalities of 10–15 % of farmed populations of *Clarias carpis, Clarias gariepinus, Clarias nigrodigitatus, 'Heteroclarias'* and *Heterobanchus bidorsalis* in Nigeria (Oladosu et al. 1994). Diseased fish were characterised by weakness, lethargy, emaciation and generalised necrotising dermatitis, with death occurring in a few days. Blood tinged fluid was present in the peritoneal cavity. Petechia and focal necrosis was evident in the liver and kidney. The spleen was enlarged, soft and friable. The myocardium was described as soft and flabby. The stomach was hyperaemic (Oladosu et al. 1994). Gram-positive rods of 1–4 μm in length were observed. It should be emphasised that skin lesions revealed the presence of *Aeromonas* and *Fla. columnare.*

Bacillary necrosis was described in farmed populations of catfish *(Pangasius hypophthalmus)* from the Mekong Delta, Vietnam. Mortalities among fish that did not otherwise respond to treatment were observed, and the disease signs centred on 1–3 mm diameter white necrotic and granulomatous areas in the kidney, liver, spleen and viscera. Apart from the presence of mostly myxosporean parasites, an organism considered as an unspeciated *Bacillus* was recovered (Ferguson et al. 2001).

Isolation

Oladosu et al. (1994) relied on nutrient agar and incubation at the comparatively high temperature of 37 °C for an unspecified period to isolate *Bacillus* spp.

Characteristics of the Pathogens

Box 3.4: *Bacillus* sp.
Using nutrient agar plates with an incubation temperature of 37 °C, cream, rough opaque colonies may be obtained. These colonies comprise non motile, fermentative Gram-positive rods of 1–4 μm in length, which contain central and oval endospores. The cells grow at 45 °C but not 50 °C, and are not haemolytic.

From these data, a link with *Bacillus* was made. However, there is insufficient information to achieve a proper identification.

A second report appertained to bacillary necrosis among catfish in Vietnam. The bacteria were described as comprising 1 mm diameter, cream coloured colonies on TSA after 24 h incubation at 28 °C. The cells were Gram-variable, long thin motile, oxidase-positive rods, that were unreactive towards sugars, and grew at 15–37 °C. H_2S was produced, and gelatin was attacked. Curiously, there was not any mention about the presence of endospores. However, by 16S rRNA sequencing, the nearest match at 95 % homology was *Bacillus fumarioli* (Ferguson et al. 2001).

Pathogenicity

Oladosu et al. 1994) infected *Clarias gariepinus* via the oral and subcutaneous routes with a comparatively low dose of 0.5 ml, which contained 1.8×10^3 cells/ml. Thus, 60 % and 30 % mortalities were achieved over a three-week period by oral and subcutaneous challenge, respectively.

Ferguson et al. (2001) reported that 2×10^7 cells of the putative *Bacillus* injected intraperitoneally led to clinical disease.

Disease Control

Use of Antimicrobial Compounds It was reported that *Bacillus* sp. was sensitive to tetracycline but not penicillin (Oladosu et al. (1994).

Bacillus cereus

There has been occasional mention of *B. cereus* as a fish pathogen causing branchionecrosis in common carp (Pychynski et al. 1981) and striped bass (Baya et al. 1992a, b). However, the supporting evidence is weak.

Bacillus mycoides

Characteristics of the Disease

An epizootic occurred in channel catfish in Alabama during 1992. The fish were darker in colour, inappetant, displayed pale areas or ulcers on the dorsal surface, focal necrosis of the epaxial muscle, and opaque muscle (Goodwin et al. 1994). Histopathological examination revealed the presence of chains of Gram-positive rods.

Isolation

Material from ulcers, brain, kidney, liver and necrotic muscle were inoculated onto a range of media, including 5% (v/v) sheep blood in blood agar base, Mueller Hinton agar (for example, as supplied by Difco or Oxoid) and BHIA within incubation at an unspecified temperature for an unstated duration (Goodwin et al. 1994). Raised, rhizoidal colonies with filamentous swirling patterns developed.

Characteristics of the Pathogen

Cultures were considered to possess the key characteristics of *B. mycoides,* as follows:

Box 3.5: *Bacillus mycoides*
Cultures are rhizoidal, and contain non motile Gram-positive rods with oval endospores. Parasporal crystals are not observed. Indole is not produced. The Voges Proskauer reaction is positive. Blood (haemolysis), casein, gelatin, lecithin and tyrosine are degraded. Acid is produced from D-glucose. Resistance is recorded to penicillin. Growth does not occur at 45 °C (Goodwin et al. 1994).

Diagnosis

Bacillus mycoides could be distinguished from other bacilli, as follows (Goodwin et al. (1994)):

	Presence of parasporal crystals	Rhizoidal growth	Motility	Growth at 45 °C
Bacillus mycoides	–	+	–	–
Bacillus anthracis	–	–	–	+
Bacillus cereus	–	–	+	+
Bacillus thuringiensis	+	–	+	+

Pathogenicity

Injection of 1.6×10^4 cells intramuscularly led to lesions in channel catfish, as described in the original outbreak (Goodwin et al. 1994). Intraperitoneal and subcutaneous injections did not lead to the development of any lesions in the infected fish.

Disease Control

Use of Antimicrobial Compounds Sensitivity was reported to erythromycin, nalidixic acid, nitrofurazone, novobiocin and oxytetracycline but not to Romet (= orthometoprim-sulphadimethoxine) (Goodwin et al. 1994).

Bacillus subtilis

There has been one reference to *B. subtilis* as a fish pathogen, causing branchionecrosis in common carp (Pychynski et al. 1981). However, the supporting evidence is weak.

Corynebacteriaceae Representatives

Corynebacterium aquaticum

Characteristics of the Disease

The organism was associated with 3-year old striped bass in an experimental aquaculture facility in Maryland, USA during December, 1990. Fish displayed pronounced bilateral exophthalmia, and contained the organism in brain tissue (Baya et al. 1992b). Fish stopped feeding, swam more slowly, and died (at this point

the eyes were ruptured). Internally, the only disease sign was that the brain was haemorrhagic, and the cranium was full of blood.

Isolation

Brain tissue samples were plated onto BHIA and TSA with incubation at 25 °C for 48–72 h (Baya et al. 1992b).

Characteristics of the Pathogen

Characteristics of the organism were, as follows:

Box 3.6: *Corynebacterium aquaticum*
Colonies are 1–3 mm in diameter and exhibit a yellow non-diffusible pigment after incubation at 25 °C for 48 h. Cultures comprise motile, non-spore-forming, non-acid fast, slightly pleomorphic (club shapes and angular arrangements) Gram-positive rods, which are neither fermentative nor oxidative, and produced alkaline phosphatase, catalase, ß-galactosidase, α-glucosidase, pyrazinamidase, pyrrolidonyl arylamidase, but not N-acetyl-ß-glucosaminidase, arginine dihydrolase, ß-glucuronidase, H_2S, indole, lysine or ornithine decarboxylase, oxidase or phospholipase. Aesculin, blood (ß-haemolysis; only at 37 °C,) casein and gelatin are degraded, but not urea. Nitrates are not reduced. Citrate is not utilised, nor is acid produced from any of the carbohydrates examined. The Voges Proskauer reaction is positive. Growth occurs at 4–42 °C and in 0–5 % but not 8 % (w/v) sodium chloride.

Identification was achieved using the API-Coryne system, and comparison to the type culture of *Cor. aquaticum* ATCC 14665. The fish isolate and reference culture agglutinated with antisera prepared against both strains. Discrepancies with the named reference culture included growth at 4 °C and in 5 % (w/v) sodium chloride, degradation of casein and gelatin, nitrate reduction, pyrrolidonyl arylamidase, and N-acetyl-ß-glucosaminidase (Baya et al. 1992b). Also, the fish isolate differed from the reference culture in the precise composition of the membrane proteins, as determined by western blotting. However, both cultures shared a 68 kDa major antigenic protein (Baya et al. 1992b).

Epizootiology

Apart from fish, the organism was recovered from water and the 'scum' forming at the air-water interface on the tank walls (Baya et al. 1992b).

Pathogenicity

The fish isolate, RB 968 BA, killed rainbow trout and striped bass, with LD_{50} doses calculated as 5.8×10^4 and 1.0×10^5, respectively (Baya et al. 1992a). Experimentally infected fish developed haemorrhaging in the cranial cavity, but did not develop any external signs of disease. ECP, which contained caseinase and gelatinase activity, was harmful to fish, with an LD_{50} dose equivalent to 1.2 μg of protein/g of fish.

Disease Control

Use of Antimicrobial Compounds The organism was sensitive to ampicillin, erythromycin, oxytetracycline and potentiated sulphonamide, one or more of which may be useful for chemotherapy (Baya et al. 1992b).

Coryneform Bacteria

Occasional mention has been made of the role of coryneforms as fish pathogens. Ajmal and Hobbs (1967) referred to *Corynebacterium* infections in rudd, salmon and trout. However, there may have been confusion with BKD, the aetiological agent of which used to be regarded as *Corynebacterium* but is now classified as *Ren. salmoninarum.* Nevertheless during a routine examination of apparently healthy rainbow trout, Austin et al. (1985) recovered an organism with some of the salient features of *Ren. salmoninarum.* Cultures were subsequently assigned to the coryneform group of bacteria.

Box 3.7: Coryneforms
Growth occurs on BHIA and plate count agar, weakly on CLED, but not on MacConkey agar or TCBS. Characteristically, isolates are non-motile, non-acid-fast rods of 0.75 × 1.5–3.0 μm in size, which contain darkly stained intracellular granules. Growth occurs at 15 and 30 °C but not at 4 or 37 °C, and in 0 % and weakly in 2 % (w/v) sodium chloride but not at all in 4 % (w/v) sodium chloride, Catalase is produced, but not arginine dihydrolase, ß-galactosidase, H_2S, indole, lysine or ornithine decarboxylase, oxidase, phenylalanine deaminase or phosphatase. The methyl red test and Voges Proskauer reaction are negative. Nitrates are reduced to nitrites, weakly. Aesculin is degraded, but not blood, DNA, gelatin, lecithin or urea. Sodium citrate is utilised slowly. Acid is not produced from glucose.

As a result of pathogenicity experiments with rainbow trout (average weight = 8 g) maintained in fresh water at 18 °C, it was established that 1.25×10^6 cells, administered by i.p. injection, were capable of killing fish within a few days (Austin et al. 1985). However, it must be emphasised that the status of this organism to fish pathology is uncertain.

Microbacteriaceae Representative

Microbacterium paraoxydans

The organism was recovered from Nile tilapia in Mexico. The disease signs included lethargy, erratic swimming, melanosis, scale loss, blindness, exophthalmia and/or red or opaque eyes. Diseased tissues were inoculated onto TSA supplemented with 0.5 % (/v) glucose, BHIA supplemented with 5 % (v/v) sheep blood, MacConkey agar, glutamate-starch-phenol red agar, and TCBS with incubation at 30 +/– 1 °C for 24–48 h. Identification was achieved by sequencing of the 16S rRNA gene. Pathogenicity was confirmed in laboratory-based infectivity experiments using sole (*Solea vulgaris*) (Soto-Rodriguez et al. 2013).

Micrococcaceae Representative

Micrococcus luteus

Characteristics of the Disease

Disease signs were consistent with the notion of RTFS. Thus, moribund fish, in the size range of 0.5–5.0 g, displayed exophthalmia, pale gills, enhanced skin pigmentation and swollen abdomen. Internally, the kidney was swollen, the spleen was pale and elongated, and some ascitic fluid was present in the peritoneal cavity (Austin and Stobie 1992).

Isolation

Cultures were recovered following incubation of swabbed material (kidney, spleen and ascitic fluid) on skimmed milk agar with incubation at 25 °C for 48–72 h (Appendix in Chap. 12; Austin and Stobie 1992).

Characteristics of the Pathogen

Conroy (1966) described a single outbreak of disease, termed micrococcosis, in farmed rainbow trout from Argentina. However, the identification of that aetiological agent is uncertain. Nevertheless, during 1990, a "micrococcus" was associated with diseased rainbow trout fry in the U.K. (Austin and Stobie 1992). On a rainbow trout farm, deemed to harbour RTFS, large (~2 μm diameter) Gram-positive cocci, displaying a characteristic tetrad arrangement, were recovered from moribund fish.

Box 3.8: *Micrococcus luteus*
The cultures (8 in total were examined) comprise yellow-pigmented non-motile, oxidative Gram-positive cocci, which display a characteristic tetrad arrangement. Acid and alkaline phosphatase, catalase, esterase, leucine arylamidase, lipase, oxidase and phosphoamidase are produced, but not so α-or ß-galactosidase, H_2S, indole, lysine or ornithine decarboxylase or tryptophane deaminase. Nitrates are not reduced, nor is the Voges-Proskauer reaction positive. Casein and gelatin are degraded.

From these characteristics, it was apparent that the organisms matched the description of *Micrococcus luteus* (Kocur 1986).

Pathogenicity

Injection of 10^5 cells, via the i.m. and i.p. routes, led to 54 % mortalities in rainbow trout fry within 14 days (Austin and Stobie 1992).

Disease Control

Use of Antimicrobial Compounds Sensitivity was recorded to chloramphenicol, streptomycin, potentiated sulphonamides and tetracycline. These compounds may be useful at controlling the progress of infections in rainbow trout fry (Austin and Stobie 1992).

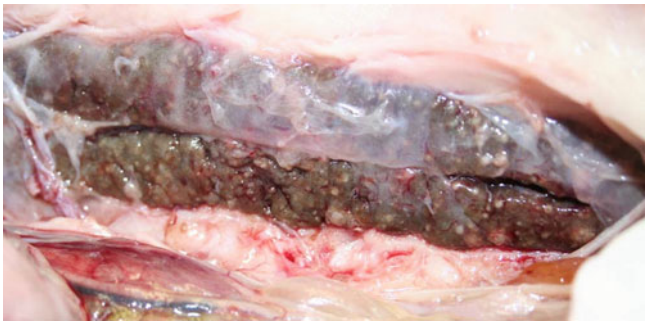

Fig. 3.7 Mycobacteriosis in yellowtail. Extensive granulomas are present on the liver and kidney (Photograph courtesy of Dr. T. Itano)

Mycobacteriaceae Representatives

Mycobacterium spp.

Characteristics of the Disease

The first report of acid-fast bacteria in freshwater fish (carp) was published by Bataillon et al. (1897)). This was followed over a decade later by an observation in marine fish (von Betegh 1910). Interest in infections caused by acid-fast bacteria continued with the isolation of *Myc. fortuitum* from diseased neon fish (*Paracheirodon innesi)* in 1953, although its identification was not reported until 6 years later (Ross and Brancato 1959). To date, mycobacteriosis (a term suggested by Parisot and Wood 1960) has been observed in >150 species of marine and freshwater fish (Nigrelli and Vogel 1963). The disease is especially troublesome in some geographical areas, such as striped bass populations in the Chesapeake Bay, USA (Latour et al. 2012).

Mycobacteriosis (fish tuberculosis) is a chronic progressive disease, with various external signs, including emaciation, inflammation of the skin, exophthalmia (Evely et al. 2011), open lesions and ulceration (e.g. Lansdell et al. 1993) and may attack many fish species, including Atlantic menhaden (Stine et al. 2005), rockfish (Whipps et al. 2003), shortfin molly (Poort et al. 2006), striped bass (Kaattari et al. 2005), turbot (dos Santos et al. 2002) and ornamental fish (Zanani et al. 2008). Internally, greyish white nodules (granulomas; Fig. 3.7) develop on various organs, particularly the liver, kidney, heart and spleen (Dulin 1979; Van Duijn 1981). The disease may take several years to progress from the asymptomatic state to clinical illness. Initially, the pigment may fade, and the fish appear sluggish with loss of appetite. If the skin is affected, blood spots develop with the ultimate formation of ulcers. In addition, fin and tail rot and the loss of scales may be seen.

Myc. abscessus was associated with 2–27 month old Japanese Meduka *(Oryzias latipes),* which had been cultured in the U.S.A. for aquatic toxicology testing (Teska

et al. 1997), and in milkfish in Taiwan (Chang et al. 2006). During a routine examination, granulomas, notably in the buccal cavity and vent, and a few acid-fast bacteria were noted in <1 % of the otherwise healthy fish. On clinically diseased fish, the disease signs would include listlessness, inappetance, swollen abdomen and visible granulomas (Teska et al. 1997). Milkfish displayed epithelial granulomas and red/grey nodules throughout the fish, which experienced 67 % mortalities (Chang et al. 2006).

Myc. avium. There has been one account of the organism associated with inappetance and granulomas on a captive epaulette shark (*Hemiscyllium ocellatum*) in The Netherlands (Janse and Kik 2012).

An organism considered to resemble *Myc. chelonei* was associated with granulomatous lesions resulting in high mortalities in cultured thread-sail filefish (*Stephanolepis cirrhifer*) in Japan during 2009 and 2010. Diseased fish displayed abdominal swellings and numerous white nodules containing thick, pale-yellow matter, which were located on the surface of internal organs (Fukano et al. 2015). Also, *Myc. chelonei* has been linked with non-tubercular, tumour-like skin and oral (cauliflower-like) lesions in 15 farmed sturgeon (*Acipenser gueldenstaedtii*) in Italy (Antuofermo et al. 2014).

Myc. fortuitum has been implicated with mycobacteriosis in farmed Nile tilapia in Mexico (Lara-Flores et al. 2014).

Myc. gordonae was recovered from guppy *(Poecilia reticulata)* in Thailand notably during rainy and/or cold periods. The fish, which experienced substantial mortalities, displayed inappetance, sluggish swimming behaviour, fin erosion, skin ulceration and the presence of systemic granulomas (Sakai et al. 2005).

Myc. marinum was first recognised from the liver, spleen and kidney of tropical coral fish kept in the Philadelphia Aquarium (Aronson 1926). As the name implies, the organism was considered to be only pathogenic to marine fish. But, it is now recognised to infect both marine and freshwater fish and also human beings (Van Duijn 1981). Currently, *Myc. marinum* is a major constraint on the farming of sea bass in Israel, leading to stunting and therefore loss of market value of the infected fish (Knibb et al. 1993), African catfish in Poland (Antychowicz et al. 2003), and in Chesapeake Bay, USA (Gauthier et al. (2004), in farmed meagre (*Argyrosomus regius*) in Turkey (Avsever et al. 2014; Timur et al. 2015), and in hybrid sturgeon in Taiwan (Chang et al. 2014). Here, an epizootic has developed, and fish experimentation has revealed the development of large aggregates of macrophages, which contain phagocytosed bacteria, with *Myc. marinum* contained exclusively within phagosomes. The organism was associated with mycobacteriosis in Brazilian flounder (*Paralichthys orbignyanus*), in barber goby (*Elacatinus figaro*) in Brazil (Romano et al. 2012), in Nile tilapia in Mexico (Lara-Flores et al. 2014), and in aquarium and freshwater fish from Central Europe (Slany et al. 2014). It is relevant to note that the first case of 'tuberculosis' reported in wild stocks, was in cod (*Gadus morhua)* landed at Fleetwood (UK), although isolation of the pathogen was not achieved (Alexander 1913). Jacobs et al. (2009) discussed a relationship between poor diet and the increased severity of infection in striped bass.

Myc. montefiorense was recovered sporadically from granulomatous skin lesions in captive moray eels in the USA (Levi et al. 2003).

Myc. neoaurum has been associated with ocular lesions (oedema; exophthalmia) in Atlantic salmon (Backman et al. 1990). Nodules may form in the muscle, where they are visible on the outside of the fish. These nodules may burst, releasing bacteria into the aquatic environment. Internally, nodules may develop on the organs, leading to emaciation, or oedema or peritonitis may ensue. Infection may spread to the skeleton, in which case deformities become apparent. Death will ultimately occur (Van Duijn 1981).

Myc. pseudoshottsii was recovered from an epizootic of mycobacteriosis in striped bass from Chesapeake Bay, USA (Rhodes et al. 2005). In addition, the pathogen has been identified in white perch (*Morone americana*) in the Corsica and Rhode rivers, Maryland and striped bass in New York Bight (Stine et al. 2009) and in diseased farmed fish in Japan (Nakanaga et al. 2012).

Myc. salmoniphilum has been recovered from burbot (*Lola lola*) in Norway. External signs included exophthalmia, cataracts, petechiae and ulceration. Internally, there were granulomas packed with acid-fast bacteria (Zerihun et al. 2011b, c). Atlantic cod was very susceptible to experimental infections, with disease signs including granuloma in the internal organs (Zerihun et al. 2012). Also, the organism has been linked to disease in farmed Russian sturgeon (Righetti et al. 2014) and in Atlantic salmon within a freshwater recirculation system in Chile (Aro et al. 2014).

Myc. shottsii was described as the cause of an epizootic in striped bass from the Chesapeake Bay. Infected fish had granulomatous lesions in the kidney and spleen, and in the skin (Rhodes et al. 2003). Since then, the organism has been recognized in striped bass from Albemarle Sound, North Carolina and the New York Bight, and white perch in the Rhode River, Maryland (Stine et al. 2009).

A range or mycobacteria was recovered from various aquarium fish in Slovenia, and based on molecular methods included *Myc. chelonae, Myc. fortuitum, Myc. gordonae, Myc. marinum, Myc. peregrinum* and *Mycobacterium* spp. Of these *Myc. gordonae* and *Myc. peregrinum* are new to fish pathology (Pate et al. 2005). Unfortunately, the authors did not address the pathogenicity of the isolates. Similarly, Rhodes et al. (2004c) recovered a range of mycobacteria from striped bass in the Chesapeake Bay, USA, and based on phenotypic traits included *Myc. interjectum, Myc. marinum, Myc. scrofulaceum, Myc. shottsii, Myc. szulgai* and *Myc. triplex* (Rhodes et al. 2004c). Again, some of these taxa are new to fish pathology, and deserve further study. The message about the diversity of mycobacteria in Chesapeake Bay fish was reinforced by work with Atlantic menhaden *(Brevoortia tyrannus),* which led to the recovery of *Mycobacterium* spp. from ulcers, and *Myc. fortuitum Myc. gordonae* and *Myc. marinum* from spleen (Stine et al. 2005). In a separate study, wild mullet with signs of mycobacteriosis from around Italy were studied, and the causal agents reported to include *Myc. fortuitum, Myc. abscessus, Myc. flavescens, Myc. chelonae, Myc. septicum* and *Myc. nonchromogenicum* (Varello et al. 2014). From a study of mycobacteriosis that occurred in Chinese sturgeon (*Acipenser sinensis*) and Amur sturgeon (*Acipenser schrencki*) during

2009 and 1010 in China, mycobacterial cultures were obtained, and equated with M*yc. arupense, Myc. chelonae, Myc. gordonae, Myc. fortuitum, Myc. marinum* (most common), *Myc. porcinum* and *Myc. szulgai* (Zhang et al. 2015). Again, the list included some newcomers to fish pathology. In their excellent review, Kaattari et al. (2006) discuss all the currently recognized mycobacterial fish pathogens, including the new but not formally named species, *Myc. "chesapeaki"*.

Isolation

With many cases of mycobacteriosis, there is no attempt made at isolation of the pathogen. Yet, great scientific conclusions seem to result from the examination of only histological material. Nevertheless, attempts at isolating the aetiological agent often fail, indicating a fastidiousness on the part of the pathogen. Some success occurs by inoculating pieces of infected tissue (especially kidney, liver or spleen) on standard mycobacterial media, including Petragnani, Löwenstein-Jensen, Middlebrook 7H10 and Dorset egg media (see Appendix in Chap. 12), or even blood agar, BHIA or TSA, whereupon growth may occur in 2–28 days at incubation temperatures of 15–30 °C in aerobic or microaerophilic, i.e. 3–5 % carbon dioxide, conditions (Dulin 1979; Lansdell et al. 1993). Most difficulty surrounds the recovery of mycobacteria from marine fish species. Clearly, more effort is required to understand the precise nutritional requirements of these organisms. *Myc. abscessus* was not isolated on Löwenstein-Jensen medium. Instead, Middlebrook 7H10 medium was modified by the addition of 10 µg/ml of amphotericin B, 500 µg/ml of chloramphenicol, 5 µg/ml of gentamicin or 30 µg/ml of cephalothin - either singly or in combination. Diseased fish were immersed in the modified Middlebrook 7H10 broth for 1 h at room temperature, before homogenisation, and inoculation of modified Middlebrook 7H10 agar with incubation at 25 °C for 14–28 days (Teska et al. 1997). Middlebrook 7H10 agar with incubation a 23 °C for 4–6 weeks but not Löwenstein-Jensen medium permitted the recovery of *Myc. shottsii* (Rhodes et al. 2003). In comparison, *Myc. montefiorense* was isolated on blood agar and Middlebrook medium after incubation at 25 °C for up to 20 weeks (Levi et al. 2003). To recover *Myc. gordonae,* Sakai et al. (2005) dipped the spleen and liver of infected guppies into 2 % (w/v) sodium hydroxide for 20 min, and then inoculated 1 % Ogawa-egg medium with incubation at 30 °C for 1 month.

There has been one account of the organism associated with inappetance and granulomas on a captive epaulette shark (*Hemiscyllium ocellatum*) in The Netherlands (Janse and Kik 2012).

Characteristics of the Pathogens

The aetiological agents have been classified, at various times, into a wide assortment of species including *Myc. anabanti, Myc. aurum, Myc. chelonei, Myc. chelonei* subsp. *piscarium, Myc. fortuitum, Myc. marinum, Myc. parafortuitum, Myc. piscium, Myc. platypoecilus, Myc. poriferae, Myc. ranae, Myc. salmoniphilum, Myc. simiae, Myc. scrofulaceum*, *Myc. simiae* and *Myc. triplex*. In addition, *Myc.*

neoaurum has been recovered as mixed culture growth from Atlantic salmon with ocular lesions (Backman et al. 1990). Additional groups have been found in granulomatous ornamental fish, and include *Myc. gordonae, Myc. triviale* and *Myc. avium* subsp. *hominissuis* (Novotny et al. 2010). *Myc. fortuitum, Myc. gordonae* and *Myc. chelonae* were identified from granulomatous ornamental fish in India (Shukla et al. 2013). *Myc. fortuitum, Myc. marinum* and *Myc. smegmatis* were found in diseased ornamental fish in Iran (Nofouzi et al. 2013). *Myc. avium* was associated with granulomas in a captive epaulette shark (*Hemiscyllium ocellatum*) (Janse and Kik 2012). Unspeciated *Mycobacterium* have also been reported by Kusuda et al. (1987; Gauthier et al. 2011b). Also, possible new species have been found (e.g. Heckert et al. 2001).

Historically, the descriptions of fish pathogenic mycobacteria have been often poor (e.g. Gomez et al. 1993; Hatai et al. 1993), with many publications based on purely morphological descriptions, which have resulted from the examination of histological sections. It has been established that the pathogens are Gram-positive, acid-fast, non-motile, pleomorphic rods of approximately 1.5–2.0×0.25–0.35 μm in size (Dulin 1979). They produce pale-cream to yellow/orange colonies on solid media. The optimum temperature for growth is 25 °C, although some isolates grow well at 37 °C. From these descriptions and a lack of molecular genetic data, it is difficult to determine whether the isolates belong in *Mycobacterium* or *Nocardia.* Conceivably the problem arises from the inherent difficulty in isolating the organisms, and a lack of interest among workers. Of the nomenspecies listed above, it is relevant to note that the taxonomic validity of *Myc. piscium* is in doubt (Van Duijn 1981), *Myc. anabanti* and *Myc. platypoecilus* are regarded as synonyms of *Myc. marinum* (Van Duijn 1981), and the slow-growing *Myc. salmoniphilum* was regarded as synonymous with *Myc. fortuitum* (Gordon and Mihm 1959), although the name has been subsequently revived, and is regarded as closely related to *Myc. chelonae* (Whipps et al. 2007). Therefore, from the early literature, it would appear that *Myc. fortuitum* and *Myc. marinum* were the only *bona fide* species of fish pathogenic mycobacteria, which could be differentiated, as follows:

	Myc. fortuitum	*Myc. marinum*[a]
Nitrate reduction	+	–
Production of nicotinamidase	–	+
Production of pyrazinamidase	–	+

[a]Data from Runyon et al. (1974)

A limited range of phenotypic tests were used to study the pathogen equated with *Myc. abscessus* (Teska et al. 1997).

Box 3.9: *Mycobacterium abscessus*
Described as homogeneous, the pathogen produces arylsulphatase, catalase and pyrazinamidase, degrades Tween 80 and urea, grows in 7 days and in 6.5% (w/v) sodium chloride, 2% (w/v) thiophenecarboxylic acid and on MacConkey agar (without crystal violet), but does not reduce nitrate, accumulates niacin and is negative for iron uptake (Teska et al. 1997).

Myc. avium. The pathogen was identified as *Myc. avium* from the results of 16S rDNA sequencing (Janse and Kik 2012).

Six isolates, recovered between 1964 and 1982, were recognised as a new subspecies, i.e. *Myc. chelonei* subsp. *piscarium,* by Arakawa and Fryer (1984), although the name was later withdrawn (Arakawa et al. 1987) because of the inability to distinguish the organisms serologically from other subspecies of *Myc. chelonei.* The resurrection of the name *Myc. salmoniphilum* and its relationship to *Myc. chelonei* is an interesting development and may well accommodate those isolates labelled as *Myc. chelonei* subsp. *piscarium* (Whipps et al. 2007*).* Essentially, the testing regime was quite extensive, but the value of using only six isolates is questionable. Colonies on Ogawa medium were off-white with a smooth texture.

Box 3.10: *Mycobacterium chelonei* subsp. *piscarium*

These comprise pleomorphic, acid-fast, weakly Gram-positive rods 1–4 × 0.3–0.6 μm in size. Neither branching nor aerial hyphae have been observed. Grow occurs at 10 and 30 °C, but not all at 37 °C, weakly in 3 % (w/v) sodium chloride but not at all in 5 % (w/v) sodium chloride, and on MacConkey agar and potassium tellurite agar, and in 250 μg/ml of azoguanine, 5 μg/ml of ethambutol, 250 μg/ml of hydroxylamine, 0.1 % (w/v) malachite green, 0.01 % (w/v) methyl violet, 0.2 % (w/v) picric acid, 0.01 % (w/v) pyronin B, 1 % (w/v) sodium deoxycholate and 0.1 % (w/v) sodium nitrate, and variably in 0.01 % (w/v) chlorophenol red and 20 μg/ml of sodium azide. Acid phosphatase, aryl sulphatase and catalase are produced, but not acetamidase, benzamidase, isonicotinamidase, nicotinamidase, pyrazinamidase or succinimidase. *p*-aminobenzoate, *p*-aminosalicylate, sodium salicylate and urea are attacked, but not allantoin, sodium hippurate or Tweens. Acid is produced from glucose and mannose, but not arabinose, dulcitol, fructose, galactose, inositol, mannitol, rhamnose, sorbitol, sucrose, trehalose or xylose. Neither sodium benzoate, sodium malonate nor sodium oxalate are utilised as the sole source of carbon, but variable responses may be recorded with sodium citrate, sodium fumarate and sodium succinate. L-serine and sodium L- glutamate but not acetamide, benzamide, glucosamine hydrochloride or nicotinamide are utilised as the sole source of carbon and nitrogen. Mycolic acids are present. The G+C ratio of the DNA is in the range of 61–65 moles %; with an average value of 63 +/− 1.7 moles % (Arakawa and Fryer 1984).

The fish isolates were considered to be related to *Myc. chelonei,* although somewhat distinct from the current subspecies, i.e. *chelonei* and *abscessus.* For example, the fish isolates were unable to grow at 37 °C, produce nicotinamidase or pyrazinamidase and degrade sodium hippurate or produce acid from trehalose, in contrast to the two validly published subspecies. However, there was an overall phenotypic

similarity in excess of 85 %. Thus, from this similarity together with the results of mycolic acid determination (the fish isolates were identical with *Myc. chelonei* subsp. *chelonei* and *Myc. chelonei* subsp. *abscessus* when examined by two-dimensional thin-layer chromatography of acid methanolysates), it was proposed to establish a new subspecies, i.e. *Myc. chelonei* subsp. *piscarium.* Nevertheless to date, this subspecies has not been formally proposed in the refereed scientific literature, and the revival of the name *Myc. salmoniphilum* may well see the subspecies disappear from the scientific literature.

Myc. chelonae is slowly increasing in significance in farmed fish. For example, *Myc. chelonae* isolates, not allocated to subspecies, were recovered from diseased Atlantic salmon in two farms in Shetland (Bruno et al. 1998). Also, the organism has ben associated with mycobacteriosis in an abscessed adult captive yellow stingray (*Urobatis jamaicensis*) (Clarke et al. 2013).

Myc. gordonae was described as comprising acid-fast rod-shaped cells, which was identified presumptively by 16S rRNA sequencing (Sakai et al. 2005).

Myc. montefiorense was regarded as a cause of granulomatous skin lesions in moray eels (Levi et al. 2003).

Box 3.11: ***Mycobacterium montefiorense***
Cultures comprise slow growing (20 weeks at 25 °C) non-chromogenic acid fast coccobacilli (on blood agar) or rods (on Middlebrook agar), which do not produce catalase or arylsulphatase, do not reduce nitrate, degrade Tween 80 or urea, or grow at >30 °C (growth occurs at 25 °C) or in 5 % (w/v) NaCl (Levi et al. 2003).

By examination of the *hsp65* gene (97.4 % similarity to *Myc. triplex)*, small-subunit rRNA genes, rRNA spacer regions and phenotypic traits, the organism was linked to *Myc. triplex*, but was regarded as sufficiently distinct to justify description as a new species, i.e. *Myc. montefiorense* (Levi et al. 2003).

Myc. neoaurum was described by Backman et al. 1990):

Box 3.12: ***Mycobacterium neoaurum***
Contains yellow-pigmented acid-fast rods, which virtually fail to be stained by the Gram's method. Growth occurs on blood agar at room temperature (but not at 37 °C) in 5–7 days, but not in 5 % (w/v) sodium chloride. Aryl sulphatase is produced. Resistance is recorded to penicillin. The cell wall chemotype is IVA. Glycolated muramic acids, mycolic acids and MK-9, as the predominant isoprenoid quinone, are present (Backman et al. 1990).

Myc. pseudoshottsi (Rhodes et al. 2005).

Box 3.13: *Mycobacterium pseudoshottsii*
Cultures develop rough colonies of 1–3 mm in diameter on Middlebrook 7H10 agar after incubation for 2 months at 23 °C, which slowly become pale yellow to golden in the light, and comprise (clumping) acid-fast cocco-bacilli, which produce niacin and urease but not arylsulphatase, ß-galactosidase or pyrazinamidase, but do not reduce nitrates or attack Tween 80, and grow slightly at 30 °C not at all at 37 °C. Resistance is recorded to 1 mg/ml of isoniazid. Cultures do not grow on Löwenstein-Jensen medium or MacConkey agar (Rhodes et al. 2005).

Myc. salmoniphilum was revived as a valid species name (Whipps et al. 2007), and has since been recognised as a cause of disease with mortalities in Norwegian farmed Atlantic salmon, which displayed greyish-white nodules in the viscera (Zerihun et al. 2011c).

Box 3.14: *Mycobacterium salmoniphilum*
Comprises acid fast slender, straight or slightly curved bacilli of 1–4 μm in length and 0.25–0.6 mm in width that grow at 20–30 °C [weakly at 10 °C] on blood agar, Löwenstein-Jensen agar, MacConkey agar and Middlebrook 7H10 agar producing smooth, shiny cream, colonies after 4–6 days. After 10 days incubation, colonies appear waxy with irregular edges [fried egg appearance]. Growth does not occur at 37 °C. Arylsulphatase is positive, but not so nitrate reduction (Whipps et al. 2007).

Myc. shottsii was named after the examination of 21 isolates recovered from mycobacteriosis among striped in the Chesapeake Bay, USA (Rhodes et al. 2003).

Box 3.15: *Mycobacterium shottsii*
Comprises slow growing, non-pigmented acid fast aggregating cocco-bacilli that grow at 23, less at 30 and none at all at 37 °C to produce small (0.5–1.0 mm) rough flat colonies becoming umbonate upon aging with slightly irregular margins after 4–6 weeks on Middlebrook 7H10 agar, that produce niacin and urease but not arylsulphatase, ß-galactosidase or pyrazinamidase, do not reduce nitrate or attack Tween 80, and are resistant to isoniazid (1 μg/ml) and *p*-aminosalicylic acid. Susceptibility is recorded to ethambutol, ethionamide, kanamycin, rifampicin and streptomycin. Growth does not occur on Löwenstein Jensen medium or on MacConkey agar with 5 % (w/v) NaCl. Mycolic acids are present, and comprise 8 peaks with similarities to the *Myc. tuberculosis* complex (Rhodes et al. 2003).

The results of 16S rRNA sequencing linked the organisms to *Mycobacterium*, and bore some affinity to *Myc. marinum* and *Myc. ulcerans* (similarity = 99.2 %).

Diagnosis

Chemical Method A simple chemical technique has been described, which may readily delineate *Nocardia* from *Mycobacterium* (Kanetsuna and Bartoli 1972). Assuming that pure cultures are available, the bacteria are saponified in 2.5 % (w/v) potassium hydroxide in a 1:1 (v/v) mixture of methanol and benzene at 37 °C for 24 h. Crude mycolic acids from *bona fide* mycobacteria may be subsequently precipitated by addition of an equal volume of ethanol to an ethereal solution of the extracted lipids. Mycobacteria give rise to copious quantities of white precipitate of melting point between 45 and 70 °C, whereas nocardias produce negligible amounts, which do not melt below 150 °C (Kanetsuna and Bartoli 1972).

Molecular Methods A noteworthy advance in diagnoses resulted from the use of PCR technology to identify *Mycobacterium* spp. in sea bass (Knibb et al. 1993), *Myc. chelonei* in a cichlid oscar *(Astronotus ocellatus)* (McCormick et al. 1995) and *Myc. marinum* in goldfish (*Carassius auratus*) (Pourahmad et al. 2014). A PCR followed by reverse cross blot hybridisation identified *Mycobacterium* sensitively (to 100 fg of DNA, which equated to 20 mycobacterial cells) to the species level (Puttinaowarat et al. 2002). *Myc. shottsii* and *Myc. pseudoshottsii* were detected in striped bass using a PCR-RFLP (Gauthier et al. 2010, 2011a). Real-time PCR, based on the polymerase ß subunit gene (*rpoß*) was used to detect *Mycobacterium* spp., albeit with cross reaction from pure DNA from *Noc. seriolae* and *Rhodococcus erythropolis* [these cross reactions were not recorded with formalin-fixed, paraffin-embedded section], with a detection limit of 10^2 CFU/g in the case of *Myc. salmoniphilum* infected tissues (Zerihun et al. 2011a). High-resolution melting analysis determined as a result of studying the melting temperature and melting profile of the 16S–23S rRNA ITS was employed to differentiate reliably 12 species of *Mycobacterium* with a detection limit of 10 genome equivalents/reaction (Phung et al. 2013). A qPCR was advocated for the detection of *Myc. marinum* (Slany 2014).

Epizootiology

Very little is known about the epizootiology of fish pathogenic mycobacteria. Undoubtedly, the reservoir for the organism is the aquatic environment (Beran et al. 2006), although the factors that lead to the development and spread of the disease condition are unknown. To illustrate the potential spread of the disease, in Oregon as many as 26 % of hatchery fish may be infected (Arakawa and Fryer 1984). Possibly, transmission may be by ingesting contaminated food or debris (Dulin 1979). Infection, via the intra-ovarian route, has been demonstrated for the Mexican platyfish (*Xiphophorus maculatus)* (Conroy 1966). However, other investigators have ruled out vertical (i.e. egg) transmission as a means of spreading the disease (Ross and Johnson 1962; Wood 1974). Using real time PCR, *Myc. pseudoshottsii* was found to be ubiquitous in water and sediment samples from the Chesapeake Bay and Rappahannock River in Virginia, respectively [where the disease is occurring]. In contrast, *Myc. shottsii* has been found only in fish, suggesting that the organism is an obligate pathogen (Gauthier et al. 2010).

The source of *Myc. avium* may well have been pre-frozen feed (Janse and Kik 2012).

Pathogenicity

Overcrowding and handling exacerbate mycobacteriosis caused by *Myc. chelonei* and *Myc. marinum* in zebra fish (Ramsay et al. 2009). At a water temperature of 12 °C, experimental infections developed in rainbow trout which were injected, via the i.p. route, with approximately 10^7 cells of *Myc. chelonei* subsp. *piscarium*. Accumulative mortalities ranged from 20 to 52 %. With juvenile chinook salmon, 98 % mortalities were recorded within 10 days at a water temperature of 18 °C (Arakawa and Fryer 1984). Goldfish have been successfully infected within 8 weeks by i.p. injection with *Myc. fortuitum* and *Myc. smegmatis* ATCC 19420 at 10^7 CFU/fish and developed granulomatous lesions, typical of mycobacteriosis (Talaat et al. 1999). Similarly, striped bass were infected using i.p. injections with ~10^5 cells of *Myc. gordonae, Myc. marinum* and *Myc. shottsii. Myc. marinum* caused peritonitis and the development of extensive granulomas particularly in the kidney, mesenteries and spleen, whereas the other two mycobacteria led to mild peritonitis, granulomas in the mesenteries which resolved with time, and persistent infections in the spleen (Gauthier et al. 2003). Zebra fish were much more susceptible, with i.p. injection of ~10^3 cells of *Myc. marinum* leading to the development of granulomatous mycobacteriosis (Swaim et al. 2006).

Evidence has indicated that a novel plasmid-encoded toxic macrolide, Mycolactone F (Ranger et al. 2006) and ECP may well be involved with the pathogenic process (e.g. Chen et al. 1997, 2001). Mycolactone F, being the smallest mycolactone recognised and has a molecular weight of 700, has been identified in *Myc. marinum* and *Myc. pseudoshottsii* (Ranger et al. 2006). Chen et al. (1997) determined the LD_{50} of ECP from *Mycobacterium* spp. as >400 μg of protein/fish to rainbow trout and Nile tilapia. Head kidney macrophages from naive rainbow trout demonstrated heightened macrophage activation when incubated with 1–100 μg/ml of ECP for 48 h (Chen et al. 2001).

Disease Control

Vaccine Development Although there are no vaccines commercially available against fish pathogenic mycobacteria, it is recognised that there is a cell-mediated response in fish, i.e. rainbow trout (Bartos and Sommer 1981). Immunisation with *Myc. salmoniphilum* mixed with Freund's adjuvant resulted in delayed hypersensitivity reactions. A DNA vaccine involving the Ag85A gene encoding for one of the major secreted fibronectin-binding proteins of *Myc. marinum* and cloned in a eukaryotic expression vector stimulated a protective (120 days after vaccination) humoral immune response, but macrophage phagocytosis or respiratory burst activities, in hybrid striped bass when administered i.m. (RPS = 80 % and 90 % for 25 μg and 50 mg doses of vaccine, respectively) and to some extent by i.p. (RPS = 20 % for the 25 μg dose) (Pasnik and Smith 2005, 2006). In an interesting approach, Kato et al. (2010) injected Japanese founder i.m. with BCG (1.2×10^8 CFU/fish) and challenged them

after 4-weeks with *Mycobacterium* sp. The resulting RPS was 31 %. Furthermore, attenuated *Myc. marinum* with impaired ability to replicate in macrophages was demonstrated to protect zebra fish against *Myc. marinum* (Cui et al. 2010). Overall, these data indicate the feasibility of eliciting protection in fish against some of the fish pathogenic mycobacteria. Therefore, there is potential for the development of vaccines.

Disinfection Ethyl alcohol (50 and 70 %), 1 % benzyl-4-chlorophenol/phenylphenol and sodium chlorite (1:5:1 or 1:18:1 in the ratio of base: water:activator) were most effective at reducing or eliminating *Myc. marinum* within 1 min. Sodium hypochlorite (50,000 mg/l) was less effective, and needed 10 min contact time to reduce bacterial numbers. Ethyl alcohol (30 %), 1:256 *N*-alkyl dimethyl benzyl ammonium chloride and 1 % potassium peroxymonosulphate – NaCl were generally ineffective even after 1 h (Mainous and Smith 2005).

Use of Antimicrobial Compounds Some workers advocate that clinically diseased fish should be destroyed, by incineration or burying in quick lime, because of the necessity for prolonged use of chemotherapeutic agents, and the potential hazard to human health (Dulin 1979; Van Duijn 1981). Other scientists have described treatments with chloramine B or T, cycloserine, doxycycline, erythromycin, ethambutol, ethionamide, isoniazid, kanamycin, minocycline, penicillin, rifampicin, streptomycin, sulphonamides and tetracycline. Of these, the most economical treatment is with chloramine B or T at 10 mg/l of tank water for an exposure period of 24 h, after which the water should be changed (Van Duijn 1981). Erythromycin, rifampicin and streptomycin appear to be highly effective against some isolates (Kawakami and Kusuda 1989, 1990).

Nocardiaceae Representatives

Nocardia spp.

Characteristics of the Disease

It is appreciated that nocardiosis may be problematical in fresh water (Valdez and Conroy 1963; Conroy 1964; Snieszko et al. 1964; Heuschmann-Brunner 1965; Campbell and MacKelvie 1968; Ghittino and Penna 1968) and marine fish (Wood and Ordal 1958), occurring in a range of fish species, including Atlantic salmon (Bransden et al. 2000). Symptoms similar to mycobacteriosis develop in affected fish. All age groups may be infected, with lesions, manifested as small white spots, present in the dermis, muscle, gills and internal organs (Fig. 3.8). *Noc. seriolae* has spread from its initial focal point in Japan, and has, for example, been diagnosed among pond cultured sea bass *(Lateolabrax japonicus)* and three striped tigerfish (*Terapon jarbua*) in Taiwan, snakehead (*Ophiocephalus argus*) in China (Wang et al. 2007, 2009), largemouth bass (*Micropterus salmoides*) in China (Jiang et al. 2012) and weakfish (*Cynoscion regalis*) in the USA [mostly likely *N. seriolae]* (Cornwell et al. 2011). In Taiwan, the cumulative mortality in sea bass reached 17.5 % within a

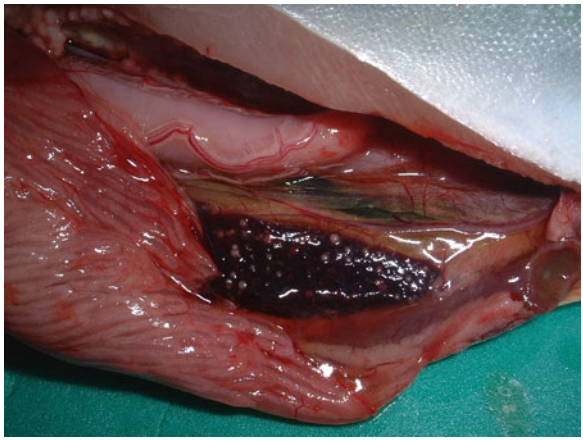

Fig. 3.8 Nocardiosis in yellowtail. Extensive granulomas are present on the liver and kidney (Photograph courtesy of Dr. T. Itano)

month, with disease signs including yellow-white nodules of 0.1–0.2 cm in diameter in the gills, heart, kidney, liver and spleen (Chen et al. 2000). In China, 35 % mortalities were reported within 30 days (Wang et al. 2007). The cumulative mortalities for three striped tiger fish was 2.4 % within 2 months (Wang et al. 2009).

There has been some confusion between distinguishing infections caused by *Mycobacterium* and *Nocardia*, the latter leading to nocardiosis. Thus it is often difficult to determine from largely histological reports the genus to which an acid-fast pathogen belongs. Conroy and Valdez (1962) isolated tubercle bacilli from neon fish (*Paracheirodon innesi),* which were also pathogenic to paradise fish (*Macropodus opercularis)* and three-spot gouramis (*Trichogaster trichopterus),* but not to goldfish. These organisms were subsequently identified by Dr. R.E. Gordon as *Nocardia asteroides.* A second species, *Noc. kampachi,* was described as a causal agent of nocardiosis, in yellowtail farmed in Japan, by Kariya et al. (1968) and Kubota et al. (1968). However, the name was not validated. Instead, *Noc. seriolae* was formally proposed, as a causal agent of fish nocardiosis (Kudo et al. 1988).

Nocardia spp. (including *Noc. asteroides)* occur in freshwater and soil. With the observation that *Noc. kampachi* grows in up to 4.5 % (w/v) sodium chloride, it was suggested that the normal habitat is terrestrial or limnetic. Moreover, in survival experiments, the organism remained viable in clean seawater for only a few days, although in polluted conditions viability was considerably extended (Kariya et al. 1968). The inference, therefore, is that fish become infected from the natural environment. Presumably these diseased animals serve as a reservoir for further infection.

Isolation

Essentially, the same isolation methods as for mycobacteria, e.g. use of Löwenstein-Jensen medium, have been employed with *Nocardia.* The initial development of colonies usually occurs within 21 days at 18–37 °C (Valdez and Conroy 1963; Conroy 1964; Snieszko et al. 1964; Heuschmann-Brunner 1965; Campbell and MacKelvie 1968; Ghittino and Penna 1968).

Disease Control

Use of Antimicrobial Compounds As with mycobacteria, it has been argued that infected fish should be destroyed so as to prevent any human health hazard. However, some success may result with chemotherapy, in particular with sulphonamides, e.g. sulphisoxazole at 2 mg/g of food (Van Duijn 1981). An improvement in the fish becomes apparent within 10 days of the commencement of treatment, but it is advisable to continue chemotherapy for 21 days. This method may be suitable for the treatment of pet fish.

Nocardia asteroides

Characteristics of the Pathogen

There is limited information available about the characteristics of fish pathogenic strains of *Noc. asteroides.*

Box 3.16: ***Nocardia asteroides***
Most of the description appertains to morphological characters. For example, it has been observed that cultures undergo a complete life cycle, including germination from resting microcysts, simple and complex fission, and branching. Thus, nocardias may appear in coccal to oval forms, and as long, slender, multiseptate rods. All these stages have been seen in infected fish (Van Duijn 1981). From Valdez and Conroy (1963), it would appear that the organisms reduce nitrates and degrade starch but not gelatin or urea. Neither H_2S nor indole is produced. The methyl red test and Voges-Proskauer reaction are negative. Acid is produced from glucose, but not from any other carbohydrate tested.

Pathogenicity

Experimental infections have been established in Formosa snakehead *(Chanos mac-ulat)* and largemouth bass *(Micropterus salmoides)* (Chen 1992). Thus, typical granulomatous lesions and mortality followed in 14 days of i.p. or i.m. injection of 8 mg of suspensions of *Noc. asteroides* (Chen 1992).

Nocardia salmonicida (= Streptomyces salmonis = Streptoverticillum salmonis)

Isolation

The organism may be isolated on glucose asparagine agar, glycerol asparagine agar and yeast extract malt extract agar, Emerson agar. Bennett agar or nutrient agar,

following incubation aerobically at 25–37 °C for an undisclosed period (Appendix in Chap. 12; Rucker 1949).

Characteristics of the Pathogen

Streptomycoccis in fish was described initially by Rucker (1949), who classified the aetiological agent in *Streptomyces,* as *Streptomyces salmonicida.* This was amended initially to *Verticillomyces salmonicida* (Shinobu 1965), then to *Streptoverticillium salmonicida* (Baldacci et al. 1966), *Streptoverticillium salmonis* (Locci et al. 1969) *Streptomyces salmonis* (Witt and Stackebrandt 1990) and finally to *Noc. salmonicida* (Isik et al. 1999). In general, a dearth of information exists about this fish pathogen. Essentially, since the work of Rucker (1949), the disease has received limited attention. Therefore, it is difficult to decide whether or not streptomycoccis represents a genuine problem.

Box 3.17: ***Nocardia salmonicida***
The organism produces brick red to orange pigmented substrate mycelia with white (with pink and yellow shades) aerial mycelia. On primary isolation, the colonies are small and, initially, smooth, but later they develop aerial mycelia that appear velvety. Characteristically, the aerial mycelia produce whorls (verticils) at frequent intervals, giving an appearance of barbed wire. The Gram-positive catalase-positive non-motile mycelia contain LL-diaminopimelic acid (DAP) and glycine but not meso-DAP, arabinose or galactose in the cell wall (i.e. Type I). Melanin, but generally not H_2S, is produced. Aesculin, DNA, gelatin, starch, testosterone, Tween 20 (some isolates), Tween 20, tyrosine and urea are degraded, but not adenine, casein, cellulose, chitin, elastin, guanine, hypoxanthine, starch, uric acid, xanthine or xanthan. Nitrates are reduced. Growth occurs at 12° and 30 °C but not at 35 °C, in 53 % (w/v) sodium chloride, 0.0001 % (w/v) bismuth citrate, 0.00001 % (w/v) crystal violet, 0.01 % (w/v) phenol and 0.01 % (w/v) potassium tellurite, but not in or 0.01 % (w/v) malachite green or 0.01 % (w/v) sodium azide. Acid is produced from glucose, glycerol, inositol, ribose and trehalose, and slightly from sucrose, but not from arabinose, cellulose, erythritol, fructose, galactose, maltose, mannose, mannitol, raffinose, rhamnose, trehalose or xylose. Butyrate, citrate, fumarate, D-fructose, D(+)-glucose, malate, D(+)-mannitol, L-proline, propionate, D(+)-sorbitol and succinate are utilised, but not amygdalin, D and L-arabinose, arbutin, D(+)-cellobiose, dulcitol, D(+)-galactose, glycogen, *m*-inositol, inulin, D(+)-melezitose, D(+)-raffinose, L-rhamnose, acetamide, acetate, anthranilic acid, benzoate, 1,4-butanediol, 2,3-butanediol, hippurate, 4-hydroxybenzoate, lactate, malonate, 2-octanol, pimelic acid or tartrate (Williams et al. 1985; Isik et al. 1999). The major cellular fatty acids are hexadecanoic, octadecanoic, octadecanoic and 10-methyloctadecanoic acid. The G+C ratio of the DNA is 67 mol% (Isik et al. 1999).

Diagnosis

A LAMP assay was developed, involving four primers designed from the 16S-23S rRNA ISR of *Noc. salmonicida*. The LAMP products were determined by gel electrophoresis, and also visually after addition of the nucleic acid stain SYBR Green I. The sensitivity was reported as 1.68×10^3 CFU/ml (i.e. 16.8 CFU/reaction), which was regarded as 10-hold higher than conventional PCRs. The LAMP detected the pathogen in diseased fish tissues (Xia et al. 2015).

Nocardia seriolae

Characteristics of the Pathogen

A good description exists for *Noc. kampachi* (Kariya et al. 1968; Kubota et al. 1968; Kusuda et al. 1974). However, in retrospect it would appear that Kariya and coworkers exercised considerable taxonomic intuition in elevating the isolates into a new species, i.e. *Noc. kampachi*. Unfortunately, the results of G+C determinations were not reported. Moreover, it is perhaps surprising that the original authors did not provide detailed reasons explaining why *Noc. kampachi* should be regarded as distinct from other species of *Nocardia.* This is especially relevant as there is some resemblance between the descriptions of *Noc. kampachi* and *Noc. caviae* (Table 3.2). Nevertheless, a more recent publication has formally proposed another nomenspecies, i.e. *Noc. seriolae,* which effectively replaces *Noc. kampachi* (Kudo et al. 1988). Excellent descriptions of fish pathogenic isolates have now been provided, with Japanese isolates displaying 99.9 % sequence homology with the type strain (Shimahara et al. 2008).

Box 3.18: ***Nocardia seriolae***
The isolates of *Noc. seriolae* contain *meso*-diaminopimelic acid, arabinose and galactose, suggesting chemotype IVA. The major components of the cellular fatty acids are *n*-$C_{16:0}$, *n*-$C_{16:1}$ and *n*-$C_{18:1}$; 10-methyl-$C_{19:0}$ is also present as a major component in four of the five isolates examined. Iso- and anteiso-branched acids have not been detected. The total number of carbon atoms in the mycolic acids is from 44 to 58. The predominant isoprenoid quinone is tetrahydrogenated menaquinone with 8 isoprene units. The G+C ratio of the DNA is 66.8–67.4 moles %.

A proposal has been made to divide Taiwanese isolates into two groups based around the reaction to α-glucosidase (Shimahara et al. 2009).

Table 3.2 Characteristics of nocardias

Character	*Nocardia asteroides*[a]	*Noc. caviae*[a]	*Noc. kampachi*[b]	*Noc. salmonicida*[c]	*Noc. seriolae*[d]
Gram-positive rods and cocci	+	+	+	+	+
(Weakly) acid-fast staining	+	+	+	+	ND
Aerial hyphae	+	+	+	+	+
Motility	–	–	–	–	–
Growth at 10 °C	v	v	–	–	–
Production of:					
Catalase	v	v	+	+	ND
H_2S	ND	ND	+	ND	ND
Indole	ND	ND	–	ND	ND
Oxidase	–	–	–	ND	ND
Nitrate reduction	+	+	+	–	ND
Degradation of:					
Casein	–	–	–	–	–
Gelatin	–	–	–	ND	ND
Hypoxanthine	–	+ (weak)	+ (weak)	–	–
Starch	ND	ND	+	–	ND
Tyrosine	–	–	+ (weak)	+	–
Urea	+ (weak)	+ (weak)	–	+	–
Xanthine	–	+	–	–	–
Acid production from:					
Fructose	+	+	+	–	ND
Glucose, glycerol	+	+	+	+	+
Utilisation of:					
Adonitol, arabinose	–	–	–	ND/–	ND
Cellobiose	–	v	–	–	ND
Dextrin, maltose	v	v	–	ND	ND
Dulcitol, Glycogen	–	–	–	–	ND
Fructose, glucose	+	+	+	+	ND
Glycerol	+	–	v	ND	ND
Inositol	–	+	–	–	ND
Inulin	–	–	v	–	ND
Lactose, salicin	–	–	–	ND	ND
Mannitol	v	+	–	+	ND
Mannose	+	v	v	ND	ND
Rhamnose	v	–	–	–	ND
Sodium acetate	+	+	+	–	ND
Sodium benzoate	–	–	–	–	–

(continued)

Table 3.2 (continued)

Character	*Nocardia asteroides*[a]	*Noc. caviae*[a]	*Noc. kampachi*[b]	*Noc. salmonicida*[c]	*Noc. seriolae*[d]
Sodium citrate	–	v	+	+	+
Sodium lactate	+	+	+	–	ND
Sodium malate	+	+	+	+	ND
Sodium malonate	–	–	v	–	ND
Sodium propionate	+	+	+	+	ND
Sodium pyruvate	+	+	+	ND	ND
Sodium tartrate	–	–	v	–	–
Sorbitol, xylose	–	–	–	+/ND	ND
Starch, trehalose	v	v	–	ND	ND

[a]From Goodfellow (1971)
[b]From Kusuda et al. (1974)
[c]From Isik et al. (1999)
[d]From Kudo et al. (1988)
ND = not determined
v = variable response

Detection/Diagnosis

Molecular Methods A PCR detected 10^2 CFUs of *Noc. seriolae* in yellowtail (Miyoshi and Suzuki 2003). A LAMP technique, which is a modern molecular approach for rapidly amplifying DNA with a high degree of specificity, has been proposed for the rapid and sensitive detection of *Noc. seriolae* amplifying up to 10^3 CFU/ml [this was 10-fold more sensitive than PCR] (Itano et al. 2006a, b, c.

Pathogenicity

Natural infections with *Noc. seriolae* have occurred in China when 15 % losses were reported in seawater cages with large yellow croakers *(Larimichthys crocea)* during 2003 (Wang et al. 2005). Yellowtail have been infected by i.p. and intradermal injection, immersion for 10 min and orally with LD_{50} values of 1.9×10^2, 4.3×10^6, 1.5×10^4/ml, 1.7×10^7, respectively (Itano et al. 2006b). Co-habitation worked also in achieving infection (Itano et al. 2006b).

Disease Control

Vaccine Development Initial research was not promising (Kusuda and Nakagawa 1978; Shimahara et al. 2005), but subsequent research directed at controlling *Noc. seriolae* infection in yellowtail by using live cells of a low virulent isolates of the same taxon (dose = 3.1×10^4 or 10^5 CFU/fish) and other nocardial species

(dose = 1.1–1.5 × 10^8 CFU/fish), i.e. *Noc. soli, Noc. fluminea* and *Noc. uniformis,* which were administered intraperitoneally, led to some benefit. This was the case with *Noc. soli* (RPS = ~65 %) and *Noc. fluminea*, and more so with the low virulent *Noc. seriolae.* The survivors were completely resistant to *Noc. seriolae* (Itano et al. 2006c). Live attenuated cells of *Myc. bovis,* which comprise the BCG vaccine for tuberculosis protected Japanese flounder (*Paralichthys olivaceus*) against *Noc. seriolae* with cumulative mortalities of only 21.4 % compared to 56.7 % of the controls with the mode of action reflecting production of bacteriolytic lysozymes (Kato et al. 2012). A sub-lethal dose of live cells and an inactivated preparation were evaluated in ginbuna crucian carp (*Carassius auratus langsdorfii*) leading to a high antibody response on the 15th day after vaccination, although the levels declined thereafter. After challenge, the RPS for the live and inactivated preparation was 62.5 % and 75 % respectively (Nayak et al. 2014). A DNA vaccine was developed using the *Antigen 85-like* gene; an expression plasmid encoding the gene and codon-optimized was injected i.m. into amberjack (*Seriola dumerili)* leading to good survival 40-days after challenge (Kato et al. 2014).

Use of Antimicrobial Compounds Isolates of *Noc. seriolae* recovered from Japan during 1999 to 2001 were susceptible to kanamycin but uniformly resistant to fosfomycin and oxolinic acid. Some isolates displayed resistance to erythromycin, kitasamycin and spiramycin. Of concern, multiple antibiotic-resistance was observed in some cultures (Itano and Kawakami 2002).

Rhodococcus sp.

Characteristics of the Disease

In Canadian farmed chinook salmon, there was evidence of melanosis and ocular oedema, leading to rupture of the cornea, and from which Gram-positive bacteria were recovered. There was no evidence of involvement of any internal organs (Backman et al. 1990). With Atlantic salmon, the presence of granulomas in the kidney was apparent (Claveau 1991). Progressive low level mortalities were recorded.

Isolation

Dense growth of two colony types was obtained from diseased tissue following inoculation of blood agar, MacConkey agar (Appendix in Chap. 12) and TSA with incubation at room temperature (19 °C) for up to 14 days. Both colony types were also recovered from the kidney and spleen of chinook salmon with ocular lesions (Backman et al. 1990; Claveau 1991).

Characteristics of the Pathogen

Organisms were cultured from Chinook salmon (Backman et al. 1990) and Atlantic salmon (Claveau 1991) and linked to *Rhodococcus* (Claveau 1991).

Box 3.19: *Rhodococcus* sp.
Isolates comprise non-acid-fast facultatively anaerobic Gram-positive rods (2–3 × 0.6 μm in size) [slightly club shaped], which grow aerobically at room temperature (but not at 37 °C) on blood agar in 3–4 days. The cell wall components include *meso*-diaminopimelic acid, arabinose and galactose, which equates with chemotype IVA, N-glycolated muramic acid, mycolic acids, and MK-8 as the predominant isoprenoid menaquinone. Neither catalase nor oxidase is produced. Urea is degraded. Xylose is fermented, but not so glucose, lactose, maltose or sucrose. Acid is produced from *meso*-inositol but not from dulcitol, mannitol or sorbitol. Growth occurs in 5 % (w/v) sodium chloride.

Epizootiology

It was considered that feeding with crude fish offal may have been the cause of infection (Claveau 1991).

Pathogenicity

Intraperitoneal injection of Atlantic salmon smolts with a very high dose of 5×10^8 cells resulted in severe peritoneal granulomatous reactions, with a low accompanying mortality rate, within 21 days (Speare et al. 1995). Unlike the natural disease where the most severe pathological changes occurred in the renal interstitium, experimental challenge resulted in damage in the direct vicinity of the injection site. Yet, the development of large bacterial colonies were common to both natural and artificial infections.

Rhodococcus erythropolis

Characteristics of the Disease

Fresh-and seawater reared Atlantic salmon, which had been previously intraperitoneally injected with oil-adjuvanted vaccine of more than one manufacturer in Norway (5 cases) and Scotland (2 cases), experienced losses of 1–35 %. The systemic infection centred on the peritoneal cavity, and moribund fish displayed scale loss and occasionally cutaneous haemorrhages in the abdomen and at the base of the fins. In addition, there was some evidence of abdominal distension. In Scotland,

bilateral exophthalmia was reported. Internally, there was bloody ascites, splenomegaly, peritonitis, the stomach filled with mucoidal contents, the presence of petechia, and fluid or pus filled cavities on the internal organs (Olsen et al. 2006b). Adhesions were present in the peritoneum, consistent with the administration of oil-adjuvanted vaccines (Olsen et al. 2006).

The organism has been recovered also from oil-adjuvant vaccinated Atlantic salmon smolts [cumulative mortality = <0.5 %] in Chile (Perdiguero et al. 2011). Here, the diseased fish displayed scale loss, some haemorrhaging at the peduncle, and tail erosion.

Isolation

Kidney and sometimes ascitic fluid was inoculated onto 4 % (v/v) bovine or horse blood agar with incubation at 15 and 22 °C for 7 days (Olsen et al. 2006b).

Characteristics of the Pathogen

Box 3.20: *Rhodococcus erythropolis*
Cultures comprise round, shiny, off-white colonies that contain strictly aerobic, non-haemolytic, Gram-positive rods that produce catalase but not oxidase, grow well at 30 rather than 15 °C and not at all at 4 or 37 °C, and grow less well in the presence of only 1.5 % (w/v) NaCl. N-acetyl-glucosamine, adipate, D-arabitol, gluconate, glucose, glycerol, inositol, malate, mannitol, phenylacetate, sorbitol, sucrose and trehalose are utilised, but not aesculin, amygdalin, D-and L-arabinose, caprate, cellobiose, dulcitol, D- and L-fucose, galactose, ß-gentibiose, glycogen, inulin, 2-keto-gluconate, lactose, D-lyxose, maltose, D-mannose, melezitose, melibiose, α-methyl-D-glucoside, ß-methyl xyloside, α-methyl-D-mannoside, D-raffinose, rhamnose, salicin, L-sorbose, starch, D-tagatose, D-turanose, D- and L-xylose (Olsen et al. 2006b).

16S rDNA sequencing revealed 99.9 % and 100 % homology between Scottish and Norwegian isolates and *Rhodococcus erythropolis,* respectively (Olsen et al. 2006).

Epizootiology

There was a link between the administration of oil-adjuvanted vaccines intraperitoneally. Olsen et al. (2006) speculated that the oil may be a source of nutrition and may give protection to the rhodococci. However, the precise source/origin of the organism was not determined.

Pathogenicity

Koch's Postulates were eventually fulfilled using previously vaccinated fish which were challenged via i.p. injection with 2×10^5, 2×10^6 and 2×10^7 cells/fish (Olsen et al. 2006b).

Rhodococcus qingshengii

Characteristics of the Disease

A condition was recognised in Chilean Atlantic salmon during 2008 whereby there were not any overt clinical signs of disease, but internally there was severe peritonitis with white, loose to compact pseudo-membranes [reddish-tan exudates were noted sometimes on the internal organs, notably the heart, liver and spleen (Avendaño-Herrera et al. 2011)].

Isolation

Bacterial isolation was achieved using TSA supplemented with 1 % (w/v) NaCl and Columbia sheep blood agar with incubation aerobically at 20 °C for 72 h (Avendaño-Herrera et al. 2011).

Characteristics of the Pathogen

One isolate, 79043–3, was studied:

Box 3.21: *Rhodococcus qingshengii*
Colonies are off white, smooth and circular with regular edges, and contain short non-motile, non-fermentative, Gram-positive cells that produce catalase but not arginine dihydrolase, lysine or ornithine decarboxylase or oxidase. Nitrates are reduced. The methyl red test is positive, but the Voges Proskauer reaction is negative. Neither casein, DNA, gelatin, elastin, urea nor Tween 80 was degraded. Growth occurs at 15–37 °C and in 0–3 % (w/v) NaCl. Growth does not occur on MacConkey agar or TCBS. With API 20STREP, positivity is recorded to aesculin, 2-naphthyl-ß-D-galactopyranoside, 2-naphthyl phosphate and L-leucine-2-naphthylamide pyruvate. Using API ZYM, 2-naphthyl phosphate, naphthol-AS-BI-phosphate, 2-naphthyl-α-D-glucopyranoside, 2-naphthyl caprylate, 6-Br-2-naphthyl-ß-D-glucopyranoside, L-leucyl-2-naphthylamide and L-valyl-2-naphthylamide were utilised. The major fatty acid methyl esters are $C_{16:0}$ (22.43 %), $C_{18:1}$ ω9c (24.48 %),10-methyl $C_{18:0}$ (13.13 %) and summed feature 3 (16.40 %; comprising $C_{16:1}$ ω6c/$C_{16:1}$ ω7c). (Avendaño-Herrera et al. 2011).

By means of the API 20STREP data base, an identification of *Str. salivarius* was obtained. However, sequencing of the 16S rRNA gene led to the association with the genus *Rhodococcus*, with the closest matches to *Rhodococcus baikonurensis* GTG 1041 T, *Rhodococcus erythropolis* DSM 43066^T, *Rhodococcus jialingiae* djl-6–2 T and *Rhodococcus qingshengii* djl-6 T, and corresponding to sequence homologies of 99.47, 99.31, 99.79 and 99.79 %, respectively (Avendaño-Herrera et al. 2011). DNA:DNA hybridization revealed that 79043–3 showed 88.7 % reassociation with with *Rhodococcus qingshengii*, but only 61.0 % and 39.3 % homology *Rhodococcus erythropolis* and *Rhodococcus baikonurensis*, respectively. Of relevance, a comparison of 79043–3 with rhodococci from diseased Norwegian and Scottish Atlantic salmon (Olsen et al. 2006) revealed almost 99.8 % sequence homology with the strains of *Rhodococcus erythropolis* 00/50/6670 and 4115, from the previous study (Olsen et al. 2006). It is questionable whether there are two pathogenic rhodococci, i.e. *Rhodococcus erythropolis* and *Rhodococcus qingshengii* or just one as seems likely from the data.

Pathogenicity

79043–3 caused mortalities within 3 days of experimental infections with Atlantic salmon [60 % mortality with a dose of 5 x 10^7 CFU/fish] with disease signs including haemorrhages particularly around the eyes, mouth and opercula. Internally, there was ascites, melanosis in the abdominal cavity, pale liver, splenomegaly, internal hemorrhaging, and the presence of pseudo-membranes on the heart, liver, spleen, and swim bladder (Avendaño-Herrera et al. 2011).

Planococcaceae Representative

Planococcus sp.

Characteristics of the Disease

Since 1988, there has been a steady increase in the incidence of motile Gram-positive cocci, tentative *Planococcus,* associated with diseases of Atlantic salmon and rainbow trout in the UK. In some cases, it appeared that the Gram-positive cocci were inhabiting fish that had previously received extensive chemotherapy to control diseases caused by Gram-negative bacteria. The organism has been associated with small off white (2–4 mm diameter) round recessed spots on the heads of Atlantic salmon (Austin et al. 1988). In large rainbow trout (average weight = 500 g), the only disease sign was the presence of watery kidney and small amounts of ascitic fluid in the peritoneal cavity (B. Austin, unpublished data). In addition during 1990, the organism was associated with two populations of rainbow trout fry deemed to

have RTFS. These animals were anaemic, with pale gills, swollen kidney, pale liver and elongated spleen (Austin and Stobie 1992).

Isolation

The pathogen was recovered from kidney swabs following incubation on BHIA, skimmed milk agar or TSA at 25 °C for up to 7 days. Cultures comprised off-white to yellow raised shiny colonies, which were 1–2 mm in diameter after 48 h (Austin et al. 1988).

Characteristics of the Pathogen

Box 3.22: *Planococcus* sp.
Cultures comprise motile (single polar flagellum) often paired Gram-positive cocci of 1–2 μm in diameter, which possess a strictly aerobic metabolism for glucose, and produce catalase, ß-galactosidase and oxidase, but not gelatinase, H_2S, indole or lysine decarboxylase. Growth occurs at 37 °C and in 0–15 % (w/v) sodium chloride.

From these traits and despite a fresh water rather than a marine origin, it was considered that the organisms belonged in the genus *Planococcus,* possibly related to *P. citreus* (Hao and Komagata 1985).

Pathogenicity

Fish, injected intraperitoneally with 10^5 cells displayed erratic swimming within 48 h. At this time, the gills were pale, the anus was protruded and abdomen was swollen. The intestine became swollen and haemorrhagic. Slight kidney liquefaction was noted. Approximately 30–40 % of the infected fish died (Austin et al. 1988; Austin and Stobie 1992).

Disease Control

Use of Antimicrobial Compounds The organism was sensitive to carbenicillin, erythromycin, penicillin G and tetracycline, which may be effective for chemotherapy (Austin et al. 1988; Austin and Stobie 1992).

Staphylococcaceae Representatives

Staphylococcus aureus

Characteristics of the Disease

During 1982 and 1983, mortalities occurred among silver carp, *Hypophthalmichthys molitrix,* at a farm in India. These mortalities were associated with pronounced eye disease, with the cornea becoming reddish, due to vascularisation, and then opaque. Thereafter, there was degeneration of the eye tissues, leaving a hollow cup. The brain and optic nerves were affected. In addition, diseased fish became lethargic and darker in colour (= melanosis). The internal organs did not appear to be affected (Shah and Tyagi 1986). Bacteria, were cultured, and Gram-positive cocci identified, and equated with *Staphylococcus aureus* (Shah and Tyagi 1986). Subsequently, jaundice in African sharp-tooth catfish, *Clarias gariepinus,* has been associated with *Sta. aureus* (Oladele et al. 2012).

Isolation

Eye and brain tissue revealed the presence of bacteria. However, the precise isolation procedures were not stated (Shah and Tyagi 1986).

Characteristics of the Pathogen

Box 3.23: *Staphylococcus aureus*
The cultures comprise Gram-positive cocci, which produce coagulase and phosphatase, degrade blood (ß-haemolysis) and DNA, and ferment glucose and mannitol. Zones of opalescence develop around (black) colonies on Baird Parkers agar.

From this description, an identification of *Sta. aureus* was achieved (Shah and Tyagi 1986). However, it is apparent that there are insufficient data to differentiate between *Sta. aureus* and *Sta. intermedius* (Kloos and Schliefer 1986).

Disease Control

Water Disinfection A bath of potassium permanganate (1 ml/l) for 5–10 min, together with treating the pond water with 250 mg/l of lime and 1 mg/l of potassium permanganate every fourth day, was considered effective at halting mortalities, except with advanced cases of the disease (Shah and Tyagi 1986).

Staphylococcus epidermidis

Characteristics of the Disease

The first reports of fish pathogenic strains of *Staphylococcus epidermidis* have emanated from Japan where, from July 1976 to September 1977, severe epizootics occurred in farmed yellowtail (*Seriola quinqueradiata)* and red sea bream (*Chrysophrus major)* (Kusuda and Sugiyama 1981; Sugiyama and Kusuda 1981a, b). The initial description of the disease was not exhaustive, but typical signs included exophthalmia, congestion, and ulceration on the tail (Kusuda and Sugiyama 1981). Later, *Sta. epidermidis* was recovered from moribund cultured grass carp in Taiwan (Wang et al. 1996). Here, the fish displayed haemorrhages on the opercula and pelvic fins. Internally, petechial haemorrhages and bloody ascites were observed. Although tapeworms were present in the lumen of the intestine, smears revealed the presence of oval bacteria, which were isolated, and considered to represent *Sta. epidermidis* (Wang et al. 1996). The organism was associated with mortalities of up to 12 % in 1 day coinciding with a sudden increase in water temperature in juvenile gilthead sea bream in Turkey during 2003. The diseased fish displayed haemorrhaging on the fins and gills, a slightly distended abdomen with ascites, and anaemic liver (Kubilay and Uloköy 2004).

Isolation

The pathogen was successfully cultured on BHIA following incubation at 37 °C for 24 h (Kusuda and Sugiyama 1981). However, the use of such a high temperature is puzzling, and it is conceivable that mesophiles, preferring lower growth temperatures, may have been overlooked.

Characteristics of the Pathogen

From the outbreaks in Japan, six isolates, identified as *Sta. epidermidis*, were recovered:

Box 3.24: ***Staphylococcus epidermidis***
All cultures comprise non-motile Gram-positive, fermentative spherical cells of approximately 0.6–1.8 μm in diameter, which form white to white/yellow colonies on BHIA. The cells occur singly, in pairs, and in irregular clusters. Catalase, ß-galactosidase and phosphatase are produced, but not arginine dihydrolase, coagulase, H_2S, indole, lysine or ornithine decarboxylase or oxidase. Nitrates are reduced. The methyl red test and Voges Proskauer reaction are positive. Blood (ß-haemolysis), gelatin, sodium hippurate, tributyrin and urea are degraded, but not starch, Tween 80, tyrosine or xanthine. Growth occurs at 45 °C and in 0–15 % (w/v) sodium chloride. Neither citrate, mucic acid nor D-tartrate is utilised (Sugiyama and Kusuda 1981a).

These isolates matched closely the species description of *Sta. epidermidis* (Kloos and Schliefer 1986), and approximated to biotypes II, V and VI of Baird-Parker (1963, 1965). It is curious that a diverse range of serotypes, i.e. five serotypes, were recognised (Sugiyama and Kusuda 1981a). The G+C content of the DNA was not assessed.

Epizootiology

Sugiyama and Kusuda considered that the bacteria originated from water (or fish) rather than from human beings, because of the pronounced antigenic differences to human - Tachikawa - strains (Sugiyama and Kusuda 1981b). This seems to be a likely possibility in view of other ecological studies which have clearly demonstrated the presence of *Sta. epidermidis* in the aquatic environment (e.g. Gunn et al. 1982).

Disease Control

Use of Antimicrobial Compounds Wang et al. (1996) reported success with erythromycin, dosed at 20 mg/kg body weight of fish/day for 10 days.

Staphylococcus warneri

Characteristics of the Disease

At a water temperature of 14–16 °C, rainbow trout of 50–100 g weight displayed ulcerations on the fins and exophthalmia. The abdomens were distended with ascitic fluid. The kidney was normal, but the liver was discoloured (Gil et al. 2000). Subsequently, the organism became associated with chronic nonlethal disease of broodstock rainbow trout held at 10 °C in Turkey (Metin et al. 2014).

Isolation

Growth was achieved from the kidney and liver of diseased fish on TSA with incubation at 22–25 °C for 48–72 h (Gil et al. 2000).

Characteristics of the Pathogen

Box 3.25: *Staphylococcus warneri*
Yellow colonies develop on Sal Mannitol medium. Cells comprise catalase positive, oxidase negative, facultatively anaerobic Gram-positive cocci, which produce arginine dihydrolase, ß-glucosidase and urease, but not alkaline phosphatase, and do not reduce nitrates. Acid is produced from sucrose and trehalose, but not from L-arabinose, lactose, mannitol, mannose, raffinose, ribose or xylose (Gil et al. 2000).

The characteristics of the organism were considered by Gil et al. (2000) to match the description of *Sta. warneri.* The culture recovered by Metin et al. (2014) displayed 99.93 % 16S rRNA gene sequence homology with the type strain in GenBank.

Pathogenicity

Infectivity of brown trout was achieved, with an LD_{50} of 1.16×10^5 cells (Gil et al. 2000).

Disease Control

Use of Antimicrobial Compounds Sensitivity was recorded to amoxicillin, erythromycin and trimethoprim-sulphamethoxazole (Gil et al. 2000), which may be worthy of examination in *in vivo* experiments.

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Chapter 4
Aeromonadaceae Representatives (Motile Aeromonads)

Abstract The motile aeromonads have traditionally been linked to *Aer. hydrophila,* but multiple species of fish pathogens have been recognized including *Aer. dhakensis* and *Aer. schubertii.* Nevertheless, *Aer. hydrophila* has been the focus of attention since the start of the twenty-first century particularly as a target for the development of diagnostic and control strategies.

Keywords Septicaemia • Motile *Aeromonas* • Pathogenicity • Vaccines • Probiotics

The scientific literature abounds with references to aeromonads as fish pathogens, and it is often difficult to distinguish the primary pathogen from opportunist or contaminant. There has been a tendency for motile aeromonads to be linked with *Aer. hydrophila* and non motile representatives with *Aer. salmonicida.* With the advent of more reliable taxonomic methods, the reliability of some of the earlier identifications may be questioned with the results that more species of fish pathogenic aeromonads are being recognised. Thus, there is evidence that *Aer. bestiarum, Aer. dhakensis* [haemorrhagic and generalized liquefaction; Fig. 4.1], *Aer. sobria* biovar sobria and *Aer. veronii* biovar sobria may pose problems to fish health (Orozova et al. 2009). In addition, aeromonads are often encountered in diseased fish, although the proof of pathogenicity may be lacking. For example, Beaz-Hidalgo et al. 2010] recovered *Aer. bestiarum, Aer. hydrophila, Aer. media, Aer. piscicola, Aer. salmonicida* and *Aer. sobria* from diseased fish but without demonstrating actual pathogenicity. *Aer. piscicola* is a newly described species from diseased fish, albeit without evidence of actual pathogenicity (Beaz-Hidalgo et al. 2009). Jagoda et al. (2014) reported the presence of *Aer. veronii* (predominated) *Aer. hydrophila, Aer. caviae, Aer. jandaei, Aer. dhakensis* and *Aer. enteropelogenes* in diseased (motile aeromonas septicaemia) freshwater ornamental fish in Sri Lanka. However, *Aer. enteropelogenes* has not been recognized before as a fish pathogen. Whereas the aeromonads used to be classified in Vibrionaceae, a new family was established to accommodate them, i.e. the family Aeromonadaceae (Colwell et al. 1986).

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Fig. 4.1 Generalised liquefaction of rainbow trout tissues resulting from infection with *Aer. aquariorum*

Aeromonas allosaccharophila

Characteristics of the Disease

A description of the exact pathology present in the diseased elvers was not provided (Martinez-Murcia et al. 1992).

Isolation

The precise isolation method was not described. However, it was mentioned that growth occurred after an unstated period on TSA at 4–42 °C (Martinez-Murcia et al. 1992).

Characteristics of the Pathogen

During an examination of 16S rRNA sequences of motile aeromonads, two isolates, which were originally recovered from diseased elvers in Spain during 1988, were considered as sufficiently distinct from existing species to warrant description as a new species, *Aer. allosaccharophila* (Martinez-Murcia et al. 1992). However, it is recognised that isolates are phenetically heterogeneous (Huys et al. 2001).

Box 4.1: *Aeromonas allosaccharophila*
Cultures comprise Gram-negative, motile fermentative rods, which produce catalase, ß-galactosidase, indole, lysine decarboxylase and oxidase but not H_2S, reduce nitrates, degrade casein, DNA, egg yolk, gelatin, starch and Tween 80 but not elastin, sodium dodecyl sulphate or urea, and grow in 0–3 % (w/v) sodium chloride, at 4–42 °C and at pH 9.0. The Voges Proskauer reaction is negative. Acid is produced from D-cellobiose, D-galactose, glucose (plus gas), glycerol, maltose, D-mannitol, D-mannose and D-trehalose, but not from adonitol, arbutin, dulcitol, *m*-erythritol, *m*-inositol, lactose, salicin, D-sorbitol or D-xylose. A wide range of compounds are utilized as sole sources of carbon for energy and growth, including L-arabinose, L-arginine, D-cellobiose, fumarate, D-galactose, D-gluconate, L-glutamate, glycerol, L-histidine, maltose, D-mannitol, D-mannose, L-proline, succinate, sucrose and D-trehalose, but not L-alanine, γ – aminobutyrate, L-citrulline, dulcitol, *m*-erythritol, ethanol, D-glucuronate, L-glutamine, glycine, DL-3-hydroxybutyrate, *m*-inositol, α-ketoglutarate, lactose, L-leucine, propionate, putrescine, salicin or L-serine. Susceptibility has been recorded to chloramphenicol, erythromycin, fosfomicin, gentamicin, kanamycin, nalidixic acid, nitrofurantoin, oxolinic acid, polymyxin B and rifampicin, but not to ampicillin, streptomycin, sulphadimethoxine or trimethoprim. The G + C content of the DNA is 59.6 moles % (Martinez-Murcia et al. 1992).

The basis of allocating the isolates to a new species stemmed from the examination of 16S rRNA sequences, where homology values of >97.7 % were exhibited to other validly described *Aeromonas* species (Martinez-Murcia et al. 1992). Three isolates were found to be highly related, i.e. 70–100 %, by DNA:DNA hybridisation (Esteve et al. 1995a). On the basis of AFLP fingerprinting, *Aer. allosaccharophila* has been determined to be genetically related to *Aeromonas* HG 8/10 (Huys et al. 1996).

Diagnosis

Phenotypic Methods

Aer. allosaccharophila isolates may be identified by the examination of key phenotypic characters. In particular, the utilisation of L-arabinose and L-histidine as sole carbon sources, acid production from D-mannitol, D-melibiose, D-raffinose, L-rhamnose, salicin and sucrose, and the Voges Proskauer reaction were considered differential (Martinez-Murcia et al. 1992). However, a word of caution is necessary, insofar as the organisms which clearly demonstrated genetic homogeneity were markedly heterogeneous phenotypically. This would complicate diagnoses.

Pathogenicity

It was not concluded that the organisms were indeed pathogenic to fish. Yet, the recovery from diseased elvers suggests a pathogenic role for the organism (Martinez-Murcia et al. 1992).

Aeromonas bestiarum

Aer. bestiarum appears to be an example of a taxon which emerged from the taxonomic chaos surrounding the understanding of *Aer. hydrophila* (Ali et al. 1996). Originally, classified in DNA HG 2 *(Aer. hydrophila),* isolates have apparently been recovered from diseased fish (Huys et al. 1996). There is certainly evidence that *Aer. bestiarum* is pathogenic to fish (Orozova et al. 2009), and isolates have been recovered from ornamental fish with mouth ulcers (Fig. 4.2) and surface haemorrhaging (Fig. 4.3).

Fig. 4.2 Surface haemorrhaging and mouth erosion on a carp which was infected with *Aer. bestiarum*

Fig. 4.3 Erosion and haemorrhaging of the mouth of a ghost carp. The aetiological causal agent was *Aer. bestiarum*

Aeromonas caviae

Characteristics of the Disease

In 1991, a septicaemic condition was diagnosed on four Atlantic salmon farms located on the Black Sea in Turkey (Candan et al. 1995). Diseased fish displayed signs of haemorrhagic septicaemia, namely haemorrhages on the body, intestine filled with bloody exudate, enlarged liver and spleen, and liquefying kidney. Subsequently, the organism has been associated with eye disease and haemorrhagic septicaemia in farmed rainbow trout from Kenya (Ogara et al. 1998). An *Aer. caviae*-like organism has been linked to ulcerations in Indian catfish (*Clarias batrachus*) (Thomas et al. 2013).

Isolation

Diseased tissues were homogenised in 0.1 % (w/v) peptone, before inoculation of TSA and blood agar with incubation at 22 °C for 48 h (Candan et al. 1995).

Characteristics of the Pathogen

Cultures were identified by recourse to the scheme in Popoff (1984):

Box 4.2: ***Aeromonas caviae***
Cultures produce arginine dihydrolase, ß-galactosidase and indole, but not H_2S, lysine or ornithine decarboxylase or tryptophan deaminase, degrade aesculin, blood and gelatin but not urea, ferment amygdalin, arabinose, glucose, mannitol, sorbitol and sucrose, but not inositol, melibiose or rhamnose, utilise potassium cyanide but not citrate, grow in 0 % (w/v) sodium chloride, and do not reduce nitrates (Candan et al. 1995).

Aeromonas dhakensis

Aer. hydrophila subsp. *dhakensis* was reclassified in *Aer. aquariorum* (Martinez-Murcia et al. 2009) and then as *Aer. dhakensis* (Beaz-Hidalgo et al. 2013). The organism has been recovered from farmed Nile tilapia in Mexico (Soto-Rodriguez et al. 2013).

Characteristics of the Disease

The organism has been associated with generalized septicaemia (Orozova et al. 2009).

Isolation

The pathogen may be recovered using the procedures for *Aer. hydrophila,* with growth on TSA at 25 °C for 24–48 h.

Characteristics of the Pathogen

Box 4.3: ***Aeromonas dhakensis***
Cultures comprise Gram-negative, motile (by polar flagella) fermentative rods. Growth occurs on MacConkey agar, optimally at 30–37 °C, and in 0–3 % (w/v) sodium chloride. Arginine dihydrolase, catalase, ß-galactosidase, H_2S, indole, lysine decarboxylase and oxidase are produced, but not ornithine decarboxylase or tryptophan deaminase. Nitrates are reduced to nitrites. The Voges Proskauer reaction is positive. Aesculin, arbutin, blood (ß-haemolysis), casein, elastin, gelatin and starch are degraded, but not urea. Acid is produced from N-acetylglucosamine, arbutin, D-fructose, D-galactose, D-glucose, D-glycerol, glycogen, maltose, D-mannitol, D-mannose, D-ribose, salicin, sucrose and trehalose, but not from adonitol, L- or D-arabinose, L- or D-arabitol, amygdalin, cellobiose, dulcitol, erythritol, L- or D-fucose, gentiobiose, inositol, inulin, 2-or 5-ketogluconate, lactose, melezitose, melibiose, methyl D-glucoside, methyl α-D-mannopyranoside, methyl ß-D-xylopyranoside, D- raffinose, L-rhamnose, D-sorbitol, L-sorbose, D-tagatose, turanose, xylitol or L- or D-xylose. Gluconate is oxidized. DL-lactate is utilized. There is resistance to the vibriostatic agent O/129) (Beaz-Hidalgo et al. 2013).

Diagnosis

Cultures may be identified by use of the API 20E rapid identification system – profile = 7047125 (Esteve et al. 2012).

Epizootiology

The organism has been recovered from environmental samples in Spain (Esteve et al. 2012).

Pathogenicity

Orozova demonstrated pathogenicity of the type strain to rainbow trout (Orozova et al. 2009). Isolates recovered from diseased eels recorded a LD_{50} dose of 3.3×10^6 CFU/fish following experimental challenge of eels (Esteve et al. 2012). The organism killed Nile tilapia; ECPs had DNase, gelatinase, haemolytic and lipase activity (Soto-Rodriguez et al. 2013).

Disease Control

Antimicrobial Compounds

Resistance was recorded to ticarcillin, amoxicillin-clavuranic acid, cefoxitin, and imipenem (Esteve et al. 2012).

Aeromonas hydrophila

Much of the historical literature describing motile aeromonads as fish pathogens identifies isolates as *Aer. hydrophila;* the validity of these identifications is now questionable.

Characteristics of the Diseases

Since its initial recognition as the causal agent of haemorrhagic septicaemia (Sanarelli 1891; Schäperclaus 1930; Haley et al. 1967), *Aer. hydrophila* has been recovered as a pathogen from a wide variety of freshwater fish species, including ornamental fish (Hettiarachchi and Cheong 1994; Pathiratne et al. 1994) and occasionally from marine fish, e.g. ulcer disease of cod (Larsen and Jensen 1977). The aetiological agent has been considered to be the dominant cause of motile aeromonas disease in China (Nielsen et al. 2001), and may well have worldwide distribution. Its presence in channel catfish aquaculture in the southeastern USA may reflect an introduction from China insofar as phylogenomic analyses point to similarities

between strains from these two countries with the suggestion that American strains have an Asian origin/ancester (Hossain et al. 2014). However, some doubt has been expressed over its precise role as a fish pathogen (Heuschmann-Brunner 1965; Eurell et al. 1978; Michel 1981) with some workers contending that it may be merely a secondary invader of already compromised hosts. Conversely, other groups have insisted that *Aer. hydrophila* constitutes a primary pathogen. To some extent, the significance of *Aer. hydrophila* and its synonyms *Aer. formicans* and *Aer. liquefaciens,* as a fish pathogen has been overshadowed by *Aer. salmonicida.*

Aer. hydrophila has been credited with causing several distinct pathological conditions, including tail/fin rot and haemorrhagic septicaemias (e.g. Hettiarachchi and Cheong 1994). The organism may be found commonly in association with other pathogens, such as *Aer. salmonicida.* There is evidence that prior infestation with parasites, namely *Ichthyophthirius multifiliis* (= Ich) in channel catfish, may lead to the development of heightened bacterial populations (*Aer. hydrophila)* in the gills, kidney, liver, skin and spleen and mortalities by *Aer. hydrophila* (Xu et al. 2012). Haemorrhagic septicaemia (also referred to as motile aeromonas septicaemia) is characterised by the presence of surface lesions [Figs. 4.4, 4.5, and 4.6] (which may

Fig. 4.4 Extensive surface haemorrhaging on tilapia infected with *Aeromonas* sp., possibly *Aer. hydrophila* (Photograph courtesy of Dr. A. Newaj-Fyzul)

Fig. 4.5 A crucian carp displaying extensive surface haemorrhaging attributed to infection with *Aer. hydrophila* (Photograph courtesy of Dr. D.-H. Kim)

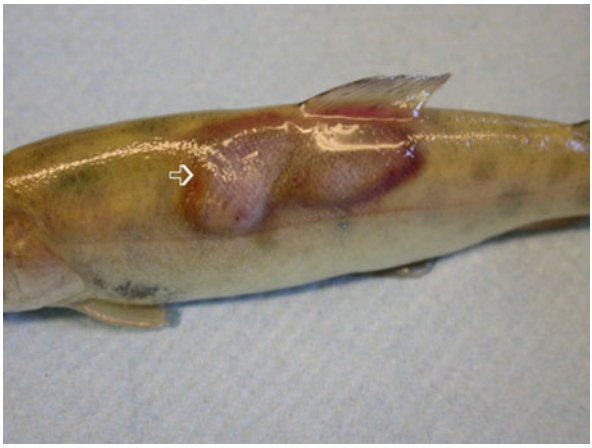

Fig. 4.6 An extensive abscess with associated muscle liquefaction in the musculature of rainbow trout. The aetiological agent was *Aer. hydrophila* (Photograph courtesy of Dr. A. Newaj-Fyzul)

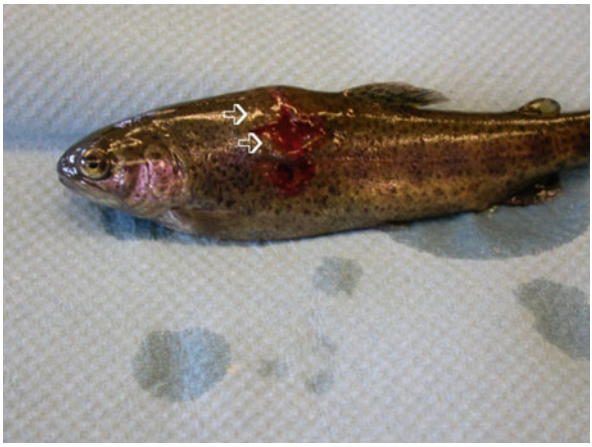

Fig. 4.7 A dissected abscess on a rainbow trout revealing liquefaction of the muscle and haemorrhaging. The aetiological agent was *Aer. hydrophila*

lead to the sloughing-off of scales), local haemorrhages particularly in the gills and vent, ulcers, abscesses (Figs. 4.6 and 4.7), exophthalmia and abdominal distension (Fig. 4.8). Llobrera and Gacutan (1987) described the presence of necrotic ulcers in a variety of fish from the Philippines. Internally, there may be accumulation of ascitic fluid (Fig. 4.9), anaemia, and damage to the organs, notably kidney and liver (Huizinga et al. 1979; Miyazaki and Kaige 1985), including generalized liquefaction of the internal organs and musculature (Fig. 4.9). Also, red sore disease in bass has been attributed to *Aer. hydrophila* (Hazen et al. 1978). This condition, which may reach epizootic proportion, is characterised by erosion of the scales and pinprick haemorrhages, which may cover up to 75 % of the body surface. There is often a high mortality rate. Hettiarachchi and Cheong (1994) described *Aer. hydrophila* as the cause of disease in freshwater ornamental fish in Sri Lanka, with disease signs including the presence of eroded fins (Fig. 4.10), haemorrhages on the skin and at the base of the caudal fin, sloughing scales and haemorrhaging in the intestinal wall.

Fig. 4.8 *Aeromonas* infection in goldfish, which is also displaying abdominal swelling (dropsy) and some surface haemorrhaging (Photograph courtesy of Dr. A. Newaj-Fyzul)

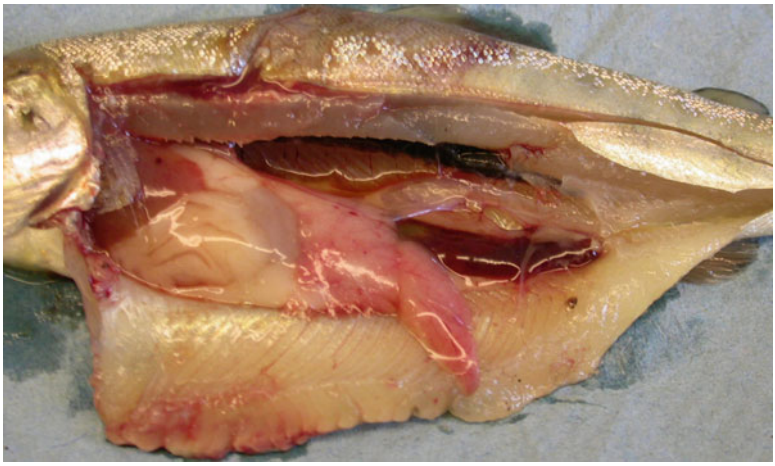

Fig. 4.9 Generalised liquefaction of a rainbow trout associated with infection by *Aeromonas*

Isolation

This is quite straightforward, involving use of kidney swabs with non-selective media, such as nutrient agar or TSA, or selective media, namely Rimler-Shotts medium (Appendix in Chap. 12; Shotts and Rimler 1973) or peptone beef-extract glycogen agar (Appendix in Chap. 12; McCoy and Pilcher 1974) with incubation at 20–25 °C for 24–48 h. Typically, on non-selective media, cream, round, raised, entire colonies of 2–3 mm diameter develop within 48 h at 25 °C.

Characteristics of the Pathogen

With improvements in the taxonomy of the "motile" aeromonads (see Carnahan and Altwegg 1996), it is speculative about whether or not the fish isolates belong as *Aer. hydrophila* or in any of the other *Aeromonas* Hybridisation Groups. To some extent,

Fig. 4.10 Extensive erosion of the tail and fins on a rainbow trout. Also, there is some evidence for the presence of gill disease. The aetiological agent was *Aer. hydrophila* (Photograph courtesy of Dr. N. Pieters)

the improvements in aeromonad taxonomy may reflect the sudden emergence of other taxa as fish pathogens. Certainly, there is marked phenotypic, serological and genotypic heterogeneity within the descriptions of fish pathogenic *Aer. hydrophila* (MacInnes et al. 1979; Leblanc et al. 1981; Allen et al. 1983a).

Box 4.4: ***Aeromonas hydrophila***

The fish pathogens comprise Gram-negative straight chemo-organotrophic (fermentative) rods of approximately 0.8–1.0 × 1.0–3.5 μm in size, which are motile by single polar flagella. Arginine dihydrolase, catalase, ß-galactosidase, indole, lysine decarboxylase (a variable response may occur), cytochrome-oxidase and phosphatase are produced, but not H_2S, ornithine decarboxylase or phenylalanine or tryptophan deaminase. Nitrates are reduced to nitrates without the production of gas. The Voges Proskauer reaction is positive, but not so the methyl red test. Growth occurs in 0–4 % (w/v) but not 5 % (w/v) sodium chloride, at 5–37 °C and in potassium cyanide. Aesculin, blood (β-haemolysis; by some isolates), casein, DNA, gelatin, lecithin, RNA, starch and Tween 80 are degraded, but not pectin or urea. There is resistance to the vibriostatic agent O/129. *N*-acetyl-ß-D-galactosamine, L-alanine, L-arabinose, *p*-arbutin, DL-lactate, D-mannitol, putrescine, D-serine, salicin and D-sucrose are utilised as the source carbon course for energy and growth, not D-cellobiose, DL-isocitrate, ß-alanine, 4-aminobutyrate or urocanic acid. Sodium citrate is utilised by some isolates. Acid is produced from cellobiose (a variable response), fructose, galactose, glucose (acid and gas), glycerol (a variable response), lactose (a variable response), maltose, mannitol, salicin, sucrose and trehalose, but not from adonitol, dulcitol, erythritol, inositol, raffinose, rhamnose, sorbitol or xylose (Paterson 1974; Larsen and Jensen 1977; Allen et al. 1983a; Popoff 1984). The G+C ratio of the DNA falls in the range of 58–61.6 moles % (Larsen and Jensen 1977; MacInnes et al. 1979; Huys et al. 2002).

In contrast to the usual characteristics of *Aer. salmonicida,* the majority of isolates of *Aer. hydrophila* are capable of growth at 37 °C and are, indeed, motile. Some isolates have also been determined to produce diffusible brown pigments, as does *Aer. salmonicida,* which could superficially confuse diagnosticians (Ross 1962; Paterson 1974; Allen et al. 1983a, b; Austin et al. 1989). Santos et al. (1991) serotyped 62 motile *Aeromonas* spp. from rainbow trout. Of these, 55 isolates (89 % of the total) were distributed between 17 serogroups, of which O3, O6, O11 and O19 were dominant. Moreover, 40 (63 % of the total) of these isolates were pathogenic to fish. Nevertheless, antigenic cross-reactivity with *Aer. salmonicida* and *Aer. sobria* has been noted (Leblanc et al. 1981). However, Shaw and Hodder (1978) reported that the core region of the LPS of *Aer. hydrophila* was distinct from that of *Aer. caviae* and *Aer. sobria.*

Diagnosis

Phenotypic Methods

The API 20E profile(s) for *Aer. hydrophila* are similar to those of *Aer. allosaccharophila* and *Aer. sobria,* and therefore use of this rapid identification system could give erroneous results. Toranzo et al. (1986) compared the API 20E rapid identification system for *Aer. hydrophila* with Kaper's medium (Kaper et al. 1979) and conventional biochemical tests. Kaper and co-workers formulated a single tube medium, which was suitable for determining motility, inositol and mannitol fermentation, ornithine decarboxylase and deamination, and the production of H_2S and indole. Thus, *bona fide* isolates of *Aer. hydrophila* gave an alkaline reaction on the top of the medium, acid production in the butt, motility, and indole but not H_2S production (H_2S production may occur on the top). Toranzo and colleagues pointed to shortcomings of the API 20E system, insofar as many environmental isolates were mis-identified or not listed by the published profile index. In contrast, Kaper's medium was effective for fast, presumptive identification. Problems were encountered with the reliability of some conventional biochemical tests, notably the Voges Proskauer reaction, fermentation and gas production from arabinose, gelatinase production, and the lysine decarboxylase test. Ironically, these tests have also been considered to be correlated with virulence in motile aeromonads.

Serology

Eurell et al. (1978) considered the effectiveness of slide agglutination especially for field use in the recognition of *Aer. hydrophila* infections, whereas tube- and macro-agglutination were useful in laboratories. Kawahara and Kusuda (1987) reported that FAT was superior to culturing for the diagnosis of *Aer. hydrophila* infections in eels. Moreover, FAT was more successful than culturing for detecting *Aer.*

hydrophila in mixed infections. A sensitivity limit of 10^4–10^5 cells/well was detailed for the *Aer. hydrophila* ELISA devised by Sendra et al. (1997). Monoclonal antibodies to *Aer. hydrophila* were used in a dot blotting method with a resultant sensitivity of 10^5–10^7 CFU/ml although this could be improved considerably to 10^2–10^3 CFU/ml by incubating the infected material in TSB for 3–6 h first (Longyant et al. 2010).

Molecular Methods

PCRs have been developed for *Aer. hydrophila* (Chu and Lu 2005).

Epizootiology

The epizootiology of *Aer. hydrophila* has not been considered in any great detail, although it has been concluded that the organism is rife in freshwater (Heuschmann-Brunner 1978; Allen et al. 1983a, b), aquatic plants and fish (Trust and Sparrow 1974; Ugajin 1979) and fish eggs (Hansen and Olafsen 1989), and may be associated with invertebrates, such as the ciliated protozoan *Tetrahymena pyriformis* (King and Shotts 1988), from where it will be readily available for infection of fish. Some isolates have been demonstrated to exhibit chemotactic responses to the mucus of freshwater fish (Hazen et al. 1982). The chemotaxic substance, which is heat stable at 56 °C, has been reported to have a molecular weight of 100 kDa.

The evidence points to a stress-mediated disease condition (Bullock et al. 1971), in which mortalities, if indeed they occur at all (Huizinga et al. 1979), are influenced by elevated water temperatures (Groberg et al. 1978; Nieto et al. 1985). Thus, Groberg and co-workers determined that deaths among fish, which were challenged via i.p. injection, occurred only at water temperatures in excess of 9.4 °C. This implies that the disease is not associated with cold water. In addition to water temperatures, the presence of pollutants, notably nitrite at 6 mg/l, increased the susceptibility of channel catfish to infection (Hanson and Grizzle 1985). It is interesting to note that survivors possess high serum titres of IgM-like antibody (Hazen et al. 1981).

During an examination of likely portals of entry of the pathogen into walking catfish, Lio-Po et al. (1996) found evidence of localisation in the muscle.

Pathogenicity

Most of the information concerning pathogenicity mechanisms of *Aer. hydrophila* appertains to isolates of medical importance and will not be considered further here. The value of using cultures, grown on nutrient rich media, has been cast into doubt following the observation that starved cells (NB: this is akin to the natural state of bacteria in the aquatic environment) are more virulent than their counterparts from

nutrient rich cultures (Rahman et al. 1997). Nevertheless as a general rule, it is apparent that the pathogen has considerable exo-enzyme potential, including haemolysins, serine (= caseinase; 68 kDa) – and metalloprotease (= elastase; 31, 44 and 60 kDa) (Esteve and Birkbeck 2004) some of which has relevance in fish pathology. The precise function of these 'toxins', which number at least six (Bernheimer and Avigad 1974; Donta and Haddow 1978; Cumberbatch et al. 1979) in fish pathology, has yet to be fully elucidated. A 21 kb plasmid has been detected in pathogenic isolates associated with ulcerative disease syndrome, and correlated with antibiotic-resistance. Curing the plasmid led to loss of virulence in Indian walking catfish *(Clarias batrachus)* whereas pathogenicity was restored when the plasmid was re-introduced into the bacterial cells (Majumdar et al. 2006). There have been more recent developments involving determination of the host response to infection. Thus, it has been documented that the stress-related genes, i.e. heat-shock protein (HSP) genes with modulation being greater in liver compared to the kidney or spleen of experimentally infected rohu particularly with regard to APG2, HSP90, glucose-regulated protein (GRP) 78, GRP75, and heat shock cognate 70 at 3–24 h after challenge; HSP 70 were down-regulated during infection. GRP 78, which is a highly conserved HSP gene essential for its regulatory role in infection and the early developmental phases of the fish, was most highly expressed in liver at 12 h after challenge (Das et al. 2015a, b). This begs the question about the nature of the host response. In the case of zebra fish skin, two-dimensional gel electrophoresis with mass spectrometry was used to determine the effect on protein expression as a result of infection with *Aer. hydrophila*. Thus, the data revealed that 17 proteins were differentially expressed 6 were up-regulated, and 11 proteins down-regulated. (Lu et al. 2014).

Surface Structures

Studies have emphasised the surface structures of *Aer. hydrophila*, which appear to be involved in autoaggregation/hydrophobicity and haemagglutination (e.g. Paula et al. 1988). There is some evidence that a capsule may be produced *in vivo* (Mateos and Paniagua 1995). The presence or absence of lateral flagella (as opposed to the more typical polar pattern) was demonstrated by electron microscopy on three isolates from catfish in Nigeria (Nzeako 1991). Del Corral et al. (1990) demonstrated the presence of pili/fimbriae, regardless of virulence. These workers considered that there was not a direct correlation between virulence and haemagglutination.

The surface array matrix, i.e. the S-layer, has been considered to influence the interaction between the bacterial cell and its environment (Esteve et al. 2004). A major function is believed to be the provision of physical protection from lytic components, including serum proteins and bacteriophages (Dooley et al. 1988). Work also links the presence of an S-layer with invasive disease in humans and mice (but not fish!) (Murray et al. 1988). As a result of studying one isolate, i.e. TF7 – isolated from a lesion on trout in Quebec, it was determined that the S-layer did not confer any increase in surface hydrophobicity or any enhanced association with macrophages, and did not specifically bind porphyrin or immunoglobulin (Murray et al.

1988). Nevertheless in *Aer. salmonicida,* the S-layer has indeed been shown to be a pre-requisite for virulence, by increasing hydrophobicity and enhancing macrophage association (Murray et al. 1988).

The detailed structure of the S-layer has been revealed in an excellent series of publications (Dooley et al. 1986, 1988; Dooley and Trust 1988). After studying 8 isolates of a serogroup with a high virulence to fish, Dooley and Trust (1988) concluded that the S-layer was tetragonally arrayed. SDS-PAGE revealed a protein of 52 kDa molecular weight, which was the major surface (protein) antigen. This protein effectively masked the underlying OMP.

Ascencio et al. (1991) investigated extracellular matrix protein binding to *Aer hydrophila.* In particular, binding of ^{125}I-labelled collagen, fibronectin and laminin is common to isolates from diseased fish. Moreover, the binding property was specific, with cultural conditions influencing expression of the bacterial cell surface binding structures. Experiments showed that calcium (in the growth medium) enhanced expression of the bacterial extracellular matrix protein surface receptors. The conclusion was reached that success in infecting/colonising a host depended on the ability of the pathogen to bind to specific cell surface receptors of the mucus layer, epithelial cells and subepithelial basement membranes.

"Adhesins"

The pathogen displays chemotaxis towards [gut] mucus, and adherence to and growth in the mucus (van der Marel et al. 2008). It appears that the pathogen has the ability to attach to selected host cells, e.g. erythrocytes, and tissue proteins, i.e. collagen, fibronectin, serum proteins and glycoproteins, via the action of 'adhesins' (Trust et al. 1980a, b, c; Toranzo et al. 1989; Ascencio et al. 1991; Lee et al. 1997; Fang et al. 2004) and become internalised (Tan et al. 1998). The adhesins, of which a 43 kDa (AHA1) adhesin has been cloned and shown to have high homology to two OMPs (Fang et al. 2004), appear to be extremely selective, recognising D-mannose and L-fucose side chains on polymers located on the surface of the eukaryotic cells. The specificity was further highlighted by the observation that human isolates of *Aer. hydrophila* failed to bind (or bound poorly) to fish tissue culture cells (Krovacek et al. 1987). Indeed using tissue culture cells from rainbow trout liver and chinook salmon embryo, Krovacek et al. (1987) demonstrated that some (~33 %) isolates of *Aer. hydrophila* from fish adhered to the tissue culture cells and glass surfaces coated with rainbow trout mucus. Adhesion and adsorption were time dependent; and the activities were lost after treatment of the bacteria with heat, proteolytic enzymes or ultra-sound.

Invasion of Fish Cells

The 43 kDa protein has been regarded as important for the invasion of epithelial cells, *in vitro* (Lee et al. 1997; Fang et al. 2004). Other workers have pointed to the relevance of capsular polysaccharides, which appear to enhance slightly adherence

to fish cells but contribute more significantly to cell invasion (Merino et al. 1997). A group II capsule gene cluster has been recognized, and the purified polysaccharide increased the ability of an avirulent culture to survive in (tilapia) serum and phagocytosis (Zhang et al. 2003). With attachment, the host cell will be at the mercy of the pathogen. Although the precise mechanism of cell damage and tissue damage remains unproven, the available evidence points to the involvement of both endo- and exo-toxins. Experiments with fish epidermal cells revealed that *Aer. hydrophila* could survive internally (Tan et al. 1998). Here, a role for tyrosine phosphorylation in the internalisation process was suggested (Tan et al. 1998). Indeed, subsequent work documented the ability of *Aer. hydrophila* to be internalized and survive in macrophages of eel (*Anguilla japonica*) with the mode of action reflecting the presence of flagella and thus motility (Qin et al. 2014).

Outer Membrane Proteins (OMPs)

Differences in the OMP according to incubation temperature has been documented, with a 40 kDa band produced following incubation at 17 and 25 °C, which also coincided with the greatest virulence and least phagocytic activity by goldfish macrophages (Rahman et al. 2001).

Extracellular Products (ECPs)

In comparison with *Aer. salmonicida,* fish pathogenic strains of *Aer. hydrophila* produce ECP, which contains considerable enzymatic activity (Shotts et al. 1984; Santos et al. 1987), including haemolysins and proteases (Angka et al. 1995; Khalil and Mansour 1997), and in particular a 64 kDa serine protease (Cascón et al. 2000) with optimum production [of protease] at 27.6 +/− 4.9 °C (Uddin et al. 1997). Interestingly, the highest mortalities were reported to occur in goldfish at 17 and 25 °C (compared to 10 and 32 °C) (Rahman et al. 2001). The relevance of the ECP was highlighted by Allan and Stevenson (1981) and Stevenson and Allan (1981), who succeeded in causing a pathology in fish as a result of injection of the material. Yet, the role of ECP is debatable with contrasting views of the importance of 'haemolysins' in virulence (Thune et al. 1986; Toranzo et al. 1989; Karunasagar et al. 1990; Paniagua et al. 1990). Stevenson and colleagues reported haemolytic (heat-labile) and proteolytic activity, the former of which was concluded to be of greater importance in pathogenesis. Kanai and Takagi (1986) recovered an a-type haemolysin which was deemed to be heat-stable and stable at pH 4–1.2, but inactivated by EDTA, trypsin and papain. The crude preparation caused swelling and reddening of the body surface following injection into carp. Previously, Boulanger et al. (1977) isolated two types of haemolysins. The reasons then for the conclusion about the

importance of haemolysins were based upon work with protease-deficient mutants, the ECP from which was more toxic to recipient fish than from wild-type cultures. Conversely, Thune et al. (1982a,b) obtained a fish-toxic fraction, which possessed proteolytic but not haemolytic activity. Moreover, in a comparison of ECP from virulent and weakly virulent isolates, Lallier et al. (1984) noted that both were haemolytic, enterotoxigenic and dermonecrotic, but the weakly virulent isolate produced twenty-fold more haemolysin than the virulent organism. Yet, only cell-free supernatants from virulent isolates produced toxic (oedematous) effects in fish. Following detailed chemical analyses, this heat-labile toxic factor was separated on Sephacryl S-200 from the haemolysin. These data suggest that factors other than haemolysins and proteases may be relevant in fish pathology. Indeed, after studying numerous isolates, Hsu et al. (1981, 1983), Shotts et al. (1984) and Paniagua et al. (1990) correlated virulence with extracellular proteolytic enzymes, notably caseinase and elastase. Santos et al. (1987) reported a relationship between virulence in fish and elastase and haemolysin (of human erythrocytes) production and fermentation of arabinose and sucrose. On this theme, Hsu et al. (1983) associated virulence with gas production from fructose, glucose, mannitol, mannose, salicin and trehalose, and the possession of resistance to colistin.

Extracellular metallo- and serine-proteases of *Aer. hydrophila* (strain B_5) have been characterised, and deemed to be heat (to 56 °C) (Leung and Stevenson 1988) and cold-stable (to −20 °C) (Nieto and Ellis 1986). Most activity was inhibited by EDTA. Overall, there were many differences in the proteases (4 or 5 were present) described by Nieto and Ellis (1986) to reports from other workers. This may be explained by the work of Leung and Stevenson (1988), who examined the proteases from 47 *Aer. hydrophila* isolates. Of these isolates, 27 produced both metallo- and serine-proteases, 19 produced only metallo-proteases, and ATCC 7966 produced only a serine-protease. The differences in these 47 isolates may well explain the apparent conflicting reports which result from the examination of only single isolates. Certainly, it seems that there are pronounced differences in the characteristics of the ECP and thus protease composition between strains.

It has been suggested that the proteases may be involved in protecting the pathogen against serum bacteriocidal effects, by providing nutrients for growth following the destruction of host tissues, and by enhancing invasiveness (Leung and Stevenson 1988). Also, proteases may be involved with the activation of haemolysin (Howard and Buckley 1985). In a significant development, it was determined that isolates with aerolysin (*AerA*), cytotoxic enterotoxin (*alt*) and serine protease genes (*ahp*) were most frequently virulent with lower LD_{50} doses in zebra fish than isolates with only one or two of these virulence genes (Li et al. 2011).

A further study identified acetylcholinesterase (a 15.5 kDa polypeptide) in the ECP, and regarded the enzyme as a major lethal factor, possibly with neurotoxic activity (Nieto et al. 1991; Rodriguez et al. 1993a, b; Peréz et al. 1998). The minimal lethal dose of the compound was given as 0.05 μg/g of fish.

Precipitation of *Aeromonas hydrophila*

The importance of precipitation after boiling is a debatable issue in screening of *Aer. hydrophila* isolates for virulence. Santos et al. (1988) considered that precipitation was not an important indicator, whereas Mittal et al. (1980) and Karunasagar et al. (1990) reported that settling after boiling was indeed an important measure of virulence.

Scavenging for Iron

The ability of a potential pathogen to scavenge successfully for iron (in iron-limited conditions) will influence the outcome of the infection process. The haemolysins of *Aer. hydrophila* are iron-regulated, and access to iron in the haemolytic destruction of the host cells may be necessary (Massad et al. 1991). The acquisition of iron from iron-transferrin in serum is dependent on the siderophore amonabactin. Many aeromonads use haem as a sole source of iron for growth. Some have evolved both siderophore-dependent (iron-transferrin) and -independent mechanisms (haem compounds) for the acquisition of iron from host tissues (Massad et al. 1991).

Enterotoxigenicity

Strains have been attributed with enterotoxigenicity, as assessed by the rabbit ileal-loop technique, and cytotoxicity (Boulanger et al. 1977; Jiwa 1983; Paniagua et al. 1990), and correlated with lysine decarboxylase production (Santos et al. 1987). Enterotoxigenic strains have been shown to produce two types of enterotoxins, which appear to be antigenically related, although the mode of action differs (Boulanger et al. 1977). This was an interesting observation because de Meuron and Peduzzi (1979) isolated two types of antigen, of which the K-antigen (this was thermolabile at 100 °C) was considered to represent a pathogenicity factor. Possibly, this corresponded to the enterotoxin or cytotoxin as described by Boulanger et al. (1977). However, the O (somatic)-antigen, which was heat stable, may have greater relevance, insofar as most virulent isolates share a common O-antigen (Mittal et al. 1980). In an excellent study, Dooley et al. (1985) used SDS-PAGE to analyses LPS (considered to constitute an O-antigen) from virulent strains, which auto-agglutinated in static broth culture. The LPS contained O-polysaccharide chains of homogeneous chain length. Two strains produced a surface protein array, which was traversed by O-polysaccharide chains and thus exposed to the cell surface. Antigenic analysis revealed that the polysaccharide of the LPS carried three antigenic determinants.

There is evidence that macrophage cytotoxicity leading to apoptosis is induced by *Aer. hydrophila* (Banerjee et al. 2012). Apoptosis commences with phagocytosis of viable bacterial cells into the head kidney macrophages; this leads to an alteration

of cytosolic calcium homeostasis initiating the activation particularly of calpain-2, which leads to caspase-3 mediated apoptosis (Banerjee et al. 2012).

Clearly, the evidence indicates the involvement of both endo- and exo-toxins in the pathogenesis of *Aer hydrophila* infections. It still remains for further work to elucidate the precise mechanism of action.

Evidence from Molecular Analyses

By comparing a virulent and avirulent culture, suppression subtractive hybridization (SSH) was used to identify genetic differences, with the results highlighting 69 genomic regions absent from the latter (Zhang et al. 2000). Genes considered to represent known virulence attributes included haemolysin, histone-like protein, oligoprotease A, OMP and multi-drug resistance protein. Other genes encoded synthesis of O-antigen (Zhang et al. 2000).

Quorum Sensing

Quorum sensing has been linked to virulence of *Aer. hydrophila* in burbot (*Lota lota*). Using quorum sensing mutants – involving N-(butyryl)-L-homoserine lactone production and receptor mutants – less mortalities developed compared to use of the wild type culture. Moreover, virulence was restored in the mutants by the addition of N-butyryl-L-homoserine lactone. Conversely, the addition of quorum sensing inhibitors protected the burbot larvae from the virulent organism (Natrah et al. 2012).

Disease Control

Disease Resistant Fish

The differential resistance of four strains of common carp to infection by *Aer. hydrophila* has been documented with crosses derived from Tata and Szarvas 15 parents being most resistant whereas fish from the wild strains Duna and Amur were the most resistant. (Jeney et al. 2011).

Vaccine Development

Some attention has been devoted to developing vaccines, with commercial products becoming available. Simple preparations of inactivated whole cells, ECPs or OMPs, which may be administered by immersion, injection or via the oral route, work well

(Schachte 1978; Acuigrup 1980; Lamers and de Haas 1983; Ruangpan et al. 1986; Rahman and Kawai 2000; Chandran et al. 2002; da Silva et al. 2013; Das et al. 2013a; Sen et al. 2014; Sirimanapong et al. 2014; Zhang et al. 2014a, b, c) with the host response including the production of superoxide anion by the head-kidney leucocytes (Basheera John et al. 2002). For example, use of ECPs achieved an RPS of 100 % in catfish, two weeks after vaccination (Zhang et al. 2014a, b, c). Schachte (1978) recorded that the most convincing immune response, measured in terms of antibody titre, was achieved after using injection techniques. A recombinant bivalent expressed OMP of *V. vulnificus* and *Aer. hydrophila* was used to vaccinate via the i.p. route American eel (*Anguilla rostrata*) with challenge after 28-days leading to RPS values of 50 % for both pathogens (Guo et al. 2015). Using formalised whole cells applied by i.p. injection, Ruangpan et al. (1986) recorded complete protection in Nile tilapia within only two weeks. Some protection, i.e. 53–61 %, occurred only one week after vaccination. The importance of dose was highlighted by Dash et al. (2011), who administered 0.2 ml amounts of formalin-inactivated cells intraperitoneally, and determined that one month later the highest dose of 10^{10} CFU/ml led to the highest antibody titre and greatest protection after challenge. The lower doses of 10^{7} and 10^{5} CFU/ml gave correspondingly less antibody and protection (Dash et al. 2011). Incorporating purified 43 kDa OMP of *Aer. hydrophila* in FCA and a booster 3 weeks later (without FCA) led to a demonstrable immune response and protection in blue gourami *(Trichogaster trichopterus)* (Fang et al. 2000). The next most promising method of application was immersion vaccination, and thence oral methods of administration, which were used successfully by Yasumoto et al. (2006), who entrapped *Aer. hydrophila* antigens (protein concentration = 33 μg/ml) in liposomes. The vaccine was fed to carp at doses of 30 μl/fish/day over 3 days leading to detectable humoral antibodies after 2 and 3 weeks (there was a decline in titre at 4 weeks) and protection (at 22 days) after subcutaneous injection with *Aer. hydrophila* at 3.0×10^{5} (RPS = 63.6 %) or 1.0×10^{6} (RPS = 55 %) CFU/fish (Yasumoto et al. 2006). OMPs with or without FCA were administered i.p. to rohu, and challenged after 2-months leading to RPS of 79–88 % (Das et al. 2013a, b). Encapsulation of OMP in biodegradable nanoparticles and administered by i.p. injection to rohu heightened the immune response and improved protection against challenge (Rauta and Nayak 2015). A recombinant OMP with a modified adjuvant was proposed as vaccine for rohu, and was efficacious in experiments both in terms of survival and immunostimulation, notably an increase in lysozyme and myeloperoxidase activities, serum natural haemolysin titre and antibody titre, and upregulation of immune-related genes, i.e. complement factor 4, IgM, interleukin-1 ß, lysozyme G, ß 2-microglobulin, and major histocompatibility complex I and II (Dash et al. 2014). The penaeidin 3–2 (these are antimicrobial peptides found in penaeids) gene was transformed into rice using *Agrobacterium*. The transgenic rice bran was fed to tilapia, which were protected against challenge (Liu et al. 2014). A recombinant haemolysin co-regulated protein, which is an important component of T6SS, was expressed, and administered to common carp leading to improved survival after challenge (survival = 46.67 %) compared to the controls (survival = 7.14 %) (Wang et al. 2015a, b).

Bivalent formalin-inactivated whole cell preparations containing *Lactococcus garvieae* and *Aer. hydrophila* with and without Montanide ISA-763 as adjuvant were administered by intraperitoneal injection (0.1 ml amounts containing 1×10^8 cells/fish) to rainbow trout, and challenged after 30 days with a RPS for *Aer. hydrophila* of 100 % and 95.3 % for non adjuvanted and adjuvanted preparation, respectively. At 90 days after vaccination, challenge resulted in decreased protection of the non-adjuvanted preparation against *Aer. hydrophila* (RPS = 85 %) but not so for the adjuvanted product (RPS = 95 %) (Bastardo et al. 2012).

Attenuated live vaccines have been evaluated. Thus a rough attenuated derivative produced by repeated sub-culturing on BHIA for 8-years was reported to confer immune protection in rohu (Swain et al. 2010). Three attenuated products with resistance to novobiocin and rifampicin were developed, and applied intraperitoneally (dose = 4×10^5 CFU) to channel catfish. The outcome was RPS values of 86–100 % (Pridgeon and Klesius 2011).

Concerning the method of vaccine inactivation, Lamers and de Haas (1983) deduced that heat-inactivated vaccines (60 °C/1 h) gave superior results to formalised products (0.3 % formalin). However, it was apparent that concentration of the vaccine, in terms of the numbers of cells, was very important in eliciting an immune response. Thus, using carp as the experimental animal, Lamers and de Haas (1983) concluded that 10^7–10^9 cells generated a distinct agglutinating response whereas 10^5 cells did not. Moreover, secondary doses of vaccine were shown to be beneficial. Nevertheless, single doses of a formalin-inactivated vaccine (containing 10^7–10^9 cells), administered via i.m. injection, were capable of eliciting an immune response which was maintained for 360 days. This demonstrates that fish have immunological memory (Lamers et al. 1985a). Continuing the work, Lamers et al. (1985b) vaccinated carp by bathing. Although a single immersion did not result in significant serum antibody levels, secondary vaccination after 1, 3 or 8 months gave rise to a dramatic immune response. In particular, the highest response resulted from using booster doses at 3 months. However at 12 months, there was no response.

The ability of *Aer. hydrophila* to develop biofilms on surfaces has been exploited, and a study with walking catfish *(Clarias batrachus)* demonstrated that cells from biofilms on chitin flakes gave a higher RPS (=91–100 %) and serum antibody titre when administered orally for 20 days compared to preparations derived from suspensions in TSB (RPS = 29–42 %) (Nayak et al. 2004). Similarly, Azad et al. (1999) used an oral biofilm vaccine (dose = 10^{10} and 10^{13} CFU/g; the bacterial cells were grown on chitin flakes) for 15 days in carp and demonstrated high humoral antibody titres and protection. A parallel study involved a biofilm oral vaccine, which was fed at 10^{10} cells/g of fish/day for 20 days, to prevent disease caused by *Aer. hydrophila* in snakehead (*Channa striatus*), and led to the development of significant antibody titre and an RPS of 88 % after challenge (Siriyappagouder et al. 2014). The question about what is so special about biofilms needs to be addressed.

Subcellular components, particularly LPS, offer promise as components of vaccines (Grochola et al. 2015). Indeed, evidence has been presented that LPS induces cell-mediated protection (= regulates T-cell like macrophage system) in carp (Baba et al. 1988). Loghothetis and Austin (1996) echoed this view about the immunoge-

nicity of LPS, but also emphasised that rainbow trout responded to exopolysaccharide. LPS from *Aer. hydrophila* administered as three doses by immersion (150 μg/ml); RPS = 49 % for 90 min on 3 days or i.p. injection (3 doses each of 50 and 100 μg/fish; RPS = 100 %) but not orally into carp led to high levels of protection following challenge with *Aer. hydrophila* (Selvaraj et al. 2009). LPS from a virulent and non virulent strain of *Aer. hydrophila* were injected intraperitoneally in 50 and 75 μg/100 g of body weight amounts into carp (*Cyprinus carpio*) leading – especially in the case of the LPS from the non virulent stain – to immunostimulation, i.e. increased leukocyte count, lysozyme and phagocytic activity, and the production of ROS, and protection against challenge (Grochola et al. 2015). LPS and OMP vaccines (derived from a culture of *Aer. hydrophila*) were effective at immunostimulation (enhanced respiratory burst, phagocytic and serum lysozyme activities) and protection against disease (RPS = 83.3 % and 72.2 % for LPS and OMP, respectively, compared with 55.6 % for a formalin-inactivated whole cell preparation) when administered to grass carp *(Ctenopharyngodon idella),* which were challenged with *Aer. hydrophila* (Sun et al. 2012). The adhesion gene from *Aer. hydrophila*, which belonged to the maltoporin group of porins coding for OMP, Omp48, was cloned and sequenced. The recombinant protein of ~ 48 kDa molecular weight was administered to rohu and upon challenge with *Aer. hydrophila* resulted in protection (RPS = 69 %) (Khushiramani et al. 2012). A recombinant S-layer protein was immunoprotective (RPS = 56–87 %) when administered in an adjuvant by i.p. injection (30 μg protein/fish) in common carp (Poobalane et al. 2010). A live *aroA* vaccine has been evaluated in rainbow trout with success. Interestingly, the growth medium was shown to have marked effect on immunogenicity, in that cultures prepared in glucose containing media, i.e. brain heart infusion (BHI), Luria broth with 0.25 % (w/v) glucose and TSB, led to a reduction in complement consumption and reduced serum susceptibility compared with BHI and Luria broth grown cells which were suspended in PBS. Indeed, these preparations led to higher and longer-lasting serum antibody titres than cells cultured in TSB (Vivas et al. 2005). Another live genetically modified auxotrophic mutant of *Aer. hydrophila* has been evaluated, and environmental concerns addressed in work which determined that the cells disappeared within 15 days, but may well enter a NCBV state (Vivas et al. 2004). Two OMPs, Aha1 and OmpW, were overexpressed in *Esch. coli,* purified, and administered to common carp when after challenge RPSs of 52 % and 71 %, respectively, were recorded (Maiti et al. 2012). An interesting study revealed the protective effect of a recombinant protein vaccine, aerA, could be enhanced by using single walled carbon nanotubes as a delivery vehicle for immersion or i.m. injection uptake in terms of increased antibody titre and protection against challenge (RPS = ~80 %) (Gong et al. 2015).

A freeze (3 min in liquid nitrogen) thaw (followed by 3 min at 37 °C) lysate was evaluated in FCA by i.p. administration (20 μg of lysate/fish in an equi-volume of FCA) in rainbow trout with a low dose challenge after 4-weeks when the RPS value was reported as 97 % (LaPatra et al. 2010).

Zhao et al. (2011) used the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene *gapA* to express *Aer. hydrophila* GAPDH in an attenuated *V. anguillarum* strain, which was injected i.p. (10^6 CFU/fish) into turbot and challenged after 4-weeks. Challenge of cytoplasm GAPDH expressing strain AV/pUC-gapA-

vaccinated fish with *Aer. hydrophila* and *V. anguillarum* led to RPS values of 42 % and 92 %, respectively.

Tu et al. (2010) used ghost cells [these were produced with a culture harbouring the lysis plasmid, pElysis, and were induced to lysis after incubation at 27–42 °C] fed orally for 5-days, normal feed for 14 days and a second dose of oral vaccine for a further 5-days at 4×10^8 cells/g of fish/day to carp, and found improved RPS of 76.8 % after challenge with *Aer. hydrophila,* compared with RPS of 58.9 % in the group administered with formalin-inactivated whole cells. In fish vaccinated with ghost cells, there was greater amounts of antigen-specific antibody in serum and the intestinal mucosa (Tu et al. 2010). In short, there is every possibility that vaccines against *Aer. hydrophila* should work (Aly et al 2015).

Research addressed the possibility of employing passive immunization methods to protect crucian carp (*Carassius auratus gibelio*) against *Aer. hydrophila* by administering specific antibodies generated in egg yolk (Jin et al. 2013). The immunoglobulins (IgY) were produced in hens immunized with inactivated cells of *Aer. hydrophila,* and once purified were used by immersion at 0.5 g/l. The result was a reduction in mortalities following challenge with *Aer. hydrophila* (Jin et al. 2013).

Immunostimulants/Dietary Supplements

Vitamins

Vitamins in diets are of value for controlling infection (Sobhana et al. 2002). Evidence has been presented, which pointed to the role of vitamin C in stimulating the humoral and cell-mediated immune response in fish vaccinated with *Aer. hydrophila* vaccines (Anbarasu and Chandran 2001). Interest in vitamin C has continued, with Misra et al. (2007) pointing to a role in immunostimulation and protection against challenge with *Aer. hydrophila.*

Yeast RNA

Furthermore, yeast RNA incorporated in diets at 0.4 % (w/v) and fed for 60 days reduced mortalities caused by *Aer. hydrophila* in rohu *(Labeo rohita)* and enhanced the phagocyte respiratory burst activity (Choudhury et al. 2005).

Levan

A 60-day feeding regime of Indian major carp with 1.25 % microbial levan incorporated into the diet led to protection (RPS = 60 %) against challenge with *A. hydrophila* (Gupta et al. 2008).

Astaxanthin

Astaxanthin, which was dosed at 50 mg/kg of feed, improved growth, enhanced immunomodulation (erythrocytes, leucocytes, haemoglobin, haematocrit, phagocytic index, antiprotease, respiratory burst, serum lysozyme, and bacteriocidal activities, serum total protein, albumin and globulin levels) and protected common carp against challenge with *Aer. hydrophila* with cumulative mortalities of only 10 % (Jagruthi et al. 2014).

Carotenoids

Carotenoids, which were fed at 50 or 100 mg/kg of diet for 2 or 4-weeks to carp (*Cyprinus carpio*) enhanced immunity (complement, lysozyme and phagocytic activities, reactive oxygen and nitrogen species) and gave protection against challenge with a virulent culture (mortality = 10 %, compared to 90 % mortalities among the controls (Anbazahan et al. 2014).

Pyridoxine

Dietary pyridoxine dosed at 0.2–12.4 mg of pyridoxine/kg of feed was fed to Jian carp *(Cyprinus carpio* var. Jian) for 80 days followed by challenge with *Aer. hydrophila* with the outcome that by increasing the dietary supplement to 5.0 mg/kg of feed led to increasing levels of survival and enhancement of the phagocytic activity of the leukocytes, acid phosphatase, haemagglutination, lysozyme, total iron-binding capacity and antibody titre (Feng et al. 2010).

Fibre

Commercial dietary fermentable fibre (= Vitacel), which was administered at 10 g/kg of feed for 45 days to rainbow trout, led to immunomodulation (enhanced bacteriocidal and serum lysozyme activities and greater serum antibody titres) and protection against challenge with *Aer. hydrophila* (Yarahmadi et al. 2014).

ß-glucans

ß-glucans enhance the non-specific resistance to *Aer hydrophila* infection (Selvaraj et al. 2005; Kumari and Sahoo 2006).

Cod Liver oil and Coconut oil

Dietary cod liver oil and coconut oil (fed for 150 days) has been useful in protecting freshwater catfish jundia (*Rhamdia quelen*) against challenge with *Aer. hydrophila* (Vargas et al. 2013).

Chinese Herbs

0.1 % (w/v) *Astragalus membranaceous* and 0.1 % (w/v) *Lonicera japonica,* with or without 0.05 % (w/v) boron fed for 4-weeks enhanced the non-specific immune response, i.e. phagocytic, plasma lysozyme and respiratory burst activities of tilapia and conferred resistance to *Aer. hydrophila* (Ardó et al. 2008). A Chinese herbal mixture of *Astragalus*, angelica, hawthorn, Licorice root and honeysuckle, fed at 0.5–2.0 % for 4-weeks resulted in immunostimulation and protection against challenge in Nile tilapia (Tang et al. 2014). *Rehmannia glutinosa* dried prepared root powder was fed at 4 % to juvenile common carp for 80-days leading to improved growth, immunostimulation (lysozyme and phagocytic activity) and protection against challenge with *Aer. hydrophila* (Wang et al. 2015a, b). Furthermore, an anthraquinone extract of *Rheum officinale bail* was effective as a dietary supplement when fed at 0.1 % for 10-weeks for controlling disease caused by *Aer. hydrophila* in Wuchang bream (*Megalobrama amblycephala*) (Liu et al. 2012).

Indian Medicinal Herbs

Azardirachta indica, demonstrated marked *in vitro* inhibitory activity against *Aer. hydrophila.* Furthermore, aqueous and ethanol extracts of equi-mixtures of *Azardirachta indica, Curcuma longa* and *Ocimum sanctum* had demonstrable *in vitro* inhibitory activity (Harikrishnan and Balasundaram 2005).

Holy Basil

The potential benefit of using holy basil (*Ocimum sanctum*) was reinforced by the study of Das et al. (2015a, b), who administered 0.2 % of an aqueous extract as a dietary supplement to rohu fingerlings, and recorded immunostimulation (enhanced total immunoglobulin levels, haemoglobin content, lysozyme activity, super oxide anion production, serum total protein, globulin, and erythrocyte and leucocyte counts) and protection (RPS = 40 %) against challenge.

Basil

100–1600 mg/kg of extract of basil (*Ocimum basilicum*) leaves fed to common carp fingerlings for 2-months led to improved growth, immunostimulations and resistance to challenge (Amirkhani and Firouzbakhsh 2015).

Andrographolide

Andrographolide, which is a labdane diterpenoid that has been isolated from the stem and leaves of the Asian herbaceous plant *Andrographis paniculata,* was added to diet at concentrations of 0.05–0.8 % and fed to Indian major carp for 42 days leading to enhanced growth, immunostimulation (enhanced serum lysozyme, myeloperoxidase, phagocytic and serum antiprotease activities) and protection after challenge with *Aer. hydrophila* (RPS = 74.06 %) (Basha et al. 2013).

Asthma Plant

The asthma plant, *Euphorbia hirta*, leaf extract was effective at enhancing immunomodulation (erythrocyte and leucocyte count, lysozyme activity, phagocytic ratio, and serum antibody and haemoglobin levels) and controlling *Aer. hydrophila* infection in common carp (Pratheepa and Sukumaran 2014).

Rhubarb

Rhubarb contains anthraquinone derivatives including aloe-emodin, chrysophanol, emodin, physcion and rhein, which have been linked with antibacterial activity against *Aer. hydrophila* [MIC = 50–200 μg/ml] (Lu et al. 2011). Unfortunately, the effect on fish disease was not determined.

Emodin

30 mg emodin/kg of diet was fed for 4 or 8-weeks to juvenile Wuchang bream (*Megalobrama amblycephala*) leading to weight gain, immunostimulation and protection against experimental challenge (Liu et al. 2014; Zhang et al. 2014a, b, c).

Prickly Chaff

Prickly chaff (*Achyranthes aspera*) seed was effective as a feed supplement at 0.5 % for 70 days in rohu to protect against challenge (mortality = 15 % compared to 50 % for the controls) with *Aer. hydrophila* (Chakrabarti and Srivastave 2012).

Garlic

Garlic (*Allium sativum*) dosed at 0.5 and 1.0 g/100 g of feed led to a stimulation in growth, feed conversion and protein efficiency, and marked reduction in mortalities (RPS = 95 %) in rainbow trout after challenge with *Aer. hydrophila* with the mode of action reflecting the stimulation of the number of erythrocytes and leucocytes, higher haematocrit and increase in anti-protease, bacteriocidal, lysozyme, phagocytic and respiratory burst activities (Nya and Austin 2009a). Of course, natural plant products are likely to contain many bioactive components, therefore, identification of the active ingredients(s) is likely to be challenging. Allicin, a component of garlic, was used separately as a feed additive dosed at 0.5 and 1.0 ml/100 g/feed for the control of *Aer. hydrophila* with great success, i.e. the RPS was 90–100 %. Again, there was evidence of immunomodulation, specifically increased phagocytic and serum lysozyme activities (Nya et al. 2010).

Ginger

Success occurred with ginger (*Zingiber officinale*), which again led to immunomodulation and protection against *Aer. hydrophila* (Nya and Austin 2009b).

Lupin, Mango and Stinging Nettle

Lupin (*Lupinus perennis*), mango (*Mangifera indica*) and stinging nettle (*Urtica dioica*) were added to feed to 1 % (w/v) for 14 days, and protected rainbow trout against challenge with *Aer. hydrophila,* with the mode of action considered to involve immune stimulation, principally enhancement in lysozyme respiratory burst and serum bacteriocidal activity, increases in the number of red and white blood cells in the blood (Awad and Austin 2010), upregulation of cytokine gene, i.e. IL-8, IL-1ß and TGF-ß] expression (Awad et al. 2011). The benefit of stinging nettle was reinforced by Ngugi et al. (2015), who noted growth, improvements, imunostimulation and protection of Victoria labeo (*Labe victorianus*) against challenge.

Neem

Dried neem leaf powder was incorporated into feed at 5 g neem/kg of diet, and fed to common carp leading to immunostimulation (enhanced superoxide anion production, lysozyme activity, serum protein and albumin levels) and reduced mortalities after challenge (35 % died compared to 85 % of the controls) (Verma and Kumari (2013)). As a further example, 4 g azadirachtin/kg, which is a secondary metabolite of neem (*Azadirachta indica*) seeds, was effective at immunomodulation (enhanced nitroblue tetrazolium and lysozyme activities, protein profiles, and leukocyte counts) and disease protection (RPS = 42.6 %) in goldfish (*Carassius auratus*) when dosed for 28-days (Kumar et al. 2013).

Indian Banyan Tree and Mimosoid/'miracle' Tree

Powdered preparations of the aerial roots and pod seeds of the Indian banyan tree (*Ficus benghalensis*) and mimosoid/'miracle' tree (*Leucaena leucocephala*) were fed at 5 % to Indian snakehead (*Channa punctata*) resulting in immunostimulation (lysozyme and phagocytic activities; increased antibody levels) and protection against challenge (Verma et al. 2015).

Willow Herb

Willow herb (*Epilobium hirsutum*) extract enhanced immunomodulation (leucocyte numbers) and resistance to challenge when fed at 3 % to common carp (Pakravan et al. 2012).

American Ginseng

American ginseng (*Panax quinquefolium*) was effective as a dietary supplement when dosed at 1.0, 2.0 [optimal dose], or 5.0 g/kg diet for 8-weeks to Nile tilapia with the data revealing improved growth and survival from challenge (Abdel-Tawwab 2012a, b).

Mangrove

Methanol extract of mangrove (*Excoecaria agallocha* leaves protected clownfish (*Amphiprion sebae*) against challenge (Dhayanithi et al. 2012).

Eugenol

Eugenol, which is a phenylpropanoid extracted from essential oils derived from basil, bay leaf, cinnamon, clove oil or nutmeg, improved the survival of infected silver catfish (*Rhamdia quelen)*, when administered by bathing at 10 mg/L for 10-days (Sutili et al. 2014).

Tetra

Feeding at 0.5–1.5 g/kg for 30 days with an extract of the woody deciduous shrub, tetra (*Cotinus coggygria*) immunostimulated (increased number of erythrocytes and enhanced lysozyme, myeloperoxidase and nitroblue tetrazolium blue activities) Koi carp (*Cyprinus carpio*), which resisted challenge (13.3–26.7 % mortalities compared with 40 % mortality of the controls) with *Aer. hydrophila* (Bilen et al. 2014).

Essential Oils of Espanta-Pulga and Basil

Plant essential oils of espanta-pulga (*Hesperozygis ringens*) and basil (*Ocimum americanum*) were evaluated therapeutically by bathing silver catfish (*Rhamdia quelen*) for 1 h daily for 5 days leading to improved survival (70 % and 75 % survival, respectively) from infection with *Aer. hydrophila* (Sutili et al. 2015).

Jungle Geranium

A purified phthalate derivative from jungle geranium (*Ixora coccinea*) was fed at 400 mg/kg for 30 days to goldfish resulting in immunostimulation (heightened erythrocyte and leucocyte numbers, increased levels of serum albumin, globulin, haemoglobin and protein, and enhanced bacteriocidal, lysozyme, and phagocytic activities) and protection (80 % survived compared with 0 % of the controls against experimental challenge (Anusha et al. 2014).

Guava

Diets containing 0.5 % guava (*Psidium guajava*) leaves were fed to rohu for 60 days better growth, immunostimulation (alternative complement and phagocytic activities, and lysozyme levels) and protection from challenge was recorded (Giri et al. 2015a, b).

Cinnamaldehyde

Liposome-encapsulated cinnamaldehyde, which is from cinnamon, improved survival of zebra fish against challenge with *Aer. hydrophila* (Faikoh et al. 2014).

3, 3′,5-triiodo-L-thyronine

Feeding with 3, 3′,5-triiodo-L-thyronine at 5 mg/kg of feed for 60 days to rohu *(Labeo rohita)* led to enhanced growth, serum protein and globulin levels, superoxide production of the neutrophils and antibodies against *Aer. hydrophila).* Moreover, there was a reduction in mortalities after challenge with *Aer. hydrophila* compared to the controls (Sahoo 2003).

Apolipoprotein A

Apolipoprotein A, recombinant goose-type lysozyme and chicken-type lysozyme have been considered as plasmid DNA immunostimulants in channel catfish with complete effectiveness against *Aer. hydrophila* (Pridgeon and Klesius 2013; Pridgeon et al. 2013a, b).

Honey Bee Pollen

Honey bee pollen, which was fed at 2.5 % for 20 or 30 days, improved growth, stimulated the immune response (phagocytic and serum bacteriocidal activity haematocrit, leucocrit, the numbers of lymphocytes, monocytes and neutrophils, serum total protein, albumin and globulin ratios), and protected Nile tilapia (survival = 93 %) against experimental challenge (El-Asely et al. 2014).

Cholesterol

Cholesterol was fed to rainbow trout at 0.9 or 1.2 % for 9-weeks leading to immunostimulation (increase of alternative complement, catalase, glutathione-peroxidase, lysozyme, respiratory burst, superoxide dismutase, and total antioxidant capacity activities, but decrease of malondialdehyde content) and resistance against challenge with *Aer. hydrophila* (Deng et al. 2013).

Mushroom Polysaccharide

Polysaccharide from the mushroom *Coriolus versicolor,* was fed at 1.0 g/kg for 56 days (but not higher amounts) to crucian carp (*Carassius auratus gibelio*) with immunostimulation (increased erythrocyte and leucocyte counts and the amount of haemoglobin) and protection against challenge (Wu et al. 2013).

Manna Polysaccharide

Manna polysaccharide was the focus of interest by Liu et al. (2013), who fed crucian carp with diets supplemented with 240 and 480 mg/kg of the compound for 10-weeks, and reported immunostimulation and resistance to challenge.

Cyanobacteria

Microcystis aueruginosa, was fed at 1.0 g/kg of feed for 90 days to Indian major carp (*Labeo rohita*) fingerlings leading to immunostimulation (enhanced levels of albumin, serum protein, lysozyme and serum bacteriocidal activity) and protection (72 % of the fish survived) against challenge (Das et al. 2013a, b).

Arginine

Dietary arginine fed at 16.1–21.9 g/kg diet. to juvenile Jian carp (*Cyprinus carpio* var. Jian) for 9-weeks led to immunostimulation (increased numbers of erythrocytes and leucocytes, enhanced IgM, lysozyme and phagocytic activity) and resistance to

challenge (Chen et al. 2015). The benefit of arginine was reinforced by Zhou et al. (2015), who fed arginine to juvenile yellow catfish (*Pelteobagrus fulvidraco*) and recorded similar health benefits.

Linolenic Acid

Linolenic acid incorporated into feed at 1.0–2.5 % and fed to darkbarbel catfish (*Pelteobagrus vachelli*) for 84 days led to immunomodulation and resistance to infection with *Aer. hydrophila* (Li et al. 2015).

N-Acylated Homoserine Lactone (AHL)

Feeding with the quorum sensing signal molecules AHL, which had lactonase activity, from *Bacillus* reduced the development of *Aer. hydrophila* infection following feeding experiments in zebra fish (Cao et al. 2012; 2014a, b). Furthermore, feeding quorum quenching *Bacillus* QSI-1 to zebra fish (*Danio rerio*) led to a reduction in the pathogenicity of *Aer. hydrophila* and improved survival of the fish after challenge (RPS = 80.8 %). Overall, there was a reduction in the accumulation of AHLs, which did not adversely affect the growth of *Aer. hydrophila* in co-cultures (although culture supernatant inhibited protease production, haemolysin activity and biofilm formation (Chu et al. 2014).

Prebiotics

A commercial prebiotic, Immunogen, was fed to common carp fingerlings at 1, 1.5 and 2.5 g Immungen/kg diet for 8-weeks led to to improved growth and enhanced resistance to *Aer. hydrophila* (Ebrahimi et al. 2012).

Probiotics

Bac. pumilus fed for 14 days at 10^7 cells/g of feed controlled infection by *Aer. hydrophila* (Aly et al. 2008). *Ps. aeruginosa* VSG-2 when fed at 10^7 or 10^9 CFU/g of feed for 60 days was reported to be effected at stimulating innate immunity (lysozyme, respiratory burst, superoxide dismutase and alternative complement pathway activities and phagocytosis) and protect rohu *(Labeo rohita)* against *Aer. hydrophila* challenge (Giri et al. 2012). *Lactobacillus plantarum* was fed at 10^7 CFU/g of diet to hybrid catfish for 45 days leading to immunostimulation (enhancement of phagocytic and lysozyme activities) and protection (0 % mortalities compared with 50 % mortalities of the controls) against experimental challenge (Butprom et al. 2013). Mixtures of live and heated-inactivated preparations containing *Bacillus subtilis, Lactococcus lactis* and/or *Saccharomyces cerevisiae* led to immunomodulation

(increase in erythrocyte number, myeloperoxidase level, and immunoglobulin, haemoglobin, albumin and globulin content, but a reduction in alanine aminotransferases, serum aspartate aminotransferases, acid and alkaline phosphatase activity) and protection of rohu (*Labeo rohita)* against challenge with *Aer. hydrophila* (Mohapatra et al. 2014). The best survival was with the group fed with the mixture of viable cells. Diets containing optimally 45 % crude protein and 2.0 g of *Sacharmyces cerevisiae*/kg of diet were fed twice a day for 5 days each week for 12 weeks to Nile tilapia fry leading to improve growth and resistance to experimental challenge with *Aer.* hydrophila (Abdel-Tawwab 2012a, b). A mixture of *Aspergillus oryzae* (2.0×10^9 CFU/g), *Bac. subtilis* (1.5×10^9 CFU/g) and *Saccharomyces cerevisiae* (10^9 CFU/g) were administered orally at 5 g/kg and 10 g/kg for 6-weeks to Nile tilapia leading to immunostimulation (enhanced leucocyte numbers, and respiratory burst activity) and protection against experimental challenge (Iwashita et al. 2015).

Bac. licheniformis KADR5 and *Bac. pumilus* KADR6 were isolated from the gut of rohu and injected intraperitoneally with subcellular components, cell wall proteins, ECPs, and whole cell proteins, and orally with live cells, i.e. 10^8 CFU/g of feed for 14 days before challenge resulting in immunostimulation (enhanced lysozyme and respiratory burst activities) and a reduction in mortalities (mortalities of 20–40 % and 23–33 % for the fish receiving subcellular components [especially whole cell and cell wall proteins], and live cells, respectively, compared with 80 % mortalities of the controls) (Ramesh et al. 2015).

Snakehead (*Channa striata*) fingerlings were fed with a commercial *Lactobacillus acidophilus* probiotic at 1 g/kg of feed together with 1 % yeast, 0.1 % ß-glucan, 1 % galacto-oligosaccharide and 0.2 % mannan oligosaccharide for 12-weeks before challenge. The results demonstrated superior growth, immunostimulation (increased numbers of erythrocytes and leucocytes; larger amounts of haemoglobin, serum protein and immunogobulin; increased lysozyme activity) and reduced mortalities after challenge (Talpur et al. 2014).

Antimicrobial Compounds

Chemotherapy of *Aer. hydrophila* infections corresponds closely to that of *Aer. salmonicida.* For example, the relevance of oxytetracycline has been well documented (Meyer 1964). Unfortunately, plasmid-mediated resistance by means of 20–30 mDa plasmids is similarly widespread in fish farms, e.g. eel ponds (Aoki 1988), and may negate the potential benefit of some antimicrobial compounds (Aoki and Egusa 1971; Toranzo et al. 1983). It is alarming that R plasmids with common sequence DNA structures have now been found in several unrelated species of fish pathogens, including *Aer. hydrophila, Aer. salmonicida* and *Edw. tarda* (Aoki 1988). Resistance in *Aer. hydrophila* has been recorded to a wide range of antimicrobial compounds, including ampicillin, chloramphenicol, erythromycin, nitrofurantoin, novobiocin, streptomycin, sulphonamides and tetracycline (Aoki 1988; De Paola et al. 1988). Indeed, it has been estimated that as many as 38 % of the *Aer. hydrophila* isolates

from diseased catfish are resistant to oxytetracycline (De Paola et al. 1988). For the future, new antimicrobial compounds, such as enrofloxacin, offer promise. This compound is anti-bacterial even at low dosages, i.e. with a MIC reported as 0.002 µg/ml (Bragg and Todd 1988).

Copper sulphate at a dose of 1 % of total alkalinity and used as a continuous bath for 18 h has been effective at controlling *Aeromonas hydrophila* infection in channel catfish (Bebak et al. 2012).

Bacteriophages

Two Myoviridae bacteriophages, designated pAh1-C and pAh6-C, were recovered, and a single administration controlled multiple-antibiotic-resistant *Aer. hydrophila* in cyprinid loaches (*Misgurnus anguillicaudatus*) (Jun et al. 2013). Subsequently, a bacteriophage was recovered from sewage, and used to control motile aeromonas septicaemia in Nile tilapia (De La Cruz-Papa et al. 2014).

Aeromonas jandaei

Characteristics of the Disease

Aer. jandaei has been reported as pathogenic to eel in Spain (Esteve et al. 1993, 1994; Esteve 1995).

Characteristics of the Pathogen

Initially, 8 isolates were recovered using unstated procedures during 1987 and 1988. Whereas initially, the method of identification was not stated (Esteve et al. 1994), a subsequent numerical taxonomy study equated isolates with *Aer. jandaei* (Esteve 1995).

Box 4.5: *Aer. jandaei*
Cultures are motile Gram-negative rods, that produce arginine dihydrolase, indole and lysine decarboxylase but not ornithine decarboxylase, degrade casein, chitin, DNA, gelatin, starch and Tween 80, grow in 0 % but not 6 % (w/v) sodium chloride and at 4–42 °C, and produce acid from galactose, glycogen, mannose, sucrose and trehalose, but not arabinose, lactose, melibiose, raffinose, rhamnose or salicin.

Pathogenicity

Esteve (1995) and Esteve et al. (1995b) reported a high LD_{50} dose of ~10^6 cells for eel. Possibly, the ECP activity, which was equated with production of caseinase, collagenase, elastase, protease, lipase and haemolysin, caused pathogenicity (Esteve et al. 1995b).

Aeromonas piscicola

Characteristics of the Pathogen

Box 4.6: ***Aer. piscicola***
Cultures are motile but do not form a brown diffusible pigment, produce ß-haemolysis, indole and lysine decarboxylase but not ornithine decarboxylase, are negative for the Voges Proskauer reaction, degrade aesculin and elastin, and produce acid from glycerol, salicin, and sucrose but not from L-arabinose, D-cellobiose or lactose (Beaz-Hidalgo et al. 2009).

Aeromonas schubertii

Characteristics of the Disease

An epizootic, with cumulative losses of 45 % over 40 days, occurred in 18-month old snakeheads (*Ophiocephalus argus)*, which were farmed in earthern ponds (water temperature = 26–28 °C) in Xianning, China during summer, 2009. The disease signs included inappetance, lethargy, listless swimming, and the presence of whitish nodules of 0.5–1.0 mm in diameter in the kidney, and blood clots in the liver (Liu and Li 2012). The possibility of *Mycobacterium* or *Nocardia* being associated with the nodules was ruled out by sequencing of the 16S rRNA gene. Necrosis and congestion in liver, kidney and spleen and damage to the heart muscle, intestine and gills has been documented (Chen et al. 2012).

Isolation

Isolation from blood, kidney, liver and spleen of moribund fish was achieved on BHIA plates with incubation at 28 °C for 48 h (Liu and Li 2012).

Characteristics of the Pathogen

The 8 bacterial cultures were identified from phenotype and sequencing of the 16S rRNA gene.

Box 4.7: *Aer. schubertii*
Cultures comprise short motile, fermentative Gram-negative rods occurring singly or in pairs, which produce arginine dihydrolase, catalase, lysine decarboxylase, oxidase and phenylalanine deaminase, but not H_2S, indole or ornithine decarboxylase. Nitrates are reduced to nitrites. The methyl red test is positive, but not so the Voges Proskauer reaction. Gelatin, lipids (Tween 80) and starch but not aesculin are degraded. Acid is produced from D-glucose, glycerol, maltose and trehalose, but not L-arabinose, cellobiose, *m*-inositol, lactose, D-mannitol, melibiose, melezitose, raffinose, L-rhamnose, ribose, D-sorbitol, sucrose or D-xylose. Growth occurs in 0 and 1 % but not 6.0 % (w/v) NaCl. Resistance occurs to the vibriostatic agent, O/129. A phylogenetic tree constructed with 16S rRNA gene sequences (99 % homology with *Aer. schubertii)* bores similarities to that derived from *gyrB-rpoD0dnaJ* concatenated sequences (100 % homology with *Aer. schubertii)* in which the snakehead isolates formed a single cluster with the type strain of *Aer. schubertii* ATCC 43700^T (Liu and Li 2012).

The snakehead isolates contained two plasmids of ~5,000 and ~10,000 bp (Liu and Li 2012).

Pathogenicity

The pathogenicity of one isolate, HYL1, was assessed intraperitoneally in healthy snakehead fingerlings (weight = ~16 g) and zebra fish (weight = ~ 2 g) maintained in static water at 25 °C [half the water was changed every second day] (Liu and Li 2012). The fish were monitored over 14 days, and HYL1 led to the development of similar disease signs to those observed in the fish farm with 100 % mortalities in snakehead and zebra fish when injected with 1.15×10^7 and 2.7×10^6 CFU/fish, respectively (Liu and Li 2012). Chen et al. (2012 reported) LD^{50} values of 1.4×10^4 and 6.4×10^6 CFU/ g of snakehead, with isolates producing elastase, haemolysin and lecithinase.

Disease Control

The oral administration of oxytetracyline led to a cessation in mortalities within two weeks (Liu and Li 2012).

Aeromonas sobria

Characteristics of the Disease

Organisms, identified as *Aer. sobria,* have been isolated from wild spawning gizzard shad (*Dorosoma cepedianum)* in Maryland, USA during 1987 (Toranzo et al. 1989), from farmed perch *(Perca fluviatilis)* in Switzerland (Wahli et al. 2006), from tail rot in tilapia (*Oreochromis niloticus*) (Li and Cai 2011) and a haemorrhagic ulcerative disease in stone loach (*Triplophya siluroides*) (Xu et al. 2014b) in China, from rainbow trout in Turkey (Ciftci et al. 2015), and from mass mortalities among the therapeutic fish *Garra rufa* in Slovakia (Majtan et al. 2012). Moribund gizzard shad did not reveal any external or internal signs of disease; perch had skin lesions and fin rot; *Garra rufa* displayed abnormal swimming, local haemorrhages and skin lesions.

Isolation

Pure culture growth was obtained from kidney, liver and spleen of moribund animals following inoculation of BHIA or TSA with incubation at 22 °C/28 °C for one or two days (Toranzo et al. 1989; Xu et al. 2014).

Characteristics of the Pathogen

Isolates identified as *Aer. sobria* biovar sobria have been found to be pathogenic to rainbow trout (Orozova et al. 2009).

Box 4.8: *Aer. sobria*
Cultures comprise motile (single polar flagellum) fermentative Gram-negative rods, which produce arginine dihydrolase, catalase, ß-galactosidase, indole, lysine decarboxylase and oxidase, but not H_2S or ornithine decarboxylase. Blood, casein, gelatin, starch and Tween 80 are degraded, but not so aesculin, elastin or urea. Acid is produced from glucose (with gas), mannitol and sucrose, but not from arabinose, inositol, rhamnose, salicin or sorbitol. Nitrates are reduced, and the Voges Proskauer reaction is positive. Growth occurs in 0-5 % but not 10 % (w/v) sodium chloride and on TCBS. Resistance has been recorded to ampicillin, novobiocin and the vibriostatic agent, O/129. The G + C ratio of the DNA is 60.4 moles % (Toranzo et al. 1989).

Overall, there was good agreement with the description of *Aer. sobria* (Martin-Carnahan and Joseph 2005). In particular, the important differentiating traits included production of acid from glucose (with gas), mannitol and sucrose but not salicin, and the inability to degrade aesculin (Martin-Carnahan and Joseph 2005). The only difference to the species description centred on the production of H_2S. The isolate recovered by Xu et al. (2014) were tentatively identified by phenotypic traits and sequencing of the 16S rRNA gene.

Epizootiology

It may be assumed that the natural reservoir is eutrophic fresh water.

Pathogenicity

Cultures were pathogenic to rainbow trout and tilapia with the LD_{50} reported as 2×10^5 cells and 4.17×10^3 CFU, respectively (Li and Cai 2011). Dead rainbow trout revealed the presence of haemorrhagic septicaemia. In addition, thermolabile ECP were cytotoxic and lethal (30 µg protein/fish) to rainbow trout. Paniagua et al. (1990) highlighted the role of caseinase, haemolysins and cytotoxins in the pathogenic process. Wahli et al. (2006) noted haemolytic activity on sheep and trout erythrocytes, and cytotoxicity to EPC cell line.

Isolates identified as *Aer. sobria* biovar sobria have been found to be pathogenic to rainbow trout (Orozova et al. 2009).

Disease Control

Probiotics

Use of *Ps. chlororaphis* JF3835 in water at 1×10^7 CFU/ml for 12 h led to a reduction in mortalities in perch (*Perca fluvialis*) caused by *Aer. sobria* (Gobeli et al. 2009).

Aeromonas veronii biovar sobria

Characteristics of the Disease

The organism was recovered from epizootic ulcerative syndrome, which is characterized by the presence of large ulcers all over the fish leading to death often within a week (Rahman et al. 2002). *Aer. veronii* biovar sobria was reported in infectious dropsy in cichlid Oscar (*Astronotus ocellat*us) (Sreedharan et al. 2011), haemorrhagic septicaemia in gourami (*Trichogaste*r sp.) and tail/fin rot in goldfish (*Carassius carassius*) (Sreedharan et al. 2013) from India. Here, there was recovery from the ascites of infected fish.

Aer. veronii has been mentioned in terms of immunization and an immune response in spotted sand bass *(Paralabrax maculofasciatus).* Infectivity data was presented but whether or not the organism is correctly identified and, indeed a fish pathogen must await further study (Merino-Contreras et al. 2001). Also, *Aer. veronii* has been included in a manuscript describing pathogens of Nile tilapia from Tanzania (Shayo et al. 2012).

Isolation

Scrapings from the ulcer were inoculated on to *Aeromonas* selective medium containing 5 μg/ml of ampicillin with unspecified incubation conditions (Rahman et al. 2002).

Characteristics of the Pathogen

The report by Sreedharan et al. (2011) provided the following description:

Box 4.9: *Aeromonas veronii* biovar sobria
Cultures comprise Gram-negative motile, fermentative rods that produce amylase, arginine dihydrolase, aryl sulphatase, caseinase, catalase, chitinase, DNase, ß-galactosidase, gelatinase, haemolysin (human blood), indole, lipase, lecithinase, lysine decarboxylase, oxidase, and gas from glucose, but not aesculin, elastase, lysine decarboxylase, or urease. Nitrates are reduced. Acid is produced from D-cellobiose, dextrin, D-fructose, D-galactose, glycerol, D-maltose, D-mannitol, D-mannose, D-ribose, sucrose, starch and trehalose, but not from adonitol, L-arabinose, inositol, inulin, D-lactose, D-melibiose, raffinose, L-rhamnose, salicin or D-sorbitol. Neither acetate, citrate, DL-lactate nor malonate is utilised. Confirmation may be obtained by sequencing the 16S RNA gene (Sreedharan et al. 2011).

Diagnosis

Identification has been achieved after examination of 14 isolates by FAME and AFLP fingerprinting, and biochemical profiling using the API 20E, API 20NE and PhenePlate system (Rahman et al. 2002).

Pathogenicity

Aer. veronii has been implicated as a potential fish pathogen but only in laboratory-based experiments, where intramuscular injection of 10^7 cells/ml resulted in muscle necrosis in Atlantic salmon (McIntosh and Austin 1990). Virulence has been demonstrated in rainbow trout (Orozova et al. 2009). Isolates have been reported to produce adhesins, cytotoxin, enterotoxin, haemagglutination (fish, human and rabbit blood) and haemolysin (Rahman et al. 2002; Sreedharan et al. 2011; 2013). In this respect, some isolates have been found to have high mucus-binding ability the relevance of which is that such bacteria employ mucus-binding as a pre-requisite to colonization and thus invasion of the intestine (Namba et al. 2012). These authors observed *Aer. veronii* around the intestinal bulb and rectum within 48 h of application to carp, suggesting that these sites are primary to invasion (Namba et al. 2012).

Control

A *Bacteriovorax* isolate has been identified as a potential biological control agent (Cao et al. 2014a, b).

Aeromonas veronii biovar veronii

There has been a report of *Aer. veronii* biovar veronii occurring in Chinese longsnout catfish (*Leiocassis longirostris*) with identification of cultures resulting from use of phenotyping and sequencing of the 16S rDNA genes. Virulence was confirmed in Chinese longsnout catfish, with the LD_{50} dose reported as 3.47×10^4 CFU/fish and 11.22 μg of ECPs/fish by i.p. injection. It was observed that the ECPs contained elastase, gelatinase, lecithinase and mostly lipase (Cai et al. 2012).

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Chapter 5
Aeromonadaceae Representative (*Aeromonas salmonicida*)

Abstract *Aeromonas salmonicida* is a significant pathogen of salmonids, and in its atypical form has spread into cyprinids and marine flatfish. Although *Aeromonas salmonicida* subsp. *salmonicida* is homogeneous, atypical isolates are more heterogeneous and do not fit into the current subspecies classification. Questions about the ecology of the organism remain but the consensus is that despite earlier work, cells exist in the aquatic environment although largely in a nonculturable form. Diagnostics have moved towards the use of sensitive and specific molecular methods. Disease control have focused on prophylaxis principally by vaccination, probiotics and immunostimulants.

Keywords Furunculosis • Ulcer disease • Pathogenicity • Vaccines • Immunostimulants

Aeromonas salmonicida

Characteristics of the Diseases

Historically, *Aer. salmonicida* was thought to have a predilection for salmonids. Over the years, however, the apparent host range of the pathogen has steadily expanded. Thus, infections are known to occur among representative of several major families of Osteicthys, including Cyprinidae, Serranidae and Anoplopomatidiae, in addition to the Salmonidae (Herman 1968), and also the Agnatha (Family Petromyzontidae) (Hall 1963). Non-salmonids which have been documented as suffering from diseases of *Aer. salmonicida* aetiology include minnow and goldfish (Williamson 1929), carp (Bootsma et al. 1977), perch (Bucke 1979) and bream, roach, dace, chub, tench, pike, bullheads, sculpins and catfish (McCarthy 1975a), wrasse (Treasurer and Cox 1991), sea bream (Real et al. 1994), sea lamprey (*Petromyzon marinus*) (Diamanka et al. 2014), and cultured marbled sole (*Pleuronectes yokohamae*) in Japan [these isolates infected Japanese flounder and spotted halibut (*Verasper variegates*)] (Kumagai et al. 2006). In some of the fish, particularly the non-salmonids, the disease may manifest itself in a different

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form to the classical furunculosis, and, the causal agent is often regarded as 'atypical'.

Traditionally, *Aer. salmonicida* has been known as the causative agent of furunculosis (Figs. 5.1, 5.2). However, it has become apparent that the pathogen manifests itself with other conditions, notably ulcerative dermatitis (Brocklebank 1998), and ulcerations especially in non-salmonids, e.g. in cod (Magnadóttir et al. 2002), black rockfish (*Sebastes schlegeli*) (Han et al. 2011), sailfin sandfish (*Arctoscopus japonicus*) (Wada et al. 2010) and turbot as a granulomatous dermatitis (Farto et al. 2011; Coscelli et al. 2014a, b). In the last mentioned, 52.5% mortalities were recorded in 2009 among fish reared in a Japanese aquarium. There was a dearth of lesions on the body surface with the exception of abrasions on the lower jaw. Internally, the kidney was swollen with white nodules (Wada et al. 2010). Furunculosis, named because of the sub-acute or chronic form of the disease, is recognised by the presence of lesions resembling boils, i.e. furuncles, in the musculature. In fact, the term furunculosis is a misnomer, because the lesions do not resemble those found in a similarly named condition of human beings (McCarthy 1975a). The name has, however, become established in the fisheries literature, so that it has been retained for convenience and to avoid the confusion that could result from a new name.

Fig. 5.1 A furuncle, which is attributable to *Aer. salmonicida* subsp. *salmonicida,* on the surface of a rainbow trout

Fig. 5.2 A dissected furuncle on a rainbow trout revealing liquefaction of the muscle

The sub-acute or chronic form of furunculosis, which is more common in older fish, is characterised by lethargy, slight exophthalmia, blood-shot fins, bloody discharge from the nares and vent, and multiple haemorrhages in the muscle and other tissues. Internally, haemorrhaging in the liver, swelling of the spleen, and kidney necrosis may occur (Snieszko 1958a; McCarthy and Roberts 1980). This form of the disease usually causes low rates of mortality, and fish may survive, although survivors have scar tissue in the vicinity of the furuncles (McCarthy 1975a). Oddly enough, the chronic form of the disease is not the most frequently occurring, nor is the presence of furuncles the most typical symptom of the disease (Snieszko 1958a).

The acute form of furunculosis, which is most common particularly in growing fish and adults, is manifested by a general septicaemia accompanied by melanosis, inappetance, lethargy, and small haemorrhages at the base of the fins. This form of the disease is of short duration, insofar as the fish usually die in 2–3 days, and causes high mortalities. The bacteria occur in the blood, disseminated throughout the tissues, and in the lesions. Internally, haemorrhaging occurs over the abdominal walls, viscera and heart. The spleen may appear enlarged. The acute disease is of sudden onset, with few, if any, external signs (McCarthy 1975a).

McCarthy and Roberts (1980) discussed a third clinical form of furunculosis, termed peracute furunculosis, which is confined to fingerling fish. The infected animals darken in colour, and may quickly die with only slight external symptoms, such as mild exophthalmia. Haemorrhages may occur at the base of the pectoral fin, if the fish manage to survive for long enough periods. Losses in farmed stock may be extremely high (Davis 1946).

Yet another form of furunculosis was discussed by Amlacher (1961), i.e. intestinal furunculosis. The symptoms were described as inflammation of the intestine, and anal inversion. This description is similar to a report by Herman (1968) of chronic furunculosis, i.e. low, relatively constant rate of mortality with intestinal inflammation and variable haemorrhages.

In addition to furunculosis, *Aer. salmonicida* has been implicated in other conditions often ulcerative, in freshwater and marine species, including wrasse (Laidler et al. 1999), Arctic charr (*Salvelinus alpinus*) and grayling (*Thymallus thymallus*) (Pylkkö et al. 2005). Atypical *Aer. salmonicida* has recently been associated with ulcerations in Atlantic salmon in Chile (Godoy et al. 2010). The best known of the ulcerative conditions is undoubtedly carp erythrodermatitis (CE; Fig. 5.3). Fijan (1972), who is credited with the name of the disease, demonstrated that CE was caused by a transmissible, antibiotic-sensitive organism, which manifested itself as predominantly a skin infection. Bootsma et al. (1977) isolated a small, Gram-negative, rod-shaped organism from skin lesions in mirror carp in Yugoslavia. This organism was subsequently identified as an "atypical" strain of *Aer. salmonicida*. CE was described as a sub-acute to chronic contagious skin disease, which varied in its morbidity and mortality (Bootsma et al. 1977). It appears that the infection often starts at the site of injury to the epidermis. A haemorrhagic inflammatory process then develops between the epidermis and dermis. This red inflammatory zone gradually extends as the infection spreads. The breakdown of tissue leads to the formation of a central ulcer, which may occur in any location on the body surface, although

Fig. 5.3 Carp erythrodermatitis. The aetiological agent is likely to be atypical *Aer. salmonicida.* Photograph courtesy of Professor H. Daskalov

it is most frequently located on the flanks. Infected fish exhibit inappetance, and appear darker in colour. Secondary invasion of the ulcer by fungi or other bacteria is common. If the fish recovers, the healed ulcer is recognisable as a grey-black scar. Frequently, contraction of the collagen of the scar tissue can result in serious deformity, which reduces the commercial value of the fish (Fijan 1972). In some instances, CE may also result in generalised septicaemia and death. Unlike furunculosis, which usually occurs only when water temperatures exceed 16 °C, CE may occur at all water temperatures.

Aer. salmonicida subsp. *masoucida* has been associated with extensive haemorrhagic septicaemia, including the presence of surface and muscle haemorrhaging, in black rockfish (*Sebastes schlegelii*) from South Korea (Fig. 5.4).

Aer. salmonicida has been reported to cause a cutaneous ulcerative disease in ornamental fish (Figs. 5.5, 5.6), especially goldfish (*Carassius auratus*), where the condition is referred at goldfish ulcer disease, which is a stress-mediated condition associated with the atypical form of the pathogen (Dror et al. 2006). However, the disease has been known for a long time previously. Ulcer disease of cyprinids, in general, has occurred in widely separated geographical locations, including the USA, Japan and England (Shotts et al. 1980). Mawdesley-Thomas (1969) studied, in detail, an outbreak of an ulcerative disease of goldfish, and recovered *Aer. salmonicida*. Symptoms included lethargy, loss of orientation, and abnormal swimming behaviour. The ulcers were of various sizes and depths, and some fish died shortly after infection. Secondary invasion of the ulcers by *Saprolegnia* was observed. More recently, we have recovered an extremely fastidious form of the pathogen from ulcerated carp, goldfish and roach in England. Here, there was evidence of secondary invasion of the ulcers by *Aer. hydrophila*. According to McCarthy and Roberts (1980), ulcer disease differed from CE in the following ways:

Fig. 5.4 Extensive skin and muscle haemorrhaging in black rockfish caused by *Aer. salmonicida* subsp. *masoucida.* Photograph courtesy of Dr. D.-H. Kim

Fig. 5.5 A well developed ulcer on a koi carp. The aetiological agent was atypical *Aer. salmonicida*

(a) the ulcers were deeper and more extensive,
(b) renal and splenic changes, such as those found in sub-acute furunculosis of salmonids, were present at an earlier stage in the course of the disease than in CE (where internal lesions were restricted to later stages in cases where septicaemia developed).

There has been some debate about whether or not atypical *Aer. salmonicida* are genuinely responsible for ulcer disease because culturing will often fail to recover the pathogen. However, Goodwin and Merry (2009) identified atypical *Aer. salmonicida* in the majority of specimens [52 out of 62 samples] of koi ulcer disease.

Fig. 5.6 An ulcerated goldfish on which the lesion has extended across the body wall, exposing the underlying organs. The aetiological agent was atypical *Aer. salmonicida*

In addition, *Aer. salmonicida* subsp. *smithia* has been recovered from ulcerative and haemorrhagic conditions in Arctic charr in Austria with identification of isolates achieved by sequencing (Goldschmidt-Clermont et al. 2009).

Another variation of *Aer. salmonicida* infection, termed "head ulcer disease", has been described in Japanese eels (Hikada et al. 1983; Ohtsuka et al. 1984; Kitao et al. 1984). An atypical strain of *Aer. salmonicida* was implicated as the aetiological agent. The progression of this disease is worthy of note because the pathogen is apparently capable of causing mortalities solely as a result of localised proliferation, with no evidence for the development of a generalised septicaemia. Results of natural and laboratory-based infections of eels with head ulcer disease revealed that *Aer. salmonicida* was not recovered in significant numbers from internal organs, i.e. brain, kidney or spleen or, indeed, blood (Ohtsuka et al. 1984; Nakai et al. 1989a). However, the pathogen proliferated substantially in the muscle of eels (Nakai et al. 1989). Similar, localised ulcerative infections caused by atypical *Aer. salmonicida* have been recognised in goldfish (Elliott and Shotts 1980) and carp (Csaba et al. 1981).

Isolation

Under ordinary circumstances, i.e. in cases of classical furunculosis caused by 'typical' strains of *Aer. salmonicida,* the pathogen may be readily recovered from diseased fish, especially from surface lesions and the kidney, by use of standard non-selective bacteriological agar media. TSA has been commonly used for this purpose. On TSA, the occurrence of colonies surrounded by a dark-brown water-soluble pigment (Fig. 5.7) after incubation at 20–25 °C for 3–4 days, is considered as indicative of the presence of *Aer. salmonicida*. However, it must be remembered that non-pigmented or slowly pigmented strains of the pathogen occur, and also that some other bacteria produce diffusible brown pigments, e.g. *Aer. hydrophila* and *Aer. media*. In addition, if the fish have succumbed to secondary infection with other micro-organisms, isolation of *Aer. salmonicida* becomes much more difficult because of overgrowth by other bacteria. Thus, growth of *Aer. salmonicida* may be

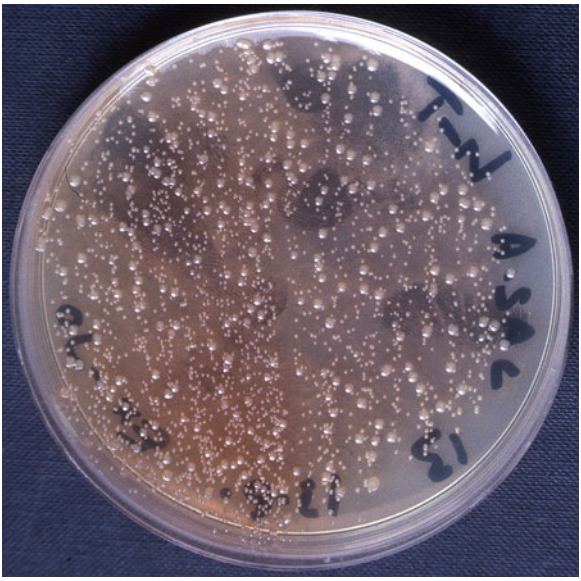

Fig. 5.7 *Aer. salmonicida* subsp. *salmonicida* producing brown diffusible pigment around the colonies on TSA. The smaller (= rough) and larger (= smooth) colonies are readily observable

suppressed or pigment production inhibited. For these reasons, McCarthy and Roberts (1980) recommended that a minimum of six fish should be sampled from any disease outbreak where the presence of *Aer. salmonicida* is suspected. Also, they suggested that samples destined for bacteriological examination should be taken from skin lesions, in all stages of development. In a similar vein, Daly and Stevenson (1985) concluded that it is advisable to sample other organs, i.e. heart, liver and spleen, in addition to the kidney in order to increase the chances of detecting *Aer. salmonicida*. Indeed, these workers found that 45 % (14/31) of successful isolations of the pathogen from brown trout, *Salmo trutta,* were from organs other than kidney. Likewise, in a study to detect carrier rates of the pathogen, Rose et al. (1989) concluded that sampling of just the kidney might result in an underestimate of the numbers of fish harbouring the pathogen. Thus, they recommended examining the intestine as well as the kidney for the presence of *Aer. salmonicida*. The problem is with the asymptomatic carriers from which recovery of the pathogen is notoriously difficult without stressing the fish (Cipriano et al. 1997).

In an attempt to improve the chances of recovering *Aer. salmonicida* from lake trout in a hatchery experiencing an outbreak of furunculosis, an enrichment procedure using TSB was evaluated by Daly and Stevenson (1985). This entailed placing swabbed material, derived from the organs of diseased animals, into TSB with incubation at 26 °C for 48 h. Then, the resulting broth cultures were streaked for single colony isolation onto plates of TSA. The results indicated that the recovery of *Aer. salmonicida* was twice that of using the conventional direct plating of swabbed material onto TSA.

The use of BHIA for isolation and maintenance of *Aer. salmonicida* has been recommended by some groups. In fact, we have observed that a greater proportion

of rough-type colonies (this trait is associated with virulence) of *Aer. salmonicida* were recovered on BHIA, than TSA.

In several instances, media supported with blood have been employed for the isolation of the pathogen, especially atypical isolates from cyprinids. McCarthy (1977a), however, stated that unsupplemented media, e.g. TSA, should be used in preference to blood-containing media, but no explanation was given, other than that the observations had resulted from extensive personal experiences.

A more recent addition to the media employed for the isolation of *Aer. salmonicida* is CBB (Appendix in Chap. 12; Fig. 5.8), as developed originally by Udey (1982). CBB was evaluated as a differential and presumptive medium for use in the identification of *Aer. salmonicida* in clinical specimens (Markwardt et al. 1989). The results showed that CBB was effective in differentiating *Aer. salmonicida* among mixed bacterial populations obtained from asymptomatic fish. In laboratory-based experiments, CBB was also successful in differentiating *Aer. salmonicida* colonies from mixed cultures containing *Aer. hydrophila* or *Y. ruckeri.* Here, *Aer. salmonicida* colonies were dark blue. An interesting development concerned the ability to detect *Aer. salmonicida* within 72 h by filtering 100 ml amounts of water through 0.45 μm pore size Millipore cellulose acetate and nitrate filters, and incubating the filters on CBB (Ford 1994). However, it is apparent that the *Aer. salmonicida* cells must possess the A-layer for the differentiating capacity of CBB to be effective. Also, other aquatic organisms, such as the purple-pigmented *Chromobacterium* and *Janthinobacterium* may produce dark blue colonies on CBB. Nevertheless, CBB is a promising addition to the narrow range of media, which may be used for the primary isolation of *Aer. salmonicida*.

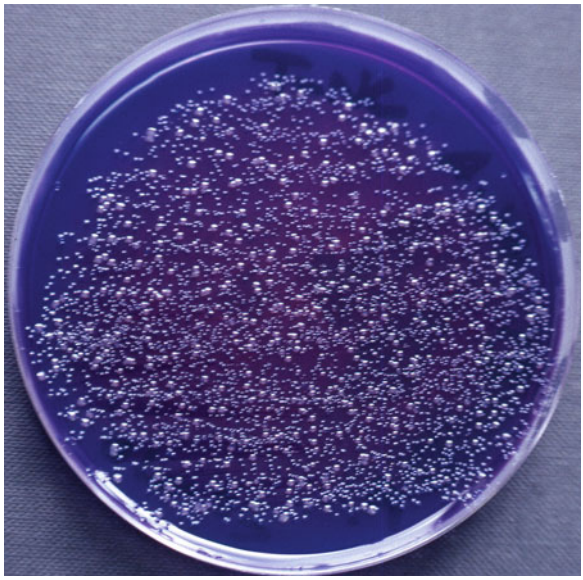

Fig. 5.8 The dark blue colonies of *Aer. salmonicida* subsp. *salmonicida* on CBB

The stress-induced furunculosis test to detect covertly infected fish has been very successful, and involves intramuscular injection with corticosteroid, namely 20 mg prednisolone acetate/kg of fish followed by increasing the water temperature typically from 12 to 18–20 °C. Cultures of *Aer. salmonicida* may be then recovered on TSA of CBB within 3–6 days (Bullock and Stuckey 1975b; McCarthy 1977b; Smith 1991; Bullock et al. 1997). Using such stressed rainbow trout, culturing was the most sensitive method for detecting *Aer. salmonicida* (detected 40 positives out of 80) followed by a direct FAT (detected 6 positive) and then a commercial ELISA system (detected 6 positives) (Bullock et al. 1997). Overall, the culture of gill and mucus was more sensitive (39 positives out of 80 fish examined) than kidney and spleen (18 positives) (Bullock et al. 1997).

In the case of CE and goldfish ulcer disease, the pathogen appears to be located more or less exclusively in the skin lesions. Thus, Bootsma et al. (1977) used an inoculating wire, which was plunged below the transparent epidermis at the edge of the ulcer, into the haemorrhagic zone. A loopful of the resulting material was streaked onto agar media. According to Bootsma, satisfactory growth occurred on tryptone-containing media supplemented with serum. Although the enrichment of a culture medium by the addition of serum has been deduced as necessary for the initial recovery of some fastidious strains, notably non-pigmented cultures, Bootsma et al. (1977) determined that the fastidiousness of the isolates decreased during maintenance *in vitro*. In our experience, strains associated with non-salmonid fish are extremely difficult to isolate. We have greatest success with blood agar (blood agar base [Oxoid] supplemented with 10 % v/v horse, sheep or bovine blood), which is inoculated and incubated at 15–18 °C for up to 7 days. Even with this method, *Aer. salmonicida* is recovered from only a small proportion of the clinically diseased fish. This begs the question about the reasons for culturability, when the pathogen is recovered from only a proportion of obviously infected animals. Microscopy will often reveal a greater number of bacterial cells than might be deduced from the results of plating experiments. Perhaps, as has been argued with L-forms (Figs. 5.9, 5.10), a threshold number of bacterial cells need to be present to enable some to be capable of producing growth in broth or on solid medium. Also, the definition of growth needs to be carefully considered, insofar as the basic criterion reflects observations with the naked eye, i.e. turbidity in broth or clearly visible colonies. The limited growth of micro-colonies may well be missed by classical bacteriological methods.

Characteristics of the Pathogen

Aer. salmonicida, which comprises the so-called non-motile taxon, is one of the most important fish pathogens because of its widespread distribution, diverse host range and economically devastating impact on cultivated fish, particularly the valuable salmonids. More has been written about *Aer. salmonicida* than any other bacterial fish pathogen. Comprehensive reviews, i.e. McCraw (1952), Herman (1968),

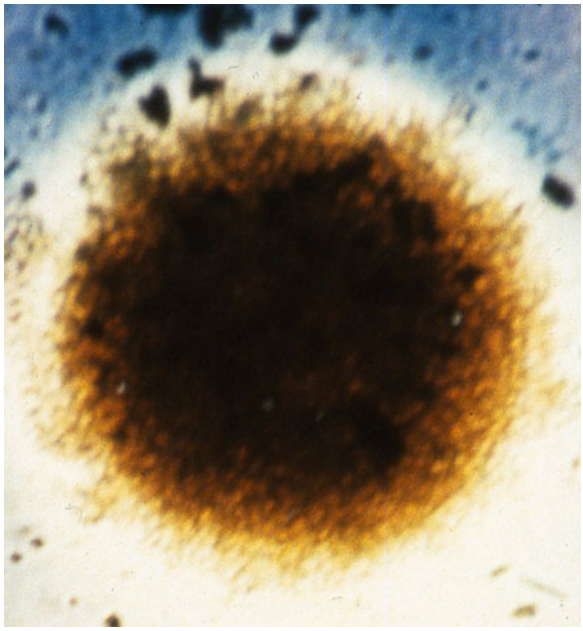

Fig. 5.9 An intensively brown pigmented L-form colony (~0.5 mm in diameter) of *Aer. salmonicida* subsp. *salmonicida* growing within L-F agar

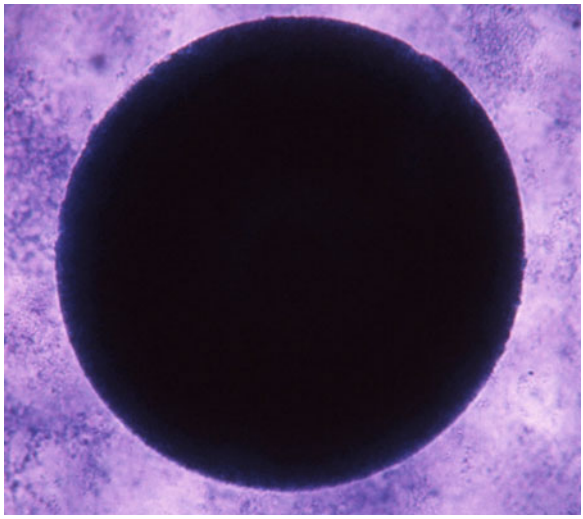

Fig. 5.10 An L-form colony (~0.5 mm in diameter) of *Aer. salmonicida* subsp. *salmonicida* growing within L-F agar and stained by Dienes method

McCarthy and Roberts (1980) and an excellent textbook (Bernoth et al. 1997) have adequately summarised the available knowledge on the pathogen in its context as a fish pathogen. The following narrative will emphasise taxonomic aspects, which have not been considered adequately by others.

Aer. salmonicida is one of the oldest described fish pathogens. It is generally accepted that the first authentic report of the organism was by Emmerich and Weibel

(1894), who isolated the pathogen from diseased brown trout, obtained from a hatchery in Germany, and named it 'Bacillus der Forellenseuche' or bacillus of trout contagious disease. Because of the importance of the organism, some authors have examined the early literature seeking evidence for the occurrence of furunculosis prior to 1894 (Williamson 1928; McCarthy and Roberts 1980). However, although several reports exist which suggest that furunculosis occurred earlier, it has proved impossible because of poor descriptions, to be certain that the bacterial isolates in question were *Aer. salmonicida* (Forel 1868; Fabre-Domerque 1890; Fischel and Enoch 1892). In North America, the first report of furunculosis was made by Marsh (1902) who described an organism, which was named as *Bacillus truttae*. This caused an epizootic in hatchery fish in Michigan, U.S.A.

The placement of *Aer. salmonicida* in the bacterial taxonomic hierarchy should be put into context. Like so many of the bacterial taxa described in the nineteenth century, the organism has undergone a series of changes in its classification. Shortly after Emmerich and Weibel (1894) reported it, the pathogen was placed in the genus *Bacterium,* as *Bacterium salmonicida,* by Lehmann and Neumann (1896). This was probably its best known epithet before its eventual transfer to the genus *Aeromonas*. To confuse the issue, however, *Bacillus devorans* (Zimmermann 1890), *Bacterium salmonica* (Chester 1897), *Bacterium truttae* (Marsh 1902) and *Bacillus salmonicida* were also names assigned to the pathogen in the past. With the publication of the 7th edition of *Bergey's Manual of Determinative Bacteriology* in 1957, the pathogen was transferred to the genus *Aeromonas,* then placed in the family Pseudomonadaceae. Later, the genus *Aeromonas* was moved again, to the family Vibrionaceae and more recently to its own family, i.e. the Aeromonadaceae (Colwell et al. 1986). The initial re-classification was based primarily on the work of Griffin et al. (1953a). This team undertook the first detailed characterisation of the organism, providing the information necessary to formulate the description, which resulted in its re-classification. However, the division of *Aer. salmonicida* strains into subspecies remains an ongoing bone of taxonomic contention. The description of *Aer. salmonicida* subsp. *pectinolytica* opens a new chapter in the understanding of the organism insofar as this is the first subspecies, which is not associated directly with fish diseases (Pavan et al. 2000). Instead, the isolates were recovered from a polluted river in Argentina (Pavan et al. 2000). To date, there is not any evidence to link this subspecies with fish pathogenicity.

Two aspects concerned with the status of *Aer. salmonicida* in the bacterial taxonomic hierarchy require discussion. One centres on the intraspecific relationships of *Aer. salmonicida* strains; the other is involved with questions that have been raised regarding the retention of the species in the genus *Aeromonas.*

Aer. salmonicida has been known by its present name since the 1950s when, on the basis of work by Griffin et al. (1953a), Snieszko (1957) in his contribution on the genus *Aeromonas* in the 7th edition of *Bergey's Manual of Determinative Bacteriology* assigned the pathogen to this genus, where it has remained. It is curious, in view of its seriousness as a pathogen, that it was over 50 years from the initial discovery of the organism to the characterisation and description of *Aer. salmonicida.* Griffin et al. (1953a) provided the first detailed description of the

organism, and were of the opinion that attempts to recognise and identify isolates were being hampered by a lack of a complete description. This resulted in confusion due to disagreement concerning the physiological and biochemical characterisation of the organism. From the results of a study of ten isolates, it was concluded that *Bacterium salmonicida* was extremely consistent in its general cultural and biochemical traits, and that problems in the past had arisen primarily from the use of media that varied in composition among laboratories. Although Griffin et al. (1953a) ended their report by recommending the re-classification of *Bacterium salmonicida* to the newly created genus *Aeromonas,* as *Aer. salmonicida,* no definite reasons were given for this move. However, with time, additional data have accumulated, and the homogeneity and authenticity of the taxon has generally been supported.

Subsequent investigators have re-examined the homogeneity of the taxon, using conventional and numerical phenotypic methods (Eddy 1960; Ewing et al. 1961; Schubert 1961; Smith 1963; Eddy and Carpenter 1964; Popoff 1969). Thus, the traditional description of *Aer. salmonicida* is of non-motile, fermentative, Gram-negative rods, which produce a brown water-soluble pigment on tryptone-containing agar, which do not grow at 37 °C, and which produce catalase and oxidase (Table 5.1). The circular chromosome is 4658 ± 30 kb (Umelo and Trust 1998). Cells are found in, and are pathogenic to, salmonids and increasingly other fish species. Traditionally, the lack of motility has been accepted as one of the reliable diagnostic traits used for the division of the aeromonads. However, this criterion has been challenged by the report of McIntosh and Austin (1991a) of motility (by polar flagella) in a strain of *Aer. salmonicida* subsp. *salmonicida* grown at elevated temperatures, i.e. 30–37 °C. The appearance of motility was also accompanied by variation in sugar fermentation patterns, the loss of ability to degrade complex molecules and an increase in antibiotic resistance. Further evidence for a motile mode of existence of *Aer. salmonicida* was provided by the recovery of eight atypical isolates from ulcers (but not from kidney tissue) on goldfish, carp and roach. The ulcerated fish were obtained from aquaria, garden ponds and rivers in England (Austin 1993). Interestingly, these isolates did not dissociate into different colony types, but grew at 37 °C. Of course, there is always the concern that motile contaminants may have been present in cultures, which were predominantly *Aer. salmonicida.* However, the isolation of flagella genes, *flaA* and *flaB, which* coded for unsheathed polar flagella at low frequency, has clinched the argument that *Aer. salmonicida* can be motile under certain circumstances (Umelo and Trust 1997).

Certain traits, such as pigment production, captured the attention of fisheries scientists particularly because they were readily observable. In an examination of pigment production, Griffin et al. (1953b) showed that its development was dependent upon medium composition, insofar as tyrosine or phenylalanine was deemed to be essential. This was confirmed by O'Leary et al. (1956). However, it was initially assumed that this pigment was related to melanin, although subsequent investigation has refuted this possibility. Thus, Donlon et al. (1983) discovered that biosynthesis of the pigment from tyrosine differed substantially from melanogenesis and not 3,4-dihydroxyphenylalanine as would have been expected of melanin synthesis. Although production of the brown, water-soluble pigment constitutes a major diag-

Table 5.1 Characteristics of *Aeromonas salmonicida*[a]

Character	*Aer. salmonicida* subsp				
	achromogenes	*masoucida*	*pectinolytica*	*salmonicida*	*smithia*
Production of:					
brown, diffusible pigment	–	–	+	+	–
Arginine dihydrolase	+	+	v	v	–
Catalase	+	+	+	+	+
ß-galactosidase	+	+	+	+	+
H_2S	–	+	–	v	+
Indole	–	–	+	–	–
Lysine decarboxylase	–	+	–	v	–
Ornithine decarboxylase	–	–	–	–	–
Oxidase	+	+	+	+	+
Phenylalanine deaminase	.	.	.	–	–
Phosphatase	.	.	.	–	+
Fermentative metabolism	+	+	+	+	+
Gluconate oxidation	–	+	–	–	–
Methyl red test	–	+	.	v	–
Motility	–	–	–	–	–
Nitrate reduction	+	+	+	+	.
Voges Proskauer reaction	–	+	v	–	–
Degradation of:					
Aesculin	–	+	–	v	–
Blood (ß-haemolysis)	–	+	v	+	–
Casein	+	–	.	+	+
Chitin	–	–	.	–	–
DNA	+	+	+	+	+
Elastin	.	.	–	+	–
Gelatin	–	+	+	+	+
Lecithin	.	.	.	+	–
Polypectate	.	.	+	.	.
RNA	+	+	.	+	+
Starch	.	.	.	+	+

(continued)

Table 5.1 (continued)

Character	*Aer. salmonicida* subsp				
	achromogenes	*masoucida*	*pectinolytica*	*salmonicida*	*smithia*
Tweens	+	+	.	+	–
Tyrosine	.	.	.	+	–
Urea	–	–	–	–	.
Xanthine	–	–	.	–	.
Growth at/on:					
4–5 °C	v	v	.	v	+
30 °C	+	+	+	+	–
37 °C	–	–	+	–	–
Cystine lactose electrolyte deficient agar	–	–	.	–	.
MacConkey agar	+	+	.	+	–
Potassium cyanide	–	–	v	–	.
TCBS agar	–	–	.	–	.
0–2 % (w/v) NaCl	+	+	.	+	+
3 % (w/v) NaCl	v	v	.	v	–
4 % (w/v) NaCl	–	–	.	–	–
Utilization of sodium citrate	–	–	+	–	–
Production of acid from:					
Adonitol	–	–	–	–	.
Amygdalin	–	–	–	–	.
Arabinose	–	+	+	+ (slow)	.
Cellobiose	–	–	+	–	–
Dulcitol	–	–	–	–	.
Erythritol	.	.	–	–	.
Fructose	.	.	.	+	.
Galactose	+	+	.	+	–
Glucose	+	+	+	+	v
Glycerol	.	.	+	–	–
Glycogen	.	.	.	+	.
Inulin	.	.	.	–	.
Lactose	–	–	+	–	–
Maltose	+	+	.	+	–
Mannitol	–	+	.	+	–
Mannose	.	+	.	+	.
Melezitose	.	.	.	–	.
Melibiose	.	.	–	–	.

(continued)

Table 5.1 (continued)

Character	*Aer. salmonicida* subsp				
	achromogenes	*masoucida*	*pectinolytica*	*salmonicida*	*smithia*
Raffinose	–	–	–	–	–
Rhamnose	–	–	–	–	–
Salicin	v	v	–	+ (slow)	.
Sorbitol	–	–	+	–	–
Sucrose	+	+	+	–	v
Trehalose	+	+	.	+	–
Xylose	–	–	–	–	.
G+C ratio of the DNA					
(Moles %)	.	.	.	57–59	56

v = variable result; . = not done

[a]Based on Griffin et al. (1953a), Schubert (1967a, b, 1974), McCarthy (1977a, 1980), Austin et al. (1989), Pavan et al. (2000) and Diamanka et al. (2013)

nostic feature of *Aer. salmonicida* (Martin-Carnahan and Joseph 2005), caution is advised against relying too heavily on the presence of pigment, insofar as there are variations among pigmented strains in the quantity of compound produced and of the time needed for its appearance (Horne 1928; Mackie and Menzies 1938). In addition, non-pigmented variants may arise (Wiklund et al. 1993), particularly upon subculture (Duff and Stewart 1933; Evelyn 1971a). It has also been observed that other *Aeromonas* species, namely *Aer. hydrophila* and *Aer. media,* may produce such pigments when grown on media containing tryptone (see Paterson 1974; Allen et al. 1983a). Obviously, this questions the reliability of using pigment production as a differential characteristic. To further complicate the issue, the existence of achromogenic or slowly pigmenting strains of *Aer. salmonicida* have been described.

Another intriguing trait of *Aer. salmonicida* is the ability to dissociate into different colony types, i.e. rough, smooth and G-phase (intermediate) colonies. This phenomenon was extensively studied by Duff (1937), and will be discussed further in connection with its relevance to pathogenicity. Electron microscopy demonstrated that 'rough' and 'smooth' forms were attributed to the presence or absence of an extracellular layer (= the A-layer), respectively.

The notion of homogeneity could be dispelled by the results of PFGE of 44 isolates of *Aer. salmonicida* subsp. *salmonicida,* which generated 30 different profiles and 40 distinct types (Chomarat et al. 1998). However, numerical analysis using the S_D coefficient revealed that all the isolates were genomically related (Chomarat et al. 1998). Yet using 17 typical, 39 atypical and three type strains, RAPD and PFGE analyses suggested heterogeneity across all the strain – notably atypical isolates-, but confirmed that typical *Aer. salmonicida* (including the type strain of *Aer. salmonicida* subsp. *salmonicida*) was homogeneous (O'hici et al. 2000).

The genomes of two isolates, A449 (4.7 Mb) and 01-B526 (4.75 Mb), have been sequenced, and the latter found to have a large plasmid, pAsa5, of 155 kb and three smaller plasmids pAsa1, pAsa2 and pAsa3 of 5424, 5247 and 5616 bases, respec-

tively whereas the former has two large plasmids of 166 and 155 kb (Reith et al. 2008; Charette et al. 2012).

Atypical Isolates of *Aeromonas salmonicida*

When compared to the so-called motile aeromonads, the description of *Aer. salmonicida* suggests a very homogeneous group of organisms. Alas, as so often happens in biology, a multitude of exceptions have disturbed the apparent idyllic situation. 'Atypical' strains deviate from the classical description of the taxon over a number of biochemical, physiological and genetic properties, e.g. AFLP fingerprints, making typing difficult (Hirvelä-Koski et al. 1994; Austin et al. 1998; Wiklund and Dalsgaard 1998; Dalsgaard et al. 1998; Høi et al. 1999; Lund et al. 2002). For example, Japanese isolates of so-called atypical *Aer. salmonicida* were recovered in four groups, which were defined after 16S rDNA sequencing. There was not any host specificity with these groups (Yamada et al. 2000). The most common reasons for describing isolates as 'atypical' are:

- lack of, weak or slow pigment production (Nakatsugawa 1994; Koppang et al. 2000)
- catalase-negativity (Kaku et al. 1999)
- oxidase-negativity (Wiklund and Bylund 1993; Wiklund et al. 1994; Wiklund and Dalsgaard 1995; Pedersen et al. 1994, 1996; Kaku et al. 1999)
- nutritional fastidiousness, i.e. for blood or blood products (Austin 1993)
- slow growth, i.e. ≥5 days to obtain visible colonies (Austin 1993; Kaku et al. 1999)
- different hosts from salmonids, i.e. cyprinids (e.g. Austin 1993; Kaku et al. 1999) and marine fish, including shotted halibut (*Eopsetta grigorjewi;* Nakatsugawa 1994), dab, plaice and flounder (*Platichthys flesus*) (Wiklund et al. 1994; Wiklund and Dalsgaard1995), common wolffish (*Anarhichas lupus*) (Hellberg et al. 1996), turbot (*Scophthalmus maximus*) (Pedersen et al. 1994), greenling (*Hexagrammos otakii*), Japanese flounder (*Paralichthys olivaceus*) and Schlegel's black rockfish (*Sebastes schlegeli*) (Iida et al. 1997), where the disease is often ulceration.

With the last mentioned example, the justification for describing the isolates as atypical was based on the host rather than the characteristics of the cultures.

One of the earliest indications that aberrant strains occurred was provided by Smith (1963), who examined six isolates of non-pigmented cultures, which were clustered as Group I from a numerical taxonomy study. These organisms were related at the 75.6 % similarity level to typical pigment-producing isolates. Smith (1963) proposed a separate new species for Group I, i.e. with the specific epithet *achromogenes,* although the recommendation was not adopted. A second non-pigmented group was recognised by Kimura (1969a) as *Aer. salmonicida* subsp. *masoucida.* This subspecies differed from typical strains on account of indole pro-

duction, Voges Proskauer reaction, H_2S and lysine decarboxylase production, and fermentation of sucrose (Table 5.1). In the eighth edition of *Bergey's Manual of Determinative Bacteriology,* Schubert (1974) regarded these non-pigmented isolates as *Aer. salmonicida* subsp. *achromogenes* and *Aer. salmonicida* subsp. *masoucida,* respectively. Typical strains were classified by him as *Aer. salmonicida* subsp. *salmonicida.* This classification into subspecies, has been retained in the recent literature (Martin-Carnahan and Joseph 2005). This is interesting, because in an earlier publication (Popoff 1970), it was contended that subspecies *achromogenes* and *masoucida* were more closely related to *Aer. hydrophila* than to *Aer. salmonicida.* In fact, Paterson et al. (1980) suggested that *'masoucida'* bridged the gap between typical *Aer. salmonicida* cultures and *Aer. hydrophila,* insofar as the subspecies possessed similar physiological and growth characteristics to the latter. However, *Aer. salmonicida* subsp. *masoucida* is non-motile, sensitive to *Aer. salmonicida* bacteriophages, possesses an antigenic component specific to *Aer. salmonicida,* and has a DNA homology of 103 % with *Aer. salmonicida* (MacInnes et al. 1979). The relationship between the subspecies is certainly not sacrosanct, insofar they could be combined or kept separate according to which methods happen to be in vogue (Austin et al. 1998). For example by PCR, there would be good reason to consider combining subspecies *achromogenes* and *masoucida,* a view that is not substantiated by ribotyping and RAPD analyses (Austin et al. 1998). Phenetic data suggested that there would be a case for combining subspecies *masoucida* with *salmonicida,* and subspecies *achromogenes* with *Haemophilus piscium.* Indeed, examination of the small subunit rRNA gene sequences revealed a profound (99.9 %) homology of an authentic strain of *Haemophilus piscium* with *Aer. salmonicida* subsp. *salmonicida* (Thornton et al. 1999). Yet, methods point to the comparative uniqueness of subspecies *smithia* (Austin et al. 1998).

Certainly, a species subdivided into four subspecies should not be considered as unworkable; however, the classification is complicated by other factors. Thus, the existence of aberrant strains from a wide range of fish hosts and geographical locations is well established, and new reports are continually being made. According to McCarthy (1980), the existence of such strains is no doubt more common than even their documentation in the published literature suggests. As Mawdesley-Thomas (1969) pointed out, there is no sound reason why a pathogen that affects one family of freshwater fishes should not infect others. He contended that emphasis had been placed on food and game fish, and that only the absence of detailed investigations of fish diseases generally had given the false impression that each fish has its own specific set of diseases. McCarthy (1980) and McCarthy and Roberts (1980) made the valid point that the original description of atypical strains, as reported by Schubert (1974), was based upon data for only a few isolates. These authors submitted that revision is now both possible and necessary. According to a comparative phenetic and genotypic analysis of 29 atypical isolates, in addition to 144 other *Aeromonas* spp., McCarthy (1977a) delineated four phenetic groups. Of these, one cluster comprised typical isolates of *Aer. salmonicida,* a second group was composed of atypical isolates of *Aer. salmonicida* derived from salmonids including representatives of subspecies *achromogenes* and *masoucida,* a third group contained

atypical isolates (*Aer. salmonicida*) from non-salmonids, and the fourth group was equated with *Aer. hydrophila.* Not surprisingly, the typical isolates of *Aer. salmonicida* formed an extremely compact group, which could not be readily differentiated from the atypical strains. However, the results of numerical phenotypic analyses were not unequivocally confirmed by G+C ratio determinations or DNA:DNA homology studies. McCarthy and Roberts (1980) proposed that, from the results of their studies, there should be three subspecies of *Aer. salmonicida,* as follows:

Group 1	*Aer. salmonicida* subsp. *salmonicida*
Group 2	*Aer. salmonicida* subsp. *achromogenes* (incorporating subsp. *masoucida*)
Group 3	*Aer. salmonicida* subsp. *nova*

However, the 'Approved Lists of Bacterial Names' (Skerman et al. 1980) retained the classification of Schubert (1974), namely of subspecies *achromogenes, masoucida* and *salmonicida.* Thus, a growing consensus of opinion suggested that it was timely to elevate certain of the better characterised atypical isolates to subspecies status; the problem concerns the number and composition of such groups (Austin et al. 1998). On the basis of DNA:DNA reassociation studies, Belland and Trust (1988), also supported the rationale of McCarthy and Roberts (1980) to create a new subspecies, i.e. *Aer. salmonicida* subsp. *nova,* to accommodate atypical isolates from non-salmonids. Yet, the subspecies was not formally proposed. Moreover, they also agreed with the suggestion of McCarthy and Roberts (1980) to combine the subspecies *achromogenes* and *masoucida.* In another (numerical) taxonomy and DNA:DNA hybridisation study, Austin et al. (1989) elevated a group of 18 non- or slowly pigmenting 'atypical' isolates into a new subspecies, as *Aer. salmonicida* subsp. *smithia.* In addition to the phenotypic studies, there is an increasing trend to employ molecular genetic techniques to elucidate inter- and intraspecific relationships within the genus. Thus, DNA:DNA and RNA:DNA hybridisation, 16S RNA cataloguing, and 5S and 16S rRNA sequencing techniques have been used. Work on DNA homologies by MacInnes et al. (1979) revealed that all isolates of *Aer. salmonicida* (including *Aer. salmonicida* subsp. *masoucida*) possessed very high homologies, i.e. 96–106 %, when hybridised against a representative strain of *Aer. salmonicida* subsp. *salmonicida.* Indeed, these authors concluded that the non-motile aeromonads comprise a genetically homogeneous taxon. In this study, the culture of *Aer. salmonicida* subsp. *masoucida* hybridised at 103 % with *Aer. salmonicida* subsp. *salmonicida.* MacInnes and co-workers determined that this homology level was also achieved with a strain of *Aer. salmonicida* subsp. *salmonicida,* which was an ultraviolet induced mutant of NCIMB 74. An interpretation was reached, therefore, that *Aer. salmonicida* subsp. *masoucida* and, perhaps, some of the biochemically atypical isolates do not warrant separate subspecies status, insofar as they may be merely mutants of other well-recognised groups. However, we emphasise that dramatic conclusions should not be made from an examination of only 11 isolates. It is interesting to note, however, that McCarthy (1980) also reported, as a result of the genotypic part of his analyses, that typical and atypical

isolates were very closely related, with minimal divergence. It is a pity that the exact homology values were not presented.

In a study of 26 typical and atypical isolates by DNA:DNA re-association methods, Belland and Trust (1988) found that typical isolates were recovered in a homogeneous group, whereas the atypical representatives were more diverse. Of these, one biotype consisted of isolates obtained from goldfish (obtained from a wide geographical range), whereas the second group accommodated isolates derived from carp in Europe. From the results of a numerical taxonomic and DNA:DNA hybridisation study, Austin et al. (1989) made similar conclusions regarding the homogeneity of typical isolates of *Aer. salmonicida.* However using 16S rRNA sequencing techniques, Martínez-Murcia et al. (1992) reported that subspecies *achromogenes* and *masoucida* were indistinguishable, and only differed from subspecies *salmonicida* by two bases.

There is overwhelming evidence that the so-called 'atypical' isolates are distinct from typical *Aer. salmonicida* (e.g. Hänninen and Hirvelä-Koski 1997; Austin et al. 1998; Wiklund and Dalsgaard 1998; Umelo and Trust 1998). Yet, it has so far proved to be impossible to include the atypical isolates into a meaningful classification. Moreover, there has been incongruence reported between the results of molecular (PCR, RAPD and ribotyping and phenotypic methods, in terms of group membership (Austin et al. 1998). Høi et al. (1999) recognised 4 PCR groups among 205 atypical isolates. The problems of inter-laboratory differences and lack of standardisation in test methods has been highlighted by Dalsgaard et al. (1998). Some studies have indicated homogeneity among atypical isolates; a sentiment which is not endorsed by others. For example, Kwon et al. 1997) carried out a RAPD analyses of 29 atypical isolates from 8 species of fish in Japan, and concluded that the profiling was identical, thereby indicating genetic homogeneity. However, heterogeneity was apparent between these atypical isolates and reference cultures from the validly described subspecies (Kwon et al. 1997); a notion which has been confirmed by others (e.g. Austin et al. 1998). After studying 51 isolates from Finland by ribotyping, plasmid profiling and phenotyping, it was concluded that pigment-producing strains could be separated from achromogenic cultures. Also, oxidase-negative isolates were distinct from oxidase-positive atypical isolates in terms of ribotypes and phenotypes (Hänninen and Hirvelä-Koski 1997).

There have been several reports of acid production in sucrose fermentation tests among typical isolates (see Fryer et al. 1988; Wiklund et al. 1992). This is relevant because hitherto this was one of the tests used to differentiate typical from atypical isolates of the pathogen (Martin-Carnahan and Joseph 2005).

Plasmid Profiles of *Aeromonas salmonicida*

Plasmids carried by typical (14 strains) and atypical (11 strains) forms of *Aer. salmonicida* have also provided additional genetic evidence for the classification of typical and atypical isolates into separate taxa (Belland and Trust 1989). These

workers found that typical isolates possessed a very homologous plasmid content comprised of a single large (70–145 kb) plasmid and three low molecular weight plasmids. Livesley et al. (1997) reported that 5 plasmids were most common among the 18 isolates examined; Giles et al. (1995) found 4 or 6 plasmids with 4 smaller plasmids of 4.3–8.1 kb being often observed in isolates from the Atlantic coast of Canada, but 6 plasmids of 4.2–8.9 kb among cultures from the Pacific coast of Canada. A total of 23 plasmids and 40 different plasmid profiles were recognised among 124 isolates from Denmark, Norway, Scotland and North America (Nielsen et al. 1993). An earlier theme was repeated insofar as all isolates had one large plasmid of 60–150 kb, and two low molecular weight plasmids of 5.2 and 5.4 kb. In addition, two plasmids of 5.6 and 6.4 kb were frequently present (Nielsen et al. 1993). A larger investigation of 383 isolates over a 6 year period concluded that 1–4 plasmids of 52–105 mDa were inevitably present, casting doubt on the relevance of plasmid typing for epizootiology (Sørum et al. 1993a, b). Again, oxytetracycline and streptomycin resistant isolates from the Atlantic and Pacific coasts contained 4 or 6 plasmids, with 4 smaller plasmids of 4.3 to 8.1 kb being often observed. Some slight variation in plasmid content was noted between sources of the isolates (Giles et al. 1995). Atypical isolates possessed two to four different plasmid types (Belland and Trust 1989). Moreover, there was a correlation between plasmid composition and source of the atypical isolates. This observation may prove useful in epizootiological studies, where plasmid content of atypical isolates could serve as useful markers (Belland and Trust 1989). In a subsequent investigation of 113 cultures of atypical isolates from a wide range of geographical locations, 7 groupings were defined; 18 cultures did not have any common plasmid profile. Of interest, the two type strains NCIMB 1110 and ATCC 27013 were recovered in different groups. For some groups, i.e. I, III, V and VII (these isolates were catalase-negative), there was an association with the origin of the cultures, i.e. the location of the farm. Also, some differences in phenotype were apparent between members of some groups. Again, the value for epizootiological investigations was stressed (Sørum et al. 2000).

The Taxonomic Dilemma

Using molecular techniques, a consistent view about the genetic relatedness of the long established subspecies of *Aer. salmonicida* emerges. The outstanding dilemma concerns the poor correlation between phenetic and genotypic data (Austin et al. 1989). This problem needs to be addressed before the definitive classification of *Aeromonas* results. Nevertheless, it may be concluded that unlike the atypical isolates, *Aer. salmonicida* subsp. *salmonicida* is extremely homogeneous; a conclusion which is supported by phenotypic and molecular data (Austin et al. 1989; Dalsgaard et al. 1994; Hänninen et al. 1995; Miyata et al. 1996; Umelo and Trust 1998).

Despite the existence of typical and atypical representatives of *Aer. salmonicida,* the current theme in operation for speciation has proved tenable. Nevertheless, new ‘atypical’ isolates, which do not fit into existing classifications of *Aer. salmonicida,*

are regularly reported. Debate has centred on the relationship of *Aer. salmonicida* within the genus *Aeromonas.* In the study by Eddy (1960), attention was focused on the inability of *Aer. salmonicida* to produce 2,3-butanediol from glucose, and the absence of motility, both characters of which contravened the genus description of Kluyver and van Niel (1936). However, Eddy did not dispute the retention of *Aer. salmonicida* within the genus. Instead, this placement was challenged by Smith (1963), who expressed doubt as to whether *Aer. salmonicida* belonged in the genus *Aeromonas.* Her recommendation was the establishment of a new genus, i.e. *Necromonas,* with two species, namely *Nec. salmonicida* for the typical isolates and *Nec. achromogenes* for the non-pigmented strains. The evidence appertained to the morphological, biochemical and metabolic traits of 42 isolates of *'Bacterium salmonicida',* six non-pigmented pathogens, and 42 other bacterial cultures. Thus, there were pronounced differences between *Bacterium salmonicida* and other *Aeromonas* cultures. It emphasised, for instance, that the production of gas from glucose was an important genus characteristic. Although many previous reports had stated that the pathogen produced gas from glucose (Griffin et al. 1953a; Eddy 1960, 1962; Ewing et al. 1961; Schubert 1961), the hundreds of isolates examined at the Marine Laboratory, Aberdeen, between 1953 and 1962 produced either very little or no gas from glucose. Instead, they produced gas from mannitol (Smith 1963). As for the production of 2,3-butanediol from glucose, Smith (1963) contended that previously this test required a tedious procedure, and consequently was often not applied to presumptive aeromonads. However, in her laboratory, *Bacterium salmonicida* isolates did not so produce the compound. Due to such discrepancies with the genus description, it was proposed that the species should be removed from the genus *Aeromonas* and placed in a new genus, i.e. *Necromonas.* Although Smith's proposal was not formally adopted, it should be mentioned that Cowan (1974) followed her suggested classification, by including *Nec. salmonicida* in the diagnostic tables. However, it is our opinion that the deviations of *Aer. salmonicida* from the initial genus description of *Aeromonas* should, for several reasons, be viewed less stringently than may be to the approval of some taxonomist purists. For example, it is often a difficult decision in bacterial systematics as to how much variation to allow within the definition of a species or a genus, before the line is drawn and relationships, or lack of, declared. If examples are taken from the characteristics of motile aeromonads, it is certainly the case that discrepancies occur for some members of these species as regards agreement with the genus description (Holder-Franklin et al. 1981; Allen et al. 1983b). Schubert (1974) included the production of 2,3-butanediol (a generic trait) as occurring in *some* species. Non-motility is also taken into account, and carbohydrates are cited as being broken down to acid or to acid and gas. These modifications to the genus description thus eliminate the major objections of Smith (1963) regarding the retention of *Aer. salmonicida* within the genus *Aeromonas*. In addition, subsequent serological and bacteriophage sensitivity data have provided strong evidence for a relationship between *Aer. salmonicida* and the motile aeromonads. The existence of a common antigen between *Aer. hydrophila* and *Aer. salmonicida* subsp. *masoucida* and some other strains of *Aer. salmonicida* was demonstrated by Kimura (1969b) and Paterson et al. (1980). In an

examination of the specificities of aeromonad extracellular antigens, Liu (1961) observed that cross-reactions occurred between *Aer. salmonicida* and the motile aeromonads. Liu suggested that the absence of serological cross-reactions with other Gram-negative bacteria indicated that organisms belonging to the genus *Aeromonas* comprise a distinct group. Studies, using bacteriophage, demonstrated the sensitivity of some *Aer. hydrophila* cultures to *Aer. salmonicida* bacteriophages, whereas organisms not belonging to *Aeromonas* showed complete resistance to the virus (Popoff 1971a, b). In particular, studies employing molecular genetic techniques support the retention of *Aer. salmonicida* in the genus *Aeromonas.* MacInnes et al. (1979) determined that percentage DNA homologies of motile aeromonads with *Aer. salmonicida* subsp. *salmonicida* ranged between 31 and 80 %, whereas those hybridised against *Aer. hydrophila* were between 31 and 100 %. The *Aer. salmonicida* strains exhibited a relatively high degree of homology when hybridised against *Aer. hydrophila.* Thus, the values for 10 out of 11 isolates were in the range of 51 to 69 %. From these results, MacInnes et al. (1979) concluded that non-motile aeromonads demonstrated a legitimate genetic relationship to the motile species of *Aeromonas.* In fact, some motile strains shared a higher level of sequence homology with *Aer. salmonicida* than with the reference motile aeromonads which according to MacInnes could be attributed to the smaller genome size of *Aer. salmonicida.* In another investigation, 56–65 % binding between *Aer. salmonicida* and *Aer. hydrophila* DNA strands was recorded (McCarthy 1978). These homology values indicate strong genetic relationships between the principal *Aeromonas* species (Paterson et al. 1980; Belland and Trust 1988).

It is well established that *Aer. salmonicida,* as a species, is phenotypically distinct from its motile counterparts. The increasing evidence from genetic and other molecular biology studies pertaining to intrageneric relationships between *Aer. salmonicida* and other aeromonad species, however, appear to support the retention of the pathogen within the genus. Certainly, the situation is not aided by ongoing manoeuvres in the classification of the motile aeromonads. Therefore, since the arrangement of the genus is in transition, and yet to be definitively resolved, we believe that nothing would be gained by the re-classification of *Aer. salmonicida.*

To reiterate, it appears to be the consensus of opinion that the area in need of further work is the intraspecific relationships between typical and atypical isolates. It is anticipated that such work would improve the classification of the genus *Aeromonas.*

Serology

Additional approaches to ascertaining the intra- and interspecific relationships of *Aer. salmonicida* have been adopted. These include serological techniques and bacteriophage typing. Certainly, the antigenicity of *Aer. salmonicida* has been the focus of much attention, primarily with a view to its significance in vaccine development. Unfortunately, the early investigations of Williamson (1929) were halted by the

persistent agglutination of the strains in saline. This problem was circumvented by Blake and Anderson (1930), who employed complement fixation for the examination of 82 isolates of *Aer. salmonicida.* All of these isolates gave a positive response. Ewing et al. (1961) examined agglutinin absorption with reference to 'O' and 'H' antigens. They concluded that the 21 strains examined were related to each other, but also to a strain of *Aer. hydrophila* (O-antigen suspensions prepared using the 21 cultures reacted to *ca.* 25% of the titre of the O-antiserum prepared with *Aer. hydrophila*). Other researchers have also found serological homogeneity among strains of *Aer. salmonicida,* but some degree of cross-reactivity with *Aer. hydrophila.* For example, common antigens among two *Aer. salmonicida* strains, as determined by gel-diffusion, and cross-reactions between *Aer. salmonicida* antiserum and three out of four isolates of *Aer. hydrophila,* but not of *Aer. salmonicida* strains and *Aer. hydrophila* antiserum, was reported by Liu (1961). Karlsson (1962), using the antigenic properties of a haemolysin from *Aer. salmonicida,* found no serological differences among the six strains tested. Although these six strains did not cross-react with other aeromonads recovered from humans, it was later established that there were indeed common thermolabile antigens between *Aer. salmonicida* and other *Aeromonas* species as assessed using precipitin, agglutination and double diffusion precipitin tests. Bullock (1966) also found evidence of cross-reactions between soluble antigens of *Aer. salmonicida* and *Aer. hydrophila.* Serological cross-reactions between casein-precipitating enzymes of these two *Aeromonas* species were reported by Snadvick and Hagan (1968). Within the *Aer. salmonicida* group, Popoff (1969) found no serological differences among large numbers of typical pigment-producing isolates. Indeed, Popoff (1984), citing the work of Karlsson (1964), Spence et al. (1965) and his own previous studies, concluded that *Aer. salmonicida* is a serologically homogeneous species. Several workers have, in contrast, reported antigenic differences within the species (Duff 1939; Liu 1961; Klontz and Anderson 1968; Kimura 1969b). Paterson et al. (1980) considered that these conflicting findings reflected the choice of cultures, because comparable methodology had been used throughout. This group also reported results similar to those of Kimura (1969a, b), suggesting that *Aer. salmonicida* may be separated serologically into two groups based upon antigenicity of a given strain. In their studies, *Aer. salmonicida* NCIMB 1110 and 1102 and *Aer. salmonicida* subsp. *masoucida* contained an extra antigenic component, termed the 'c' component. Kimura (1969b) demonstrated the heat-sensitivity of an additional antigenic component (shared with *Aer. hydrophila*) in the subspecies *masoucida.* Klontz and Anderson (1968) observed smears prepared from 24 cultures of *Aer. salmonicida* with three antisera, by means of an indirect FAT. They postulated the existence of at least seven different serotypes, based upon non-reactivity of certain strains with one or more of the antisera. However, McCarthy and Roberts (1980) questioned the suitability of this technique for serological analysis of laboratory cultures, due to the potential for technical difficulties.

McCarthy and Rawle (1975) carried out an extensive serological study of both thermolabile and thermostable somatic antigens of *Aer. salmonicida* and their relationship to other bacteria. They employed whole-cell agglutination and double

cross-absorption of smooth strains, and passive haemagglutination and double cross-absorption of rough colony types. It was determined that cross-reaction titres for both antigen types were, in general, high, and cross-reactions between the *Aer. hydrophila* isolate and three out of six thermostable *Aer. salmonicida* antisera were very weak. In contrast, a strain of *V. anguillarum* and *Ps. fluorescens* gave no reaction with the *Aer. salmonicida* antisera. The passive haemagglutination method was more sensitive than whole-cell agglutination, as titres obtained for positive reactions were ten-fold higher in the former. When a double-diffusion method was employed to study cell-free extracts, prepared from the bacteria used for the somatic antigen study, strong cross-reactions among *Aer. salmonicida*, *Aer. hydrophila* and *V. anguillarum,* and, to a lesser extent, with *Ps. fluorescens* occurred. McCarthy and Rawle (1975) concluded that no qualitative differences in serological composition among *Aer. salmonicida* strains had been demonstrated, but noted that laboratory maintenance of some *Aer. salmonicida* cultures resulted in progressive loss of serological reactivity, giving negative responses with their antisera. Therefore, they suggested that, when embarking on serological (or vaccination) studies, it is important to include only fresh isolates. Hahnel et al. (1983) used micro-agglutination and double-diffusion precipitin tests to study serological relatedness among virulent and avirulent forms of eight isolates of *Aer. salmonicida* subsp. *salmonicida.* No serological differences were detected in the virulent isolates, but antigenic differences were observed between the virulent and avirulent form of each culture. Thus, in double-diffusion precipitin tests, the antigens of virulent sonicated cells formed an additional precipitin line when compared with the homogeneous avirulent form.

Bacteriophage Typing

Bacteriophages have been used to study taxonomic relatedness between strains. The first isolation of bacteriophage specific for *Aer. salmonicida* was made by Todd (1933), although the usefulness for typing purposes was not demonstrated until the work of Popoff (1971a, b). It is considered that phage typing has value in epizootiological studies (Popoff 1984; Bast et al. 1988; Belland and Trust 1989). Essentially, the bacteriophages may be divided into three morphological groups and ten serological types (Popoff 1984). Thus, using a set of eight phages, Popoff (1971b) recognised 14 phage types. Also, Paterson et al. (1980) studied phage sensitivity as a means of determining relationships between typical and atypical cultures. Pigmented and achromogenic as well as aggregating and non-aggregating strains showed a high sensitivity to two out of the three bacteriophages. In a further investigation by Rodgers et al. (1981), 27 groups of *Aer. salmonicida* were defined on the basis of sensitivity patterns to 18 bacteriophage isolates. Significantly, the morphological characteristics of the host bacterium, i.e. whether a rough, smooth or G-phase form, influenced attachment of the bacteriophage. This was apparently attributed to the varying quantities of LPS in the cell wall of the different morphological types.

Haemophilus piscium

What about *Haemophilus piscium,* the causal agent of ulcer disease? The name was coined by Snieszko et al. (1950). However, the detailed taxonomic study of Kilian (1976) showed that the organism did not belong in the genus *Haemophilus.* In particular, the strains did not exhibit requirements for haemin or NAD, which contrasted with the genus description. *H. piscium* differed from the type species, *H. influenzae,* in the inability to reduce nitrate or alkaline phosphatase and to grow at 37 °C, together with a relatively high G+C ratio of the DNA. Unfortunately, Kilian did not establish the most appropriate taxonomic position of the pathogen. The validity of the taxonomic position was similarly questioned by Broom and Sneath (1981), as a result of a detailed numerical taxonomic study. The low similarity of *H. piscium* with other *Haemophilus* spp., i.e. only 65 %, suggested that the organism should be excluded from the genus. From examination of DNA, biochemical, serological and bacteriophage sensitivity data, it is apparent that *H. piscium* represents an atypical, achromogenic variant of *Aer. salmonicida* (Paterson et al. 1980). Evidence for this conclusion consists of the G+C ratio of the DNA (55.1 moles %), which is well within the range reported for *Aer. salmonicida* by McCarthy (1978). Moreover, Paterson et al. (1980) regarded *H. piscium* to be serologically indistinguishable from *Aer. salmonicida.* Also, the pathogen was sensitive to several *Aer. salmonicida* bacteriophages, and exhibited biochemical reactions similar to those expected for some achromogenic variants of *Aer. salmonicida.* Trust et al. (1980a) also concluded, on the basis of bacteriophage sensitivity, that *H. piscium* is, in fact, atypical *Aer. salmonicida.* Thus, a virus that produced lysogeny in *Aer. salmonicida* but displayed no such activity in *Aer. hydrophila,* caused plaque formation in several isolates of *H. piscium.* On the basis of one strain, Austin et al. (1998) concurred with the view that *H. piscium* should probably be classified with *Aer. salmonicida*. However its precise relationship to the four subspecies reflected the nature of the phenotypic and molecular methods used.

Diagnosis

Culturing and Phenotypic Characters Diagnosis is readily achieved by culturing techniques, usually on TSA or BHIA (the preferred medium in the view of many scientists) in which case 'typical' isolates produce a characteristic brown, diffusible pigment. Also, CBB may be employed as a differential medium (Markwardt et al. 1989). However as a primary isolation medium, CBB appears to be less sensitive and gives a poor recovery of the pathogen compared to BHIA (B. Austin, unpublished data).

Aer. salmonicida may be distinguished from other fish pathogens on the basis of a small number of phenotypic tests, notably the Gram-staining reaction (small Gram-negative rods), motility (usually appears to be non-motile), growth at 37 °C

(usually a negative response), fermentative metabolism, catalase and oxidase production (both positive) and acid production from sucrose and xylose (both negative; recently, acid production from sucrose has been attributed to some isolates [Wiklund et al. 1992]). These tests will result in a provisional identification of *Aer. salmonicida* (McCarthy 1976). In addition, it is recommended that pathogenic isolates should be examined for degradation of gelatin (positive), starch (positive) and urea (negative), arginine dihydrolase (positive), gluconate oxidation (negative) and ornithine decarboxylase production (negative). Unfortunately, this apparently simple state of affairs may be complicated by the increasing presence of 'atypical' isolates, particularly in non-salmonid fish. In particular, these may be non- or slow-pigmenting.

Serodiagnosis Whole cell agglutination is effective with *Aer. salmonicida,* but only for smooth (non-auto-agglutinating) colonies (Rabb et al. 1964). This is a pity since the majority of isolates recovered from clinical cases of disease are, in fact, rough and auto-agglutinating (McCarthy 1976). Subsequently, Kawahara and Kusuda (1987) reported that FAT was superior to culturing for the diagnosis of atypical *Aer. salmonicida* infections in eels. In a comparative study of serodiagnostic techniques, Sakai et al. (1986) reported that iFAT and the peroxidase-antiperoxidase enzyme immunoassay (PAP) were more sensitive (capable of detecting 10^3 CFU/ml) than the latex agglutination and co-agglutination techniques. These required 10^7 CFU/ml for positive results to be recorded. Nevertheless with latex agglutination and co-agglutination techniques, more (15/15 = 100 %) positive samples were detected than by iFAT (10/15 = 67 %) or PAP (1/15 = 73 %). We have successfully married monoclonal antibodies to *Aer. salmonicida* with ELISA for a test, which has proven suitable for use on fish farms. Indeed, experiments demonstrated that reliable diagnoses were achieved within 30 min (Austin et al. 1986). It is noteworthy that ELISA systems appear to be more sensitive than culturing for the detection of *Aer. salmonicida* (Hiney et al. 1994). However, a subsequent development has involved the use of polyclonal antibody-coated gold nanoparticles in an immunoassay, which enabled the specific, sensitive (1×10^4 CFU/ml) and rapid [within 45 min] detection of *Aer. salmonicida* cells in tissues. Here, the clearly visible red-purple agglutination of the gold particles indicated the presence of the pathogen (Saleh et al. 2011).

Molecular Techniques Molecular techniques have been used with *Aer. salmonicida* (e.g. Mooney et al. 1995; Miyata et al. 1996; Oakey et al. 1998; Keeling et al. 2013). Barry et al. (1990) suggested that such probes have the potential to detect the pathogen in environmental and clinical samples. These workers found that specific probes for micro-organisms could be developed, even if only two base pair differences existed in the target sequence. Hiney et al. (1992) continued with developmental work leading to the isolation of a DNA fragment specific to *Aer. salmonicida,* which when incorporated into a polymerase chain reaction technique enabled a sensitivity of detection of approximately two cells of *Aer. salmonicida.* Mooney et al. (1995) examined the blood from 61 wild Atlantic salmon from 3 rivers in Ireland,

and recorded 87 % positives, with 100 genome equivalents/fish, using a specific DNA probe for *Aer. salmonicida*. Høie et al. (1997) designed primers and probes from 16S rRNA and plasmid DNA; the former of which amplified *Aer. hydrophila, Aer. salmonicida* subsp. *achromogenes, Aer. salmonicida* subsp. *masoucida* and atypical isolates, whereas the latter detected only *Aer. salmonicida* subsp. *achromogenes* and *Aer. salmonicida* subsp. *salmonicida.* Based on an examination of 100 ml volumes of kidney suspension and gill swabs, the PCR detected 20 and 200 colony forming units in 10 µl of PCR template by 16S rRNA and plasmid primers, respectively. The numbers corresponded to 10^3 and 10^4 colony forming units in 100 ml of kidney suspension, respectively (Høie et al. 1997). A conclusion was reached that the PCR detected *Aer. salmonicida* more often than culturing. Terminal-RFLP permitted the detection of ~30 CFU/mg of artificially inoculated kidney tissue (Nilsson and Strom 2002). A multiplex PCR was developed for the simultaneous detection *Aer. salmonicida, Pis. salmonis, Str. phocae* and *V. anguillarum*. The detection limit using purified total bacterial DNA was 5 pg/ µl (=5.33×10^4 CFU/ml). The limits of detection using spiked tissues, i.e. kidney, liver, muscle or spleen, were $3.8 \pm 0.78 \times 10^3$ CFU/mg (Tapia-Cammas et al. 2011). A possible drawback for use of DNA probes, however, has been proposed by Hennigan et al. (1989). Using four DNA probes in combination with seven restriction enzymes and seven strains of *Aer. salmonicida,* their data suggested that the DNA sequences of the species is very strongly conserved. They emphasised that the use of DNA probe technology to identify different strains of *Aer. salmonicida* may be limited. Using reverse transcription-multiplex PCR with primers, SV1/SV2 and SF1/SF2, which were specific to *vapA* and *fstB* genes, respectively, Rattanachaikunsopon and Phumkhachorn (2012) detected 10 CFU in pure culture and 30 CFU in tissue. Moreover, the method distinguished viable from non-viable cells, and typical from atypical representatives of *Aer. salmonicida.* Keeling et al. (2013) developed and validated a highly specific and reproducible real-time PCR, which detected the surface array protein, vapA, with reported sensitivity of 5 fg of DNA, 2.2×10^4 CFU/g of kidney tissue without enrichment and 40 CFU/g with enrichment.

In a comparison of the sensitivity of culturing with DNA probes, the former detected *Aer. salmonicida* from the kidney of only dead or moribund farmed Atlantic salmon smolts in Ireland whereas probe technology allied to a PCR assay was capable of recognising the pathogen in water, faeces and effluent (O'Brien et al. 1994). The benefit of species-specific primers and a nested PCR was demonstrated over universal eubacterial primers when the detection limit improved from 1.4×10^4 CFU/reaction to <14 CFU/sample (Taylor and Winton 2002). The importance of the primer set was further highlighted in a comparative study by Byers et al. (2002a,b).

Denaturing Gradient Gel Electrophoresis (DGGE) A method has been proposed and was evaluated in wild spawning coho salmon (*Oncorhynchus kisutch*) and cultured lake trout (*Salvelinus namaycush*) for the nonlethal detection of the pathogen from mucus involved DGGE of 16S rDNA, which led to a reproducible 4-band pattern. This was distinctive from other aeromonads, and *Aer. salmonicida.* The technique recognised 36 out of 52 coho salmon that were positive for *Aer. sal-*

monicida, compared with 31 positives achieved by culturing the mucus, and 16 positives by culturing the organs (Quinn and Stevenson 2012).

Epizootiology

The Ecology of *Aeromonas salmonicida* McCarthy (1980) performed detailed experiments concerned with the ecology of *Aer. salmonicida* and also reviewed the work carried out by others. According to his report, contact with infected fish or contaminated water and fish farm materials, and transovarian transmission have all been cited as probable routes of infection. Also, carrier fish, which show no overt signs of disease but harbour the pathogen in their tissues, appear to be implicated in horizontal or vertical transmission. Such carrier fish are presumed to provide a reservoir which retains the pathogen in fish populations. Sea lamprey have been found to harbour typical *Aer. salmonicida*, and it may well be that this fish species is a possible source of infection for salmonids (El Morabit et al. 2004).

To understand how *Aer. salmonicida* is transmitted both among and within fish populations it is necessary to know the source of the pathogen and its capacity to survive in the environment. In fact, most of the work done on epizootiological aspects of fish diseases caused by *Aer. salmonicida* has focused on investigations of potential sources of infection. The role of water, mud and detritus, contaminated implements on fish farms, animals other than fish themselves, and particularly, carrier fish, (i.e. salmonids as well as non-salmonids) as potential sources of infection with *Aer. salmonicida* have been examined. The popular approach to the study of this subject has been to determine the presence of and survival capabilities of *Aer. salmonicida* in the variety of habitats listed above. Certainly, there is evidence that the pathogen can survive without a significant change in numbers in transport systems, such as containing Stuart's medium, at 18–20 °C for 48 h (Cipriano and Bullock 2001). This opens up the possibility of transporting samples from field to laboratory without greatly influencing the populations of *Aer. salmonicida.*

***Aeromonas salmonicida* – Survival Studies** The survival of *Aer. salmonicida* in water has been thoroughly examined by numerous investigators (Williamson 1929; Smith 1962; Lund 1967; McCarthy 1980; Sakai 1986a, b; Rose et al. 1990a, b; Morgan et al. 1991; Effendi and Austin 1991, 1994; Table 5.2). Unfortunately, caution must be used in the interpretation of some of the data as many of the studies employed pre-sterilised water or types of water in which *Aer. salmonicida* would not normally be present, e.g. distilled or tap water. Thus, the information gleaned from such studies does not necessarily reflect the behaviour of the pathogen in the natural aquatic environment. However, enough work has been done to allow tentative conclusions to be drawn. Based on the survival data accumulated, it appears that *Aer. salmonicida* is capable of surviving for a prolonged period in fresh, brackish and sea water, although contradictory results as to the exact time interval involved abound. Thus for unsterilised fresh water, including river water, recovery of the

Table 5.2 Experimental data concerning the survival of *A. salmonicida* in water

Type of experimental system	Temperature (°C)	Survival time	References
Sterilised water:			
Distilled water	20	35 days	Lund (1967)
Distilled water	20	<7 days	Sakai (1986b)
Lake water	10	8 days	Morgan et al. (1991)
	10	21 days	Morgan et al. (1993)
	20	60 days	Deere et al. (1996a)
Physiological saline	20	<7 days	Sakai (1986b)
Tap water	–	5 days	Arkwright (1912)
	–	3 days	Williamson (1929)
	20	12 h	Lund (1967)
Reservoir water, low inoculum of *A. salmonicida* (10^1–10^2 cells/ml), in mixed culture with other aquatic bacteria	15	*ca.* 3 days	Allen (1982)
Reservoir water, as above, supplemented with 0.005 % (w/v) brain heart infusion	15	>3 days	Allen (1982)
River water	–	5 day	Williamson (1929)
	20	8 days	Lund (1967)
	10	63 days	Cornick et al. (1969)
	20–25	28 days	Cornick et al. (1969)
Seawater		19 h	Arkwright (1912)
	–	3 days	Williamson (1929)
	20	24 days	Lund (1967)
	–	<10 days	Rose et al. (1990b)
	20	>24 days	Effendi and Austin (1991)
	5–25	≤28 days	Effendi and Austin (1994)
Unsterilised water:			
Brackish water		16–25 days	Smith (1962)
	11–13	24 days	McCarthy (1980)
Distilled water	–	14 days	Horne (1928)
	–	4 days	Williamson (1929)
	–	7 days	Duncan (1932)
	20	9 days	Lund (1967)
Fresh water		24–30 h	Duncan (1932)
	11–13	17 days	McCarthy (1980)
River water	–	2 days	Williamson (1929)

(continued)

Table 5.2 (continued)

Type of experimental system	Temperature (°C)	Survival time	References
Sterilised water:			
		7–19 days	Smith (1962)
	20	2 days	Lund (1967)
10^1–10^2 *A. salmonicida* cells/ml	15	*ca.* 3 days	Allen (1982)
10^5–10^6 *A. salmonicida* cells/ml	15	>3 days	Allen (1982)
Seawater	–	2 days	Williamson (1929)
	–	24–30 h	Duncan (1932)
	20	5–6 days	Lund (1967)
	11–13	8 days	McCarthy (1980)
	–	<10 days	Rose et al. (1990b)
	20	6 days	Effendi and Austin (1991)
Tap water		3–4 days	Horne (1928)
	–	3 days	Williamson (1929)
	–	4 days	Duncan (1932)
	20	3 days	Lund (1967)
Mixtures of water types:			
50 % sea water + 50 % tap water		19 h	Arkwright (1912)
25 % sea water + 75 % tap water		45–67 h	Arkwright (1912)

pathogen from as little as 24 h to as long as 19 days had been reported by different studies. Survival in unsterilised brackish water was between 16 and 25 days (Smith 1962; McCarthy 1980). In unsterilised sea water, the organism could be recovered from between 24 h and 8 days (McCarthy 1980). If sterilised water samples were used, survival time in the absence of competing –antagonistic –organisms was invariably greatly increased. For example, survival times in fresh water of up to 63 days were reported (Cornick et al. 1969), and in sea water up to 24 days (Lund 1967). Using whole cells and DNA released into lake water micocosms, with media and PCR for detection, Deere et al. (1996a) cultured *Aer. salmonicida* for <4 weeks, but found the DNA remained intact for >13 weeks. This discrepancy between the results of culturing and other techniques opens a veritable Pandora's box. Why should intact DNA be found 2+ months after culturing techniques indicated that the population of *Aer. salmonicida* had disappeared?

Using a laboratory-based microcosm and culturing, direct counts, respiratory activity (the reduction of tetrazoliums to coloured formazans; after Effendi and Austin 1993), iFAT, epifluorescence microscopy and the direct viable count techniques (by incorporating yeast extract and nalidixic acid; after Kogure et al. 1979), Effendi and Austin (1994) confirmed the emerging view that cells of *Aer. salmonicida* remained after plates counts reached zero. These workers determined that survival was maximal in brackish conditions, i.e. salinity = 25‰, notably on substrates – especially on wood but also in sediment – rather than in the water col-

umn. Similarly, using an oxidase-negative atypical isolate, sterilised microcosms and culturing techniques, Wiklund (1995a) deduced that survival was better at 4 °C than 15 °C in brackish rather than sea or fresh water, and in the presence of particulates, i.e. sand. The addition of nutrients did not resuscitate cells after colony counts declined to zero. This is important, insofar as workers have been generally unsuccessful at retrieving culturable cells after plate counts declined to zero, regardless of the method that indicated cells or cellular components remained.

McCarthy attributed the discrepancies among the various investigations to the technical difficulty of isolating *Aer. salmonicida* from mixed cultures. The temperature at which the experiments were run may well have also influenced the results. For instance, McCarthy (1980) conducted his experiments between 11 and 13 °C, and reported longer survival times for the pathogen in fresh, brackish and seawater (17, 24 and 8 days, respectively), than had been recorded by most other investigators (Table 5.2). Also, the differences may reflect inherent variations between cultures.

In the majority of reports about the survival of *Aer. salmonicida,* a large initial inoculum of the bacterium, usually 10^6 to 10^7 cells/ml of sample, was used. It is unlikely, however, that the pathogen would occur in these numbers even in the event of a free-living existence in the natural environment, except perhaps during epizootics where moribund and dead fish were releasing large numbers of *Aer. salmonicida* into the immediate vicinity. Therefore, studies were undertaken using a low number of cells, *ca.* 10^1 to 10^2/ml, as an inoculum (Allen 1982). When the pathogen was placed in sterilised reservoir water in such minimal numbers, and incubated at 15 °C, the organism underwent a severe reduction in numbers within 72 h, such as to be virtually unrecoverable by plating methods on solid non-selective medium. From these results, which are in contrast to other studies, it becomes apparent that several factors, including the size of the inoculum and the temperature at which the experiments are conducted, are crucial in determining the outcome of survival studies. In contrast, when an inoculum of approximately 10^5 to 10^6 cells/ml was placed into sterilised reservoir water, *Aer. salmonicida* survived and multiplied with up to 10^8 cells/ml, in the system in 72 h. It could still be recovered in substantial numbers (10^7 cells/ml) at 55 days, when the experiment was concluded. In addition, nutrient conditions appeared to have an effect as it was observed that supplementation with low concentrations of nutrient, e.g. 0.005 % (w/v) brain heart infusion broth, caused an increase in the number of *Aer. salmonicida* cells within 24 h. This increase was maintained until the end of the sampling period. The addition of nutrient, moreover, caused the increase in numbers of the pathogen regardless of whether the initial inoculum of cells was large or small. Supplementation with nutrient was also reported to increase survival time by McCraw (1952), who observed that the addition of 0.1 % (w/v) peptone to seawater enabled *Aer. salmonicida* to survive up to 80 days, a much longer time than recorded for unsupplemented sea water.

The ability of *Aer. salmonicida* to persist in mud (sediment) or detritus in the fish farm environment has also been examined. McCarthy (1980) demonstrated that the pathogen was able to survive in numbers of *ca.* 10^5 viable cells in fish pond mud and

detritus at least up to 29 days, and probably longer as the experiments had to be terminated prematurely due to decomposition of the dialysis bags containing the bacteria. He further pointed out that the 10^5 viable cells remaining after 29 days are significant, since from his studies it was shown that if this number of cells was released into fresh water, their survival time would be 14 days. Michel and Dubois-Darnaudpeys (1980) investigated the persistence of *Aer. salmonicida* in sediments and reported that the pathogen survived and grew in sterilised river sediments for over 10 months. However, pathogenicity of the two isolates tested was lost after 8 or 9 months. They concluded that in natural conditions such a length of time would enable the pathogen to be released from sediment into the water, and that the behaviour of bottom-feeding fishes would allow direct contamination of fish, possibly becoming carriers. A reduction in pathogenicity, subsequent to prolonged incubation in river sediments, was also noted by Sakai (1986a, b). He offered an explanation whereby avirulent cells (with a positive electrical charge), which originate from virulent cells (negatively charged) attached to sediment, spontaneously detach from the sediment particles (river sand in survival experiments) thus decreasing the number of virulent cells recovered. Michel and Dubois-Darnaudpeys (1980) conceded that competition of *Aer. salmonicida* with large numbers of other bacteria in streams, some with an ability to synthesise bacteriocins, may act as a regulatory mechanism and limit the proliferation of the pathogen. However, previous work by Dubois-Darnaudpeys (1977b) supports the concept that *Aer. salmonicida* is genuinely capable of survival and multiplication in natural sediments, hence providing a reservoir of infection, even though direct contact with diseased fish is likely to remain the primary route of transmission. In addition, the regular detection of bacteriophages specific for *Aer. salmonicida* in samples of river sediments was taken as an indirect demonstration that the pathogen was present throughout the year (Dubois-Darnaudpeys 1977b). Sakai (1986b) reported extended survival times (>15 weeks) for virulent cultures of *Aer. salmonicida* if placed in the presence of dilute humic acid (10 μg/ml), tryptone (10 μg/ml) and cleaned river sand (100 g/100 ml of medium). Without the addition of the sand, detection of viable cells ceased within 5 weeks. However, avirulent strains of the pathogen did not survive more than 2 weeks regardless of whether or not sand was included in the experimental system. Sakai (1986b) determined that humic acid and amino acid-humic acid complexes were absorbed onto the sand, which led to a build-up of 30–50 times the environmental concentration of amino acids on the surface of the sand particles. This, in turn, allowed only colonisation of/attachment by bacterial cells with net negative electrical charges (virulent cells of *Aer. salmonicida* in this instance), which resulted in their enhanced survival in the presence of the sand. Thus, Sakai (1986b) concluded that the electrostatic interrelationship occurring among humic acid, river sand and the bacteria explain the ability of virulent *Aer. salmonicida* strains to survive for extended periods in river sediments.

McCarthy (1980) contended that during epizootics of furunculosis there existed a strong possibility that fish farm implements could become contaminated with *Aer. salmonicida.* In a study emphasising survival of *Aer. salmonicida* on fish nets which would be used both to remove dead infected fish and to move healthy fish, it was

established that the pathogen survived up to 6 days on both dry and wet contaminated nets. In addition, wet and dry contaminated netting was disinfected using three compounds, i.e. acriflavine, Teepol-sodium hydroxide and hypochlorite solutions. *Aer. salmonicida* was not recovered from either wet or dry netting disinfected with the acriflavine or Teepol-sodium hydroxide solutions, but the hypochlorite solution failed to disinfect dry nets. McCarthy (1980) concluded from these results that the use of contaminated and improperly disinfected nets is potentially dangerous to healthy stock as it is known that netting abrades fish to some extent, and such abrasions can facilitate bacterial invasion. In addition, *Aer. salmonicida* has been reported to attach in higher numbers to plastic rather than stainless steel surfaces, which opens up the possibility that the pathogen may have a preference for certain substrates/surfaces in the aquaculture environment (Carballo et al. 2000).

More recently, it has been found that wrasse (these are small inshore benthic fish which have gained popularity as a means of controlling sea lice populations among infested Atlantic salmon) are also susceptible to furunculosis (Treasurer and Cox 1991). These investigators reported the recovery of typical *Aer. salmonicida* from the liver and kidney, and the presence of skin lesions reminiscent of chronic furunculosis in golsinny, rock cook and cuckoo wrasse of fish farm origin. But could salmon lice harbour and transmit *Aer. salmonicida?* By means of recovery techniques with immunomagnetic beads coated with monoclonal antibodies to LPS and culturing techniques, it was determined that *Aer. salmonicida* was recoverable from lice (~10^4 *Aer. salmonicida* cells/louse) and also marine plankton 600 *Aer. salmonicida* cells/g of homogenised plankton) (Nese and Enger 1993). Perhaps more worrisome is the report by Frerichs et al. (1992) of the recovery of atypical *Aer. salmonicida* from apparently healthy wild wrasse captured in the open sea. Fortunately in this instance, the isolates were proven to be non-pathogenic to Atlantic salmon smolts. Consequently, both groups have cautioned against the arbitrary stocking of wrasse in fish farms without first checking for the possible presence of *Aer. salmonicida* in the fish. Furthermore, Treasurer and Cox (1991) recommended that wrasse should not be released back into the wild, or transferred between fish farms at the end of the production cycle.

Only a few investigators have examined animals, other than fish, as a potential source of infection. The extensive study carried out on this topic was that of Cornick et al. (1969); a total of 2954 vertebrate and invertebrate specimens, collected from fish ponds during an epizootic of furunculosis, were examined for the presence of *Aer. salmonicida.* No isolates of the pathogen were recovered despite this heroic attempt. This study is cited time and time again as evidence against the likelihood of animals, other than fish, acting as reservoirs of infection. Williamson (1928) was also unsuccessful in isolating the pathogen from water snails under similar conditions. Allen (1982) examined macroscopic algae and zooplankton taken from fish tanks prior to, during, and after a furunculosis epizootic at a fish-rearing unit in Essex, UK, in an unsuccessful attempt to recover *Aer. salmonicida* from these organisms. In contrast, King and Shotts (1988) determined that viable cells of *Aer. salmonicida* survived and, indeed, multiplied (two-fold) within the digestive tract of the ciliated protozoan, *Tetrahymena pyriformis.* It may be concluded from some of

these studies that the pathogen may be found in association with other aquatic animals, but it is apparent that many of the existing methods available for detection of the pathogen are inadequate.

Data have pointed to the possibility that *Aer. salmonicida* may be disseminated in aerosols (Wooster and Bowser 1996). In particular, experiments demonstrated that the pathogen travelled 104.1 cm (the limit of the test chamber), via the airborne route (Wooster and Bowser 1996). Thus, another possible means of spreading the pathogen needs to be considered.

Difficulties in Recovering *Aeromonas salmonicida* from the Aquatic Environment It is relevant to digress, at this point, from the discussion of sources of infection, in order to comment upon the difficulties besetting the isolation of *Aer. salmonicida* from environmental samples, other than fish. A dependable isolation procedure for the pathogen is of critical importance to an understanding of the epizootiology of diseases caused by *Aer. salmonicida* . For example, if the pathogen is capable of a free-living existence outside a fish host, the prevention and control of diseases of *Aer. salmonicida* aetiology would be rendered much more difficult if not impossible. However, currently *Aer. salmonicida* is defined as an obligate fish pathogen not found in surface waters (Popoff 1984). This definition has no doubt been formulated due to the paucity of conclusive evidence for a free-living existence of the pathogen. The organism, for instance, often cannot be isolated from water on fish farms even during an epizootic of the disease (Cornick et al. 1969; Kimura 1970; Allen 1982). Several reasons have been put forward to explain this disconcerting phenomenon. One is that *Aer. salmonicida* is notoriously difficult to isolate from mixed microbial populations as it is quickly outcompeted in growth by most other commonly occurring aquatic bacteria. In addition, pigment production on agar plates, heavily relied upon as a first indication that *Aer. salmonicida* is present, is inhibited by the close proximity of colonies of other bacterial types. Therefore, there is the perceived problem of recognising *Aer. salmonicida* in large mixed microbial communities. Many of the problems with habitat and survival studies on *Aer. salmonicida* are blamed on lack of adequate methodology. Both McCarthy (1980) and Michel and Dubois-Darnaudpeys (1980) stressed contamination difficulties when employing non-selective media, e.g. TSA, for isolation of the pathogen. Cornick et al. (1969) reported that *Aer. salmonicida* was isolated most frequently from environments containing few, if any, other bacterial species, particularly representatives of the genus *Pseudomonas.* Their preliminary experiments suggested that some pseudomonad taxa obtained from water and fish inhibited the growth of *Aer. salmonicida* in liquid and on solid culture media. Both cell-free filtrates and extracts of disrupted cells of the pseudomonads caused the inhibition, believed to be due to antimicrobial activity. Dubois-Darnaudpeys (1977a) also examined the effects of the bacterial flora commonly occurring in surface water, such as the *Pseudomonas-Achromobacter* and *Flavobacterium* groups, on the survival and growth of *Aer. salmonicida.* She found that the pathogen was inhibited at all temperatures if the experiments were run in the presence of the other bacteria. The ability of other micro-organisms, e.g. *Acinetobacter, Aer. hydrophila,*

Chromobacterium, Esch. coli, Flavobacterium and *Pseudomonas,* and their metabolites, to inhibit the survival of *Aer. salmonicida* in non-sterile seawater was also reported by Effendi and Austin (1991). Thus, because the isolation and study of the viability of the pathogen is complicated by competition and inhibition by other organisms, it is not surprising that investigations into survival of the pathogen, which could help to establish whether or not it is capable of a free-living existence outside of fish, have invariably retreated to laboratory-based experiments using sterilized water. A filtration method tested by Maheshkumar et al. (1990) attempted, with some degree of success, to overcome the difficulties of isolation of *Aer. salmonicida* posed by the presence of other bacteria, i.e. the possible overgrowth by small numbers of cells of the pathogen. In their studies, up to 5 l of hatchery water was seeded with *Aer. salmonicida* and passed through 1^{-MDS} electropositive filters. This technique was used in combination with removal of the filters after backwashing, soaking them in a small volume of 3 % beef extract solution, and followed by the scraping of the filters to remove trapped bacteria. When all the eluates from the filters were combined, recovery of *Aer. salmonicida* was determined to be 35 %. Thus, Maheshkumar et al. (1990) concluded that the filtration technique demonstrated greater sensitivity than the direct examination of water. Also, the enumeration of *Aer. salmonicida* was not overly effected by the presence of other bacteria because in the water samples the pathogen retained the ability to produce a brown pigment. However, these authors noted that biochemical/serological tests would be necessary for the detection of atypical non-pigmenting isolates. Nonetheless the classic dilemma of many ecological studies persists, i.e. how does the response of an organism in laboratory-based experiments relate to its performance in the natural environment where it needs to interact in a diverse and heterogeneous community? It is a problem still in search of a reliable solution.

It has often been stated that ecological investigations of *Aer. salmonicida* are hampered by the lack of an effective selective isolation medium specifically formulated for the pathogen. It was, for example, the opinion of Cornick et al. (1969) that the development of a selective medium for *Aer. salmonicida* could quite possibly change the present views on the habitat and viability of the pathogen. McCarthy (1980) also believed a selective medium would greatly assist ecological work. No doubt such a medium would be extremely useful; however, it is unlikely that its existence alone would cause all the remaining difficulties concerning ecological work on *Aer. salmonicida* to evaporate. It should be noted, however, that although a selective medium for *Aer. salmonicida* has not yet been formulated, CBB serves well as a differential growth medium, and is increasingly employed for this purpose. This medium is especially useful in the detection of *Aer. salmonicida* in fish tissues. For instance using CBB, Cipriano et al. (1992) recovered the pathogen from 56 % of mucus samples, but interestingly from only 6 % of kidney material, taken from salmonids. This was reinforced by a later study, which pointed to the presence of the organism in gills and well as mucus, i.e. external carriage (Cipriano et al. 1996a, b). The data has been summarised, as follows:

Number of Samples Revealing *Aer. salmonicida*

15/100 gill samples revealed the pathogen at 6.3×10^2–1×10^4/g
19/100 mucus samples revealed the pathogen at 9.1×10^2–1.7×10^4/g

Using CBB, Cipriano et al. (1996b) reported *Aer. salmonicida* in higher numbers from the mucus than kidney of six salmon. Thus, the populations of *Aer. salmonicida* were in the range of 1.1×10^3 to 1.8×10^7/g and 1.0×10^3 to 1.4×10^7/g for mucus and kidney, respectively. Hiney et al. (1994) reiterated the view that *Aer. salmonicida* may colonise mucus, gills and also fins. Also, these workers considered that the intestine may well be the primary location of *Aer. salmonicida* in Atlantic salmon with asymptomatic infections. In addition, there are other considerations that may contribute to the problems experienced. For instance, parallels might be drawn from studies on the isolation methods used for other micro-organisms, i.e. notably coliforms and enteric pathogens such as *Salmonella* spp., where it has been observed that a pre-enrichment step must be employed prior to use of selective media that impose too stringent conditions on these organisms which have been stressed, injured or are too sensitive to selective agents, and, thus, are rendered unrecoverable by selective methods alone (Geldreich 1977; Kaper et al. 1977; Olson 1978). Alternatively, it can be postulated that *Aer. salmonicida* follows the pattern of certain other types of micro-organisms which are extremely difficult to detect in the natural environment by means of routine bacteriological procedures (i.e. plate counts) and, hence, have been assumed to be absent from these environments. Stevenson (1978) commented on adjustments made by bacteria, which enable the organisms to survive in the variable and often stressful conditions imposed upon them by existence in natural aquatic systems. He suggested that bacteria surmount changes in their environment, including varying degrees of solar input, temperature, availability of nitrogen and dissolved oxygen, by entering a state of dormancy defined as 'any rest period or reversible interruption of the phenotypic development of an organism' (Sussman and Halvorson 1966). Thus, the possibility that *Aer. salmonicida,* in the natural environment outside a fish host, assumes a physiological state such that it cannot be recovered on agar plates used for primary isolation should not be discounted. There is tentative evidence that the situation for *Aer. salmonicida* is similar to that of a related pathogen, *V. cholerae.* In the survival of *V. cholerae* in aquatic microcosms, Singleton et al. (1982a, b) reported that certain combinations of environmental parameters, i.e. sub-optimal salinities and low nutrient concentrations, not only affected multiplication of *V. cholerae* populations but also recoverability of the cells. Thus, these investigators found that *V. cholerae* cells were observed using acridine orange staining in conjunction with epifluorescence microscopy when culturable cells were not detected. Further work on the theme of a 'non-recoverable' stage of existence for bacterial populations which, however, remain viable was done by Xu et al. (1982), also for *V. cholerae.* They used direct viable counting, a procedure allowing estimation of substrate-responsiveness, i.e. viable cells, using microscopy. This method revealed that a significant proportion of the non-culturable cells were, in fact, viable.

Survival of *Aeromonas salmonicida* in the Absence of Culturing The observations for *V. cholerae* were of especial interest because we found that cells of *Aer. salmonicida* could be enumerated by microscopic direct counts when no colony-forming units were recovered on agar plates, raising the possibility that problems concerning the recovery of *Aer. salmonicida* may bear similarities to *V. cholerae.* Our experiments had shown that if low numbers of *Aer. salmonicida* were inoculated into sterilised reservoir water the organism could not be recovered using plating methods, after 72 h. However, when microscopic direct counts were done the number of bacteria underwent an initial increase 6 h post-inoculation, and maintained these numbers until the conclusion of the experiment. Unfortunately, it was not possible to know from these results whether the cells observed by microscopy were, indeed, viable. Thus, in a follow up study, the hypothesis that *Aer. salmonicida* may enter a non-recoverable but viable state was tested. However, these data bore some similarity to survival studies with other pathogens, namely that there is often is an initial increase in the number of cells as visualised by microscopic methods despite culturing indicating a progressive decline in numbers.

The Non Culturable But Viable (NCBV) State It was confirmed by Allen-Austin et al. (1984) that small inocula of cells rapidly declined in filter-sterilised river water, when enumerated using total viable count procedures on agar plates, such that the system appeared to be totally devoid of viable cells by day 17. However, the microscopic procedures showed that, after an initial decrease, the number of *Aer. salmonicida* cells remained constant at approximately 8.0×10^2/ml. TSB to 0.01 % (v/v) was added to the experimental system 7 days after the plate counts reached and remained at zero, and the sample was split into three equal volumes, incubated at 22 and 18 °C in addition to 15 °C. At 22 °C, 150 colony-forming units of *Aer. salmonicida/* ml of sample were recovered on TSA 6 h after supplementation with the nutrient. There was no apparent increase in the direct microscopic count at 6 h, but by 24 h the microscopic fields contained too many cells to count. This result demonstrated that 6 h after nutrient addition, a proportion of the cells had regained the ability to produce colonies on TSA, despite the fact that for the previous 7 days none had been capable of colony formation. At 18 °C, the response to the added nutrient was much slower, insofar as colonies were not detected until after 4 days had elapsed. There was, however, a pronounced increase in the direct counts at 24 h after nutrient was added. This continued up to 4 days, when the first colonies were cultured on solid medium. Similarly, there was a lag of 5 days before colonies were recoverable at 15 °C. This coincided with an increase in the direct count. In comparison, it is emphasized that plate counts remained at zero in unsupplemented river water at 15, 18 and 22 °C. The factors involved in triggering the return of *Aer. salmonicida* to a culturable state need careful evaluation. As demonstrated in the experiment reported here, temperature and nutritional changes appear to be responsible for reactivating cells of *Aer. salmonicida* (Allen-Austin et al. 1984).

Sakai (1986b) also proposed a mechanism for the long-term survival of *Aer. salmonicida* in the aquatic environment based on electrostatic charge differences on

individual cells, with net negative charges reported on virulent, agglutinating cells, and net positive charges on avirulent non-agglutinating strains. He suggested that the negatively charged virulent form of *Aer. salmonicida* is able to persist, albeit under starvation conditions, retaining viability in river sediments. It was also proposed that the decline in negatively charged virulent cells in sediments over prolonged periods, also noted by other investigators (e.g. Michel and Dubois-Darnaudpeys 1980), may be caused by the spontaneous occurrence of positively charged avirulent free-living cells of *Aer. salmonicida.* These cells originate from the virulent ones, attached to sediment particle surfaces, and subsequently detach from the sediment/sand particles. This free-living form could be considered to enter a dormant phase, according to Sakai (1986b), because the viability of these bacteria declines due to a lack of nutrients. It was further proposed that the free-living cells represent a transitional life stage of the pathogen, which would ultimately lose viability (Sakai 1986b).

Subsequently, Rose et al. (1990a) re-examined the possibility that *Aer. salmonicida* may enter a dormant state in water, using methods modified from the work of Allen-Austin et al. (1984), as described above. However in their experiments, the addition of 0.1 % (w/v) TSB to aliquots withdrawn from microcosms after viable counts of *Aer. salmonicida* had reached zero, did not result in renewed growth of the organism. Thus, Rose et al. (1990a) concluded that the most probable explanation for the results obtained in the previous study (when there appeared to be resuscitation of dormant cells by added nutrients) was the presence of small numbers of viable culturable cells, which were too few in quantity to be detected by the sampling protocol employed. This conclusion was based on the observation that the addition of 0.1 % (w/v) TSB to microcosms after the viable count had reached zero resulted in the re-appearance of viable culturable cells within 48 h of incubation at 22 °C. However in both studies, bacteria enumerated by microscopic techniques remained at levels of approximately 10^4/ml in water samples retrieved from the experimental microcosms containing *Aer. salmonicida,* even after viable counts had apparently reached zero. It is curious that Rose et al. (1990a) proffered no explanation which account for the level of bacteria that were observed microscopically (were the cells alive or could they have been dead?). In a later study, which again addressed the issue of dormancy/NCBV for *Aer. salmonicida,* Morgan et al. (1991) assessed the survival of the pathogen in lake water, employing an extensive range of techniques, including epifluorescence microscopy, respiration, cell culture, cell revival, flow cytometry, plasmid maintenance and membrane fatty acid analysis. These workers found that *Aer. salmonicida* became unculturable in sterile lake water, but microscopic and flow cytometric methods revealed the continued presence of cells. However, attempts to revive these cells by the addition of TSB were unsuccessful. Despite this, it was found that both genomic and plasmid DNA, and also RNA, were maintained in the cells, even though they could not be cultured on conventional media. Morgan et al. (1991) concluded that morphologically the cells remained intact, although their viability could not unfortunately be definitively demonstrated. In addition, they commented (and we strongly agree) that non-culturability of some bacteria from environmental samples maybe as much a func-

tion of the ignorance of the parameters necessary for their recovery, as the occurrence of a truly non-culturable, specialised survival state. Subsequently by means of flow cytometry with rhodamine 123, they established a NCBV state in sterile lake water (Morgan et al. 1993). However, flow cytometry indicated that cellular properties related with viability was lost shortly after culturability disappeared in distilled water, but not so in lake water (Deere et al. 1996b). Additionally, these workers marked an isolate of *Aer. salmonicida* with the *xylE* gene, using the plasmid pLV1013. This isolate was culturable on TSA from normal (non sterile) lake water for 3 weeks, after which non-culturability developed, with the NCBV cells retaining chromosomal and plasmid DNA. The NCBV state can be postponed (60 days was mentioned by Pickup et al. 1996) by the addition of high levels of nutrient, especially 125 μM quantities of the amino acids arginine and methionine, to experimental microcosms. *Aer. salmonicida* decreased in size and became rounded, but were still culturable (Pickup et al. 1996).

The development of a dormant, nonculturable state of *Aer. salmonicida* in seawater at 4 °C was apparent from the work of Ferguson et al. (1995). These workers incorporated a luciferase gene, *luxAB*, from *V. fischeri* into *Aer. salmonicida* and followed the fate of the cells. As before, intact nonculturable cells could not be resurrected.

There has always been a dilemma about the relevance of cells that cannot be cultured, to fish. It is worth heeding the results of Stanley et al. (2002), who determined that only culturable cells in laboratory microcosms could induce furunculosis upon injection into fish, and not PCR or ELISA positive samples which could not be supported by culturing evidence.

Ecology of *Aeromonas salmonicida* – An Explanation To develop some previous points, there is tentative evidence to support the possibility that *Aer. salmonicida* undergoes sufficient modifications to its morphology in seawater so as to be only recoverable on specialised media. Thus while conducting experiments on the survival of *Aer. salmonicida* in seawater, Effendi and Austin (1991) found that samples where the pathogen was believed to be absent (or unculturable) actually contained cells which passed through 0.22 and 0.45 μm pore size porosity filters. These isolates grew on specialised media designed for the recovery of L-forms (Fig. 5.8), and showed agreement with the characteristics of *Aer. salmonicida* L-forms as reported by McIntosh and Austin (1988, 1990, 1991b). Subsequently, *Aer. salmonicida* colonies developed on basal marine agar (BMA) plates inoculated with material from turbid L-form broth medium. On this basis, Effendi and Austin (1991) recorded populations of *ca.* 10^3 *Aer. salmonicida* cells/ml in the microcosms after corresponding enumeration of colonies on BMA had reached zero. Thus, they suggested that the existence of specialised forms, e.g. L-forms, of *Aer. salmonicida,* may be a factor in the difficulties previous researchers have experienced in attempts to recover the pathogen from environmental samples. Continuing this theme, Effendi and Austin (1995a) examined the characteristics of the so-called NCBV cells. Using a marine microcosm, it was observed that these NCBV cells became much smaller and coccoid while retaining respiratory activity as measured by the

reduction of tetrazoliums to insoluble formazans. There was not any alteration in the LPS composition of the cells, but an alteration in the protein composition was recorded, with a reduction in some (15, 17, 22, 30 and 70 kDa proteins) and an increase in a 49 kDa protein. This was accompanied by a loss in DNA. That these cells were still alive was indicated by the development of large bizarre shapes following the addition of yeast extract and nalidixic acid (after Kogure et al. 1979).

To summarise, the question as to whether *Aer. salmonicida* is able to persist in a free-living form outwith a fish host is still outstanding. An equally important corollary concerns the pathogenicity of such forms, assuming that they exist, i.e. can they retain the ability to infect fish? Traditionally, *Aer. salmonicida* has been defined as an obligate fish pathogen. However, there appears to be an increasing trend in ecological studies to at least consider the possibility that this definition may no longer hold true at all times. On the basis of the available data obtained from numerous survival studies, it may be stated that *Aer. salmonicida* has the ability to persist in the aquatic environment for protracted periods. It is the mechanism of this survival and its effects on the organism in the natural environment around which the debate now centres. It is possible that *Aer. salmonicida* could exist in a so-called non-culturable stages (perhaps due to the presence of an altered morphological state, such as L-forms). After all, the reason for the occurrence of explosive outbreaks of furunculosis among fish populations, particularly salmonids, which have not been previously expose to the disease, has yet to be explained. The answer to this important question of facultative versus obligate pathogen still awaits the development of methods for the more efficient and refined detection of the organism in the natural environment. As one astute scientist has said, 'absence of evidence is not evidence of absence'; hence further efforts to breach this gap in our understanding of *Aer. salmonicida* epizootiology are essential.

***Aeromonas salmonicida* – Transmission by Fish** Fish undoubtedly play a major role in the transmission of disease among themselves. One early study Blake and Clark (1931) stated that furunculosis was only spread by infected fish or by material which has come into contact with them. Fortunately, the recovery of *Aer. salmonicida* from fish tissues is generally less troublesome than seeking it in other environmental sources. Fish may act as a source of infection in two ways: those which have died or are ill with furunculosis are heavily contaminated with the pathogen, alternatively they may be carriers which, although appearing healthy, harbour the pathogen in their organs where it can be released if the fish eventually succumb to the disease. Both these aspects have received attention. McCarthy (1980) established that material from a furuncle could contain up to 10^8 viable cells/ml of necrotic tissue, and was interested to assess the viability of *Aer. salmonicida* in dead fish and the degree to which they could contaminate pond water. He found that *Aer. salmonicida* remained viable in fish (muscle) tissue for 32 days, and for 40 days in the tank water where the dead fish had been kept, thus providing a possible source of infection for healthy fish. Another study showed that *Aer. salmonicida* remained viable in infected trout tissues and internal organs (heart, liver, spleen and kidney) for 49 days when the fish were stored at −10 °C (Cornick et al. 1969). However, the patho-

gen was isolated only from the kidney of infected trout held at 4 °C for 28 days. McCarthy (1980) pointed out that the prolonged survival of *Aer. salmonicida* in dead diseased fish shows the risk of using scrap fish, which might be infected with a chronic form of furunculosis, as food, particularly in view of the finding of Cornick et al. (1969) on the survival of the pathogen at low temperatures. Some investigations, which have examined the survival of *Aer. salmonicida* in water, have also commented on the potential of the non-culturable cells, detected microscopically in the experimental systems, to infect fish. However, these studies have generally concluded that *Aer. salmonicida* in this form does not appear to be pathogenic, as re-infection of fish does not occur (Rose et al. 1990a; Morgan et al. 1991).

Trash fish used in the production of feed may well be implicated in the spread of furcunculosis. In this respect, Kim et al. (2013) attributed outbreaks of disease in Korean rockfish (*Sebastes schlegeli*) to the low-value fish, which included big head croaker, Japanese anchovy and Konoshiro gizzard shad, used in the production of feed.

***Aeromonas salmonicida* – Osmotically Fragile Forms/L-Forms** Another line of investigation examined L-forms (spherical, filterable cells) of *Aer. salmonicida.* The L-forms were induced experimentally, and were found to be unculturable by conventional plating methods. Subsequently, a stable L-form was induced with benzylpenicillin, and determined to contain more OMP of ~40 kDa molecular weight than its parental cell (Gibb et al. 1996). This stable L-form did not require specialised media, and could grow on BHIA at 0–5 °C (Gibb and Austin 1994). It could be argued that the development of an L-form state could enable it to persist in tissues of infected fish, although not causing clinical disease (McIntosh and Austin 1991b). However, attempts to infect fish using L-forms did not result in recovery of the pathogen from fish, even after the administration of immunosuppressants. In addition, it was reported that small numbers of 'natural' L-form colonies were observed, albeit infrequently, on specialised medium (containing horse serum and high quantities of sucrose), which had been inoculated with kidney and spleen samples taken from farmed Atlantic salmon suffering with furunculosis. This suggests that L-forms may have implications in the disease process. Such findings certainly merit further investigation to more conclusively establish the role of this form of *Aer. salmonicida* in disease processes. Obviously, if *Aer. salmonicida,* in a dormant or NCBV or indeed in any other altered state it may undergo outside a fish host, is genuinely unable to transmit infection, then control of the diseases is vastly simplified and rendered less difficult. It is not possible, on the basis of the limited data available, to draw grand, definitive conclusions about this crucially important aspect of the pathogens behaviour. Further work will, hopefully, clarify the situation.

***Aeromonas salmonicida* – The Role of Carriers** The second mechanism by which fish may provide a source of infection is by becoming carriers of *Aer. salmonicida.* The existence of such fish and their role in transmission of the disease was recognised early in the history of furunculosis. In a study of furunculosis in trout in the River Kennett, Horne (1928) found that *Bacterium salmonicida* was

recovered in the blood of 17 % (3 out of 18) of trout examined, and it was concluded that carriers provided a source of infection in the river. He also sampled fish from fish farms and found them to be generally healthy and thus contended that these results indicated that trout farms were not harbouring the disease. Horne (1928) additionally commented that knowledge of carrier rates in fish populations before, during and after the furunculosis season would be of great epizootiological value.

Because of the obvious importance carrier fish have in the epizootiology of furunculosis, it is essential that the methods used to detect their presence are effective. However, research into the carrier state has been hampered by technical difficulties concerned with detecting such fish with certainty, as present culture isolation methods appear to be too insensitive (McCarthy 1980). Blake and Clark (1931) reported that raising the temperature of the water in which suspected carriers (usually survivors which had been previously exposed to infection) were maintained from 5 to 18 °C induced the disease. At present, a combination of increasing the water temperature to 18 °C and the injection of corticosteroids is employed to activate the carrier state. This method is based on the work of Bullock and Stuckey (1975b), who tested corticosteroid injection and heat stress as means of producing overt furunculosis in carrier trout. They reported that although heat stress alone produced mortality, the pathogen could not be recovered from the majority of test fish which died. Direct kidney cultures to isolate *Aer. salmonicida* were found to be ineffective for carrier detection. McCarthy (1980) reported success with the method of Bullock and Stuckey (1975b), although he stated that prednisolone acetate was the most effective corticosteroid of the several he tested, for inducing furunculosis in fish. In further experiments, he assessed the prevalence of carriers in fish populations, examining 1-year-old brown trout from four different commercial fish farms. The fish had a high carrier rate of 40–80 %; however, similar populations of rainbow trout tested had a very low incidence of <5 % or were possibly free of carriers. The technique has also been applied by McCarthy (1975a) to non-salmonids, e.g. silver bream (*Blicca bjoerkna*), infected with a non-pigmented aberrant *Aer. salmonicida* strain.

The ELISA technique has also been assessed for its effectiveness in the detection of carrier fish. Rose et al. (1989) compared a commercial ELISA kit (obtained from Stirling Diagnostics Ltd.), the commonly used corticosteroid/ heat protocol of McCarthy (1980) and plating of rectum and kidney samples from Atlantic salmon onto BHIA and TSA. The Atlantic salmon were obtained from a site where outbreaks of furunculosis had previously occurred. From the results, it was apparent that the ELISA was the most successful technique, with 56.17 % (14/26) of the fish shown to be carriers. This compared with a carrier rate of 26.4 % as determined by means of the corticosteroid/heat test. Yet, plating techniques failed to reveal *Aer. salmonicida* in any of the fish. However, some disadvantages of ELISA are apparent, namely the inability to distinguish living from dead cells, and, for that matter, to differentiate viable, pathogenic cells from those resulting from use of living, attenuated or dead, virulent cells from vaccines. Moreover, ELISA does not enable the provision of cultures, which could be used for additional investigation, such as

the determination of antibiogrammes. Notwithstanding such drawbacks, ELISA is a valuable tool for the rapid detection of *Aer. salmonicida* in clinically diseased and asymptomatic carrier fish.

It has not been conclusively established which organs of carrier fish serve as the site of carriage. From experiments using immunofluorescence techniques, Klontz (1968) concluded that the intestine is a primary site of infection, leading to the establishment of asymptomatic carriers. There is also some evidence to suggest that the kidney is involved, but the intestinal tract has again been mentioned (McCarthy 1980). This is a subject which requires further work to clarify it.

Another problematic aspect of the carrier state is that antibiotic therapy to control furunculosis outbreaks does not necessarily completely remove the bacterium from the tissues of fish, at least when sulphamerazine, furazolidone and potentiated sulphonamides are used. McCarthy (1980), however, reported that fish treated with tetracycline hydrochloride (i.m. injection of 50 mg/kg) survived attempts to induce the disease. On the basis of such findings, McCarthy (1980) warned that it must be anticipated that fish populations treated for furunculosis will remain carriers.

However more recently, other antibacterial compounds have been investigated for their ability to reduce or eliminate the carrier state of *Aer. salmonicida* in fish. Examples include erythromycin phosphate (Roberts 1980), flumequine, and an aryl-fluoroquinolone (Scallen and Smith 1985) in combination with 0.01 % Tween 80 to enhance the assimilation of the antimicrobial compound into fish (Markwardt et al. 1989). The last mentioned group of workers found that the aryl-fluoroquinolone/surfactant combination was effective in eliminating the asymptomatic carrier state of *Aer. salmonicida* within 48 h of treatment. Markwardt and Klontz (1989) also pointed out the advantages of the use of the aryl-fluoroquinolone, such as its broad spectrum activity, low *in vitro* minimal inhibitory concentration for many bacterial pathogens (Stamm et al. 1986), and lack of occurrence of resistant strains (Fernandes et al. 1987).

***Aeromonas salmonicida* – Transmission; What Does It All Mean?** Several possible routes for the transmission of furunculosis have been propounded and investigated. It is commonly accepted that the disease is disseminated by lateral transmission of *Aer. salmonicida* which includes contact with contaminated water and infected fish in addition to possible infection via the gastro-intestinal tract. Also, vertical transmission has been considered in several investigations. A waterborne route where water contamination with *Aer. salmonicida* has occurred initially from moribund infected fish or from overtly healthy carriers shedding the pathogen is favoured as the common means of transmission. Once released into the aquatic environment, the organism is then able to persist for a prolonged period of time and the disease spread in this way. Early studies demonstrated that trout placed into water that had contained diseased fish contracted the infection (Emmerich and Weibel 1894; Horne 1928; Blake and Clark 1931). The Furunculosis Committee of the UK concluded, on the basis of available data, that both food and diseased fish could constitute sources of infection. McCarthy (1980) has examined in detail the likely transmission mechanisms, specifically with regard to the ability of *Aer. sal-*

monicida to penetrate fish tissues (invasiveness), a pre-requisite for the occurrence of infection. Transmission by contaminated water was tested by McCarthy (1980) in laboratory-based experiments by seeding water in a tank containing six brown trout with a suspension of *Aer. salmonicida* to a final concentration of 10^6 cells/ml. Five of the six fish died of furunculosis, and the sixth succumbed when given an injection of prednisolone acetate. In a subsequent large-scale experiment using 50 brown trout placed in a pond on a fish farm experiencing a summer epizootic of furunculosis, 41 fish had died within 28 days, and the remaining 9 succumbed after injection with corticosteroid. McCarthy (1980) concluded from these experiments that the disease is readily disseminated through water and also that brown trout surviving infection probably become carriers. However, both Blake and Clark (1931) and McCarthy (1980) reported failure in attempts to infect rainbow trout by co-habitation with infected brown trout or the addition of *Aer. salmonicida,* respectively. It is known that rainbow trout are more resistant to the disease than are brown trout. In experiments, which examined different routes of exposure to *Aer. salmonicida* subsp. *salmonicida* in Atlantic salmon in seawater, Rose et al. (1989) noted that a minimum dose of 10^4 colony forming units/ml by bath was required to initiate infection.

***Aeromonas salmonicida* – Uptake Into Fish** Another unresolved aspect of the transmission of furunculosis is the uptake of *Aer. salmonicida* into a fish host. It is possible that the pathogen may gain entry to a new host through the gills, lateral line, mouth, anus or a surface injury (e.g. Effendi and Austin 1995b). McCarthy (1980) demonstrated that rainbow trout that resisted the disease subsequently died from furunculosis after their flanks had been abraded with sandpaper. Also, Lund (1967) found that infection was acquired by fish, that had been scarified and experimentally challenged with the pathogen. However, these injuries were artificially induced. Effendi and Austin (1995b) evaluated many different routes for the possible uptake of *Aer. salmonicida* into fish. The data may be summarised, as follows:

Route	Recovery of *Aer. salmonicida* from:
Gill	blood and kidney
Oral	blood
Lateral line	blood and spleen (but not kidney)
Ventral surface	blood and spleen (but not kidney)
Flank	blood and spleen (but not kidney)
Anus	blood (but not kidney or spleen)

Generally, *Aer. salmonicida* remained at the site of administration for 24 h. The most effective route of uptake leading to mortalities was the gill and anus. In contrast, fewer deaths resulted from challenge via the lateral line, flank or ventral surface (Effendi and Austin 1995b). Yet, despite all this work, the natural mode of uptake remains unresolved.

In a study of uptake of *Aer. salmonicida* by rainbow trout, it was observed that the pathogen could be detected in the blood and kidney within 5 min of immersion in a suspension containing 10^5 cells/ml (Hodgkinson et al. 1987). Interestingly, it was also found that uptake of the pathogen was enhanced by the addition of particulates, e.g. latex, to the bacterial suspension. If latex was indeed added, *Aer. salmonicida* was isolated from blood at 12 min, and from kidney, spleen and faeces at 4 h post-challenge. The organism was also cultured from the skin, gills, blood and faeces for up to 48 h. In the absence of latex, the pathogen could be again recovered at 12 min, but from a wider range of sites including kidney, spleen and the lower intestine. However by 24 h, the pathogen was no longer recovered from the fish. From culturing methods alone, it may not, however, be assumed that *Aer. salmonicida* had been totally removed from the animals. In fact, cultures of the pathogen were isolated from kidney, spleen and faeces within 1 to 4 h of immunosuppression of the fish at 7 days post-challenge. In addition, the method of challenge yielded different results. Thus, when the entire fish was immersed in the bacterial suspension, superior uptake occurred compared to exposing only the head or tail regions. The explanation of this phenomenon is unclear, but such results suggest that uptake may occur through several locations rather than a single site, e.g. mouth, nares, gill or anus. It is possible that the pathogen gains entry via all these sites or additionally through the lateral line and/or skin (Hodgkinson et al. 1987). Perhaps, the most significant observation resulting from these experiments was the rapidity by which *Aer. salmonicida* entered the rainbow trout. Other investigators have not sampled so close to the initial time of challenge. McCarthy (1980) reported uptake to occur from the oral route within 5 h, with the organism found in the kidney. Tatner et al. (1984) first sampled the fish at 24 h post-challenge. However, all these studies indicate the localisation of *Aer. salmonicida* principally within the reticulo-endothelial systems of fish (Tatner et al. 1984). Notwithstanding, evidence continues to be firmly pointed at the role of gills and skin/mucus in the uptake of *Aer. salmonicida* into fish (Ferguson et al. 1998).

Another route of infection that has been proposed is via the gastro-intestinal tract, due to intake of contaminated food. However, there is disagreement as to whether or not this in fact occurs. Plehn (1911) and Blake and Clark (1931) reported success in experimentally infecting fish by feeding contaminated food. However, Krantz et al. (1964a, b) and McCarthy (1980) failed to infect brown trout by feeding with food containing the pathogen. Klontz and Wood (1972) reported clinical furunculosis in the sable fish apparently caused by ingestion of carrier coho salmon. Evidence has been published that shows *Aer. salmonicida* may translocate across the intestinal epithelia (Jutfelt et al. 2006). Here, the authors exposed intestinal segments of rainbow trout for 90 min to isothiocyanate-labelled cells of virulent *Aer. salmonicida* and demonstrated translocation. In addition, in laboratory-based experiments that compared various methods designed to induce the carrier state of *Aer. salmonicida* in juvenile spring chinook salmon, Markwardt and Klontz (1989) observed that gastric intubation (of *ca.* 1×10^8 bacteria) resulted in a 65 % carrier state. This result was a significantly higher percentage than those recorded for exposure to a broth culture as a bath, ingestion of broth culture coated food, and exposure

to intraperitoneally injected fish (40, 20 and 10 % carrier rates, respectively). Markwardt and Klontz (1989) also commented that the exposure to clinically diseased fish and bathing in broth cultures most probably simulated the natural routes of infection (Paterson 1982).

***Aeromonas salmonicida* – Transmission; The Role of Eggs** Vertical or transovarian transmission of *Aer. salmonicida* as a possible route of infection has been widely studied with inconclusive results. Smith (1939) claimed the pathogen could be carried on the egg surface, in contrast to Plehn (1911) and Mackie et al. (1930), who were of the opinion that the organism was unable to infect fish eggs. The possibility that the pathogen could be transmitted at fertilisation was examined by the Furunculosis Committee in the UK. The experimental evidence gathered for their report indicated that furunculosis was not transmitted in such a fashion. Lund (1967), however, contended that since the conclusions were based on the results of a sole set of experiments, further work was necessary to confirm this point. In a detailed series of experiments aimed at clarifying the situation regarding transmission of *Aer. salmonicida* at fertilisation, Lund (1967) examined ovaries, testes and ova of experimentally infected fish for the presence of the pathogen. *Aer. salmonicida* was recovered in pure culture from the ovaries and testes of infected fish. Results of isolation of the pathogen from ova were decisive as, of 500 ova sampled, only three obtained from the same fish yielded the bacterium in pure culture from the interior of the ovum. In further experiments, using wild spawners, ova were contaminated with *Aer. salmonicida* on the external surface at the time fertilisation was effected, and the eggs then planted out in a river bed. It was observed that these ova died quickly and were subjected to *Saprolegnia ferax* infection. *Aer. salmonicida* was not isolated from dead or living ova, and Lund (1967) concluded that the experiment had been unsuccessful. Ova taken from parents experimentally infected with *Aer. salmonicida* also failed to yield the bacterium. Continuing the study of vertical transmission, McCarthy (1980) examined the fertilised ova of mature brood stock brown trout taken from a known carrier population (5/8 proved to be carriers when challenged with prednisolone acetate). However, *Aer. salmonicida* was not recovered from the fertilised egg sample. When artificially infected brood stock were stripped as soon as signs of clinical furunculosis had developed, both fish organs and fertilised eggs were positive for *Aer. salmonicida.* However, the high numbers of viable cells initially present, i.e. 10^6 cells/ml of egg macerate, rapidly declined and could not be detected 5 days after incubation began. Based upon these experiments, McCarthy (1980) concluded that vertical transmission of *Aer. salmonicida* was not a significant means of disseminating the disease, and, moreover, in the improbable event that overtly infected fish were used for stripping, the pathogen was unlikely to survive to the eyed-egg stage at which the eggs are marketed. Neither Lund (1967) nor McCarthy (1980) recovered *Aer. salmonicida* from fry derived from experimentally infected parents or of known carrier brood stock, respectively. However, both these authors pointed out the possibility that the negative results obtained may have been due to the inadequacy of techniques used for detection and isolation of the pathogen in the face of inhibition or overgrowth by commensal bacteria, or the presence of only small numbers of *Aer. salmonicida.*

***Aeromonas salmonicida* – Transmission in Seawater** A remaining aspect of the epizootiology of *Aer. salmonicida* diseases which requires consideration is the transmission of the infection in sea water. This is an important topic for the aquaculture industry, as salmonids are not infrequently placed in seawater for on-growing. In addition, early in the study of the pathogen, the possibility that migratory Salmonidae could spread the infection was considered. Lund (1967) investigated the possibility that the disease could be carried by salmon or sea trout smolts (previously infected in fresh water) when they migrated to the sea. The Furunculosis Committee had not agreed with this theory because examination of large numbers of smolts taken from the River Coquet in 1928 and 1929 had given no evidence for the presence of *Aer. salmonicida,* although the reverse process, i.e. salmon or sea trout contracting the infection upon migration into rivers containing infected trout, had become generally accepted. In an examination of 234 smolts from the River Coquet, Lund (1967) isolated and confirmed *Aer. salmonicida* from four smolts (two salmon and two sea trout), and believed the findings to be significant as such fish would possibly develop the disease on exposure to suitable conditions or remain resistant, possibly transmitting the infection upon contact with healthy fish in sea or brackish waters. Lund (1967) could not offer a definitive reason for the results differing from those of Williamson and Anderson (see Mackie et al. 1930), who examined 1339 smolts taken from the Coquet without recovering any isolates of *Aer. salmonicida.* Certainly, mortalities attributed to *Aer. salmonicida* in anadromous fish in sea water and in trout grown-on in sea water have been reported (Evelyn 1971a; Håstein and Bullock 1976; Novotny 1978). However, it has not been determined whether the disease outbreaks resulted from stress experienced by fish carrying a latent infection initially contracted in fresh water, or whether they represented a case of lateral transmission of the pathogen via sea water. Smith (1962), for example, had established that *Aer. salmonicida* survived in sea water for a prolonged period of time. It had also been demonstrated that *Aer. salmonicida* is capable of infecting sea and brown trout by contact with infected fish in sea and brackish waters (Scott 1968). She found that the infection was transmitted between salinities of 2.54 and 3.31 % (w/v) at water temperatures ranging from 5.6 to 14.5 °C. Smith et al. (1982) reported on mortalities of Atlantic salmon from two marine fish farms in Ireland, presenting evidence for the lateral transmission of *Aer. salmonicida* in sea water to a group of fish not known to be carriers. They also provided data suggesting that subsequent to the stocking in spring 1978, and removal of carrier fish in summer 1979, at a marine fish farm, the pathogen became established and persisted in the fish farm environment for at least 6 months after the removal of the carrier fish. Thus, a carrier-free population placed on the site in the spring of 1980 was infected. Unfortunately, it was not determined whether the pathogen persisted in feral fish outside the cages or in the sediments under the cages. To lend support to a sea water transmission of furunculosis, Evelyn (1971) has documented isolation of *Aer. salmonicida* from a strictly marine host, the sable fish, although probably the route of infection was by ingestion of moribund or dead salmonid carrier fish (Klontz and Wood 1972). Obviously, *Aer. salmonicida* has wider potential for causing disease problems than has been hitherto suspected. The study of the epizootiol-

ogy of *Aer. salmonicida* diseases in aquatic environments other than freshwater, again, as in so many other aspects of the pathogen, demands additional attention to unravel it complexities.

***Aeromonas salmonicida* – The Summary** In summary, although substantial efforts have been made to reach an understanding of the natural disease cycle in the environment, there still remain many issues to be resolved. Some of the data provide partial answers, some are contradictory. Lack of appropriate methodology appears to constitute a major hindrance to progress. In the face of the several unresolved questions concerning *Aer. salmonicida* epizootiology, McCarthy (1980) has valiantly put forth several hypotheses aimed at deriving an overall view of *Aer. salmonicida* epizootiology. The first of these is that furunculosis is introduced to a fish farm by importation of healthy carrier fish or that non-carrier resident fish may be infected from a water supply contaminated by wild or imported carrier fish. Accordingly, if contact with carriers of *Aer. salmonicida* can be prevented, furunculosis should not arise in the population. The second hypothesis McCarthy (1980) advanced is that the mechanisms by which carriers disseminate the disease to non-carriers is dependent upon environmental parameters at a given fish farm site. He postulated that if stressful conditions are absent, resident fish may become carriers without developing clinical signs of disease, and that under such conditions a high carrier rate could be maintained without clinical evidence of furunculosis. This, in the opinion of other workers, would be an arguable premise, as some believe all carriers will eventually succumb to the disease. It is reasonable to suppose that once carrier fish are present on a fish farm they are responsible for initiation of epizootics, although infection also occurs by the water-borne route. The third hypothesis is that the bacterium in the carrier state provides a measure of protection to the fish in return for shelter from the ravages of the aquatic environment. Were this the case, the observation by McCarthy (1980) that non-carrier and carrier fish exhibit marked differences in their susceptibility to furunculosis would be explained.

Finally, it is our opinion that while a firm understanding of the interactions of the pathogen in its milieu and in its fish host is lacking, fisheries scientists will remain at a serious disadvantage when tackling the problems of infectious diseases of *Aer. salmonicida* aetiology that currently beset fish cultivation.

Pathogenicity

The Spread of the Pathogen

Historically, *Aer. salmonicida* was regarded as a risk primarily to salmonids (e.g. Mackie et al. 1930, 1933, 1935). Then, cyprinids followed by other freshwater and marine fish became recognised to be vulnerable to infection (e.g. Herman 1968; Austin et al. 1998).

Could farmed salmonids pose a realistic risk to native marine fish species?

The data on this topic are confusing. Certainly, marine fish larvae have been infected with *Aer. salmonicida* subsp. *salmonicida,* with turbot regarded as being more susceptible than halibut (Bergh et al. 1997). Using co-habitation and injection challenges, experiments suggested that *Aer. salmonicida* subsp. *salmonicida* could be transmitted rarely from Atlantic salmon to Atlantic cod, halibut and wrasse (Hjeltnes et al. 1995). Interest in turbot has spurred some exciting work on understanding the virulence genes associated with the different stages of the infection process. Thus, attention focused on the genes known to occur in salmonids, i.e. *vapA, tapA, fla, ascV, ascC, aexT, satA* and *aspA*, using a PCR and challenge by i.p. injection and bathing. The result was that the genetic profile linked to virulence and challenge by i.p. injection was more frequent in cultures derived from turbot rather than salmonids or the external environment but it was not possible to predict virulence based solely on the presence of these genes (Lago et al. 2012).

Could atypical isolates, which are appearing with increasing frequency in wild fish, pose a threat to cultured salmonids?

Wiklund (1995b) using an atypical isolate from ulcerated flounder concluded that there was not any risk to rainbow trout.

What about the risk of transferring *Aer. salmonicida* from the freshwater to seawater stage of salmonids?

Eggset et al. (1997) concluded that the susceptibility of Atlantic salmon to furunculosis in seawater possibly reflected the overall quality of the smolts.

Pathogenicity – Historical Aspects

Although the factors conferring pathogenicity on *Aer. salmonicida* strains have been the subject of speculation since early in the study of the pathogen, it is only relatively recently that the details concerning pathogenesis and virulence have begun to be elucidated. The initial investigations, carried out in the 1930s, resulted in several key observations, notably that prolonged laboratory maintenance of *Aer. salmonicida* isolates was frequently responsible for a loss of virulence, and that histopathological examinations of infected fish suggested the occurrence of leucopenia and proteolysis in certain tissues. Among the first studies concerned with virulence mechanisms of the organism was the extensive work of the Furunculosis Committee in the UK (Mackie et al. 1930, 1933, 1935). This group did not detect any toxin production by *Aer. salmonicida* when either ultra-filtrates of broth cultures or diseased fish tissue was injected into healthy fish. Based on their failure to demonstrate toxin production, they hypothesised as a result of detailed clinical observations that the pathogenic processes caused by *Aer. salmonicida* could be explained by the prolific growth in the blood and tissues of its host which, in turn, interfered with blood supply resulting in anoxic cell necrosis and ultimately death. Additional evidence for a possible contribution to virulence, in the form of a leucocytolytic component, was provided by Blake (1935), who described the presence of ‘free’ bacteria and little phagocytosis in the blood of diseased fish, with no definite leucocytic

infiltration at the foci of infection. Mackie and Menzies (1938) confirmed the production of a leucocytolytic substance, as did Field et al. (1944), who determined the absence of leucocytosis by performing repeated blood counts on experimentally infected carp. Perhaps, a more significant finding of their study, however, was the rapid decline in blood sugar levels resulting in hypoglycaemic shock, which was sufficient in some instances to cause acute mortalities. They suggested that the hypoglycaemic shock was the outcome of rapid utilisation of blood glucose by the multiplying pathogen. Regarding virulence mechanisms of *Aer. salmonicida,* Griffin (1953) theorised that leucocidin production *in vivo* by *Aer. salmonicida* would account for the observations by previous workers that marked cytolytic tissue necrosis did not seem to be accompanied by leucocytic infiltration. Another aspect of *Aer. salmonicida* pathogenicity, which eventually proved to be extremely important, was discussed by Duff (1937). He reported a loss in pathogenicity among strains after 6 or more months of maintenance on artificial culture in the laboratory. The loss was accompanied by a change in the appearance of colonies on nutrient agar from glistening, convex and translucent to strongly convex, distinctly opaque and cream-coloured. Because of such observations, Duff further investigated this phenomenon of dissociation into different colony types. Subsequently, he discovered that dissociation could be induced by culturing the pathogen in nutrient broth with the addition of either 0.25 % lithium chloride or 0.1 % phenol. Use of this procedure gave rise to several distinct colony forms. One of these resembled the original stock culture, a second corresponded to the 'new' type, and a third was intermediate between the other two forms. The colonies resembling those of the original stock culture were described as opaque, strongly convex, cream-coloured and friable, whereas the new colony form appeared translucent, slightly convex, and a bluish-green in colour with a butyrous consistency. When the two different colony types were inoculated intraperitoneally into goldfish, the blue-green, translucent dissociant caused the deaths of the fish and was accompanied by lesions typical of the disease. In contrast, the original type of colony did not adversely affect fish, which survived for the 30-day duration of the experiment without any signs of illness. Thus, Duff concluded that the cream-opaque form which produced friable colonies was non-pathogenic, and more stable on prolonged storage. Duff designated this colony type as 'rough'. The 'smooth' form (i.e. the blue-green-translucent dissociant, which produced butyrous colonies on agar media) was pathogenic, but less stable in prolonged storage. In the subsequent study, Duff (1939) also reported the presence of an extra antigen in the rough strains. Although Duff (1937) was the first worker to report the ability of *Aer. salmonicida* to dissociate into several distinct colony types with differences in pathogenicity, a phenomenon which is now widely accepted, it is curious that he ascribed pathogenicity to the smooth colony type. This is in contrary to the view currently held that the rough colony type is, in fact, virulent. Interestingly, the Furunculosis Committee had also reported a variation in colony morphology among isolates (it may be assumed that these corresponded to the rough and smooth variants) but contended that this phenomenon was not accompanied by a difference in virulence. It is regrettable that this initial confusion over dissociation occurred, preventing an earlier realisation of its significance. In fact,

the relevance of dissociation of *Aer. salmonicida* colonies and the relationship to virulence was not made apparent until the work of Udey (1977), almost 40 years later. Early studies provided tentative evidence for a variety of possible pathogenic mechanisms, but there is no doubt that progress in the understanding of *Aer. salmonicida* pathogenesis and virulence has been accelerated by rapid advances in the knowledge of cell biology and the development of sophisticated biochemical techniques. It is the application of such techniques that continues to yield considerable new information about the manner in which *Aer. salmonicida* may effect its disease processes in fish.

Pathogenicity – The Value of Intraperitoneal Chambers

An intriguing and significant development concerned the description of intraperitoneal chambers, which could be implanted into fish (Garduño et al. 1993a). These chambers could be filled with pathogens – or for that matter a range of other objects -, implanted into fish, and measurements made with time. Garduño and colleagues placed *Aer. salmonicida* into a chamber, and studied its fate in the peritoneal cavity of rainbow trout. In one set of investigations, these workers observed that when the pathogen was contained in the chamber killing occurred rapidly as a result of host-derived lytic activity (in the peritoneal fluid). In contrast, free cells had a better chance of survival (Garduño et al. 1993a). Moreover, within the peritoneal chamber, *Aer. salmonicida* produced novel antigens, as determined by western blots (Thornton et al. 1993). In another publication using the peritoneal chamber, evidence was presented that the capsular layer around *Aer. salmonicida* permitted the pathogen to resist host-mediated bacteriolysis, phagocytosis and oxidative killing (Garduño et al. 1993b).

Pathogenicity – Cell-Associated Versus Extracellular Components

A variety of pathogenicity mechanisms and virulence factors have been proposed for diseases caused by *Aer. salmonicida,* namely possession of an extracellular (A) layer (= the surface or "S" layer), a type III secretion system (e.g. Dacanay et al. 2006) and the production of ECP, with the latter involved with the effects on macrophages (Ewart et al. 2008), although there is confusion and even contradiction about the relative merits of the various components in pathogenicity (see Ellis et al. 1988b). Yet, ironically fish may mount an antibody response during infection (Hamilton et al. 1986). Indeed, complement and non-a_2 m-antiprotease activity have been considered important host defence mechanisms against *Aer. salmonicida* (Marsden et al. 1996c).

Munro (1984) has grouped the virulence/pathogenicity factors into cell-associated and extracellular components, a division which is convenient for the purpose of this narrative. The best-studied cell-associated factor is the additional layer, external to the cell wall, termed the A-layer:

The A-Layer

The A-layer is now thought to be the product of a single chromosomal gene (Belland and Trust 1985), is produced *in vivo* (Ellis et al. 1997) and contributes to survival in macrophages (Daly et al. 1996). The virulence array protein gene A (*vapA*), which encodes the A-protein has been sequenced, and differences noted in the amino acids between typical and atypical isolates, with homogeneity among the former but heterogeneity with the latter. These differences undoubtedly lead to antigenic differences among atypical isolates (Lund and Mikkelsen 2004). First reported by Udey and Fryer (1978), and resulting from detailed electron microscopic studies, the A-layer was determined to be correlated with virulence (e.g. Madetoja et al. 2003); an insulin-binding capacity for the A-layer has been documented (Nisr et al. 2012). It was observed that virulent strains possessed the A-layer, whereas avirulent isolates did not. In addition, the presence of the A-layer was found to correspond with strong auto-agglutinating properties of the organism, and to the adhesion to fish tissue culture cells. The auto-agglutination trait has been found to be influenced by temperature, with weak and strong auto-agglutination at 25 and 15–20 °C, respectively (Moki et al. 1995). The presence of the A-layer may confer protection against phagocytosis and thus destruction by macrophages (Olivier et al. 1986; Graham et al. 1988). Essentially, these workers noted that avirulent cells, i.e. those without an A-layer, were phagocytosed and destroyed when virulent cells with A-layer were more resistant. Moreover, the bacteriocidal activity of macrophages was stimulated by prior exposure to low doses of *Ren. salmoninarum,* but inhibited by high amounts of living or dead renibacterial cells or the p57 antigen (Siegel and Congleton 1997). Interestingly, it was deduced that living and formalised virulent cells, in the absence of serum, attracted macrophages more readily than avirulent cells after a period of 90 min (Weeks-Perkins and Ellis 1995). The surface layer may inhibit growth at 30 °C, enhance cell filamentation at 37 °C, and enhance uptake of the hydrophobic antibiotics streptonigrin and chloramphenicol (Garduño et al. 1994). Following the intravenous injection of purified A-layer protein into Atlantic salmon, the protein located to the epithelial cells in renal proximal tubules of the head kidney (Stensvåg et al. 1999).

For its formation, Belland and Trust (1985) reasoned that the A-layer subunits pass though the periplasm and across the outer membrane for assembly on the cell surface. A requirement for the presence of O-polysaccharide chains, for which the AbcA protein is involved in biosynthesis (Noonan and Trust 1995) on the LPS was reported as necessary for the assembly of A-layer (Dooley et al. 1989). These virulent, auto-agglutinating forms produce characteristic deep blue colonies on CBB agar (Wilson and Horne 1986; Bernoth 1990). Sakai (1986a, b) postulated that a possible mechanism for auto-agglutination and adhesion could be attributed to the presence of net negative electrical charge in the interiors or on the surfaces of cells. In particular, pathogenic cultures were highly adhesive (Sakai 1987). It should be emphasised that Udey and Fryer (1978) determined that strains maintained for long periods in laboratory conditions were not auto-agglutinating, and demonstrated reduced virulence. Conversely, it was observed that fresh isolates, obtained from

epizootics, were of the aggregating type. From the results of experiments, Udey and Fryer (1978) concluded that the presence of the A-layer was necessary for virulence. However, they contended that more work was needed to establish whether or not the A-layer alone could confer virulence. The discovery of the A-layer generated much interest, resulting in further study of its chemical composition and its specific role in fish pathology. Kay et al. (1981) succeeded in purifying the A-layer from virulent isolates, and concluded that it was composed of a surface-localised protein with a molecular weight of 49 kDa. Phipps et al. (1983) continued with work on purification and characterisation of the substance, determining that it was hydrophobic in nature, present on the entire cell surface, did not possess any enzymic activity, but instead constituted a macromolecular refractive protein barrier which was essential for virulence. Meanwhile, an independent parallel investigation of Evenberg et al. (1982) highlighted the relationship between auto-agglutination and the presence of the A-layer. This group examined the cell envelope protein patterns of a variety of isolates obtained from a wide range of geographical locations and different fish species (i.e. carp, minnow, goldfish and salmonids). These fish were suffering from either furunculosis, CE or ulcer disease. A major protein (molecular weight = 54 kDa) was found in all auto-aggregating strains, but little or no trace occurred in isolates that were not auto-agglutinating. When examinations for the presence of the protein were carried out after a change of growth medium, i.e. replacement of horse serum by synthetic sea salt, it was observed that an almost complete loss of the additional cell envelope and the auto-agglutinating ability of the isolate had occurred. Using gel immunoradio assays, it was also determined that the extra cell envelope proteins of all the isolates, irrespective of fish host, type of infection or geographical source, were immunologically related. Evenberg and Lugtenberg (1982) pursued this topic, and described the protein as water insoluble with an amino acid composition similar to those of the additional surface layers of other bacteria, e.g. the adhesive K88 fimbriae of enteropathogenic strains of *Esch. coli*. It is particularly relevant that the findings of Evenberg et al. (1982), concerning the auto-agglutinating ability of 'atypical' strains from cases of CE and ulcer disease, and the presence of the A-layer, were in excellent agreement with the work of Trust et al. (1980b, c) and Hamilton et al. (1981). These earlier studies deduced the presence of an outer layer protein, which was estimated to have a molecular weight of 50 kDa. Evidence was provided by Ishiguro et al. (1981) that loss of the A-layer and loss of auto-agglutinating properties resulted in decreased virulence. After examining the effects of temperatures on the growth of *Aer. salmonicida,* it was shown that in cells, cultured at 30 °C (the generally accepted upper limit for the organism), virulence was restricted to <10 % of the population. The avirulent attenuated cells that resulted from use of the higher growth temperature, did not auto-agglutinate and, for that matter, did not possess the A-layer. It is interesting to note that higher maximum growth temperatures were recorded for the attenuated strains, in comparison to their virulent counterparts. Perhaps, this is explained by their selection at high temperatures. Because of this observation, Ishiguro et al. (1981) hypothesised that the A-layer is important in determining physical properties of the cell envelope, and that these properties undergo a change when the A-layer is lost,

permitting growth at higher than normal temperatures. If the A-layer is a prerequisite for virulence, it may be assumed that its presence confers advantages on the bacterial cell in its role as a pathogen. Indeed, several prime functions for the A-layer have been proposed. Thus, evidence exists that the extracellular layer protects *Aer. salmonicida* cells from the action of protease (Kay and Trust 1991) and bacteriophage, by shielding its phage receptors (Ishiguro et al. 1981). In addition, the layer may protect the cell from serum complement, insofar as Munn and Trust (1984) demonstrated that virulent strains (with A-layer) were resistant to complement bacteriocidal activity in the presence (and indeed absence) of specific antibody in rainbow trout serum. Other investigations have revealed that hydrophobicity is conferred upon the bacterial surface by the A-layer (Trust et al. 1983; Van Alstine et al. 1986). These workers reported that the hydrophobic A-layer provided *Aer. salmonicida* cells with an affinity for fatty acid esters of polyethylene glycol and an enhanced ability to associate with rainbow trout and mouse phagocytic monocytes (macrophages), in the absence of opsonising antibody. Although Trust et al. (1983) conceded that the advantages to the pathogen of the increased association with macrophages remained to be determined, they suggested as a tentative explanation, the possibility that *Aer. salmonicida* is a facultative intracellular pathogen able to survive within phagocytes. Indeed, Munn and Trust (1984) demonstrated that A-layer$^+$ bacteria (i.e. bacteria with A-layer) were able to multiply within the principal phagocytic organs, e.g. the spleen, following experimental infection. Subsequently, it has become established that *Aer. salmonicida* is capable of internalization and replication in macrophages (Ewart et al. 2008), where the pathogen is presumed to be able to resist reactive radicals (Garduño et al. 1997). It has been argued that the surface layer constitutes the first line of defence for *Aer. salmonicida,* with an inducible catalase and manganese superoxide dismutase as second defensive systems against macrophage-mediated killing via reactive oxygen species.

The A-layer has also been implicated in a role concerning adhesion to fish tissues. By means of *in vitro* experiments, Parker and Munn (1985) examined the ability of avirulent (A-layer$^-$) cells to adhere to cells of baby hamster kidney and rainbow trout gonad in tissue culture. Attachment of A-layer$^+$ *Aer. salmonicida* to both types of cells was greater than for the A-layer$^-$ derivative. As a result, Parker and Munn (1985) proposed that since attachment to epithelial cells may be the primary step in the pathogenic process, their observations could account for the association of virulence with the presence of an extra outer membrane layer.

Another function of the A-layer is a possible interference with the antibacterial peptides, namely magainin, cecropins and defensins (Henry and Secombes 2000).

To summarise, the accumulating body of evidence indicts the A-layer as a principal virulence determinant, even though its precise functions and the mechanism of action obviously require further clarification. However, blithe acceptance of an absolute relationship between virulence and possession of an A-layer must unfortunately be cautioned against. This is in view of reports by Johnson et al. (1985) and Ward et al. (1985) on the occurrence of virulent, auto-agglutinating strains that have no detectable A-layer. Conversely, Olivier (1990) recovered non-virulent A-layer$^+$ isolates. Thus, the association between presence of the extracellular layer and virulence, but not between auto-agglutination and virulence, appears to be open to ques-

tion. It is important that the extent of this problem should be determined, particularly because the use of A-protein as an antigenic component of a potential vaccine for control of diseases caused by *Aer. salmonicida* has been advocated. This is due to the apparent immunological relatedness of the A-protein among isolates from different locations and a variety of fish hosts (Evenberg et al. 1982). In view of the existence of virulent, auto-agglutinating *Aer. salmonicida* strains apparently lacking the A-layer, the effectiveness of such a vaccine would possibly be subject to severe limitations.

Type I Pilus System

Using knockout mutants, it was concluded that the type I pilus system was not important for invasion but was for subsequent colonization (Dacanay et al. 2010).

Type III Secretion System

A type III secretion system, which utilises a 140 kbp pAsa5 plasmid and chromosome-encoded transmembrane injection devise incorporating membrane proteins and a needle-like structure to translocate the effector protein AexT toxin from the cytosol into the host cell, has been linked to the virulence of *Aer. salmonicida* subsp. *salmonicida* and in particular the ability to achieve a systemic infection (Burr et al. 2002, 2003a, b, 2005; Stuber et al. 2003; Dacanay et al. 2006; Ebanks et al. 2006; Tanaka et al. 2012). In the case of one culture A449, the expression of the type III secretion system was temperature dependent, being active within 30 min at 28 °C especially followed by exposure to low levels of calcium but not 17 °C, which is more usual for the outbreak of disease (Ebanks et al. 2006). However, expression was induced at 16 °C in the presence of 0.19 to 0.38 M NaCl. A second effector protein, AopP, has been identified, and found to inhibit the NF-kB pathway downstream of 1kB kinase activation. The gene was found to encoded on a small ~6.4 kb plasmid (Fehr et al. 2006). The effector genes, *aexT;* this codes for ADP (ribosyltransferase; Burr et al. 2003a, b)*, oapH* and *aopO,* and *ascC,* which is the gene encoding the outer membrane pore of the secretion system, were inactivated by deletion and the effects examined in Atlantic salmon. The outcome was that the Δ *ascC* mutant was not virulent. However, i.p. injection of Δ *aexT,* Δ aopH *and* Δ *aopO* resulted in disease, which was regarded as being indistinguishable from the parental wild-type culture. The conclusion was that whereas the type III secretion system was essential for virulence, the individual effectors were less significant for virulence but were for colonisation (Dacanay et al. 2006).

Type IV Pilin

Type IV pili are regarded as important virulent determinants among Gram-negative bacterial pathogens, participating as adhesins. A four gene cluster, *tapABCD*, from a virulent *Aer. salmonicida* has been found to encode proteins with homology to those necessary for biogenesis of type IV pili (Masada et al. 2002). *TapA*, which was regarded as ubiquitous among *Aer. salmonicida* isolates, encoded a protein with homology to type IV pilin subunits in common with other Gram-negative bacterial pathogens, e.g. *Aer. hydrophila* and *V. vulnificus.* A mutant *Aer. salmonicida* defective in *tap*A was less pathogenic to rainbow trout following i.p. injection. *Tap*B is part of the ABC-transporter family with nucleotide-binding regions; *Tap*C homologues are cytoplasmic membrane proteins that exert a role in the anchoring and/or assembly of pili; *Tap*D has homology with type IV prepilin leader peptidases (Masada et al. 2002). *Tap*D was capable of restoring type IV pilin assembly and type II extracellular protein secretion (albeit in *Ps. aeruginosa*) and was presumed to have a similar function in *Aer. salmonicida* (Masada et al. 2002).

Outer Membrane Proteins

In many Gram-negative bacterial pathogens, OMPs exert an important role in virulence. A proteomic analysis of *Aer. salmonicida* identified 76 unique proteins including the dominant S-layer Vap protein, >10 porins, phosphoglycerate kinase, enolase and receptors involved in nutrient acquisition (Ebanks et al. 2005).

Capsules

Capsular polysaccharides have been found to develop around cells of *Aer. salmonicida* in the presence of glucose, phosphate, magnesium chloride and/or trace elements. Production of this material was improved in the presence of yeast extract (Bonet et al. 1993). Interestingly, a striking difference in cells grown *in vitro* or *in vivo* reflected the presence or absence of capsules. Using intraperitoneal chambers, it was observed that *Aer. salmonicida* produced capsules with virulence functions (Garduño and Kay 1995). Adherence to fish cell cultures was slightly higher in cultures of *Aer. salmonicida* grown in conditions to promote capsule formation. Also, invasion of fish cells was more pronounced in the capsulated cells (Merino et al. 1996). Another role for the capsule concerns resistance to complement. Thus, it was recorded that when grown under conditions promoting capsule development, *Aer. salmonicida* were partially resistant to complement (Merino et al. 1997).

Agglutination of Fish Cells

Another cell-associated factor, possibly relevant to virulence of *Aer. salmonicida,* is the ability of the pathogen to agglutinate trout and mammalian erythrocytes (Mellergaard and Larsen 1981). The haemagglutination capability is proported to be related to the presence of adhesins, which are structures on the bacterial surface that mediate the attachment of the pathogen to the host's cell surface. Thus, the interest in haemagglutination is due primarily to its use to provide semi-quantitative information on the adhesive potential of a bacterial strain, while the sugar inhibition of haemagglutination has allowed adhesive specificity to be demonstrated (Duguid and Old 1980). There is good agreement in the literature on the haemagglutinating ability of *Aer. salmonicida.* For instance, Jiwa (1983) reported mannose-resistant agglutination of bovine, chicken, human group A and guinea-pig erythrocytes by two *Aer. salmonicida* isolates which were recovered from diseased brown trout. It has also been demonstrated that smooth strains were unable to agglutinate erythrocytes, whereas rough strains showed a broad spectrum of haemagglutinating activity. Similarly, Parker and Munn (1985) observed that virulent, auto-agglutinating A-layer$^+$ cells agglutinated trout erythrocytes as well as a range of mammalian erythrocytes. They also noted that the process was not inhibited by specific sugars, and thus concluded that adhesion was a relatively non-specific process, attributable to the hydrophobic properties of the A-layer.

Extracellular Products

Researchers interested in the biology of *Aer. salmonicida* have been aware for some time that extracellular substances (produced by the organism) presumably exerted a role in virulence. However, ECPs are not always harmful to fish. For example, Madetoja et al. (2003) reported that ECPs, which lacked caseinase and gelatinase and had low cytotoxic activity in cell culture, from an atypical strain isolated from Arctic charr did not cause mortalities. Nevertheless, the ECP have been the focus of numerous investigations. Unfortunately, the work has been rendered more difficult by the complexity of the substance(s), which, at present, is known to include an ADP-ribosyltransferase toxin (AexT) (Braun et al. 2002), acetylcholinesterase (an ichthyotoxin with neurotoxic activity; Pérez et al. 1998), several proteases namely two metalloproteases, i.e. the 37 kDa leucine aminopeptidase and the 30 kDa metalloprotease 3 (Arnesen and Eggset 1999), P1, GCAT, metalloendopeptidase AsaP1, P2 metallo-gelatinase and a serine caseinase (Gudmundsdóttir and Gudmundsdóttir 1997; Schwenteit et al. 2015), phospholipase, haemolysins and a leucocidin (Munro et al. 1980; Sheeran and Smith 1981; Shieh and MacLean 1975; Titball and Munn 1981; Cipriano et al. 1981; Fuller et al. 1977; Rockey et al. 1988; Huntly et al. 1992; Lygren et al. 1998), as well as LPS. Work with monoclonal antibodies has shown heterogeneity in the LPS (Rockey et al. 1991). Ellis et al. (1981) reported that ECP of the pathogen, prepared by a cellophane overlay method, reproduced the lesions normally associated with the chronic form of furunculosis, e.g. muscle necrosis and

oedematous swelling at the site of injection. This suggests that the toxins and aggressins released by the bacteria *in vivo* are responsible for much of the pathology of the disease. In addition, when injected intraperitoneally into rainbow trout, the ECP proved to be fatal for the fish (Munro et al. 1980). In preparations of fish cells, the ECP exhibited cytotoxic effects, and at higher concentrations was leucocytolytic and haemolytic. These investigators concluded that most of the virulence factors were produced extracellularly, with most strains of *Aer. salmonicida* producing similar compounds, although the quantities varied. However, a detailed chemical analysis was not carried out.

Other studies have also indicated that injection of ECP closely reproduce the pathological condition attributed to furunculosis (Sakai 1977; Cipriano et al. 1981). Cipriano et al. (1981) attempted to determine the role between ECP and virulence by extracting the compounds from culture supernatants. The ECP was resolved into four fractions by ion-exchange chromatography. It was deduced that fraction II possessed leucocytolytic activity, although this fraction was not associated with virulence. Rather, a link between virulence and the toxicity of crude material, and fractions II and III, to cultured rainbow trout was observed. In this experiment, the extracted material from virulent isolates was more toxic to tissue culture cells than preparations derived from the avirulent strains. Fraction II also demonstrated proteolytic activity. Furthermore, results of *in vivo* toxicity studies revealed that three of the fractions were toxic to fish, although their activities varied according to the nature of the fish species used. Thus, mortalities, accompanied by haemorrhaging at the vent and fins, and inflammation at the site of injection, occurred in Atlantic salmon and brook trout that received fractions I and II. Fraction III also caused haemorrhaging at the base of the fins, injection site, and in the mouth; however, the majority of fish administered with this fraction survived. In contrast, rainbow trout were relatively resistant to the effects of all four fractions, insofar as no mortalities resulted. However, administration of fraction II resulted in the development of characteristic furuncle-like lesions at the inoculation site. Fractions I, III and IV did not cause any obvious pathology. The results of Cipriano et al. (1981) supported the previous findings of Sakai (1977) who, on the basis of work with crude ECP preparations, considered that a protease was the most pathogenic substance produced by *Aer. salmonicida.* The effects ascribed to proteolytic activity by Sakai (1977) were analogous to those noted by Cipriano et al. (1981) for fraction II. In fact, the muscle necrosis and degeneration of connective tissue associated with furunculosis indicates proteolytic enzyme activity. Yet, Fyfe et al. (1986) recorded that protease preparations were less effective than equivalent amounts of ECP (with similar amount of proteolytic activity) at causing lesions, i.e. furuncles, following i.m. injection of juvenile Atlantic salmon. This team identified three major components with molecular weights of 70 kDa (a serine protease; Ellis et al. 1997), 56 kDa (a haemolysin) and 100 kDa (unidentified protein) in the ECP (Fyfe et al. 1987a); the first mentioned of which was produced in greater quantities after incubation for 18 h at 25 °C compared to 125 h at 10 °C (Fyfe et al. 1987b). Haemolysin production was similar at both temperatures, but ten-fold more of the 100 kDa protein was produced at the lower temperature.

Proteases, as prime candidates for exerting a significant role in disease pathogenesis, have aroused substantial interest as a research topic. Indeed, a variety of investigators have performed detailed analyses, and suggested heterogeneity among isolates. In particular, Gudmundsdóttir (1996) described 6 protease groups, but this information might have greater value for taxonomy than an understanding of pathogenicity. Thus by examining 5 typical and 25 atypical isolates, it was determined that the proteases produced by the type strains of *Aer. salmonicida* subsp. *achromogenes* (this produced a metalloendopeptidase = AsaP1; Schwenteit et al. 2015) and *Aer. salmonicida* subsp. *salmonicida* were different to those of the fresh isolates. Moreover, all the typical isolates belonged to one protease group with proteolytic activities comparable to P1 and P2 proteases, whereas the atypical cultures were different. With the exception of three atypical oxidase-negative isolates, which secreted a protease reminiscent of P1, the others produced metallo-gelatinase. Ten of the atypical isolates produced AsaP1 (Gudmundsdóttir 1996).

Shieh and MacLean (1975) purified a proteolytic enzyme, which was determined to have a molecular weight of 11 kDa, and an optimum pH range of 8–11. Because the enzyme was inhibited by PMSF, these workers concluded that it was a serine protease. Mellergaard (1983) also isolated and purified a proteolytic enzyme (molecular weight = 87.5 kDa; optimum pH of 9.0), as did Tajima et al. (1984), who reported the presence of an extracellular protease with a molecular weight of 71 kDa, and a pH range of 5–10. This enzyme was deduced to be an alkaline serine protease. Sheeran et al. (1984) described two extracellular proteolytic activities that differed in their susceptibility to inhibitors and substrate specificity. One of the enzymes, designated P1, hydrolysed casein, elastin and gelatin, and showed a low non-specific activity against collagen. The second enzyme (P2) hydrolysed collagen and gelatin, but not casein or elastin; a pattern that suggested it is a specific collagenase. Also, Rockey et al. (1988) described two proteases, coined P1 and P2, and a haemolysin (T-lysin) in the ECP. P1 and T-lysin were shown to work separately in the complete lysis of (rainbow trout) erythrocytes. T-lysin interacted with the outer membrane of the erythrocytes, whereas P1 destroyed the nuclear membrane. A role for P2 was not described. Hastings and Ellis (1985) recorded differences in the pattern of extracellular protein production, according to the origin of the bacterial isolates. For example, isolates from Iceland (achromogenic) and the USA lacked caseinase and gelatinase activity in the ECP. Indeed, an isolate of *Aer. salmonicida* subsp. *achromogenes* from Iceland has been credited with the production of a novel metallo-protease (Gudmundsdóttir et al. 1990). For other strains, caseinase and gelatinase activities were inhibited by PMSF and EDTA. These data suggested that both enzyme activities could be attributed to a single serine protease, which depends upon divalent cations for activity. An extracellular metallo-caseinase, AsaP1, has been linked with lethal toxicity of atypical *Aer. salmonicida* in Atlantic salmon, with furuncles being produced by ECP with AsaP1 (Gunnlaugsdóttir and Gudmundsdóttir 1997).

Using the "P1" and "P2" terminology, Lygren et al. (1998) discussed differences in protease secretion according to the age of the culture of *Aer. salmonicida* subsp. *salmonicida.* Essentially, two different proteolytic activities were found in early and

late exponential phase of growth. The P2 activity, found in culture supernatants in the early exponential phase was described as a metalloprotease (molecular weight = 30–40 kDa) with activity against casein and gelatin. This caseinase activity was regarded as novel for metalloproteases of *Aer. salmonicida* subsp. *salmonicida.* The second and major protease, P1, appeared in the culture supernatant during late exponential phase of growth. This protease was regarded as identical to the 70 kDa serine protease (Lygren et al. 1998).

Intramuscular injection of the proteases into brown trout resulted in the development of gross symptoms similar to those occurring in natural outbreaks of furunculosis, i.e. muscle liquefaction along the flanks adjacent to the lesion and swelling at the site of injection (Sheeran et al. 1984). From these data, Sheeran and co-workers concluded that their observations differed from those of other workers. They stated that the absence of significant mortalities, or haemorrhaging of the fins and anus in the experimentally infected fish contrasted with the reports of others, suggesting that, in previous work, enzyme preparations may have contained toxic material other than proteases. Alternatively, it seems possible that Sheeran used insufficient quantities to achieve the pathological changes in question. However, the latter probability seems to be unlikely insofar as the levels of P1 enzyme injected by Sheeran et al. (1984) were equivalent to those of Sakai (1978), who reported furuncle formation, haemorrhaging and mortalities in kokanee salmon (*Oncorhynchus nerka*) following administration via i.m. injection. Further evidence for the role of protease production in the pathogenesis of furunculosis was provided by Sakai (1977), who reported a reduction in virulence for a proteolytically deficient mutant strain of *Aer. salmonicida* compared with its isogenic wild-type. In his later article, Sakai (1985) considered a role for proteases in reproduction of the pathogen by making available small peptides and amino acids from proteolysis. Of course, this would considerably benefit the nutrition of the pathogen. It should be emphasized that serum from salmonids is capable of neutralizing lethal doses of proteases (Ellis et al. 1981), possibly through the action of an a-migrating antiprotease (Grisley et al. 1984). Nevertheless, it is apparent that proteases play an important role in the pathogenicity process. Indeed, a serine protease, which was reported to have a molecular weight of 64 kDa, suppressed the immune response of Atlantic salmon (Hussain et al. 2000). A complication concerns the recovery of a pathogenic non-protease secreting strain (Tajima et al. 1987). Therefore, much work is still required to clarify the precise mode of action of proteases in the pathogenic process.

The greater susceptibility of brown trout (compared to rainbow trout) to furunculosis has long been recognised (e.g. McCarthy 1975a, b). An explanation for this difference has been provided by Ellis and Stapleton (1988), who found that at low ratios of exotoxin to serum, brown trout serum considerably enhanced *Aer. salmonicida* proteolytic activity. Yet at similar ratios, rainbow trout serum demonstrated some inhibition of the bacterial protease activity. The interpretation of these data is that during the initial stages of infection, *Aer. salmonicida* would have greater potential to multiply in brown trout rather than rainbow trout. Furthermore, Rockey et al. (1988) determined that serum from rainbow trout protected the erythrocytes from the haemolysins of *Aer. salmonicida.*

A LPS-free phospholipase has also been recovered from the ECP, and demonstrated to cause disease signs, upon injection, into Atlantic salmon (Wong et al. 1989; Huntly et al. 1992). Death resulted overnight following injection of 10 μg/g body weight of fish. Disease signs included lethargy, melanosis, and other defects characteristic of furunculosis. In addition, erythema was noted on the undersurfaces, particularly around the vent, at the bases of the pectoral and pelvic fins, and head (Huntly et al. 1992). Erythrocyte membranes were degraded (= haemolytic activity). It was concluded that this phospholipase exhibited GCAT activity.

Production of haemolysins by *Aer. salmonicida* may also contribute to the pathogenesis of furunculosis, insofar as it has been established that ECP contain components with pronounced haemolytic activity for trout erythrocytes (Munro et al. 1980). Titball and Munn (1981) carried out the first extensive study of haemolysin production by *Aer. salmonicida.* These authors reported the existence of two distinct haemolytic activities. Essentially, they determined that the supernatant from unshaken broth cultures contained haemolytic activity against erythrocytes from a diverse range of vertebrate species, with maximal activity against horse red blood cells. Titball and Munn termed this 'H' activity. If cultures were shaken, however, the resulting supernatant yielded an activity against trout erythrocytes only (this was designated the 'T' activity). Furthermore, the H lysin was reported as unstable in culture supernatants, sensitive to heat after exposure to 56 °C for 5 min, and became membrane bound when solutions were filtered. In contrast, the T lysin was stable in supernatants, and was inactivated by normal rainbow trout serum. Nomura and Saito (1982) also studied the extracellular haemolytic toxin, which was recorded as cytotoxic for sheep and salmonid erythrocytes. These investigators observed that the production of haemolysin was stimulated by the addition of enzymic hydrolysates of protein, but suppressed by carbohydrates, such as glucose or sucrose. Moreover, bivalent metal ions, e.g. Ca^{2+}, Co^{2+} and Mn^{2+}, and phosphate ion ($(HPO_4)^{2-}$) were necessary for production of the haemolysin. The optimum pH range and optimum temperature for toxin production was 7.5–8.0 and 20 °C, respectively. Nomura and Saito (1982) concluded that the haemolysin was produced during the stationary phase of the growth cycle, and was relatively heat labile, being inactivated at 60 °C. These observations coincided with those of Titball and Munn (1981).

In continued studies of the T and H lysins, Titball and Munn (1983, 1985a) purified the components, and examined properties of the haemolytic activity. Thus the T lysin activity was separated into two factors, namely a caseinase and another, apparently membrane-associated (T_1) activity, which by itself caused only incomplete lysis. In fact, complete lysis of trout erythrocytes occurred only in the presence of both T_1 activity and the caseinase (also see Rockey et al. 1988). Titball and Munn (1983) believed that this phenomenon was due to the co-operative effect of both activities on the red blood cell membrane, rather than the conversion of T_1 to T lysin by caseinase. This opinion was reached because the inhibition of caseinase resulted in the loss of complete lytic potential from supernatant fluids containing T lysin. Titball and Munn (1985a) regarded the H lysin to be a proteinaceous substance, on the basis of results of the ultraviolet absorption spectrum. Additionally, they

observed that the partially purified H lysin contained detectable levels of GCAT. This enzyme possesses some similarities to the H lysin, e.g. molecular weight of 23.2 and 25.9 kDa, respectively (Buckley et al. 1982). Yet, the molecular weights were much smaller than the 200 kDa size of "salmolysin", the haemolytic toxin described by Nomura et al. (1988). The ionic strengths needed for the elution of GCAT and H lysin from ion-exchange gels were similar. These factors may complicate the isolation of pure H lysin, assuming that GCAT and the haemolysins are separate entities, which appears to be the case. Thus, GCAT has not been reported to possess haemolytic activity, and is stable at room temperature (Buckley et al. 1982). Membrane filtration of the preparation failed to remove GCAT, whereas H lysin activity was lost after the procedure (Titball and Munn 1981). Other observations of H lysin activity have indicated that haemolysis of horse erythrocytes occurs in two steps, namely a first stage in which there is no detectable cell lysis (this was termed the pre-lytic stage), followed by a second phase involving haemoglobin release and disruption of the cell membrane. Binding of the H lysin to the erythrocytes during the pre-lytic stage does not occur. Together with the observation of an optimum temperature of 25–33 °C for lysis, this suggests that the H lysin has enzymic action on the erythrocyte membrane. Nevertheless, fish injected with H lysin appeared to be unaffected, despite an apparent toxicity to rainbow trout gonad tissue cell lines. Titball and Munn (1981) concluded that the failure of H lysin to elicit a response in the fish experiments was explained by the use of an unsuitable route of administration or the injection of too low a quantity of the material. In addition, these authors argued that possibly H lysin is non-toxic to fish, with no important role in the pathological process.

Several investigators have explained the relationship of haemolysins and proteases to virulence by using different strains of *Aer. salmonicida.* For example, Hackett et al. (1984) studied the possibility of a plasmid-encoded origin for these extracellular enzymes. Significantly, the team concluded that the loss of proteolytic and haemolytic activity in variants of wild-type *Aer. salmonicida,* obtained by treatment with ethidium bromide, did not correlate with loss of plasmid DNA. Moreover, there was no apparent change in the LD_{100} between the virulent wild-type strain and its protease-haemolysin deficient variant. This implied that the extracellular activities were not essential for virulence or, indeed, pathogenicity, at least with regard to the acute form of furunculosis in rainbow trout. Two clones derived from another virulent strain, one of which was negative for protease and haemolysin production whereas the second derivative was positive for these attributes, were avirulent (LD_{50} increased by greater than four orders of magnitude). This was an important observation, denoting that attenuation of a virulent strain occurred without loss of the A-layer, plasmids or extracellular proteolytic and haemolytic activities. For this reason, Hackett et al. (1984) concluded that virulence was attributable to other, as yet unknown, factors. Titball and Munn (1985b) also studied the effects of quantitative differences in virulence on the production of potential toxins by *Aer. salmonicida.* The release of ECP, i.e. proteases and haemolysins, by virulent strains and their avirulent attenuated derivatives (differing only in the presence or absence of the A-layer) could not be linked directly to virulence.

Insofar as no appreciable differences were recorded between the levels of ECP from virulent (possessing an A-layer) and avirulent (no A-layer) cells, it appears that these compounds are not virulence determinants.

Hastings and Ellis (1985) reported that there was a marked variation in the production of haemolysins and proteases among different strains of *Aer. salmonicida.* In their study, four isolates, recovered from Atlantic salmon in Scotland, produced caseinase and gelatinase. Both of these enzymes were inhibited by PMSF (a serine protease inhibitor) and to a lesser extent by EDTA (a divalent metal ion chelator). This finding indicated that both enzyme activities could be attributed to a single serine protease, which was dependent upon divalent cations for activity. In contrast, an achromogenic isolate that was obtained from Iceland did not produce detectable quantities of haemolysin or gelatinase. It is noteworthy that the caseinase from this isolate differed from that of the Scottish strains, insofar as it resembled a metalloprotease. Hastings and Ellis (1985) noted that this enzyme appeared to be unique to fish pathogens. It is not known, however, if other achromogenic strains of *Aer. salmonicida* share similar properties regarding their ECPs. Nevertheless, it is relevant to note that yet another strain, recovered from the USA, differed from the Scottish isolates insofar as it lacked both caseinase and gelatinase activity in the ECP. Moreover, its haemolysin production was notably lower. Hence, it seems that there is a marked variation in the nature of the precise components of the ECPs from different strains of the pathogen. Thus, there may be some variation in the modes of pathogenesis.

Yet another factor with a potential role in virulence and pathogenicity is the leucocytolytic component of the ECP. Although this component was recognised in the 1930s, many years passed before detailed study ensued. Thus, Klontz et al. (1966) reported leucopenia in rainbow trout, following injection of either viable cells or a saline soluble extract of the culture. On the basis of these results it was hypothesised that a leucocytolytic compound was responsible for the limited leucocyte activity observed in infected fish. This group did not comment, however, on the biochemistry of the leucocytolytic compound. Nevertheless, this aspect was examined by Fuller et al. (1977), who deduced that the compound was a glycoprotein, which was distinct from the endotoxin, i.e. LPS, as previously studied by Ross (1966), Anderson (1973) and Paterson and Fryer (1974a, b). The leucocytolytic factor was present in the supernatants from broth cultures; moreover, virulent strains produced more than the avirulent counterparts. Furthermore, the glycoprotein was cytolytic for leucocytes *in vivo,* and produced a pronounced leucopenia when injected intravenously into adult rainbow trout. In addition, the factor appeared to enhance pathogenicity, presumably by increasing susceptibility of the host. This opinion was reached after experiments in which small coho salmon were inoculated with the leucocytolytic compound in combination with live *Aer. salmonicida* (approximately a quantity sufficient to achieve a LD_{50}). The result was that 36/40 fish succumbed, in contrast to the death of only 14/40 animals injected with just the pathogen. So, the conclusion was reached that the glycoprotein constituted a virulence factor of *Aer. salmonicida.* However, the results of Cipriano et al. (1981) were not in accord with the findings of Fuller's team. Essentially, Cipriano and colleagues deduced that there was no

correlation between virulence and the leucocytolytic properties. Therefore according to Cipriano et al. (1981), leucocytolytic factors could not be considered as a principal virulence mechanism. Alternatively, the activity may not solely relate to the attack on leucocytes. Instead, Cipriano and colleagues suggested that the leucocytolytic factor, contained within fraction II of the ECP, contributed to virulence not only by way of its leucocytolytic properties, but also through its role as a generalised cytotoxin capable of generating pathological changes. Indeed, these investigators recorded that intramuscular injection of fraction II (produced by a virulent isolate) into brook trout caused haemorrhaging at the mouth, base of the fins and site of inoculation. Death occurred within 24 h. Such deleterious changes did not ensue in fish that received fraction II derived from an avirulent isolate.

It must be emphasised that the lack of a leucocytic response, apparent in the majority of salmonids with furunculosis, has not been substantiated in infections of coarse fish. In one example, a chronic leucocytosis was observed in goldfish (Mawdesley-Thomas 1969).

Ellis et al. (1981) outlined a hypothesis for the role of ECP in pathology. Moreover, they highlighted some of the difficulties involved in reaching a complete understanding of the pathogenic process. Importantly, they emphasised that in many respects furunculosis is an inconsistent disease, insofar as a variety of lesions have been associated with invasion of the aetiological agent. Yet, virtually none of the symptoms may be considered as unique to the disease (Wolke 1975). Consequently, it is hardly surprising that inconsistencies have resulted in conflicting opinions over the pathogenicity mechanisms. Ellis and co-workers formulated a tentative explanation for the lesions caused by *Aer. salmonicida.* Thus, they reported that nearly all of the lesions normally associated with the disease may be achieved by i.p. or i.m. injection of ECP. However, it would appear that artificially high doses are required to accomplish such lesions. Munro et al. (1980) suggested that the presence of an a-globulin in normal trout serum may have the ability to neutralise ECP activities. Indeed, other workers have confirmed such effects of fish serum on ECP. Rockey et al. (1989) published an article detailing the inhibition of haemolysin activity by salmonid serum. Sakai (1984) mentioned a decrease in, or absence of, mortality among rainbow trout that had received ECP first treated with large volumes of rainbow trout serum prior to injection. These results indicated involvement of complement in the detoxification of ECP. Continuing this theme, Grisley et al. (1984) reported the presence of an a-migrating protein (a possible homologue of mammalian a_2-macroglobulin) in normal rainbow trout serum. This protein apparently exerts a role in a non-specific defence function against microbial proteolytic toxins. Ellis and Grisley (1985) pursued the theme, concluding that normal trout serum inhibits ECP protease but neutralisation is effected by different antiproteases and less efficiently than trypsin. They contended that the data, to some extent, explained the potency of ECP in causing disease. Ellis et al. (1981) assumed that in natural infections lesions would be produced after the ECP had exhausted any inhibiting factors, either locally or systemically. They thought that the various symptoms of furunculosis were explained by the colonization of different host tissues by the pathogen. It was concluded that the pathological effects resulting from infection by

Aer. salmonicida were probably caused by the ECP released by the pathogen. Thus, the leucocytolytic component might act against leucocytes, eventually resulting in leucopenia, and preventing the destruction of the bacterial colonies, thus allowing microbes to be transmitted to other organs via the circulatory system where they may initiate the development of more colonies. It was further submitted by these authors that lesions and mortalities are due to the collagenolytic activity of the ECP (this is one of the notable features of furunculosis), with haemorrhaging resulting in the vicinity of bacterial colonization. Generalised circulatory failure could ensue if the ECP subsequently entered the circulatory system.

Just when the role of ECP and proteases was becoming clarified, some elegant work with deletion mutants caused a fundamental re-think. It was obvious that to be sure of the role of a specific component, eliminate the genes from the bacteria and determine the effect on the host. Using this approach, Vipond et al. (1998) confirmed that mutants lacking GCAT or serine protease (AspA) were not less virulent than the parental cell following i.p. of cohabitation challenge of Atlantic salmon.

Finally, it is appropriate to recall the words of Munn et al. (1982), who commented that the interrelationships of ECP suggest that the pathogen exerts its toxic effects *in vivo* by means of multiple factors that interact synergistically.

Scavenging for Iron

A current theme, which has prompted some excellent work, concerns the ability of *Aer. salmonicida* cells to successfully scavenge for iron in iron-limited conditions. These would be created in the host, and function as a defence mechanism against invasion by pathogens. Indeed, there is evidence that IROMP are produced *in vivo* (Ellis et al. 1997). Thus, free iron would be bound to proteins, such as transferrin, resulting in iron-restricted conditions in the host. Initially, Chart and Trust (1983) demonstrated that typical strains of *Aer. salmonicida* were capable of sequestering iron. Then, Kay et al. (1985) determined that the A-layer was implicated as a component of an iron-uptake mechanism. The conclusion was that the A-layer functioned as the initial stage of iron-uptake, being a binding site for porphyrins, i.e. haemin and protoporphyrin. The difference between typical and atypical isolates was reinforced by the conclusion that there was a fundamental difference in the mechanism of utilisation of non-haem bound sources of iron. Hirst et al. (1991) and Hirst and Ellis (1996) described an inducible siderophore (these are soluble low molecular weight iron-chelators)-dependent iron-chelating system in typical strains and an unidentified siderophore-independent system in atypical *Aer. salmonicida.* Among *Aer. salmonicida* subsp. *salmonicida* (17 isolates from Scotland and Spain were examined), the siderophore is regarded as homogeneous (Fernandez et al. 1998).

Six genes have been studied that showed similarity with haem uptake genes of other Gram-negative bacteria, and other genes of unknown function. Mutation of *hutB,* which encodes a periplasmic haemin-binding protein led to a marked effect on the ability of the pathogen to use haemin as a source of iron. Mutation of *hutB*,

which encodes the outer membrane haemin receptor, led to an initial reduction in the ability to grow on haemin as a sole source of iron, but after 24 h there was a recovery to that of the parental strain (Najimi et al. 2008).

In summary, a variety of cell-associated and extracellular factors have been investigated in order to determine their role in virulence and pathogenicity of diseases of *Aer. salmonicida* aetiology. Unfortunately, the overview that emerges for the current understanding of pathogenicity mechanisms is confused. Much of the evidence, about the various factors suspected to be involved with virulence, is contradictory, or is based solely upon *in vitro* studies. Thus, although the presence of an A-layer is firmly believed to be a primary determinant of virulence, reports of avirulent isolates with A-layer (Udey 1978) raises further questions. Conversely, ECP contain such a diverse array of different factors implicated with virulence and pathogenicity, that to pin-point the function of each *in vivo* has proved difficult. Hence, a definitive assessment of the role of the various haemolysins, proteases and leucocidins in the natural disease process still eludes us. However, it is conceded that substantial progress has been made in the isolation, purification and biochemical characterisation of the ECP. Moreover, strong evidence exists that the ECP are capable of eliciting a pathology reminiscent of the natural disease (Cipriano et al. 1981; Ellis et al. 1981). However, the interrelationships between the various subcomponents remain unclear. Thus, Cipriano et al. (1981) believed that the leucocytolytic and proteolytic activities were dual expressions of a component, i.e. the chromatographic fraction II; a notion which requires more information for confirmation. This group opined that the generalised cytotoxicity for rainbow trout gonad cell lines by ECP was a better indicator of virulence. There was, however, agreement with the suggestion of Sakai (1977) that proteases constituted the most pathogenic element of the ECP. Yet, it is apt to recall the warnings of Sheeran et al. (1984), who emphasised that it is vital to establish the levels of the proteolytic enzymes in naturally infected fish tissues. Until this can by done, even conclusions drawn from *in vivo* experiments remain speculative. Results from some investigations have demonstrated that there is some degree of variation in the quantities of potential virulence factors produced by different isolates of *Aer. salmonicida.* Thus, there may be some variation in the precise mode of pathogenesis (Hastings and Ellis 1985). Yet, Titball and Munn (1985b) did not find any appreciable differences in the levels of ECP between virulent and avirulent isolates. Nevertheless, these authors admitted that this did not exclude a role for the substances as aggressins, although it was paradoxical that a delayed release of ECP by A-layer$^+$ strains was observed. As a possible explanation, the disadvantage of late release of toxic material may be counterbalanced by the role of A-layer in conferring resistance to host defence mechanisms (Munn et al. 1982). In yet another comment, Hackett et al. (1984) proposed that in peracute or acute forms of furunculosis, virulence is independent of the presence of protease and haemolysin. Accordingly, these workers suggested that death of the fish may result from organ dysfunction, due principally to massive growth of the pathogen. Alternatively, it was speculated that there may be involvement by an as yet unidentified component of the ECP.

The Fate of *Aeromonas salmonicida* Following Infection

Workers have addressed the questions concerning the fate of *Aer. salmonicida* after infection by various routes. Some of this information is discussed elsewhere. Using radio-active methods, Svendsen et al. (1999) published evidence that following infection by immersion, the pathogen could be readily found around surface wounds (the Atlantic salmon had been artificially wounded prior to use), the gills and hindgut (radioactivity increased here from 2 to 24 h). Two hours after challenge, bacteria were detected in the blood; at 24 h *Aer. salmonicida* was in the kidney but not the blood (Svendsen et al. 1999). It is clear from recent work that the pathogen is able to translocate across the salmonid intestinal epithelium. Using modified Ussing chambers and intestinal segments with fluorescein-isothiocyanate labeling and fluoremetry, Jutfeldt et al. (2008) considered the role for viable and heat-inactivated cells, ECP and LPS, determining that live cells translocated better than inactivated cells.

Disease Control

Disease Resistant Fish Embody and Hayford (1925) increased resistance in brook trout to furunculosis by selective breeding. Subsequently, Wolf (1954) reported the start of an investigation aimed at developing ulcer disease and furunculosis-resistant strains of brook trout and brown trout. Five years later, Snieszko et al. (1959) concluded that disease was, indeed, genetically determined. Ehlinger (1964, 1977) echoed this opinion by determining resistance to furunculosis in the progeny of brook trout. Thereafter, a substantial leap forward in knowledge occurred following a publication by Cipriano (1982c), who reported varying degrees of resistance to furunculosis among 11 different strains of rainbow trout, and correlated this with the serum neutralisation titre. Cipriano determined that the McConnaughy strain was the most susceptible, with 83 % of the animals dying within 14 days of challenge with 1.2×10^9 cells administered in a 1 min bath. The serum neutralisation titre was 1:80 against one of the extracellular fractions of *Aer. salmonicida.* In contrast, there was no mortality among the Wytheville strain, which demonstrated a serum neutralisation titre of 1:2560. Cipriano (1983) concluded that serum from rainbow trout (which are naturally resistant to furunculosis) could protect passively immunised brook trout from challenge with a virulent culture. In contrast, the administration of serum from susceptible Atlantic salmon was unsuccessful in conferring resistance upon brook trout. The protective effect of rainbow trout serum was believed to be attributed to the neutralisation of toxic components produced by the pathogen. Some unpublished data have pointed to the ability of certain strains of rainbow trout to tolerate the rigours of furunculosis. Genetic variation in susceptibility of Atlantic salmon has been examined in the study of one-year-old fish (Gjedrem and Gjoen 1995). The differential resistance of four turbot families to *Aer.*

salmonicida has been indicated, and may have significance for breeding disease resistant fish (Rodriguez-Ramilo et al. 2011).

Vaccine Development The development of an effective vaccine against the rigours of *Aer. salmonicida* infections remains one of the great challenges to researchers. Interest in vaccine development may be traced to the pioneering work of Duff (1942), who produced an orally administered, chloroform-inactivated whole-cell preparation. It is enigmatic that his reported success has not been surpassed, and indeed often not equaled, by subsequent workers. Unfortunately, efforts concerning vaccine development languished as chemotherapy became established as the principal means of disease control. Eventually, however, aforementioned resistance problems with chemotherapeutants led researchers to recognise the need for alternative control measures, and, thus, a resurgence of interest in vaccines ensued. However, it now appears that *Aer. salmonicida* is an inefficient antigen, in terms of its overall capability of stimulating a protective immune response (Tatner 1989). There is some controversy over the effectiveness of formulations based on ECP. Some studies indicate that they may well be immunosuppressive (Sövényi et al. 1990), whereas others describe their benefit in terms of immunogenicity (Kawahara et al. 1990). Notwithstanding, modern molecular techniques, principally the PCR, have demonstrated that vaccine antigens do get taken up into the body of fish, namely the head kidney and spleen (Høie et al. 1996).

Some of the problems associated with vaccine development have been summarised below. Essentially, the problems reflect economics, i.e. the perceived need for low-cost products on the part of the fish farmer, versus the desire for substantial profit margins on the part of the vaccine manufacturer/suppliers. Scientific problems exist due to an incomplete understanding of the biology of *Aer. salmonicida.* Specifically, progress has been hindered by the uncertainty surrounding the nature of the antigenic components of *Aer. salmonicida,* the effect of strain differences [n.b.: Gudmundsdóttir and Gudmundsdóttir 1997 while examining the cross protection of vaccines against typical and atypical isolates of *Aer. salmonicida,* concluded that the best protection resulted with autogenous products], and the lack of a consistent and reliable challenge method, although the latter has been improved by the development of effective cohabitation and bath methods (e.g. Bricknell 1995; Nordmo et al. 1998). Injectable vaccines based on microencapsulation with *V. anguillarum* LPS led to significantly higher oxygen consumption, lysozyme activity, specific growth rates and antibody titre to *Aer. salmonicida* in rainbow trout than fish which received inactivated whole cells with or without levamisole or emulsified oil as adjuvants, or microencapsulated with or without muramyl dipeptide or ß-1,3-glucan (Ackerman et al. 2000). Severe side effects have resulted from the i.p. injection of oil-adjuvanted vaccines, with the ECP component contributing to inflammation (Mutoloki et al. 2006) Intra-abdominal adhesions have been reported in Atlantic salmon following the i.p. injection of oil-adjuvanted vaccines (Gudmundsdóttir et al. 2003) with damage occurring from 10-weeks to 14-month after injection (Villumsen et al. 2015). Also, there is evidence of temporary immu-

nosuppression following the administration of some vaccines (Inglis et al. 1996). One solution to this problem has been the use of antibiotics, namely amoxicillin dosed at 0.1 ml containing 150 mg/fish, which are administered by injection with the vaccine (Inglis et al. 1996). However compared to the problems with salmon, rainbow trout have been successfully protected using a 5 min bath in a formalin-inactivated whole cell preparation with or without a booster (>93 % survival compared to 70 % survival of the controls 10-weeks later) (Villumsen and Raida 2013).

The precise composition of the vaccine is of critical importance. To date, scientists have evaluated inactivated whole cells (including those based on IROMP, inactivated L-forms, soluble extracts, attenuated live cells (such as those lacking A-layer and O-antigen; Thornton et al. 1994), inactivated cells supplemented with toxoids and/or purified sub-cellular components, immune serum (for passive immunisation) and polyvalent preparations, usually including inactivated whole cells of *Aer. salmonicida* and *Vibrio* spp. (e.g. Hoel et al. 1997). Most of the early formulations yielded poor or equivocal results (Table 5.3). The notable exceptions are passive immunisation and the use of attenuated live vaccines (Cipriano and Starliper 1982; Ellis et al. 1988a, b; Vaughan et al. 1993). The latter was particularly effective in Atlantic salmon, in which experimental use resulted in 12.5 % mortalities in the vaccinated group compared to 87.5 % mortality among control fish, after challenge with a virulent culture of *Aer. salmonicida.* A live aromatic-dependent *Aer. salmonicida* vaccine, *aroA* was administered intraperitoneally at 2×10^6 to 2×10^9 live bacteria/fish, resulting in a 253-fold increase in LD_{50} (Vaughan et al. 1993). This live vaccine stimulated T-cells rather than B-cell responses in rainbow trout (Marsden et al. 1996a). But how long did this live vaccine remain in fish tissues? The evidence revealed that following i.p. injection, the live vaccine became widely distributed throughout fish (in this case rainbow trout) tissues, with clearance taking 7–9 d at 16 °C. Lower temperatures led to more prolonged retention of the bacterial cells within the vaccinated fish (Marsden et al. 1996b).

The novel approaches of using IROMP and inactivated L-forms have met with success (Durbin et al. 1999; McIntosh and Austin 1993). Using formalin-inactivated cells of *Aer. salmonicida* subsp. *salmonicida* grown in iron-depleted conditions administered to rainbow trout intraperitoneally followed by an oral boost, antibodies were produced against OMP (maximum titre = 1:2560 at day 105) and IROMPs (maximum titre = 1:12,800 at day 105) and conferred protection (RPS = >80 %; Durbin et al. 1999). A complex formulation of atypical *Aer. salmonicida* cells (with, but not without. A-layer) grown in iron-deplete and iron-supplementation plus cells of *Aer. bestiarum* grown in TSB successfully protected goldfish against ulcer disease (RPS = >90 %) when administered by immersion (~5×10^7 cells/ml for 60 s) followed by oral boosting over 7 days after 28 days (5×10^7 cells/g of feed) (Robertson et al. 2005). Avirulent cells, with altered A-layer, have also been proposed as candidates for live vaccines (Thornton et al. 1991). However, a complication to the various developmental studies comes from the fascinating work of Norqvist et al. (1989), who used live attenuated cells of a different bacterial taxon, namely *V. salmonicida,* and reported their effectiveness at controlling infections by *Aer. salmonicida.*

Table 5.3 Vaccines for *A. salmonicida*

A. salmonicida strain used for vaccine and/or challenge	Nature of vaccine	Method of administration	Nature of challenge	Type of fish used	Water temp. (°C)	Ability of vaccine to protect fish	Ability of vaccine to induce antibody response	Reference
Whole cell virulent	Chloroform inactivated	Oral	Immersion/i.p./ co-habitation	Cutthroat trout (1–2 years)	19	+	+ (Average 1:80)	Duff (1942)
NG	Heat inactivated	Oral	i.p.	Brook trout	–	–	–	Snieszko and Friddle (1949)
NG	Heat inactivated	Oral	Natural	As above	–	+	–	As above
Ex-brook trout (vaccine)	Formalised	i.p.	i.p.	Brook and brown trout (0 to 2+ years)	11	–	+ (1:160)	Krantz et al. (1964b)
Ex-brook trout (vaccine)	Formalised + adjuvant	i.p.	i.p.	As above	11	+	+ (1:10520)	Krantz et al. (1964b)
ATCC 14174 + 6 strains from hatcheries	Formalised	Oral	Water-borne	Coho salmon (0 to 1+ years)	13	–	–	Spence et al. (1965)
As above	Formalised + FCA	i.p.	NG	Rainbow trout (2 to 4+ years)	12	ND	+ (1:5120)	Spence et al. (1965)
AS 67	Formalised + FCA	i.p.	ND	Coho salmon (1+ years)	13	ND	+ (1:20480)	Cisar and Fryer (1974)
Ex-salmon	Formalised + FCA	i.p.	i.p.	Coho salmon (juveniles)	12	+	+ (1:40960)	Paterson and Fryer (1974a)
SS-70 (virulent)	Formalised	Oral	Natural	Coho salmon (juveniles)	NG	–	–	Udey and Fryer (1978)
SS-70 (virulent)	Formalised + Al(OH)3	Oral	Natural	Coho salmon (juveniles)	NG	–	–	Udey and Fryer (1978)
S-70 (virulent)	Formalised + FCA	i.p.	Natural	Coho salmon (juveniles)	NG	+	+ (NG)	Udey and Fryer (1978)
36/75	Formalised	Oral	i.m.	Rainbow trout	15	–	–	Michel (1979)

36/75	Formalised	i.p.	i.m.	Rainbow trout	15	–	+ (1:80000)	Michel (1979)
FD-2-75	Formalised + FCA	i.p.	ND	Atlantic salmon (1+ years)	12-15	ND	+ (1:0–1:640)	Weber and Zwicker (1979)
A47R	Formalised + FCA	i.p.	Natural	Atlantic salmon (1+ years)	ND	-	+ (1:32)	Palmer and Smith (1980)
A47R	As above	h.i.	Natural	As above	ND	–	+ (1:16)	As above
A	Formalised	Oral	Natural	Brown trout (0+ years)	Ambient	+	+ (1:40)	Smith et al. (1980)
Virulent	Formalised	Oral	Natural	Brown trout (0+ years)	Ambient	+	+ (NG)	Austin and Rodgers (1981)
Virulent	Unwashed cells, formalised	i.p.	Water-borne	Brook trout (0+ years)	12.5	–	+ (1:205)	Cipriano (1982a)
Virulent	Washed cells, formalised	i.p.	Water-borne	Brook trout (0+ years)	12.5	–	+ (1:14)	Cipriano (1982a)
Avirulent	Attenuated, live	Immersion/dip	Water-borne	Brook trout, Atlantic salmon	12.5	+	+ (NG)	Cipriano and Starliper (1982)
Virulent	Formalised + FCA + Al(OH)3	i.p.	Immersion, injection, or co-habitation	Salmon	10–12	+	ND	McCarthy et al. (1983)
Avirulent	As above	i.p.	As above	Salmon	10–12	–	ND	As above
Virulent	Formalised	Immersion	Immersion	Chinook and coho salmon	8–18	+	ND	Johnson and Amend (1984)
Virulent	Formalised	i.p.	i.p.	Coho salmon (0+ years)	13–15	+	+ (1:5120)	Olivier et al. (1985a)
Avirulent	Formalised	i.p.	i.p.	As above	13–15	+/–	+ (1:2560)	As above
Virulent (MT004; = A-layer–)	Formalised + FCA or FIA	i.p.	Water-borne	Atlantic salmon, rainbow trout	NG	–	ND	Adams et al. (1988)
Virulent	Attenuated live cells of *V. anguillarum*	Immersion	i.p.	Rainbow trout	18	+	ND	Norqvist et al. (1989)

(continued)

Table 5.3 (continued)

A. salmonicida strain used for vaccine and/or challenge	Nature of vaccine	Method of administration	Nature of challenge	Type of fish used	Water temp. (°C)	Ability of vaccine to protect fish	Ability of vaccine to induce antibody response	Reference
Virulent (TG 36–75)	Culture superna- tant + purified antigen	i.p.	i.m.	Rainbow trout	15	–	+ (1:~844)	Michel et al. (1990)
MT 423	Formalised + mineral oil adjuvant	i.p.	i.p.	Atlantic salmon parr	9–15	+	+	Inglis et al. (1997)
S24-92, V341-95	Formalised autogenous + mineral oil adjuvant	i.p.	i.m.	Atlantic salmon fingerlings	10	+	ND	Gudmundsdóttir and Gudmundsdóttir (1997)
AL2017 (with A-Layer)	Formalised adjuvanted with Montanide	i.p.	Cohabitation	Atlantic salmon	12	+	+	Lund et al. (2003)
Atypical	Cell fractions + oil adjuvant	i.p.	i.p.	Spotted wolffish	12	+	?	Lund et al. (2003a)
Aer. hydrophila aroA	Live, mutant	i.p.	i.p.	Rainbow trout	16	+	+	Vivas et al. (2004a)
ORN2; ORN6	Formalised; IROMP + Iron supplemented	Immersion + oral boost	i.m.	Goldfish	17	+	+ (1:39=1:396)	Robertson et al. (2005)
Atypical Commercial, and autogenous	Contained in liposomes killed	Oral	Immersion	Carp	23	+	+	Irie et al. (2005)
		i.p.	Immersion	Turbot	15	+	+	Santos et al. (2005)

Disrupted cells/sub-cellular components								
Virulent	Water-soluble extract, toxoided with alum	oral	Natural	Coho salmon (juveniles)	Ambient	–	ND	Klontz and Anderson (1970)
Virulent (AS-Sil 67 to AS SS 70)	LPS endotoxin	i.p.	ND	Coho salmon (juveniles)	7–18	ND	+ (1:10000)	Paterson and Fryer (1974b)
NG	Toxoid	oral/i.p.	Natural	Coho salmon (juveniles)	Ambient	+/–	+ (1:16–1:2048)	Udey and Fryer (1978)
B	Diluted with SDS	h.i.	Natural	Brown trout	Ambient	+/–	+ (1:40)	Smith et al. (1980)
B	Dsrupted with ultra-sonication	h.i.	Natural	Brown trout	Ambient	+/–	ND	Smith et al. (1980)
Virulent	Toxoid, formalised	Oral	Natural	Brown trout (0+ years)	Ambient	–	–	Austin and Rodgers (1981)
Virulent	ECP, precipit-ated (NH4)2SO4 + ethanol	i.p.	Water-borne (juveniles)	Brook trout	12.5	+	+ (1:122)	Cipriano (1982a)
Virulent/avirulent	Lysed broth cult-ures + FCA + Al(OH)3	i.p.	Immersion	Salmon	10–18	+	ND	McCarthy et al. (1983)
Virulent	Toxoid, formal-ised and with chloroform	Oral	Natural	Rainbow trout	Ambient	–	ND	Rodgers and Austin (1985)
Virulent	ECP, precipit ated with (NH4)2SO4	i.p.	i.p.	Coho salmon (juveniles)	13–15	+/–	+ (1:1280)	Olivier et al. (1985a)
Avirulent	As above	i.p.	i.p.	As above	13–15	–	+ (1:1280)	As above
Avirulent	Protease	i.m.	i.m.	Atlantic salmon (0+ years)	NG	+	NG	Shieh (1985)

(continued)

Table 5.3 (continued)

A. salmonicida strain used for vaccine and/or challenge	Nature of vaccine	Method of administration	Nature of challenge	Type of fish used	Water temp. (°C)	Ability of vaccine to protect fish	Ability of vaccine to induce antibody response	Reference
Virulent (MT004, = A-layer−)	ECP, toxoided	Immersion	Water-borne	Atlantic salmon, rainbow trout	NG	+/−	ND	Adams et al. (1988)
265-87, M108-91, S24-92	Formalised ECP + FIA	i.p.	i.m.	Atlantic salmon fingerlings	10	+	+ (1:102,400)	Gudmundsdóttir and Gudmundsdóttir (1997)
Various	Heated or formalised + adjuvant	i.p.	Immersion (MT16)	Rainbow trout	13	NG	NG	Lutwyche et al. (1995)
Mixed	Polyvalent formalised + adjuvant	i.p.	Waterborne	Atlantic salmon	7–9	+	+	Hoel et al. (1997)
VI 88/09/03175	Formalised + adjuvant	i.p.	Cohabitation	Atlantic salmon	11	+	+ (1:20)	Hoel et al. (1998)
Avirulent	Formalised	i.p.	Immersion (MT 26)	Rainbow trout	13	+	+ (1:128)	Thornton et al. (1994)
Virulent	Monovalent + trivalent + adjuvant	i.p., oral + immersion	Cohabitation	Atlantic salmon pre-smolts	10	+	NG	Midtlyng et al. (1996)
NG	Formalised + mineral oil adjuvant	i.p.	Natural	Atlantic salmon pre-smolts	6–9	+	NG	Midtlyng et al. (1996)
Virulent (Linne, LL, S24, 256/91) Avirulent (AS 14)	Formalised L-forms	Immersion	i.p.	Atlantic salmon + rainbow trout	NG	+ (varying)	+ (0–1:256)	McIntosh and Austin (1993)

Compound vaccines/approaches								
Avirulent	Whole cells, chloroform inactivated + supernatant	Immersion	Natural	Brown trout	NG	+	ND	Cipriano (1983)
Virulent	Polyvalent whole cells, formalised + toxoid, formalin + chloroform inactivated	Oral	Natural	Rainbow trout (0+ years)	Ambient	–	ND	Rodgers and Austin (1985)
Virulent	Whole cells, formalised with A-layer + toxoid, formalin, chloro-form + lysine inactivated	oral	Natural	Rainbow trout (0+ years)	Ambient	+	ND	Rodgers and Austin (1985)
Virulent	Whole cells, formalised + A-layer	oral	Natural	Rainbow trout (0+ years)	Ambient	+/–	ND	Rodgers and Austin (1985)
Virulent, 3SA	Whole cells, formalised + toxoid + LPS +/– liposomes	immersion	Natural	Rainbow trout, fry	Ambient	+	ND	Rodgers (1990)
Derived from 644RB (Brivax II)	Formalised or sonicated	i.p.	–	Rainbow trout	16	+	+ (1:162,755)	Marsden et al. (1996a)
Various virulent	Cloned aromatic dependent mutant	i.p.	i.m.	Rainbow trout, Atlantic salmon	5–14	+	NG	Vaughan et al. (1993)

(continued)

Table 5.3 (continued)

A. salmonicida strain used for vaccine and/or challenge	Nature of vaccine	Method of administration	Nature of challenge	Type of fish used	Water temp. (°C)	Ability of vaccine to protect fish	Ability of vaccine to induce antibody response	Reference
Virulent	Formalised + therapy with oxolinic acid	i.p.	Natural	Atlantic salmon	NG	+	NG	Ford et al. (1998)
Passive immunisation								
Virulent	Immune serum	i.p.	Scarification/ water-borne	Coho salmon (0+ years)	NG	ND	NG	Spence et al. (1965)
Virulent	Immune serum – rainbow trout	i.p.	Water-borne	Brook trout	12.5	+	ND	Cipriano (1983)
virulent	Immune serum – Atlantic salmon	i.p.	Water-borne	Brook trout	12.5	–	ND	Cipriano (1983)
R, virulent strain	Immune serum – rainbow trout	i.p.	Water-borne	Sockeye salmon	10–18	+	+ (1:2048)	McCarthy et al. (1983)
R, virulent – boiled	As above	i.p.	Water-borne	As above	10–18	–	+ (1:2048)	As above
Virulent	As above	i.p.	Water-borne	As above	10–18	–	+ (1:512)	As above
Virulent	Immune serum – rabbit	i.p.	i.p.	Coho salmon	NG	+	ND	Olivier et al. (1985a)
Virulent (MT028, MT048)	Immune serum- rabit	i.p.	i.p.	Rainbow trout	11–14	+	ND	Ellis et al. (1988a)

NG not given, *ND* not done, *h.i.* hyperosmotic infiltration

A detailed study revealed that a 28 kDa outer membrane pore forming protein (= porin) from *Aer. salmonicida* led to the development of protective immunity in rainbow trout (Lutwyche et al. 1995). Moreover, the immunogenicity of T3SS was evaluated in rainbow trout by administering two formalin-inactivated whole cell products one of which was an isogenic strain with a deletion of the T3SS gene by i.p. injection with challenge 8-weeks later. Survival was better with the with the product based on the isogenic strain with which proteomics revealed that deletion resulted in expression of all the components of T3SS, some of which were immunosuppressive. The conclusion reached was that vaccines containing T3SS proteins led to decreased protection (Bergh et al. 2013). However, Schwenteit et al. (2015) constructed mutant strains secreting AsaP1-toxoid instead of the toxin (AsaP1 = a metalloendopeptidase, which is expressed as a 37-kDa pre-pro-peptide and processed to a 19-kDa active peptide). An inactivated whole cell vaccine based on the AsaP1(Y309F)-toxoid mutant (this had weak caseinolytic activity and was processed to the 19-kDa peptide) protected Arctic charr against challenge, with the level of protection stated to be comparable with a commercial product (Schwenteit et al. 2015).

The commercial interest in polyvalent vaccines has resulted in several products, which are regularly used in Europe and elsewhere. The benefit of this approach to controlling furunculosis may be illustrated by the observation that *Vibrio* antigens, particularly *V. salmonicida,* appear to enhance the humoral immune response to *Aer. salmonicida* (Hoel et al. 1997). Moreover, vaccination with *V. salmonicida* antigens led to protection against *Aer. salmonicida* following challenge by cohabitation (Hoel et al. 1998). This approach could well overcome the perceived problem that *Aer. salmonicida* is a weak antigen (Tatner 1989). Also, this cross protection may explain the often superior protection afforded by polyvalent vaccines (Hoel et al. 1998).

Concerning the use of rough and smooth strains for vaccine preparation, discrepancies are apparent among results obtained by different groups of investigators. Michel (1979) reported that there was no difference in the effectiveness of vaccines prepared with either rough or smooth cultures, when administered orally or via i.p. injection to rainbow trout. In fact, neither type of vaccine was protective. Yet, circulating antibodies were present in fish that received the vaccines via injection. Cipriano (1982a), examining the effectiveness of vaccines prepared from virulent and avirulent cultures, determined an equal level of protection from passive immunisation of brook trout. Similar agglutinin titres, i.e. 1:512, were found in both groups of vaccinated fish. He concluded, therefore, that protective immunogens were common to both virulent and avirulent cultures. Chicken egg yolk powder containing immunoglobulin, IgY, was used in the fish-rearing water to successfully control to ulcer disease in koi carp (Gan et al. 2015).

McCarthy et al. (1983) reported that, in general, only rough variants conferred protective immunity. A parallel result emanated from the work of Olivier et al. (1985a), who ascertained that avirulent cells were less effective immunogens than their virulent counterparts. Both of these groups regarded the A-layer protein as the antigen that probably conferred a protective response by the fish. In another devel-

opment, Hastings and Ellis (1988) recorded that rainbow trout responded to A-protein and LPS O-antigen and some of the components of the ECP (including proteases; Ellis et al. 1988b). So, it is not surprising that Shieh (1985) demonstrated protection with protease fractions. Others have also demonstrated that the A-layer protein is an important protective antigen in non-oily Montanide adjuvanted injectable whole cell inactivated vaccines (RPS = 51–78 %), with preparations without A-layer lacking efficacy in Atlantic salmon, as did those with purified LPS. Again, there was no correlation between protection and antibody production (Lund et al. 2003a). Interestingly with atypical *Aer. salmonicida* and Atlantic cod, there was a correlation reported between vaccine efficacy and the presence of cross-reacting LPS-specific antibodies (Lund et al. 2008a). Furthermore, Villumsen et al. (2012) reported a correlation between antibody titre, which were measured with an ELISA, and protection 18-weeks after vaccination of rainbow trout with an oil-adjuvanted vaccine. Yet within 3-days of challenge, there was a significant decrease in antibody titre, but nevertheless, it was concluded that antibodies have an important and central role in explaining the protective nature of vaccines in rainbow trout. The correlation between antigen dose in the vaccine, antibody titre as determined by ELISA, and protection against challenge was further reinforced by Romstad et al. (2012, 2013).

Striving to protect spotted wolffish against atypical *Aer. salmonicida,* Lund et al. (2003b) confirmed the need for A-layer in vaccine preparations, but highlighted the necessity of incorporating atypical rather than typical cells (RPS = 82–95 %) . The explanation given was that atypical *Aer. salmonicida* had genetically (by AFLP) and serological different A-layer than their typical counterparts (Lund et al. (2003b). Unfortunately, the desired immersion vaccination strategy did not work insofar as high levels of mortalities resulted after challenge, even when adopting an immersion boost (Grøntvedt et al. 2004). A later publication by Lund et al. (2008a) reinforced the importance of A-layer in vaccine preparations designed to protect Atlantic cod against atypical isolates. This group used oil adjuvanted preparations administered by i.p. injection containing formalised cultures with different cell surface components, specifically A-layer (including an A^- isolate with re-attached A-protein) and LPS. The outcome was that whole cell preparations with A-layer elicited better protection than those without. The nature of the host on the success of the product has been demonstrated in a comparison of a whole cell atypical furunculosis product in spotted wolffish (*Anarhichas minor*) and Atlantic halibut (*Hippoglossus hippoglossus*) (Lund et al. 2008b). Using genetically-different A-layer proteins, it was determined that only vaccines containing whole cells were reattached A-layer protein genetically homologous with the challenge strain resulted in protection comparable with the homologous vaccine (Arnesen et al. 2010).

Researchers should consider the interesting work of Olivier et al. (1985b), who noted protection to *Aer. salmonicida* in coho salmon after i.p. injection of formalised cells as well as after injection with FCA. Undoubtedly, the use of adjuvant stimulated non-specific immunity, probably involving macrophage activity. Certainly, i.p. injection has led to activation of leucocytes (Köllner and Kotterba 2002). From the

study of Norqvist et al. (1989), it is necessary to question the need for incorporation of *Aer. salmonicida* cells or their cellular components into furunculosis vaccines.

Injection techniques appear to be the most efficacious, whereas the oral route is least promising (Midtlyng et al. 1996). The use of adjuvants, especially mineral oil, in injectable vaccines is clearly beneficial (Midtlyng 1996) with 16S rRNA and LPS being detected in the head kidney and spleen at 2 weeks (and in the head kidney at 12 weeks) after injection with a commercial oil adjuvanted, formalin-inactivated vaccine (Grove et al. 2003). Indeed, Midtlyng (1996) determined from a field study in Norway that i.p. administration of furunculosis vaccine in a mineral oil adjuvant gave the best protection in Atlantic salmon. Apart from the obvious benefits of FCA (Olivier et al. 1985b), the use of ß-1,3 glucan (Vita-Stim-Taito), lentinan and formalin-killed cells of *Ren. salmoninarum* have enhanced the effectiveness of vaccines based on formalised *Aer. salmonicida* cells (Nikl et al. 1991). Possibly with oral uptake, degradation of the vaccine in the gastro-intestinal tract may occur. In this case, there may be potential for the use of micro-encapsulation techniques to avoid such pitfalls. One interesting and relevant approach, which is reminiscent of the probiotic saga, involved the i.p. administration of 10^7 cells/fish of a live auxotrophic *aroA* mutant of *Aer. hydrophila* that protected rainbow trout 30 days later against furunculosis (RPS = >60 %) and stimulated the humoral and cellular immune response (Vivas et al. 2004a).

Immersion techniques have generated much useful data. Rodgers (1990) reported the benefits of using inactivated whole cells, toxoided ECP and LPS for the protection of juvenile salmonids. Moreover, the vaccinated animals grew better than the controls. Work has indicated that the duration of the immersion vaccination process does not affect the uptake of the vaccine, providing that the antigens are not in low concentrations (Tatner 1987). Therefore, there appears to be some promise for the widely used immersion vaccination technique with furunculosis vaccines.

Ultrasound has been used as a method to administer vaccines for the control of goldfish ulcer disease. Thus, soluble A-protein was applied by immersion (100 µg A-protein/ml for 10 min) after ultrasound (1 MHz frequency of ultrasound/1 min) pre treatment, and led to promising results in goldfish against challenge (Navot et al. 2011). Furthermore, low frequency sonophoresis at 37 kHz has been used to increase the uptake of antigens across rainbow trout skin by threefold, and without any significant side effects (Cobo et al. 2014).

Traditionally, oral vaccines were considered to be the least successful insofar as it was reasoned that the antigens became degraded during passage through the stomach and possibly there were issues regarding access to the antibody-producing sites. Liposome-entrapped antigens of atypical *Aer. salmonicida* were fed to carp with the result that there was a stimulation of the immune response, specifically the presence of antibodies in bile, intestinal mucus and serum, and greater protection (less mortalities) and a reduction in ulceration compared to the controls (Irie et al. 2005). Gradually, however, oral vaccines have attained favour, and commercial products are now available.

Some of the difficulties with ascertaining the efficacy of vaccines have been ascribed to methods of experimental challenge. Indeed, it is not unusual for vac-

cines to appear to work in laboratory conditions but to fail dismally in field trials. Under such circumstances, it is questionable whether or not meaningful challenge techniques have been used. For example, the precise dosage of cells to be employed remains undetermined. Apparently, there is substantial variation in virulence among strains. In addition, the most effective means of administering the challenges remains to be elucidated. In this respect, Michel (1980) and Cipriano (1982b) suggested standardized methods of challenge. However, the effectiveness of these techniques awaits clarification.

It is readily admitted that much effort has been expended on the development of furunculosis vaccines. Yet, after 40 years the quest continues. Most studies, to date, have measured effectiveness in terms of the humoral antibody response (e.g. Michel et al. 1990). Unfortunately, there is now some doubt as to whether the presence of humoral agglutinins actually correlates with protection. Maybe, it would be preferable to emphasise other aspects of fish immunology, such as cell-mediated immunity, a notion, which has been suggested by McCarthy and Roberts (1980).

Ford et al. (1998) treated sea run salmon broodstock with oxolinic acid and vaccinated with a formalised whole cell vaccine in an attempt to reduce the impact of furunculosis. Encouraging results were obtained insofar as of 2552 fish captured from the rivers Connecticut and Merrimack and treated in 1986–1992, only 362 died of which 65 (18 %) were diagnosed with furunculosis. In comparison, 206 fish served as untreated controls, with just over half, i.e. 109, dying, of which 63 (=58 %) had furunculosis.

There is ongoing concern about the value of furunculosis vaccines developed for use in salmonids, and containing antigens of *Aer. salmonicida* subsp. *salmonicida,* for application in other groups of fish, which may be affected by atypical isolates of the pathogen. For example, a commercial polyvalent product for salmon failed to protect turbot from experimental challenge with *Aer. salmonicida* subsp. *achromogenes* (Björnsdóttir et al. 2005). However, Santos et al. (2005) appear to have experienced better success with turbot, although the specific pathogen was not equated with subsp. *achromogenes*. Nevertheless, the commercial vaccine, Furovac 5, and an autogenous vaccine resulted in RPS of 72–99 % when challenged 120 days after administration intraperitoneally. Even after 6-months, there was still reasonable protection (RPS = 50–52 %). In contrast, vaccination by immersion did not lead to significant protection. Interestingly, an oral booster dose did not improve protection (Santos et al. 2005).

Immunostimulants/Dietary Supplements Kitao and Yoshida (1986) found that synthetic peptides could enhance resistance of rainbow trout to *Aer. salmonicida.* Administration of Baypamum to rainbow trout led to a reduction in symptoms and mortalities attributed to furunculosis (Ortega et al. 1996). Dimerised lysozyme, which is regarded as less toxic than the monomer, was injected into rainbow trout at a dose of 10 or 100 μg/kg, and stimulated cellular and humoral mechanisms giving protection against furunculosis (Siwicki et al. 1998). One and three injections of lysozyme led to 45 % and 25 % mortalities following challenge with *Aer. salmonicida.* This compares to 85 % mortality among the untreated controls (Siwicki et al.

1998). Use of ß-1,3- glucan and chitosan for 30 min immersion in 100 µg/ml or as single i.p. injections with 100 µg led to protection in brook trout against *Aer. salmonicida* from 1 to 3 days after administration (Anderson and Siwicki 1994), with the former reducing the inflammatory response (in common carp) (Falco et al. 2012). Generally, injection was superior to immersion (Anderson and Siwicki 1994). Furthermore, dietary glucan, which was fed at 6 mg/kg body weight/day for 14 days, stimulated C-reactive protein and complement in common carp during experimental infection with *Aer. salmonicida* (Pionnier et al. 2013).

The potential for use of plant products was demonstrated by Breyer et al. (2015), who reported the benefit of using 0.5–1.0 % of garlic (*Allium sativum*) extract to control a *Aer. salmonicida* challenge in rainbow trout.

The potential benefit of vitamin B_6 as a nutritional supplement for Atlantic salmon has been examined, albeit without success (Albrektsen et al. 1995). Thus, fish of 14 g weight were fed with diets supplemented with 0–160 mg of vitamin B_6/kg of feed for 20 weeks. However, challenge with *Aer. salmonicida* revealed that increased dietary levels of vitamin B_6 did not increase resistance to furunculosis.

Synergism between low levels of iron and high amounts of long-chain polyunsaturated fatty acids led to and RPS of 70 after challenge with *Aer. salmonicida* (Rørvik et al. 2003).

Probiotics An isolate of *V. alginolyticus,* previously used as probiotic in Ecuadorian shrimp hatcheries, has been effective at controlling diseases caused by *Aer. salmonicida* (Austin et al. 1995).

Bacteriophage Consideration has been given to the use of bacteriophages for biocontrol purposes. In this connection a representative of the *Myoviridae*, which was recovered from sediment in a rainbow trout farm in Korea, demonstrated broad host range across the taxonomic divide of *Aer. salmonicida,* and was considered for use in aquaculture (Kim et al. 2012). Moreover, bacteriophage PAS-1 was administered to rainbow trout, and demonstrated the ability to protect the fish against virulent cells of the pathogen. Furthermore, the virions were cleared from the fish within 200 h of administration (Kim et al. 2015).

Disinfection Methods Adequate husbandry practices such as maintenance of good water quality, disinfection of fish farm equipment and utensils especially when disease outbreaks occur, and routine disinfection policies for eggs upon arrival at receiving sites (Herman 1972; McCarthy and Roberts 1980).

Use of Antimicrobial Compounds A variety of inhibitory agents have been applied with varying degrees of success to the treatment of furunculosis. Early studies established that sulphonamides, notably sulphamerazine, were successful in controlling furunculosis when administered orally with food at a dose of 22 g of drug/100 kg of fish/day (Gutsell 1946; Snieszko 1958a). Among the antibiotics, Snieszko (1958a) showed the usefulness of chloramphenicol and oxytetracycline, when dosed at 5–7 g/100 kg of fish/day. Furazolidone was also briefly mentioned as

having promise. Subsequently, polymyxin B nonapeptide has been found to inhibit *Aer. salmonicida,* probably by disrupting the A-layer (McCashion and Lynch,1987).

Curiously, the use of some compounds follows a geographical pattern, e.g. in France flumequine (a quinolone) is favoured (Michel et al. 1980), whereas in England and Japan, oxolinic acid has been used extensively (see Endo et al. 1973; Austin et al. 1983b). It is debatable as to whether flumequine or oxolinic acid has been more successful at combating furunculosis (Barnes et al. 1991a). Additionally in England, potentiated sulphonamides have been used widely (McCarthy et al. 1974). Within the UK, four compounds, namely amoxicillin, oxolinic acid, oxytetracycline and potentiated sulphonamides, are currently licensed for fisheries use in the treatment of furunculosis. For other diseases caused by *Aer. salmonicida,* i.e. CE and goldfish ulcer disease, less information is available. It appears, however, that potentiated sulphonamides and oxytetracycline are generally effective against the pathogen, regardless of the disease manifestation and assuming that treatment begins at an early stage in the disease cycle (Gayer et al. (1980). Unfortunately despite its comparatively recent arrival in the armoury of fisheries chemotherapeutants, resistance to amoxicillin has been documented in Scotland (Barnes et al. 1994). For the future, florfenicol, dosed at 10 mg/kg body weight of fish/day for 10 days, offers promise, insofar as it has already been used with some success against furunculosis in Norway (Nordmo et al. 1994; Samuelsen et al. 1998).

Since the initial work with oxolinic acid (Endo et al. 1973; Austin et al. 1983), it is apparent that substantial quantities have been used in aquaculture within many countries. Consequently, it is hardly surprising that resistant strains of *Aer. salmonicida* have emerged (Hastings and McKay 1987; Tsoumas et al. 1989; Barnes et al. 1990a, b). Yet, the widespread usefulness of the compound at controlling furunculosis has prompted a search for other related compounds. The fruits of this research may be illustrated by the apparent success of 4-quinolones/fluoroquinolones at inhibiting the pathogen (Barnes et al. 1990a, b, 1991b; Bowser and House 1990; Lewin and Hastings 1990; Martinsen et al. 1991; Inglis and Richards 1991; Stoffregen et al. 1993; Elston et al. 1995). Thus, enrofloxacin and sarofloxacin have been found to be more effective than oxolinic acid, in terms of MIC at inactivating *Aer. salmonicida.* The effectiveness of enrofloxacin at 10 mg/kg body weight of fish/day for 10 days has been attested by field trials with lake trout (*Salvelinus namaycush*) (Hsu et al. 1995). In addition, enrofloxacin has been effective in controlling atypical *Aer. salmonicida* in tom cod, insofar as a single injection with 5 mg of enrofloxacin/kg of fish stopped furunculosis (Williams et al. 1997). Difloxacin, dosed at 5 or 10 days at 5 mg/kg body weight, and 10 days at 1.25 or 2.5 mg/kg body weight resulted in significantly lowered mortalities compared to controls following i.m. injection with a virulent culture of *Aer. salmonicida.* Of relevance, there was little difference in the results between 5 and 10 day treatment regimes with 5 mg/ kg body weight (Elston et al. 1995).

With the realisation that *Aer. salmonicida* occurs on the external surfaces, i.e. gills and mucus, attempts have been made at disinfection. Cipriano et al. (1996c, d) evaluated chloramine T, dosed at 15 mg/l for 60 min on three consecutive days, but

the infection was not controlled. Success occurred with 77 mg of oxytetracycline/kg body weight of fish/day for 10 days with mortalities stopping within 4 days of starting treatment (Cipriano et al. 1996c).

There can be no dispute that chemotherapeutic agents are (and will continue to be) invaluable for preventing heavy mortalities during outbreaks of furunculosis. Nonetheless, there are substantial reasons for avoiding total reliance upon such compounds. For instance, the development of resistance by the pathogens to some widely used drugs is cause for serious concern (Wood et al. 1986). Thus, it is disquieting that plasmids carrying antibiotic resistance factors (R factors) have been isolated from *Aer. salmonicida* strains (Aoki et al. 1971). Indeed, a strain resistant to sulphathiazole and tetracycline was recovered as early as 1959. Moreover, Snieszko and Bullock (1957) reported the occurrence of cultures that were resistant to sulphonamides, although at the time the mechanism of resistance was not known. Now, it seems that resistance may reflect alterations in the outer membrane of *Aer. salmonicida* (Barnes et al. 1992). Aoki et al. (1983) examined 175 isolates, which had been isolated from cultured and wild salmonids in Japan, for susceptibility to a wide range of antimicrobial agents. They noted that 96% of the isolates from cultured fish were resistant to at least one, and up to six, of the drugs, particularly nalidixic acid and nitrofuran derivatives. In addition, transferable R plasmids, coding for resistance to chloramphenicol, streptomycin and sulphonamides, and non-transferrable plasmids conveying resistance against tetracycline, were found in several strains. It was concluded from these results that drug resistant strains of *Aer. salmonicida* had increased in direct proportion to the enhanced use of antimicrobial compounds in fish culture. This was particularly evident in view of the observation that few isolates recovered from wild salmonids exhibited drug resistance. Toranzo et al. (1983a, b) characterised the plasmids, determining that the organism frequently possessed more than one plasmid. In particular, five strains possessed six plasmids of varying molecular weights. However, these workers did not observe any correlation between loss of plasmids, or changes in plasmid mobilities, and the loss of resistance to sulphadiazine, in the case of one strain that was studied in detail. Nevertheless, the molecular genetic studies of Mitoma et al. (1984) identified gene sequences in R plasmids coding for either chloramphenicol or tetracycline resistance. Hedges et al. (1985), using aeromonad isolates obtained from France, Ireland, Japan and the UK, determined that plasmids from *Aer. hydrophila* and *Aer. salmonicida* were similar. These workers reported that some plasmids were transmissible to *Esch. coli,* whereas others were unstable in this recipient organism. The R plasmids of *Aer. salmonicida* were considered to confer upon the pathogen the potential to withstand the onslaught of a wide variety of inhibitory agents, thus diminishing the effectiveness of chemotherapy. It may be hoped that the development of new compounds, particularly synthetic or semi-synthetic molecules, and the strict rotation in the use of currently available drugs may assist with the problem of resistance in the pathogen. Obviously, a constant awareness of the problem must be maintained.

Another aspect of chemotherapy concerns the presence and retention of the compounds in fish tissues. In fact, difficulties of this nature were recognised as early as 1951 with a report by Snieszko and Friddle who expressed concern with tissue

levels of sulphamerazine in trout. McCarthy and Roberts (1980) pointed out that in some countries, such as the USA, there existed legislation restricting the number of antimicrobial compounds that may be used on fish destined for human consumption. In many countries, drugs may only be obtained on veterinary prescription. The caveat to the use of chemotherapeutants is that a suitable period of time must lapse following the conclusion of treatment, before the fish may be sold for human consumption. This should allow for the purging from the fish of all traces of the active compound and the metabolites. It is worth remembering the opinions of Snieszko (1958a), who cautioned that drug therapy should only be considered as a stopgap measure until the sources of infection by *Aer. salmonicida* could be eliminated, or disease-resistant strains of fish introduced.

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Chapter 6
Enterobacteriaceae Representatives

Abstract A wide range of enteric bacteria, including representatives of *Edwardsiella,* and *Yersinia,* have been associated with fish diseases; some of the pathogens, e.g. *Y. ruckeri,* being responsible for large scale losses in aquaculture. Some new species have been described, e.g. *Edw. anguillarum* and *Edw. piscicida*.

In addition to the organisms detailed below, there has been fleeting mention of a fish pathogenic role for *Erwinia* sp. (Starr MP, Chatterjee AV, Annu Rev Microbiol 26:289–426, (1972)) and *Salmonella enterica* serovar Typhimurium (Morse EG, Greenwood DE, Meyers EP, Anderson VL, Duncan MA, J Exp Sci Health Part A Environ Sci Eng 13:325–335, (1978)).

Keywords Enterics • Edwardsiellosis • ERM • Vaccines • Immunostimulants

Citrobacter freundii

Characteristics of the Disease

Historically, there have been brief references to *Cit. freundii* as a fish pathogen (see Conroy 1986). Yet, definitive evidence was not forthcoming until a report of the organism as a pathogen of sunfish, *Mola mola* in a Japanese aquarium (Sato et al. 1982). Subsequently, *Cit. freundii* has been implicated with disease in Atlantic salmon and rainbow trout in Spain and the USA (Baya et al. 1990a; Sanz 1991) and with carp in India (Karunasagar et al. 1992). In addition, the organism has been recovered in mixed culture from diseased salmonids in the UK (B. Austin, unpublished data). Consequently, it appears that *Cit. freundii* is an emerging fish pathogen.

Diseased sunfish displayed erratic swimming, inappetance, eroded skin, surface haemorrhages, enteritis, deep red spleen, pale liver and tumorous masses (granulomas) on the kidney (Sato et al. 1982). Similar signs have been observed in salmonids within the UK (B. Austin, unpublished data). In the initial disease outbreak, 25/29 animals (=86 % of the total) died (Sato et al. 1982).

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Isolation

Pure culture growth was recovered from kidney on rabbit blood agar (Sato et al. 1982). Subsequently, similar organisms have been obtained from kidney homogenates spread over the surface of BHIA and TSA with incubation at 25 or 37 °C for 24–48 h.

Characteristics of the Pathogen

Box 6.1: *Citrobacter freundii*
Cultures contain Gram-negative motile, fermentative rods, which produce catalase, ß-galactosidase and H_2S but not arginine dihydrolase, indole, lysine or ornithine decarboxylase, oxidase or tryptophan deaminase. Neither gelatin nor urea is attacked, nor is citrate utilised. The Voges Proskauer reaction is negative. Acid is produced from arabinose, glucose, mannitol, melibiose, rhamnose, sorbitol and sucrose but not from amygdalin or inositol.

From these traits, mostly obtained with the API 20E rapid identification system, an identification as *Cit. freundii* was obtained (Sato et al. 1982; Baya et al. 1990a; Sanz 1991). Certainly, these characteristics match the description of *Cit. freundii,* except in the inability to utilise citrate (Frederiksen 2005).

Epizootiology

The organism is common in eutrophic freshwater, from which spread to fish is possible (Allen et al. 1983). In addition, the organism was widespread in the seawater in the Japanese aquarium (Sato et al. 1982).

Pathogenicity

The pathogenicity of isolates was not confirmed in laboratory experiments by Sato et al. (1982). Nevertheless, Baya et al. (1990a) and Karunasagar et al. (1992) demonstrated pathogenicity following i.p. injection of rainbow trout and carp, respectively. Here, the LD_{50} was in the range of 10^5–10^6 cells. Injection of cell-free extracts did not result in mortalities (Karunasagar et al. 1992).

Disease Control

Disinfection Outbreaks of the disease abated following adoption of water disinfection (with chlorine) practices (Sato et al. 1982).

Antimicrobial Compounds

According to Sato et al. (1982) and Baya et al. (1990), isolates were resistant to chloramphenicol, potentiated sulphonamides and tetracycline. Indeed, chemotherapy was unsuccessful at controlling the disease in the Japanese aquarium.

Edwardsiella anguillarum

Cultures were obtained from diseased eels, sea bream and red sea bream in China (Shao et al. 2015).

Characteristics of the Pathogen

Box 6.2: *Edwardsiella anguillarum*
Cultures comprise non-pigmented colonies with irregular margins that contain Gram-negative facultatively anaerobic rods that are motile by peritrichous flagella. Growth occurs at 18–37 °C (optimally at 28–30 °C) but not 12 °C, in 1–3 % (w/v) sodium chloride, and at pH 6–9 (optimally at pH 7–8). Catalase, H_2S and indole are produced, but not arginine dihydrolase, ß-galactosidase, oxidase or tryptophan deaminase. Neither gelatin nor urea is degraded. The principal fatty acids of the type strain are $C_{14:0}$, $C_{16:0}$, $C_{17:0}$ cyclo, summed feature 2 ($C_{14:0}$ 3OH/iso-$C_{16:1}$I), summed feature 3 ($C_{16:1}$ ω6c/ω7c) and summed feature 8 ($C_{16:1}$ ω6c/ω7c). The respiratory quinone of the same culture is menaquinone MK-8; also present is phosphatidylethanolamine, phosphatidylglycerol, aminolipid, phospholipid and one unknown lipid. The G+C ratio of the type strain 58.72 mol% (Shao et al. 2015).

Pathogenicity

Pathogenicity was demonstrated in a laboratory infectivity experiment using turbot in which the LD_{50} dose was reported as 5.7×10^2 CFU/g (of fish). Three sets of distinct T6SSs and 2 T3SSs were present in the type strain (Shao et al. 2015).

Edwardsiella ictaluri

Characteristics of the Disease

Enteric septicaemia of catfish was initially recognised in 1976 among populations of pond-reared fingerlings and yearling fish in Alabama and Georgia, USA (Hawke 1979). Later, reports described its presence in Mississippi, Arkansas, Idaho, Colorado, Indiana and Maryland. Although *Edw. ictaluri* is primarily a disease of catfish, with time other fish groups have been reported to become infected, for example, brown bullhead (*Amieurus nebulosus*) in the USA (Iwanowicz et al. 2006) and wild ayu (*Plecoglossus altivelis*) in Japan, particularly in September and October when the temperature declined and the fish were sexually mature (Hassan et al. 2012). The disease has spread from its original focal point in the USA to include cultured striped catfish (*Pangasius hypophthalmus*) in Sumatra, Indonesia (Yuasa et al. 2003) and catfish in Vietnam (Fig. 6.1; Crumlish et al. 2002), yellow catfish (*Pelteobagrus fulvidraco*) (Ye et al. 2009) and southern catfish (*Silurus soldatovi meridionalis*) in China (Geng et al. 2013) and ayu in Japan (Sakai et al. 2008). It is apparent that there is a great variability in the clinical signs associated with the disease. Just prior to death, the fish may hang listlessly in an almost vertical position at the water surface, spin rapidly in circles, or exhibit spiral swimming. External signs, often absent in fish over 15 cm in length, include the presence of petechial (pin-prick) haemorrhages on the skin in the vicinity of the throat and mouth, pale gills, exophthalmia and open lesions on the head, particularly on the frontal bone of the skull between the eyes and on the lateral body surface. Internally, there may be swelling of the kidney and spleen, haemorrhaging and necrotic areas in the liver, blood-filled ascitic fluid in the peritoneum, and petechial haemorrhages throughout the internal muscle walls (Hawke 1979). In Vietnam, the disease is referred to as "bacillary necrosis of *Pangasius*" with which there are irregular white lesions on the kidney, liver and spleen, and the involvement of parasites and bacteria including *Bacillus* and *Edw. ictaluri* (Crumlish et al. 2002). With wild ayu, mortalities occurred from August to early October in 2007, with haemorrhagic ascites as a common disease sign. Some fish demonstrated exophthalmia, and reddening along the body surface, anus or the base of fins (Sakai et al. 2008).

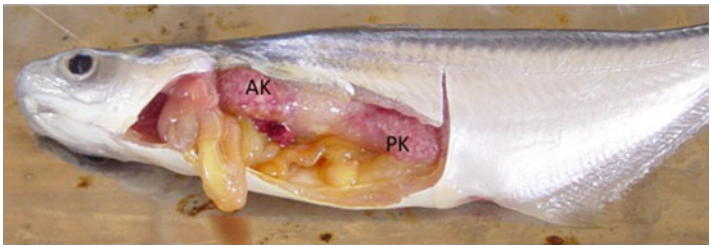

Fig. 6.1 *Edw. ictaluri* infection in *Pangasianodon hypophthalmus.* Pale areas of cellular necrosis are evident in the anterior (AK) and posterior (PK) kidney. Photograph courtesy of Dr. M. Crumlish

Isolation

Isolation has been readily achieved from kidney, liver, spleen, intestine, brain and skin or muscle lesions by inoculation of material into BHIA or blood agar. Following incubation at 26 °C for 48 h, smooth circular (2 mm diameter), slightly convex, entire, non-pigmented colonies develop (Hawke 1979). A selective medium has been described by Shotts and Waltman (1990) [Appendix in Chap. 12].

Characteristics of the Pathogen

Box 6.3: *Edwardsiella ictaluri*
Cultures comprise Gram-negative rod-shaped fermentative organisms (strains with a limited tolerance for oxygen have been recovered from channel catfish in the USA; Mitchell and Goodwin 2000), which are motile by peritrichous flagella. Catalase, ß-galactosidase (a variable response has been recorded between various laboratories) and lysine and ornithine decarboxylase are produced but not H_2S, indole, oxidase or phenylalanine deaminase. The methyl red test is positive, but not so the Voges Proskauer reaction. Nitrates are reduced. Growth occurs in 1.5 % but not 2 % (w/v) sodium chloride. The optimum growth temperature is between 20 and 30 °C, which coincides with the water temperature during severe outbreaks of the disease. Blood is degraded, but not casein, DNA, elastin, gelatin, Tween 20, 40, 60 and 80 or urea. Acid is produced from fructose, galactose, glycerol, maltose, mannose and ribose but not from adonitol, aesculin, amygdalin, arabinose, arbutin, cellobiose, dulcitol, erythritol, inositol, inulin, lactose, melezitose, raffinose, rhamnose, salicin, sodium malonate, sorbitol, sorbose, starch or sucrose (Waltman and Shotts 1986a). The G+C ratio of the DNA is 53 moles % (Hawke et al. 1981). The complete genome sequence of strain 93–146, which was recovered from an outbreak of disease in Louisiana, USA in 1993, was 3812315 bp with 3783 predicted protein coding genes (Williams et al. 2012).

All the published reports point to the presence of a very homogeneous group of bacteria phenotypically, including use of isozyme analysis (Starliper et al. 1988; Bader et al. 1998), although subgroups may be recognised by molecular methods. Thus, Bader et al. (1998) recognised 4 subgroupings among 20 isolates by use of arbitrary primed PCR. Using AFLP, a single clone of Japanese isolates from ayu was recognised, suggesting a single point source of origin of the organism [in Japan] (Sakai et al. 2009).

Similarities have been observed with *Edw. tarda,* except that isolates of *Edw. ictaluri* did not produce H_2S or indole, or ferment glucose with the production of gas at 37 °C. Moreover, the isolates did not agglutinate with antiserum to *Edw. tarda* (Hawke 1979). However, in terms of DNA relatedness, the causal agent of enteric

septicaemia of catfish was determined to be most similar to *Edw. tarda,* i.e. 56–62 % DNA homology, but sufficiently distinct to warrant separate species recognition (Hawke et al. 1981). Yet, comparison of 23S rRNA gene sequences confirm a close phylogenetic relationship between the two species (Zhang and Arias 2007). It remains for further work to elucidate the relationship, if any, with *Edwardsiella* phenon 12 of Johnson et al. (1975).

Cryptic plasmids, i.e. pCL1 and pCL2 of 5.7 kb and 4.9 kb respectively, have been found in isolates of *Edw. ictaluri* (Lobb et al. 1993). However, an isolate from green knife fish differed insofar as 4 plasmids, of 3.1, 4.1, 5.7 and 6.0 kb, were present. Of these, the 4.1 and 5.7 kb plasmids hybridised strongly (Lobb et al. 1993).

Diagnosis

Plasmid Profiling A novel diagnostic approach concerns determination of plasmid profiles for *Edw. ictaluri* (Lobb and Rhodes 1987; Speyerer and Boyle 1987).

Serology iFAT with monoclonal antibodies in an enzyme immunoassay has shown promise for the recognition of *Edw. ictaluri* (Rogers 1981; Ainsworth et al. 1986). A relevant development has been the use of a rapid iFAT to simultaneously detect two pathogens, i.e. *Edw. ictaluri* and *Fla. columnare* using fluorochromes with two different spectra properties, Alexa Fluor 488 and 594 emitting green and red fluorescence, respectively (Panangala et al. 2006). An indirect ELISA has been effective at detecting the presence of antibodies to *Edw. ictaluri* in fish serum (Waterstrat et al. 1989; Swain and Nayak 2003). A development of this approach involved the use of tissue homogenisation (using 0.5 % v/v Triton X-100 in 0.05 M PBS [pH 7.2]), filtration and then the ELISA (Earlix et al. 1996). This approach was used successfully to detect asymptomatic carriers, and permitted live bacteria to be filtered from 1 g quantities of tissue slurries, with a sensitivity of <10 colony forming units/g of tissue. The filter-ELISA system detected *Edw. ictaluri* in 80 % of 98 channel catfish compared to a detection of 24 % by culturing (Earlix et al. 1996).

Molecular Methods Real time PCR, which produces a result in 4–5 h, has been developed for *Edw. ictaluri,* and detected the equivalent of 2.5 cells using DNA samples from cultures and fish blood (Bilodeau et al. 2003). A later study confirmed a PCR for the detection of *Edw. ictaluri* and its differentiation from *Edw. tarda* (Williams and Lawrence 2009).

Epizootiology

The natural reservoir for infection has not yet been firmly established. It is known that the organism survives for limited periods, i.e. up to 8 days, in sterile pond water (Hawke 1979). This suggests that *Edw. ictaluri* has only limited ability to survive in

the aquatic environment, although there is some evidence that the pathogen may be present in invertebrates that could act as vectors for transmission. A recent study demonstrated that *Edw. ictaluri* could be introduced onto theronts and replicate during tomont division of fish parasite *Ichthyophthirius multifiliis.* Theronts arriving on fish (in this case channel catfish) could be observed by fluorescent microscopy with *Edw. ictaluri* on the skin and gills (Xu et al. 2012). A carrier state in channel catfish has been documented (Klesius 1992). Also, data have shown that cells can pass from dead fish to non-infected channel catfish (Klesius 1994). Of course, the results of such laboratory-based survival experiments need to be treated cautiously. It is conceivable that the organism comprises part of the normal microflora of fish, lurking perhaps in the digestive tract.

Lytic bacteriophage, notably ΦeiAU and ΦeiDWF, have been recovered (Hossain et al. 2012), and it is speculative if such bacteriophage impact on populations of the pathogen in the environment.

Pathogenicity

Mortalities of up to 50 % have been recorded. In one comparative study, an injection of 1.5×10^3 cells of the pathogen was sufficient to cause 100 % mortality among a group of channel catfish; tilapia only demonstrated slight susceptibility, whereas golden shiner, bighead carp and largemouth bass were completely resistant (Plumb and Sanchez 1983), with daily feeding (of channel catfish) leading to less mortalities than groups that were fed less often or starved (Lim and Klesius 2003). The LD_{50} of ayu was reported at 10^4 CFU/fish, with dead animals displaying similar signs to the natural infection (Sakai et al. 2008). Survival of natural outbreaks has led to high humoral antibody levels and protection from fresh onslaught with *Edw. ictaluri* (Vinitnantharat and Plumb 1993). The aetiological agent has been also associated with disease outbreaks in non-ictalurid fish, e.g. danio (*Danio devario*) (Waltman et al. 1985).

Knowledge about the pathogenicity mechanisms of *Edw. ictaluri* is steadily increasing, with genome sequencing identifying various virulence mechanisms including type I fimbriae, type III and type IV secretion systems, a twin arginine translocation system, and flagellins (Williams et al. 2012). Extracellular products have been associated with virulence (Stanley et al. 1994; Williams et al. 2003). By comparing virulent with attenuated (these had been subcultured repeatedly in liquid medium) isolates, the latter lacked a 55 kDa OMP, showed markedly less haemolytic activity, and had differences in the composition of core oligosaccharide sugars of the LPS compared with the former (Williams et al. 2003). Lawrence et al. (2001) using transposon mutagenesis and O side chain mutants, deduced that the LPS O side chains were important for virulence. Rabbit antibodies to 3 (22, 31 and 59 kDa) of 4 major OMPs blocked invasion of cells from fathead minnow (*Pimephales promelas*) by *Edw. ictaluri* demonstrating that some if not all of these OMPs are involved in initial host-pathogen interactions (Skirpstunas and Baldwin 2003).

Saeed (1983) showed that cells are highly piliated, and inferred that the pili might be associated with virulence. By means of intragastric intubation and a comparatively high dose of 1×10^9 cells, *Edw. ictaluri* crossed the intestinal mucosa of channel catfish in 15 min (Baldwin and Newton 1993). Using 1×10^6 cells/ml and an application directly into the olfactory organs of channel catfish, light and electron microscopy revealed damage after 1 h (Morrison and Plumb 1994). The importance of entry across the intestinal epithelium is becoming widely accepted, with actin polymerization and receptor-mediated endocytosis as likely mechanisms of uptake (Li et al. 2012). It has been firmly established that channel catfish are highly susceptible to the organism, with an injected dose of 1.5×10^3 cells capable of killing the host within 10 days at a water temperature of 26 °C (Plumb and Sanchez 1983). It does not appear that the organism produces abundant exo-enzymes, which would function as exotoxins in fish. It has been argued that both gut and nares are primary sites for the invasion of *Edw. ictaluri* in natural outbreaks of disease (Shotts et al. 1986). Fluorescence microscopy evidence pointed to the localisation of the organism on the gill within 5 min and within gill epithelia after 45 min and to the kidney within 4 h of a waterborne route (Nusbaum and Morrison 2002); the outcome was a bacteraemia within 24 h (Wise et al. 1997). By 72 h, the pathogen was recoverable from the blood. Then by 216 h, there was evidence of the pathogen clearing from the blood, with all survivors developing agglutinating antibodies to *Edw. ictaluri*. Entry, survival (many organisms were present in vacuoles) and replication in head kidney macrophages of channel catfish has been observed microscopically. Opsonisation with normal serum led to even greater internalization of *Edw. ictaluri* at 0 h, but did not affect replication once internalized (Booth et al. 2006). Uptake of the pathogen into host (epithelial) cells may well involve actin polymerization and receptor-mediated endocytosis (Skirpstunas and Baldwin 2002). The infection process was accompanied by shedding of the pathogen into the water; a process contributing to transmission of the disease (Wise et al. 1997). It does not appear that the level of dietary iron affected antibody production and thereby influenced the course of an infection (Sealey et al. 1997).

Another approach to understanding pathogenicity is to determine the nature of the host's response to infection. Thus using qPCR, the transcriptional levels of 7 key channel catfish antimicrobial peptides genes was followed with data revealing a total lack of significant upregulation at 2 h post infection (Pridgeon et al. 2012). At 4 h onwards, hepcidin was upregulated. Additionally at 24 h after infection, upregulation of NK-lysin type 1 and type 3, and cathepsin D occurred. Administration of lethal but not sub-lethal doses of *Edw. ictaluri* led to upregulation of hepcidin in the posterior kidney, the significance of which may be related to the inhibition of replication of the pathogen. The suggestion was that hepcidin may well be important as a defence mechanism against *Edw. ictaluri* infection in channel catfish (Pridgeon et al. 2012).

Disease Control

Disease Resistant Fish A difference in susceptibility of channel catfish to *Edw. ictaluri* infection was reported, and attributed to genetic factors (Camp et al. 2000). Whereas both sensitive and resistant fish produced antibodies to *Edw. ictaluri,* the later produced more T lymphocytes in peripheral blood and more macrophage aggregations in the posterior kidney and spleen (Camp et al. 2000).

Vaccine Development Studies have been carried out to demonstrate the feasibility of developing a vaccine against *Edw. ictaluri* (Plumb 1984). Fortunately, the organism is highly immunogenic, with agglutination titres of 1:10 000 found in the serum of channel catfish after receiving only single injections of *Edw. ictaluri* cells mixed in Freund's adjuvant (cited in Plumb 1984). Furthermore, Saeed (1983) and Saeed and Plumb (1987), using a LPS extract, demonstrated protection following i.p. injection. In these experiments, 0.2 mg of LPS injected into channel catfish (individual weight = 60 g) induced agglutination titres of >1:500, which was sufficient to confer ≥80 % survival of the population. This compared with <30 % survival of the unvaccinated controls. Antigenicity of the LPS extract was enhanced in FCA, with protection conferred by single or multiple injections. Similarly, it should be emphasised that an inactivated whole-cell vaccine administered with FCA also showed promise. Immersion and oral boosting of channel catfish fry with a commercial vaccine was successful at controlling mortalities (Plumb and Vinitnantharat 1993). With this study, mortalities were as follows:

controls:	96.7 % mortalities
immersion vaccinated group	6.7 % mortalities
Immersion vaccinated + oral boosted group	3.3 % mortalities

In a comparison of techniques (immersion, immersion and oral in combination, and injection), injection led to the highest antibody titre after 10 weeks in channel catfish fry (Thune et al. 1997). However, a booster by immersion after 25 weeks gave a significant increase in titre. A modern approach reflected the development of an adenine-auxotrophic strain, the virulence of which was attenuated (Lawrence et al. 1997). Following injection, fish were protected against challenge with a virulent culture. An *aroA* mutant achieved success as a live vaccine (Thune et al. 1999). Also, an isogenic transposon-generated O polysaccharide mutant strain was evaluated as a vaccine administered intraperitoneally and immersion to channel catfish, and achieved useful protection (Lawrence and Banes 2005). A novobiocin-resistant attenuated strain has been proposed as a vaccine, and applied to channel catfish by i.p. injection with 4.2×10^6 CFU/fish of by immersion. Challenge led to an RPS of >90 % [100 % for immersion vaccination] (Pridgeon and Klesius 2011). Interest in live attenuated vaccines continued with the work of Wise et al. (2015), who attenuated a virulent isolate, S97-773, by repeated sub-culturing on laboratory media containing increasing concentrations of rifamycin. The cells were applied to feed at a

rate of 100 ml of diluted (1:10 or 1:100) vaccine/454 g of feed to achieve doses estimated at 5×10^7 and 5×10^6 CFU/g of feed, respectively, which was fed to channel catfish fingerlings with challenge after 30-days. The results revealed the RPS of 82.6–100 % for the lower dose, with even few mortalities occurring as a result of feeding with the higher dose. Also, there was enhanced antibody production and feed conversion (Wise et al. 2015).

Dietary supplementation of channel catfish with 4 % arginine and/or 2 % glutamine enhanced vaccine efficiency, which was measured in terms of cellular and humoral immunity, notably antibody titre and B-cell proportion in the head kidney (Pohlenz et al. 2012).

Immunostimulants/Dietary Supplements Enriched, i.e. 2 % (w/v) levels of dietary lysine fed to demand over 2 weeks led to resistance of channel catfish to *Edw. ictaluri* (Alejandro-Buentello and Gatlin 2001). Eya and Lovell (1998) reported the beneficial effect of dietary phosphorus at enhancing resistance of channel catfish to infection by *Edw. ictaluri.* Diets supplemented with 1–3 % of astaxanthin were fed to olive flounder for 15-days leading to immunostimulation and less mortalities after challenge (Kim et al. 2012).

Vitamins, i.e. A, C and E, in diets are of value for controlling infections by *Edw. ictaluri* (Lim et al. 2000). For example, vitamin E and iron sulphate, dosed at 2500 mg/kg and 60 mg/kg respectively, have been reported to be beneficial in enhancing the immune response of channel catfish, especially by improved phagocytosis, to *Edw. ictaluri* (Wise et al. 1993; Lim et al. 1996; Sealey et al. 1997).

A phytogenic feed additive (Digestarom), which contained the essential oils carvacrol, thymol, anethol, and limonene, was fed to cannel catfish for 6-weeks leading to greater survival after challenge (69.5 % survival compared with 48.4 % of the controls) (Peterson et al. 2015). Also, LPS and levamisole have been useful for immunostimulation and for conferring resistance against challenge albeit when administered by injection (Hang et al. 2014).

Antimicrobial Compounds Clinical cases of disease have been greatly reduced, although not completely eliminated, using oxytetracycline, dosed at 2.5 g of drug/45.4 kg body weight of fish/day for 4–5 days (Hawke 1979). Certainly, isolates have been found to be susceptible to a wide range of inhibitory compounds, including cefaperazone, cinoxacin, florfenicol, kanamycin, moxalactam, neomycin, nitrofurantoin, oxolinic acid, streptomycin, ticarcillin and trimethoprim (Waltman and Shotts 1986a; Gaunt et al. 2003). Yet, plasmid mediated resistance to antimicrobial compounds (including tetracycline) has been detected (Waltman et al. 1989). Therefore, problems with chemotherapy may be envisaged in the future.

Edwardsiella piscicida

Characteristics of the Disease

Edw. piscicida has been recognized in Asia, Europe and in catfish culture in the southeastern USA (Griffin et al. 2014). The organism was recovered from septicaemic farmed whitefish (*Coregonus lavaretus*) in Finland during 2000, 2002 and 2013 (Shafiei et al. 2016). The fish displayed exophthalmia, haemorrhaging on the skin, at the base of the fins, the gills, and in the muscle, swelling and hyperaemia of the anal area, and internal haemorrhages, and enlarged kidney and spleen (Shafiei et al. 2016).

Isolation

Isolation was achieved using TSA and 5 % (v/v) blood agar with incubation at 15 or 20 °C for 10 days (Shafiei et al. 2016).

Characteristics of the Pathogen

Box 6.4: *Edwardsiella piscicida*
The organism produces pinprick slightly convex, glistening colonies, that are slightly ß-haemolytic on TSA + blood agar. Optimum growth is at 85–30 °C, and not at 12 or 42 °C, and in 1–5 % (w/v) but not 6 % (w/v) sodium chloride. Cultures contain Gram-negative, facultatively anaerobic, straight motile rods. Catalase, H_2S, indole, and lysine and ornithine dcarboxylases are produced, but not arginine hydrolase, ß-galactosidase, oxidase or tryptophan deaminase. The methyl red test is positive, but the Voges Proskauer reaction is negative Acid is produced from glucose, but not from amygdalin, arabinose, inositol, mannitol, melibiose, rhamnose, saccharose or sorbitol. Citrate is not utilized. Neither gelatin nor urea is degraded (Abayneh et al. 2012).

Multilocus variable number tandem repeat analysis of 37 isolates resulted in the delineation of five major clusters consistent with their geographical origins (Abayneh et al. 2014).

Diagnosis

Examination of *gyrB* sequences revealed a >99.6 % homology between US isolates and the type strain leading to the development of a PCR to differentiate two genotypic groups, i.e. *Edw. piscicida* and *Edw. piscicida*-like organisms (Griffin et al. 2014). Individual RT-PCRs were developed to detect *Edw. tarda, Edw. piscicida* and *Edw. piscicida*-like organisms in channel catfish leading to sensitive (5 copies of the DNA in a 15 µl reaction; 10^2 to 10^3 CFU in ~25 g amounts of kidney tissue, 35 ml of pond water, or 20 l of broth culture), specific and reproducible assays (Reichley et al. 2015).

Pathogenicity

Pathogenicity experiments utilized i.m. injection of 3 µl of suspensions containing 10^6 CFU/ml of the cultures into zebra fish with 11.1-100 % mortalities recorded after 7-days (Abayneh et al. 2012).

Edwardsiella tarda (= Paracolobactrum anguillimortiferum, Edw.anguillimortifera)

Characteristics of the Disease

Edw. tarda has emerged with some frequency in the Far East, particularly Japan, Taiwan (Wakabayashi and Egusa 1973; Miyazaki and Egusa 1976; Kou 1981) and China (Xu and Zhang 2014; Qin et al. 2014; Mo et al. 2015). The history of edwardsiellosis, as the disease is called, may be traced to two separate parallel developments, i.e. those of Wakabayashi and Egusa (1973) in Japan, and Meyer and Bullock (1973) in the USA. The situation is further complicated by an earlier report of Hoshina (1962), who described *Paracolobactrum anguillimortiferum* as a pathogen of pond-cultured eels. This organism is probably synonymous with *Edw. tarda.*

Excellent descriptions of the clinical manifestation of *Edw. tarda* infections in channel catfish were published by Meyer and Bullock (1973). These authors reported that with mild infections, the only external signs of disease are the presence of small cutaneous lesions of approximately 3–5 mm in diameter, which are located in the postero-lateral region of the body. With progression of the disease, abscesses develop in the muscle of the body and tail. These abscesses may enlarge, and develop into gas-filled hollow areas. From the surface, these are seen as poorly pigmented, convex swollen areas which, if punctured, emit a foul odour. This condition has given rise to the name emphysematous putrefactive disease of catfish.

Although catastrophic losses of catfish have not been recorded, the disease has a severe economic effect on infected sites. When infected fish enter processing plants, the noxious odours effectively stop production by necessitating disinfection and deodorisation. Thus, heavy financial losses to the processors may result from the presence of only a small number of infected animals (Meyer and Bullock 1973).

Examination of naturally diseased tilapia has indicated a range of symptoms including loss of pigmentation, the presence of a swollen abdomen filled with ascitic fluid, protruded haemorrhaged anus and opaqueness to the eyes. Internally, small white nodules may be observed in the gills, kidney, liver and spleen, and occasionally the intestine. These nodules are packed with bacteria (Kubota et al. 1981).

Diseased turbot in Spain revealed the presence of eye tumefaction, haemorrhaging, inflammation (kidney, liver and spleen) and ascites (Padrós et al. 2006). Subsequently, the organism became associated with disease in farmed Senegalese sole in Spain (Castro et al. 2012).

In China, turbot exhibited one of two set of external signs – blackening of the dorsal surface on the posterior part of the body; and red cutaneous foci on the ventral surface. The kidneys were enlarged (Qin et al. 2014). Giant mottled eel (*Anguilla marmorata*) developed extreme ulceration with haemorrhaging, swelling of the kidney and liver, and liquefactive necrosis in the brain leading to high mortalities (Mo et al. 2015).

Isolation

Isolation of *Edw. tarda* from diseased fish is a straightforward procedure involving the use of commonly available media, such as TSA (Meyer and Bullock 1973; Alcaide et al. 2006) or BHIA (Amandi et al. 1982). On such media, small round (0.5 mm in diameter) raised, transparent colonies develop in 48 h at 24–26 °C (Meyer and Bullock 1973). The use of thioglycollate broth followed by subculturing on BHIA has also been used successfully (Appendix in Chap. 12; Amandi et al. 1982). Indeed, this two-step enrichment procedure has proved to be more sensitive than BHIA used alone. In one experiment, this two-step procedure enabled the recovery of *Edw. tarda* from 19 % of a group of chinook salmon, compared to only 2 % recovery on BHIA used alone (Amandi et al. 1982). A selective and differential medium, ET, which was originally published by Lindquist (1991), has enabled the recovery of *Edw. tarda* from mixed cultures following incubation at 25 °C for 24–48 h when transparent colonies with black centres were indicative of the pathogen (Appendix in Chap. 12; Castro et al. 2011).

Characteristics of the Pathogen

The organisms from fish match closely the description of *Edw. tarda* (Ewing et al. 1965; Cowan 1974; Farmer and McWhorter 1984), insofar as they are fairly reactive (Table 6.1) Gram-negative rods, motile by means of peritrichous flagella (Qin et al. 2014 reported nonmotile isolates from turbot in China). The original isolates of Meyer and Bullock (1973) were identified definitively as *Edw. tarda* by the CDC. Thereafter, each successive report emphasised the similarity to the species description of Ewing et al. (1965), highlighting the homogeneity of the fish isolates. In fact, it is astonishing how little variation has been recorded in the phenotypic characters; despite recovery from as far afield as Japan (Wakabayashi and Egusa 1973; Nakatsugawa 1983) and the USA (Amandi et al. 1982). The only difference reflects serology. Thus, four serotypes (A, B, C and D) have been recognised from O-agglutination analyses of 270/445 isolates recovered from eel ponds in Japan during 1980 and 1981 (Park et al. 1983). Among the cultures, serotype B predominated (22–35 % of the total), followed by serotype A (13–17 % of the isolates), serotype C (4–13 % of the isolates) and serotype D (2–4 % of the total). Most of the isolates in serotype A were derived from kidney samples. Moreover, this group was by far the most virulent as assessed from experimental infections of eels, loach and tilapia (Park et al. 1983) and natural outbreaks in Japanese flounder (Rashid et al. 1994a, b). It has been argued that isolates from turbot in Spain constitute a separate serological group (Castro et al. 2006). Examination of genomic diversity by BOX-A1R-based repetitive extragenic palindromic PCR (BOX-PCR) and PCR-ribotyping recovered 51 isolates into 32 and 27 genotypes, respectively. These were correspondingly defined in 9 and 8 clusters (Maiti et al. 2008).

The G+C ratio of the DNA was calculated as 59 moles % (Amandi et al. 1982).

Confusion has enveloped the taxonomic status of this pathogen, insofar as the 'Approved Lists of Bacterial Names' (Skerman et al. 1980) include two specific epithets for what is apparently the same organism, *Edw. tarda* and *Edw. anguillimortifera. Edw. tarda* was proposed initially by Ewing et al. (1965), and it would appear that there was a lack of familiarity with the earlier work of Hoshina (1962), who described *Paracolobactrum anguillimortiferum.* The characteristics of these two nomenspecies are identical (Table 6.1). Recognising the efforts of Hoshina (1962), Sakazaki and Tamura (1975) proposed that *Paracolobactrum anguillimortiferum* should be reclassified in the genus *Edwardsiella*, as *Edw. anguillimortifera.* Furthermore, Sakazaki and Tamura (1975) emphasised that priority should be attached to the specific epithet of *anguillimortiferum* rather than *tarda*. Thus two names have resulted for what is surely the same organism. Clearly, this is an unacceptable situation and, despite the pioneering work of Hoshina (1962), it is our contention that, to avoid further confusion in medical and veterinary microbiology, the name of *tarda* should be retained.

Table 6.1 Characteristics of *Edwardsiella tarda*[a] and *Paracolobactrum anguillimortiferum*[b]

Character	*Edw. tarda*	*Paracolobactrum anguillimortiferum*
Fermentative metabolism	+	+
Production of:		
Arginine dihydrolase	–	NS
Catalase	+	+
β-galactosidase	–	NS
H_2S	+	+
Indole	+	+
Lysine decarboxylase	+	NS
Ornithine decarboxylase	+	NS
Oxidase	–	NS
Phenylalanine deaminase	–	NS
Methyl red test	+	NS
Nitrate reduction	+	+
Voges Proskauer reaction	–	–
Degradation of:		
Aesculin, casein, gelatin	–	–
Tributyrin, urea	–	NS
DNA, elastin, lecithin	–	–
Tween 20, 40, 60 and 80	–	–
Chitin	–	–
Blood	+	+
Utilisation of:		
Sodium citrate, sodium malonate	–	NS
Growth at 42 °C	+	+
Growth in 3 % (w/v) sodium chloride	+	+
Growth in 8 % (w/v) sodium chloride	–	–
Acid production from:		
Adonitol, aesculin, erythritol	–	NS
Arabinose, cellobiose, dulcitol	–	–
Fructose, galactose, glycerol	+	NS
Glucose, maltose,	+	+
Inositol,	–	NS
Lactose, mannitol, raffinose	–	–
Mannose	+	NS
Rhamnose, salicin, sorbitol	–	–
Sucrose, trehalose, xylose	–	–

[a]From Sakazaki and Tamura (1975), Wakabayashi and Egusa (1973), Amandi et al. (1982) and Nakatsugawa (1983)
[b]From Hoshina (1962)
NS not stated

Diagnosis

Serology FAT has been found useful for diagnosing *Edw. tarda* and determining the presence of the pathogen in infected fish tissues (Amandi et al. 1982). Horiuchi et al. (1980) considered FAT as being extremely valuable for field diagnoses. iFAT with monoclonal antibodies in an enzyme immunoassay is also useful for diagnosis (Rogers 1981). An ELISA, which detects OMPs, particularly a common 44 kDa protein, has been described (Kumar et al. 2007).

Molecular Methods PCR has been developed for the detection of *Edw. tarda* and its differentiation from *Edw. ictaluri* (Williams and Lawrence 2009). LAMP has been proposed for the rapid and sensitive detection of *Edw. tarda* (Savan et al. 2004; Xie et al. 2013). Developed to detect the pathogen in Japanese flounder kidney and spleen (and seawater), results were achieved in 45 min. An indication of sensitivity is that the LAMP technique was positive for seawater containing 3.2×10^2 CFU of *Edw. tarda* (Savan et al. 2004). Xie et al. (2013) targeted the upstream region of the *hlyb* gene, and reported that their test was 100-times more sensitive than a conventional PCR, i.e. detected 10 compared to 10^3 CFU/test, could be recorded visually or by the addition of a fluorescent marker, and was specific to *Edw. tarda*. Individual RT-PCRs were developed to detect *Edw. tarda, Edw. piscicida* and *Edw. piscicida*-like organisms in channel catfish leading to sensitive (5 copies of the DNA in a 15 µl reaction; 10^2 to 10^3 CFU in ~25 g amounts of kidney tissue, 35 ml of pond water, or 20 l of broth culture), specific and reproducible assays (Reichley et al. 2015).

Epizootiology

The precise source of infection is often unknown. However, between 2002 and 2004, *Edw. tarda* was recovered from seawater and the kidney and spleen of healthy Japanese flounder when disease was occurring in farmed fish populations. It was apparent that the antibody titre increased rapidly in the run-up to outbreaks of clinical disease. Interestingly, bacteriophage was found in the seawater at least 1 month before the onset of and during the period of the disease outbreaks, but not afterwards. It was speculated that the presence of bacteriophage could be used as an indicator for *Edw. tarda* cells (Matsuoka and Nakai 2004). Also, there has been speculation that snakes or faecal contamination from humans or other animals may have been involved in the first documented outbreak (Meyer and Bullock 1973). Certainly, experimentally infected fish (= Japanese flounder) were determined to shed the pathogen 1–6 days before death, with the number of bacterial cells shed from newly dead (and for several days afterwards) fish reaching 10^7–10^8 CFU/min. Of relevance these cells were more virulent than their counterparts from TSA. Such bacteria, which are discharged from diseased/dead fish, may well have importance in the spread of disease among fish populations (Matsuoka 2004). Environmental

parameters, namely water temperature and the quantity of organic matter in the water, undoubtedly influence the severity of outbreaks. In particular, it is noteworthy that most disease outbreaks occur at high water temperature, i.e. 30 °C, when high levels of organic matter are present. However, data showed that the incidence of disease in catfish ponds rarely exceeded 5 %, except when infected fish were moved to holding tanks. During these periods, edwardsiellosis became rampant with total mortalities approaching 50 % of the population (Meyer and Bullock 1973).

There is some evidence that *Edw. tarda* occurs in sediment (Rashid et al. 1994a) and water within the vicinity of fish farms (Minagawa et al. 1983; Park et al. 1983; Rashid et al. 1994a). It has been reported to inhabit aquatic invertebrates, such as the freshwater aquarium snail (*Ampullaria* sp.; Bartlett and Trust 1976) and sea-urchin (Sasaki and Aita 1975), and has been associated with vertebrates, namely snakes (Iverson 1971), frogs (Sharma et al. 1974), turtles (Otis and Behler 1973), gulls (Berg and Anderson 1972) and human beings (Koshi and Lalitha 1976). Any one of these hosts may serve as a reservoir for infections of fish. However, it is unclear whether *Edw. tarda* should be regarded as a primary or opportunistic pathogen of fish. Indeed, it may comprise part of the normal microflora of fish surfaces, insofar as Wyatt et al. (1979) have indicated the widespread occurrence of the micro-organism in and around channel catfish. A suggestion has been made that the organism becomes non-culturable in the aquatic environment (Sakai et al. 1994). Experimentally, a VBNC state was induced following culturing in aged seawater at 4 °C when total counts remained constant over 28 days [cells became coccoid, and decreased in size) whereas culturability declined to undetectable levels. Resuscitation was possible in chick embryos at higher temperatures, and these cells killed turbot following i.p. injection (Du et al. 2007).

The presence of sub-lethal concentrations of copper (100–250 µg/l) in water leads to increased susceptibility of Japanese eels to infection (Mushiake et al. 1985). This increased susceptibility seems likely to be attributed to a reduction in lymphocytes and granulocytes in the blood, leading to lowered phagocytosis (Mushiake et al. 1985).

Pathogenicity

To date, the disease has been recorded in a diverse array of fish species, including chinook salmon (Amandi et al. 1982), channel catfish (Meyer and Bullock 1973), mullet (Kusuda et al. 1976), carp (Sae-Oui et al. 1984), eels (Wakabayashi and Egusa 1973; Mo et al. 2015), tilapia (Kubota et al. 1981), olive flounder (Han et al. 2006) and flounder (Nakatsugawa 1983; Mekuchi et al. 1995a; Pakingking et al. 2003). From laboratory-based experiments, pathogenicity has also been demonstrated in steelhead and rainbow trout (Amandi et al. 1982), yellowtail (Nakatsugawa 1983) and loach (Park et al. 1983). Co-infection of *Edw. tarda* with aquabirnavirus has led to higher mortalities in Japanese flounder (Pakingking et al. 2003).

There has been no difficulty achieving experimental infections of fish with *Edw. tarda.* Thus, using channel catfish, Meyer and Bullock (1973) established an infection by using the i.p. route of administration. At a water temperature of 27 °C, deaths in 80 % of the population of 5–10 cm long fingerling fish followed within 10 days of injecting an artificially high dose of 8.0×10^7 cells. Similar experiments, with 8×10^6 and 8×10^5 cells resulted in only 40 % cumulative mortalities within 10 days. However, these workers pointed to the host specificity of *Edw. tarda*, insofar as brown trout, held at a water temperature of 13 °C, did not show any mortalities following injection with the pathogen. Subsequent investigations, however, showed that *Edw. tarda* could indeed infect salmonids. Thus Amandi et al. (1982) demonstrated that the LD_{50} for chinook salmon and steelhead trout, was 4.1×10^6 and 5.6×10^6 cells, respectively. It is worth emphasising that ictalurids were determined to be more sensitive than salmonids, insofar as these workers determined the LD_{50} for channel catfish to be only 4.0×10^5 cells. At lower water temperatures, e.g. 12 °C, the LD_{50} was in the region of one order of magnitude higher. Ironically, a water-borne challenge proved to be a failure, although this may reflect the inability of cells, grown in nutrient-rich media and therefore not typical of the natural physiological state, to survive in the aquatic environment. Nevertheless, Song et al. (1982) deduced that of 110 isolates, the lowest LD_{50} (by water-borne challenge) was 3.1×10^7 cells/ml. Clearly, this is not conducive to the notion of a particularly virulent pathogen.

Using immersion, oral, and i.p. and i.m. routes, Mekuchi et al. (1995a) succeeded in infecting and killing Japanese flounder (by all routes). The LD_{50} dose was calculated at 7.1×10^1/fish (via i.m. injection), 1.2×10^2/fish (via the i.p. route), 3.6×10^6/fish (by immersion) and 1.3×10^6/fish (orally) (Mekuchi et al. 1995a)

The pathogenic mechanisms were investigated by Ullah and Arai (1983a,b), who reported that in contrast to *Edw. ictaluri,* pili were absent. Instead, cells were observed to be surrounded by a slime layer. This may help with the adhesion to host cells, and also protect the bacteria from host defences. Conversely, Sakai et al (2004) pointed to a role for fimbriae on haemagglutination (this could also be induced or increased by 3 % w/v NaCl; Yasunobu et al. 2006). A relation between motility and virulence was indicated by Matsuyama et al. (2005), who observed that non-motile (and not motile) cells were pathogenic by i.p. injection to red sea bream (there was not any difference between motile and non-motile cultures recorded in Japanese flounder or yellowtail). Following immersion challenge, Japanese flounder and red sea bream died as exposure to motile and non-motile cultures, respectively (Matsuyama et al. 2005). Opsonised virulent, but not avirulent, cells adhered, survived and even replicated in phagocytes whereas non opsonised avirulent cells could also replicate intracellularly in phagocytes (Rao et al. 2001). Furthermore, only avirulent cells enabled higher production of ROS intermediates by phagocytes, which suggests that they are more likely to be susceptible to inactivation by this means (Rao et al. 2001). A link to LPS, namely the O-antigen ligase gene *waaL,* has been made insofar as deletions mutants were attenuated in terms of virulence, and with markedly increased LD_{50} dose (Xu et al. 2010). Furthermore, a comparison of virulent and avirulent cultures pointed to the importance of LPS in pathogenicity,

with lipid A as a biologically active determinant (Wang et al. 2010). Mutation of the tryptophanase gene, *tnaA,* has been correlated with reduced virulence in zebra fish, with a *tnaA* deletion mutant leading to a ~55 increase in LD_{50} dose (Han et al. 2012). A type III secretion system has been documented (Xie et al. 2010) and its presence linked to virulence notably in connection with the intracellular replication of the pathogen in phagocytes (Rao et al. 2004; Okuda et al. 2009*;* Li et al. 2011c); defective mutants having reduced virulence (Tan et al. 2005). Secreted proteins of the type III secretion system were of 22, 25 and 55 kDa, being identified as EseD, EseC and EseB, and may function as a translocon (Okuda et al. 2009). Pathogenic and non-pathogenic cultures have been differentiated on the basis of detection of a type III secretion system using a PCR targeting the type III component gene *esaV* with the result that 18 pathogenic cultures were positive but not so the same number of non-pathogenic isolates (Li et al. 2011). It has been proposed that the type III secretion system (T3SS), which is involved in translocating proteins involved with virulence into the host cell, facilitates survival and replication of *Edw. tarda* in macrophages with the pathogen causing caspase-1-mediated macrophage death (= pyroptosis) and enhanced secretion of interleukin 1ß (Xie et al. 2014). Deletion of the flagellin gene, *fliC,* decreased macrophage cytotoxicity, but did not decrease virulence. The proposal was made that *Edw. tarda* evades the innate immune response of the host by downregulating the expression of *FliC* (Xie et al. 2014). Also, a type VI secretion system (T6SS) has been reported (Nakamura et al. 2014).

ECPs have been attributed with stimulating a chemotactic and chemokinetic response by macrophages that may be involved in the inflammatory response by infected fish (Widenemayer et al. 2008). Variable OMP patterns, including some major (25–40 kDa) and many minor proteins (~10-120 kDa), have been identified in isolates when cultured at 25 °C. Interestingly, salinity affected OMP composition in some cultures, suggesting heterogeneity in the taxon (Darwish et al. 2001). Four immuno-reactive OMPs have been detected, and using matrix-assisted laser desorption/ionization mass spectrometry analyses, two of these were identified as OmpA and murein lipoprotein (Yu et al. 2013).

Haemolysins and dermatotoxins, but not lipases or proteolytic enzymes, were produced *in vitro*, and it was postulated that these exo-enzymes may confer pathogenicity on *Edw. tarda* (Ullah and Arai 1983a,b). Furthermore, the pathogenic role of dermatotoxins was highlighted in additional experiments (Ullah and Arai 1983b). This work concluded that two high molecular weight, heat sensitive dermatotoxins were produced, which in rabbits (not fish!) were found to have separate functions. Thus, one toxin caused erythema within 3–8 h of intracutaneous injection, whereas the second caused oedema followed by necrotic erythema in 5–7 days. Hopefully at some point, this work will be repeated in fish. Proteolytic toxins (molecular weight = 37 kDa), from ECP, have been purified from avirulent cultures (the toxin is not present in avirulent isolates) and the LD_{50} equated to 1.6 g of toxin/g of fish (Suprapto et al. 1996). The organism has been associated with the development of liver hypertrophy following experimental infection of Japanese flounder (Miwa and Mano 2000).

On iron-deficient medium, many *Edw. tarda* cultures, notably those associated with virulence, produced siderophores (Kokubo et al. 1990; Mathew et al. 2001; Igarishi et al. 2002) and OMPs of which one was considered to be the receptor for the siderophore under iron-limited conditions. Such components permit the pathogen to scavenge for iron in the blood of the host. Certainly, it appears that the ability of *Edw. tarda* to acquire iron is an important part of the infection process (Park 1986; Iida and Wakabayashi 1990), and it is relevant that an iron-regulated haemolysin gene has been reported (Hirono et al. 1997a). The virulent strains are more resistant to the bacteriostasis of iron-chelating reagents than their avirulent counterparts. There is an indication that exposure to copper attenuate virulence (Hu et al. 2010).

A 70 kb plasmid, which codes antibiotic resistance has been linked to pathogenicity insofar as curing of the plasmid led to attenuation of virulence in goldfish and zebra fish models (Yu et al. 2012).

Typical of many bacterial pathogens, *Edw. tarda* adheres to host cells before internalization, which involves microfilaments and protein tyrosine kinase (Ling et al. 2000). Using green fluorescent protein (GFP) tagged cells, the portals of entry after immersion challenge were identified as the digestive tract, i.e. anterior intestine, gills and body surface of blue gourami (Ling et al. 2000). The bacteria located in these sites plus blood, heart, kidney, liver, muscle, posterior intestine and spleen after 3 days, but declined substantially by 7 days, with only substantial populations remaining in the intestine. Bacterial OMPS, of which 8 have been recognised as interacting with 12 fish gills proteins, have been described (Liu et al. 2012). The *evpP* gene, which encodes the T6SS secretory protein, EvpP, has been described as essential for invasion of host cells (Zhang et al. 2014).

Disease Control

Disease Resistant Fish The differential resistance of rohu families has been discussed (Mohanty et al. 2012).

Vaccine Development Prophylaxis by vaccination has been successful (e.g. Gutierrez and Miyazaki 1994; Kwon et al. 2006, 2007; Sun et al. 2010a; Takano et al. 2011), with oral administration of immunostimulants, namely ß-glucan, levamisole and vitamins C an E heightening protection further, especially if harmful (aflatoxin B_1 was identified) conditions prevailed (Sahoo and Mukherjee 2002). Eels responded to the administration of heat- or formalin-killed cells (preferably by injection) by producing humoral antibodies with titres of up to 1:4096 (Song and Kou 1981). Song et al. (1982) vaccinated eels by immersion for periods of 20 s to 3 min in suspensions containing 10^5 to 10^8 bacterial cells/ml. A trivalent formalin-inactivated vaccine comprising *Edw. tarda, Str. iniae* and *Str. parauberis,* protected olive flounder when administered intraperitoneally in 0.1 ml amounts (Han et al. 2011). Furthermore, Takano et al. (2011) described the success of using

formalin-inactivated whole cells of atypical *Edw. tarda,* administered intraperitoneally without or without oily adjuvant, to protect red sea bream. Following the i.m. injection of 10 mg amounts of formalised cells/100 g of Japanese eels and a booster after 7 days, an oral challenge resulted in 60–87.5 % mortalities in the vaccinates, compared to 80–100 % deaths among controls (Gutierrez and Miyazaki 1994). Rather better protection resulted from use 1 mg of LPS/100 g body weight of Japanese eels. With this vaccine, 40–57 % mortalities were recorded compared to 80–90 % of the controls (Gutierrez and Miyazaki 1994). Oral vaccination at an equivalent of 5×10^8 cells/fish/day for two 5-day periods separated by 2 weeks using ghost cells, which were produced from lysed cultures with the lysis plasmid pλ PR cI-Elysis, protected olive flounder (*Paralichthys olivaceus*) (Kwon et al. 2007). ECPs have been found to modulate innate immunity, and may well become a candidate for future vaccine studies (Lee et al. 2007).

Unfortunately, Mekuchi et al. 1995b) did not find any clear sign of protection in Japanese flounder, that had been vaccinated with formalised cells via the i.m., oral or immersion routes. Other work has doubted the importance of cell-mediated immunity in the protection of fish against disease (Miyazaki and Egusa 1976). This is perhaps surprising in view of the current opinions concerning fish immunology.

Intramuscular injection of eels and red sea bream with LPS resulted in protection from challenge with a virulent culture of *Edw. tarda* (Salati et al. 1987a, b). Moreover, there was a demonstrable humoral immune response (titre = 1:2048) and phagocytosis by T-lymphocytes. Phagocytic activity in the eels peaked 3 weeks after vaccination (Kusuda and Taira 1990). Indeed, the evidence showed that LPS was much more successful as an immunogen than vaccination with a formalised culture (Salati et al. 1987a, b).

Igarashi and Iida (2002) used live attenuated and formalin-inactivated cells of a mutant, SPM31, constructed with transposon Tn5 with reduced siderophore producing capability. Tilapia were vaccinated intraperitoneally (0.1 mg of vaccine/100 g of fish) whereupon antibodies were produced and protection recorded after challenge for the live (0 % mortality) but not the formalin-inactivated (mortality = 80–100 %) preparation (Igarashi and Iida 2002). Interest in live attenuated vaccines continued with the work of Li et al. (Li et al. 2015), who deleted *esrB, esaC, evpH, rpoS* and *purA* genes thereby establishing 5 double- and 3 triple gene mutants, which were markedly less virulent in blue gourami (*Trichogaster trichopterus*). Delta *aroA Delta esrB* mutant led to the highest RPS of 82.6 %, and thus was evaluated by immersion or i.m. injection in flounder (*Paralichthys olivaceus*) leading to persistence in the animals for 14–35 days with higher antibody titres compared with the controls. After challenge, the i.m. and immersion vaccinated fish achieved RPS values of 14.3–66.7 % and 100 %, respectively, pointing to the value of live attenuated vaccines for the control of edwardsiellosis (Li et al. 2015). Work has been carried out to determine the host response to administered live attenuated cells. Thus using zebra fish as a model, Yang et al. (2013) During the immunization phase, the toll-like receptor (TLR) 5 signaling and the MHC-I antigen processing pathways and cytotoxic T-lymphocyte responses were activated, in succession.

However, there was down-regulation of the MHC-II antigen processing pathway and the markers of CD4(+) T-lymphocyte activation. IgM antibody titres were not significantly induced following this phase (Yang et al. 2013).

Jiao et al. (2009) described the use of two antigens, Eta6 (= ecotin precursor) and FliC, (= FliC flagellin), the former of which was moderately protective in Japanese flounder (RPS = 53 %) when administered in 100 µg amounts by i.p. injection with *Bacillus* sp. B187 as an adjuvant with boosters after 20 days. Challenge was only 14 days later. DNA vaccines, centred around Eta6 and FliC, i.e. *pEta6*, and *pFliC* respectively, gave protection in terms of RPS of 50 % and 33 % respectively, when injected i.m. as 100 µg amounts in PBS (Jiao et al. 2009). A chimeric DNA vaccine, i.e. *eta6* covalently linked to *FliC,* led to superior protection, i.e. RPS = 72 % (Jiao et al. 2009). A fraction of flagellin, FliC, termed N163, which is the conserved N-terminal 163 residues of FliC, has been proposed as an effective adjuvant for use in Japanese flounder (Jiao et al. 2010a). Sun et al. (2010a) proposed use of a recombinant surface protein, Esa1, of 795 amino acid residues, which led to protection of Japanese flounder when administered orally in alginate microspheres (RPS = 52 %) and by i.p. injection (RPS = 79 %). Furthermore, Kwon et al. (2006) used ghost cells, which were generated by gene *E* mediated lysis, in tilapia, and demonstrated high protection.

By multiple passaging through laboratory media, Sun et al. (2010b) isolated a poorly virulent (= attenuated) strain, TX5RM, which was immunoprotective in Japanese flounder following application by i.p. injection (100 µl containing 10^8 CFU/ml), immersion, (8 h in 10^8 CFU/ml) orally (10^{10} cells/g of feed for 5 days), and orally plus immersion. With the exception of injection uptake, the other groups were administered booster after 3-weeks. Challenge was after 5 or 8 weeks. Oral administration plus immersion led to the highest RPS of 80.6 % at 5-weeks after vaccination, which decreased to 69.4 % at 8-weeks (Sun et al. 2010b). In comparison, the injectable vaccine led to an RPS of 67.7 % at 5-weeks. By the oral route and immersion alone, the RPS values were 61.3 % and 71 %, respectively at 5-weeks with a decline in protection at 8-weeks. (Sun et al. 2010b).

Eta2 is a protein with sequence identity to OMP and was prepared as a recombinant protein in *Esch. coli.* Eta2 was administered in aluminium hydroxide adjuvant by i.p. injection as a subunit vaccine to Japanese flounder and elicited protection against challenge at 4-weeks of RPS = 83 % (Sun et al. 2011). Meanwhile, using Eta2 as a DNA vaccine (plasmid pCEta2) was administered by i.m. injection and also protective (RPS = 67 %) after challenge at 4-weeks (Sun et al. 2011). The adhesion gene from *Aer. hydrophila*, which belonged to the maltoporin group of porins coding for OMP, Omp48, was cloned and sequenced. The recombinant protein of ~ 48 kDa molecular weight was administered to rohu and upon challenge with *Edw. tarda* resulted in protection (RPS = 60 %). Thus, it was apparent that *Aer. hydrophila* conferred broad spectrum protection against *Edw. tarda* (Khushiramani et al. 2012).

A natural avirulent isolate ATCC 15947 was successful when applied by i.p. injection [100 µl containing ~4×10^8 CFU/ml] [RPS = 79 %] especially with a booster 3 weeks later [RPS = 100 %] to Japanese flounder. High levels of protection

were maintained for 12 weeks [RPS = 81 %]. Furthermore, some success resulted from oral application for 5 days [RPS = 56 %] when incorporated in alginate microspheres [dosed at 10^9 CFU/fish/day], with challenge 5 weeks later, but less so for immersion vaccination at 10^8 CFU/ml for 15 h [RPS = 21 %] (Cheng et al. 2010b). Hu et al. (2011) continued the work by developing a recombinant product that expressed *V. harveyi* DegQ as a soluble antigen that elicited significant protection against both *Edw. tarda* and *V. harveyi* in laboratory-based experiments with turbot when administered by i.p. (RPS = 89.2 %) orally (RPS = 60.1 %) or immersion (RPS = 42.1 %) or a combination of oral plus immersion (RPS = 66.1 % after 1 month in a mock field trial; RPS = 75 % after 2 months).

Interest in live attenuated vaccines continued with the study of Xiao et al. (2011) who used a deletion mutant of the *aroC* gene, which is involved in the biosynthesis of chorismic acid. This was applied intramuscularly in 5 µl amounts [4×10^7 CFU/fish] to zebra fish with RPS of 68.3–81 % resulting after challenge 5-weeks later. A temperature-sensitive mutant was generated by replacement of the *alr* gene promoter, and upon use as a primary and booster vaccine in olive flounder gave 100 % protection after challenge (Choi et al. 2012).

An auxotrophic mutant (*Δalr Δasd E. tarda*) was administered orally (10^9 CFU/fish) to olive flounder which were completely protected against challenge (Choi et al. 2011).

A pressure (600 kgf/cm^2 for 5 s using a French press) inactivated whole cell preparation adjusted with 0.85 % (w/v) saline to contain 10^9 cells/ml administered as 0.1 ml amount by i.p. led to an RPS of >85 % in Japanese eels, 6-months after vaccination (Hossain and Kawai 2009).

Consideration has been given to the comparative efficacy of adjuvants in injectable vaccines based on a weakly protective antigen, Et49, with research evaluating the effects of aluminium hydroxide, aluminium phosphate and FIA in Japanese flounder. Results demonstrated that the adjuvants increased the RPS by 19 %, 35 % and 47 %, respectively (Jiao et al. 2010b).

Pseudomonas P1SW and *Vibrio* V3SW were recovered from seawater and administered live separately or together by immersion and orally (the bacteria were embedded in sodium alginate microspheres) to turbot (*Scophthalmus maximus*) at doses equivalent to 2×10^8 CFU with evidence that both organisms became distributed in the internal organs, and some protection became apparent after challenge. Better protection was reported with both bacteria administered together (Wang et al. 2013). Vaccines or probiotics?

Immunostimulants/Dietary Supplements Yano et al. (1989) published data that showed ß-1,3 glucans, when applied by i.p. injection at 2–10 mg/kg of fish, enhanced resistance to infection by *Edw. tarda.* This effect was measured by heightened phagocytic activity. Durve and Lovell (1982) and Misra et al. (2007) pointed to a role for vitamin C in immunostimulation and protection against challenge with *Edw. tarda.*

Incorporated into a purified basal medium (Table 6.2), vitamin C (dosed at 150 mg/kg of food) dramatically increased the resistance of catfish to *Edw. tarda*

Table 6.2 Composition of the purified basal medium to which different concentrations of vitamin C at 0–150 mk/kg were added[a]

Ingredient	% Composition
Carboxymethyl-cellulose	3.0
Cellulose	10.0
Cod liver oil (contains 850 IU of vitamin A and 85 IU of vitamin D/g)	3.0
Dextrin	33.05
Gelatin	9.4
Mineral mix of Williams and Briggs (1963)	
Supplemented with cobalt chloride (1 mg/kg of diet), aluminium	
Potassium sulphate (0.7 mg/kg of diet) and sodium selenite (0.08	
mg/kg of diet)	4.0
Soybean oil	4.0
Vitamin-free casein	32.6
Vitamin mix[b] (minus vitamin C)	0.95

[a]from Durve and Lovell (1982)

[b]this contains thiamin (10 mg/kg of diet), riboflavin (20 mg/kg of diet), pyridoxine (10 mg/kg of diet), folic acid (5 mg/kg of diet), calcium pantothenate (40 mg/kg of diet), choline chloride (3000 mg/kg of diet), niacin (150 mg/kg of diet), vitamin B_{12} (0.6 mg/kg of diet), retinyl acetate (5000000 IU/kg of diet), α-tocopherol (50 mg/kg of diet), cholecalciferol (1000000 ICU/g) (6 mg/kg of diet), menadione sodium bisulphite (80 mg/kg of diet), inositol (400 mg/kg of diet), biotin (2 mg/kg of diet) and ethoxyquin (200 mg/kg of diet)

infections. This was carried out at a water temperature of 23 °C, but curiously this observation was not confirmed at higher temperatures, e.g. 33 °C, when less resistance to infection was noted (Durve and Lovell 1982). Control groups of fish held at 23 °C and fed with diets devoid of vitamin C, all died within 96 h of infection with 10^3 cells administered via the i.p. route. With only 30 mg of vitamin C/kg of diet, 85 % mortality resulted in the recipient fish after challenge. These mortalities were reduced to 60 % and 20 % following administration of 60 mg and 150 mg of vitamin C/kg of diet, respectively. However, it is relevant to enquire whether or not the effective dose should be higher. Studies with channel catfish have shown than only 30 mg of vitamin C/kg of diet was sufficient to prevent vitamin deficiencies, which manifest themselves as scoliosis and lordosis. Doubling the dose to 60 mg of vitamin C/kg of diet enabled the maximum rate of wound repair in channel catfish (Lim and Lovell 1978). As might be expected, there is some variation in the precise levels of vitamin C needed for nutritional requirement among various species of fish. Among the salmonids, rainbow trout needed 100 mg of vitamin C/kg of diet for normal growth, but a tenfold increase enabled maximum wound repair to occur (Halver et al. 1969). The precise effect of vitamin C on retarding bacterial infections remains largely unknown, although several mechanisms have been postulated. Perhaps, leucocytic or phagocytic activity is stimulated, or synthesis and release of glucocorticoids enhanced (Durve and Lovell 1982).

Injection of 0.25 or 0.5 μg/fish of synthetic cytidine-phosphate-guanosine (CpG) oligodeoxynucleotide (ODN) into olive flounder led to higher chemiluminescence by phagocytes; supernatants from leucocytes, which received CpG ODN as a pulse, induced much higher respiratory burst activity after 3–7 days. Additionally, the fish which received CpG ODN were better protected against challenge with *Edw. tarda* (mortality = 17 %) compared to the controls (mortality -= 92 %) (Lee et al. 2003).

The blue green algae, *Spirulina pacifica*, was added to diet to achieve levels of 3, 6 or 9 %, and fed to olive flounder for 15 days before challenge when there was evidence of immunostimulation and protection against *Edw. tarda* (Kim et al. 2015).

Probiotics Citrus byproducts were fermented with probiotics, particularly *Bac. subtilis*, *Enterococcus faecium,* and fed to juvenile olive flounder for 10-weeks leading to resistance with challenge with a pathogenic culture and immunostimulation (lysozyme and myeloperoxidase activities) (Lee et al. 2013).

Antimicrobial Compounds It was reported that isolates were highly susceptible to cinoxacin, nitrofurantoin, oxolinic acid, kanamycin, moxalactam, trimethoprim, piperacillin, potentiated sulphonamides, neomycin, mezlocillin and streptomycin, but not to colistin, cloxacillin, clindamycin, bacitracin, erythromycin, lincomycin, methicillin, penicillin G, novobiocin or spectinomycin (Waltman and Shotts 1986b). Chemotherapy by means of oxytetracycline, dosed at 55 mg of drug/kg body weight of fish/day for 10 days, has been recommended (Meyer and Bullock 1973). Assuming that infected fish consume the medicated diet, mortalities apparently dwindle away within 48–72 h of initiating treatment. However, recovery is slow, and the survivors may exhibit scar tissue. A complication with chemotherapy concerns R plasmids which have been demonstrated in cultures of *Edw. tarda* isolated from eels (Aoki et al. 1977). Conceivably, this may cause problems for chemotherapy in the future.

Enterobacter cloacae

Characteristics of the Disease

The organism was recovered from diseased mullet (*Mugil cephalus*) from India (Sekar et al. 2008).

Isolation

Recovery was on TSA and MacConkey agar with incubation at 30 and 37 °C for 48 h (Sekar et al. 2008).

Characteristics of the Pathogen

Box 6.5: *Enterobacter cloacae*
Cultures comprise Gram-negative rod-shaped fermentative organisms motile rods. Catalase, arginine dihydrolase and ornithine decarboxylase is produced but not H_2S, lysine decarboxylase or oxidase (Sekar et al. 2008).

By 16S rRNA sequencing there was 100 % homology with *Ent. cloacae* (Sekar et al. 2008).

Pathogenicity

Bath challenge resulted with 8.6×10^7 cells/ml for 1 h when 100 % of the mullet died within 5 days at 30 °C. An unspecified cationic factor was linked to pathogenicity (Sekar et al. 2008).

Escherichia vulneris

Characteristics of the Disease

An organism, subsequently identified as *Esch. vulneris,* was first isolated in 1994 from naturally infected balloon moly (*Poecilia* sp.), silver moly (*Poecilia* sp.) and Caucasian carp (*Carassius* sp.) from Turkey (Aydin et al. 1997). Clinical signs included haemorrhagic lesions on the skin, pale gills, digestive tract full of bloody exudate, haemorrhaging in the gonads, and yellow liver with hyperaemic areas (Aydin et al. 1997).

Isolation

Isolates were recovered on TSA with incubation at 25 °C for 48 h (Aydin et al. 1997).

Characteristics of the Pathogen

Box 6.6: *Escherichia vulneris*
Cultures comprise Gram-negative, motile, fermentative rods, which produce arginine dihydrolase, catalase and lysine decarboxylase but not H_2S, indole, oxidase or ornithine decarboxylase, degrade aesculin and blood, but not casein, DNA, gelatin, starch or urea, reduce nitrates, utilise malonate, and produce acid from arabinose, glucose, lactose, maltose, mannitol, melibiose, rhamnose, salicin, trehalose and xylose, but not adonitol, cellobiose, dulcitol, glycerol, inositol, sorbitol or sucrose. The methyl red test is positive, but the Voges Proskauer reaction is negative. Growth occurs at 37 °C but not at 5 or 42 °C.

The organisms coincided with the description of *Esch. vulneris* (Brenner et al. 1982).

Pathogenicity

There is scant information about the pathogenicity of *Esch. vulneris.* It would appear that infection was achieved in rainbow trout of 120 g in weight with death ensuing in 175 h (Aydin et al. 1997). Details about dosages were not included in the original publication.

Hafnia alvei

Characteristics of the Disease

The disease was described as a haemorrhagic septicaemia (Orozova et al. 2014). Fish became darker, moved slowly, developed exophthalmia, haemorrhaging in the eye and petechial haemorrhages on the body surface. Internally, petechial haemorrhages were apparent on the spleen (enlarged) and kidney (hyperemic). The disease appeared in fish after transportation or during cultivation in inappropriate conditions (Gelev and Gelev 1988).

A second outbreak occurred in cherry salmon (*O. masou*) on Japanese farms. Here, the disease was characterised by melanosis, swollen abdomen, and slow swimming. There were grey/white furuncles on the kidney (Teshima et al. 1992).

Isolation

Cultures were obtained from the internal organs and subcutaneous tissues at the base of the pelvic fins on BHIA and nutrient agar supplemented with 10 % (v/v) sheep blood following incubation at an unspecified temperature for 48 h, whereupon small, round, smooth colonies developed. Teshima et al. (1992) isolated the organism on heart infusion agar following incubation at 30 °C for 2 days.

Characteristics of the Pathogen

Box 6.7: *Hafnia alvei*
The isolate comprises small coccoid or slightly elongated motile Gram-negative rods which produce catalase, ß-galactosidase and lysine and ornithine decarboxylase, but not arginine dihydrolase, H_2S, indole or oxidase. The methyl red, nitrate reduction and citrate utilisation tests and Voges Proskauer reaction (weakly positive) are positive, but not malonate utilisation or phenylalanine deaminase. Neither aesculin, DNA, gelatin, lipids nor urea is degraded. Acid is produced from arabinose, cellobiose, glucose (but not gas), glycerol, maltose, mannose, mannitol, rhamnose, trehalose and xylose, but not from adonitol, dulcitol, erythritol, lactose, melibiose, raffinose, salicin, sorbitol or sucrose. Growth occurs at 41 °C.

In 1988, an apparently new *Brucella* – like bacterium was described as a pathogen of rainbow trout in Bulgaria (Gelev and Gelev 1988). Although antigenic relationships to *Brucella abortus* were reported, the organism displayed marked similarities to the Enterobacteriaceae. Subsequent investigation led to a realisation that the organism was, in fact, *Hafnia alvei* (Gelev et al. 1990). Subsequently, *Haf. alvei* was recognised as the cause of mortalities among cherry salmon (*O. masou*) in Japanese farms (Teshima et al. 1992). Isolates matched the species description, with the exception of lack of motility and utilisation of D-tartrate. As a word of caution, *Haf. alvei* may be confused with *Y. ruckeri* especially if reliance is based on some phenotypic diagnostic procedures.

It was deemed that there was a common antigenic determinant in the LPS with *Brucella abortus* and *Y. ruckeri* (Gelev and Gelev 1988). However, the presence of motility and the negative reaction in the oxidase test precludes a relationship with *bona fide Brucella* taxa. There was 82–100 % re-association between the fish isolates and *Haf. alvei* (Gelev et al. 1990).

Pathogenicity

The culture caused clinical disease in laboratory experiments with rainbow trout. Injection (subcutaneous) of rainbow trout, weighing 150–200 g, and maintained at a water temperature of 4–6 °C, resulted in clinical disease, with mortalities occurring between 3 and 10 days.

In the subsequent study by Teshima et al. (1992), it was reported that disease took 3 months to develop at 15 °C following i.p. injection with 5×10^6 to 3×10^7 cells/ml. So, the inference was that the organism was not very aggressive. Yet, brown trout appeared to be more susceptible with LD_{50} doses of 21.5×10^4 (an isolate from human enteritis, UK) to 7.4×10^7 cells (an isolate from rainbow trout in Spain) depending on the culture (Acosta et al. 2002).

Using 23 isolates that were at best of extremely low virulence to gilthead sea bream, pathogenicity was correlated with the bacteriocidal effect of serum. However in the absence of any clinical signs of disease, the pathogen was capable of remaining viable within the gilthead sea bream for up to 3 months (Padilla et al. 2005).

Klebsiella pneumoniae

Characteristics of the Disease

The organism was recovered from the diseased tails and fins of 12 rainbow trout in Scotland. No other disease signs were noted (Daskalov et al. 1998).

Isolation

Pure cultures were obtained after shaking tails and pectoral fins in 0.9 % (w/v) saline for 5 min, and thereafter spreading 0.1 ml volumes on TSA with incubation at 15 °C for 7 days (Daskalov et al. 1998).

Characteristics of the Pathogen

Twelve pure cultures were obtained. The characteristics were, as follows:

Box 6.8: *Klebsiella pneumoniae*
Cultures comprise fermentative Gram-negative encapsulated rods that produce catalase but not arginine dihydrolase, H_2S, indole, lysine or ornithine decarboxylase, oxidase nor phenylalanine deaminase, produce acid from arabinose, cellobiose, glucose, maltose and raffinose, reduce nitrates to nitrite, and do not degrade gelatin (Daskalov et al. 1998).

Pathogenicity

Cultures could induce fin and tail rot by immersion for 5 min in 10^7 bacteria/ml, but only after prior abrading the surface of the fins. Disease signs became evident after 3 days, with mortalities being recorded after a further 2 days. Within 5 days of injecting 10^4 cells/fish, there was some reddening of the muscle in animals injected intramuscularly. Seven days after i.m. and i.p. injection, mortalities began, with disease signs including gastro-enteritis, liquefaction of the kidney, and the presence of ascitic fluid in the peritoneal cavity. All fish were dead within 12 days (Daskalov et al. 1998).

Pantoea agglomerans

Characteristics of the Disease

Juvenile mahi mahi (average weight = 1 g) were transported from Florida, USA to Bermuda for ongrowing in sea cages. During January 1986, mortalities were noted with dead specimens displaying marked haemorrhaging in the eyes. Pronounced haemorrhaging was noted in the eyes of dead and moribund animals. Haemorrhages were also recorded in the musculature. Otherwise, there was an absence of disease signs in the internal organs (Hansen et al. 1990).

Isolation

Pale yellow colonies were recovered from kidney blood on TSA supplemented with 2 % (w/v) NaCl following incubation at 16 °C for 7 days. Subculturing was possible on marine 2216E agar (Hansen et al. 1990).

Characteristics of the Pathogen

There was excellent agreement between the characteristics of the fish isolate and the description of *Pantoea agglomerans* (Grimont and Grimont 2005a).

Box 6.9: *Pantoea agglomerans*
Cultures comprise pale yellow pigmented, motile, fermentative Gram-negative rods, which produce catalase and ß-galactosidase but not arginine dihydrolase, H_2S, indole, lysine or ornithine decarboxylase, or oxidase. The methyl red test, Voges-Proskauer reaction, and nitrate reduction test are positive. Gelatin, but not blood, chitin or starch, is degraded. Acid is produced from glucose, D-mannitol and sucrose. Citrate is utilised. Growth occurs at 4–37 °C but not at 40 °C, and in 0–6 % (w/v) but not 8 % (w/v) NaCl (Hansen et al. 1990).

There has been some debate over the precise taxonomic relationship of the organism, which has been classified as *Enterobacter agglomerans*, *Erwinia herbicola* and now as *Pantoea agglomerans* (see Grimont and Grimont 2005a).

Epizootiology

The source of the pathogen was unknown (Hansen et al. 1990).

Disease Control

Antimicrobial Compounds

The pathogen was sensitive to ampicillin, chloramphenicol, streptomycin and tetracycline but not to novobiocin or penicillin (Hansen et al. 1990). Based on these data, it would be prudent to evaluate tetracycline as a chemotherapeutant on fish farms, if the disease recurs.

Plesiomonas shigelloides

Characteristics of the Disease

Although there have been unauthenticated verbal reports that *Plesiomonas shigelloides* may be pathogenic to fish, it was not until the summer of 1984 that the organism was definitely recovered from diseased rainbow trout in northern Portugal (Cruz et al. 1986). Subsequently, the organism was recovered in Germany from catfish, which had originated in Nigeria, sturgeon fingerlings, which had been sent from Russia, gourami imported from Thailand and eels (Klein et al. 1993). Symptoms included emaciation, reddening of the anus with yellow exudation, petechial haemorrhages in the internal muscle wall, and sometimes the accumulation of ascitic fluid in the peritoneal cavity (Cruz et al. 1986). Inappetance was noted by Klein et al. (1993). More recently, the organism was isolated from diseased cichlids in India (Nisha et al. 2014).

Isolation

This was accomplished by inoculating samples of kidney and liver onto plates of TSA with incubation at 22–37 °C for an unspecified period whereupon round, raised, off-white, circular colonies developed (Cruz et al. 1986).

Characteristics of the Pathogen

Many of the characteristics of the pathogen were derived from use of the API 20E rapid identification system (Cruz et al. 1986) and API 20NE system (Klein et al. 1993).

Box 6.10: *Plesiomonas shigelloides*
Colonies contain motile, fermentative Gram-negative rods, which produce arginine dihydrolase, catalase, ß-galactosidase, indole, lysine and ornithine decarboxylase and oxidase, but not H_2S, phenylalanine deaminase, tryptophan deaminase or urease. Nitrates are reduced to nitrite, but negative reactions are recorded for gelatin liquefaction, the methyl red test, utilisation of citrate or malonate, and the Voges Proskauer reaction. Acid is produced from glucose, inositol and trehalose, but not from adonitol, aesculin, amygdalin, L-arabinose, dulcitol, lactose, mannitol, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose or xylose. Growth occurs at 22 and 37 °C but not at 4 °C, and in 0 % (w/v) sodium chloride (Cruz et al. 1986; Klein et al. 1993).

It should be emphasised that phylogenetic studies have indicated that the taxon should really belong in the genus *Proteus* as *Proteus shigelloides* (MacDonell et al. 1986).

Pathogenicity

At 10^5 CFU/ml, an isolate caused 100 % mortalities of cichlids within 48 h (Nisha et al. 2014).

Epizootiology

The outbreak in 1984 affected mostly 1–2 year old fish, in which the total mortality approximated 40 % of the population. There was a correlation with water temperature, which increased in June from 10 to 17 °C. Moreover, there was a pronounced increase in organic matter from food within the water. These factors would undoubtedly contribute to stress in the fish. It has been noted that *P. shigelloides* may be normally resident in the gastro-intestinal tract of warm-water fish (Vandepitte et al. 1980), from where it could serve as a reservoir of infection.

Disease Control

Antimicrobial Compounds A 10-day treatment regime with potentiated sulphonamides (sulphadiazine at 200 mg/kg of body weight of fish/day and trimethoprim at 50 mg/kg body weight of fish/day) was effective in reducing mortality levels (Cruz et al. 1986).

Providencia rettgeri

Characteristics of the Disease

During 1976, there was a mass mortality among farmed silver carp (*Hypophthalmichthys molitrix*) in Israel. The fish displayed large red ulcers on the abdomen, base of the pectoral fin, and around the head. At 20–23 °C, mortalities occurred in 3 days, whereas at 12–20 °C, the deaths ensued in 8 days (Bejerano et al. 1979).

Isolation

Pure culture growth was recovered from the heart, kidney and base of the lesions following inoculation of nutrient agar, blood agar and TSA with incubation at 15–35 °C for an unspecified period (Bejerano et al. 1979).

Characteristics of the Pathogen

Box 6.11: ***Providencia rettgeri***
Cultures comprise fermentative Gram-negative rods, which are motile by means of peritrichous flagella. Catalase, indole, phenylalanine deaminase and tryptophan deaminase are produced, but not β-galactosidase, H_2S or oxidase. DNA and urea are degraded, but not casein, gelatin, starch or the Tweens. The methyl red test is positive. Nitrates are reduced. Acid is produced from adonitol, aesculin, erythritol, galactose, inositol, mannitol, mannose, melezitose, rhamnose and salicin, but not from amygdalin, arabinose, cellobiose, dulcitol, glycerol, glycogen, inulin, lactose, maltose, melibiose, raffinose, ribose, sorbitol, sucrose, trehalose or xylose. The G+C ratio of the DNA is 39.2 moles % (Bejerano et al. 1979).

Generally, the isolates matched the description of *Pr. rettgeri* (Cowan 1974; Johnson et al. 1975; McKell and Jones 1976; Penner 2005).

Epizootiology

Inference was made that the source of infection was from poultry faeces, which were used extensively to fertilise the carp ponds (Bejerano et al. 1979). In this connection, it is relevant to note that *Pr. rettgeri* has been associated with the digestive tract of poultry.

Pathogenicity

Experimental infection of silver carp by i.m. injection of 5×10^2 bacteria, or by scarification of the fish surface and subsequent exposure to a broth culture, resulted in mortalities of 50 % at a water temperature of 18–20 °C. Experimentally infected fish showed lesions typical of the farmed stock (Bejerano et al. 1979).

Providencia vermicola

Characteristics of the Disease

The organism was recovered from diseased farmed Indian major carp (*Labeo rohita*) with ulceration and reddish colouration on the abdominal surface, base of the pectoral fins and around the head. The disease occurred during August 2012 to July 2013, and resulted in heavy mortalities (Ramkumar et al. 2014).

Isolation

Liver, gill and ulcerative skin tissue was inoculated onto *Aeromonas* agar, nutrient agar and TSA with incubation at room temperature for 24–48 h. Dominant growth on TSA from ulcerative skin was used in the subsequent investigation (Ramkumar et al. 2014).

Characteristics of the Pathogen

Box 6.12: *Providencia vermicola*
Cultures exhibit brown soluble diffusible pigment, and contain Gram-negative motile rods. Catalase but not arginine dihydrolase is produced. Nitrates are reduced. Urea is not attacked. Acid is produced from L-arabinose, glucose, inositol an D-mannose, but not from amygdalin, rhamnose or sucrose. There is 99 % 16S rRNA homology with *Providencia vermicola* (Ramkumar et al. 2014).

Epizootiology

The source of the pathogen was considered to be from handling, and from nearby poultry and human faeces. This could mean the the organism was an opportunist pathogen (Ramkumar et al. 2014).

Pathogenicity

Cultures were pathogenic in infectivity experiments using Indian major carp, and challenge by immersion and i.m. injection (Ramkumar et al. 2014)

Salmonella enterica subsp. *arizonae* (*Salmonella choleraesuis* subsp. *arizonae* = *Salmonella arizonae*)

Characteristics of the Disease

Sal. enterica subsp. *arizonae* was recovered during 1986 from a single dead pirarucu, *Arapaima gigas,* which had been exhibited in an aquarium at Sapporo, Japan. The pirarucu was deemed to have died of a septicaemic condition. Externally, there was minimal evidence of disease, with the eyes (corneas) displaying opacity. A bloody exudate was found in the body cavity. Lesions and congestions were recorded in the mucus membranes of the stomach and intestine (Kodama et al. 1987).

Isolation

Samples of internal organs were inoculated onto plates of blood agar, BHIA and MacConkey agar with incubation at 25 °C for 48 h. Thereupon, an organism was recovered as pure culture growth from heart, kidney, liver and spleen (Kodama et al. 1987).

Characteristics of the Pathogen

Box 6.13: *Salmonella enterica* subsp. *arizonae*
Cultures contain Gram-negative fermentative, motile rods, which produce catalase, ß-galactosidase, H_2S and lysine decarboxylase but not indole or oxidase. Neither aesculin, blood, casein, gelatin, starch nor urea is degraded. Citrate is utilised, nitrate is reduced, and the methyl red test is positive. The Voges Proskauer reaction is negative. Acid is produced from glucose (plus gas), lactose, maltose, mannitol, raffinose, sorbitol, sucrose, trehalose and xylose, but not arabinose or salicin. Growth occurs at 15–41 °C in 0–6 % (w/v) NaCl and on MacConkey agar (Kodama et al. 1987).

In a previous study by Austin et al. (1982), a possible taxonomic relationship was discussed between *Y. ruckeri* and *Sal. choleraesuis* subsp. *arizonae*. Therefore, it is ironic that the latter organism has now been involved in fish pathogenicity. To date, this is the only report implicating *Sal. enterica* subsp. *arizonae* as a fish pathogen (Kodama et al. 1987). With the exception of acid production from arabinose, raffinose and sucrose, there is good agreement with the description of *Sal. enterica* subsp. *arizonae* (Popoff and Le Minor 2005). Yet, agglutination was not recorded

with commercial *Salmonella* antisera, although not specifically a product to *Sal. enterica* subsp. *arizonae* (Kodama et al. 1987).

Epizootiology

Presumably, the pathogen had been derived from the aquarium water or from fish (carriers) (Kodama et al. 1987).

Pathogenicity

The pathogenicity of the single isolate was not confirmed (Kodama et al. 1987).

Disease Control

Antimicrobial Compounds Although control regimes were not adopted, the isolate was sensitive to chloramphenicol, fradiomycin, gentamicin, kanamycin, nalidixic acid, oxytetracycline, streptomycin and tetracycline, but resistant to erythromycin, spiramycin and sulphadimethoxine (Kodama et al. 1987).

Serratia liquefaciens

Characteristics of the Disease

During spring and early summer of 1988, heavy mortalities, i.e. up to 30 % of the stock, were noted among three separate populations of Atlantic salmon (*Salmo salar* L.), two of which (average weight = 450 g) were in Scottish marine cage sites, and the third population (average weight = 25 g) was in freshwater. Since the initial occurrence, the disease has been recognised on another two marine sites in Scotland during 1990. Also in 1990, the organism was recognised as the cause of low continuous mortalities among farmed turbot in France (Vigneulle and Baudin-Laurençin 1995). Moribund fish did not display any external signs of disease. Yet, internally the kidney was swollen, and nodules were present on both the kidney and spleen; the liver appeared speckled, and some ascitic fluid was present in the peritoneal cavity. Initially, it was considered likely that the animals were infected with *Ren. salmoninarum*, the causal agent of BKD, but the presence of Gram-negative bacteria and complete absence of any Gram-positive micro-organisms precluded this possibility (McIntosh and Austin 1990a, b). Microscopic examination of formalin-fixed

kidney sections indicated the presence of Gram-negative bacteria which exhibited bipolar staining. In a separate development during 1990, the organism was attributed to low level mortalities in turbot, farmed in floating cages in France. With this outbreak, the disease signs included swelling and liquefaction of the kidney and spleen, which were also characterised by the presence of yellowish nodules (Vigneulle and Baudin-Laurencin 1995). In 1999, Arctic charr in the USA were also found to be infected with the pathogen (Starliper 2001). The external signs centred on a slight redness and swelling around the anus, but internally there was evidence of severe hemorrhaging with bloody ascites

Isolation

Isolation was readily achieved from kidney by inoculation of swabbed material onto BHIA and TSA with incubation at 25 °C for 24–48 h (McIntosh and Austin 1990b).

Characteristics of the Pathogen

These organisms were recovered as dense pure culture growth from diseased tissues, and were examined bacteriologically (McIntosh and Austin 1990b).

Box 6.14: ***Serratia liquefaciens***
Cultures comprise Gram-negative fermentative catalase positive rods of 2–3 μm in length which, in contrast to the normal characteristics of the Enterobacteriaceae (Grimont and Grimont 2005b), are motile by single polar flagella. Intracellular bipolar staining properties are exhibited. Catalase, ß-galactosidase and lysine and ornithine decarboxylase are produced, but not H_2S, indole or tryptophan deaminase. The results of the oxidase test are variable, i.e. from negative to weakly positive depending to some extent on the age of the culture. The methyl red test and Voges Proskauer reaction is negative. Nitrates are not reduced. Blood (ß-haemolysis), casein, DNA, lecithin (weak), tributyrin (slow), Tween 20, 40, 60 and 80, and tyrosine are degraded, but not chitin, elastin, starch or urea. Growth occurs at 4–37 °C, in 0–5 % (w/v) but not 7 % (w/v) sodium chloride, and on deoxycholate citrate agar, eosin methylene blue agar and MacConkey agar, but not in 40 % (v/v) bile salts. Sodium citrate but not sodium malonate is utilised. Acid is produced from amygdalin, arabinose, inositol, mannose, melibiose and saccharose, but not rhamnose. Acid and gas are produced from fructose, galactose, glucose, maltose, mannitol, melibiose, raffinose, sorbitol, sucrose, trehalose and xylose. The G+C ratio of the DNA is 55 moles % (McIntosh and Austin 1990b).

Initially, it was considered that the organisms bore similarities to *Aer. veronii* (Hickman-Brenner et al. 1987) and *Ser. liquefaciens* (Grimont and Grimont 2005b). Similarities with the former included the presence of Gram-negative fermentative, catalase (and oxidase) positive rods, which were motile by single polar flagella, and with a G+C ratio of the DNA of 55 moles %. Indeed, the only discrepancies with the definition of *Aer. veronii* concerned some sugar fermentation reactions. Yet, similarities were also apparent with *Ser. liquefaciens*. However, the use of the API 20E rapid identification system (profile=5305363 or 5305367) and whole cell agglutination using a polyclonal antiserum raised against the type strain, confirmed an identification as *Ser. liquefaciens*.

Epizootiology

The natural reservoir of the organism was probably polluted waters (McIntosh and Austin 1990b).

Pathogenicity

Injection of 10^3 cells killed Atlantic salmon within 72 h. Typically, there was pronounced muscle liquefaction within the vicinity of i.m. injections. ECP resulted in death within 48 h (McIntosh and Austin 1990b).

Disease Control

Vaccine Development Whole cell formalised vaccines and toxoid preparations were effective for prophylaxis in laboratory-based experiments with Atlantic salmon (McIntosh and Austin 1990b).

Antimicrobial Compounds There was contradictory evidence regarding the value of chemotherapy with oxytetracycline. Nevertheless, the disease could be controlled with oxolinic acid (McIntosh and Austin 1990b).

Serratia marcescens

Characteristics of the Disease

The initial association of *Ser. marcescens* with fish diseases stemmed from a publication by Clausen and Duran-Reynals (1937). More detailed work waited for over half a century when in July 1990 during a survey of white perch (*Morone*

americanicus) from the Black river, a tributary of Chesapeake Bay (USA), a red-pigmented organism, i.e. *Ser. marcescens,* was recovered, and, on subsequent examination, deemed to be potentially pathogenic for fish. It was considered likely that the presence of the organisms reflected the polluted nature of the river (Baya et al. 1992). However, it should be emphasised that the organisms were recovered only from apparently healthy fish, which were devoid of overt signs of disease (Baya et al. 1992).

Isolation

Pure cultures resulted from inoculation of kidney swabs onto plates of BHIA and TSA with incubation at 22 °C for 48–72 h (Baya et al. 1992).

Characteristics of the Pathogen

Box 6.15: *Serratia marcescens*
Cultures comprise red (prodigiosin) pigmented fermentative motile Gram-negative rods, which produce catalase, ß-galactosidase and lysine and ornithine decarboxylase but not arginine dihydrolase, H_2S, indole, oxidase or tryptophan deaminase. Nitrate is reduced; but the methyl red test and the Voges Proskauer reaction are negative. Growth occurs at 4–45 °C, and in 0–8 % (w/v) sodium chloride. Blood (sheep), casein, gelatin, starch and Tween 80 are degraded, but not so urea. A wide range of carbohydrates are attacked, including amygdalin, fructose, galactose, glycerol, inositol, maltose, mannitol, mannose, salicin, sorbitol, sucrose and trehalose, but not so arabinose, cellobiose, dulcitol, lactose, melibiose, raffinose, rhamnose or xylose (Baya et al. 1992).

From these characteristics, it is apparent that there is reasonable agreement with the description of *Ser. marcescens* (Grimont and Grimont 2005b).

Epizootiology

Seemingly, the organism may be associated with fish, and possibly comprise part of the ‘normal’ microflora of eutrophic waters (Baya et al. 1992).

Pathogenicity

Laboratory-based studies confirmed pathogenicity for striped bass ($LD_{50} = 1 \times 10^5$ cells) [LD_{50} in rainbow trout $= 5 \times 10^3$ cells] with death occurring 1–3 or 1–7 days following administration of the cells via the i.m. or i.p. route, respectively (Baya et al. 1992). Experimentally infected fish displayed muscle necrosis and some signs of haemorrhagic septicaemia. Further work indicated a role for ECP, which possessed marked proteolytic and phospholipase activity. In fish, the ECP caused a cytotoxic response, with mortality occurring 24–48 h after administration. Here, the LD_{50} dose for rainbow trout and striped bass was 0.4 and 4.8 μg of protein/g of fish, respectively (Baya et al. 1992).

Disease Control

Antimicrobial Compounds Isolates were sensitive to flumequine, oxolinic acid and potentiated sulphonamides (Baya et al. 1992).

Serratia plymuthica

Characteristics of the Disease

During September to December 1987, a new pathogen was associated with diseased rainbow trout fingerlings (average weight = 7 g) in a hatchery in Northwestern Spain. Progressive low level mortalities (cumulative total = 35 % of the stock) were reported, with which there appeared to be a correlation with rainfall (Nieto et al. 1990). Then in 1992, a similar organism was associated with skin lesions in farmed rainbow trout in Scotland (Austin and Stobie 1992). In this disease outbreak, there was an association with pollution by domestic sewage, i.e. leakage from a septic tank. Nieto et al. (1990) noted that diseased fish did not display any external or internal clinical signs. However in Scotland, the diseased fish possessed extensive skin lesions over the entire flank, from the operculum to the tail (Austin and Stobie 1992). In Poland, the organism has been recovered from 42 Atlantic salmon, rainbow trout and sea trout farms since 1996 (Grawinski and Antychowicz 2001).

Isolation

Pure culture growth was recovered from the kidney and liver following inoculation of TSA with incubation at 22 °C for 7 days (Austin and Stobie 1992).

Characteristics of the Pathogen

Box 6.16: *Serratia plymuthica*
Nieto et al. (1990) reported that cultures comprise red (prodigiosin) pigmented fermentative non-motile Gram-negative rods, producing catalase and ß-galactosidase but not arginine dihydrolase, H_2S, indole, lysine or ornithine decarboxylase, or oxidase. The nitrate reduction, citrate utilisation and Voges Proskauer reaction tests are positive. A negative response is recorded for the methyl red test. Gelatin is degraded, but not so blood, casein or urea. Acid is produced from L-arabinose, D-fructose, D-galactose, D-glucose (but not gas), inositol, D-maltose, D-mannitol, D-mannose, melibiose and sucrose, but not from D-adonitol, lactose, L-rhamnose or D-sorbitol.

From these results, it was considered that the cultures resembled *Ser. plymuthica* and *Ser. rubidaea* (Grimont and Grimont 2005b). Yet as a result of acid production from inositol but not adonitol, it was considered that the pathogen should be assigned to *Ser. plymuthica.* Similar traits were reported by Austin and Stobie (1992).

Epizootiology

Possibly, the pathogen comprises a component of the freshwater bacterial community, particularly where pollution with organic material is rife (Austin and Stobie 1992).

Pathogenicity

Laboratory experiments revealed that the LD_{50} dose for rainbow trout was 10^4–10^5 cells. Moreover, such infected fish displayed discoloration and abdominal swelling (Nieto et al. 1990) and extensive surface lesions (Austin and Stobie 1992). Isolates were strongly hydrophobic (Rodriguez et al. 1990); a property that may be involved in the adherence of the organism to surfaces.

Disease Control

Antimicrobial Compounds Isolates were sensitive to chloramphenicol, flumequine, oxolinic acid, oxytetracycline, potentiated sulphonamides and streptomycin, but not to nitrofurantoin or sulphadiazine (Nieto et al. 1990; Austin and Stobie 1992).

Presumably, effective chemotherapy could be achieved with one or more of these compounds.

Yersinia intermedia

Characteristics of the Disease

Affected Atlantic salmon were of 40–50 g in weight, and were held at a water temperature of 5 °C. Disease signs included lazy movement with the fish congregating at the surface of the water, darkening of the body pigment, tail erosion, haemorrhaging on the flank and abdominal inflammation (Carson and Schmidtke 1993).

Isolation

Blood samples were cultured on Oxoid blood agar base supplemented with 7 % (v/v) defibrinated sheep's blood at 18 °C for 7 days (Carson and Schmidtke 1993). After 48 h, the kidneys of two fish revealed the presence of dense growth, equated with *Yersinia intermedia.* In addition, other bacteria were recovered including CLB and *Ps. fluorescens.*

Characteristics of the Pathogen

Characteristics matched the species description (Farmer and Kelley 1991) at 36 °C, except that the fish-isolate utilised sodium citrate and was weakly positive in the Voges Proskauer reaction (Carson and Schmidtke 1993).

Box 6.17: ***Yersinia intermedia***
The culture comprises fermentative, motile (at 25 °C but not 36 °C) cells that produce ß-galactosidase and indole, but not arginine dihydrolase, H_2S, lysine or ornithine decarboxylase or oxidase. Aesculin and urea are degraded. The methyl red test and Voges Proskauer reaction (at 36 °C but not 25 °C) are positive. Nitrates are reduced. Acid is produced from glycerol, inositol, mannitol, melibiose (at 36 °C but not 25 °C), rhamnose, sorbitol, sucrose, trehalose and xylose, but not adonitol or lactose. Sodium citrate is utilised at 36 °C but not 25 °C (Carson and Schmidtke 1993).

Epizootiology

The organism has been associated with water and the intestines of apparently healthy fish (e.g. Kapperud 1981; Shayegani et al. 1986; Zamora and Enriquez 1987), which are likely to be the source.

Pathogenicity

It was considered that the organism was of endogenous origin, being a pathogen of cold-stressed (possibly immunocompromised) fish (Carson and Schmidtke 1993). Pathogenicity experiments have not been carried out.

Yersinia ruckeri

Characteristics of the Diseases

Enteric redmouth (ERM, Hagerman redmouth disease, redmouth, salmonid blood spot) was initially diagnosed as a systemic infection among farmed rainbow trout in the Hagerman Valley of Idaho, USA during the early 1950s, and subsequently described in detail by Ross et al. (1966). The disease is mostly restricted to salmonids (Bullock and Snieszko 1975) within the geographical locations of North and South America (Ross et al. 1966; Stevenson and Daly 1982; Bravo and Kojagura 2004), Denmark (Dalsgaard et al. 1984), Great Britain (Austin 1982; Roberts 1983), France (Lesel et al. 1983), Germany (Fuhrmann et al. 1983), Italy (Busch 1978), Ireland (McCormick and McLoughlin 1993), Norway (Richards and Roberts 1978) and Australia (Bullock et al. 1978; Llewellyn 1980). With the recent upsurge in the number of cases of the disease, particularly in Europe, it would appear that the disease is still spreading. So far, it has been a severe problem mostly in rainbow trout, although outbreaks of disease have been reported among populations of brown trout, brook trout, chinook salmon and coho salmon (Dulin et al. 1976) and Atlantic salmon and Pacific salmon (Bullock et al. 1978). A few non-salmonid fish species have also been reported to harbour the pathogen, and these include channel catfish (Danley et al. 1999), emerald dace (*Notropis atherinoides;* Mitchum 1981), goldfish (McArdle and Dooley-Martin 1985), carp (B. Austin, unpublished observation), lake herring or cisco (*Coregonus artedii;* Bullock and Anderson 1984), minnows (*Pimephales promelas*) (Michel et al. 1986) and pompano (*Trachinotus marginatus*) (Romano et al. 2012). Also, sea bass and turbot appear to be susceptible (Vigneulle 1990). In the case of rainbow trout, there is experimental evidence that significantly lower mortalities occur with increases of salinity. Thus, mortalities were reduced from 96.5 % in freshwater to 75 % in 9‰ salinity (Altinok and Grizzle 2001a). Much excellent work has certainly been accomplished, but there are many basic facets of the biology of the pathogen, which remain unknown or unclear.

The name of the disease, i.e. enteric redmouth (ERM), is fairly descriptive insofar as one of the most common symptoms is reddening of the mouth and throat, which is caused by subcutaneous haemorrhaging (Fig. 6.2; Busch 1973). Other external signs include inflammation and erosion of the jaws and palate, melanosis, haemorrhaging around the base of the fins, bilateral exophthalmia which may lead to blindness in the survivors (Fig. 6.3), and a tendency for sluggishness (Fuhrmann et al. 1983; Bullock and Anderson 1984; Danley et al. 1999). In channel catfish, the disease was characterized by swollen haemorrhaged rings around the eyes and raised haemorrhaged areas overlying the frontal foramens (Danley et al. 1999). Internally, there may be haemorrhaging in the muscle, body fat and in the intestine, which may also contain a yellow fluid. A generalised bacteraemia occurs in the principal organs, with slight enlargement of the kidney and spleen. The disease has been held responsible for greater financial loss of the trout farming industry in west-

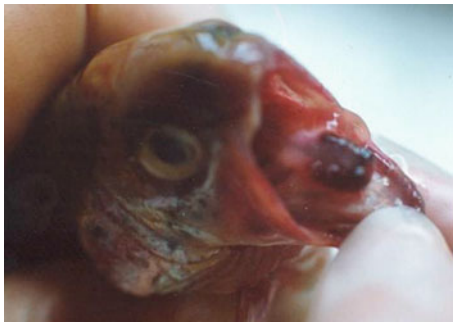

Fig. 6.2 A rainbow trout displaying extensive haemorrhaging in the mouth caused by ERM. Photograph courtesy of Professor V. Jencic

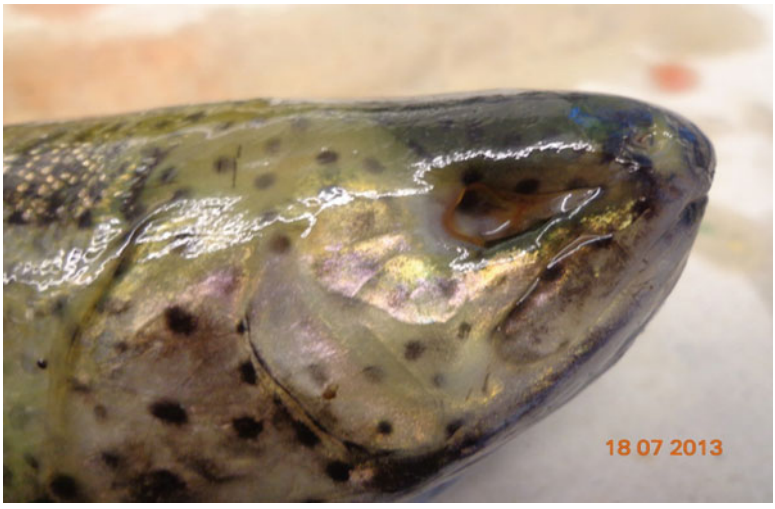

Fig. 6.3 A blind rainbow trout that survived infection with ERM. Photograph courtesy of Dr. P. Orozova

ern areas of the USA than any other disease (Hester 1973). Onset of an epizootic is often gradual with resulting heavy losses (Newman and Majnarich 1982). It is relevant to note that a second disease, known as salmonid blood spot, in Australia (Llewellyn 1980) is attributable to the same aetiological agent (Green and Austin 1982).

Isolation

The pathogen may be readily recovered from kidney on routine bacteriological media, e.g. BHIA or TSA, following incubation at 20–25 °C for 48 h whereupon round, raised, entire, shiny, off-white colonies of 2–3 mm diameter develop (e.g. Ross et al. 1966). Three selective media have been devised (Appendix in Chap. 12; Waltman and Shotts 1984; Rodgers 1992; Furones et al. 1993). Tween 80 hydrolysis, which occurred commonly among isolates from the USA, resulted in the precipitation of insoluble calcium salts around colonies on Waltman-Shotts medium. However, according to Hastings and Bruno (1985), there were some limitations insofar as there is some growth variation among *bona fide* strains of *Y. ruckeri.* Moreover, non-motile isolates, which did not degrade Tween 80 have been recovered in Europe, e.g. Germany (Klein et al. 1994). Ribose ornithine deoxycholate medium of Rodgers (1992) appears to have overcome the problems of the earlier formulation, and laboratory use points to its value for the recovery of *Y. ruckeri.* Furones et al. (1993) supplemented TSA with 1 % (w/v) SDS, 100 µg/ml of coomassie brilliant blue and 100 µg/ml of Congo red. On this medium, *Y. ruckeri* produced a creamy deposit around the colonies.

Characteristics of the Pathogen

Box 6.18: ***Yersinia ruckeri***

The organisms comprise a homogeneous group of fermentative Gram-negative, slightly curved rods of 1.0×2.0–3.0 µm in size, which are motile usually by means of 7 or 8 peritrichously arranged flagella, or nonmotile. Catalase, ß-galactosidase, and lysine and ornithine decarboxylase are produced, but not H_2S, indole, oxidase, phenylalanine deaminase or phosphatase. The methyl red test is positive, but not the Voges Proskauer reaction. Nitrates are reduced. Gelatin, and Tween 20, 40 and 60 are degraded, but not aesculin, chitin, DNA, elastin, pectin, tributyrin, Tween 80 or urea. Growth occurs in 0–3 % (w/v) sodium chloride. Sodium citrate is utilised. Acid is produced from fructose, glucose, maltose, mannitol and trehalose, but not from inositol, lactose, raffinose, salicin, sorbitol or sucrose. The G+C content of the DNA is 47.5–48.5 moles % (Ewing et al. 1978).

The precise taxonomic position of the causal agent of ERM has intrigued bacteriologists since the initial isolation of the organism. Ross et al. (1966) realised that heated O-antigens prepared from 14 cultures of the ERM organism agglutinated strongly (titre = 1:320 or 1:640) with the corresponding antigens of *Sal. enterica* subsp. *arizonae* O group 26, and weakly (titre = 1:20) with O group 29. Conversely, there was no reaction with O-antigens prepared from *Salmonella, Ent. liquefaciens, Citrobacter* or *Serratia.* In addition, this team pointed to the biochemical similarities with *Ent. liquefaciens, Ser. marcescens* subsp. *kiliensis* and *Sal. enterica* subsp. *arizonae.* Furthermore, Stevenson and Daly (1982) indicated serological cross-reactions with *Haf. alvei*. However after an examination of the phenotypic and molecular traits of 33 isolates, Ewing et al. (1978) elevated the pathogen to species status, as *Y. ruckeri.* It is interesting to note that the pathogen was included in the genus *Yersinia* because of only a 30–31 % DNA homology to *Y. enterocolitica* and *Y. pseudotuberculosis.* This compares to DNA homologies of 24–28 % and 31 % with *Ser. marcescens* and *Ser. liquefaciens,* respectively (Steigerwalt et al. 1976). Therefore from the DNA hybridisation experiments, it is difficult to determine the reasons for including the ERM organism with *Yersinia* rather than *Serratia.* However, it would be relevant to extend the existing data bases by comparing the homology with *Sal. enterica* subsp. *arizonae.* This is especially relevant because Green and Austin (1982) have shown a greater phenotypic relationship of *Y. ruckeri* with *Sal. enterica* subsp. *arizonae* than with *Y. enterocolitica* or *Y. pseudotuberculosis.* In fact, the causal agent of ERM may belong in a new genus of the Enterobacteriaceae, an idea which has been mooted by Bercovier and Mollaret (1984). The distinctiveness was confirmed by an examination of phylogeny based on 16S rRNA sequences, when *Y. ruckeri* formed a distinct node, albeit associated with other yersinias (Ibrahim et al. 1993).

Whereas the taxon is phenotypically homogeneous, it is serologically diverse insofar as five serotypes have been recognised (O'Leary 1977; Bullock et al. 1978; Stevenson and Airdrie 1984a). Three serotypes have been referred to colloquially as the "Hagerman strain" (the most common and the most virulent), the 'Big Creek strain' (relatively avirulent) and the 'Australian strain' (appears to be avirulent) (Busch 1981). These serotypes have been designated as Type 1 (Hagerman), Type 2 (O'Leary) and Type 3 (Australian) (Bullock and Anderson 1984) and the newly described serovars IV and V (Stevenson and Airdrie 1984a). Serotype 2 may be distinguished from serotype 1 by its ability to ferment sorbitol (O'Leary 1977). However, caution needs to be advocated since this may well be a plasmid-mediated trait. Nevertheless, serotypes/serovars 2, 3, 4 and 5 appear to be highly related in terms of DNA homology (De Grandis et al. 1988). Also, two LPS profiles have been recognised among 23 Portuguese isolates, corresponding to serotypes O1 and O3. Greater heterogeneity was recorded for OMP, with 7 profiles recognised, and there was 10 ribotypes, 6 of which accommodated serotype O1 isolates (Sousa et al. 2001).

During the early 1990's in England, a new form of the pathogen was recovered among rainbow trout that had been previously vaccinated by immersion with commercial enteric redmouth vaccines. This form, which was initially considered to

share some of the characteristics of *Haf. alvei,* was confirmed as *Y. ruckeri* by 16S rRNA sequencing (homology = 100 %), and was regarded as a new biotype, i.e. biotype 2. In contrast to the species description, cultures often appeared to be non-motile (although *flgA* and *flhA* flagellar genes have been found in German isolates; Huang et al. 2014), lacked phospholipase activity, were positive for the Voges Proskauer reaction and displayed a unique LPS profile (Tinsley et al. 2011a). Similar non-motile variants were also recovered from previously vaccinated rainbow trout in Spain (Fouz et al. 2006), Denmark, Finland (Ström-Bestor et al. 2010), France (Calvez et al. 2014), Germany (Huang et al. 2014) and the USA (Welch et al. 2011). In the case of Germany, during June 2011 to June 2012, 48 *Y. ruckeri* isolates were collected from 12 different rainbow trout hatcheries in Germany. Of these isolates, the majority (44 = 91.7 %) were nonmotile (Huang et al. 2015). Indeed, one study pointed to four independent natural mutations in *fliR*, *flhA* or *flhB* genes that led to lack of motility due to their effect on the flagellae secretion system (Evenhuis et al. 2009). These authors pointed to the similarity between UK and US strains in terms of the same mutant allele, suggesting a common origin of biotype 2. However, evidence has been presented which suggests that isolates from Denmark, Spain and the UK emerged separately because of the difference in pulsotypes (Wheeler et al. 2009). Notwithstanding, the non-motile isolates were more active at lower temperatures than their motile counterparts. This may explain why non-motile cultures were more common I Germany during winter and were responsible for more outbreaks then than motile forms (Huang et al. 2014).

Diagnosis

Phenotypic Methods Diagnosis of *Y. ruckeri* may be achieved by isolation of the pathogen, such as on the selective media of Waltman and Shotts (1984) or Rodgers (1992), and thence identification. According to Waltman and Shotts (1984), 53/60 isolates hydrolysed Tween 80 but none fermented sucrose. Therefore, typically on the selective medium, *Y. ruckeri* colonies were green with a zone of hydrolysis (indicated by the presence of insoluble calcium salts) around them. Unfortunately in our experience with this medium, UK isolates rarely hydrolysed Tween 80. Therefore, interpretations should be made carefully.

Bacteriophage Typing Development of a bacteriophage typing scheme may be of considerable value for diagnosis in the future. Reference is made here to a collection of tailed icosahedral bacteriophages which are specific to *Y. ruckeri* (Stevenson and Airdrie 1984b).

Serology iFAT has a proven track record with the diagnosis of ERM (Johnson et al. 1974). An indirect ELISA has been effective at detection (Cossarini-Dunier 1985).

Molecular Methods There is evidence that molecular techniques are finding use for the identification of infections caused by *Y. ruckeri* (e.g. Argenton et al. 1996; Taylor and Winton 2002; Sakai et al. 2006; Saleh et al. 2008). A PCR was successful in detecting *Y. ruckeri* in artificially and naturally diseased trout tissues, with a sensitivity of 60–65 cells/PCR tube (Gibello et al. 1999). The value of PCR was echoed by Altinok et al. (2001), who detected the pathogen in the blood of rainbow trout within 1 h of immersion in a suspension containing 4.5×10^6 CFU of *Y. ruckeri*/l. Indeed, the approach was more reliable than culturing at detecting the organism (Altinok et al. 2001a, b). Detection of the *yruR/yru1* genes involved with quorum sensing has been regarded as sensitive (i.e. 1 pg; 12 CFU) specific for the 6 isolates of *Y. ruckeri* tested but not to representatives of 5 other *Yersinia* species (Temprano et al. 2001). Others have proposed a PCR and RFLP targeting the *aroA* gene (Yugueros et al. 2001). A nested PCR had a detection limit of 1.4×10^5 CFU/reaction. However, use of species-specific primers improved detection to <14 CFU/sample (Taylor and Winton 2002). LAMP, amplifying the *yruI/yruR* gene, which encodes the quorum sensing system, was regarded as tenfold more sensitive that PCR, detecting 1 pg of genomic DNA (Saleh et al. 2008). Real-time PCR (RT-PCR), which targeted the glutamine synthetase (glnA) gene, was developed and proposed for the detection of *Y. ruckeri* (Keeling et al. 2012). The RT-PCR was determined to be absolutely specific for the pathogen, and detected 5 fg of bacterial DNA and 3×10^3 CFU/g of seeded kidney tissue (Keeling et al. 2012). RT-PCR based on the r*ecA* gene was developed, and determined to be specific, sensitive and reproducible. The sensitivity was given as 1.7 CFU/ ml for purified DNA 3.4 CFU/g in the case of tissues, i.e. liver, kidney, that had been seeded with the pathogen, and 0.34 CFU/100 µl for blood (Bastardo et al. 2012).

Spectroscopy Fourier transform infrared spectroscopy has been regarded to be extremely reliable and effective for the recognition of *Y. ruckeri* including the newer non-motile varieties (Wortberg et al. 2012).

Epizootiology

In rainbow trout, ERM most commonly affects fish of approximately 7.5 cm in length. The disease is less severe but more chronic in larger fish, i.e. of 12.5 cm in length. Severity peaks with a water temperature of 15–18 °C, and decreases when it drops to 10 °C or below. Overly fat or debilitated (stressed) fish are thought to be more susceptible to severe epizootics (Rucker 1966). Moreover, occurrence of ERM may be cyclic, suggesting the presence of asymptomatic carrier fish in the population (Busch and Lingg 1975). Such fish would be capable of shedding the organism in the faeces. Alternatively, it may be argued that the pathogen is present normally in the water and/or sediment (Rucker 1966). From the results of some fascinating experiments, Hunter et al. (1980) demonstrated that unstressed carrier fish did not

transmit *Y. ruckeri* to recipient fish. In fact, it was necessary to stress these carriers, such as by heat (i.e. a water temperature of 25 °C), in order to enable release and thus transmission, of the pathogen to other fish. Even under these conditions, colonisation of the lower intestine took place in the recipient fish without any mortalities. However, the handling of fish, which may appear to be extremely healthy, and crowded conditions causing excess ammonia and metabolic waste products in the water and, thus, a decrease in oxygen levels, may precipitate outbreaks of clinical disease (Bullock and Snieszko 1975). Nevertheless, outbreaks generally occur only after the fish have been exposed to large numbers of the pathogen (Ross et al. 1966). Mortalities tend to start 5–19 days after exposure, depending on the size of the inoculum, and last for 30–60 days (Rucker 1966; Busch 1973). Cumulative losses due to ERM may account for as much as 30–35 % of the rainbow trout population (Klontz and Huddleston 1976).

There is controversy over some aspects of the ecology of *Y. ruckeri,* insofar as the organism has been alternatively considered as well adapted as a normal aquatic saprophyte (McDaniel 1972) and, secondly, as not capable of free-living for any extended period in water (Klontz and Huddleston 1976). Nevertheless, these authors conceded that survival for up to 2 months was possible in mud. Further experiments indicated that the pathogen could survive for 4 months in unsupplemented water at a salinity of 0–20‰; survival being reduced in seawater (salinity = 35‰) (Thorsen et al. 1992). Romalde et al. (1994) reported survival for 3 months. Therefore, the implication is that *Y. ruckeri* is capable of surviving in the (fresh) water column for a long time after an outbreak of disease has occurred. Survival may be influenced by the ability of virulent cultures to adhere to surfaces, a characteristic linked to the presence of flagella. Interestingly, oxolinic acid was better able to inactivate planktonic rather than attached cells of the pathogen (Coquet et al. 2002). Perhaps, the organism is a normal inhabitant of the digestive tract of fish or in contaminated waters. In this respect, *Y. ruckeri* has been isolated from sewage sludge (Dudley et al. 1980) and a bacteriophage specific for *Y. ruckeri* found in sewage (Stevenson and Airdrie 1984b). Moreover, aquatic invertebrates, notably crayfish, and even terrestrial mammals, namely muskrats (Stevenson and Daly 1982) may harbour large numbers of the pathogen, thereby serving as a reservoir of infection. It is noteworthy that McArdle and Dooley-Martin (1985) recovered *Y. ruckeri* from the digestive tract of an apparently healthy goldfish following inspection of a consignment upon importation into Ireland. The isolate was subsequently demonstrated to be pathogenic for rainbow trout. Thus it would appear that ornamental fish could pose a reservoir of infection into salmonids. If Dulin et al. (1976) are correct in asserting that *Y. ruckeri* is not ubiquitous in nature, it is puzzling why ERM should be found in remote fish stocks, without prior history or association with the disease (Janeke, personal communication). Under these conditions, it is doubtful if movement of infected fish could account for the spread of ERM. Yet, isolations have been made from wild salmonids. In one example, *Y. ruckeri* type I was recovered from a mature wild Atlantic salmon in freshwater [in Scotland] (Petrie et al. 1996). Whether or not this suggests a natural reservoir of the pathogen in wild fish or reflects a transfer from aquaculture remains to be established.

There is some evidence that *Y. ruckeri* may well be capable of surviving in the environment in a form (the so-called dormant or non-culturable state) that is not readily culturable on conventional media (Romalde et al. 1994). These workers found that the number of culturable cells increased for the first 15 days after *Y. ruckeri* was seeded into an experimental system. Thereafter, a decline in numbers was recorded over a 100-day period. Culturable cells persisted in sediment more so than water at 6 and 18 °C. Yet, by means of acridine orange staining and fluorescence microscopy, a so-called dormant state was considered to develop. There were slight changes in the LPS of the dormant cells, but not in membrane proteins or plasmid composition. Interestingly, virulence was maintained during the period of non-culturability. Unlike the situation with *Aer. salmonicida,* cells were resuscitated to a culturable state following the addition of nutrients (Romalde et al. 1994).

A serotyping scheme, based on O-antigens, has been proposed (Davies 1990), and may have value in epizootiological investigations.

PFGE and MLST were used to study isolates from outbreaks on French fish farms. For the PFGE analysis, two enzymes, NotI and AscI, were used either separately or together, with the latter approach revealing the great homogeneity among the isolates with 43 pulsotypes defined. The dominant pulsotypes have been described elsewhere, i.e. in Denmark, Finland, Germany, Italy and Spain. MLST revealed two dominant sequence types, i.e. ST31 and ST36; this is an epidemic within France (Calvez et al. 2015).

Pathogenicity

Uptake from an aqueous suspension is likely to be across the epithelial cells in the secondary gill lamella, with recovery of the pathogen from blood possible after 1 min from challenge. With the development of septicaemia, the pathogen was widespread in all organs, including the brain (Ohtani et al. 2014). Some interesting experiments were carried out with rainbow trout, which were immersed in a suspension containing a luminescent culture. This was developed by using the pCS26-Pac plasmid, which contains the lux operon from *Photorhabdus luminescens.* The fate of this culture in the digestive tract was followed with bioluminescence imaging, in which dissemination and the development of infection were studied. *In vivo* expression technology reveled that there was expression of promotors, i.e. *yhlBA, cdsAB*, *yctCBA* and *yrp1,* which are involved/associated with haemolysin production, cysteine and citrate uptake, and the encoding of serralysin metalloprotease, respectively, during the infection process (Méndez and Guijarro 2013). Other studies using immunohistochemistry and *in situ* hybridization techniques, followed the fate of *Y. ruckeri* cells, in which the lateral lines, dorsal fin, epidermis and mucosal tissue in gastro-intestinal tract were determined to be the primary areas of uptake (Khimmakthong et al. 2013).

In terms of pathogenicity to rainbow trout, Type 1 (Hagerman) is the most virulent, followed by Type 2 (O'Leary) and then Type 3 (Australian) (Bullock et al.

1983). The LD_{50} dose has been established to be 3.0×10^5 cells/ml for Type 1, 1.0×10^7 cells/ml for Type 2 and an as yet undetermined dose for Type 3. The degree of pathogenicity of serovars IV and V needs clarification. The new biogroup, biotype 2, described by Austin et al. (2005) killed rainbow trout within 4-days at a dose of 10^5 cells/fish. It is possible that the BarA-UvrY two component system contributes to pathogenesis by regulating invasion of epithelial cells and sensitivity to oxidative stress induced by immune cells (Dahiya and Stevenson 2010a). Also, the ZnuABC high affinity zinc transporter has a role in the infection cycle (Dahiya and Stevenson 2010b). It should be emphasised that exposure to sublethal concentrations of copper, i.e. 7 µg/l for 96 h, rendered the fish more susceptible to infection by *Y. ruckeri* (Knittel 1981). Moreover, the nature of the virulence factors is incompletely understood. The role of plasmids in virulence has been indicated insofar as the more pathogenic Type 1 isolates possess a large 40–50 mDa plasmid, and some contain a smaller 20–30 mDa plasmid. The larger plasmid is absent from Type 2 cultures (Cook and Gemski 1982; De Grandis and Stevenson 1985). Further work is necessary, however, to resolve the precise role of this large plasmid in pathogenicity, especially as it is not thought to carry virulence factors (Guilvout et al. 1988).

The O antigen, from the LPS of serogroup O1, has been determined to comprise a branched tetrasaccharide, with repeating units containing 2-acetamidino-2,6-dideoxy-L-galactose, 2-acetamido-2-deoxy-D-glucose and 7-acetamido-3,5,7,9-tetradeoxy-5-(4-hydroxybutyramido)-D-glycero-L-galacto-nonulosonic acid (Beynon et al. 1994), and is regarded as the dominant immunogenic molecule (Costa et al. 2011).

ECP have been recovered, and demonstrated to have an LD_{50} of 2–9.12 µg of protein/g of fish. The ECP has been found to contain amylase, caseinase, gelatinase, haemolytic (salmon, sheep and trout erythrocytes) lipase and phospholipase activity. Haemolysin Yh1A is expressed more so at 18 °C [the temperature of many disease outbreaks] than 28 °C, and is further increased in iron-depleted conditions (Fernández et al. 2007). A novel 47 kDa azocasein hydrolyzing protease, which was produced during the end of the exponential growth phase, was recovered from culture supernatants (Secades and Guijarro 1999). Later, a serralysin metalloprotease (= metalloendopeptidase), termed Yrp1, that hydrolyses actin, fibrinogen, gelatin, laminin and myson (but not type II and type IV collagen) has been linked to pathogenicity (Fernández et al. 2002, 2003). Also, aesculin has been attacked (Romalde and Toranzo 1993). Adhesin activity, at least to the CHSE-214 cell culture, has been recorded (Romalde and Toranzo 1993).

Evidence has been presented that *Y. ruckeri* may have a siderophore mediated iron uptake system (Romalde et al. 1991; Fernández et al. 2004), in parallel to *Aer. salmonicida* and *V. anguillarum.* Romalde and colleagues revealed that >3 OMP were induced in iron limiting conditions. Ruckerbactin, a catecholate siderophore iron acquisition system, has been described (Fernández et al. 2004). As a result of a commendable study, Davies (1991) reported on the OMP of 135 *Y. ruckeri* isolates. Several 36.5–40.5 kDa peptidoglycan associated proteins and a 36.5 or 38 kDa heat-modifiable protein were characterised. This 39.5 kDa peptidoglycan-associ-

ated protein was apparently not produced during logarithmic growth, but increased quantitatively in the stationary phase.

A publication has reported that a L-cysteine uptake system together with a L-cysteine desulphidase-encoding gene constitutes a novel *cdsAB* operon important in virulence (Méndez et al. 2011).

An example of the new approach in examining pathogenicity involved an inbred fish model, i.e. platyfish (*Xiphophorus maculatus*) and cultured fish cells. In particular, infection with *Y. ruckeri* involved bathing in suspensions containing 10^6 to 10^8 cells (Kawula et al. 1996). The result, in terms of invasion of key tissues, may be summarised, as follows:

Cell type	% invasion
rainbow trout gonad	2.6
rainbow trout kidney	2.3
minnow epithelial cells	10.2

Using a strain which had been genetically tagged with green fluorescent dye and by means of immersion and i.p. infectivity experiments, it was observed that the pathogen moved extracellularly and to a less extent intracellularly to the kidney, spleen and peripheral blood (Welch and Wiens 2005). Further evidence pointed to the ability of the pathogen to adhere to and invade gill and gut epithelia of rainbow trout (Tobback et al. 2009, 2010).

Y. ruckeri may be able to outcompete other micro-organisms, which may be an advantage for any potential pathogen, by the production of water soluble antimicrobial compounds (Michel and Faivre 1987).

Disease Control

Electromagnetic Fields Low frequency electromagnetic induction (15 Hz) at 0.1, 0.5, 5 and 50 μT for 1 h daily for 60 days enhanced protection of rainbow trout against *Y. ruckeri* (Nofouzi et al. 2015).

Vaccine Development The development of vaccines is a story of success until the advent of the new biogroup (Austin et al. 2005), with commercially available products being marketed for use in aquaculture. Recently, there has been research concerned with how vaccines are taken up by the fish, and the exact mechanism of their action. In terms of the latter, humoral and cellular immunity has been recognized to be involved in protection of rainbow trout as a result of vaccination. The latter were more prominent in the spleen rather than head kidney, and was more closely related to protection (than humoral immunity). Protection early in infection was associated mostly with the expression of genes encoding complement factors, lysozyme, and acute phase proteins. Later in infection, there was the dominant expression of adap-

tive immune genes, comprising the transformation of resident cells into macrophage-like cells, and the increased occurrence of CD8 α and IgM cells (Deshmukh et al. 2013).

The initial attempts to produce vaccines for ERM may be traced back to the work of Ross and Klontz (1965). These workers used a phenol inactivated vaccine, which was administered orally, via the food. Success was most encouraging, insofar as 90 % of the vaccinated fish survived subsequent infection with *Y. ruckeri*. A comparison of different methods of bacterial inactivation convinced Anderson and Ross (1972) that 3 % chloroform was better than sonication, 1 % formalin, or 0.5 % or 3 % phenol. Passive immunisation confirmed that the fish produced humoral antibodies to chloroform inactivated cells (Busch 1978). Commercial interest in *Y. ruckeri* vaccines grew with the involvement of the now defunct Tavolek Company. Amend et al. (1983), examining factors affecting the potency of preparations, reported that potency was not affected by pH values of 6.5 to 7.7, or by cultivation for up to 96 h in TSB at room temperature. This team concluded that inactivation, whether by formalin or chloroform, did not matter. However, there was good evidence that protection was enhanced by culturing the cells for 48 h at pH 7.2, lysing them at pH 9.8 for 1–2 h, and then adding 0.3 % (w/v) formalin. A live auxotrophic *aroA* mutant was evaluated by i.p. injection in rainbow trout, and an RPS of 90 % recorded after challenge (Temprano et al. 2005).

Application of these vaccine formulations may be by the oral route, i.e. on food (Klontz 1963; Ross and Klontz 1965; Anderson and Nelson 1974; Villumsen et al. 2014), by injection (Anderson and Nelson 1974; Cossarini-Dunier 1986), by immersion, shower or spray (Johnson and Amend 1983a, b), nasally (LaPatra et al. 2015) or by anal intubation (Johnson and Amend 1983b). Immersion vaccination of rainbow trout using a inactivated whole-cell vaccine with and without Montanide IMS 1312 VG led to better protection when the adjuvant was present. Thus without Montanide, the RPS after challenge was 80–82 % whereas with adjuvant, the corresponding RPS was 93.8–100 % (Soltani et al. 2014). Researching vaccination to *Y. ruckeri* O1 biotype 2 in rainbow trout, Chettri et al. (2013) used a commercial product, which was used at recommended or a greater dilution by immersion or injection with or without boosters. Challenge was 3–7 months after the initial vaccination. At 3-months, the best results in terms of protection were the single injection where the group had a mortality of 2 %, and the immersion in the manufacturer's recommended dose plus a booster by injection 1 month later where there was 0 % mortality, in comparison the unvaccinated controls with 76 % mortalities. At 5-months after the initial vaccination, the corresponding mortalities levels were 5 % and 0 %, and 26 % for the controls. At 7-month, the injection vaccinated fish demonstrated 17 % mortalities compared to 44 % mortalities of the controls. At this this time, there was not any evidence of protection for the fish that received a single immersion dose. Clearly, the use of vaccination by injection and the use of a booster dose resulted in long term health benefits to rainbow trout (Chettri et al. 2013).

Problems have been recorded previously with the oral method, insofar as protection was short-lived although progress on oral delivery has been made. Thus in a comparison of injection and oral methods of uptake with a chloroform inactivated vaccine, Anderson and Nelson (1974) did not find any antibody in fish fed with a vaccine for 7 days; whereas a low titre of 1:16 and 1:32 resulted in trout which were injected. Moreover, injected fish were protected for 12 weeks compared to only 6 weeks in the group which received the oral preparation. Similarly, a comparison of injection, immersion, shower and spray methods showed that injection offered the best protection against artificial challenge with *Y. ruckeri* (Johnson and Amend 1983a). In a comparison of the efficacy of injection, oral uptake and anal intubation, Vigneulle (1990) favoured the first mentioned in terms of protection. The nasal and intraperitoneal delivery of a combined live attenuated infectious hematopoietic necrosis virus (IHN) and inactivated whole cell ERM vaccine was evaluated in rainbow trout, and challenged after 7 and 28 days. The best protection was recorded after 28 days in the i.p. vaccinated group, although early protection was demonstrated following nasal administration (LaPatra et al. 2015). Moreover, interestingly, antibodies were found in the serum of rainbow trout vaccinated by injection and anal intubation, but not by the oral route. Oral administration of rainbow trout with a commercial formalin-inactivated whole cell vaccine or a locally made experimental product was carried out (single and ×50 doses) with or without booster doses 4 months later. A further group received a single dose anally. All groups were challenged 6 months after the primary administration with heavy mortalities in groups apart from the anally and orally (×50 dose) vaccinated fish; all fish in these groups survived challenge. Thus, anal uptake gave full protection as did the very dose administered orally, which suggested that there is significant degradation (= digestion) of the antigens in the stomach before reaching the intestine where it is available to the immune system, and specifically the lymphocytes (Villumsen et al. 2014). Unfortunately, it must be emphasised that injection is only feasible for large and/or valuable fish and not for the millions of fry/fingerlings which abound on the typical fish farm. However, ERM vaccines have been used successfully on fish farms when administered by bathing (Tebbit et al. 1981). In one investigation, 22959239 rainbow trout were vaccinated by a 90 s dip in a commercial vaccine preparation. The results were very encouraging with significantly reduced losses attributable to ERM. In this particular trial, the vaccine effected an 84 % reduction in mortalities due to ERM (Tebbit et al. 1981). Of additional benefit, there was a concomitant decrease in the use of medication by 77 %, and an increase in food conversion of 13.7 %. Analogous findings have been reported by Amend and Eschenour (1980) and Newman and Majnarich (1982).

Optical projection tomography and immunohistochemistry was used to visualize the uptake and processing of a whole cell vaccine by bath in rainbow trout. Tomography showed that vaccine was initially taken up via secondary gill lamellae from within 30 s of vaccination. At 5 to 30 min, vaccine cells were found on other mucosal surfaces, namely the skin and olfactory bulb; at 3-h, the digestive tract was filled with a complex of vaccine and mucus, remaining there for >24 h. At 24 h, vaccine cells were located in the blood, spleen and trunk kidney (Ohtani et al. 2015).

Addressing the question of the interval necessary for the onset and duration of immunity to develop, Johnson et al. (1982a) reported that a 5 s immersion in a vaccine suspension was sufficient to induce protection within 5 days at 18 °C, or 10 days at 10 °C. The minimum size of salmonids necessary for maximal protection was estimated to be in the range of 1.0–2.5 g. In fact, these authors concluded that protection was correlated with size of the fish, and not their age. Thus with 1.0, 2.0 and 4.0 g fish, immunity lasted for approximately 4, 6 and 12 months, respectively (Johnson and Amend 1983b). However, the water temperature was important, with 15 °C being completely effective but not so 5 or 25 °C (Raida and Buchmann 2008). There was some variation in results between species, with coho salmon and sockeye salmon retaining immunity for longer than pink salmon. Lamers and Muiswinkel (1984) concluded that a secondary immune response occurred as long as 7 months after primary contact with the antigen, indicating the presence of a fairly long-lived memory. Cossarini-Dunier (1986) found protection lasted for 445 days after intraperitoneal injection of a formalised culture which was suspended in saline or oily adjuvant. Thus, after challenge, 88.5 % of the controls died but only a few vaccinates. Interestingly, booster vaccination using 1: 100, 1: 1000 and 1: 2000 dilutions of inactivated whole cell vaccine with 1 h or 2 h exposure resulted in higher and longer protection although the typical 30 s in a 1:10 dilution was better (Chettri et al. 2015).

The commercial immersion vaccines were less successful at controlling the new biogroup (RPS = 47 % compared to 95 % for the Hagerman strain). This prompted an approach to supplement a current commercial vaccine with formalised whole cells of the new biogroup (RPS = 56 % compared to 97 % for the Hagerman strain) or by using an autologous vaccine comprised exclusively of formalin-inactivated cells of the new biogroup (RPS = 76 % compared to 58 % for the Hagerman strain) (Austin et al. 2005). A parallel situation occurred with Atlantic salmon culture in Tasmania whereby the standard ERM vaccine lost effectiveness, and led to an improved product involving the trypsinisation of the component culture to expose the O-antigen and thereby improving antigenicity. The end result was improved protection [the RPS increased from 37 % to 55.6 %] (Costa et al. 2011). A new commercial vaccine, which contains antigens of the Hagerman strain and the new biogroup, has been marketed, and its use documented to protect rainbow trout against biogroup 1 and 2 (Deshmukh et al. 2012). A comparison of commercial vaccines, one of which contained biogroup 1 and the second that was formulated with both biogroups, revealed better protection for rainbow trout with the latter 4 and 6-months after the standard 30 s immersion (Deshmukh et al. 2012).

Use of non-adjuvanted flagellin as a sub-unit vaccine in the native and recombinant form resulted in protection from challenge with biotype 1 and biotype 2 cultures of *Y. ruckeri*. Moreover, there was evidence of protection against pathogens, e.g. *Aer. salmonicida* (Scott et al. 2013).

Immunostimulants/Dietary Supplements Vigneulle and Gérard (1986) reported that 48 000 IU/kg, 8 650 mg/kg and 500 mg/kg of vitamin A, C and E for 5 days respectively enhanced resistance to ERM.

Antimicrobial Compounds Control of clinical cases of ERM may be mediated by means of antimicrobial compounds, including sulphamerazine and oxytetracycline (Rucker 1966), methylene blue and oxytetracycline (Llewellyn 1980), potentiated sulphonamides (Bullock et al. 1983), tiamulin (Bosse and Post 1983) and oxolinic acid (Rodgers and Austin 1982). Early success was obtained by administering medicated diet containing sulphamerazine (200 mg/kg body weight of fish/day for 3 days; Rucker 1966; Klontz and Huddleston 1976), followed by oxytetracycline (50 mg/kg body weight of fish/day for 3 days). A parallel therapy worked against salmonid blood spot (Llewellyn 1980). This involved treatment with methylene blue dosed at 1 g of dye/kg of food for 5 days), followed by oxytetracycline (66 mg/kg body weight of fish/day for 10 days) and then a repeat dose of methylene blue. Llewellyn (1980) reported that in hatchery conditions, the disease cleared up in 10 to 14 days.

Two groups of workers demonstrated success with potentiated sulphonamides. Bullock et al. (1983) described the beneficial effects of a mixture of sulphadimethoxime and ormetroprim, dosed at 50 mg/kg body weight of fish/day for 5 days, whereas Bosse and Post (1983) discussed the usefulness of a combination of sulphadiazine and trimethoprim at the very low dose of 1 mg/kg body weight of fish/day for 14 days. This latter group also emphasised the benefit of using tiamulin at 5 mg/kg body weight of fish/day for 14 days. Finally, on the basis of laboratory experiments, Rodgers and Austin (1982) reported the effectiveness of oxolinic acid (10 mg/kg body weight of fish/day for 10 days). Unfortunately, the presence of R plasmids may reduce the effect of some antibiotics (De Grandis and Stevenson 1985).

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Chapter 7
Flavobacteria and Cytophagas

Abstract An increasing number of Gram-negative chromogens have become associated with fish diseases particularly involving the gills and external surfaces [fin/tail rot]. New additions to the list of pathogens include *Chryseobacterium piscicola, Flavobacterium oncorhynchi, Tenacibaculum dicentrarchi, T. discolor, T. gallaicum* and *T. soleae* with research interest focusing on molecular diagnoses and disease control by vaccination, probiotics, immunostimulants and bacteriophages.

Keywords Gill disease • Flavobacteriosis • RTFS • Coldwater disease • Columnaris

Numerous reports have centred around the role of Gram-negative chromogens as agents of fish disease, particularly associated with gill disease involving hyperplasia, hypertrophy and fusion of the gill lamellae (Fig. 7.1). Genera that have been mentioned frequently include *Cytophaga, Flavobacterium, Flexibacter, Myxobacterium, Myxococcus* and *Sporocytophaga.* The common factor is that these genera comprise difficult-to-identify species, a taxonomic re-evaluation of which has at long last occurred. Moreover from the early literature it is often uncertain into which of these genera unknown isolates should have been placed. In particular, the distinction between *Cytophaga* and *Flexibacter* was confusing (Christensen 1977; Allen et al. 1983). The authenticity of *Flavobacterium* has been questioned repeatedly, insofar as it became a recipient of problematical pigmented bacteria. Fortunately, more recent work has improved the taxonomy of *Flavobacterium* (Holmes et al. 1984; Bernardet et al. 1996). *Myxobacterium,* considered to be a causal agent of gill disease (Ghittino 1967; Wood 1968; Bullock and McLaughlin 1970; Ashburner 1978), is not included in the 'Approved Lists of Bacterial Names' (Skerman et al. 1980) or their supplements. Therefore, this genus lacks taxonomic meaning. It is possible that the organisms, identified as *Myxobacterium* (and referred to as myxobacters), belong in either *Cytophaga, Flavobacterium* or *Flexibacter.*

Historically, interest in chromogenic Gram-negative bacteria started with a publication by Davis (1922), who reported serious mortalities (columnaris) among warm-water fish, namely small mouth bass and common perch, from the Mississippi River. These fish were held at the US Biological Station at Fairport, Iowa, when the disease occurred. Davis recognised two important features of the disease, namely that occurrence was primarily in injured (damaged/stressed) fish, and that the water temperature was high, i.e. >21.1 °C. Unfortunately, Davis did not succeed in isolat-

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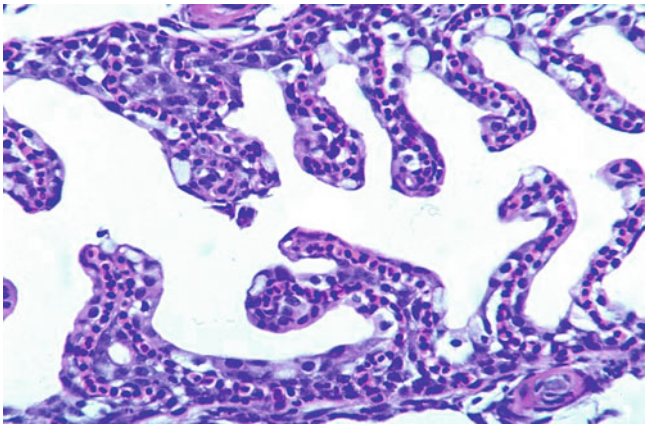

Fig. 7.1 Hyperplasia, hypertrophy and fusion of the gill lamellae in rainbow trout (The photograph, courtesy of Professor J.F. Turnbull, was taken at a magnification of × 400)

ing the pathogen. In fact, this was not achieved for two decades, until Ordal and Rucker (1944) succeeded in 1943 during an outbreak in hatchery-reared sockeye salmon.

Fish pathogenic flexibacters have been recognised (see Masumura and Wakabayashi 1977; Hikida et al. 1979; Pyle and Shotts 1980, 1981; Wakabayashi et al. 1984). For example, during 1976 and 1977, a bacterial disease developed in juvenile (usually ≤60 mm in length) red sea bream and black sea bream maintained at marine sites in Hiroshima Prefecture, Japan. The outbreak occurred 1–2 weeks after the fish were transported from the hatchery to sea cages. An organism was isolated by Masumura and Wakabayashi (1977), and later considered to be a new species of *Flexibacter,* for which the name of *Fle. marinus* was coined (Hikida et al. 1979). This organism was subsequently re-named as *Fle. maritimus* (Wakabayashi et al. 1986) and thence as *Tenacibaculum maritimum* (Suzuki et al. 2001). However, it has been suggested that this taxon is synonymous with *Cyt. marina* (Holmes 1992). *Fle. ovolyticus* was named as a new pathogen of halibut eggs and larvae (Hansen et al. 1992), and then reclassified as *Tenacibaculum ovolyticum* (Suzuki et al. 2001). Additional reports of fish pathogenic *Flexibacter* spp. have been noted. In particular, the genus has been found in cases of fin rot (Bullock and Snieszko 1970; Ziskowski and Murchelano 1975; Schneider and Nicholson 1980) and general 'myxobacterial' diseases (e.g. Pyle and Shotts 1980, 1981).

Flavobacterium became the second genus of yellow-pigmented Gram-negative fish pathogens to be recognised, with the description of *Fla. balustinum* by Harrison and Sadler (1929). This taxon was re-classified into a newly established genus, as *Chryseobacterium balustinum* (Vandamme et al. 1994). From the original publication, we believe that this organism was really a fish spoilage agent rather than a pathogen, insofar as the only work describing 'infectivity' referred to dead not living fish. However, there are indications that the organism is now becoming associ-

ated with disease. Originally, the organism was first described as being a problem on freshly landed halibut, on which it produced a yellowish slime (Harrison and Sadler 1929). It would seem likely that the first *bona fide* report of fish pathogenic flavobacteria was by Bein (1954), who described *Fla. piscicida* as the causal agent of mass mortalities (referred to as 'red tide') in marine fish from Florida, USA. Incidence of the disease appeared to be associated with the proliferation of phytoplankton and may, consequently, be considered as influenced by water quality. The name of *Fla. piscicida* does not appear in the 'Approved Lists of Bacterial Names' (Skerman et al. 1980) or their supplements, and is, therefore, of dubious taxonomic standing.

A filamentous Gram-negative organism, isolated from gill lesions in rainbow trout and Yamame salmon, formed the basis of an article by Kimura et al. (1978). These isolates from Japan, together with similar strains recovered from Oregon, USA, were included in a detailed investigation by Wakabayashi et al. (1980). The outcome was recognition of a novel group of *Flavobacterium,* i.e. *Fla. branchiophila* (Wakabayashi et al. 1989). This name has now been corrected to *Fla. branchiophilum* (von Graevenitz 1990), and its authenticity verified (Bernardet et al. 1996). *Fla. branchiophilum* appears to be spreading as a fish pathogen in the Far East e.g. in Korea (Ko and Heo 1997).

There have been other reports of 'flavobacteriosis' caused by unknown species of *Flavobacterium* (e.g. Brisou et al. 1964; Roberts 1978; Acuigrup 1980; Farkas 1985). Whereas there has been reticence to equate these organisms with existing nomenspecies (or to name new species), the descriptions of the aetiological agents have been quite reasonable. For example, Acuigrup (1980) discussed flavobacteriosis among coho salmon held in Spanish seawater sites. This disease, which resembled a generalised septicaemia, caused 20–25 % mortality in the fish population during the summer of 1978. The subsequent description of the organism was superior to the initial work with *Fla. balustinum* or *Fla. piscicida. Fla. succinicans* has been suggested as a fish pathogen (see Zamora et al. 2012b) whereas *Fla. chilense* and *Fla. araucananum* have been recovered from diseased salmonids in Chile but pathogenicity has not been proven (Kämpfer et al. 2011). Similarly, *Fla. collinsii*, *Fla. branchiarum* and *Fla. branchiicola* have been found in and around the aquaculture environment, including fish gills and liver, but pathogenicity has not been addressed (Zamora et al. 2013).

Cytophagas became implicated as fish pathogens with the work on coldwater (low-temperature) disease. The causal agent was initially isolated and described by Borg (1948), and subsequently named as *Cyt. psychrophila* (Borg 1960), and then to *Fla. psychrophilum* (Bernardet et al. 1996). Coldwater disease affects predominantly juvenile salmonid fish, notably coho salmon in the northwest USA, and is most prevalent in winter and spring when the water temperature is <10 °C. Cranial and vertebral lesions may occur (Kent et al. 1989). More recently, the organism has been associated with systemic disease in eels and cyprinids in Europe (Lehmann et al. 1991) and with an anaemic condition of juvenile rainbow trout in Chile (Bustos et al. 1995) and Europe (e.g. Lorenzen et al. 1991), referred to as rainbow trout fry syndrome [RTFS] (Baudin-Laurençin et al. 1989; Lorenzen et al. 1991).

A causal mention was given initially to the role of *Cyt. johnsonae* and *Cyt. rosea* as fish pathogens (Christensen 1977). *Cyt. johnsonae* has emerged as a problem in Australia. However, only scant information is available about *Cyt. rosea*; therefore, this organism will not be considered further.

An organism was recovered initially from the gills of diseased hatchery-reared salmon, trout and suckers in Michigan, USA (Strohl and Tait 1978). Thirteen isolates were recovered, and although similarities were noted to organisms previously described by Borg (1960), Pacha and Porter (1968) and Anderson and Conroy (1969), it was decided to elevate them into a new species, as *Cyt. aquatilis* (Strohl and Tait 1978), and thence to *Fla. hydatis* (Bernardet et al. 1996). It must be emphasised that Strohl and Tait (1978) did not prove that the organisms were capable of causing disease. Nevertheless, we have recovered similar organisms from outbreaks of gill disease in farmed rainbow trout from England.

Cytophaga sp. has been associated with skin and muscle lesions on Atlantic salmon in the U.S.A. (Kent et al. 1988), and a previously undescribed *Cytophaga* – like bacterium (CLB) has been associated with a gill and systemic disease in turbot (Mudarris and Austin 1989). This organism was described as a new species, as *Fla. scophthalmum* (Mudarris et al. 1994), which was re-classified to *Chrys. scophthalmum* (Vandamme et al. 1994).

Some new species have been recovered from diseased fish, but pathogenicity has not been confirmed. These include:

- *Chrys. arothri* was recovered from the kidneys of puffer fish (*Arothron hispidus*) caught off the coast of Hawaii (Campbell et al. 2008).
- *Chrys. viscerum* was described from the gill and liver of diseased rainbow trout in Spain (Zamora et al. 2012a).
- *Chrys. oncorhynchi* was described from the gills and liver of rainbow trout (Zamora et al. 2012c).
- *Chrys. tructae* was recovered from rainbow trout (Zamora et al. 2012d).
- *Chrys. shigense* was recovered from diseased rainbow trout (Zamora et al. 2012e).

Little is known about the role of *Sporocytophaga* as a fish pathogen. Mixed infections attributed to *Sporocytophaga* and *V. anguillarum* occurred as surface lesions, termed saltwater columnaris, on salmon and trout held in marine conditions (Wood 1968). However, apart from discussing the presence of microcysts which began to form at 2–7 days, there is little information about this suspected pathogen (Pacha and Ordal 1970).

The precise reservoir of most of these fish pathogens remains unclear. Yellow and orange-pigmented bacteria occur in large numbers in fresh water (Allen et al. 1983) and seawater (Austin 1982), and comprise part of the normal microflora of gills of healthy salmonids (Trust 1975) and possibly even eggs (Hansen et al. 1992). Therefore, it seems likely that the taxa comprising the fish pathogens occur naturally in the aquatic environment.

Isolation Generally, the aetiological agents may be readily recovered from diseased tissues on low-nutrient media, with incubation at 10–25 °C for 4–14 days. Specialised media have been devised, of which cytophaga agar (Appendix in Chap. 12; after Anacker and Ordal 1959) has received greatest use. This is suitable for the isolation of *Fla. hydatis, Fla. johnsoniae* (specifically with incubation at 27 °C for 7 days; Carson et al. 1993), *Fla. psychrophilum, Fla. branchiophilum* and *Fla. columnare.* The problem of overgrowth/out-competition by saprophytes has been raised in connection with the recovery of the causal agent of flavobacteriosis with the advise that serial dilutions of the (external) samples are needed before plating techniques are used in order to maximise the possibility of recovering the actual disease causing agent (Tiirola et al. 2002).

Chryseobacterium spp.

Chryseobacteria have been described that infected fish from the Great Lakes of North America, and which were regarded as novel (Loch and Faisal 2015).

The isolation of 17 cultures was achieved from kidney, gills or fins using Hsu-Shotts medium (Bullock et al. 1986) supplemented with 4 mg of neomycin/ml with incubation at 22 °C for up to 7-days (Loch and Faisal 2015). Two of these isolates were regarded as comprising novel taxa (Loch and Faisal 2015).

Chryseobacterium aahli

Characteristics of the Disease

Cultures were obtained during disease surveillance in 2009. The disease signs were as expected of chryseobacteria, and included the presence of necrotic finage (Loch and Faisal 2014b).

Isolation

Diseased tissue, i.e. kidney and necrotic fin, was inoculated onto Hsu–Shotts medium (Bullock et al. 1986) with incubation at 22 °C for 72 h (Loch and Faisal 2014b).

Characteristics of the Pathogen

Box 7.1: *Chryseobacterium aahli*
The two cultures examined comprise semi-translucent, golden yellow colonies with flexirubrin-type pigment, 1.0–1.5 mm in diameter, and convex with entire margins. The organisms are Gram-negative nonmotile, non-gliding rods of 1.5–2.0 μm in length, that produce acid and alkaline phosphatase, N acetyl-ß –glucosaminidase, α-chymotrypsin, catalase, ß-glucosidase, naphthol-AS-BI-phosphohydrolase, and oxidase, but not arginine dihydrolase, α–fucosidase, α– or ß-galactosidase, ß-glucuronidase, H_2S, indole, esterase, esterase lipase, cysteine, leucine and valine arylamidase, lysine or ornithine decarboxylase, α–mannosidase or tryptophan deaminase. Growth occurs on TSA, but not on cetrimide agar, MacConkey agar or marine 2216E agar, at 4–22 °C but not at 37 °C, on 0–2 % (w/v) sodium chloride (only weakly at 2 %), and at pH 6.0–8.0. Aesculin, casein, elastin, gelatin and Tween 20 and 80 are degraded, but not agar, starch, L-tyrosine or urea. The Voges Proskauer reaction is negative. Nitrates are not reduced. Citrate is utilized. L arabinose, D-glucose and D–mannose are utilized weakly, but not N acetylglucosamine, adipic acid, capric acid, malic acid, maltose, D-mannitol, phenylacetic acid, potassium gluconate nor trisodium citrate. Acid is produced from aesculin, gentiobiose (weakly), D-glucose (weakly) and trehalose (weakly), but not from D-adonitol, amygdalin, D- or L-arabinose, D- or L-arabitol, arbutin, cellobiose, dulcitol, erythritol, D- or L-fucose, D-galactose, glycerol, glycogen, inositol, inulin, lactose, maltose, D-mannitol, melezitose, melibiose, potassium gluconate, raffinose, rhamnose, D-ribose, L-sorbose, D-sorbitol, starch, turanose, xylitol or D- or L-xylose. The major fatty acids comprise iso-$C_{15:0}$, summed feature 3($C_{16:1}$ ω6c and/or $C_{16:1}$ ω 7c), iso-$C_{17:0}$ 3-OH, $C_{16:0}$ and $C_{16:0}$ 3-OH. The G+C ratio of the DNA of the type strain is 34.1 mol% (Loch and Faisal 2014b).

Pathogenicity

Pathogenicity of one culture, T68^T, was established for multiple Great Lakes salmonid species (Loch 2012).

Chryseobacterium balustinum (= Flavobacterium balustinum)

Isolation

'Halibut jelly' was used to culture *Chrys. balustinum* as yellow-green ('fluorescent') pigmented colonies (Harrison and Sadler 1929).

Characteristics of the Pathogen

The original description was extremely brief, referring to the growth of colonies at very low temperatures, i.e. 1–3 °C, rod-shaped micromorphology, motility, and the ability to degrade gelatin (Harrison and Sadler 1929). The revised description of *Flavobacterium* excludes motile organisms (Holmes et al. 1984); therefore the validity of *Fla. balustinum* was doubtful. The organisms were re-classified to *Chryseobacterium,* as *Chrys. balustinum* on the basis of rRNA clustering (Vandamme et al. 1994). Interestingly, the extensive list of characters in *Bergey's Manual of Systematic Bacteriology* (Holmes et al. 1984) precluded reference to motility or, for that matter, gliding movement among representative strains of any species. Other characteristics of the taxon included:

Box 7.2: *Chryseobacterium balustinum*
The organism comprises yellow-pigmented, mucoid colonies with non-motile, oxidative rods of 1.0–1.8 × 0.5 μm in size. Catalase, indole, oxidase and phosphatase, but not ß-galactosidase, arginine dihydrolase, H_2S or phenylalanine deaminase, are produced. Nitrates are reduced. Aesculin, casein, DNA, gelatin, tributyrin, Tween 20 and 80, but not starch, tyrosine or urea, are degraded. Growth occurs at 37 °C, but not at 5 or 42 °C. This is in contrast to the original work of Harrison and Sadler (1929), who reported growth at almost the freezing point of water. Acid is produced from ethanol and glucose, but not from arabinose, cellobiose, lactose, mannitol, raffinose, salicin, sucrose or xylose. The G + C ratio of the DNA is 33.1 moles %.

Chryseobacterium indologenes

Characteristics of the Disease

In January 2012, mortality was recorded in farmed yellow perch (*Perca flavescens)* in the USA. The disease signs centred on the presence of yellowish skin lesions, which were below the dorsal fin and on the caudal peduncle (Pridgeon et al. 2012).

Isolation

Skin lesion samples were inoculated onto Shieh agar supplemented with 1 μg of tobramycin/ml (Decostere et al. 1997) with overnight incubation at 19 +/− 1 °C (Pridgeon et al. 2012).

Characteristics of the Pathogen

Box 7.3: *Chryseobacterium indologenes*
Fifteen isolates were recovered from skin lesions. All but two of these isolates produce yellow pigmented colonies that comprise Gram-negative rods, which produce catalase, ß-glucosidase and oxidase. Gelatin is attacked. Sequencing of the 16S rRNA gene revealed 99 % homology with the reference culture of *Chrys. indologenes* in GenBank (Pridgeon et al. 2012).

Pathogenicity

Laboratory based infectivity experiments with yellow perch by i.p. injection recorded LD_{50} values of $1.5–3.2 \times 10^8$ CFU/fish (Pridgeon et al. 2012).

Chryseobacterium piscicola

Characteristics of the Disease

There was an association with external lesions/ulcerations on rainbow trout and Atlantic salmon farmed in Chile (Ilardi et al. 2009, 2010).

Characteristics of the Pathogen

In the initial study, which proposed a new species, 8 isolates were recovered from farmed Atlantic salmon and rainbow trout in Chile. Examination of the 16S rRNA gene sequences revealed that the closest neighbours with homologies of 96.9 and 97.1 %, respectively, were *Chryseobacterium soldanellicola* and *Chryseobacterium soli* (Van Gelderen et al. 2009a).

Box 7.4: *Chryseobacterium piscicola*
Colonies are yellow [produces flexirubin pigment], smooth, shine and circular, and comprise Gram-negative, non-motile, non-gliding, non-fermentative α– haemolytic rods of 1.6–3.5 μm in length and 0.8–1.2 mm in diameter that produce catalase and oxidase. Growth occurs at 4–28 °C but not at 37 °C and in 0–3 % (w/v) sodium chloride. Nitrates are not reduced. Aesculin, DNA (weakly) and gelatin are degraded, but not agar, casein, starch, Tween 80, tyrosine or urea. H_2S and indole are not produced. Neither arginine dihydrolase, or lysine or ornithine decarboxylase is produced. The methyl red test and Voges Proskauer reaction are negative. Acid and alkaline phosphatase, esterase, esterase lipase, lipase, cysteine leucine and valine arylamidase, α–chymotrypsin, naphthol-AS-BI-phosphohydrolase, ß-glucuronidase, α– and ß-glucosidase and *N*-acetyl-ß-glucosaminidase were produced in the API ZYM system, but not α–fucosidase, α– or ß-galactosidase, α–mannosidase or trypsin. The G + C ratio of the DNA of the two cultures examined is 32.5- and 32.3 moles %. Examination of the 16S rRNA gene sequences confirmed membership of the genus *Chryseobacterium* (Ilardi et al. 2009, 2010).

Pathogenicity

It was stated that infectivity experiments confirmed pathogenicity of *Chry. piscicola* for Atlantic salmon (Ilardi et al. 2010).

Chryseobacterium scophthalmum (= Flavobacterium scophthalmum)

Characteristics of the Disease

This organism caused lethargy, gill hyperplasia, haemorrhaging in the gills, distended abdomen, surface haemorrhaging, and extensive internal haemorrhaging (Mudarris and Austin 1989, 1992).

Isolation

The pathogen may be recovered from infected tissues on medium K (Appendix in Chap. 12; Mudarris and Austin 1989).

Characteristics of the Pathogen

Box 7.5: *Chryseobacterium scophthalmum*
Colonies (2–3 mm in diameter after 48 h at 25 °C) contain orange-pigmented (flexirubin pigment) uniformly shaped, short, fermentative Gram-negative rods of approximately 2.0×0.8 μm in size. Gliding movement is exhibited. Catalase and oxidase are produced, but not H_2S, indole, or lysine or ornithine decarboxylase. Nitrates are not reduced. The Voges Proskauer reaction is negative. Aesculin, casein, DNA, gelatin, tributyrin and tyrosine are degraded, but not chitin or starch. Acid is produced from cellobiose and glucose, but not arabinose, mannitol, raffinose, sucrose or xylose. Thin sections reveal the presence of a thick (43.5 nm) cell envelop, which could be mistaken for an extracellular layer. The G + C ratio of the DNA is 31.7–34.2 moles % (Mudarris and Austin 1989; Mudarris et al. 1994).

From these characteristics, the 50 isolates from turbot were equated with a new species of *Flavobacterium,* as *Fla. scophthalmum* (Mudarris et al. 1994). With subsequent improvements in taxonomy, the species was transferred to the newly established genus *Chryseobacterium,* as *Chrys. scophthalmum* (Vandamme et al. 1994).

Epizootiology

Chrys. scophthalmum was found in the water, from which it is surmised that spread to turbot occurred (Mudarris and Austin 1989).

Pathogenicity

Intraperitoneal injection of cell-free culture supernatants of *Chrys. scophthalmum* resulted in clinical disease in turbot, with swollen oedematous gill lamellae, and haemorrhaging in the stomach, gastro-intestinal tract, kidney and liver (Mudarris and Austin 1989).

Disease Control

Antimicrobial Compounds Furazolidone, used by i.p. injection at 50 mg/kg body weight of fish or by immersion (50 mg/l for 30 min daily for 10 days) was effective at controlling mortalities caused by the organism (Mudarris and Austin 1989).

Flavobacterium sp.

Characteristics of the Diseases

Many of these organisms cause gill disease, which may be characterised histologically as hyperplasia (swelling) of the gill epithelia. Frequently, the condition in juvenile fish involves fusion at the distal tips of adjoining gill lamellae. The involvement of hyperplasia-inducing agents has also been implicated in some cases by *Flavobacterium* spp. (Kudo and Kimura 1983a, b).

Isolation

TSA supplemented with 0.5–3.0 % (w/v) sodium chloride was used for the isolation of *Flavobacterium* sp. (Acuigrup 1980).

Characteristics of the Pathogen

The organisms, discussed by Acuigrup (1980), were characterised, as follows:

Box 7.6: *Flavobacterium* sp.
Isolates produce yellow-orange colonies after 24 h (the temperature of incubation was not stated). Cultures comprise motile, pleomorphic, Gram-negative rods, which produce arginine dihydrolase and oxidase but not ß-galactosidase, H_2S, indole or lysine or ornithine decarboxylase, and are neither oxidative nor fermentative. The Voges Proskauer reaction is positive. Gelatin is degraded. Acid is produced from amygdalin, arabinose, inositol, mannitol, melibiose and rhamnose, but not from glucose or lactose. Sodium citrate is not utilised (Acuigrup 1980).

Most of the data originate from use of an unstated API product, possibly the API 20E rapid identification system. The presence of motility contrasts with the revised definition of *Flavobacterium* (Holmes et al. 1984). Therefore, it appears that the organisms are not *bona fide Flavobacterium,* but probably represent a closely allied taxon.

Diagnosis

The API 20E rapid identification system has been used for diagnosing *Flavobacterium* sp. (Acuigrup 1980).

Disease Control

Antimicrobial Compounds Apparently, some level of control was exercised with oxytetracycline (Acuigrup 1980). *In vitro* experiments have also pointed to the value of oxytetracycline and other drugs commonly used in aquaculture (Farkas 1985).

Flavobacterium branchiophilum

Isolation

Isolation may be achieved using cytophaga agar (Appendix in Chap. 12; after Anacker and Ordal 1959).

Characteristics of the Pathogen

Box 7.7: *Flavobacterium branchiophilum*
This pathogen forms yellow, translucent, smooth colonies of 0.5–1.0 mm in diameter after incubation at 18 °C for 5 days. Isolates (10 isolates from Oregon, 5 from Japan and one from Hungary; Farkas 1985) comprise slender, strictly aerobic, non-motile, Gram-negative rods of 5–8×0.5 µm in size, which are surrounded by pili (Heo et al. 1990). Usually, they occur as short chains, each comprising 2 to 3 cells. Catalase and oxidase are produced, but not H_2S or indole. Nitrates are not reduced. Casein, gelatin, lecithin, starch, tributyrin, Tweens and tyrosine were degraded, but not cellulose or chitin (Bernardet et al. 1996). Growth occurs at 5 and 30 °C but at 37 °C, and in 0 and 0.1 % (w/v) sodium chloride. It is noteworthy that Japanese isolates grow at 30 °C, whereas American strains do not. Acid is produced from a wide range of compounds, including cellobiose, fructose, glucose, inulin, maltose, melibiose, raffinose, sucrose and trehalose, but not from adonitol, arabinose, dulcitol, galactose, inositol, lactose, mannitol, salicin, sorbitol or xylose (Ko and Heo 1997). The G+C ratio of the DNA is 29–34 moles %. Another geographical difference concerns serology, insofar as the Japanese isolates are antigenically distinct from the American and Hungarian cultures (Huh and Wakabayashi 1989). The complete genome sequence, which revealed a small genome compared with other flavobacteria, has been published of strain FL-15, which was recovered from diseased sheatfish in Hungary (Touchon et al. 2011).

Diagnosis

Serology Rapid identification of *Fla. branchiophilum* was achieved by ELISA, which detected 1×10^3 cells/ml from gills [= the threshold value] (MacPhee et al. 1995).

Molecular Methods PCR targeted 16S rRNA has been successful with recognising *Fla. branchiophilum, Fla. columnare* and *T. maritimum* (Toyama et al. 1994, 1996; Avendaño-Herrera et al. 2004e; Yeh et al. 2006). Similarly, a DNA array-based multiplex assay permitted the simultaneous specific and sensitive (<1 pg of bacterial DNA) recognition and identification/differentiation of *Fla. branchiophilum, Fla. columnare* and *Fla. psychrophilum*, and viruses (Lievens et al. 2011).

Epizootiology

Fla. branchiophilum may be fatal to small fish following infection via water, i.e. adding a bacterial suspension to tank water (Wakabayashi et al. 1980). Certainly, the pathogen is present in water at times of disease outbreaks (Heo et al. 1990).

Pathogenicity

Kimura et al. (1978) established that fatal infections by *Fla. branchiophilum* were only induced following water-borne challenge in salmonids weighing ≤1.1 g each. Pathogenicity experiments with juvenile rainbow trout demonstrated that the organism occurred abundantly in the gills within 18–24 h after exposure to a dilute bacterial suspension, i.e. 10–20 ml of a 48 h broth culture in 2 l of fresh water. The gill lamellae became very swollen, but the reasons for subsequent fatalities remain unknown (Wakabayashi et al. 1980). Initial attachment of the pathogen to host cells may be by means of pili (Heo et al. 1990). Scrutiny of the complete genome sequence revealed adhesins and a cholera-like toxin; the first time that this has been found in non-*Proteobacteria* (Touchon et al. 2011).

Flavobacterium columnare (=Flexibacter/Cytophaga columnaris)

Characteristics of the Disease

Columnaris has been recognised to have worldwide distribution in a wide range of freshwater fish, including Arctic charr, bass, black bullheads, carp, channel catfish, chub, eel, goldfish, killifish, loach, perch, rainbow trout, roach, Atlantic salmon, chinook salmon, sheatfish, squawfish, tilapia, white crappie, whitefish and white-suckers (Nigrelli 1943; Nigrelli and Hutner 1945; Wakabayashi and Egusa 1966; Ajmal and Hobbs 1967; Fijan 1969; Bowser 1973; Wobeser and Atton 1973; Chun 1975; Bootsma and Clerx 1976; Ferguson 1977; Farkas and Oláh 1980; Kuo et al. 1981; Morrison et al. 1981; Chen et al. 1982; Koski et al. 1993; Welker et al. 2005). The disease was considered to be sufficiently serious to warrant inclusion in the list of notifiable fish diseases, as defined by the (British) Diseases of Fish Act, 1937. However, columnaris was omitted from the 1983 Act.

Infection with *Fla. columnare* may result in several discrete disease conditions. In young fish, there is often negligible pathology before death ensues. The gill is usually the major site of damage. Typically, congestion (blockage) of the blood vessels supplying the gills occurs, with dissociation of the surface epithelium of the lamellae from the capillary bed. There may be scattered areas of haemorrhaging (Pacha and Ordal 1967). In adult fish, the lesions may occur on the gills, skin and/or in the musculature. Systemic infections may develop (Wolke 1975). Skin discoloration/fading and muscle lesions have been documented on neon tetra (*Paracheirodon innesi)* (Michel et al. 2002). Gill lesions consist normally of yellow-orange areas of necrosis. These start usually at the periphery of the gill, and extend towards the base of the gill arch. Eventually, extensive erosion may completely destroy the gill filament (Pacha and Ordal 1970). On the body, small lesions start as areas of pale discoloration at the base of the dorsal fin or occasionally at the base of the pelvic fin, and lead to deterioration of the fins. These areas increase in size and may become as large as 3–4 cm in diameter, covering as much as 20–25 % of the total surface area of the fish. This may have the characteristic appearance of a saddle, and hence the descriptive term, ‘saddleback’. Frequently, the skin becomes completely eroded away, exposing the underlying muscle. Large numbers of bacteria are present at the advancing edge of the lesion. It is not uncommon for the fish to die within 48 h of the appearance of the skin discoloration (Pacha and Ordal 1970; Becker and Fujiwaha 1978; Morrison et al. 1981).

Isolation

Cytophaga agar (Appendix in Chap. 12; after Anacker and Ordal 1959) and Bootsma and Clerx’s medium for *Fla. columnare* (Appendix in Chap. 12; Bootsma and Clerx 1976) are useful for the recovery of the pathogen. Antibiotic-containing selective

media have been formulated for the selective recovery of the pathogen (Fijan 1969). On this medium, the pathogen produces characteristically yellow-orange pigmented colonies.

Characteristics of the Pathogen

Box 7.8: *Flavobacterium columnare*
Colonies appear to be flat, spreading, yellowish green (flexirubin-type pigment), with rhizoidal edges, and contain slender, strictly aerobic, Gram-negative rods of 4–8 × 0.5–0.7 μm in size, which move by gliding. Catalase, H_2S and oxidase are produced, but not indole, or lysine or ornithine decarboxylase. Nitrates are reduced. The methyl red test and Voges Proskauer reaction are negative. Casein, gelatin and tributyrin are degraded, but not aesculin, agar, cellulose, chitin, starch and tyrosine. Growth occurs at 4–30 °C (some isolates grow also at 37 °C), and in 0–0.5 % (w/v), but not 1 % (w/v), sodium chloride (Bernardet 1989; Bernardet and Grimont 1989; Bernardet et al. 1996). Dead bacterial cells, notably *Esch. coli* are lysed. Carbohydrates are generally not utilised. Moreover, acid is not produced from arabinose, cellobiose, glucose, lactose, mannitol, raffinose, salicin, sucrose or xylose. The G + C ratio of the DNA is 32.9–35.9 moles % (Wakabayashi and Egusa 1966; Pacha and Porter 1968; Pacha and Ordal 1970; Chun 1975; Bootsma and Clerx 1976; Morrison et al. 1981). Four major serological groups and one miscellaneous group have been recognised among the 325 strains examined by Anacker and Ordal (1959). Moreover, separate and distinct DNA homology groups have been described (Song et al. 1988). Two major genetic groups were recognised by PFGE using MluI restriction enzyme digestion among 30 isolates from channel catfish in the USA (Soto et al. 2007).

The taxonomic status of the pathogen has undergone radical change since the pioneering work of Davis (1922). Davis coined the name *Bacillus columnaris* because, in wet preparations of infected material, the bacteria congregated in column-like masses. According to Ordal and Rucker (1944), pure cultures of the organism exhibited gliding, and therefore should be associated with the myxobacteria. The taxonomy became further complicated by the observation in cultures of oval and spherical structures, which were thought to be microcysts (this notion was subsequently rejected). This led to a re-classification, as *Chondrococcus columnaris.* Shortly afterwards, Garnjobst (1945) suggested that the pathogen was actually a *Cytophaga,* and thus, the transition was made to *Cytophaga columnaris.* With further deliberation, the organism was transferred to *Flexibacter,* as *Flexibacter columnaris* (Leadbetter 1974), and from phylogenetic data as *Fla. columnare* (Bernardet et al. 1996).

The intraspecific diversity of 10 isolates was examined by means of 16S rRNA gene sequencing and RFLP, and three genomic groups recognised of which most cultures were recovered in Group 1. Interestingly, the 3 isolates in Group 3 contained a ~370 bp fragment that was absent from the other cultures (Schneck and Caslake 2006). Arias et al. (2004) evaluated 30 fish isolates and reference cultures by 16S rDNA sequencing, AFLP and intergenic spacer region sequencing, and defined two predominating genomovars (I and II) although the intergenic spacer region (ISR) sequencing revealed a higher diversity among genomovar I representatives, and by AFLP 22 profiles were recognised. Of interest, 4 isolates from tilapia in Brazil were recovered in a separate group, albeit related to genomovar I and II. Using a similar approach and 30 Finnish isolates plus the type strain, (Suomalainen et al. 2006a), the outcome was that the isolates were recovered in a genomovar although 8 ARISA profiles were defined of which three were similar. In a later study, Olivares-Fuster et al. (2007) studied 90 isolates predominantly from Alabama, USA, and defined genomovar status on the basis of RFLP analyses and other genotyping approaches, i.e. ALFP, MLSA and ISR-SSCP with the outcome that two groupings [genomovar I and II] were recognised of which there was higher genetic diversity in the latter. Interestingly, most of genomovar I was from threadfin shad whereas genomovar II isolates were mostly from catfish (Olivares-Fuster et al. 2007). In catfish farms in southeastern USA, *Fla. columnare* were recovered from columnaris outbreaks during spring 2010 to summer 2012 and determined to be of genomovar II (Mohammed and Arias 2014).

Fla. columnare may display Rhizoidal and Rough colony morphotypes, which are associated with virulence and resistance to starvation/bacteriophages, respectively (Zhang et al. 2014). The Rough colonies may arise spontaneously, or are induced by infection with bacteriophages, and have been demonstrated to be more susceptible to predation by amoebae and ciliates, and produced more biofilm (in the presence of amoebae) (Zhang et al. 2014). A third morphotype has been described as "Soft" (Laanto et al. 2014). By electron microscopy, the cells of Rhizoid and Soft but not so Rough morphotypes displayed an organised structure, which may be related to gliding motility; a reflection of virulence properties. Planktonic cells of the Rhizoid and Rough but not Soft colonies produced large membrane vesicles that contained two proteins, i.e. OmpA and SprF, and may be associated with adhesion and possibly invasion, and gliding motility, respectively. Also, the Rhizoid morphotype secreted a small, unidentified 13 kDa protein, possibly related to virulence (Laanto et al. 2014).

Diagnosis

Phenotypic Methods A simplistic approach has involved the observation of wet preparations, prepared from lesion material, which were supposed to demonstrate the presence of 'column' – like masses of bacteria (Snieszko 1958b).

Chemotaxonomy Methods Chemotaxonomic characters, namely whole cell fatty acid profiles and a commercial system, i.e. MIS – Microbial ID, have been used, with the dominant fatty acids including 11-methyl-dodecanoic acid, 13-methyl tetradecanoic acid, pentadecanoic acid, 14-methyl-pentadecanoic acid, 3-hydroxy-13-methyl tetradecanoic acid, 15-methyl *cis* 9-hexadecanoic acid, 3-hydroxy 14-methyl pentadecanoic acid, 15-methyl *cis* 9-hexadecanoic acid, 3-hydroxy 14-methyl pentadecanoic acid and 3-hydroxy-15-methyl hexadecanoic acid (Shoemaker et al. 2005).

Serology Whole cell agglutination is effective for the detection of *Fla. columnare* (Morrison et al. 1981). A relevant development has been the use of a rapid iFAT to simultaneously detect two pathogens, i.e. *Edw. ictaluri* and *Fla. columnare* using fluorochromes with two different spectra properties, Alexa Fluor 488 and 594 emitting green and red fluorescence, respectively (Panangala et al. 2006).

Molecular Methods PCR targeted 16S rRNA has been successful with *Fla. branchiophilum, Fla. columnare* and *T. maritimum* (Toyama et al. 1994, 1996; Avendaño-Herrera et al. 2004e; Yeh et al. 2006). LAMP was used successfully to diagnose *Fla. columnare* (Yeh et al. 2006; Suebsing et al. 2015). A highly specific and sensitive (2.2×10^2 CFU) LAMP assay with the pre-addition of calcein was developed, in which positivity in gill, gonad, blood, fermilised eggs and water, was recorded as colour change from orange to green within 45 min at 63 °C (Suebsing et al. 2015). A PCR targeting 16S rRNA has shown promise for the specific (negative for other flavobacteria) and sensitive (~100 cells) detection of *Fla. columnare* from culture and fish tissues within 5–8 h. Also PCR based on the 16S-23S rDNA intergenic spacer region has demonstrated promise for the detection of *Fla. columnare* from catfish in terms of specificity and sensitivity (capable of detecting 7 CFU). The technique was regarded as more sensitive than culturing (Welker et al. 2005).

Epizootiology

The consensus is that the disease is problematic only in the warmer periods of the year. Generally, epizootics occur when the water temperature is in the region of 18–22 °C, and the disease is rarely troublesome at <15 °C (Amend 1970). Thus, most outbreaks of columnaris occur between May and October (Bowser 1973; Kuo et al. 1981). To illustrate the dramatic effects of water temperature on the level of mortalities, the investigation of Holt et al. (1975) is especially relevant. This team challenged steelhead trout, chinook salmon and coho salmon with *Fla. columnare,* via the water-borne route. At a water temperature of 9.4 °C there were no mortalities attributable to columnaris. By increasing the temperature to 12.2 °C, 4–20 % mortalities ensued; whereas at 20.5 °C all the steelhead trout and coho salmon died, together with 70 % of the chinook salmon.

The level of mortality may be extremely high, and figures of 60–90 % are not uncommon. For example, columnaris was considered to be the most important contributing factor for 72.3–97.6 % and 75.4–95.4 % mortality among populations of adult sockeye salmon and adult chinook salmon, respectively (Fish and Hanavan 1948). Similarly, Chen et al. (1982) reported 77 % and 88.3 % losses among groups of carp and goldfish, respectively. Certainly, there are good data demonstrating the seasonal effects of mortalities due to columnaris, insofar as Bowser (1973) reported 60 % infection of bullheads in mid May whereas only a few months later the incidence had dropped to only 10 %.

In addition to water temperature, the severity of columnaris is influenced by a multiplicity of environmental (stress) and host-related factors. Chen et al. (1982) described the highest eel mortality levels to be associated with stagnant running water, whereas the lowest losses occurred in running water. Interestingly with aeration, the total losses fell between these two extremes. In this respect, the mortality rate has been inversely correlated with the level of dissolved oxygen in the water. Moreover, with adequate dissolved oxygen, deaths increased with a concomitant rise in the level of ammonia.

Rucker et al. (1953) isolated highly virulent strains of *Fla. columnare,* which were capable of killing fish within 24 h, from the water in the upper Columbia River basin. Furthermore, it has been demonstrated that large numbers of *Fla. columnare* cells are present in water during epizootics (McCarthy 1975), with good survival occurring over a wide range of pH and harness values (Fijan 1968). Moreover, in one study, Collins (1970) reported a relationship between eutrophication and the numbers of *Fla. columnare* cells in lake water.

The susceptibility of juvenile Chinook salmon has been correlated with the age of the fish and the stocking density as well as water temperature (Fujihara et al. 1971). These workers concluded that rainbow trout of 1 g average weight and Chinook salmon of 3 g average weight were less susceptible to columnaris than smaller fish. Therefore, it was concluded that age was more important than weight in determining susceptibility to infection.

Fourteen isolates were recovered from a river by the intake to a fish farm and 400 m upstream of the site. One isolate was from a lake without any association with aquaculture. Generally, these isolates were less virulent than those from a disease outbreak. The isolates survived for months outside of fish, and changed their colony morphology. These data indicate that the pathogen occurs upstream of fish farms, and may be a potential source for outbreaks of disease (Kunttu et al. 2012).

Pathogenicity

Initially, *Fla. columnare* is attracted chemotactically to fish mucus (Peatman et al. 2013), and grows in and colonises the skin, mucus and gills (Shoemaker and LaFrentz 2015). Certainly, there is a marked variation in the virulence of isolates of *Fla. columnare,* with PFGE genetic group A, comprising isolates from channel

catfish in the USA, regarded as leading to more mortalities than group B (Soto et al. 2007). Genetic differences between high and low virulent strains have been determined by suppression subtractive hybridization, and include ferrous iron transport protein, TonB-dependent receptor, transposases and ABC transporter permease protein (Li et al. 2010). Virulent and non-virulent cultures display variation in colony morphology and chondroitin AC lyase activity with the former being rhizoidal, moderately adherent [temperature dependent, with increased adhesion up to 20 °C] with higher chondroitin AC lyase activity whereas the latter are non-rhizoidal and rough with less chondroitin AC lyase activity or soft colony types which are poorly adherent (Kunttu et al. 2011). However, use of gene deletion mutants revealed that chondroitin lyases were not essential virulence factors (Li et al. 2015a).

Pacha and Ordal (1963) classified cultures into four grades of virulence, from high to low. Using a highly virulent culture, it was possible to achieve infection of chinook salmon and sockeye salmon following a 2-min dip in a diluted broth culture. Possibly, the organism entered the host through damaged areas of skin, especially if physically abraded or hot branded (Bader et al. 2003b, 2006), although it recognized that *Fla. columnare* attaches to the gill. Thus using a common carp gill perfusion approach with bivalent ion-rich water, the presence of nitrite or organic material, and high temperatures (=28 °C), it was noted that a highly virulent culture adhered more readily than a culture of low virulence (Decostere et al. 1999a). The adherence receptor was considered to be composed at least partially of carbohydrate, with the adherence ability of *Fla. columnare* correlated with haemagglutination and the capsule (Decostere et al. 1999b). Fujihara et al. (1971) achieved 100 % mortality in chinook salmon following exposure for 25 min in a suspension containing 2.5×10^5 cells/ml. Deaths followed within 96 h and 8 h at water temperatures of 10 and 22 °C, respectively. Adhesion and infection is inversely proportional to the salinity, specifically there was a decline in mortalities from freshwater (98 % mortalities) to 3‰ (0 % mortalities) for channel catfish (Altinok and Grizzle 2001). Also, the concentration of bivalent, i.e. calcium and magnesium, cations in water influences pathogenesis with removal by ion-exchange filtration decreasing flavobacterial adhesion to gill tissue and thus mortalities (Straus et al. 2015). However, the pathogenic mechanism is unclear, but work has pointed to an involvement of adhesion principally insofar as an adhesion-defective mutant had reduced virulence (Bader et al. 2005). A preceding stage in an infection cycle is the arrival of the pathogen at the cells/tissues. Whereas, the event may be random, in many cases there is a direct attraction of the pathogen to the host cells. There is evidence that *Fla. columnare* is attracted chemotactically to skin mucus, with carbohydrate-binding receptors important in the process (Klesius et al. 2010). Using a high virulent culture, there was a high level of colonization and destruction; invasion of gill tissue was observed to spread from the filament tips towards the base, with outer membrane vesicles surrounding most of the flavobacterial cells. Higher virulent cultures led to significantly higher apoptotic cell counts in carp and rainbow trout (Declercq et al. 2015). Using RNA-seq expression profiling, a rhamnose-binding lectin was found that was dramatically upregulated in gills of channel catfish during infection. In a susceptible channel catfish family, there was a dramatic upregulation

of the lectin for >24 h. Exposure to lectin ligands, i.e. D-galactose and L-rhamnose, protected the catfish against columnaris (Beck et al. 2012).

Extracellular proteases have been implicated with *Fla. columnare* (Newton et al. 1997), including collagenase, chondroitinase and proteases (Stringer-Roth et al. 2002; Olivares-Fuster and Arias 2008). Using cell-free extracts (culture supernatants) of *Fla. columnare*, Pacha (1961) obtained muscle damage following injection with the material. However, the result could not be substantiated by detailed *in vitro* chemical analyses of the supernatant. Later, two proteases of 53 and 58 kDa were isolated from *Fla. columnare* (Newton et al. 1997), and we assume that these are responsible for tissue damage. Iron may influence the pathogenicity of *Fla. columnare,* insofar as the presence of 0.35–1.4 mg of iron/100 g of fish reduced the survival time following experimental challenge with the pathogen from 20 days to 1 day (Kuo et al. 1981). In contrast, transferrin exerted a negligible effect. *Fla. johnsoniae* has infected barramundi and goldfish via waterborne challenge (Soltani et al. 1994).

Fla. columnare cells have been detected in the gill, mucus and skin within 5 min of immersion challenge of previously abraded fish (15 min without abrading) (Bader et al. 2003a). There is evidence that mucus promotes the growth of *Fla. columnare,* increasing extracellular protease production and the ability to form biofilms (Staroscik and Nelson 2008).

A siderophore-mediated iron uptake system has been found. With iron-limitation, synthesis of an OMP, a TonB-dependent ferrichrome-iron receptor precursor, of 86 kDa was upregulated. A putative ferric uptake regulator protein was also identified in the genome (Guan et al. 2013).

Disease Control

Management Techniques Columnaris is most severe at high water temperatures; it has been suggested that control may be exercised by keeping the water as cool as possible, although this approach may be difficult for many fish farms. Using a high virulent culture, there was a high level of colonization and destruction; invasion of gill tissue was observed to spread from the filament tips towards the base, with outer membrane vesicles surrounding most of the flavobacterial cells. Higher virulent cultures led to significantly higher apoptotic cell counts in carp and rainbow trout (Declercq et al. 2015).

Vaccine Development Fujihara and Nakatani (1971) experimented with heat-killed cells, which were administered via food to juvenile coho salmon. The fish responded with the production of antibody (titre = 1:5120). Schachte and Mora (1973) concurred with the general view by demonstrating agglutinating antibody in channel catfish. Survivors of infection experiments resisted re-infection, suggesting the presence of a protective immune response (Fujihara et al. 1971). Formalised cells of *Fla. columnare* were administered to eel by immersion and injection, result-

ing in an immune response (in the skin) two weeks later, and survival of 60 % and 20 %, respectively (Mano et al. 1996). Interestingly, 14 days following vaccination by immersion and injection of eel with formalin killed cells of *Fla. columnare* agglutinating antibody could not be detected in the serum or mucus. Instead, there was inhibition of bacterial adhesion in the skin of the immersion vaccinated eel (Mano et al. 1996). Formalin-inactivated sonicated and whole cell preparations of *Fla. columnare* were applied intraperitoneally and by immersion to tilapia with booster doses after 4 weeks (Grabowski et al. 2004). The data revealed that use of formalised sonicated cells in FCA injected by i.p. led to a significant humoral immune response (titre = 1: 11,200 by ELISA after 2 weeks; titre = 1:30,600 after boosting). However, there was no information about protection. It has been suggested that rifampicin mutants of high virulence strains may well become vaccine candidates (Olivares-Fuster and Arias 2011).

A modified live *Fla. columnare* vaccine and a 1:1 bivalent product with a commercial vaccine for *Edw. ictaluri* was evaluated in channel catfish eggs. The live vaccine was administered by immersion (1.35×10^7 CFU/ml for 15 min) with a booster on day 34 (2.17×10^7 CFU/ml for 15 min) leading to RPS values of 50–76.8 % after challenge. Similarly, the bivalent product was applied by immersion for 15 min, and challenged with RPS of 33–59.7 %. Overall, the approach was successful in protecting fish from the eyed egg stage against challenge with *Fla. columnare* (Shoemaker et al. 2007). Avirulent mutants derived from genomovar I and the more virulent genomovar II were administered to channel catfish fry and Nile tilapia, with the results pointing to protection with both genomovar mutants (Mohammed et al. 2013).

Another approach to developing vaccines could be to determine which components of the pathogen stimulate immune responses. By use of two-dimensional electrophoresis immunoblotting of antiserum from grass carp, Liu et al. (2012) reported that 14 proteins of *Fla. columnare* were immunogenic, and included alcohol dehydrogenase, dihydrolipoamide succinyltransferase, fructose-biphosphate aldolase, 3-hydroxybutyryl-CoA dehydrogenase, regulator protein, 30S ribosomal subunit protein S1, succinyl-CoA synthetase, SpoOJ, translation elongation factors G and Tu, two conserved hypothetical proteins, and chaperonins DnaK, GroEL and trigger factor. The suggestion was made that some among these bacterial proteins would be appropriate candidates for vaccine development (Liu et al. 2012).

A ghost vaccine was produced involving a specific *Flavobacterium* lysis plasmid pBV-E-cat that was electroporated into *Fla. columnare* G4cpN22 after curing of its endogenous plasmid. The ghosts were by gene E-mediated lysis, and administered by i.p. injection to grass carp (*Ctenopharyngodon idellus*) leading after challenge to a RPS of 70.9 % (Zhu et al. 2012).

Probiotics/Biocontrol The progress of infection of *Fla. columnare* was mediated by *Aer. hydrophila* and *Cit. freundii* (Chowdbury and Wakabayashi 1989). Subsequently, Boutin et al. (2012) recovered bacteria from the skin of brook charr that were inhibitory *in vitro* to *Fla. columnaris,* and when mixed and administered to fish led to a decrease in mortality after challenge.

Immunostimulants/Dietary Supplements An aqueous extract of Asiatic pennywort (*Centella asiatica*) has been proposed for the control of columnaris in Nile tilapia when used as a bath at 100 mg/l for what appeared to be a continuous exposure (Rattanachaikunsopon and Phumkhachorn 2009). AlkoSel [inactivated *Saccharomyces cerevisiae;* dosed at 0.25 g/kg of feed*]* has been attributed with immunostimulatory activity when fed to rainbow trout for 7 whereupon challenge with *Fla. columnare* led to demonstrable protection (Suomalainen et al. 2009).

Disinfection Diquat was effective as a bath treatment for channel catfish, effectively stopping mortalities caused by the pathogen. Also, there was some reduction in mortalities following bathing in chloramine-T, hydrogen peroxide and potassium permanganate, but not so with copper sulphate and hydrogen peroxide (Thomas-Jinu and Goodwin 2004; Bowker et al. 2013). Experiments with rainbow trout fingerlings have demonstrated a potential for the use of sodium chloride baths in reducing mortalities caused by columnaris following waterborne challenge (Suomalainen et al. 2005).

Antimicrobial Compounds Farkas and Oláh (1980) suggested the use of a salt (sodium chloride) bath for controlling infections. We agree with this suggestion, insofar as a 30 s dip in 8 % (w/v) sodium chloride cleared up an infection in rainbow trout fingerlings within a few days. Other remedies, which have met with varying degrees of success, include:

arsenic, cadmium, copper, lead and selenium mixture, dosed at 1–3 µg/l for 1 day (MacFarlane et al. 1986);
copper sulphate, used at a dilution of 1:2000, for a 1–2 min dip (Snieszko 1958b);
malachite green, used at a dilution of 1:15,000, for a 1–30 s dip (Snieszko 1958b);
pyridylmercuric acetate, used at 2 mg/l for 1 h (Snieszko 1958b);
diquat, used at 1–2 mg/l for 30–60 min (McCarthy 1975);
quaternary ammonium compounds, used at 2 mg/l for 1 h (McCarthy 1975);
oxytetracycline, used at 50–100 mg/kg body weight of fish/day for 10 days (Snieszko 1964; Ferguson 1977). For external infections, oxytetracycline or chlortetracycline may also be used as a bath, i.e. 26–60 mg/l for 1 h (Snieszko and Hoffman 1963; Wood 1968);
oxolinic acid, used at 10 mg/kg of body weight/day for 10 days (Soltani et al. 1995)
chloramphenicol, used at 5–10 mg/l, has been suggested for aquarium fish (Snieszko 1958b);
Florfenicol dosed at10mg/kg of body weight for 10 consecutive days (Matthews et al. 2013).
sulphadiazine, sulphamerazine or sulphamethiazone, used at 220 mg/kg body weight of fish/day for 10 days (Snieszko 1954; Wolf and Snieszko 1963). Sulphamerazine has been used successfully to treat rainbow trout, but not chinook salmon (Johnson and Brice 1952).

Bacteriophage Bacteriophage were recovered from fish farms and freshwater in Finland with a view for using them in phage therapy (Laanto et al. 2011). Experiments with rainbow trout and zebra fish were successful insofar as a single application of bacteriophage FCL-2 into the water supply of a flow-through system protected the fish against challenge (Laanto et al. 2015).

Flavobacterium hydatis (= Cytophaga aquatilis)

Isolation

Isolation from infected tissues may be achieved on cytophaga agar (Appendix in Chap. 12; after Anacker and Ordal 1959) and Pacha and Ordal's medium (Pacha and Ordal 1967) (Strohl and Tait 1978).

Characteristics of the Pathogen

Box 7.9: *Flavobacterium hydatis (= Cytophaga aquatilis)*
Following incubation at 20 °C for 14 days, yellow-orange colonies develop, which contain Gram-negative facultatively anaerobic rods of 8.0×0.5 μm in size. Copious quantities of extracellular slime are produced. Cells demonstrate gliding movement. Microcysts and fruiting bodies are absent. Catalase is produced, but not H_2S, indole, lysine or ornithine decarboxylase, oxidase or phenylalanine deaminase, and the methyl red test and Voges Proskauer reaction are negative. A wide range of complex molecules are degraded, including aesculin, blood, casein, DNA, gelatin, pectin, starch, tributyrin, Tween 40, 60 and 80, and tyrosine (slowly), but not cellulose or urea. Some strains attack chitin. Growth occurs at 5 to 35 °C but not 42 °C, in 0–2 % (w/v) sodium chloride, which is an indication that the organism is unlikely to be present in full strength seawater. Growth occurs also at pH 5.5–11.0. Nitrates are reduced to ammonia. Acid is produced from arabinose, cellobiose, glucose, lactose, mannitol, raffinose, sucrose and xylose. The G+C content of the DNA is 33.7 moles %. Chemical analyses have established that the major pigments are similar to the 'flexirubin' of *Fle. elegans* (Strohl and Tait 1978).

On the basis of the micromorphology, gliding movement, G+C ratio, and the ability to degrade complex molecules, this group was considered to represent a previously undescribed species of *Cytophaga,* for which the name of *Cyt. aquatilis* was

proposed (Strohl and Tait 1978). However, on the basis of phylogenetics, the species was transferred to *Flavobacterium,* as *Fla. hydatis* (Bernardet et al. 1996).

Pathogenicity

Fla. hydatis, although not a proven fish pathogen, produces extracellular, thermostable, glucose-repressible collagenases, which could be involved in pathogenicity (Strohl, Gibb and Tait, personal communication).

Disease Control

Antimicrobial Compounds Antibiogrammes confirmed sensitivity to chloramphenicol (30 µg), erythromycin (15 µg), nalidixic acid (30 µg), streptomycin (10 µg), sulphathiazole (1 mg) and tetracycline (30 µg), but not to lincomycin (2 µg), methicillin (5 µg), penicillin (10 IU) or sulphadiazine (300 µg) (Strohl and Tait 1978). From the uniform sensitivity to nalidixic acid, sulpha drugs and tetracycline, these could be considered for chemotherapeutic use, should the need arise.

Flavobacterium johnsoniae (= Cytophaga johnsonae)

Characteristics of the Disease

In the initial outbreak in Queensland, Australia, 2–5 % mortalities occurred in farmed barramundi, *Lates calcarifer,* over a two week period when the water temperature was 27–28 °C. The fish were listless, and displayed elevated scales and superficial erosion of the skin (particularly on the posterior flank). Some fish presented eroded pectoral fins and lower jaws (Carson et al. 1993). *Flavobacterium johnsoniae*-like organisms have been recovered from diseased fish (gill necrosis, skin ulcers, systemic disease) in South Africa (Flemming et al. 2007).

Isolation

Cytophaga agar (Appendix in Chap. 12; after Anacker and Ordal 1959) is suitable for the isolation of the pathogen with incubation at 27 °C for 7 days (Carson et al. 1993).

Characteristics of the Pathogen

Fla. johnsoniae was identified by an examination of phenotypic tests, and associated with mortalities in salmonids (Rintamäki-Kinnunen et al. 1997). Subsequently, an organism with similarities to *Flavobacterium johnsoniae* has been recovered from a variety of fish and disease conditions in South Africa (Flemming et al. 2007). The characteristics for the fish isolates were as follows:

Box 7.10: *Flavobacterium johnsoniae*
Colonies are yellow (flexirubin pigment) and contain Gram-negative gliding, filaments. Catalase, ß-galactosidase and oxidase are produced, but not H_2S or indole. Nitrates are reduced. Acid is produced from glucose. Growth occurs at 10–30 °C but not at 37 °C, and in 0.5–1.0 % (w/v) sodium chloride. Casein, chitin, DNA, gelatin, starch, tributyrin, Tween 20, Tween 40 and tyrosine (with pigment production) are degraded, but not Tween 80. Ammonium, asparagine, glutamate, potassium nitrate and urea are utilised as sole sources of nitrogen (Carson et al. 1993).

Disease Control

Vaccine An attenuated vaccine was developed by using a streptomycin-resistant strategy leading to protection of grass carp (*Ctenopharyngodon idella*) to challenge (RPS = 34.8 % to 100 %) depending on where infected occurred at 28 or 240 days after vaccination, and whether the challenge was by i.p. injection or by bath. The best results involved i.p. challenge at 28 days (RPS = 100 %) (Li et al. 2015b).

Antimicrobial Compounds Acriflavine and oxolinic acid were considered to be effective treatments (Carson et al. 1993).

Flavobacterium oncorhynchi

Eighteen isolates were recovered from diseased rainbow trout in Spain, and named as a new species, *Fla. oncorhynchi* (Zamora et al. 2012b). Although pathogenicity was not confirmed in the initial publication, unpublished work has determined a [systemic] pathogenic role in rainbow trout.

Isolation

Cytophaga agar was used with gill, kidney, liver and samples from diseased rainbow trout with incubation at 22 °C for 7 days. For these plates, 18 yellow-pigmented isolates formed the basis of detailed taxonomic work leading to the proposal of a new species, i.e. *Fla. oncorhynchi* (Zamora et al. 2012b).

Characteristics of the Pathogen

The isolates were 99.2–100 % related by 16S rRNA sequence homology, with the nearest neighbor as *Fla. chungangense* (98.6 % sequence homology), although the corresponding DNA:DNA hybridization value was only 18.5 ± 7.8 % (Zamora et al. 2012b).

Box 7.11: *Flavobacterium oncorhynchi*
Cultures comprise non-diffusible pale yellow-pigmented [flexirubin-type pigment] colonies with Gram-negative rods of 2–3 × 0.5 μm in size on TSA and nutrient agar aerobically at 15–30 °C. Growth does not occur at 37 °C. Gliding does not occur. Nitrates are reduced, catalase, ß-galactosidase and oxidase but not H_2S or urea are produced, and aesculin, casein [variable result] and starch but not DNA, sheep blood gelatin, tyrosine [but a brown pigment is produced] or urea are degraded. Arabinose, mannose, N-acetyl-glucosamine and maltose but not adipate, caprate, citrate, malate or mannitol are utilized as the sole source of carbon for energy and growth. Acid is not produced from carbohydrates. Growth does not occur in 3 % (w/v) NaCl. The predominant fatty acids were iso-$C_{15:0}$, $C_{15:0}$ and $C_{16:1}$ ω7*c*. Mk-6 was the major respiratory quinone. The G + C content of the DNA of the type strain is 33.0 mol% (Zamora et al. 2012b).

Flavobacterium psychrophilum (=Cytophaga psychrophila = Flexibacter psychrophilum)

Characteristics of the Diseases

Although this organism is mostly associated with infections in salmonids, it is becoming increasingly associated with disease in other fish groups. For example, it has become linked to tissue necrosis in the region of the mouth and with 40–60 % mortalities in farmed perch (*Perca fluviatilis*) in Finland (Lönnström et al. 2008). Also, sea lamprey have become infected (Elsayed et al. 2006), and ayu has been challenged successfully by immersion (Miwa and Nakayasu 2005). Mostly, this

organism causes skin lesions, which are often described as saddle-like lesions, containing myriads of organisms, near the dorsal fin. The fish may darken and, in advanced cases, develop bacteraemia with the pathogen ramifying throughout the animal (Wood and Yasutake 1956; Winton et al. 1983; Lehmann et al. 1991). A systemic infection of rainbow trout has been described in Norway, the outcome of which was mortalities of ~90 % (Nilsen et al. 2011a). Here, the disease in fry was characterized by anorexia, distended abdomen, and darkened pigmentation in the region of the caudal peduncle. Larger fish were lethargic, and often displayed skin ulceration (Nilsen et al. 2011a). The organism has also been linked with septicaemia and necrotic myositis (accumulated loss of 7 %) in Atlantic salmon smolts of 60—100 g in size from a freshwater site in Norway during winter (water temperature = <5 °C) (Nilsen et al. 2011b). The fish displayed swollen darkened spleen, pale live, bloody ascites and haemorrhaging in the abdominal fat and muscle (Nilsen et al. 2011b).

Isolation

Specialised media have been devised, of which cytophaga agar (Appendix in Chap. 12; after Anacker and Ordal 1959) has received greatest use for the recovery of *Fla. psychrophilum.* An improved growth medium has been published with the outcome that more rapid and luxuriant growth occurred; the recipe was based on cytophaga agar/broth supplemented with galactose, glucose, rhamnose and skimmed milk (Appendix in Chap. 12; Daskalov et al. 1999). Then, Cepeda et al. (2004) reported that a glucose, salt, tryptone and yeast extract based medium, coined FLP, was superior for speed and abundance of growth of the pathogen (Appendix in Chap. 12). Cytophaga agar supplemented with 0.2 % skimmed milk and 1 % horse serum gave a slight improvement in growth (Oplinger and Wagner 2012). An alternative approach was to add 5 μg/l of the aminoglycoside antibiotic tobramycin, which was stated to improve recovery of *Fla. psychrophilum* especially from the external surfaces of carrier ayu (Kumagai et al. 2004a). Caution has been suggested over the source of ingredients used to make some media. For example, the importance of the brand of beef extract (the Difco product was reported as superior to Gibco or Oxoid) for the growth of *Fla. psychrophilum* on cytophaga agar was highlighted by Lorenzen (1993). Improved recovery of viable cells of *Fla. psychrophilum* from dilute samples was achieved using nutrient agar supplemented with activated charcoal (Appendix in Chap. 12; Álvarez and Guijarro 2007).

Characteristics of the Pathogen

The best of the descriptions of this organism stems from the work of Pacha (1968), Bernardet and Kerouault (1989), Bernardet and Grimont (1989b) and Schmidtke and Carson (1995). Opinion is that this pathogen comprises a phenotypically

homogeneous group (Madsen and Dalsgaard 2000: Madetoja et al. 2001; Valdebenito and Avendaño-Herrera 2009), although two biovars have been identified by use of the API ZYM system (Hesami et al. 2009) although genetic diversity has been recognised (Chen et al. 2008; Del Cerro et al. 2010). Two distinct smooth, hydrophobic, adhesive (autoagglutinating) and rough (non-agglutinating) hydrophilic colony phenotypes have been described (Högfors-Rönnholm and Wiklund 2010; Sundell et al. 2013) with the smooth developing into the rough phenotype in broth, but never the reverse. Both phenotypes were virulent to rainbow trout (Högfors-Rönnholm and Wiklund 2010). Plasmid profiling was carried out to evaluate genetic variability among 104 isolates, most of which were recovered from Japan. Most (72/104 = 69 %) possessed plasmids of 2.8, 3.4, 4.1 and 5.6 kb. The 3.4 kb plasmid was mostly recovered from cultured obtained from rainbow trout; the 4.1 and 5.6 kb plasmids were from Japanese isolates (Izumi and Aranashi 2004). The plasmid profile may reflect the rough or smooth morphotype (Sundell et al. 2013).

Box 7.12: *Flavobacterium psychrophilum*
Cultures produce non-diffusible yellow-pigmented (flexirubin pigment) colonies with thin spreading margins. Cells are strictly aerobic, Gram-negative, slender, flexible rods of 1.5–7.5 × 0.75 μm in size. With increasing age, the cells appear to be shorter. As with *Fla. hydatis,* gliding movement is exhibited, and fruiting bodies and microcysts are absent. Catalase is produced, oxidase may appear to be positive, but H_2S, indole, and lysine and ornithine decarboxylase are not produced. Nitrates are not reduced, and the Voges Proskauer reaction is negative. Casein, gelatin and tributyrin are degraded; tyrosine is attacked by some isolates, and aesculin, chitin, starch and xanthine not at all. Generally, growth occurs at 4–23 °C, but not at 30 °C, and in 0.8 % but not 2 % (w/v) sodium chloride. This demonstrates clearly that the organisms are suited to low-temperature, freshwater environments. The organisms are capable of degrading autoclaved cells of *Esch. coli* (Pacha 1968). The G + C ratio of the DNA is 32.5–34 moles %.

The original 10 isolates of Pacha (1968) were deemed to be serologically homogeneous. Yet, later work identified two major serogroups, with O-1 accommodating isolates from Japan and the USA and O-2 comprising only Japanese cultures (Wakabayashi et al. 1994). This number increased to three, with European isolates included in one major serogroup (Lorenzen and Olesen 1997). Seven antigenic types have been recognised among Finnish isolates, with results suggesting a new serogroup (Madetoja et al. 2001). Furthermore, a novel serotype has been described for cultures recovered from amago in Japan (Izumi et al. 2003). Several ribotypes have been recognised among 85 isolates, with most harbouring one or more plasmids [11 plasmid profiles defined] (Chakroun et al. 1998). In comparison, 13 ribotypes were recognised among Finnish cultures (Madetoja et al. 2001). PCR and

RFLP led to the recognition of genetic heterogeneity (Izumi et al. 2003), with four RFLP pattern types and 9 sequence types recognised among isolates from Ontario, Canada (Hesami et al. 2009). MLST of 560 *Fla. psychrophilum* isolates from various Nordic countries collected between 1983 and 2012 revealed 81 different sequence types belonging to 12 clonal complexes and 30 single member sequence types. The largest clonal complex, CC-ST10, included mostly isolates from rainbow trout and comprised the dominant genotype, ST2. In Norway, ST2 was the only isolated CC-ST10 genotype, which suggested a recent introduction of an epidemic clone. Most of the single member sequence types were isolated from the environment or non-rainbow trout species. It was considered likely that dissemination of the putatively virulent clonal complex, CC-ST10, was associated with the movement of fish or their products (Nilsen et al. 2014). In the case of Switzerland, MLST was used to study the relationships among 112 isolates from rainbow trout and brown trout, with the outcome that 27 sequence types were defined. Most isolates were recovered in two clonal complexes, CC-ST2 and CC-ST90, which were represented by several sequence types. Eight single member sequence types were unique, and could not be accommodated in any clonal complex (Strepparava et al. 2013). Using MLST with Japanese isolates, 35 mostly unique sequence types were delineated, suggesting a comparatively recent introduction of *Fla. psychrophilum* into Japan (Fujiwara-Nagata et al. 2013). Moreover, MLST was used to examine 66 isolates from rainbow trout in France with the recognition of 15 sequence types, of which 14 were regarded as being unique. These sequence types were grouped in one clonal complex of 8 genetically-related sequence types plus 7 single members (Siekoula-Nguedia et al. 2012).

Evidence has been presented that isolates may well contain prophage. Thus, PCR examination of 49 isolates from Chile, Denmark and the USA revealed the presence of four bacteriophage genes, i.e. integrase, tail tape protein and two hypothetical proteins, in the majority (80 %). Therefore, it would appear that temporate bacteriophage has lysogenized in a large proportion of *Fla. psychrophilum* isolates (Castillo et al. 2014).

On the basis of the phenotypic characteristics, the conclusion was reached that the causal agent of coldwater disease could be equated with the genus *Cytophaga,* as defined by Stanier (1942), but was sufficiently distinct from existing species to warrant description as a new species. Hence, *Cyt. psychrophila* was named (Borg 1960). However, Lewin and Lounsberry (cited in Leadbetter 1974) disagreed with this notion, and proposed that the organisms would be better classified in the genus *Flexibacter,* as *Fle. aurantiacus.* Bernardet and Grimont (1989b) agreed with the opinion that the organisms belong in the genus *Flexibacter,* but proposed *Fle. psychrophilum.* Then with general improvements in the understanding of the yellow-pigmented bacteria resulting from phylogenetic data, the taxon was transferred to *Flavobacterium,* as *Fla. psychrophilum* (Bernardet et al. 1996). It is agreed that the taxon comprises a homogeneous group of bacteria (Lorenzen et al. 1997).

Diagnosis

Phenotypic Methods Congo red has been used successfully to differentiate *Fla. psychrophilum* from other flavobacteria (Crump and Kay 2008). For this, 100 μg/ml of Congo red was incorporated into tryptone yeast extract salt (TYES) medium, and inoculated with a culture with incubation at 15°C for 4-days. *Fla. psychrophilum* was inhibited by Congo red, and did not grow on the medium, whereas other flavobacteria grew well (Crump and Kay 2008).

Serology Pacha (1968) implied that whole-cell agglutination reactions were effective for differentiating *Fla. psychrophilum* from other (unnamed) myxobacteria. Also together with ELISA, the approach allowed a useable typing scheme to be devised (Mata et al. 2002). Rapid identification of *Fla. psychrophilum* was achieved by ELISA, which detected $\geq 1 \times 10^4$ cells/ml from infected spleen (Rangdale and Way 1995). Indeed, a detection level of 1.6×10^3 CFUs was reported in spiked kidney homogenates (Lindstrom et al. 2009). By use of ELISA, a useful typing scheme was devised for *Fla. psychrophilum*, recognising 7 serogroups with host specificity (Mata et al. 2002). Lorenzen and Karas (1992) detailed an immunofluorescence technique for detecting *Fla. psychrophilum* in the spleen of rainbow trout suffering with RTFS.

Immunomagnetic separation and flow cytometry was developed, and involved use of fluorescent magnetic beads and 5-cyano-2,3-ditolyl tetrazolium chloride. The pathogen was harvested using fluorescent magnetic beads with a single antigen-antibody reaction. Then, the tetrazolium dye developed into a red fluorescent coloured formazan that was detectable by flow cytometry. In use, it took <3 h to achieve a result (Hibi et al. 2012).

Molecular Methods A PCR was sensitive enough to detect 1.5 CFU/PCR reaction tube of *Fla. psychrophilum* from apparently healthy coho salmon eggs and juvenile ayu (Izumi and Wakabayashi 1997). *In situ* hybridization using digoxigenin-labelled 16S rDNA probes led to the detection of *Fla. psychrophilum* in experimentally (after immersion or subcutaneous injection – in gills, heart, kidney, muscle and spleen, but not in the brain, intestine, liver, pyloric caeca or stomach) and naturally (intestine, liver, pancreas, pyloric caeca and stomach) infected ayu (Liu et al. 2001). Similarly, PCR technology has proven to be successful for the detection of *Fla. psychrophilum* and *T. maritimum*, and was considered to be more sensitive than culturing (Wiklund et al. 2000; Avendaño-Herrera et al. 2004a). In experiments using brain tissue seeded with the pathogen, the detection limit was 0.4 CFU/PCR tube, which corresponded to 17 CFU/g of brain tissue. Using normal freshwater, i.e. containing a mixed microbial population, seeded with *Fla. psychrophilum*, the detection limit was 1.7 CFU/PCR tube, which corresponded to 110 CFU/ml of water. The PCR detected the pathogen in water from a fish farm, whereas culturing did not reveal the presence of the organism (Wiklund et al. 2000). A nested PCR enabled a detection limit of 1 cell/PCR tube, which was equivalent to 10 cells/g of

spleen and 5 cells/g of ovarian fluid (Baliarda et al. 2002). Ovarian fluid eggs and gill washings and benthic diatoms were used with a nested PCR by Taylor (2004) and Izumi et al. (2005), respectively, with again commendable results. A *Taq*Man-based PCR used primers amplifying a 971 bp fragment of the 16S rRNA, and detected 1.1 pg of *Fla. psychrophilum* DNA, which equated with 4.7 CFU/PCR reaction (del Cerro et al. 2002). Terminal-RFLP permitted the detection of ~30 CFU of *Fla. psychrophilum*/mg of artificially inoculated kidney tissue (Nilsson and Strom 2002). The benefit of species-specific primers and a nested PCR was demonstrated over universal eubacterial primers when the detection limit improved from 1.4×10^5 CFU/reaction to <14 CFU/sample (Taylor and Winton 2002). A specific and rapid LAMP assay, which takes only 70 min to complete, has been proposed for the detection of *Fla. psychrophilum* with detection in the range of 2.0×10^1 to 2.0×10^9 DNA copies/reaction (Fujiwara-Nagata and Eguchi 2009). A highly specific quantitative PCR was published, and stated to be effective in detecting only two bacteria/reaction, which corresponded to 800 bacterial cells/fish or 20 flavobacterial cells/mg of tissue (Orieux et al. 2011). A RT-PCR involving a sequence that encodes a hypothetical protein of unknown function had a detection limit of 3.1 genome equivalents/reaction, and successfully recognized *Fla. psychrophilum* in naturally occurring outbreaks of disease on rainbow trout farms (Marancik and Wiens 2013). In a comparison of PCR methods, Suzuki et al. (2011) identified issues with false positives when targeting DNA gyrase subunit gene *gyrA* but not so for *gyrB* and peptidyl-prolyl cis-trans isomerase C gene *ppiC*. However, the PCR targeting 16S rDNA was more sensitive. An ideal situation would involve techniques that could recognise and differentiate between multiple diseases, and this has been achieved with multiplex PCR. Del Cerro et al. (2002) detected simultaneously *Aer. salmonicida, Fla. psychrophilum* and *Y. ruckeri* in fish tissues, recognising the equivalent of 6, 0.6 and 27 CFU, respectively. A highly sensitive and specific quantitative RT-PCR based on the single copy gene ß DNA-dependent RNA polymerase revealed a detection limit of 20 gene copies and a quantification limit of 10^3 gene copies/reaction (Streppaeava et al. 2014). High-gradient immunomagnetic separation with a PCR was regarded as more sensitive than PCR alone, and permitted the recording of results in <3.5 h (Ryumae et al. 2012).

Which Method is Best? A comparison was made of the sensitivities of various methods to detect the pathogen in tissue and ovarian fluid, which were collected from 224 fish at five hatcheries. The methods used included ELISA, a membrane-filtration FAT, culture and nested PCR, with the first mentioned being regarded as the appropriate technique for the screening of broodstock, and for indicating the severity of infection (Long et al. 2012).

Epizootiology

Although the precise reservoir of *Fla. psychrophilum* has not been established, we have recovered some organisms, with the key characteristics of *Fla. psychrophilum,* from fresh water in England. However, it is uncertain whether or not the organisms were native, or merely transient, in the aquatic environment. Cells may be able to enter a VBNC state following 2-days incubation at 28 °C although the relevance to pathogenicity is questionable insofar as the cells did not cause clinical disease (Sugahara et al. 2010b). Starvation over 14-days resulted in morphological changes with cells maintaining their length but the majority (80 %) changed into coiled forms that remained culturable, and subsequently developed an extracellular matrix that covered the cells; starved cells were avirulent. Revival in the presence of nutrients led to cells returned to their original shape (Arias et al. 2012). The organism forms biofilms, and of significance is that attached cells are less susceptible to inhibitory agents (Sundell and Wiklund 2011). Recovery has been achieved from overwintering ayu in a Japanese river, with the suggestion that the fish may be the source of infection during the following spring (Miyazaki 2008). *Fla. psychrophilum* was isolated from the internal organs (spleen) and/or sexual products (gonad but not the eggs at the eyed stage) in 7/50 (=14.0 %) Baltic salmon *(Salmo salar)* brood fish sampled at capture and 63/272 (=23.2 %) of fish examined at stripping. Also, the pathogen was recovered from the spleen or gonads in 2/19 (=10.5 %) of the fish with abnormal wiggling behaviour. Overall, the possibility exits that brood fish could transfer the pathogen to their progeny (Ekman et al. 1999). Additionally, Kumagai et al. (2011) found the organism in wild ayu from four Japanese rivers that had been artificially stocked with ayu. In this study, the isolation rate from apparently healthy fish was in the range of 58–100 % with the isolates demonstrating virulence to juvenile ayu (Kumagai et al. 2011). Kumagai and Nawata (2011a) reported the presence of the pathogen in the ovarian fluid [544 out of 3276 fish; but less so intra-ovum (Kumagai and Nawata 2011b)] and milt [248 out of 1434 fish] of farmed salmonids in Japan, and suggested that the organism is widely distributed. There is other evidence that *Fla. psychrophilum* may be transmitted within salmonid eggs (Brown et al. 1997), possibly entering during water hardening (Kumagai et al. 2000). This same team reported the presence of the organism on the surface of but not inside ayu eggs but not after surface sterilization with 5 mg/l of povidone-iodine for 10 min or 150 mg/l hydrogen peroxide for 30 min (Kumagai et al. 2004b). Also, the organism has been found in wild fish, namely perch and roach, from the ovarian fluids and milt of rainbow trout broodstock, and from waters (in close proximity to farmed trout or their eggs) in rainbow trout farms in Denmark (Madetoja et al. 2002; Madsen et al. 2005), in the eggs, gill or kidney tissue of wild ayu and other feral fish in Japan (Amita et al. 2000) and from water in Finnish trout farms as determined by nested PCR and iFAT but not culturing which undoubtedly lacked sensitivity (Madetoja and Wiklund 2002). More than likely, wild fish and broodstock are a possible source of infection.

Laboratory-based studies using sterilised fresh water have shown that *Fla. psychrophilum* has the capacity to survive for 300 days at 15 °C; in 30 % sodium chloride culturability was <1 day (Madetoja et al. 2003). However in the absence of a normal microflora, the relevance of the data to explaining survival of the pathogen in the natural environment is debatable. Survival studies revealed that the organism stopped multiplying but the numbers remained fairly constant in stream water for 16 weeks, and then declined. The cells became smaller and more rounded. Culturable cells could not be recovered on *Cytophaga* agar after 19 weeks, but resuscitation in *cytophaga* broth was possible for up to 36 weeks. In distilled water, culturability was lost after 1 h (Vatsos et al. 2003). The pathogen has been detected on algae growing on the surface of stones in rivers that contain diseased (with *Fla. psychrophilum)* fish (Amita et al. 2000).

Colonisation of fish may be a forerunner for the development of disease. For example, colonisation of eyed rainbow trout ova with *Fla. psychrophilum* has been considered to lead to the development of RTFS (Rangdale et al. 1997b). Using a modified Anacker and Ordal's medium, the pathogen was recovered from ovarian fluid of 2/15 hen fish, egg surfaces 14 days after fertilisation, but not from milt (Rangdale et al. 1996).

Ribotyping and plasmid profiling appear to be useful for epizootiology. In particular, several ribotypes have been recognised among 85 isolates, and determined to be associated with the fish species from which the cultures were obtained (Chakroun et al. 1998).

There is no dispute that these organisms are capable of causing severe losses to fish populations. Coldwater disease may cause losses of up to 50 % of the fish population, as determined for coho salmon fry (Rucker et al. 1953). This disease is especially troublesome at low water temperatures (Holt et al. 1989), i.e. ≤15 °C.

Pathogenicity

Fla. psychrophilum has been shown to attach to and even colonise the surface of fish (rainbow trout) eggs (Vatsos et al. 2001, 2006), and is certainly capable of inducing infection of eggs (Atlantic salmon) (Cipriano 2005). Exposure of disinfected eyed ova of rainbow trout with an extremely high dose of 10^{10} cells of *Fla. psychrophilum*/ml in PBS for 30 and 60 min at 10 °C led to the development of clinical signs of RTFS (Rangdale et al. 1997b). Rainbow trout unfertilized eggs were immersed in lesser doses of 10^7 CFU/ml and above, leading to the presence of the pathogen in the eggs. Furthermore, when mature hen fish were injected i.p. with 3.3×10^9 CFU/fish some 5–9 days before ovulation, the resulting intra-ovum infection revealed heavy contamination in the ovarian fluid, i.e. $>10^6$ CFU/ml. The conclusion was reached that the pathogen could contaminate eggs in ovarian fluid becoming internalized during water hardening (Kumagai and Nawata 2010). Nanoinjection of newly fertilized rainbow trout eggs with $10^1 - 10^3$ CFU/egg led to higher mortalities (95–100 % mortalities of the eggs) than the uninfected controls (0 % mortality) at 70 days

post-hatch (Eckman et al. 2003). Moreover, bathing rainbow trout fry for 24 in a logarithmic-phase culture, i.e. 24 h, led to higher mortalities than use of other phases of the culture growth cycle (Aoki et al. 2005). Bathing (also with stress caused by treatment with formalin) and i.p. injection of 10^4 CFU/rainbow trout (average weight = 1 g) [10^7 cells were needed for larger fish] led to reproducible infections, with results reflecting the nature of the bacterial strain, the stocking density, and the source and weight of the fish (Madsen and Dalsgaard 1999). Bathing yellowtail in a suspension of the pathogen led quickly to adherence of the organism to external surfaces, i.e. lower jaw, pectoral fin, gills and skin. Then, there was an increase in pathogen numbers in the gills followed by increase internally, i.e. in the blood, kidney and spleen (Nagano et al. 2011a). These workers concluded that gills are the primary target for invasion by the pathogen (Nagano et al. 2011a, b). Abrasion of skin and its mucus enhanced invasion of *Fla. psychrophilum* via immersion and cohabitation, with the shedding rate from infected fish reflecting water temperature, i.e. 15 rather than 4 °C, and the presence of dead fish (Madetoja et al. 2000). Highly virulent cultures are better capable of adhering to intestinal explants and gill tissue in gill perfusion models from rainbow trout than less virulent isolates (Nematollahi et al. 2003, 2005b). The presence of 2 g/l of organic material or 5 mg/l of nitrite increased adhesion to gills (Nematollahi et al. 2003). Adherence and temperature-mediated (greater at 5 than 15 °C) agglutination of yeast cells and erythrocytes as a function of serology, i.e. serotype, has been documented, with haemagglutination inhibited by sialic acid, heating to 65 °C or treatment with proteinase K (Møller et al. 2003). Electron microscopy of haemagglutinating and non-haemagglutinating cultures revealed the presence of a thin capsule (in both types of cultures), an absence of pili, but the presence of long, tubular blebs particularly in iron-restricted media that released membrane vesicles into the supernatant. Only the membrane vesicles of haemagglutinating cultures had haemagglutinating activity. The conclusion reached by the workers (Møller et al. 2005) was that via surface blebbing the pathogen releases membrane vesicles with some proteolytic activity that may somehow impede the immune response of the host.

Following infection, the bacteria may be seen embedded in the mucus along the fins which is where necrosis starts (Martínez et al. 2004). Although the precise pathogenicity mechanism of the organism eluded scientists for many years, it is now appreciated that *Fla. psychrophilum* produces extracellular components, including the ability to degrade gelatin and type II but not type I and IV collagen and has zinc metalloprotease like activity (Ostland et al. 2000) which in other organisms would be associated with virulence. Moreover, a psychrophilic metalloprotease, termed Fpp2 and with a molecular weight of ~62 kDa, has been described (Secades et al. 2001, 2003). This protease is different from Fpp1, which is a 55 kDa metalloprotease and cleaved actin and myosin, i.e. components of muscle (Secades et al. 2003). An OMP of 18 kDa, termed P18, was described, and linked with the S-layer (Massius et al. 2004). Once inside fish, *Fla. psychrophilum* appears to associate with kidney macrophages for which there is a role for sialic acid in the binding process (Wiklund and Dalsgaard 2003). Moreover, the pathogen has been found to become internalized in spleen phagocytes of rainbow trout, with bacterial numbers

increasing with time (Decostere et al. 2001). Within macrophages, the pathogen is cytotoxic and resists ROS thereby overcoming the bacterial killing abilities of these cells. Spleen macrophages were found to have lower antibacterial activity compared to those from head kidney therefore it was reasoned that the former will be a safer location for *Fla. psychrophilum* to exist (Nematollahi et al. 2005a). Additional evidence has been published that shows that the addition of *Fla. psychrophilum* cells or their metabolites to head kidney phagocytes from rainbow trout leads to immediate oxidative activity as determined by chemiluminescence (Lammens et al. 2000). Furthermore, the ability of isolates to grow in fish serum correlates well with pathogenicity, particularly in the case of ayu (Nagai and Nakai 2011). There is a clear relationship between early body weight of rainbow trout and resistance to infection, but not to genetic diversity of the host (Overturf et al. 2010).

The impact of bacteriophage on the host pathogen resulted in a study of four cultures that were exposed to three virus isolates. Using zebra fish, the ancestral rhizoid morphotype were associated with mortalities of 25–100 %, whereas the bacteriophage-resistant rough morphotypes that had lost virulence and the ability to glide did not affect the fish. Both morphotypes maintained their colony morphologies over 10- serial passages in broth, except for one low-virulence culture, Os06, which changed morphology with each passage (Laanto et al. 2012).

Also, research has indicated a link between the presence of oxidized lipids in diets with the development of RTFS (Daskalov et al. 2000). In this work fish that were fed with diets containing oxidized lipids developed dystrophic changes in the kidney, liver and muscle.

A genotyping method analysing two SNPs in the *gyrA* gene distinguished [ayu] virulent from avirulent isolates. In total, 232 isolates from four families of fish were equated with four genotypes, G-C, A-T, A-C and G-T, of which the first mentioned was highly pathogenicity to ayu. Th A-C genotype was at most weakly virulent, but A-T and G-T types not at all (Fujiwara-Nagata et al. 2012).

Disease Control

Management Practices Madsen and Dalsgaard (2008) were of the opinion that bore-hole water recirculation systems and good management, i.e. egg disinfection were important to the control of RTFS infections in rainbow trout. Use of warm water (28 °C compared with the norm of 18 °C) for 3 days has been successful for controlling coldwater disease in ayu (Sugahara et al. 2010a). This was explored further when protective immunity was found to be induced in ayu by warmed, i.e. 28 °C, water treatment, for 3-days. Thus, ayu subjected to warm water at 1, 6 and 24 h after infection by immersion experienced cumulative mortalities of 36 %, 30 %, and 18 %, respectively, compared to 90 % mortalties of the controls. Moreover, the warmed fish had higher antibody titres to the pathogen (Sugahara and Eguchi 2012).

Reduction of outbreaks of disease in fry may occur by implementation of a management strategy in which progeny of heavily infected broodstock are removed from the population. Thus, broodstock screening may well be an effective means of evaluating infection levels, which in turn could feed into disease management strategies (Long et al. 2014).

Disease Resistant Fish Amphidromous stock of ayu challenged with *Fla. psychrophilum* experienced reduced mortalities after challenge, than domesticated, hybridized or landlocked fish (Nagai et al. 2004; Nagai and Sakamoto 2006). The comparative resistance of the amphidromous stock could not be correlated with innate immune parameters, for example respiratory burst, serum-killing, and phagocytic activity of leucocytes.

Vaccine Development *Fla. psychrophilum* has been investigated as a vaccine candidate, by passive immunisation (LaFrentz et al. 2003), with formalin inactivated cells administered orally at 0.1–02 g/kg body weight of fish for 2 weeks or on 5 days over 2 weeks which led to good protection of ayu after immersion challenge (Kondo et al. 2003), formalin inactivated cells with water soluble adjuvant, i.e. Montanide IMS1312 administered i.p. to ayu which led to an RPS of 33 % and 39.6 % (Nagai et al. 2003), by use of surface antigens (Dumetz et al. 2006) and by use of an auxotroph, i.e. an *aroA* mutant (Thune et al. 2003). Administration of a formalin-killed whole vaccine in FCA intraperitoneally led to high serum and mucosal antibody titres in 9 weeks, and commendable protection (RPS = 83 %) (LaFrentz et al. 2002). Two autogenous multivalent or divalent water-in-oil vaccines comprising whole cells were administered intraperitoneally to rainbow trout with significant protectiom resulting after challenge (Fredriksen et al. 2013). In parallel, formalin- and heat-inactivated whole cell preparations of two serotypes in oily adjuvant led to high antibody titres, but not in the skin mucus, and protection (Madetoja et al. 2006). Similarly, use of OMP administered intraperitoneally led to a demonstrable immune response and protection of ayu (RPS = 64 and 71 %) and rainbow trout (RPS = 93 and 95 %) (Rahman et al. 2002). A surface protein, coined P18, was purified and the responsible gene identified which encoded a 166 amino acid OmpH – like protein. In vaccine trials using rainbow trout and intraperitoneal administration with FCA, a high antibody titre developed and protection ensued (RPS = 88 %) (Dumetz et al. 2006). The auxotroph, which has a mutation in the shikimate pathway, was used successfully by injection and immersion with hybrid striped bass (RPS = 85 %) (Thune et al. 2003). A live attenuated vaccine was administered by immersion or orally with or without alginate microencapsulation to rainbow trout, and conferred protection from challenge (Ghosh et al. 2015). A live attenuated strain, CSF259-93B.17 grown under conditions of iron-limitation or in the presence of iron was used as a vaccine administered by injection or immersion in coho salmon. Following vaccination by injection, the RPS values after challenge were 98 % and 90 % for the iron-limited and iron-replete preparations, respectively. By immersion, the corresponding RPS values were 73 % and 46 % (Long et al. 2013). Similarly, the use of subcellular components, specifically fractions of 18–28, 41–49

and 70–100 kDa were identified by western blotting in rainbow trout immune serum, and adjuvanted in FCA. Commendable protection was reported after i.p. injection of rainbow trout fry for the 41–49 (RPS = 58 %) and 70–100 kDa (O-proteins and O-polysaccharide) fractions with an RPS (for the latter fraction) of 94 % (LaFrentz et al. 2004). A total of 15 immunogenic proteins, that may be important in protection, were identified in cells of the pathogen, and equated with elongation factor G, gliding motility protein GldN, OmpA, trigger factor, ClpB, and a conserved hypothetical protein (LaFrentz et al. 2011). There is evidence that fish respond by producing antibodies to LPS and a ~20 kDa surface protein; the latter of which could be considered for any future vaccine development (Crump et al. 2001, 2005). A low molecular weight fraction of 25–33 kDa identified from immunoblotting with specific antiserum was applied as 5 and 10 µg quantities in FCA by i.p. injection to rainbow trout with booster doses 3-weeks later, and challenge 6-weeks afterwards with promising results (Högfors et al. 2008). Recombinant [purified from *Esch. coli]* and DNA vaccines to HSP60 and HSP70 have been evaluated in rainbow trout following administration by i.p. injection in FCA, although protection was not observed (Plant et al. 2009). A live attenuated vaccine based on the isolation of rifampicin-resistant cells as been detailed by LaFrentz et al. *(*2008*)*. The basis of the work is that when pathogens are passaged in increasing concentrations of rifampicin there is an attenuation of virulence, and thus the resultant cultures may serve as live vaccines. Thus, i.p. injection of 50 µl quantities containing ~8.3×10^6 CFU/ml with boosters (50 µl containing ~6.9×10^6 CFU/ml) 5-weeks later, and challenge after 8 (RPS = 45.2 % with a lower dose of the challenge strain) and 15 weeks. Similar results followed vaccination by immersion (LaFrentz et al. 2008). An attenuated vaccine, achieved by mutagenesis in which a mutant FP1033 was obtained with an inability to grow in iron-depleted medium, was protective of rainbow trout after challenge (Álvarez et al. 2008). Recombinant proteins of 3-hydroxyacyl-CoA dehydrogenase, ATP synthase ß subunit, and glutamate dehydrogenase were expressed in *Esch. coli* BL21 cells, and used to vaccine ayu by i.p. injection leading to better survival after challenge (Kato et al. 2014).

Immunostimulants/Dietary Supplements Ayu were reported as protected against *Fla. psychrophilum,* both in terms of mortalities and development/severity of disease signs, following oral administration of 1–10 % aqueous humus extracts (Nakagawa et al. 2009).

Probiotics/Biocontrol Success resulted from use of *Pseudomonas* M174 (Korkeaaho et al. 2011) and *Enterobacter* C6-6 and C6-8 (Burbank et al. 2011; Schubiger et al. 2015) for controlling *Fla. psychrophilum* infections. Boutin et al. (2012) recovered bacteria from the skin of brook charr that were inhibitory *in vitro* to *Fla. psychrophilum,* and when mixed and administered to fish led to a decrease in mortality after challenge.

Bacteriophage Twenty-two lytic bacteriophage cultures of 5–90 kb genome size with activity against *Fla. psychrophilum* have been recovered Danish fish farms,

and suggested for consideration in future biocontrol programmes (Stenholm et al. 2008). Fifteen bacteriophages were recovered from Chile, and under laboratory conditions determined to reduce mortalities in salmonids caused by *Fla. psychrophilum* (Castillo et al. 2012). The fate of bacteriophage has prompted some fascinating work by Madsen et al. (2013) and Christiansen et al. (2014). After i.p. injection, bacteriophage rapidly spread throughout the tissues, and could be isolated from the organs, particularly the kidney and spleen, of rainbow trout for up to 10-days (Madsen et al. 2013). With oral application, virus particles were detected in the intestine, kidney and spleen of rainbow trout within 1 h of administration. Thus, the data revealed that bacteriophage could survive passage through the stomach and be taken up into the internal organs (Christensen et al. 2014).

Antimicrobial Compounds Chemotherapy with oxytetracycline (Winton et al. 1983a), sulphonamides (Amend et al. 1965) and furanace (Holt et al. 1975) has been advocated. In particular with infected fry, furanace dosed at 0.5 μg/ml for 1 h on every third day has been useful. From a comparison of 48 isolates from RTFS, sensitivity was recoded to doxycycline, enrofloxacin, florfenicol and sarofloxacin (Rangdale et al. 1997a).

Flavobacterium piscicida

Only a poor description of *Fla. piscicida* exists in the fisheries literature. Therefore, further discussion will not be attempted.

Flavobacterium spartansii

Two isolates were recovered from the kidneys of feral spawning Chinook salmon and the gills of farmed Chinook salmon fingerlings, that were part of an outbreak of disease leading to mortalities. However, pathogenicity was not confirmed (Loch and Faisal 2014a).

Characteristics of the Disease

The cultures were derived from Chinook salmon with necrotic gill lamellae, and from a specimen with mild to severe unilateral exophthalmia, muscular ulcerations, pale liver and friable kidneys (Loch and Faisal 2014a).

Isolation

Isolation was achieved on Hsu-Shotts medium (Bullock et al. 1986) with incubation at 22 °C for 72 h (Loch and Faisal 2014a).

Characteristics of the Pathogen

Box 7.13: *Flavobacterium spartansii*
Cultures comprise semi-translucent almost flat dark yellow colonies with irregular, spreading margins that possessed a flexirubin-type pigment. The cells are Gram-negative, gliding rods of 3.0–5.0 μm in length, and do not contain cell-wall associated galactosamineglycans. Growth occurs on cytophaga agar, Hsu-Shotts medium, nutrient agar, sheep's blood agar and TSA, but not on marine 2216E agar, cetrimide agar or MacConkey agar at 22 °C. Casein and gelatin are degraded. Growth occurs at 4–22 °C, but not at 37 °C, in 0–2 % w/v (weakly at 2 %) but not in 3 % (w/v) sodium chloride, and at pH 6.0–8.0. Nitrates are reduced. N acetyl-ß–glucosaminidase, acid and alkaline phosphatases, catalase, cysteine, leucine and valine arylamidases, esterase, esterase lipase, ß-galactosidase (variable), α-glucosidase naphthol-AS-BI-phosphohydrolase and oxidase but not arginine dihydrolase, α-fucosidase, ß-glucuronidase, ß-glucosidase, H_2S, indole, lysine or ornithine decarboxylase, α-mannosidase, phenylalanine deaminase or tryptophane deaminase are produced. The Voges Proskauer reaction is variable. Do not produce acid from glucose. D- or L-arabinose. Degrade aesculin, casein, elastin, gelatin, starch and Tween 20 but not agar. Tween 80 or urea. Produces acid from cellobiose, D-galactose, gentobiose, D-glucose, glycogen, maltose, starch and trehalose, but not from D-adonitol, amygdalin, arabinose, dulcitol, erythritol, D-fructose, glucose, glycerol, inositol, inulin, lactose, D-mannitol, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, salicin, D-sorbitol, sucrose or D- or L-xylose. Utilises citrate as the sole source of carbon. Assimilates N-acetylglucosamine, D-glucose, maltose and D-mannose, but not adipic acid, L-arabinose, capric acid, malic acid, D-mannitol, phenylacetic acid or potassium gluconate. The major fatty acids comprise iso-$C_{15:0}$, $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$, iso-$C_{17:0}$3-OH, and iso-$C_{15:0}$3OH. The G + C ratio of the DNA was 35.6 mol%. Sequencing of the 16S rRNA gene reveals synonomy with *Flavobacterium,* but at a homology of <99 % with the closest neighbours, i.e. *Fla. aquidurense, Fla. araucananum* and *Fla. frigidimaris* (Loch and Faisal 2014a).

Flavobacterium succinicans

The organism has been associated with rainbow trout in recirculation systems in the USA. From these fish, the presence of *Fla. branchiophilum* and *Fla. succinicans* was determined by sequencing of the 16S rRNA gene (Good et al. 2015).

Flectobacillus roseus

Characteristics of the Disease

The disease, coined flectobacillosis, was recognized in captive rohu (*Labeo rohita)* in India. Fish displayed discolouration, loss of mucus and scales in the region of the caudal peduncle, and tail rot (Adikesavalu et al. 2015).

Isolation

Inocula from the caudal peduncle were streaked onto cytophaga agar supplemented with 5 mg of neomycin/ml and 200 IU of polymyxin B/ml with incubation at 30 °C for 48 h leading to the development of pale pink-rose colonies (Adikesavalu et al. 2015).

Characteristics of the Pathogen

Box 7.14: *Flectobacillus roseus*
Cultures are pale pink-rose pigmented, round, convex and smooth with entire edges, and contain Gram-negative nonmotile, non-fermentative long thin rods, that produce catalase, α- and ß-galactosidase, ß-glucoronidase, α- and ß-glucosidase, oxidase and phosphatase, but not H_2S, lysine or ornithine decarboxylase. Urea is not degraded. Nitrate reaction is variable. Citrate is not utilized. Sequencing of the 16S rRNA gene reveals a homology of 96 % with *Flectobacillus roseus* in GenBank (Adikesavalu et al. 2015), which is below the level for an acceptable identification (Adikesavalu et al. 2015).

Pathogenicity

Experimental challenges with scarified rohu involving i.m. injection with 0.1 ml containing 2.0×10^7 cells/fish led to the development of tail erosion within 36 h, with mortalities recorded 24 h later (Adikesavalu et al. 2015). Additional groups were bathed in a suspension of the *Flectobacillus* at ~2.0×10^6 bacteria/ml.

Flexibacter spp.

The fish pathology literature abounds with references to unspeciated *Flexibacter.* Pyle and Shotts (1980, 1981) studied 17 strains for phenotypic traits, as determined by use of the API 20E rapid identification system, and by DNA homology. The conclusion reached was that the isolates from warm-water fish were distinct to those recovered from cold-water fish. At least three separate groups were recognised. However, whether or not these organisms belong in existing species or represent new taxa must await further study.

Myroides odoratimimus

In this case, cultures obtained from the gut of healthy grey mullet *(Mugil cephalus),* and which demonstrated pathogenicity in laboratory experiments with healthy fish (Ravindran et al. 2015). Time will tell if this going to be emerging pathogen.

Characteristics of the Disease

Experimental challenge resulted in anorexia and abnormal swimming behaviour with rapid breathing within 1–2 days. This was followed by lethargy with the fish stationary at the bottom or swimming close to the surface. Skin haemorrhaging was observed, and reddening on the abdomen, mouth and pectoral fins. Later, black patches developed on the skin, and eye haemorrhages were seen.

Isolation

Gut contents were inoculated onto marine 2216E (marine/Zobell's) agar, nutrient agar and TSA with incubation at an undisclosed temperature for an unstated period, the outcome of which was the recovery of pure cultures.

Characteristics of the Pathogen

Box 7.15: *Myroides odoratimimus*
Colonies are yellow-orange, circular and convex with entire margins, and contain aerobic Gram-negative nonmotile rods that produce catalase and oxidase, but not H_2S. Sequencing of the 16S rRNA gene led the authors to report a close relationship (= high sequence similarity) to *Myroides odoratimimus* (Ravindran et al. 2015).

Epizootiology

It was considered that the feeding habits of gray mullet, which include detritus, dead plant material and zooplankton, may be an important source of *Myroides* (Ravindran et al. 2015).

Pathogenicity

The LC_{50} dose was 1.3×10^6 CFU/fish (Ravindran et al. 2015).

Tenacibaculum spp.

Characteristics of the Disease

There has been a report of the involvement of a difficult-to-isolate *Tenacibaculum* with winter ulcers in Atlantic salmon in Norway (Olsen et al. 2011).

Characteristics of the Pathogen

The fatty acid methyl esters (FAMEs) of *T. gallaicum, T. maritimum, T. ovolyticum* and *T. dicolor* were different in terms of iso-$C_{15:0}$3- OH, iso-$C_{16:0}$3-OH, iso-$C_{15:1}$OH, summed feature 3 (a component that contains $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), iso-$C_{16:0}$, $C_{17:1}\omega6c$, $C_{15:0}$3-OH and iso-$C_{17:0}$3- OH (Piñeiro-Vidal et al. 2008a).

Tenacibaculum dicentrarchi

Characteristics of the Disease

Although recovered from diseased sea bass and proposed as new species, the authors did not prove that the organism was actually pathogenic in laboratory-based infectivity experiments (Piñeiro-Vidal et al. 2012).

Isolation

Recovery was achieved on *Fle. maritimus* medium following incubation at 25 °C for 48 h (Piñeiro-Vidal et al. 2007, 2008c, 2012).

Characteristics of the Pathogen

The culture was linked to *Tenacibaculum* as a result of sequencing the 16S rRNA gene; the homology of 93.1–97.3 % indicating a new species (Piñeiro-Vidal et al. 2012).

Box 7.16: *Tenacibaculum dicentrarchi*
Cultures comprise flat pale yellow-pigmented [not flexirubin] strictly aerobic straight Gram-negative rods of 2–40 [filaments of up to 150 μm in length may occur] × 0.3–0.5 μm in size, which demonstrate gliding movement. Older cells may become spherical. Growth occurs at 4–30 °C and pH 6.0–9.0, and in media containing 30–100 % seawater [optimally 70 %] but not in media supplemented only with sodium chloride. Catalase, oxidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C4), cystine leucine and valine arylamidases are produced, but not α–chymotrypsin, H_2S, indole or trypsin. Casein and gelatin are degraded, but not starch or Tween 80. The Voges Proskauer reaction is negative. Acid is not produced from carbohydrates. Susceptible to O/129. The G + C ratio of the DNA was reported as 31.3 moles % (Piñeiro-Vidal et al. 2012).

Tenacibaculum discolor

Characteristics of the Disease

Diseased fish were described as displaying eroded mouth, rotted fins, shallow skin lesions and pale internal organs (Piñeiro-Vidal et al. 2008c).

Isolation

Recovery was achieved on *Fle. maritimus* medium following incubation at 25 °C for 48 h (Piñeiro-Vidal et al. 2007, 2008c; 2012).

Characteristics of the Pathogen

A single isolate was recovered from diseased sole in Spain:

Box 7.17: ***Tenacibaculum discolor***
Cultures comprise flat bright yellow-pigmented [not flexirubin] strictly aerobic straight Gram-negative rods of 2–30 × 0.5 μm in size, which demonstrate gliding movement. Older cells may become spherical. Growth occurs at 14–38 °C (optimally at 25–30 °C) and pH 6.0–8.0, and in media containing 30–100 % seawater but not in media supplemented only with sodium chloride. Catalase and oxidase are produced, but not H_2S or indole. Acid and alkaline phosphatase, α-chymotrypsin, esterase, esterase lipase, lipase, cystine, leucine and valine arylamidases, naphthol-AS-BI-phosphohydrolase and trypsin are produced. Nitrates are reduced. Casein and gelatin are degraded, but not starch or Tween 80. The Voges Proskauer reaction is negative. Acid is not produced from carbohydrates. L-proline and L-glutamate are utilized, but not D-galactose, D-glucose, D-ribose, D-sucrose or L-tyrosine. The G + C ratio of the DNA was reported as 32.1 moles % (Piñeiro-Vidal et al. 2008c).

By sequencing the 16S rRNA gene, the closest neighbour was *T. litoreum* (homology = 99.4 %); the corresponding DNA:DNA hybridization value was 39.6 % (Piñeiro-Vidal et al. 2008c).

Pathogenicity

The descriptions of *T. discolor* contained a statement that pathogenicity had been achieved in turbot and sole (Piñeiro-Vidal et al. 2007, 2008c).

Tenacibaculum gallaicum

Characteristics of the Disease

Diseased fish were described as displaying eroded mouth, rotted fins, shallow skin lesions and pale internal organs (Piñeiro-Vidal et al. 2008c).

Isolation

Recovery was achieved on *Fle. maritimus* medium following incubation at 25 °C for 48 h (Piñeiro-Vidal et al. 2007, 2008c; 2012).

Characteristics of the Pathogen

A single isolate was recovered from diseased turbot in Spain:

Box 7.18: *Tenacibaculum gallaicum*
Cultures comprise flat bright yellow-pigmented strictly aerobic Gram-negative rods of 2–30 × 0.5 μm in size, which demonstrate gliding movement. Older cells may become spherical. Growth occurs at 14–38 °C (optimally at 25–30 °C) and pH 6.0–8.0, and in media containing 30–100 % seawater but not in media supplemented only with sodium chloride. Catalase and oxidase are produced, but not H_2S or indole. Acid and alkaline phosphatase, α-chymotrypsin, esterase, esterase lipase, lipase, cystine, leucine and valine arylamidases, naphthol-AS-BI-phosphohydrolase and trypsin are produced. Nitrates are reduced. Casein and gelatin are degraded, but not starch or Tween 80. The Voges Proskauer reaction is negative. Acid is not produced from carbohydrates. L-proline and L-glutamate are utilized, but not D-galactose, D-glucose, D-ribose, D-sucrose or L-tyrosine. The G+C ratio of the DNA was reported as 32.7 moles % (Piñeiro-Vidal et al. 2008a).

By sequencing the 16S rRNA gene, the closest neighbour was *T. litoreum* (homology = 98.4 %); the corresponding DNA:DNA hybridization value was 40.2 % (Piñeiro-Vidal et al. 2008c).

Pathogenicity

The descriptions of *T. gallaicum* contained a statement that pathogenicity had been achieved in turbot and sole (Piñeiro-Vidal et al. 2007, 2008c).

Tenacibaculum maritimum (= Flexibacter maritimus = Cytophaga marina)

Characteristics of the Diseases

Principal signs of disease caused by *T. maritimum* include mouth erosions, gill erosion and tail rot (Handlinger et al. 1997), especially in juvenile fish. In older animals, lesions develop initially as grey-white cutaneous areas on the fins, head and trunk. These lesions degenerate into ulcers (Hikida et al. 1979; Wakabayashi et al. 1984). Two stages of disease appear to develop in salmonids in Australia: acute and chronic. The acute form developed 2–3 days after challenge with high numbers of cells 1×10^8 cells/ml, and was characterized by disintegration of the epithelium. The chronic form started as small blisters on the epidermis, which led to ulceration exposing the underlying musculature. Lesions developed on the fins and jaw; gill necrosis occurred. There was lack of an inflammatory response at higher challenge doses, suggesting a role for toxins (Van Gelderen et al. 2011) The organism caused ulcers in wedge sole (*Dicologoglossa cuneata*) in Spain (López et al. 2009). Black patch necrosis of Dover sole in Scotland and bacterial stomatitis (= mouth rot) of Atlantic salmon in Canada has been attributed to this pathogen (Bernardet et al. 1990; Ostland et al. 1999).

Isolation

Cytophaga agar, prepared in 70 % seawater, and TCY medium (Appendix in Chap. 12; Hikida et al. 1979) have achieved success with the isolation of *T. maritimum* (Appendix in Chap. 12; Hikida et al. 1979).

Characteristics of the Pathogen

Box 7.19: *Tenacibaculum maritimum*
A very homogeneous group (Bernardet et al. 1994), with cultures forming pale yellow flat, thin colonies, containing strictly aerobic Gram-negative rods of 2–30 × 0.5 μm in size, which occasionally form filaments of up to 100 μm in length. In older cultures, cells become spherical (~0.5 μm in diameter). Microcysts do not occur. Gliding movement is a characteristic feature of all isolates, and a non-gliding strain has been reported from diseased puffer fish (*Takifugu rubripes*) in Japan (Rahman et al. 2014). Catalase and oxidase are produced, but not H_2S or indole. Nitrates are not reduced. The methyl red test is negative. Casein, gelatin, tributyrin and tyrosine are degraded, but not aesculin, agar, cellulose, chitin starch or urea. Growth occurs from 14.6–34.3 °C, and at pH 6–9. There are requirements for potassium chloride and sodium chloride. Acid is not produced from arabinose, cellobiose, glucose, lactose, mannitol, raffinose, salicin, sucrose or xylose. Isolates are capable of lysing dead cells of *Aer. hydrophila, Edw. tarda, Esch. coli and V. anguillarum,* but not *Bacillus subtilis.* The G+C ratio of the DNA is 31.3–32.5 moles % (Hikida et al. 1979; Wakabayashi et al. 1986). All isolates share a common antigen (Wakabayashi et al. 1984).

From these traits, Hikida et al. (1979) proposed that the organism belonged in an as yet undescribed species of *Flexibacter,* for which the name of *Fle. marinus* was mooted. Subsequently, Wakabayashi et al. (1986) formally proposed the name of *Fle. maritimus* to accommodate the pathogen. Independently, Reichenbach (1989) proposed the name of *Cyt. marina* for the same organism. However, the organism was subsequently reclassified in a newly described genus *Tenacibaculum,* as *T. maritimum* (Suzuki et al. 2001). The intraspecific diversity of 29 fresh isolates and 3 reference cultures has been addressed using RAPDs with the outcome that two principle (and distinct serological) groups were recognised, of which one contained all the cultures from sole and gilthead sea bream, and the second group comprised isolates from Atlantic salmon, turbot and yellowtail. Interestingly, the reference strains were not recovered with the fresh isolates, and may reflect the problem of using archived cultures which have been long removed from their natural habitat (Avendaño-Herrera et al. 2004c). Further publications by this group (Avendaño-Herrera et al. 2004d; 2005b) defined three serotypes, i.e. O1, O2 and O3 which were regarded as host specific, with serotype O1, O2 and O3 comprising isolates from sole and gilthead sea bream, turbot and sole, respectively.

The nonmotile strain described by Rahman et al. (2014) formed slightly yellow round colonies with rhizoidal edges, and was not pathogenic for Japanese flounder when challenged by immersion. There was reduced adhesion ability to glass surfaces in shaking broth cultures and to the body surface of Japanese flounder. By gel immunodiffusion, the cell-surface antigen associated with gliding strains was absent, although the potential virulence traits, i.e. chondroitinase and gelatinase activities, were present (Rahman et al. 2014).

Diagnosis

Serology Whole cell agglutination was effective for the detection of *T. maritimum* (Wakabayashi et al. 1984). Also, FAT is useful for detecting *(=T. maritimum)* in fish tissues (Baxa et al. 1988a).

Molecular Methods PCR targeted 16S rRNA has been successful with *Fla. branchiophilum, Fla. columnare* and *T. maritimum* (Toyama et al. 1994, 1996; Avendaño-Herrera et al. 2004e; Yeh et al. 2006) although freezing-thawing destroyed the cells leading to the DNA being undetectable by PCR (Suomalainen et al. 2006a, b, c). However, a detection limit of 10^2–10^4 cells/reaction tube was reported (Avendaño-Herrera et al. 2004b). Nested-PCR gave even greater sensitivity, detecting 1–250 cells/PCR reaction particularly in the skin and mucus (Avendaño-Herrera et al. 2004b). This is compatible with the sensitivity of 75 CFU/g reported for the nested-PCR by Cepeda et al. (2003). A nested-PCR increased sensitivity to 10 bacterial cells in asymptomatic fish (Bader et al. 2003a). A quantitative real-time PCR, which incorporated primers amplifying a 155-bp fragment of the 16S rRNA gene, had a detection limit of 4.8 DNA copy number/µl and was applicable to use with formalin-fixed paraffin-embedded samples from the field (Fringuelli et al. 2012). DNA from the pathogen was detected in most (89 %) of the tissue blocks, which did not have clear sign of gill disease, and in 95 % of the blocks from fish with mild-to-severe gill pathology (Fringuelli et al. 2012). Similarly, PCR technology has proven to be successful for the detection of *Fla. psychrophilum* and *T. maritimum*, and was considered to be more sensitive than culturing (Wiklund et al. 2000; Avendaño-Herrera et al. 2004b).

Epizootiology

T. maritimum may also be responsible for heavy losses, i.e. 20–30 % of the population, among juvenile red sea bream and black sea bream (Hikida et al. 1979). In common with many organisms, extended survival of *T. maritimum* occurs in sterile but not in natural seawater (~5 days), i.e. with a resident microflora (Avendaño-Herrera et al. 2006).

Pathogenicity

In laboratory-based experiments, black sea bream were more susceptible to *T. maritimum* than red sea bream following i.m. injection of 0.02 ml of culture per fish, or infection by bathing or direct application of cultures to the tail or mouth. Mortalities, of up to 10 %, occurred in 3 days (Wakabayashi et al. 1984). An infection model has also been established for turbot in which the fish were immersed for 18 h with the

LD_{50} corresponding to containing 5×10^3 and 5×10^4 cells/ml for isolates from sole (serogroup O3) and turbot (serogroup O2), respectively (Avendaño-Herrera et al. 2004b). Abrading the dorsal skin of Japanese flounder (*Paralichthys olivaceus*) with a blade or by clipping the tip of dorsal fin followed by immersion in 10^6 CFU/ml led to100% mortality (Rahman et al. 2015). In contrast, i.p. injection failed to establish an infection (Avendaño-Herrera et al. 2004b). Other evidence has pointed to a role for the direct introduction of high numbers (4×10^{11} cells/fish) of bacteria onto gill abrasions for establishing infection (Powell et al. 2004, 2005). ECP, subcellular components (Wakabayashi et al. 1984), the ability to take up iron via siderophores and by utilization of heme groups (Avendaño-Herrera et al. 2005a) and the adherence [clumping ability] of cells (van Gelderen et al. 2010) have been implicated with pathogenesis. Haemolysin (26.5 μg/fish) and ECP (25.5 μg/fish) killed black sea bream. Pathological signs included the presence of ascitic fluid, enlarged spleen, petechial haemorrhages in the visceral fat and intestine, and suppurating (= pus filled) liver. Also, mortalities ensued following i.p. injection of crude LPS with protease (but not when administered separately) (Wakabayashi et al. 1984). ECPs of *T. maritimum* have been linked to pathology in Atlantic salmon (Van Gelderen et al. 2009b).

Disease Control

Vaccine Development A formalised suspension of *T. maritimum* containing ~1.79×10^{10} cells/ml with (RPS = 79.6 %) and without (RPS = 27.7 %) FIA was used to vaccinate Atlantic salmon (0.1 ml amounts by i.p. injection) followed by challenge (Van Gelderen et al. 2009a, b). Apart from the success of the adjuvanted version at combating mortalities, side effects of the use of FIA were noted, and included the development of black/brown pigment, most likely melanin, on the stomach with inflammation in the form of granulomas and cysts (Van Gelderen et al. 2009a).

Plant-based Immunostimulants 1.0–2.0 mg amounts of water and ethanol extracts of the Asian plant, big blue lilyturf (*Liriope platyphylla*) were administered to olive flounder (*Paralichthys olivaceus*) leading to immunostimulation (enhanced erythrocyte, lymphocyte and monocyte counts; increased haemoglobin content, and heightened complement and phagocytic activities) (Harikrishnan et al. 2012). Whereas these data are encouraging, the effect on protection against challenge awaits determination.

Antimicrobial Compounds From an examination of 75 isolates, it was determined that *T. maritimum* was highly susceptible to ampicillin, erythromycin, josamycin, nifurpirinol, penicillin G and sodium nifurstyrenate, moderately sensitive to chloramphenicol, doxycycline, oleandomycin, oxytetracycline, sulphamonomethoxine and thiamphenicol, weakly sensitive to nalidixic acid, oxolinic acid, spiramycin and sulphisoxazole, and resistant to colistin and streptomycin (Baxa et al.

1988b). In *in vivo* experiments, the efficacy of sodium nifurstyrenate was confirmed for chemotherapy. Thus, immersion (0.5 µg/µl for 1 h) or oral administration (30 mg/kg body weight of fish/day for 4 days) reduced mortalities in yellowtail to 60.5 %, compared to 100 % among the untreated controls (Baxa et al. 1988b). Trimethoprim and amoxicillin (dosed at 80 mg/kg body weight of fish) have been recommended in Australia (Soltani et al. 1995).

Tenacibaculum ovolyticum (=Flexibacter ovolyticus)

Characteristics of the Disease

T. ovolyticus led to mortalities among halibut eggs and larvae. The chorion became dissolved, and the underlying zona radiata was damaged by exotoxins resulting in puncturing of the egg, leakage of cell constituents and larval death (Hansen et al. 1992).

Isolation

Cytophaga agar, prepared in 70 % seawater, has been used for the recovery of the pathogen (Hansen et al. 1992).

Characteristics of the Pathogen

The 35 isolates were described as comprising:

Box 7.20: ***Tenacibaculum ovolyticum***
Pale yellow-pigmented long, slender (0.4 × 2–20 µm) Gram-negative rods, which demonstrate gliding movement. Catalase and oxidase are produced, but not so arginine dihydrolase, ß-galactosidase, H_2S, indole or lysine or ornithine decarboxylase. Nitrates are reduced. DNA, Tween 80 and tyrosine but not agar, cellulose, chitin, starch or urea, are degraded. Acid is not produced aerobically from arabinose, cellobiose, glucose, lactose, mannitol, raffinose, salicin, sucrose or xylose. Growth occurs at 4–30 °C but not 35 °C, and in >3 % (w/v) sodium chloride. The G+C ratio of the DNA was reported as 31.0 moles % (Hansen et al. 1992).

On the basis of DNA:DNA hybridisation, there is 26–42 % homology with *T. maritimum.* Certainly, the strains are distinct from *T. maritimum,* but it would be relevant to enquire about possible relationships with other cytophagas-flexibacteria.

Tenacibaculum soleae

Characteristics of the Disease

The pathogen has been recovered from Senegalese sole (*Solea senegalensis*), wedge sole (*Dicologoglossa cuneata*), brill (*Scophthalmus rhombus*) and turbot (*Psetta maxima*) (López et al. 2010) and sea bass (Castro et al. 2014) in Spain. In brill and wedge sole, heavy mortalities of up to 90 % occurred in juveniles; with 7–9 % deaths in adults with disease signs centring on ulcers on the body and tail. In addition, affected fish displayed haemorrhagic or pale livers, petechial on the finage, jaw, and ventral body surface, anaemia, and erratic swimming (López et al. 2010).

Characteristics of the Pathogen

A single culture from sole (*Solea senegalensis*) in Spain was used to describe a new species:

Box 7.21: *Tenacibaculum soleae*
Yellow-pigmented [not flexirubin pigment], strictly aerobic Gram-negative rods of 2–25 × 0.5 μm in size, which demonstrate gliding movement. Optimal growth occurs at 22–25 °C. Growth occurs in media containing 50–100 % seawater, but not in media supplemented with sodium chloride. Catalase and oxidase are produced, but not H_2S or indole. Casein and gelatin are degraded, but not starch or Tween 80. Neither L-proline, L-glutamate, sucrose, D-ribose, D-galactose, D-glucose no L-tyrosine is utilised. Alkaline phosphatase, esterase lipase, cystine, leucine and valine arylamidase, lipase is produced on the API ZYM system, but not α-chymotrypsin or trypsin. The G + C ratio of the DNA was reported as 29.8 moles % (Piñeiro-Vidal et al. 2008b).

From 16S rRNA sequencing, the closest neighbours were *T. ovolyticum* and *T. aestuarii* with 96.7 % homology to *T. soleae* (Piñeiro-Vidal et al. 2008b).

Identification/Diagnosis

PCR technology involving primers that amplified a 248 bp fragment of the 16S rRNA gene has been used, and permitted the detection of 1–10 bacterial cells/reaction within 3 h. The PCR discriminated *T. soleae* in mixed plate culture (García-González et al. 2011). López et al. (2011) described a PCR involving a 16S-23S internal spacer region (ISR) that was regarded as useful for discriminating the pathogen, and had a detection limit of 1 pg of DNA/reaction (= <30 bacterial cells) for pure cultures. In the presence of fish DNA or from other bacteria, the detection limit was 10 pg.

Pathogenicity

It has been stated that experimental infections of *T. soleae* in sole and turbot have been achieved (Piñeiro-Vidal et al. 2008b).

Sporocytophaga spp.

Negligible information is available about these Gram-negative microcyst-forming organisms (see Pacha and Ordal 1970), isolation was achieved using a seawater based medium (Appendix in Chap. 12; Anderson and Conroy 1969). Data have indicated that control may be exercised by use of pyridylmercuric acetate, ethylmercuric phosphate, oxytetracycline or chlortetracycline, at 1 mg/l for 1 h (Wood 1968), or a 2-min dip in copper sulphate (repeated on 3 consecutive days) at 1:2000 (Anderson and Conroy 1969).

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Chapter 8
Francisellaceae Representatives

Abstract *Francisella noatunensis* and *Fr. noatunensis* subsp. *orientalis* are intracellular pathogens which cause granulomatous disease. Isolation has been achieved initially in cell culture with subsequent growth on cysteine heart agar. Disease control has been achieved by vaccination and by use of antimicrobial compounds, notably florfenicol.

Keywords *Francisella* • Granulomas • Intracellular • Vaccines • Diagnosis

Isolation

Initially, francisellas were grown in cell cultures at 20 °C. Subculturing onto cysteine heart agar at 22 °C resulted in dense growth of small opaque colonies, which increased to 2–3 mm in diameter (Olsen et al. 2006). However, Kamaishi et al. (2005) managed to recover an organism from the spleen of an infected three-line grunt using cysteine heart agar supplemented with 1 % (w/v) haemoglobin. Also, isolation has been achieved on Thayer Martin agar within 4-days of incubation (Soto et al. 2014) and on modified cysteine heart agar (Lewisch et al. 2014).

Francisella noatunensis

Characteristics of the Disease

The pathogen has been found in both wild and farmed Atlantic cod in Norway with heavy losses in many farms (Ottem et al. 2008). Mature farmed cod, with an average weight of 3 kg, and which were contained in sea cages in Norway, developed mortalities in July 2005 when the water temperature was 14.5 °C. Mortalities peaked in August, and over the 5-month period to November ~40 % of the stock had died. Initially, clinical signs of disease were not noted, but later the fish were observed to be swimming sluggishly, were generally inappetant, and became emaciated. Some fish displayed dermal haemorrhagic nodules, and corneal opacity and perforation.

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There were white nodules in the heart, kidney, liver (swollen) and spleen (swollen). The intestinal mucosa was thickened. Some fish had bloody ascites (Olsen et al. 2006). Francisellosis has become associated with a chronic inflammatory granulomatous disease (= visceral granulomatosis) in which the affected fish contain large numbers of intracellular Gram-negative cocco-bacilli (Mikalsen et al. 2007; Zerihun et al. 2011; Gjessing et al. 2011). Since the 1980s wild caught cod have been fished sporadically from the southern North Sea, and thought at the time to be infected with mycobacteria. Farmed cod with similar disease signs have been subsequently recovered. Therefore, it is concluded that the disease in wild fish pre-dates that in farmed animals (Zerihun et al. 2011).

In Japan, the marine fish species, three-line grunt *(Parapristipoma trilineatum)* was observed with white granulomas. Intracellular bacteria were found in the kidney and spleen from which DNA was extracted and small subunit rRNA amplified by PCR, and sequenced. The outcome was 97.3–98.5 % homology to *Francisella,* with *Francisella philomiragia* as the closest neighbour (Kamaishi et al. 2005).

In a third study, *Francisella*-like bacteria were associated with mortalities in freshwater-reared (water temperature = 20–29 °C) hybrid striped bass. Here, the fish became lethargic, darker pigmented and demonstrated skin haemorrhages over the abdomen, mild to moderate bilateral exophthalmia, swollen kidney and spleen, and interstitial granulomas (Ostland et al. 2006).

550–60 % mortalities have been attributed to farmed tilapia in Costa Rica when infected fish displayed a range of non-specific signs including anaemia, anorexia, erratic swimming and exophthalmia (Soto et al. 2009).

Characteristics of the Pathogen

A Gram-negative intracellular cocco-bacillary organism was recovered from farmed Atlantic cod in Norway during 2005, and equated with *Francisella* (Olsen et al. 2006). Seven cultures were examined by phenotypic and molecular methods resulting in a proposal for a new subspecies, i.e., *Francisella philomiragia* subsp. *noatunensis* to accommodate them (Mikalsen et al. 2007). This became re-classified as *Fr. noatunensis* (Ottem et al. 2009; Mikalsen and Colquhoun 2010).

Box 8.1: *Francisella noatunensis*
Cultures develop initially small convex, opaque colonies on cysteine heart agar at 22 °C but not at 37 °C. These colonies comprise non-motile, small (variable sizes), weakly Gram-negative strictly aerobic, weakly catalase positive, oxidase negative, non-haemolytic, H_2S producing, facultatively intracellular cocco-bacilli, which grow at 10–30 °C optimally at 22 °C, and only slightly at 30 °C. Acid and alkaline phosphatase, esterase C4, esterase lipase C8, ß-lactamase and naphthol-AS-BI-phosphohydrolase are produced. Acid is not produced from a variety of carbohydrates, namely arabinose, glucose, maltose, mannitol, ribose, sorbitol, sucrose or trehalose.

Cultures were examined by 16S rRNA sequencing for which the nearest match was *Francisella philomiragia* (99.3 % identity) and several apparent *Francisella* from tilapia in Taiwan and three-lined grunt in Japan (Olsen et al. 2006). It was considered that the isolate from Atlantic cod was more fastidious that *Francisella philomiragia* (Olssen et al. 2006). Organisms were equated with two new species, i.e. *Fr. asiatica (= Fr. noatunensis* subsp. *orientalis)* and *Fr. noatunensis* (Ottem et al. 2009; Mikalsen and Colquhoun 2010). *Fr. piscicida* has a 99.8 % 16S rRNA sequence homology with *Fr. noatunensis,* and was regarded as a heterotypic synonym of *Fr. noatunensis* (Mikalsen and Colquhoun 2010).

Separately, intracellular bacteria were found in the kidney and spleen of three-line grunt from which DNA was extracted and small subunit rRNA amplified by PCR, and sequenced. The outcome was 97.3–98.5 % homology to *Francisella,* with *Francisella philomiragia* as the closest neighbour (Kamaishi et al. 2005). Originally, *Francisella philomiragia* was classified in *Yersinia* as *Y. philomiragia,* which comprised bacteria first recovered from dying muskrat in Utah, USA (Jensen et al. 1969). The link to *Yersinia* resulted from the micro-morphology of the cells and supposed DNA relatedness to *Y. pestis* (Ritter and Gerloff 1966). However, subsequent evaluation led to a transfer to *Francisella* (Hollis et al. 1989).

Diagnosis

Molecular Methods A specific quantitative real-time PCR has been developed for the recognition of *Fr. noatunensis* subsp. *orientalis,* with a stated detection limit of 50 fg of DNA/reaction (= ~25 genome equivalents) (Soto et al. 2010a).

Epizootiology

Experimental data suggested that *Fr. noatunensis* could pass through the digestive tract of blue mussels (*Mytilus edulis*), and cells in the resulting faeces caused disease upon intraperitoneal injection of cod (Wangen et al. 2012). However, there was not evidence that the pathogen remained in or even colonized blue mussel tissues. Moreover, cohabitation of Atlantic cod with artificially infected blue mussels did not lead to disease (Wangen et al. 2012).

Pathogenicity

Experimentally infected Atlantic cod did not develop any overt signs of disease and only comparatively low numbers of fish challenged intraperitoneally succumbed and died (Mikalsen et al. 2009). The organism replicated in adherent cells from head kidney, and inhibited respiratory burst activity (this is probably important to intracellular survival) in cod but not salmon leucocytes (Vestvik et al. 2013).

Control

Susceptibility was reported to florfenicol, flumequine, oxolinic acid and rifampin, and less so to ciprofloxacin, erythromycin, oxytetracycline, streptomycin and trimethoprim/sulphadiazine (Isachsen et al. 2012).

Francisella noatunensis subsp. *orientalis*

Characteristics of the Disease

Francisella was found in diseased [granulomatous] tilapia fry in an English recirculation site, with sequence homology confirming the pathogen (Jeffrey et al. 2010). The fry were lethargic, displayed exophthalmia, pale gills and petechial haemorrhaging in and around the pectoral fins. The spleens were enlarged and granular; some kidneys were enlarged, and the intestines were empty. The gall bladders were large (Jeffrey et al. 2010). Furthermore, the organism was recovered from granulomatous disease in Nile tilapia farmed in Brazil (Leal et al. 2014) and Mozambique tilapia, Koilapia (*Oreochromis hornorum*), blue tilapia (*Oreochromis aureus*) and Nile tilapia hybrids from Oahu, Hawaii (Soto et al. 2013).

The organism has been associated with disease in wild caught French grunt (*Haemulon flavolineatum*) and Caesar grunt *(Haemulon carbonarium)* from the Florida Keys, USA. Dead fish revealed the presence of granulomas particularly in the kidney and spleen (Soto et al. 2014). Also, Malawi ornamental cichlids from Austria displayed disease, i.e. anorexia, dyspnea, and abdominal distension, leading to mortalities (Lewisch et al. 2014).

Characteristics of the Pathogen

Although the organisms were first recognised in tilapia in Taiwan, it is thought that they may have been introduced on imports from South America, and identified initially as *Rickettsia*-like organisms (Chern and Chao, 1994).

Box 8.2: *Francisella noatunensis* subsp. *orientalis*
Small (1 mm diameter) colonies develop after 3 days incubation at 22 °C, and comprise non-motile, weakly catalase positive, oxidase negative, strictly aerobic weakly Gram-negative cocco-bacilli, that produce H_2S on cysteine containing media. Growth occurs at 10–30 °C but not at 37 °C. Acid is not produced from carbohydrates in phenol red broth base. Alkaline and acid phosphate, esterase C4, esterase lipase C8 and naphthol-AS-BI-phosphohydrolase are produced (Mikalsen and Colquhoun 2010).

DNA:DNA hybridisation of one strain (PQ1104) revealed 60.3 % homology with *Fr. noatunensis* (Mikalsen and Colquhoun 2010).

Pathogenicity

As an intracellular pathogen located within tight vacuoles [in infected macrophages], the organism is able to resist serum killing, and invade head kidney macrophages and replicate within them causing apoptosis and cytotoxicity [within 24–36 h after infection] (Soto et al. 2010b).

Disease Control

Vaccine Development An attenuated mutant, Δ*igl*c, was protective in tilapia following administration by immersion with 10^7 CFU/ml for 30 min (RPS = 68.75 %) or 180 min (RPS = 87.5 %) and immersion challenge with isolate WT (Soto et al. 2011).

Antimicrobial Compounds Florfenicol has been reported to be effective for the treatment of tilapia when dosed orally at 15 mg/kg of fish/day for 10 days (Soto et al. 2010c).

Francisella spp.

Kamaishi et al. (2005) succeeded in establishing an experimental challenge, and re-isolating the same organism, labeled as *Francisella* spp., from diseased fish.

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Chapter 9
Pseudomonads

Abstract Fish pathogenic pseudomonads include *Pseudomonas anguilliseptica, Ps. baetica, Ps. chlororaphis, Ps. fluorescens, Ps. koreensis, Ps. luteola, Ps. plecoglossicida, Ps. pseudoalcaligenes* and *Ps. putida,* which are the causes of Sekiten byo (= red spot), a disease of wedge sole without external or internal disease signs, distended abdomen and haemorrhaging on the body surface, fin/tail rot with or without the presence of external haemorrhaging, haemorrhagic septicaemia, bacterial haemorrhagic ascites of ayu, extensive skin lesions, and exophthalmia with external ulceration, respectively. Molecular diagnosis has been achieved with *Ps. anguilliseptica.* Formalin inactivated whole cell vaccines have been developed for *Ps. anguilliseptica* and *Ps. plecoglossicida.*

Keywords Sekiten-byo • Red spot • *Pseudomonas* • Fin rot • Haemorrhagic ascites

Pseudomonas aeruginosa

Ps. aeruginosa has been included in an article describing pathogens (including *Aer. hydrophila* and *Aer. veronii)* of ulcerated Nile tilapia from Tanzania (Shayo et al. 2012), and has been reported as pathogenic for Mozambique tilapia (*Oreochromis mossambicus)* in India (Thomas et al. 2013). Infection experiments using 0.1 ml volumes of high doses (2.4×10^8 CFU/ml) resulted in disease with signs including darkening of the skin, detachment of the scales, fin necrosis, exophthalmia cataract/trachoma and blindness, and presence of hemorrhaging on the body surface (Shayo et al. 2012). Pathogenicity was also reported towards Mozambique tilapia (Thomas et al. 2013). Disease control was possible by use of a lime oil nanoemulsion (Thomas et al. 2013) and neem oil nanoemulsion (Mishra et al. 2014).

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Pseudomonas alcaligenes

A fatal disease was recognized in a breeding station for Chinese sturgeon (*Acipenser sinensis*) summer (July) when the water temperature was 28 +/− 2 °C (Xu et al. 2015).

Characteristics of the Disease

The fish appeared to be normal before floating to the water surface. There were small numbers of dead fish observed daily, but the overall number of mortalities was high. Diseased fish were anaemic, the gills were pale with swollen and haemorrhagic filaments. Blood spots were present in the oral cavity, and petechiae were present in the pale livers and the digestive tract; haemorrhaging was observed in the gonads and kidney. The spleen was swollen, and reddish in colour. Some ascites was present (Xu et al. 2015).

Isolation

Isolation was achieved from the liver and kidney of moribund fish using BHIA with incubation at 28 °C for 10 days (Xu et al. 2015).

Characteristics of the Pathogen

Box 9.1: *Pseudomonas alcaligenes*
Colonies are white, translucent, circular and entire, and contained Gram-negative rods of ~ 2.4 × 0.8 μm that occur singly or in pairs, and are motile by single polar flagella. Phenotypic traits were determined using BIOLOG-GN, and key characters that met the description of *Ps. alcaligenes* include utilization of lactate, L-alanine, L-arginine, L-glutamic acid, pyruvate, L-lactic acid, L-malic acid, succinic acid and acetic acid, but not D-maltose, D-trehalose, sucrose, stachyose, α-D-glucose, D-mannose, D-fructose, D-galactose or L-serine. There was a 99 % homology with *Ps. alcaligenes* by sequencing the 16S rRNA gene (Xu et al. 2015).

Pathogenicity

Hybrid sturgeon (*Huso dauricus* x *Acipenser schrenckii)* were used for experimental infections, and a dose of 2×10^6 CFU/fish resulted in 33 % mortalities at day 5 after infection. Ten- and 100-fold increases in inocula resulted in 60 % and 100 % mortalities by day 15 (Xu et al. 2015).

Pseudomonas anguilliseptica

Characteristics of the Disease

Originally observed in eels, when the disease was referred to as Sekiten byo (= red spot), the pathogen has been identified in a wide range of other species, including Baltic herring *(Clupea harengus membras)* (Lönnström et al. 1994), gilthead sea bream (Doménech et al. 1999), black spot sea bream *(Pagellus bogaraveo)* (López-Romalde et al. 2003), orange-spotted grouper *(Epinephelus coioides)* (Al-Marzouk 1999) and cod (Ferguson et al. 2004). Typically, the disease manifests itself by the presence of petechial (pinprick) haemorrhages in the skin of the mouth region, opercula and ventral side of the body (Fig. 9.1). Haemorrhaging in the eye has been seen in infected Baltic herring (Lönnström et al. 1994). Reddening of the fins (as with vibriosis or *Aer. hydrophila* infections) does not usually occur. Small petechial haemorrhages may develop in the peritoneum, and the liver may be pale and haemorrhaged. The kidney may be soft and liquefying. Alternatively, in some cases of disease, there may be a dearth of internal signs of distress (Wakabayashi and Egusa 1972). Winter disease of gilthead sea bream, in which affect fish displayed slow erratic swimming before sinking to the bottom of the water and dying, has been linked to this pathogen (Doménech et al. 1999). Other disease signs included abdominal distensions on some animals, haemorrhaged kidney, pale liver, and the

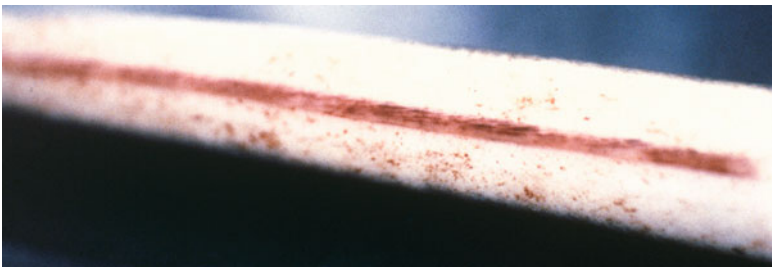

Fig. 9.1 Petechial haemorrhages on the surface of an eel with Sekiten-byo (Photograph courtesy of Dr. G. Dear)

intestine full of yellowish exudate. Low level mortalities, albeit in the absence of external signs of disease, were reported in black spot sea bream in Spain (López-Romalde et al. 2003).

Isolation

Isolation of *Ps. anguilliseptica* is readily achieved from blood, kidney, liver and spleen samples by use of nutrient agar supplemented with 10 % (v/v) horse blood or nutrient agar containing 0.5 % (w/v) sodium chloride, and adjusted to a pH of 7.4. Incubation should be at 20–25 °C for at least 7 days, when small (≤1 mm in diameter) round, raised, entire, shiny, pale-grey colonies develop (Wakabayashi and Egusa 1972).

Characteristics of the Pathogen

Box 9.2: *Pseudomonas anguilliseptica*
A homogeneous group of Gram-negative asporogenous rods, which are motile by means of single polar flagella. Electron microscopy of 18-h-old cultures on TSA reveal the presence of long, slightly curved rods with rounded ends. The size of these cells has been estimated as 5–10 × 0.8 μm. In addition, many bizarre forms have been observed. Fluorescent pigment is not produced. There is no reaction in the oxidative-fermentative test. Catalase and oxidase are produced, but not arginine dihydrolase, ß-galactosidase, H_2S or indole. Nitrates are not reduced. Gelatin, Tween 20 (variable result) and Tween 80 are degraded, but not blood, DNA, starch (variable result) or urea. Acid is not produced from arabinose, fructose, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, salicin, sucrose or xylose. Citrate is utilised by some isolates. Growth occurs at 5–30 °C but not 37 °C, in 0–4 % (w/v) sodium chloride, and at pH 5.3–9.7. The G + C ratio of the DNA is 56.5–57.4 mol% (Wakabayashi and Egusa 1972; Muroga et al. 1977; Nakai and Muroga 1982; Stewart et al. 1983; López-Romalde et al. 2003).

On the basis of phenotypic traits, evidence suggests that isolates are homogenous (López-Romalde et al. 2003). However, other approaches have detected some variation. Thus, a comprehensive examination of 96 isolates indicated the presence of two antigenic groups. Type I was not agglutinated in unheated antisera (this was prepared against heat-killed cells), although clumping (agglutination) of the cells subsequently occurred after the antiserum was heated to 100 °C for 2 h (or 121 °C

for 30 min). Type II lacked this inhibition. It was speculated that this thermolabile agglutination – inhibition antigen corresponds to the so-called K-antigens of coliforms (Nakai et al. 1981, 1982a). Molecular traits based on PFGE have revealed four types among 54 isolates from sea bream in Portugal and Spain (Blanco et al. 2002). Results with RAPD revealed two groups related to the host of origin of the cultures, with most of the isolates from eels in one cluster and the second grouping comprising isolates from other fish species (López-Romalde et al. 2003).

From the phenotypic traits, Wakabayashi and Egusa (1972) concluded that the causal agent of Sekiten-byo corresponded to a new centre of variation within 'Group III' or 'Group IV' of the genus *Pseudomonas*. This opinion was reached because the pathogen was Gram-negative, rod-shaped, motile by polarly located flagella, insensitive to the vibriostatic agent (O/129), and produced catalase and oxidase, but not acid from glucose or, for that matter, diffusible (fluorescent) pigment. Because the strains were dissimilar to other fish pathogenic pseudomonads, namely, *Ps. fluorescens*, a new taxon was proposed, i.e. *Ps. anguilliseptica*. We are sceptical about the validity of this proposal because the description could equally fit *Alcaligenes* or *Deleya* as well as *Pseudomonas* (See Cowan 1974; Kersters and De Ley 1984; Palleroni 2005). In some respects, the G+C ratio and the inability to produce acid in peptone water sugars is more conducive to the concept of *Alcaligenes or Deleya*, although the pathogen is clearly distinct from existing nomenspecies (Kersters and De Ley 1984). Moreover, it may not be ruled out that the causal agent of Sekiten-byo should be classified in a newly described genus. Certainly, the distinctive micromorphology adds weight to this supposition. Maybe this explains the pronounced dissimilarity of *Ps. anguilliseptica* to other species of *Pseudomonas* as revealed by analyses of fatty acids and outer-membrane proteins (Nakajima et al. 1983).

Diagnosis

Phenotypic Methods Wakabayashi and Egusa (1972) proposed an identification scheme for *Ps. anguilliseptica* based on a small number of phenotypic traits, principally motility, growth at 37 °C, presence of soluble pigment, production of H_2S, indole and oxidase, nitrate reduction, gelatin degradation, susceptibility to the vibriostatic agent (O/129), and the ability to attack glucose. According to these workers, the tests were sufficient to differentiate *Ps. anguilliseptica* from *Ps. fluorescens*, *Ps. alcaligenes*, *V. anguillarum*, *Aer. liquefaciens* (= *Aer. hydrophila*), *Ph. damselae* subsp. *piscicida* and *H. piscium*. By API 20 E, *T. maritimum* and *Ps. anguilliseptica* may be indistinguishable.

Serology *Ps. anguilliseptica* may be rapidly diagnosed by slide agglutination. However, slight cross-reactions may also occur with other organisms, including *Ps. putida* and *V. anguillarum*, but these interfering cross-agglutinations may be effectively eliminated by using diluted antiserum (Nakai et al. 1981).

Molecular Methods A PCR has been developed for the detection of *Ps. anguilliseptica*, with a detection limit of 170–200 cells/PCR tube within 8 h (Blanco et al. 2002). A *TaqMan* probe RT-PCR targeted the 16S rRNA gene, and was specific and sensitive, detecting 300 pg of *Ps. anguilliseptica* genomic DNA (Kang et al. 2015).

Epizootiology

Red spot disease, also known as 'Sekiten-byo', was first recognised in pond-cultured eels (*Anguilla japonica*) in Japan (Wakabayashi and Egusa 1972). Since then, it has developed into one of the most destructive diseases of eels in Japan (Nakai and Muroga 1979). During 1981, the disease was recognised in European eels (*A. anguilla*) within Scotland (Nakai and Muroga 1982; Ellis et al. 1983; Stewart et al. 1983). Conceivably, the disease is spreading, and this may reflect the natural migratory patterns of wild eel populations, or the rapid increase in intensive eel cultivation during the mid 1970s. A spread to other fish groups has now occurred. The pathogen was recovered from striped jack at water temperatures of 14–16 °C during February to April, 1993 (Kusuda et al. 1995), diseased rainbow trout, sea trout and whitefish *(Coregonus* sp.) in Finland during 1986–1991 (Wiklund and Lönnström 1994), wild Baltic herring *(Clupea harengus membras)* (Lönnström et al. 1994), sea bass, sea bream and turbot in France (Berthe et al. 1995), and as a winter disease outbreak in sea bream in Spain (Doménech et al. 1997). However, in Finland, there was some evidence also for the presence of *V. anguillarum* and *Aer. salmonicida.*

Very little has been published about the epizootiology of Sekiten-byo. The disease is recognised, however, to be prevalent in brackish water, when the water temperature is between 20 and 27 °C (Muroga et al. 1977). Indeed, temperature is considered to be the major factor in influencing epizootics. All the available information points to the seriousness of outbreaks in terms of high mortalities. Thus in one outbreak of the condition in Scotland, 67,000 elvers (96 % of the total) plus 154 adult eels (3.9 % of the total) died (Stewart et al. 1983). These authors observed that generally the large adult eels fared worse than smaller adults. Nevertheless, the greatest losses occurred with elvers. It is interesting to note that the disease eliminated 14 % of the total weight of the farm stock. This represents a significant investment; therefore, Sekiten-byo has the potential to be a severe economic problem.

Molecular typing techniques have been evaluated using 52 isolates, with three major genomic groups recognized by repetitive extragenic palindromic PCR (REP-PCR) and repetitive intergenic consensus PCR (ERIC-PCR). These groups comprised isolates from eels, turbot and blackspot sea bream, and other fish species (gilthead sea bream, sea bass and salmonids) (Beaz-Hidalgo et al. 2008).

Pathogenicity

Eels, challenged by i.p. injection with 3-day-old broth cultures, eventually displayed the same symptoms attributed to natural outbreaks of the disease. Thus, they became gradually inactive, and developed petechial haemorrhages prior to death, which usually occurred in 6–10 days (Wakabayashi and Egusa 1972). This is a short period for death to ensue. Of course, this depends upon the number of cells in the initial challenge. We consider that this is indicative of the effect of exotoxins, probably exoenzymes, although Dear (1985) could not obtain mortalities following injection of European eels with ECP. Of course, *Ps. anguilliseptica* is not metabolically very active, but most isolates attack proteins (gelatin) and lipids (Tween 80). Therefore, it is suggested that the pathogenicity mechanism involves proteases and lipases. There is no evidence for the presence of an extracellular layer in virulent isolates as occurs in *Aer. salmonicida.* There is some evidence of species-based susceptibility to Sekiten-byo, with Japanese eels seemingly more prone to the disease than European eels.

It is speculative whether or not this infers that the organism may be more common in and around European eels (or may have originated with this species). A comparative observation is that brown trout are more susceptible than rainbow trout to furunculosis caused by *Aer. salmonicida*. Certainly, *Ps. anguilliseptica* is capable of infecting a greater range of species than represented by the genus *Anguilla*. Thus, experimental infections have been achieved in ayu, bluegill, carp, goldfish and loach (Muroga et al. 1975). The organism is only of low pathogenicity to rainbow trout (Lönnström et al. 1994).

The presence of sublethal concentrations of copper (100–250 mg/l) in water exacerbates the disease (Mushiake et al. 1984). Evidence points to a reduction in lymphocytes and granulocytes, which leads to lowered phagocytosis (Mushiake et al. 1985).

Disease Control

Management Techniques Control of Sekiten-byo is possible by means of raising the water temperature in the fish holding areas to 26–27 °C. By keeping the water at this temperature for 2 weeks, followed by reducing it to approximately 21 °C, there was no further outbreak of the disease for 5 months (Wakabayashi and Egusa 1972).

Vaccine Development Attempts have been made to develop formalin-inactivated vaccines. It is encouraging that fish are capable of eliciting an immune response against *Ps. anguilliseptica*, insofar as experimentally vaccinated eels developed

agglutinating antibody within 2 weeks at water temperatures of between 15 and 28 °C. The maximum titre recorded was 1:256, which was reached during the 7-week period that an immune response could be detected. Although injection in Freund's adjuvant produced the highest immune response in terms of production of agglutinating antibody (titre = 1:4096), all the commonly used vaccination techniques protected the recipient fish against experimental challenge with virulent cells (Nakai and Muroga 1979). Field trials with batches of eels, each comprising 2000 animals, Nakai et al. (1982b) confirmed the efficacy of injectable heat-killed vaccine. Using a formalin-inactivated whole cell vaccine in olive flounder, Jang et al. (2014) reported commendable success (RPS = 89 %).

Antimicrobial Compounds Jo (1978) discussed the usefulness of nalidixic acid, oxolinic acid and piromidic acid for chemotherapy.

Pseudomonas baetica

Characteristics of the Disease

In the single outbreak, dying wedge sole lacked external or internal signs of disease (López et al. 2011).

Isolation

Isolation was achieved on *Fle. maritimus* medium with incubation at 20 °C for 24–96 h (López et al. 2011).

Characteristics of the Pathogen

Five isolates were recovered from the liver of diseased adult wedge sole in Spain, and on the basis of 16S rRNA sequencing equated with *Pseudomonas.* Examination of the housekeeping genes *gyrB* and *rpoD* permitted differentiation from other *Pseudomonas,* a conclusion supported by DNA:DNA hybridisation (López et al. 2011).

Box 9.3: *Pseudomonas baetica*
Cultures are whitish and up to 0.5 mm in diameter after incubation for 48 h, and comprise fluorescent [with fluorescein] large irregularly shaped haemolytic Gram-negative strictly aerobic, motile rods that produce acid and alkaline (mostly) phosphatase, α-chemotrypsin, arginine dihydrolase, catalase, cystine arylamidase, esterase (C4) (mostly), esterase lipase (C8), leucine arylamidase (mostly) and oxidase, but not N-acetyl-ß-glucosaminidase, α-fucosidase, α– or ß-galactosidase, ß-glucuronidase,, α– or ß-glucosidase, H_2S, lipase (C14), α-mannosidase, lysine or ornithine decarboxylase, naphthol-AS-BI-phosphohydrolase, trypsin or valine arylamindase. Casein and gelatin are degraded, but not aesculin, starch, Tween 80 or urea. Nitrates are not reduced to nitrite. The Voges Proskauer reaction is negative. Citrate is utilised. Growth occurs at 4–30 °C and in 0–6 % (w/v) sodium chloride. Acid is produced from glucose and mannose, but not from amygdalin, arabinose, inositol, lactose, mannitol rhamnose, salicin or sorbitol (López et al. 2011).

Pathogenicity

The original description by López et al. (2011) included confirmation that one culture, A390^T, caused mortalities in wedge sole following i.p. (10^6 cells/fish) but not bath challenge (18 h in 10^7 cells/ml). In comparison to the natural infection, experimentally challenged fish did not reveal any overt signs of disease.

Pseudomonas chlororaphis

Characteristics of the Disease

In moribund fry, it was observed that symptoms included the presence of distended abdomen with ascitic fluid, and haemorrhages on the body surface (Hatai et al. 1975).

Isolation

This may be accomplished by inoculating homogenates, prepared from the entire fish, onto the surface of nutrient agar plates, with incubation at 25 °C for 5 days. It is surprising that this simple method enabled the recovery of pure cultures, because contaminants may be expected from the intestine and body surface (Hatai et al. 1975).

Characteristics of the Pathogen

To date, there has been only one report of *Pseudomonas chlororaphis* as a fish pathogen. This involved a heavy mortality among farmed Amago trout (*Oncorhynchus rhodurus*) in Japan (Hatai et al. 1975). For the present, it is uncertain whether *Ps. chlororaphis* represents an emerging problem, or a secondary (opportunistic) invader of already diseased hosts.

The isolates matched the description of *Ps. chlororaphis*, insofar as cultures comprised Gram-negative motile rods, which produced distinctive colonies. These produced green pigment, which crystallised as needles in the colonies (Stanier et al. 1966; Palleroni 1984). Other phenotypic traits were not reported, although the authors inferred that further tests had been carried out, and that these agreed with the definition of *Ps. chlororaphis*.

Epizootiology

Ps. chlororaphis occurs in water (Palleroni 1984), which is assumed to be the reservoir of infection. No other information is available.

Pathogenicity

The isolates from Amago trout and the neotype culture of *Ps. chlororaphis* were pathogenic to carp, eels and trout, following challenge by i.m. injection. Total mortalities occurred within 48 h at a water temperature of 22 °C, with disease symptoms paralleling those on the naturally infected fish. However, the pathogenicity mechanism is unknown (Hatai et al. 1975).

Pseudomonas fluorescens

Characteristics of the Diseases

Ps. fluorescens is a dominant component of the freshwater ecosystem (Allen et al. 1983). At various times, *Ps. fluorescens* has been considered as a fish spoilage organism (Shewan et al. 1960), a contaminant or secondary invader of damaged fish tissues (Otte 1963), as well as a primary, but poor pathogen (Roberts and Horne 1978). It has been reported to cause disease in a wide range of fish species, including silver carp (*Hypophthalmichthys molitrix*) and bighead (*Aristichthys nobilis*) (Csaba et al. 1981; Markovic et al. 1996), goldfish (*Carassius auratus*) (Bullock

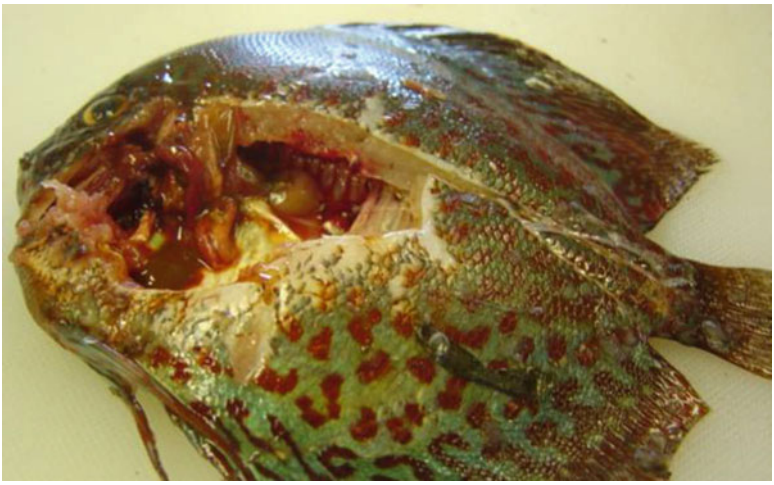

Fig. 9.2 Clown fish with systemic *Pseudomonas* infection (Photograph courtesy of Dr. A. Newaj-Fyzul)

1965), tench (*Tinca tinca*) (Ahne et al. 1982), grass carp (*Ctenopharyngodon idella*) and black carp (*Mylopharyngodon piceus*) (Bauer et al. 1973), Indian major carp (*Catla catla*) (Darak and Barde 2015), unnamed species of carp (Schäperclaus 1959; Schäperclaus and Brauer 1964; Heuschmann-Brunner 1978) and rainbow trout (Li and Flemming 1967; Li and Traxler 1971; Sakai et al. 1989). Generally, *Ps. fluorescens* is associated with fin or tail rot in which the infected area is eroded away (Schäperclaus 1979). In tench fry, high mortalities (up to 90 % of the population) have been reported, in which visual signs of disease included haemorrhagic lesions on the skin and at the base of the fins. Ascitic fluid accumulated in the peritoneal cavity, and petechial haemorrhages were evident in the gills, kidney, liver and in the lumen and submucosa of the gut, i.e. a typical generalised bacterial septicaemia (Fig. 9.2) (Ahne et al. 1982). Similar symptoms were apparent in silver carp and bighead (Csaba et al. 1981). Stress, including a lowered water temperature, may trigger outbreaks of disease (Markovic et al. 1996). With rainbow trout the presence of ulcers at haemorrhages on the gills and fins were reported (Sakai et al. 1989).

Isolation

Ps. fluorescens was recovered from most organs as pure culture growth on standard bacteriological media, such as *Pseudomonas* F agar (Appendix in Chap. 12), blood agar, TSA and nutrient agar, following incubation at 22–28 °C for 24–28 h (Csaba et al. 1981; Ahne et al. 1982).

Characteristics of the Pathogen

All the published descriptions of the organism (e.g. Bullock 1965; Csaba et al. 1981; Ahne et al. 1982) agree closely with the definition of *Ps. fluorescens* (Stanier et al. 1966; Palleroni 2005).

Box 9.4: *Pseudomonas fluorescens*
Cultures comprise Gram-negative, oxidative, arginine dihydrolase, catalase and oxidase producing rods, which are motile by polar flagella. Growth occurs at 4 °C but not at 42 °C. Fluorescent pigment (fluorescein) and gelatinase, but not ß-galactosidase, H_2S, indole, amylase or urease, are produced. The Voges Proskauer reaction is negative. Citrate is utilised, and acid is produced from arabinose, inositol, maltose, mannitol, sorbitol, sucrose, trehalose and xylose, but not from adonitol or salicin.

It seems likely that other fish pathogenic pseudomonads, as discussed by Li and Flemming (1967) and Li and Traxler (1971), correspond to *Ps. fluorescens*.

Epizootiology

In view of its widespread occurrence in the aquatic environment, *Ps. fluorescens* is likely to be spread through water, which will serve as the primary reservoir of infection. The disease is especially troublesome at low water temperatures, i.e. at ~1 °C. In one experiment, Ahne et al. (1982) achieved 100 % mortality in tench fry within 10 days at a water temperature of 10 °C. Challenge was by i.p. injection of a bacterial suspension.

Ahne and co-workers noted that initial occurrence of disease, with 30 % of the population developing skin haemorrhages, was 7 days after transfer of the tench to laboratory aquaria. Mortalities began on day 14, and by 2 weeks later, 90 % of the fish had died. It is noteworthy that the problem in silver carp and bighead developed after a stressful period during winter, when the water temperature fluctuated around freezing. In this outbreak, the mortality rate was at 5 % of the population per day (Csaba et al. 1981).

Pathogenicity

Following invasion of the fish, extracellular proteases are probably responsible for the ensuing damage (Li and Fleming 1967; Li and Jordan 1968). Sakai et al. (1989) reported the LD_{50} for rainbow trout as 4.2×10^5 cells at 18 °C and 1.1×10^5 cells at 12 °C.

Disease Control

Antimicrobial Compounds Bath treatments with benzalkonium chloride (1–2 mg/l of water/1 h), furanace (0.5–1 mg/l of water/5–10 min) or malachite green (1–5 mg/l of water/1 h) may help control early clinical cases of disease (Austin 1984). In one study, isolates showed susceptibility to kanamycin, nalidixic acid and tetracycline (Sakai et al. 1989). A second investigation reported widespread susceptibility to gentamicin, kanamycin and neomycin, less to amikacin and oxytetracycline, and total resistance to chloramphenicol, erythromycin, penicillin and potentiated sulphonamides (Markovic et al. 1996).

Pseudomonas koreensis

Ps. koreensis was described as the causal of eye lesions in golden mahseer (*Tor putitora*) from India. Between March and May, 2012, farmed adult golden mahseer were observed with opaque eyes, which led to mortalities. The clinical signs included haemorrhaging in the periphery of the eyes, corneal opacity, and pale livers. Material from the diseased eyes were inoculated onto TSA with incubation at 28 °C for 48 h when dense growth of yellowish-green colonies were observed. Identification was achieved by phenotyping and by 99 % homology with *Ps. koreensis* by sequencing of the 16S rRNA gene. Infectivity experiments using 100 µl volumes of 7.6×10^6 CFU/ml, which were injected i.p. in golden mahseer fingerlings, resulted in 55 % mortalities within 2-days albeit in the absence of eye pathology (Shahi and Mallik 2014).

Characteristics of the Pathogen

Box 9.5: *Pseudomonas koreensis*
Cultures produce a yellowish-green pigment on *Pseudomonas* P agar, and comprise Gram-negative, motile asporogenous rods of 1.6–2.0 µm in size that grow between 8 and 30 °C, and in <6 % (w/v) sodium chloride. Catalase and oxidase are produced, but not ß-galactosidase, H_2S or indole. Blood (ß-haemolysis), casein, lecithin, Tween 80 and urea are degraded, but not aesculin, DNA, gelatin or starch. Citrate is utilised. The methyl red test and Voges Proskauer reaction are negative. Nitrates are not reduced (Shahi and Mallik 2014).

Pseudomonas luteola

Characteristics of the Disease

A disease outbreak occurred during 2004 in a rainbow trout farm in Turkey when 40 % of the fish population died, with signs including exophthalmia, melanosis, haemorrhaging at the base of the fins and around the vent, pale liver, enlarged spleen, and the intestine filled with yellowish fluid (Altinok et al. 2007).

Isolation

The organism was recovered on TSA with incubation at 25 °C for 2 days (Altinok et al. 2007).

Characteristics of the Pathogen

Box 9.6: *Pseudomonas luteola*
Cultures comprise Gram-negative, motile, oxidative, oxidase bacteria that produce arginine dihydrolase and ß-galactosidase but not H_2S, indole, lysine or ornithine decarboxylase, or tryptophan deaminase. Capric acid, citrate, glucose, malate and potassium gluconate are utilised, but not adipic acid or phenylacetic acid. The Voges Proskauer reaction is negative. Aesculin, and gelatin are degraded, but not urea. Acid is produced from arabinose, glucose, inositol, D-mannose, mannitol and sucrose but not rhamnose (Altinok et al. 2007).

By means of sequencing of the 16S rDNA gene, a 99.8 % homology was recorded to *Ps. luteola* (Altinok et al. 2007).

Pathogenicity

Experimental challenge was established by immersion of rainbow trout in 6.4×10^6 CFU/ml for 1 h (Altinok et al. 2007).

Pseudomonas mosselii

The organism was recovered from Nile tilapia in Mexico. The disease signs included lethargy, erratic swimming, melanosis, scale loss, blindness, exophthalmia and/or red or opaque eyes. Diseased tissues were inoculated onto TSA supplemented with 0.5 % (/v) glucose, BHIA supplemented with 5 % (v/v) sheep blood, MacConkey agar, glutamate- starch-phenol red agar, and TCBS with incubation at 30 +/− 1 °C for 24–48 h. Identification was achieved by sequencing of the 16S rRNA gene. Pathogenicity was confirmed in laboratory-based infectivity experiments using tilapia (*Oreochromis mossambicus*). ECPs displayed cytotoxic effects to HeLa cells (Soto-Rodriguez et al. 2013).

Pseudomonas plecoglossicida

Characteristics of the Disease

A new bacterial disease emerged during the 1990s, and caused mass mortalities in pond-cultured ayu in Japan (Wakabayashi et al. 1996) and subsequently with diseased large yellow croaker (*Larimichthys crocea*) in China (Zhang et al. 2014). The term bacterial haemorrhagic ascites is descriptive, with affected ayu displaying lesions in the gills, heart, intestine, kidney, liver and spleen. In particular, lesions in the spleen and haematopoietic tissues were profound. Those in the kidney, liver and spleen were necrotic. Abscesses were apparent in the liver. In contrast, the brain did not reveal the presence of any lesions (Kobayashi et al. 2004). In olive flounder, the pathogen caused visceral granulomas (Zhang et al. 2014).

Characteristics of the Pathogen

The pathogen was regarded as having phenetic similarities with *Ps. putida* biovar A, but on the basis 16S rRNA sequencing was regarded as distinct, and elevated into a new species, as *Ps. plecoglossicida* (Nishimori et al. 2000).

Box 9.7: *Pseudomonas plecoglossicida*
The 6 cultures (a brown-pigmented culture has been subsequently recovered; Park et al. 2000) examined in the initial study comprises a homogeneous group of strictly aerobic Gram-negative motile (several polar flagella) rods that produce catalase and oxidase, and reduce nitrate to nitrite, and grow at 10–30 °C but not at 4 or 41 °C, in 0–5 % (w/v) NaCl. Arginine dihydrolase is produced, but not lysine or ornithine decarboxylase. Blood is degraded, but not gelatin, lecithin, starch or Tween 80. Caprate, citrate, D-fructose, 2-ketogluconate, L-alanine, glucose, D-malate, propylene glycol, L-lysine, succinate and L-citrulline are utilised, but not L-arabinose, *m*-inositol, mannitol, D-mannose, sucrose, D-tartrate, testosterone, trehalose, L-tryptophan or D-xylose. A weak fluorescent pigment is produced on King medium B. The G+C ratio of the DNA is 62.8 mol% (Nishimori et al. 2000).

DNA:DNA hybridisation levels were <50 % with reference *Pseudomonas* spp. (Nishimori et al. 2000).

Epizootiology

Immersion leads to infection of ayu, with gills and gill epithelia being the likely portals of entry as determined by real-time quantitative PCR. At 6 h after infection, the pathogen was located in the kidney, liver and spleen; septicaemia was apparent after 48 h when the organism was found in the blood (Sukenda and Wakabayashi 2000).

Pathogenicity

During surveys of dead ayu (with bloody ascites) in Japan during 1999 and 2001 with the exception of one isolate, all the others were non-motile. Moreover, non-motile cells were injected intramuscularly into ayu leading to the recovery of both motile and non-motile cells from the kidney. However, motile cells were recovered after the injection of motile cultures (Park et al. 2002). By use of GFP-labelled cells, the pathogen has been observed to adhere predominantly to the site of microscopic injuries in the fins and skin (Sukenda and Wakabayashi 2001).

Disease Control

Vaccine Development Formalin inactivated cells administered in oily adjuvant, i.e. Montanide-ISA711 or Montanide-ISA763A, or saline to ayu followed by challenge after 22 and 52 days led to reasonable to excellent protection. Thus, the RPS for the Montanide-ISA711, Montanide-ISA763A and saline vaccines were 17–58 %, 57–92 % and 65–86 %, respectively (Ninomiya and Yamamoto 2001). An acetone-killed (37 °C for 2 h), dehydrated oral whole cell vaccine was developed and fed to ayu at 2 week intervals before challenge (RPS = 40–79 %) (Kintsuji et al. 2006).

Biological Control Two lytic bacteriophage – Podoviridae PPp-W4 and Myoviridae PppW-3 –, which were recovered from diseased ayu and pond water used to rear fish (Park et al. 2000) have been considered for use against *Ps. plecoglossicida* in ayu (Park and Nakai 2003). In *in vitro* work, PPp-W4 was the more successful at inhibiting the pathogen. In fish experiments involving challenge with feed supplemented with *Ps. plecoglossicida* at 10^7 CFU/fish followed by use of feed containing bacteriophage (10^7 PFU/fish), the resulting mortalities were greatly reduced. Thus, by use of groups with PPp-W3, PPp-W4 and a mixture of both, mortalities of 53, 40 and 20 % were recorded compared to 93 % mortalities among the controls (Park and Nakai 2003). Field trials were equally successfully, and there was no evidence for the development of bacteriophage resistance by *Ps. plecoglossicida* (Park and Nakai 2003).

Pseudomonas pseudoalcaligenes

Characteristics of the Disease

During 1992 at a site in the UK, rainbow trout (average weight = 100 g) were observed with extensive skin lesions and signs of ERM (from which *Yersinia ruckeri* was recovered). The fish displayed extensive skin lesions, which extended over the entire flank from the operculum to the tail. The skin and underlying muscle to a depth of approximately 1 mm were totally eroded (Austin and Stobie 1992).

Isolation

Ps. pseudoalcaligenes was recovered as mixed cultures with *Serratia plymuthica* from the ascitic fluid and surface lesions following incubation on TSA at 22 °C for 3–4 days (Austin and Stobie 1992).

Characteristics of the Pathogen

Box 9.8: *Pseudomonas pseudoalcaligenes*
The cream-coloured colonies (with a "gummy" consistency) comprise motile oxidative (alkali is produced) short Gram-negative rods, which produce arginine dihydrolase, catalase, ornithine decarboxylase and oxidase, but not ß-galactosidase, H_2S, indole, lysine decarboxylase or tryptophan deaminase, degrade gelatin, tyrosine (with the production of melanin) and Tween 80, but not DNA, starch or urea, and grow at 15 and 25 °C, but not at 4 or 40 °C. Acid is produced from arabinose and glucose, but not from amygdalin, inositol, mannose, melibiose, rhamnose or sorbitol. Citrate is utilised. The Voges Proskauer reaction is negative.

The organism matched the description of *Pseudomonas* (Palleroni 2005), and approximated *Ps. pseudoalcaligenes* as determined from the probability matrix of Holmes et al. (1986). The only discrepancies concerned the degradation of gelatin and starch, and the production of ornithine decarboxylase.

Epizootiology

Pseudomonads occur in polluted/eutrophic freshwater, which is considered to be the source of *Ps. pseudoalcaligenes* (Austin and Stobie 1992). Moreover, it was apparent that the fish farm waters received sewage from a neighbouring septic tank (B. Austin, unpublished data).

Pathogenicity

Injection of 10^5 cells by i.p. or i.m. injection into rainbow trout (average weight = 12 g), held at 15 °C, resulted in total mortalities within 7 days. Moribund fish revealed the presence of haemorrhaging (internal and around the vent) and ascitic fluid in the peritoneal cavity (Austin and Stobie 1992).

Disease Control

Antimicrobial Compounds Antibiogrammes revealed sensitivity to oxytetracycline, oxolinic acid and potentiated sulphonamides (Austin and Stobie 1992).

Pseudomonas putida

Characteristics of the Disease

Altinok et al. (2006) described exophthalmia, melanosis and ulcers on the dorsal surface of rainbow trout in grow out ponds in Turkey. The internal organs appeared normal, but the intestine was full of yellowish liquid.

Characteristics of the Pathogen

There has been a casual mention of *Ps. putida* as a fish pathogen in Japan (Muroga 1990). The organism was recovered from diseased (the disease was described as bacterial haemorrhagic ascites) ayu in Japan, and equated with *Pseudomonas* (Wakabayashi et al. 1996). Similarities were noted to *Ps. putida,* but there was an absence of fluorescent pigment and a lack of agglutination with antiserum prepared to the type strain (of *Ps. putida).* The profile, obtained with the API 20NE rapid identification system, was "1140452" (Wakabayashi et al. 1996). In a subsequent study, Altinok et al. (2006) identified a pathogen as *Ps. putida* on the basis of 16S rRNA sequencing (homology = 99.8 %) and phenotypic characteristics.

Box 9.9: *Pseudomonas putida*
Cultures are fluorescent, motile Gram-negative rods, which produce arginine dihydrolase and oxidase but not ß-galactosidase, H_2S or indole, grow at 4 but not 41 °C, do not attack aesculin, gelatin or urea, and produce acid from arabinose, capric acid, glucose, malate, potassium gluconate and trisodium citrate but not adipic acid, maltose, mannitol, D-mannose, rhamnose, sorbitol or sucrose (Altinok et al. 2006).

Stenotrophomonas maltophilia

The organism was recovered in India during 2014 from African catfish (*Clarias gariepinus*), which were farmed in earthern ponds (Abraham et al. 2016) and may be an emerging pathogen of catfish (*Ictalurus punctatus*) in China (Geng et al. 2010a).

Characteristics of the Disease

The fish were lethargic with surface discolouration, and evidence of fin/tail rot, hemorrhages on the surface, and distended abdomen, which was filled with transparent jelly-like material. There was inflammation of internal organs, and visceral hemorrhages (Abraham et al. 2016).

Isolation

Kidney material was inoculated onto BHIA, glutamate starch phenol red agar supplemented with 100 IU/ml penicillin G sodium salt, and *Edw. ictaluri* agar with incubation at 30 +/− 2 °C for 24–48 h (Abraham et al. 2016).

Characteristics of the Pathogen

Box 9.10: *Stenotrophomonas maltophilia*
Cultures comprise Gram negative, motile, non-fermenting rods, that produce catalase, L-proline arylamidase, and phosphatase, but not ß-galactosidase, α- or ß-glucuronidase, α—glucosidase, H_2S, indole, lysine or ornithine decarboxylase, or oxidase. Nitrates are reduced. Aesculin and blood (α-haemolysis) but not starch or urea is attacked. Citrate is utilized. Sequencing of the 16S rRNA gene resulted in 97–98 % homology with *Stenotrophomonas maltophilia* (Abraham et al. 2016), which is below the 99 % threshold for an acceptable identification.

Pathogenicity

A culture was weakly pathogenic to African catfish juveniles with 35 ± 5 % mortalities resulting from challenge intraperitoneally with 1.4×10^7 cells/fish. By intramuscular injection, only 10 % mortalities resulted (Abraham et al. 2016). However, Geng et al. (2010a) reported 90–100 %, 65–70 %, 40–50 % and 0–5 % mortalities after i.p. injection of channel catfish with doses of 1.5×10^6 cells/fish, 1.5×10^5 cells/fish,1.5×10^4 cells/fish and 1.5×10^3 cells/fish, respectively. ECPs were harmful to albeit to mice (Du et al. 2011), and may well induce apoptosis in the lymphocytes, multiple organ failure and ultimately and death (Geng et al. 2010b).

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Chapter 10
Vibrios

Abstract The vibrios have been major problems to aquaculture in marine, coastal and estuarine water. Some species, e.g. *V. anguillarum,* have virtually a worldwide distribution; others have been focused on either colder, e.g. *Ali. salmonicida,* or warmer, e.g. *Ph. damselae,* environments. Because of their role in large scale mortalities, much effort has been expended on developing suitable diagnostic and control measures. Thus, a wide range of modern molecular diagnostic methods have been developed. Also, some pathogens have been the target of vaccine development, with commercial products available for some diseases, such as those caused by *Ali. salmonicida, Ph. damselae* subsp. *piscicida* and *V. anguillarum.*

Keywords Vibriosis • Coldwater disease • Photobacteriosis • Pasteurellosis • Winter ulcer disease

Vibrios have emerged as the scourge of marine fish (Figs. 10.1 and 10.2) and shellfish. Renewed interest has resulted in the description of new species and a better understanding of the biology of long-recognised taxa. To date, many species have been described as fish pathogens. In addition, hard-to-speciate *Vibrio* have been regularly recovered (e.g. Yasunobu et al. 1988; Masumura et al. 1989; Muroga et al. 1990; Nagai et al. 2008) and may represent new taxa. There is controversy over the role of *V. parahaemolyticus* as a fish pathogen, and we are not entirely satisfied with the evidence. One article has mentioned challenging tilapia with *V. parahaemolyticus,* but there was insufficient information about the authenticity of the isolates (Balfry et al. 1997). However, organisms with intermediate characteristics between *V. alginolyticus* and *V. parahaemolyticus* have been recovered from diseased milkfish in the Philippines (Muroga et al. 1984a). A publication has suggested that *V. campbellii, V. nereis and V. tubiashii* may be associated with disease in gilt-head sea bream in Spain (Balebona et al. 1998). However, confirmation is desirable before these taxa become recognised as *bona fide* fish pathogens. In the taxonomic study of Austin et al. (1997), a single isolate from Atlantic salmon in Tasmania was equated with *V. aestuarianus* using the diagnostic scheme of Alsina and Blanch (1994a, b). By phenotypic means, more isolates were associated with disease in gilt-head sea bream in Spain (Balebona et al. 1998). *V. chagassi* has been associated

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Fig. 10.1 Extensive surface haemorrhaging on a turbot with vibriosis (Photograph courtesy of Professor X.-H. Zhang)

Fig. 10.2 An ulcer, caused by *Vibrio* sp., on the surface of olive flounder (Photograph courtesy of Dr. D.-H. Kim)

with skin haemorrhages of sand smelt (*Atherina boyeri*) in Italy, albeit in a mixed community with *V. anguillarum* and *V. harveyi,* but pathogenicity was not proven (Fabbro et al. 2011).

Apart from the established vibrio fish pathogens, a study of the type strains of newly described and some older known vibrios and their ECPs revealed that *V. brasiliensis* (LD_{50} = ~2×10^4), *V. coralliilyticus* (LD_{50} = 7.5×10^1 – 2.5×10^3),

V. ezurae ($LD_{50}=7.3\times10^3$), *V. fortis* ($LD_{50}=\sim10^2$), *V. kanaloaei* ($LD_{50}=<2\times10^2$), *V. neptunius* ($LD_{50}=10^2$), *V. rotiferianus* ($LD_{50}=<10^2-5.0\times10^5$) and *V. tubiashi* ($LD_{50}=2.5\times10^2$) were pathogenic in laboratory-based experiments with rainbow trout with mortalities of up to 100 % (Austin et al. 2005). It will be interesting to see if these organisms become recognised as pathogens in aquaculture. Thus, the study of Wu et al. (2015) is relevant because an epidemic in fugu (*Takifugu rubripes*) during 2011 in Shandong Providence, China resulted in high mortalities. The diseased fish were anorexic, and displayed haemorrhaging and swollen internal organs. Twelve bacterial species were recovered and identified by sequencing of 16S rRNA gene. Pathogenicity experiments using zebra fish revealed that several newcomers to fish pathology were recognised, including *Enterovibrio nigricans*, *Ph. swingsii, V. owensii*, *V. harveyi* and *V. rotiferianus*, with LD_{50} values of 7.8×10^4 CFU/g to 8.4×10^6 CFU/ g (Wu et al. 2015). Along with other well recognized fish pathogens, *V. rotiferianus* was also recovered from diseased sea bass in the southeastern Black Sea (Uzun and Ogut 2015). A massive mortality of weak post-larvae red conger eel (*Genypterus chilensis*) in Chile revealed a range of bacteria, including V. *anguillarum, V. ordalii, V. tapetis* and *V. toranzoniae* (Levican and Avendaño-Herrera 2015).

As a general comment, most vibrios may be routinely isolated on marine 2216E agar (supplied by Difco) with incubation at 25 °C for 2–7 days (e.g. Ishimaru et al. 1996; Zhang et al. 2011b).

Photobacterium damselae subsp. *damselae*

Characteristics of the Disease

The organism was associated initially with ulcerative lesions along the flank of blacksmith *(Chromis punctipinnis),* one of the damselfish.

Characteristic skin lesions, i.e. ulcers, are formed, particularly in the region of the pectoral fin and caudal peduncle. These ulcers may reach a size of 5–20 mm in diameter. Typically, muscle lysis occurs. The results of histopathological examination suggests the presence of granulomatous ulcerative dermatitis.

Isolation

Isolation may be readily achieved by swabbing ulcerative material onto the surface of BHIA supplemented with 5 % (v/v) sheep blood, or TCBS (Appendix in Chap. 12) with incubation at 25 °C for an unspecified period (probably 2–5 days) yellowtail (Fujioka et al. 1988; Sakata et al. 1989; Fouz et al. 1991).

Characteristics of the Pathogen

The validity and distinctiveness of *Ph. damselae* has been confirmed, with isolates homogeneous by BIOLOG-GN fingerprints and API 20E profiles LPS profiles, but heterogeneous by ribotyping and serology (4 serogroups were defined) (Austin et al. 1997).

Box 10.1: *Photobacterium damselae* subsp. *damselae*
Cultures comprise facultatively anaerobic Gram-negative, weakly motile (by one or more unsheathed polar flagella) rods. Arginine dihydrolase, catalase and oxidase are produced, but not ß-galactosidase, H_2S, indole, lysine or ornithine decarboxylase or phenylalanine deaminase. Chitin, DNA, starch and urea, but not corn oil (lipids) or gelatin, are degraded. The methyl red test and Voges Proskauer reaction are positive. Nitrates are reduced. Growth occurs in 1–6 % (w/v) but not 0 % or 7 % (w/v) sodium chloride. Acid is produced from D-glucose, maltose and mannose, but not D-adonitol, arabinose, cellobiose, dulcitol, erythritol, inositol, lactose, mannitol, melibiose, raffinose, L-rhamnose, salicin, D-sorbitol, sucrose, trehalose or D-xylose. Acetate, citrate and malonate are not utilised. Sensitivity is recorded to the vibriostatic agent, O/129. The G+C ratio (for one strain) is 43 moles %.

Although Love et al. (1981) did not publish detailed reasons for the dissimilarity of '*V. damsela*' to other species of *Vibrio*, they did mention that DNA: DNA hybridisation studies had been completed. Unfortunately, the results were not published. This situation was corrected by Grimes et al. (1984b), who demonstrated low DNA homology values with other vibrios. Therefore, it is not surprising that the pathogen was re-classified out from *Vibrio,* initially to *Listonella* (MacDonell et al. 1986), then to *Photobacterium,* as *Photobacterium damsela* (Smith et al. 1991) and finally corrected to *Ph. damselae* (Trüper and De'Clari 1997).

Diagnosis

Serology Magnetic beads incorporating polyclonal antibodies with an enzyme immunoassay have found success for the rapid diagnosis of pasteurellosis caused by *Ph. damselae* subsp. *piscicida,* in which the commercial kit had a detection limit of 10^4 cells albeit with a problem of specificity, i.e. other photobacteria namely *Ph. damselae* subsp. *damselae* and *Ph. histaminum*, were also detected (Romalde et al. 1999).

Molecular Methods It may be necessary to discriminate subspecies *piscicida* from *damselae,* which has been achieved using TCBS on which the former did not grow (Rajan et al. 2003). However, this situation regarding discrimination between the two subspecies had been previously resolved by Osorio et al. (2000), who used multiplex-PCR to detect and differentiate subsp. *damselae* and *piscicida.* The former produced two amplification products, i.e. of 267 (fragment of 16S rRNA) and 448 bp (fragment of *ureC* gene) whereas the latter revealed only the 267 bp product. This suggests – and was confirmed by dot blot hybridisation – that subsp. *piscicida* lacks the *ureC* gene. A multiplex PCR was developed with a detection limit of 500 fg, which equals 100 genome equivalents (Amagliani et al. 2009). RT-PCR and PCR-DGGE of the *toxR* gene fragment were developed, and detected 1797.85 +/− 376.15 (*ca.* 0.71 fg) and 2976.68 +/− 1253.63 (ca. 1.76 fg) gene copy numbers of *toxR* genes in aquaculture samples (Martins et al. 2015).

Epizootiology

The organism was associated initially with ulcerative lesions along the flank of blacksmith *(Chromis punctipinnis),* one of the damselfish. These ulcers were noted in summer and autumn among fish populations in the coastal waters of southern California. Surveys of wild fish populations led to a conclusion that the ulcers were restricted to species of damselfish. Additional information pointed to a role in human pathogenicity, insofar as the organism has been isolated from human wounds (Love et al. 1981). Subsequent work demonstrated this organism in sharks (Grimes et al. 1984a, b; Fujioka et al. 1988), turbot (Fouz et al. 1991, 1992), yellowtail (Sakata et al. 1989) and nurse sharks and rainbow trout [in Denmark] (Pedersen et al. 1997a).

Ulcerated fish, presumably the result of disease caused by *Ph. damselae,* accounted for 10–70 % of the population in King Harbor, Redondo Beach, California, during August to October, and at a second site (Ship Rock, Catalina Island) during June to October. This suggests a seasonal distribution in the incidence of disease, and possibly coincides with warmer water temperatures and lower resistance caused by physiological changes in the host during sexual maturity. Conceivably, *Ph. damselae* occurs normally in the marine environment, where it could pose a constant threat to susceptible fish species. Indeed, seawater is the likely mode of transmission of virulent cells of the pathogen, with cells adhering to and resisting the bacteriocidal effects of mucus. This suggests that skin is a site of entry into the host (Fouz et al. 2000).

Pathogenicity

The pathogen has been implicated with disease in sharks, turbot and yellowtail (Fujioka et al. 1988; Sakata et al. 1989; Fouz et al. 1991) and rainbow trout in marine sites in Denmark (Pedersen et al. 2009). Laboratory infections of *C.*

punctipinnis have been established by removing 4 to 6 scales from the flank, scarifying the dermis, and swabbing the wound with 10^7–10^8 viable cells of *Ph. damselae.* At water temperatures of 16.0–16.5 °C, the fish developed large ulcers in 3 days, with death following 24 h later. Similar data were recorded after experimental challenge of unscarified animals. However in the initial study, fish from other families appeared to be unaffected by *Ph. damselae,* pointing to host specificity of the pathogen. Thus, representatives of Atherinidae, Clinidae, Cottidae, Embiotocidae, Girellidae and Gobiidae resisted experimental challenge. This is interesting because representatives of these families co-habited the reefs with blacksmith (Love et al. 1981). However, Grimes et al. (1984a) and Labella et al. (2006) successfully infected dogfish and red banded sea bream ($LD_{50} = 3.9 \times 10^5$ CFU/g of fish) by i.p. injection with *Ph. damselae.* Death ensued in the dogfish within 18 h at an unspecified water temperature. Virulence to rainbow trout was highly variable, with LD_{50} doses of 3.9×10^3 to 1.5×10^8 CFU at 20 °C that was more pronounced than at 13 °C (Pedersen et al. 2009). The LD_{50} dose for Asian sea bass was determined as 8.1×10^5 CFU/g of fish (Kanchanopas-Barnette et al. 2009). Grimes et al. (1984a) reported that the organism was highly cytotoxic. A neurotoxic acetylcholinesterase has been described (Peréz et al. 1998). The most virulent strains to rainbow trout were haemolytic (Pedersen et al. 2009), for which the haemolysins damselysin (= a cytotoxin encoded by the *dly* gene with lethality to mice) and the pore-forming toxin HlyA are encoded within a newly described 153,429 bp virulence plasmid pPHDD1, the presence of both toxins being necessary for full virulence (Rivas et al. 2011).

ECPs, such as amylase, lipase, phospholipase, alkaline phosphatase, esterase lipase, acid phosphatase and β-glucosaminidase but not damselysin, have been implicated with cytotoxicity (Labella et al. 2010), with the LD_{50} dose ranging from 0.02 to 0.43 μg of protein/g of fish within death occurring between 4 and 72 h after administration (Fouz et al. 1993). The ECP were considered to have low proteolytic activity, without evidence of any caseinase, elastinase or gelatinase. In contrast, pronounced phospholipase and haemolytic activity was recorded for turbot (and human and sheep) erythrocytes. It was possible that LPS contributed to heat stability of the toxic fractions (Fouz et al. 1993).

A siderophore-mediated iron sequestering system has now been described, and almost certainly contributes to the pathogenicity of the organism (Fouz et al. 1994, 1997).

Photobacterium damselae subsp. *piscicida* *(= Pasteurella piscicida)*

Characteristics of the Disease

During the summer of 1963, an epizootic was reported in white perch and striped bass in the upper region of the Chesapeake Bay, USA from which 30 cultures of an organism were recovered which possessed some of the salient features of *Pasteurella*

Fig. 10.3 Surface haemorrhaging on a tongue sole (*Cynoglossus semilaevis*) infected with *Ph. damselae* subsp. *piscicida* (Photograph courtesy of Professor X.-H. Zhang)

(Snieszko et al. 1964). Hence the condition was termed 'pasteurellosis'. However, the literature became confusing, insofar as the disease is also referred to in Japan as 'pseudotuberculosis' because of the distinctive pathology. (Snieszko et al. 1964). The disease has been responsible for heavy losses among menhaden and striped mullet in Galveston Bay, Texas (Lewis et al. 1970). However, it is in Japan that the disease has become of considerable economic importance, causing significant losses in farmed yellowtail (Egusa 1983). Since its initial recognition in yellowtails, pasteurellosis appears to have spread to other fish species, including gilt-head sea bream (Balebona et al. 1998), red sea bream (Yasunaga et al. 1983), black sea bream (Muroga et al. 1977; Ohnishi et al. 1982), Atlantic bluefin tuna (Mladineo et al. 2006), sole *(Solea senegalensis)* (Zorrilla et al. 1999) and golden pompano (*Trachinotus ovatus*) (Snieszko et al. 1964). More recently, the disease seems to have spread to farmed and wild fish stocks in the Mediterranean area, notably Croatia, France, Italy and Spain (Magariños et al. 1992; Mladineo et al. 2006).

Essentially, pasteurellosis is a septicaemia in which acute cases exhibits only a few pathological signs (Fig. 10.3). Internally, granulomatous-like deposits, which have led to the coining of the descriptive name of pseudotuberculosis, may develop on the kidney and spleen. These deposits comprise many greyish-white bacterial colonies of 0.5–1.0 mm^2 in size (Kusuda and Yamaoka 1972). Purulent material may accumulate in the abdominal cavity (Lewis et al. 1970).

Isolation

The organism may be isolated by inoculating swabs of kidney and/or spleen material onto marine 2216E agar (Difco), nutrient agar or blood agar, with incubation at 25 °C for 48–72 h. An improved liquid medium has been described, which may be solidified by the addition of 1 % (w/v) agar (Appendix in Chap. 12; Hashimoto et al. 1989). On conventional media, shiny, grey-yellow, entire, convex colonies develop,

which are approximately 1–2 mm in diameter after 72 h (Kusuda and Yamaoka 1972). Another approach, which has met with success, has enabled the recovery of *Ph. damselae* subsp. *piscicida* from water in the vicinity of fish. The method involved filtering 250 ml volumes of water through 0.45 µm cellulose nitrate filters, before transfer (of the filters) to 2216E agar supplemented with 1 % (w/v) mannitol and 0.5 % (w/v) phenol red. The non-fermenting *Ph. damselae* subsp. *piscicida* produced red colonies (Reali et al. 1997). With this method, the pathogen was detected on sea bass 8-days before the outbreak of disease.

Characteristics of the Pathogen

In addition to the articles about pasteurellosis/pseudotuberculosis, there are also some reports indicating the presence of fish pathogenic *Pasteurella* in Great Britain (Ajmal and Hobbs 1967) and Norway (Håstein and Bullock 1976). However, it is possible that these organisms should have been identified as atypical *Aer. salmonicida* (see Paterson et al. 1980).

Box 10.2: *Photobacterium damselae* subsp. *piscicida*
Cultures comprise fairly unreactive, Gram-negative, non-motile, fermentative rods of 0.5 × 1.5 µm in size, with pronounced bipolar staining. Pleomorphism may be evident, especially in older cultures. Catalase and oxidase are produced, but not alanine deaminase, ß-galactosidase, H_2S, indole, lysine or ornithine decarboxylase or phenylalanine deaminase. Nitrates are not reduced. The methyl red test is strongly positive, whereas the Voges Proskauer reaction is weakly positive. Arginine and Tween 80 are degraded, but not blood, casein, chitin, gelatin, starch or urea. Growth occurs at 25–30 °C but not 10 or 37 °C, in 0.5–3.0 % (w/v) sodium chloride and at pH 5.5–8.0, but not on MacConkey agar or in potassium cyanide broth. Uniform turbidity is recorded in broth cultures. Sodium citrate is not utilised. Acid is produced from fructose, galactose, glucose (weak) and mannose, but not amygdalin, arabinose, dulcitol, inositol, lactose, maltose, mannitol, melibiose, rhamnose, salicin, sorbitol, sucrose or trehalose. Unfortunately, the G+C ratio of the DNA has not been determined for any *bona fide* strains. So far, only one serotype has been recognised. In general, the organism possesses one heat-stable and four heat-labile somatic antigens, and three heat-labile extracellular antigens (presumably enzymes) (Kusuda et al. 1978a). The LPS comprises <1 % protein, 18–24 % sugar and 34–36 % fatty acids. The sugar component includes hexose, heptose, pentose, 6-deoxyhexose, 2-keto-3-deoxyoctonate and hexosamine. The fatty acids include lauric acid, 3-hydroxy lauric acid, myristic acid and palmitic acid (Salati et al. 1989a, b; Hawke et al. 2003).

The results of many investigations led to the conclusion that the pathogen consists of a phenotypically and serologically homogeneous taxon (e.g. Magariños et al. 1992) but is genetically heterogeneous as determined by results of substractive hybridisation (Juíz-Río et al. 2005). By ribotyping of 29 isolates, 2 major ribotypes were recognised which effectively separated European and Japanese isolates. A third ribotype accommodated a unique strain (Magariños et al. 1997b).

The morphology and physiology of this pathogen led Snieszko et al. (1964) to suspect a similarity to the genus *Pasteurella.* This view was reinforced by cross-precipitin reactions with *Pasteurella (Yersinia) pestis.* From this deduction, Janssen and Surgalla (1968) realised that, from an examination of 27 isolates, the organism was very homogeneous and different from existing species of *Pasteurella.* Therefore, the name of *Pa. piscicida* was coined. Independently, Kusuda gave it an alternative name, i.e. *Pa. seriola,* but quickly realised its synonymy with *Pa. piscicida,* which was accorded preference. However, *Pa. piscicida* was not included in the 'Approved Lists of Bacterial Names' (Skerman et al. 1980) or their supplements. Consequently, the name of *Pa. piscicida* lacked taxonomic validity.

A detailed taxonomic evaluation based on small-subunit rRNA sequencing and DNA: DNA hybridisation revealed that the organism was highly related to *Ph. damselae* (there was >80 % relatedness of the DNA), and it was proposed that the organism be accommodated in a new subspecies, as *Ph. damsela* subsp. *piscicida* (Gauthier et al. 1995), the epithet of which was corrected to *damselae* (Trüper and De'Clari 1997), as *Ph. damselae* subsp. *piscicida.* AFLP analysis revealed that the two subspecies are indeed distinct and separate entities (Thyssen et al. 2000).

To complicate matters, there is controversy over interpretation of the Gram-staining reaction. The majority opinion is that the organism is Gram-negative. However, Simidu and Egusa (1972) considered that cells displayed Gram-variability when young, i.e. in 12–18 h cultures incubated at 20–25 °C. In addition, they presented photographic evidence which showed that cells shortened with age. In fact, the suggestion was made that the pathogen is related to *Arthrobacter.* It is ironic that a similar phenomenon, concerning the interpretation of Gram-stained smears, was reported by Kilian (1976) and Broom and Sneath (1981) for *Haemophilus piscium,* the causal agent of ulcer disease.

Diagnosis

Phenotypic Methods Kent (1982) reported that *Ph. damselae* subsp. *piscicida* gave positive responses in the API 20E rapid identification system for arginine dihydrolase and weak acid production from glucose; all other tests were negative. Of course, it is necessary to modify the protocol for use with marine bacteria. Thus, it was essential to suspend cultures in 2–3 % (w/v) saline rather than distilled water, and the inoculated test strips were incubated at 25 °C (not 37 °C) for up to 48 h.

Serology An ELISA was developed, which successfully identified *Ph. damselae* subsp. *piscicida* albeit in artificially infected fish tissue within 4 h. By visually recording the ELISA the threshold for positivity was 10^5 cells/ml. However, use of a reader cut this level to only 10^3 cells/ml (Bakopoulos et al. 1997a). Magnetic beads incorporating polyclonal antibodies with an enzyme immuno assay have found success for the rapid diagnosis of pasteurellosis, in which the commercial kit had a detection limit of 10^4 cells albeit with a problem of specificity, i.e. other photobacteria namely *Ph. damselae* subsp. *damselae* and *Ph. histaminum*, were also detected (Romalde et al. 1999).

Molecular Methods Specific DNA hybridisation probes for *Ph. damselae* subsp. *piscicida* offer promise for the future. Already, initial studies with a ^{32}P-labelled DNA probe indicated a minimum detection limit of 3.9 ng of DNA or 10^5 bacterial cells (Zhao and Aoki 1989). Aoki et al. 1997) emphasised the value of a 629 base pair DNA fragment from the subspecies specific plasmid pZP1 for PCR. Kvitt et al. (2002) reported a detection limit of 0.35 pg, which equated to ~40 bacterial cells. A nested PCR detected 10 fg to 1 pg of DNA, which was considered to be equivalent to 20 to 200 cells, being sensitive enough to detect the pathogen in asymptomatic fish (Osorio et al. 1999). In comparison, PCR-RFLP approach detected <180 fg of purified DNA, and was useful for use with sea bream and sea bass (Zappulli et al. 2005). In one case, it was necessary to discriminate subspecies *piscicida* from *damselae,* which was achieved using TCBS on which the former did not grow (Rajan et al. 2003). However, this had been previously resolved by Osorio et al. (2000), who used multiplex-PCR to detect and differentiate subsp. *damselae* and *piscicida.* The former produced two amplification products, i.e. of 267 (fragment of 16S rRNA) and 448 bp (fragment of *ureC* gene) whereas the latter revealed only the 267 bp product. This suggests – and was confirmed by dot blot hybridisation – that subsp. *piscicida* lacks the *ureC* gene. A multiplex PCR was developed with a detection limit of 500 fg, which equals 100 genome equivalents (Amagliani et al. 2009).

Epizootiology

There is no doubt that pasteurellosis is a serious condition of both farmed and wild fish populations (Snieszko et al. 1964; Kusuda and Yamaoka 1972; Ohnishi et al. 1982; Yasunaga et al. 1983). Heavy mortalities, in the range of 40–50 % of the stock, have occurred during summer months. Unfortunately, the reasons for these outbreaks are largely unknown. Likewise, the precise nature of the pathogenicity mechanism remains to be elucidated.

It is thought that infection takes place in sea water at temperatures of approximately 25 °C (Yasunaga et al. 1983). Toranzo et al. (1982) devised a series of survival experiments, and concluded that *Ph. damselae* subsp. *piscicida* was short-lived in freshwater and estuarine conditions. Thus in freshwater, the organism could not be cultured after 48 h at 20 °C. Survival in estuarine water (salinity = 12‰) was

slightly longer, i.e. 4–5 days. These results support the earlier findings of Janssen and Surgalla (1968) that the organism does not appear to survive well away from fish. However, there is some evidence that has demonstrated the discharge of viable cells from experimentally infected yellowtail for 1–2 days before death (Matsuoka and Kamada 1995). The survival of starved cells in seawater needs to be clarified (Magariños et al. 1997a). Furthermore, it was speculated that transmission of the disease is likely to be fish to fish. Data suggest that the gill is key to the development of the disease cycle insofar as the pathogen has been located in the gills of apparently healthy amberjack (Nagano et al. 2011b). Although the arguments are reminiscent of the debate about the spread of furunculosis, it should not be overlooked that *Ph. damselae* subsp. *piscicida* may survive in water, albeit in a non-culturable, dormant or altered form. Indeed, a view was expressed that the pathogen may well exhibit a dormant phase (Magariños et al. 1994a). Plate count data revealed that *Ph. damselae* subsp. *piscicida* could survive in seawater and sediment for 6–12 days, with metabolism being reduced by 80 %. Indeed, in terms of numbers, culturable cells persisted in sediment better than in seawater. However, when culture techniques inferred a reduction in bacterial numbers, microscopy using acridine orange suggested that the populations remain at 10^5 (Magariños et al. 1994a).

A possibly useful tool for epizootiology is ribotyping, which has already successfully discriminated between European and Japanese isolates (Magariños et al. 1997b).

Pathogenicity

Experimental infection may be achieved by i.m. injection, oral uptake or immersion, with maximum mortalities at 18 and 20 but less at 15 °C (Magariños et al. 2001). Medium composition, and in particular the presence of yeast extract and/or (fish) peptone, enhanced the toxicity of ECPs and the virulence of cells administered via immersion or i.p. injection (Bakopoulos et al. 2002). The fate of the pathogen has been examined by FAT (Kawahara et al. 1989). Thus following i.m. injection, the pathogen became located initially in the kidney and spleen, before spreading to the gills, heart, intestine and pyloric caeca. The pathogenic has the ability to adhere to, invade and survive within cells, and has been observed to adhere to and invade the epithelial cell line SAF-1 (Acosta et al. 2009). Following oral uptake, the pathogen appeared in the stomach, before spreading to the internal organs. After immersion, *Ph. damselae* subsp. *piscicida* located in the gills, and then spread widely to the heart, kidney, liver, pyloric caeca and spleen (Kawahara et al. 1989). Within the tissues of infected fish, *Ph. damselae* subsp. *piscicida* was seen to accumulate and multiply in the macrophages (Nelson et al. 1989; Elkamel et al. 2003) perhaps after an initial cell adherence stage (Magariños et al. 1996a, b), which appears to involve capsular polysaccharide [this is dependent on the presence of iron and younger, i.e. logarithmic rather than lag phase cultures] (Magariños et al. 1996b; do Vale et al. 2001), which has a minor role in the binding of haemin (do

Vale et al. 2002). The surface-located 22–38 kDa sialic acid, which may inhibit the complement cascade and thus protect the pathogen from the host's antibodies, has a possible role in cell adhesion and survival in the host (Jung et al. 2000). Another study reported that cells of the pathogen were killed by macrophages *in vitro* in 3–5 h (Skarmeta et al. 1995). Yet, the ability to induce apoptosis and thence lysis of sea bass macrophages and neutrophils has been linked to a plasmid-encoded 56 kDa protein, coined AIP56, which was secreted by virulent but not avirulent cultures (do Vale et al. 2002, 2005). Interestingly, passive immunisation with rabbit antiserum against this protein led to protection (do Vale et al. 2005). Using the EPC cell line, bacterial cells were seen to adhere to and become internalized by the cells within vacuoles possibly by endocytosis, remaining intracellular for 6–9 h. Intracellular multiplication was not recorded (López-Dóriga et al. 2000). Increased catalase activity, which is inversely related to the quantity of iron, has been detected in virulent compared to non-virulent cultures leading to the thought that this enzyme may well be involved in survival within the host (Díaz-Rosales et al. 2006).

Resistance to the pathogen may well reflect the size of the fish and the efficiency of the phagocytes (Noya et al. 1995). There is a distinct role for the capsule to protect against phagocytosis (Arijo et al. 1998) and, in immunised fish, protection against complement-mediated killing (Acosta et al. 2006). Comparing five capsulated, virulent and 1 non-capsulated, avirulent culture, Arijo et al. (1998) recorded significant differences in phagocytosis with the former resisting being engulfed and killed by the macrophages. In a separate development, it was considered that mucus from turbot – thought to contain a glycoprotein – inhibited *Ph. damselae* subsp. *piscicida,* but less so mucus from sea bass and sea bream (Magariños et al. 1995). Perhaps, such observations explain the comparative sensitivity of some fish species, e.g. sea bass and sea bream, to the pathogen.

A siderophore-mediated iron sequestering mechanism has been found in *Ph. damselae* subsp. *piscicida*, with IROMPs of 105, 118 and 145 kDa in size (Magariños et al. 1994b). Some variability has been detected insofar as isolates expressed a 75 kDa IROMP. Also, others have reported different sizes for the novel proteins associated with iron limitation. Thus, European isolates were considered to express four novel proteins of 63 kDa and three at ≥200 kDa, whereas Japanese isolates did not form any different proteins (Bakopoulos et al. 1997b). Bakopoulos et al. (2004) reported a novel >206 kDa protein associated with iron sequestration. Indeed, high levels of iron, i.e. 200 mg of iron/kg of feed and 2.5 % carbonyl iron to separate groups, in the diet were found to adversely influence the pathogenicity of *Ph. damselae* subsp. *piscicida* in sea bass (Rodrigues and Pereira 2004). In the carbonyl iron treated group, 64 % of the fish died after challenge compared to only 9 % of the controls.

The pathogen utilizes haem compounds as the sole source of iron. Work has determined the presence of a gene cluster with 10 haem uptake and utilization genes, of which *hutC* and *hutD* are iron-regulated, and are expressed during infection (Osorio et al. 2010).

Disease Control

Vaccine Development Much effort has been expended on vaccine development, with recent research highlighting major antigenic proteins of 7 kDa and 45 kDa (Hirono et al. 1997). Programmes have included the use of passive immunisation (Fukuda and Kusuda 1981a), which is of dubious practical value, the more conventional approach of using formalin-inactivated whole-cell preparations (Kusuda and Fukuda 1980; Fukuda and Kusuda 1981b; Afonso et al. 2005) and the more modern approaches of genetic engineering. Of relevance, the salinity of the growth medium composition appears to have an effect on the subsequent immune response after vaccination, with 2.5 % (w/v) rather than 0.5 % NaCl being the more effective (Nitzan et al. 2004). Bacteriological media containing peptones, yeast extract and salt led to the synthesis of a wider range of cellular components, (including novel compounds of ~14 and ~21.3 kDa) than those produced in more *in vivo* type conditions. These compounds were recognised by post-disease sea bass serum (Bakopoulos et al. 2003b). It was demonstrated that administration of a formalin-inactivated preparation in Freund's complete adjuvant by i.p. injection induced agglutinating antibodies in yellowtail. Thus titres of 1:256–1:2048 were achieved 5 weeks after vaccination (Kusuda and Fukuda 1980). Vaccination enhances the nitric oxide response, i.e. the production of reactive nitrogen intermediates with their antimicrobial activities, to infection with the pathogen, and is correlated with the level of protection (Acosta et al. 2005). Further work, using a variety of vaccines and application methods, demonstrated conclusively that fish could be protected against subsequent infection by *Ph. damselae* subsp. *piscicida,* although this has been refuted by some workers (e.g. Hamaguchi and Kusuda 1989). Toxoid enriched whole cells applied by immersion led to a low antibody response an RPS of 37–41 % in sea bream (Magariños et al. 1994c). An improved RPS of >60 % after 35 days resulted from use of an LPS mixed chloroform-killed whole cell vaccine (Kawakami et al. 1997). Using formalin-inactivated cells with or without FCA and a range of application methods, namely i.p. injection, 5–7 s spray, hyperosmotic infiltration and oral uptake via food, Fukuda and Kusuda (1981b) reported encouraging results within 21 days following artificial challenge with *Ph. damselae* subsp. *piscicida.* The best results, conferring 100 % protection to the fish, were obtained by use of i.p. injection or by spraying. The titre of agglutinating antibodies was measured at between 1:4 and 1:128. A subsequent study by these authors has pointed to the value of vaccinating with sub-cellular components, notably bacterial LPS (Fukuda and Kusuda 1982). In this connection, a whole cell vaccine in combination with ECPs was used more successfully than a commercial product by immersion for 1 h and i.p. injection in sea bass (Bakopoulos et al. 2003a). However, formalin-inactivated whole cells administered intraperitoneally achieved an RPS of 96 % in sea bream (Hanif et al. 2005). The question about the nature of the immune response after i.p. vaccination with or without a booster after 4 weeks with a FIA adjuvanted inactivated whole cell vaccine was addressed by Arijo et al. (2004), who demonstrated a

humoral response to ECPs, OMP, outer (extremely immunogenic) and cytoplasmic membranes, LPS and O-antigen.

A bivalent vaccine (with *V. harveyi)* based on formalised cells and ECP administered to sole by immersion with booster or by i.p. injection led to high levels of protection (RPS = ~82 %) for 4 months after which the benefit declined (Arijo et al. 2005).

A ribosomal vaccine has been evaluated following administration by i.p. injection into yellowtail. Certainly, the initial evidence pointed to success with ribosomal antigen P (Kusuda et al. 1988; Ninomiya et al. 1989). In a further development, this group experimented with a potassium thiocyanate extract and acetic acid treated "naked cells" obtained from a virulent culture (Muraoka et al. 1991). Yellowtail were vaccinated twice i.p., at one week intervals with the extract (with or without the naked cells), and were challenged two weeks after the second injection. Results indicated partial success for the extract when used alone. However, the extract used in conjunction with naked cells led to good protection (RPS = 36.5). Yet, the corresponding antibody levels were low, suggesting to the researchers that humoral antibodies did not play an important role in protection (Muraoka et al. 1991).

A more modern approach has involved examination of 370 ORFs, which enabled the identification of eight possible antigens that wer expressed as recombinant proteins. After purification a lipoprotein, PDP_0080, which was involved in adherence of the pathogen to epithelial cells, was injected in 25 μg amounts into sea bass leading to the production of high antibody titres (Andreoni et al. 2013).

Dietary Supplements Garlic powder was fed at 0.5 and 1.5 g/kg body weight to cobia *(Rachycenron canadum)* for 28-days, leading to higher growth, and after challenge with *Ph. damselae* subsp. *piscicida* lower mortalities were recorded (Guo et al. 2012, 2015a).

Probiotics Sugita et al. (1997) isolated a *Vibrio*, coined strain NM10, from ponyfish *(Leiognathus nuchalis*) intestines, and determined antagonism of *Ph. damselae* subsp. *piscicida* by a heat-labile proteinaceous compound of 5 kDa.

Antimicrobial Compounds Little is known about the value of chemotherapeutants. An *in vitro* study highlighted the value of ampicillin (Kusuda and Inoue 1976), but field trials were not carried out. In a further study, Kusuda et al. (1988) reported marked sensitivity to ampicillin and oxolinic acid, moderate sensitivity to nalidixic acid and sodium nifurstyrenate, but resistance to chloramphenicol, chlortetracycline, oxytetracycline and tetracycline. Again, field evidence was not supplied. Sano et al. (1994) noted the value of fosfomycin (MIC = 1.56 – 3.13 μg/ml) at controlling laboratory infections. An effective dose was 40 mg of fosfomycin/kg body weight of fish/day for 5 days, albeit administered only one hour after infection. This dose reduced mortalities by *Ph. damselae* subsp. *piscicida* to 0 % (Sano et al. 1994). R plasmids have been identified among isolates, conferring resistance to chloramphenicol, kanamycin, sulphamonomethoxine and tetracycline (Aoki and Kitao 1985) and florfenicol (Kim et al. 1993). Although the isolates described by Aoki and

Kitao (1985) were confined to one locality in Japan, there is the likelihood that the resistance will spread quickly to other sites.

Aliivibrio (= Vibrio) fischeri

During Autumn 1988, visceral tumours (neoplasia) and skin papillomas were observed in juvenile turbot, farmed in northwest Spain. Although viral involvement was suspected, bacteria were evident in the majority of affected fish. Most diseased fish possessed whitish nodules on the skin (dorsal surface), haemorrhagic ulceration, and tumours involving the pancreas and bile duct. Within a year, 39 % losses occurred in the fish population (Lamas et al. 1990). Bacteria were recovered by use of TSA supplemented with 2 % (w/v) sodium chloride, marine 2216E agar (Difco) and TCBS (Oxoid), when virtual pure culture growth of bacteria was recovered from the kidney and liver, following incubation at an unstated temperature for an unspecified duration (Lamas et al. 1990), and approximated the description of *V. fischeri,* albeit with similarities to *V. harveyi* (Lamas et al. 1990). However, detailed characteristics of the cultures were not presented. Interestingly, other isolates have been recovered from gilt-head sea bream, also in Spain (Balebona et al. 1998). The taxon was transferred to a newly created genus *Aliivibrio* as *Ali. fischeri* on the basis of phylogeny principally involving sequencing of the 16S rRNA gene (Urbanczyk et al. 2007). Chemotherapy was ineffective at reducing mortalities (Lamas et al. 1990).

Aliivibrio logei

Characteristics of the Disease

An organism, with similarities to *Ali. logei,* was associated with shallow skin lesions of Atlantic salmon farmed in Iceland at low temperatures, i.e. ~10 °C (Benediktsdóttir et al. 1998).

Isolation

Benediktsdóttir et al. (1998) used 5 % (v/v) horse blood agar supplemented with 1.5 % (w/v) sodium chloride with incubation at 15 °C for 7 days.

Characteristics of the Pathogen

Fifteen Icelandic and one Norwegian isolates were recovered and equated with *V. logei* (Benediktsdóttir et al. 1998). Then, the species was transferred to a newly created genus *Aliivibrio* as *A. logei* on the basis of phylogeny principally involving sequencing of the 16S rRNA gene (Urbanczyk et al. 2007).

Box 10.3: *Aliivibrio logei*
Cultures do not produce arginine dihydrolase, indole or lysine or ornithine decarboxylase. Blood (haemolysis) and chitin are degraded, but not starch. Acid is produced from N-acetyl glucosamine, glycerol, maltose, mannose, ribose, sucrose and trehalose.

Epizootiology

It may be assumed that the source of the bacteria was seawater, although *Ali. logei* has been recovered from the digestive tract of larval cod (Reid et al. 2009).

Aliivibrio salmonicida

Characteristics of the Disease

With the tremendous increases in production of Atlantic salmon in Norway, it was perhaps inevitable that at some time a new or emerging disease would cause havoc to the industry. Then in 1979, such a "new" disease appeared in salmon farms located around the island of Hitra, south of Trondheim in Norway. In 1983, the disease appeared in Stavanger and, in particular, the large number of fish farms in the Bergen region. The disease, coined coldwater vibriosis or Hitra disease (Egidius et al. 1981), occurs mainly during the period of late autumn to early spring. The disease is now widespread throughout Norway, and there are some reports of outbreaks in Scotland, Shetland (Bruno et al. 1985) and Canada. The disease resembles a generalised haemorrhagic septicaemia. Externally, haemorrhaging may be evident around the abdomen (Holm et al. 1985). Internally, there is often evidence of anaemia, haemorrhaging on the organs, swim bladder and abdominal wall and posterior gastro-intestinal tract (Poppe et al. 1985; Holm et al. 1985; Egidius et al. 1986). Microscopy suggests that bacteria are rampant throughout infected fish, and especially in the blood and kidney of moribund and freshly dead specimens.

Isolation

Pure cultures may be readily recovered from blood and kidney samples on TSA supplemented with 1.5 % (w/v) sodium chloride following incubation at 15 °C for up to 5 days (Holm et al. 1985; Egidius et al. 1986). Colonies are small, i.e. ≤1 mm in diameter, round, raised, entire and translucent. Unexperienced personnel could easily miss the colonies upon cursory glances at inoculated plates. To some extent, the organism is fragile, and will quickly die at supra-optimum temperatures or by failure to carry out regular sub-culturing.

Characteristics of the Pathogen

An organism, named originally as *V. salmonicida*, was recovered from diseased salmon (Egidius et al. 1986), and the validity and distinctiveness confirmed (Austin et al. 1997). However, the organism was transferred to the newly created genus *Aliivibrio* as *Ali. salmonicida* on the basis of phylogeny principally involving sequencing of the 16S rRNA gene (Urbanczyk et al. 2007).

Box 10.4: ***Aliivibrio salmonicida***
Cultures contain motile (~9 polar flagella) fermentative Gram-negative curved pleomorphic rods of 2–3 × 0.5 μm in size. Catalase and oxidase are produced, but not arginine dihydrolase, ß-galactosidase, H_2S or indole. Nitrates are not reduced, nor is the Voges Proskauer reaction positive. Citrate is not utilised. Neither blood, chitin, gelatin, lipids nor urea are degraded. N-acetylglucosamine, glucose, glycerol (slowly), maltose, ribose, sodium gluconate and trehalose are utilised, but not adonitol, amygdalin, D- or L-arabinose, D- or L-arabitol, arbutin, D-cellobiose, dulcitol, erythritol, D- or L-fucose, inositol, ß-gentobiose, lactose, D-lycose, D-mannose, melezitose, melibiose, D-raffinose, rhamnose, salicin, sorbitol, sucrose, L-sorbose, D-tagatose, D-turanose or D- or L-xylose. Growth occurs at 1–22 °C, optimally at 15 °C but not at 37 °C, and in 0–4 % but not 7 % (w/v) sodium chloride. Sensitivity is displayed to the vibriostatic agent, O/129, but not to novobiocin. The G+C ratio of the DNA is 44 moles % (Holm et al. 1985; Egidius et al. 1986).

Strains have been divided into four different categories on the basis of plasmid profiles (Wiik et al. 1989). The plasmids were 2.6, 3.4 and 24 mDa in size; with the largest plasmid being common to all four groups. There was no apparent difference in biochemical traits among these four plasmid groups. In a separate study, Sørum et al. (1990) described plasmids of 2.8, 3.4, 21 and 61 mDa from isolates recovered from Atlantic salmon and cod. These authors reported 11 plasmid profiles for *Ali.*

salmonicida. A similar plasmid composition has been indicated for isolates from the Faroe Islands (Nielsen and Dalsgaard 1991). Comparing isolates from Canada, Faroe Islands, Norway and Shetland, Sørum et al. (1993) noted a similarity in plasmid profile, with three plasmids of 2.8, 3.4 and 21 mDa revealed. Furthermore, a conclusion has been reached that all strains carry plasmids (Valla et al. 1992). Differences have also been implied by serological studies, which have indicated the presence of two serotypes (Schrøder et al. 1992).

DNA hybridisation of four cultures confirmed homogeneity (DNA homology = 82 – 100 %), but low relatedness to *V. anguillarum* (30 %), *V. ordalii* (34 %) or *V. parahaemolyticus* (40 %) (Wiik and Egidius 1986). Although these data were used to justify the uniqueness of *Ali. salmonicida,* the relationship to other representatives of the family Vibrionaceae was not considered. The validity of the species is not, however, questioned, and in the detailed study of Austin et al. (1997), *Ali. salmonicida* formed a discrete taxon among the other fish-pathogenic vibrios. Its transfer to *Aliivibrio* was an appropriate taxonomic move (Urbanczyk et al. 2007).

Epizootiology

It has been demonstrated that *Ali. salmonicida* survives for >14 months in laboratory-based experiments with sea water, when seeded at ~10^6 cells/ml (Hoff 1989). Thus, there is the potential for long term survival in the vicinity of fish farms, as confirmed by Husevåg et al. (1991). Moreover, the pathogen has been detected in the sediment (12–43 cells/ml) below fish farms, several months after an outbreak of Hitra disease. In addition, *Ali. salmonicida* has been detected in the sediments from fish farms which were not experiencing clinical disease (Enger et al. 1989, 1991). Clearly, there will be a reservoir of the pathogen around farmed fish, from which further infections may occur. Attachment and colonisation of surfaces to surfaces to form biofilms is influenced by quorum sensing (Hansen et al. 2014).

Pathogenicity

Intraperitoneal injection and immersion of Atlantic salmon with broth cultures led to clinical disease, with the pathogen appearing quickly, i.e. within 2 h, in the blood suggesting the rapid development of a septicaemia (Bjelland et al. 2012), and in which *Ali. salmonicida* either inhibits or resists the host's innate immune system (Bjelland et al. 2013). The intestine became colonized more slowly after immersion challenge (Bjelland et al. 2012). Atlantic salmon were more susceptible than rainbow trout (Egidius et al. 1986; Hjeltnes et al. 1987). The LD_{50} dose ranges from $4 \times 10^6 - 1 \times 10^8$ cells/fish (Wiik et al. 1989). The presence of other acute disease diseases, such as infectious pancreatic necrosis, has exacerbated infections in Atlantic salmon caused by *Ali. salmonicida* (Johansen and Sommer 2001). In addition, *Ali.*

salmonicida has caused mortalities in cod (Jørgensen et al. 1989). The plasmids, which are regarded as being present in all strains, do not appear to be related to virulence (Valla et al. 1992). Using isolated macrophages from Atlantic salmon and rainbow trout with immunofluorescence techniques, the pathogen has been observed to be internalised (Brattgjerd et al. 1995). The pathogen has been found to express a luciferase, but does not produce detectable luminescence. Yet, there appears to be an association between a mutation in the *lux* gene and virulence (Nelson et al. 2007).

The pathogen has the ability to adhere to mucosal surfaces, (Ræder et al. 2007) i.e. foregut, gills, hindgut, pyloric caeca and skin (Knudsen et al. 1999). Indeed, there is evidence that skin is the major route of infection (Kashulin and Sørum 2014). In the presence of fish mucus, there was evidence of increased levels of *Ali. salmonicida* proteins notably those flagellin and those involved with oxidative-[peroxidase] and general [heat shock protein and chaperonin] stress responses (Ræder et al. 2007).

What about the risk of disease after transferring salmon from fresh to seawater? Eggset et al. (1997) concluded that the susceptibility of Atlantic salmon to Hitra disease in seawater possibly reflected the overall quality of the smolts.

Disease Control

Disease Resistant Fish Gjedrem and Aulstad (1974) noted significant variation in resistance among strains of Atlantic salmon.

Vaccine Development There has been success with vaccines for the prophylaxis of Hitra disease, with the protective antigen possibly including the presence of a 20 kDa peptidoglycan-associated lipoprotein, Pal (Karlsen et al. 2011). Immersion of Atlantic salmon in vaccine preparations resulted in protection, even after 6 months (Holm and Jørgensen 1987). It has emerged that *Ali. salmonicida* vaccines exert adjuvant activities on T-dependent and T-independent antigens in salmonids, namely rainbow trout. Essentially, vaccine preparations enhance antibody responses notably to LPS (Steine et al. 2001). Thus, the inclusion of inactivated *Ali. salmonicida* antigens in vaccines preparations may have an overall beneficial effect on the recipient fish (Hoel et al. 1998). The incubation temperature used to culture *Ali. salmonicida* is an important aspect of vaccine production with 10 °C (this coincides with the upper range of water temperatures at which coldwater vibriosis is most likely to occur) rather than 15 °C giving a higher yield of cells in broth media (Colquhoun et al. 2002).

At least one vaccine has been commercialised in a polyvalent form.

Immunostimulants/Dietary Supplements Synergism between low levels of iron and high amounts of long-chain polyunsaturated fatty acids led to and RPS 96 % after challenge with *Ali. salmonicida* (Rørvik et al. 2003). Carvacrol, which in part of the essential oil of plants including oregano (*Origanum vulgare*) was fed at

0.025 % and 0.05 % to sea bass for 9-weeks leading to an impact on immunity, i.e. a significant reduction in serum proteins, immunoglobulins and lysozyme activity, and an increase in phagocytosis, and enhanced resistance to experimental challenge (RPS = 75 % (Volpatti et al. 2013)

Antimicrobial Compounds Oxolinic acid controls mortalities in Atlantic salmon.

Aliivibrio wodanis

Two groups of bacteria were recovered from Atlantic salmon with so-called winter ulcer disease/syndrome (Lunder et al. 2000), of which one comprised a comparatively heterogeneous assemblage of cultures, i.e. *Ali. wodanis.* Our hypothesis is that the two organisms [the second is *Moritella viscosa*] work together synergistically to give the single disease condition.

Characteristics of the Disease

Ulcers, of indeterminate cause, have been appearing on the flanks of Atlantic salmon in seawater during winter (= winter ulcer disease), principally in Iceland and Norway (Salte et al. 1994; Lunder et al. 1995; Benediktsdóttir et al. 1998), and more recently in Scotland. Since its first recognition, a view has emerged that two new vibrios*, Ali. wodanis* and *Moritella viscosa,* may be responsible (Benediktsdóttir et al. 2000).

Characteristics of the Pathogen

Box 10.5: ***Aliivibrio wodanis***
The taxon is regarded as not being especially homogeneous, phenotypically. Nevertheless, the yellow, opaque colonies contain Gram-negative, motile fermentative rods that produce alkaline phosphatase, caprylate esterase, catalase, indole and oxidase but not arginine dihydrolase, chemotrypsinase, α-fucosidase, α- or ß-glucosidase, lysine decarboxylase, α-mannosidase, ornithine decarboxylase, degrade bovine blood (ß-haemolysis only in the presence of 2 % w/v NaCl), DNA, starch and Tween 80 but not aesculin, casein or lecithin, grow at 4–25 but not 30 °C in 1–4 % (w/v) NaCl, produce acid from galactose, glycerol, glucose and mannose but not L-arabinose, cellobiose, inositol, lactose, melibiose, raffinose, L-rhamnose or xylose, and are susceptible to the vibriostatic agent, O/129. The methyl red test is positive, but not the Voges Proskauer reaction. The G + C ratio of the DNA is 40.0 mol% (Lunder et al. 2000).

By DNA: DNA hybridisation and 16S 3RNA sequencing, the closest neighbour was *Ali. logei* with 57 % re-association (Lunder et al. 2000) and 98.8 % sequence homology (Benediktsdóttir et al. 2000), respectively. The organism was transferred to a newly created genus *Aliivibrio* as *Ali. wodanis* on the basis of phylogeny principally involving sequencing of the 16S rRNA gene (Urbanczyk et al. 2007).

Vibrio aestuarianus

Characteristics of the Disease

During 2008, cultured tongue sole in China suffered high mortalities with disease signs including inappetance, erratic swimming, haemorrhages on the head, opercula and base of the fins, dorsal fin rot, swollen abdomen filled with ascitic fluid, and the presence of hernias in the intestine (Zhang et al. 2011b).

Characteristics of the Pathogen

Box 10.6: ***Vibrio aestuarianus***
Translucent greyish-white colonies on marine 2216E agar comprise motile, fermentative Gram-negative slightly curved rods of 1.2–2.0×0.5–1.0 μm in size that produce catalase, arginine dihydrolase, ß-galactosidase, indole and oxidase, but not acetamidase, H_2S or phenylalanine deaminase. Acid is produced from galactose, lactose, maltose, mannitol, mannose, sorbitol and sucrose, but not adonitol, amygdalin, arabinose, dulcitol, erythritol, inositol, salicin or xylose. Citrate, malonate, mucate and tartrate are utilised, but not acetate. The methyl red test is positive, but not the Voges Proskauer reaction. Blood (slight ß-haemolysis), DNA, lecithin, lipids and urea are attacked, but not gelatin. Nitrates are reduced. Growth occurs in 1–3 % but not 0 or 6 % (w/v) sodium chloride, and at 28 and 37 °C. Sensitivity is recorded to the vibriostatic agent, O/129.

Identification of the four cultures to *V. aestuarianus* was largely on the sequencing of the 16S rRNA and *gyrB* genes when homologies of 98–99 % were recorded with the entry [for *V. aestuarianus]* in GenBank (Zhang et al. 2011b). This level of homology is low to be completely convinced of the justifiable inclusion in the species, and the identification is tentative although the phenotypic evidence is more supportive.

Pathogenicity

Isolates caused disease and mortalities when injected i.p. into tongue sole, albeit at high doses, i.e. 10^5 to 10^7 CFU/fish, with putative pathogenicity factors including DNase, ß-haemolysin, lecithinase, lipase and protease (Zhang et al. 2011b).

Vibrio alginolyticus

Characteristics of the Disease

The most extensive study of the role of *V. alginolyticus* as a fish pathogen concerned the observations of mortalities in farmed sea bream *(Sparus aurata)* in Israel. Mortalities were recorded after extensive handling of the fish (Colorni et al. 1981). However, these workers were unable to reproduce the infection under laboratory conditions, which casts some doubt on the importance of this organism in fish pathology. Nevertheless, there is additional evidence linking this organism with a pathogenic mode. From the description of Colorni et al. (1981) and Austin et al. (1993), the disease may be classified as a typical bacterial septicaemia. Infected fish were observed to become sluggish, the skin darkened, scales loosened and sloughed off, and ulcers developed. The liver, capillaries in the intestinal wall, air bladder, and peritoneum became congested. Simultaneously, the intestine and gall bladder became distended with clear fluid and bile, respectively. Anaemia and gill rot were also reported. Austin et al. (1993) attributed the organism with gill disease leading to progressive low-level mortalities in turbot, which were maintained at supra-optimal temperatures in a recirculating aquarium. Lee (1995) recovered one isolate, which was identified phenotypically, from diseased grouper *(Epinephelus malabaricus),* and attributed the organism with causing exophthalmia and corneal opaqueness; signs that are reminiscent of the pathology caused by *V. harveyi.* Also, occasional isolations have been made from ulcers (Akazaka 1968). Woo et al. (1995) and Ye et al. (1997) considered that *V. alginolyticus* was responsible for heavy mortalities in silver sea bream *(Sparus sarba)* in Hong Kong. The organism has also been associated with disease in gilt-head sea bream in Spain (Balebona et al. 1998). Additionally, *V. alginolyticus* has been reported as a secondary invader of sea mullet suffering with ‘red spot’ (Burke and Rodgers 1981). *V. alginolyticus* has also been implicated with mortalities in cultured black sea bream fry (Kusuda et al. 1986) and in cobia (*Rachycentron canadum*) in the latter case the disease signs focused on sluggish swimming and bilateral exophthalmia leading to death (Rameshkumar et al. 2014). Overall, there is some evidence that *V. alginolyticus* may constitute an opportunistic invader of already damaged tissues, or a weak pathogen of stressed fish.

Isolation

This may be readily achieved from blood by inoculation onto TSA prepared with seawater, TCBS, or seawater agar with incubation at 15–25 °C for 2–7 days. The precise conditions employed by Colorni et al. (1981) were not stated. However, this group succeeded in isolating *V. alginolyticus, V. anguillarum* and *V. parahaemolyticus* from blood, and long thin, flexible rods from cases of gill rot. In addition, we have isolated pure culture growth of *V. alginolyticus* from moribund eels. A selective and differential medium, termed *Vibrio alginolyticus* agar (VAL) has been described, and which incorporates bile salts, high sodium chloride concentration with a incubation at 37 °C (Appendix in Chap. 12). Using this approach, *V. alginolyticus* developed green-yellow colonies (Chang et al. 2011).

Characteristics of the Pathogen

Box 10.7: *Vibrio alginolyticus*
Typically, swarming growth develops on the surface of solid media. Cultures comprise motile, fermentative Gram-negative rods that produce catalase, H_2S, indole, lysine and ornithine decarboxylase, and oxidase, but not arginine dihydrolase or ß-galactosidase. Blood (haemolysis), chitin, gelatin, lipids, starch and urea are degraded, but not aesculin. Nitrates are reduced. The methyl red test and Voges Proskauer reaction are positive. Acid is produced from glycerol, maltose, mannitol, mannose, salicin and sucrose, but not arabinose, inositol or lactose. Growth occurs at 37 °C, and in 7 % but not 0 % or 10 % (w/v) sodium chloride. Sensitivity is recorded to the vibriostatic agent, O/129. The G + C ratio of the DNA is 45–47 moles%.

Generally, fresh isolates matched the species description of *V. alginolyticus* (Farmer et al. 2005).

Diagnosis

Molecular Methods A loop mediated isothermal amplification PCR (LAMP) with primers targeting the *gyrB* gene, was successful for the rapid and sensitive detection [detection limit of 3.7 × 102 CFU/ml] of *V. alginolyticus* in diseased marine fish (Cai et al. 2010).

Epizootiology

Vibrios abound in the marine and estuarine environments (see Kaneko and Colwell 1974), and therefore present a constant threat for any susceptible host. In particular, *V. alginolyticus* has been recovered from the water in marine fish tanks (Gilmour 1977).

Pathogenicity

V. alginolyticus adheres to intestinal mucus of the large yellow croaker (*Pseudosciaena crocea*), possibly involving glycoproteins, with adhesion influenced by environmental parameters, namely pH, salinity and temperature. Maximal adhesion occurred in acid conditions, at 30 °C and at marine salinities [35‰] (Yan et al. 2007). The portal of entry into large yellow croaker has been identified as the intestinal tract rather than gill or skin (Chen et al. 2008). A type III secretion system has been determined to rapidly cause cell death by inducing rapid apoptosis, characterized by membrane blebbing, nuclear condensation and DNA fragmentation, rounding of the cells, and lysis (Zhao et al. 2010).

ECPs, growth in iron-limited conditions, and survival in fish serum have been associated with virulence (Kahla-Nakbi et al. 2009). Lee (1995) revealed that the organism produced ECP, which was lethal at 0.52 μg/g of fish. The ECP contained a 44 kDa toxic protease, for which the minimum lethal dose was 0.17 μg/g of fish. In comparison, the LD_{50} for ECP to silver sea bream was reported as 0.92 μg/g of fish, with haemolysins and proteases featuring in pathogenicity (Li et al. 2003). Also, administration of ECP led to a reduction in one of the hepatic heat shock protein (HSP), specifically HSP90 during the latter stages of acute infection in silver sea bream (Deane et al. 2004). The LD_{50} of the pathogen to silver sea bream was reported as 4.85×10^4, 5.01×10^5, 3.16×10^6 and $>2.5 \times 10^8$ CFU/ml for i.m., i.p., injury coupled with immersion and immersion, respectively (Li et al. 2003). From, these data, the impact of injury leading to infection is apparent. Following infection of sea bream, there was a rapid and substantial decline in Na^+, K^+ and ATPase activity in the kidney, and a decreased expression of HSP70 in the kidney and liver (Deane and Woo 2005). Subsequently, virulence of *V. alginolyticus* was linked to genotype, viz. the collagenase gene (Yishan et al. 2011). A virulence-associated *toxR* gene has been described in a pathogenic isolate, coined ZJ51-O, and linked to resistance to bile salts and the ability to form a biofilm and thus colonise the surface of fish intestine. A deletion mutant, lacking *toxR*, lacked a 37 kDa OMP [a homolog of OmpT] whereas a second OMP of 43 kDa [a homolog of OmpN] was reduced (Chen et al. 2012).

A thermostable haemolysin, TLH, has been described, and shown to induce caspase-3, 8, −9/6 activities followed by apoptotic DNA fragmentation, membrane

vesiculation, i.e. protrusions on the plasma membrane which became detected as particles, and necrosis in nucleated sea bream erythrocytes (Wong et al. 2012).

The LuxS quorum sensing system, which regulates the expression of some virulence factors, has been studied in *V. alginolyticus* when mutations in the *luxS* gene led to reduced virulence. The mutants had reduced growth rates, defective flagellar synthesis, decreased protease production but enhanced extracellular polysaccharide production and biofilm development. The interpretation was that the LuxS quorum sensing system regulated the expression of virulence factors in the pathogen (Ye et al. 2008).

Disease Control

Vaccine Development A DNA vaccine was constructed containing the flagellin *flaA* gene from a culture of *V. alginolyticus* and used i.m. (8 μg/fish) in red snapper (*Lutjanus sanguineus*) with results showing a wide distribution of the antigens to the gill, kidney, liver, spleen and muscle around the injection site with expression of the *flaA* gene after 7–28 days with a RPS after challenge of 88 % (Liang et al. 2011). Also, recombinant *flaC* was a worthy vaccine candidate with a resulting RPS of 84 % (Liang et al. 2010). A combination vaccine with inactivated cells of *V. alginolyticus*, *V. harveyi*, *V. vulnificus* and infectious spleen and kidney necrosis virus was evaluated in orange-spotted grouper (*Epinephelus coioides)* with an overall RPS of 80 % (Huang et al. 2012).

A conserved OMP, OmpK, administered i.p. at 100 μg protein/fish and boosted after 2-weeks protected large yellow croakers (RPS = 70–79 %) against challenge 4-weeks later (Qian et al. 2008).

Immunostimulants/Dietary Supplements Aloe has been found to increase resistance to *V. alginolyticus* infections in rockfish when fed at 5 g aloe/kg of diet for 6 weeks (Kim et al. 1999). Chitin and chitosan enriched (1 % and 2 %) diets were fed to kelp grouper (*Epinephelus bruneus*) leading to immunostimulation (enhanced albumin, globulin, haematocrit, haemoglobin, erythrocyte and leucocyte count) and some evidence of protection against challenge, more so with chitosan than chitin (Harikrishnan et al. 2012a, b, c, d, e). Aqueous leaf extract of grey/white mangrove (*Avicennia marina*) was fed at 1–8 % to clownfish (*Amphiprion sebae*) leading to immunostimulation and improved survival after challenge. Specifically, feeding 1, 4 and 8 % extracts led to survival of 70 %, 85 % and 80 % compared to 10 % surviving in the controls (Dhayanithi et al. 2015a). Extract of leaves from another mangrove *Rhizophora apiculata* was fed at 5 % and 10 % also to clownfish, (*Amphiprion sebae*) when improved growth, immunostimulation (superoxide anion production, and anti-protease, bacteriocidal, complement, lysozyme, phagocytic and respiratory burst activities) and protection against challenge (with 5 % mangrove extract, the survival rate was 85 %) were reported (Dhayanithi et al. 2015b). Katuk (*Sauropus androgynous*) was fed at 1.0 and 2.5 g/kg for 30 days to grouper juveniles leading

to improved growth, immunostimulation and resistance to challenge (Samad et al. 2014).

Antimicrobial Compounds Colorni et al. (1981) achieved success with chloramphenicol, dosed at 50 mg of drug/kg body weight of fish/day for an unspecified period, and nitrofurantoin (50 mg/l of water/1 h), both of which alleviated mortalities. However, we would caution against the use of chloramphenicol in fisheries, in view of the report of the Swann Committee (Report 1969). In essence, chloramphenicol should be restricted to use in human beings.

Vibrio anguillarum

Characteristics of the Disease

The causal agent of 'red-pest' in eels was first isolated by Canestrini (1893), who designated the organism as *Bacterium anguillarum.* A further case among eels in Sweden during 1907 was investigated by Bergman (1909), and it was directly attributable to this scientist that the name of *V. anguillarum* was coined. 'Red-pest' (referred to historically as *pestis rubra anguillarum erysipelosis anguillarum)* caused catastrophic losses among eels held in sea water sites within Italy during the eighteenth and nineteenth centuries. The excellent description of an outbreak of 'red-pest' in eels during 1718 is undoubtedly the first reference to a bacterial fish disease in the European literature (Bonaveri 1761). For a detailed account of the early narratives, reference is made to the splendid review of Drouin de Bouville (1907). However, confusion may result from the multiplicity of names used to describe the disease. Thus, references may be found to 'salt-water furunculosis' (Rucker 1963), 'boil-disease' (Kubota and Takakuwa 1963), 'ulcer-disease' (Bagge and Bagge 1956) and 'Hitra-disease' (Egidius et al. 1981), as well as to the universally accepted name of 'vibriosis'.

Apt but gory descriptions have been made about the nature of vibriosis in fish. To microbiologists, the disease may be regarded as yet another haemorrhagic septicaemia (Fig. 10.4). Typically, infected fish show skin discoloration, the presence of red necrotic lesions in the abdominal muscle, and erythema (bloody blotches) at the base of the fins, around the vent, and within the mouth (in this respect there is a resemblance to ERM, caused by *Yersinia ruckeri).* The gut and rectum may be distended, and filled with clear viscous fluid. Exophthalmia may be evident (Anderson and Conroy 1970).

In Pacific salmon fingerlings, a bacteraemia occurs in the initial stages of disease. From histological examination, it may be concluded that there are pathological changes in the blood, connective tissue, gills, kidney, liver (an anaemia) and posterior gastro-intestinal tract, and swelling in the spleen. The bacterial cells appear to be uniformly distributed throughout the affected tissues, although the

Fig. 10.4 Haemorrhaging on the fins and around the opercula of a sea bass. The aetiological agent was *V. anguillarum* (Photograph courtesy of Professor V. Jencic)

greatest concentration is in the blood (Tajima et al. 1981; Ransom et al. 1984). Usually infected fish become inactive, cease feeding (this may cause problems for chemotherapy), and suffer heavy mortalities.

Fin rot is another condition attributed to *V. anguillarum.* For example, the pathogen has been blamed for causing fin rot in juvenile turbot principally in Northern China (Lei et al. 2006). Here, the infection led to mortalities of 90–100 % (Lei et al. 2006). The organism has been recovered in mixed communities with *V. chagassi* and *V. harveyi* from sand melts with skin haemorrhages (Fabbro et al. 2011).

Isolation and Detection

The pathogen may be readily recovered from infected tissue by use of TSA (Traxler and Li 1972), nutrient agar (Muroga et al. 1976a, b) and BHIA (Tajima et al. 1981) supplemented with sodium chloride at 0.5–3.5 % (w/v), seawater agar and TCBS (Bolinches et al. 1988), with incubation at 15–25 °C for periods of up to 7 days.

The presumptive identification of *V. anguillarum* has been achieved using a specially designed medium, designated VAM, which combined bile salts with a high sodium chloride concentration, ampicillin, sorbitol and a high pH (Appendix in Chap. 12; Alsina et al. 1994). On VAM, *V. anguillarum* produced bright yellow colonies with yellow haloes. Its usefulness was attested by its ability to recognise the majority, i.e. 197/227 = 87 % of *V. anguillarum* isolates. However, some erroneous results occurred insofar as VAM recognised 3/66 = 4 % of other vibrios as *V. anguillarum* (Alsina et al. 1994).

A RT-PCR has been developed for the detection and quantification of *V. anguillarum* in sea bass tissues, targeting *16S rDNA* and *toxR* genes. The approach was specific and sensitive, detecting 1–10 bacterial cells/reaction in pure culture and 2×10^2 – 2×10^3 cells/g of fish tissue (Crisafi et al. 2011). A subsequent publication reported a multiplex RT-PCR that was useful for detecting the pathogen in seawater. The primers used targeted *vah1, empA* and *rpoN* genes, and the method detected 3 CFU/ml in sea water within 70 min without the need for need for culturing (Hickey et al. 2015).

Characteristics of the Pathogen

The taxonomy of the pathogen has had a chequered history, which culminated in the description of a second species, i.e. *V. ordalii.* This accommodated strains previously regarded as biotype II of *V. anguillarum* (Schiewe 1981). However, there is cross reactivity with the LPS of *V. anguillarum* serogroup O2 and *V. ordalii* (Muthiara et al. 1993). The complexity in the taxonomic understanding of the pathogen began with the recognition by Nybelin (1935) of two biotypes. These were differentiated on the basis of a few biochemical reactions. A further group, i.e. biotype C, was recognised by Smith (1961). These biotypes were distinguished, as follows:

- type A, known as *V. anguillarum forma typica*, produced indole and acid from mannitol and saccharose.
- type B, referred to as *V. anguillarum forma anguillicida,* did not produce indole or acid from mannitol or saccharose.
- type C, coined as *V. anguillarum forma ophthalmica,* produced acid from mannitol and saccharose, but did not produce indole.

Two further biotypes, i.e. D and E, were later described, both of which produced indole, but not acid from mannitol. Biotype D, but not E, produced acid from saccharose. In view of modern thought and approaches on bacterial taxonomy, these descriptions are inadequate. Nevertheless, they heralded an appreciation of heterogeneity within the species. Type C deserves special mention because this was proposed for Japanese strains derived from rainbow trout, and labelled as *V. piscium* var. *japonicus* (David 1927; Hoshina 1956). Interestingly, they were originally recognised as dissimilar to *V. anguillarum.* Alternatively, it must be conceded that the separate name may have reflected ignorance of the existence of *V. anguillarum* as described by Bergman (1909). The relationship of these so-called biotypes with *V. ichthyodermis,* as described by Wells and ZoBell (1934), and the organism tentatively assigned as *V. anguillicida* (Nishibuchi and Muroga 1977) needs clarification. Indeed, the latter was considered to resemble both *V. anguillarum* and *Ali. fischeri.*

The multiplicity of studies of Harrell et al. (1976), Ohnishi and Muroga (1976), Håstein and Smith (1977), Schiewe et al. (1977), Baumann et al. (1978), Kusuda et al. (1979), Ezura et al. (1980), Lee et al. (1981), Kaper et al. (1983) and West et al. (1983) demonstrated very clearly the heterogeneity within *V. anguillarum.* Håstein and Smith (1977) distinguished two subgroups after a principal components analysis on data collected for 163 isolates and 28 tests. A similar conclusion, i.e. two subgroupings, was voiced by Schiewe et al. (1977), Baumann et al. (1978), Ezura et al. (1980) and Lee et al. (1981). Of the 50 isolates studied by numerical taxonomy, Kusuda et al. (1979) defined three groups. These were equated with *V. anguillarum* (divided into three subgroups), a group closely related to *V. parahaemolyticus,* and a cluster considered to have affinity with *V. ichthyodermis.* Kaper et al. (1983) recognised four homogeneous phena among isolates received as *V. anguillarum.* This view was reinforced in a later study by West et al. (1983). Pazos

et al. (1993) studied 46 isolates of *V. anguillarum*-like organisms from diseased fish and shellfish and the environment by numerical phenetic methods, and recognised 4 phena. The apparent heterogeneity was reinforced by the results of ribotyping, with 44 ribotypes recognised among isolates of *V. anguillarum* (Olesen and Larsen 1993). Yet, Austin et al. (1995a, 1997) recognised a single taxon, homogeneous by ribotyping – isolates were recovered in a single ribotype – and outer membrane protein patterns, but heterogeneous in terms of LPS profiles, plasmid composition, serogrouping, and BIOLOG-GN fingerprints and API 20E profiles.

The taxon was re-classified to *Listonella,* as *Listonella anguillarum* (MacDonell and Colwell 1985). However, the name change was not widely accepted, and it has been proposed that *Listonella* is a heterotypic synonym of *Vibrio,* and that the epithet *anguilllarum* should be included in *Vibrio* (Thompson et al. 2011). The pathogen has now been fully sequenced, and it is appreciated that there are two chromosomes on which there are 8 genomic islands on chromosome 1 and two on chromosome 2 (Naka et al. 2011).

Box 10.8: *Vibrio anguillarum*
Cultures comprise cream-coloured (a water-soluble brown-pigmented culture has been reported in Japanese flounder; Sakai et al. 2006), round, raised, entire, shiny colonies [dissociation into two or three colony types may occur; Austin et al. 1996] comprising short (0.5 × 1.5 μm), fermentative, Gram-negative rods, which are motile by single polar flagella. Arginine dihydrolase, catalase, ß-galactosidase, indole and oxidase, but not H_2S, lysine or ornithine decarboxylase, phenylalanine deaminase or urease, are produced. Along with most other vibrios, sensitivity is displayed to the vibriostatic agent, O/129. A positive result is usually recorded for the Voges Proskauer reaction, but not for the methyl red test. Chitin, gelatin, DNA, lipids and starch, but not aesculin, are degraded. Nitrates are reduced. Growth occurs at 15–37 °C, and in 0.3–3.0 % (w/v) but not 0 % and 7 % (w/v) sodium chloride. Citrate, malonate and tartrate are utilised. The organisms produce acid from amygdalin, arabinose, cellobiose, galactose, glycerol, maltose, mannitol, sorbitol, sucrose and trehalose, but not from adonitol, dulcitol, erythritol, inositol, lactose, melibiose, raffinose, rhamnose, salicin or xylose. Cultures comprise a single dominant ribotype. The G+C ratio of the DNA is 45.6–46.3 moles % (Smith 1961; Kiehn and Pacha 1969; Evelyn 1971; Muroga et al. 1976a, b; Schiewe et al. 1981; Austin et al. 1995a; Farmer et al. 2005).

It has been observed that some so-called *bona fide* isolates of *V. anguillarum* possess fascinating micromorphologies, insofar as broth cultures appear to contain two types of cells. Apart from the typical short, motile rods, we have observed very small, highly motile cells, some of which are capable of passing through 0.22 μm porosity filters. From transmission electron microscopy, we believe that these cells comprise extremely small, spherical bodies, each attached to a single polar flagel-

lum. The significance of these small cells is unclear, but they may represent a stage in a life cycle, a laboratory artefact, or some form of survival mechanism.

The results of serology further complicated the understanding of *V. anguillarum* (Bolinches et al. 1990). The establishment of serotypes has traversed species (or phenetic) boundaries. Initially, three serotypes were recognised for isolates from north western (USA) salmonids, Europe, and Pacific-north west (USA) (Pacha and Kiehn 1969). This was supported by the work of Japanese scientists (Aoki et al. 1981; Muroga et al. 1984b). With further study, the number of serotypes increased to six (Kitao et al. 1983). Thus in a mammoth study of 267 isolates from ayu, eel and rainbow trout, Kitao and co-workers defined serotypes A, B, C, D, E, and F as a result of cross-agglutination and cross-absorption tests with thermo-stable 'O' (somatic) antigens. The majority (243) of these Japanese isolates were recovered in serotype A. It is noteworthy, however, that avirulent isolates were not recovered in any of these serotypes (Muroga et al. 1984b). This reflects the nature of the LPS in the cell wall, which accounts for both the nature of the serotype (Johnson 1977; Aoki et al. 1981) and the immunogenicity. Later, Sørensen and Larsen (1986) reported the presence of 10 O-antigen serotypes, based upon examination of 495 isolates, representatives of which shared a common 40 kDa protein (Simón et al. 1996). A common 47 kb plasmid was reported by Giles et al. (1995). Serovars 01 and 02 contained plasmids, but there was no apparent correlation between the presence of such extrachromosomal DNA and biochemical properties (Larsen and Olsen 1991), although serogroup O1 was regarded as biochemically homogeneous (Pedersen and Larsen 1995). The same 10 serogroups together with 6 nontypeable groups were described by Olsen and Larsen (1993). The number of serogroups was now increased to 23, i.e. O1 to O23 (Silva-Rubio et al. 2008).

A consensus view would be that serogroup O1 has dominated both in the number of isolates available for study and the relative importance to fish pathology (Austin et al. 1995a; Pedersen et al. 1996a, b). The homogeneity of serogroup O1 has been established (Austin et al. 1995a), yet data have pointed to variability among isolates. For example, after studying 75 isolates of serogroup O1, 8 plasmid profiles – with one predominating – and 6 ribotypes were recognised (Skov et al. 1995). An even larger examination of 103 isolates of serogroup O1 recognised 15 plasmid profiles (Pedersen and Larsen 1995). PFGE had high discriminatory power, recognising 35 profiles.

It is a personal view that isolates of serogroup O2 have seemed to be more aggressive than serogroup O1, with the former developing into a significant pathogen of farmed cod in Norway. Serogroup O2, which has been subdivided into serogroup O2a and O2b (e.g. Mikkelsen et al. 2007), has revealed heterogeneity in LPS profiles – six different profiles have been recognised among 129 isolates (Tiainen et al. 1997). By western blotting and slide agglutination, four different patterns have emerged. By comparing LPS profiling, western blotting and slide agglutination, nine different groupings were formed (Tiainen et al. 1997). A view was expressed that additional sub-groups within serogroup O2 remain to be described (Tiainen et al. 1997). Serotype O3, which was comparatively uncommon, appears to be

spreading, and has certainly reached Atlantic salmon production in Chile where it has caused losses (Silva-Rubio et al. 2008).

Diagnosis

Phenotypic Methods Kent (1982) reported that *V. anguillarum* produces a characteristic profile in API 20E, i.e.

$$+v--+---v++++-+-+-+v+$$

whereas Maugeri et al. (1983) published a slightly different pattern, i.e.
$v+--v---vvv++---v-+v+$, where 'v' indicated a variable result.

Of course, it is necessary to modify the protocol for use with marine bacteria. Thus, it was essential to suspend cultures in 2–3 % (w/v) saline rather than distilled water, and the inoculated test strips were incubated at 25 °C (not 37 °C) for up to 48 h. Maugeri et al. (1983) considered that it was essential to carry out some additional tests with putative *V. anguillarum*, namely motility and sensitivity to O/129, in order to confirm that the isolates, were indeed, motile and were inhibited by the vibriostatic agent. However, some cultures may be resistant to the action of O/129 (Muroga et al. 1979) and appear to be non-motile. The latter phenomenon may result from exposure to the partial inhibitory activity of some antimicrobial compounds.

A simplified diagnostic test for *V. anguillarum*, involving 'glucose motility deeps' (GMD) has been reported (Walters and Plumb 1978). Essentially, GMD is a much modified version of the oxidation-fermentation test medium, comprising:

phenol red broth base (Difco) 1.6 % (w/v)
glucose 1.0 % (w/v)
yeast extract 0.3 % (w/v)
agar 0.3 % (w/v)

Stab-inoculated media are incubated at 25 °C for 24–48 h, when acid production and motility (indicated as a carrot-like diffuse growth around the stab mark) are recorded. It remains for further work to confirm the specificity of the reaction for *V. anguillarum.*

Serology An interesting development concerns the detection of a thermolabile *V. anguillarum* O-antigen, termed the k-1 antigen by slide agglutination (Tajima et al. 1987). So far, the data indicate that this antigen is specific to *V. anguillarum.*

Molecular Methods Molecular methods have been invoked to improve the identification of *V. anguillarum.* Using partial 16S rRNA sequences, a specific 16S rRNA oligonucleotide probe detected a minimum of 5×10^3 cells/ml in culture or tissue extracts (Rehnstam et al. 1989). Detection of 1–10 bacterial cells in culture or

10–100 cells (equivalent to 2×10^3 to 2×10^4 cells/g of tissue) in turbot tissue per PCR reaction was detailed by Powell and Loutit (1994a) used a 310 base pair DNA fragment as a probe for *V. anguillarum.* This system detected 100 (but not 10) ng of purified genomic DNA of most serogroups (not serogroup O7) of *V. anguillarum* but did not react with other vibrios. Using the species specific. probe in combination with membrane filtration, *V. anguillarum* could be detected in water (Powell and Loutit 1994b). In a parallel development, an oligonucleotide (VaV3) detected 150 ng by DNA:DNA slot blot hybridisation. The system did not cross react with other species, and was capable of detecting 8 out of 10 serogroups of *V. anguillarum* (Martínez-Picardo et al. 1994). Using the *empA* gene, which encodes a zinc metalloproteinase, Xiao et al. (2009) detected 3.3×10^2 CFU/ml of pure culture, and 4.1×10^2 in seeded turbot kidney homogenates. The *rpoS* gene, which is involved with regulating stress, was incorporated into a PCR which led to the detection of 6 CFU/ml for cultures but was less sensitive when used with genomic DNA from infected flounder (50 ng/g of tissue) and prawn (10 ng/g of tissue) (Kim et al. 2008). González et al. (2004) used a multiplex PCR and DNA microarray, and achieved the simultaneous and differential diagnosis of *Aer. salmonicida, Ph. damselae* subsp. *damselae, V. anguillarum, V. parahaemolyticus* and *V. vulnificus,* with a minimum detection limit of <20 fg per reaction, which equates to 4–5 bacterial cells. A multiplex PCR was developed for the simultaneous detection of *Aer. salmonicida, Pis. salmonis, Str. phocae* and *V. anguillarum*. The detection limit using purified total bacterial DNA was 50 pg/µl ($=3.69\times10^5$ CFU/ml). The limits of detection using spiked tissues, i.e. kidney, liver, muscle or spleen, were $2.64\pm0.54\times10^7$ CFU/g (Tapia-Cammas et al. 2011).

Epizootiology

Vibriosis had gained considerable notoriety in mariculture, where it has become a major limiting factor in the successful rearing of salmonids (Mahnken 1975). To cite one example, in Denmark, the disease has resulted in cumulative losses of 30 % among eel populations (Bruun and Heiberg 1935). This represents a significant economic loss. An authoritative publication reported that vibriosis occurs in more than 14 countries, where it has ravaged approximately 48 species of marine fish. Vibriosis appears to have been confined initially in European waters. North America escaped the ravages of the disease until 1953 (Crosa et al. 1977). Its arrival in Japan in 1975 may have resulted from the importation from France of contaminated eels (Muroga et al. 1976a, b). Evidence is also accumulating that the disease may occur in freshwater conditions (Muroga 1975; Ghittino and Andruetto 1977). This suggests that vibriosis is an extremely widespread problem. Consequently, the literature abounds with reports of ‘new’ isolations and titbits of gossip, which slowly contribute to an overall saga. However, it would appear that vibriosis is, in fact, a syndrome caused by a multiplicity of vibrios (see Schiewe 1981). Here, emphasis will be placed on *V. anguillarum.*

V. anguillarum constitutes part of the normal microflora of the aquatic environment (e.g. West and Lee 1982; Muroga et al. 1986), particularly associated with rotifers, which may become colonized (Tatani et al. 1985; Muroga and Yasunobu 1987; Mizuki et al. 2006; Prol-Garćia et al. 2010) and has been found in the digestive tract of cod larvae (Reid et al. 2009), with maximal and minimal numbers in summer and winter, respectively (Larsen 1982). Experiments have suggested that the pathogen survives in seawater (Prol-Garćia et al. 2010). Thus, Hoff (1989) reported survival for >50 months in a seawater microcosm. Additionally, experiments were conducted with *V. anguillarum* O2ß to determine a role for blue mussels (*Mytilus edulis*) to harbour and release the pathogen into the environment. Thus, 10^4 CFU of *V. anguillarum*/ml was introduced to mussels, which concentrated the pathogen in the digestive gland tissues, with subsequent release in faeces (10^7 CFU/g). These released bacteria were potentially harmful, and in challenge experiments with cod by immersion resulted in substantial mortalities, i.e. 58–70 %. However when the oysters were depurated, the pathogen could not be detected in faeces within 72 h of depuration (Pietrak et al. 2012).

The organism may also constitute part of the normal microflora of marine fish (Oppenheimer 1962; Mattheis 1964). Some elegant work, albeit with only one isolate, has addressed the precise changes to the organism, i.e. starvation-stress responses, in the marine environment (Nelson et al. 1997) where Na^+ is essential for starvation-survival (Fujiwara-Nagata and Eguchi 2004). When starved of carbon, nitrogen and phosphorus, the number of colony forming units (note: this is a dubious measure of viability) dropped rapidly over an initial 5–7 day period, and then gradually declined over 3–4 weeks. Some cells became small and spherical, corresponding to the notion of ultamicrobacteria (see Austin 1988), whereas others elongated to short spirals. Protein synthesis, as measured by incorporation of [^{35}S]-methionine declined during the first 6 h of starvation, and increased to >70 % of the rate in exponentially growing cells by 5 days into the starvation regime (Nelson et al. 1997).

The precise origin of an isolate has importance for epizootiology. In this respect, Olsen and Larsen (1990) detailed a seemingly useful method, namely restriction fragment length polymorphism of the 65–70 kb plasmid. This method should have value for epizootiological investigations.

The exact mode of infection is unclear, but undoubtedly involves colonisation of (attachment to) the host, and thence penetration of the tissues. Ransom (1978) postulated that infection probably begins with colonisation of the posterior gastrointestinal tract and rectum. This conclusion resulted from the observation that *V. anguillarum* was seen initially in these sites. Horne and Baxendale (1983) reported adhesion of *V. anguillarum* to intestinal sections derived from rainbow trout. All regions of the intestine were colonised (approximately 10^3 cells/cm^2), with maximum attachment occurring within 100 min. The skin appears to become colonised within 12 h of immersion in a virulent culture (Kanno et al. 1990). Then, invasion of the liver, spleen, muscle, gills and intestine follows (Muroga and De La Cruz 1987).

It has been well documented that epizootics occur in the warm summer months when water temperatures exceed 10 °C, the water is depleted of dissolved oxygen, and the fish stressed by overcrowding and poor hygiene (Anderson and Conroy 1970). There are exceptions to the norm insofar as outbreaks have been documented in fresh water (e.g. Rucker 1959) and at low temperature, i.e. 1–4 °C (Olafsen et al. 1981). It is perhaps ironic that isolates recovered from rainbow trout in fresh water have an obvious salt requirement for growth (Rucker 1959). Perhaps, the organisms were contained in a protected ecological niche such as within the fish body, prior to the manifestation of the disease. However, it should be remembered that the pathogen has been recovered sporadically from fresh water (West and Lee 1982). The determination of plasmid profiles may have value for epizootiological investigations (Wiik et al. 1989).

The presence of heavy metals, notably copper and iron, contributes to an exacerbation of vibriosis. Yet sublethal concentrations of chlorine do not appear to promote the development of infections (Hetrick et al. 1984). Levels of only 30–60 μg copper/ml and 10 μg of iron/ml have caused severe problems (Rødsaether et al. 1977; Nakai et al. 1987). Further investigation demonstrated the susceptibility to vibriosis was dependent upon concentration and time of exposure to copper (Baker et al. 1983). The debilitating effect has been attributed to coagulation in the mucus layer of the gills, and thus the inhibition of oxygen transport leading to respiratory stress (Westfall 1945). The practical outcome from this information is that fish holding facilities should not be coated with copper-containing anti-fouling compounds, which could trigger vibriosis.

Pathogenicity

AHL signal molecules, which have been recognized in *V. anguillarum,* may well have a role in the expression of virulence factors, e.g. biofilm formation and protease production (Buchholtz et al. 2006). Cultures produced two dominant molecules, i.e. *N*-(2-oxodecanoyl)-L-homoserine lactone and *N*-(3-hydroxy-hexanoyl)-L-homoserine lactone. Smaller amounts of other molecules were also present. Apart from production associated *in vitro* with laboratory cultures, there was evidence that infected fish produce the two dominant AHLs, although there may be some overall differences in balance between the molecules in *in vitro* and *in vivo* conditions (Buchholtz et al. 2006).

The exact mode of infection undoubtedly involves colonisation of (attachment to) the host starting with the skin (Spanggaard et al. 2000) and/or gills (Avci et al. 2012), and thence penetration of the tissues. It is regarded that chemotactic motility is necessary for virulence (O'Toole et al. 1999; Larsen et al. 2004), particularly invasion of the host. The pathogen is attracted to amino acids and carbohydrates particularly in intestinal and to a lesser extent to skin mucus (O'Toole et al. 1999). Chemotaxis to serine, more so at higher, i.e. 25 °C, than lower, namely 5 and 15 °C, temperatures has been documented (Larsen et al. 2004). Also, chemotaxis was

heightened when the bacterial cells were starved for 2 and 8 days (Larsen et al. 2004). Evidence points to a 40.1 kDa flagellin A protein (encoded by the *flaA* gene) being essential for virulence (Milton et al. 1996). Thus, loss of flagella by transposon mutagenesis led to a 500-fold reduction in virulence following an immersion challenge (O'Toole et al. 1996). Flagellum production and virulence by the water-borne but not i.p. route was correlated with RpoN (O'Toole et al. 1997). As a cautionary note, it is possible that other changes to the bacterial cells may have occurred with the loss of flagella. Ransom (1978) postulated that infection probably begins with colonisation of the posterior gastro-intestinal tract and rectum. This conclusion resulted from the observation that *V. anguillarum* was seen initially in these sites. Using GFP-labelled *V. anguillarum* cells and immersion challenge, the gastrointestinal tract of zebra fish was the first site where the pathogen was observed, with chemotactic motility being regarded as essential for the association with the host surface (O'Toole et al. 2004). Horne and Baxendale (1983) reported adhesion of *V. anguillarum* to intestinal sections derived from rainbow trout. All regions of the intestine were colonised (approximately 10^3 cells/cm^2), with maximum attachment occurring within 100 min. Using gnotobiotic sea bass larvae and 10^8 CFU/ ml of GFP-labelled *V. anguillarum* HI-610 serovar O2a, which was originally isolated from cod, Rekecki et al. (2012) observed colonisation of gut enterocytes within 2 h or oral uptake; it took 48 h for the bacterial cells to be recognised in the swim bladder. *V. anguillarum* made contact with the host in the oesophageal mucosa, with likely attachment to the microvilli of the mid- and hindgut enterocytes (Rekecki et al. 2012). *V. anguillarum* cells were detected in the gut of experimentally infected gnotobiotic sea bass larvae by TEM and immunogold labelling. In particular, bacteria were observed to be in close contact with the apical brush border in the gut lumen. Some luminal cells were invaginated, and demonstrated likely engulfment of the pathogen by pseudopod-like formations. Immunogold positive thread-like structures secreted by the pathogen were suggestive of the presence of outer membrane vesicles, which may transport virulence factors to gut cells (Rekecki et al. 2013). It is interesting to note that serogroup O1 but not O2 isolated demonstrated the ability to adhere to mucus from Atlantic salmon epithelial surfaces, i.e. foregut, gills, hindgut, pyloric caeca and skin (Knudsen et al. 1999). Orally administered *V. anguillarum* survived in the stomach of juvenile turbot for several hours, persisted in the intestine, and proliferated in faeces (Olsson et al. 1998). This view has been reinforced by a study, which concluded that >50 % of the spleens of turbot contained cells of *V. anguillarum* after infection via the oral and rectal routes (Olsson et al. 1996). The skin appears to become colonised within 12 h of immersion in a virulent culture (Kanno et al. 1990). Then, invasion of the liver, spleen, muscle, gills and intestine follows (Muroga and De La Cruz 1987). Resistance to the potential debilitating effect of fish serum (Trust et al. 1981) may hasten the invasion processes. Some degree of host specificity has been indicated, insofar as strains from rainbow trout were poorly pathogenic to saithe, and *vice versa* (Egidius and Andersen 1978). This raises the question concerning the size of inoculum necessary to achieve clinical disease. Levine et al. (1972) reported lesions at the site of infection in winter flounder after exposure to only 640 cells. These were administered by

intradermal injection. Much larger inocula resulted in sizeable mortalities. For example, Evelyn (1971) determined that *Oncorhynchus keta* and *O. nerka* died within 48 h of receiving, by i.p. injection, 0.1 ml containing 10^7 viable cells of *V. anguillarum.* In a much more spectacular demonstration of virulence, Sawyer et al. (1979) established 80–100 % mortality in a population of Atlantic salmon following exposure to $1–2.5 \times 10^5$ cells/ml as a bath for 1 h. In this demonstration, the fish were maintained at a water temperature of 10–15 °C. However, temperature shocking does exacerbate mortality. Thus in one series of experiments using rainbow trout, the temperature was decreased from 23 to10°C resulting in a significantly increased level of mortality; an increase which was not correlated with an impairment in immune parameters (Aoshima et al. 2005).

Turbot larvae have been successfully challenged with *V. anguillarum* orally via live feed (Grisez et al. 1996; Planas et al. 2005). Using 10^3 *Artemia* nauplii/ml and 10^9 *V. anguillarum* cells/ml, the recipient fish died within 4 days (Grisez et al. 1996). Similarly, feeding with rotifers containing *V. anguillarum* cells led to a successful infection of turbot larvae (Planas et al. 2005).

The precise nature of the virulence mechanism of *V. anguillarum* has prompted some excellent work. With the advent of random genome sequencing, a strain, H775-3, was examined, and 40 genes, which may well be related to virulence identified, of which 36 genes were considered to be novel to *V. anguillarum,* and included genes for capsule biosynthesis, enterobactin, haemolysin, flagella, LPS biosynthesis, pilus and protease (Rodkhum et al. 2006). Rainbow trout skin epithelial cells were shown to phagocytize the pathogen. Moreover, use of mutants has shown that *V. anguillarum* requires the LPS O-antigen to evade phagocytosis. By adding mannose to epithelial cells, phagocytosis was inhibited, which indicates that a mannose receptor is involved with uptake of the pathogen. Interestingly, O-antigen transport mutants did colonize skin but did so the intestines of rainbow trout (Lindell et al. 2012). The highlight of the early studies was the discovery that *bona fide* strains of serogroup O1 of the pathogen contained a virulence plasmid, which was associated with an iron-uptake system expressed under iron-limited conditions (Crosa et al. 1977; Crosa 1980; Crosa 1980; Wolf and Crosa 1986; Chen et al. 1996). This plasmid, designated pJM1 and of 67 kb, has been fully sequenced (Di Lorenzo et al. 2003), and is always present in virulent isolates (Pedersen et al. 1996a, b, 1997b) and may be included on a transposon-like structure (Tolmasky and Crosa 1995), but absent from those of low virulence. The genes involved in the biosynthesis of anguibactin are located on the plasmid and chromosome. However, the genes for the outer membrane receptor, FatA, are located only on the plasmid (López et al. 2007). Interestingly, the pJM1 plasmid has been found in some avirulent isolates (Pedersen et al. 1997b). Yet, virulence may be attenuated by curing this plasmid (Crosa 1980) or by deleting three plasmid encoded gene products (Singer et al. 1991). The role for pJM1 concerns specifying an iron-sequestering mechanism, i.e. the low molecular weight siderophore anguibactin for which the precursor is chromosome-mediated 2,3-dihydroxybenzoic acid (Chen et al. 1994), and specific iron-transport proteins, of which the angR protein (this is regulated by the regulatory gene *angR* [Salinas and Crosa 1995], which has been reported as similar to bacteriophage P22; Farrell

et al. 1990) acts as a positive regulator of anguibactin biosynthesis and the transcription of the iron-transport genes *FatA* and *FatB* (Actis et al. 1995; Chen et al. 1996). Also, *V. anguillarum* has a plasmid-encoded histamine decarboxylase gene *angH,* which is essential for the biosynthesis of anguibactin (Barancin et al. 1998). The overall effect is that the system enables the bacterial cell to compete for available iron in the fish tissues. Two OMP have been designated as OM2 (molecular weight = 86 kDa) and OM3 (molecular weight = 79 kDa). The siderophore and OM2 are coded by plasmid pJM1, whereas OM3 is a function of chromosomal involvement. The basic mechanism involves diffusion of the siderophore into the environment, and the formation of iron complexes that attach to OM2, presumably leading to transport of the iron into the bacterial cell (Crosa and Hodges 1981; Crosa et al. 1983; Tolmasky and Crosa 1984; Actis et al. 1985; Mackie and Birkbeck 1992). Thus, invading bacteria may multiply in the host by scavenging successfully for the iron that is bound by high-affinity iron-binding proteins, such as transferrin, lactoferrin and ferritin. These are present in the serum, secretions and tissues, respectively (Bullen et al. 1978). Toranzo et al. (1983a, b) complicated the issue by publishing data that showed that virulent strains, obtained from striped bass, did not contain plasmids. Yet, all the isolates grew in iron-limiting conditions, during which new OMP and a siderophore were found. Chromosomal DNA sequences, which hybridised with pJM1, were present. Thus, it seems likely that the plasmid DNA had become integrated into the bacterial chromosome.

A pJM1-like plasmid, pEIB1, has been sequenced, and determined to comprise 66,164 bp encoding 44 ORFs (>400 bp) containing genes for biosynthesis and regulation of anguibactin, transport of ferric-anguibactin complexes and DNA replication (Wu et al. 2004).

Work proved that a separate iron-uptake system was contained on the chromosome. This differed from the plasmid-mediated system, insofar as the diffusible siderophore is not utilised as an external siderophore, and different OMP are synthesised (Lemos et al. 1988; Conchas et al. 1991; Mackie and Birkbeck 1992). Siderophores of the phenolate class, possibly related to enterobactin, have been found in New Zealand isolates (Pybus et al. 1994). Interestingly, iron-uptake mechanisms have been reported in non-pathogenic cultures (Lemos et al. 1991), casting some doubt on the precise relevance of the mechanism to pathogenicity.

A catechol-type siderophore, termed vanchrobactin, has been reported in some strains (e.g. Balado et al. 2008). The genes, *vabA, vabB, vabE, vabF, vabG* (encodes a 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase)*, vabS, vabH,* vabR (encodes a Lys-R-family transcriptional regulator) and *fvtA* (a putative siderophore receptor) have been identified. Deletion of *vabG* or *vabD* led to a reduction in growth in iron-limitation. *A vabD* mutant did not produce any vanchrobactin; a *vab* mutant demonstrated the ability for residual production. By PCR, it was deduced that the gene cluster is arranged into 6 iron-regulated transcriptional units, with ferric uptake regulator protein (Fur) as the principle ion-responsive regulator of the genes (Balado et al. 2008).

But what about the other serogroups? The virulence plasmid, pJM1, has not been found in representatives of any other serogroup (Austin et al. 1995a). Yet, we regard

that other serogroups, especially serogroup O2, which produces a 50 kDa porin (Davey et al. 1998) may be more aggressive to fish than serogroup O1.

The presence of common antigens between *V. anguillarum* and other taxa would explain the cross protection observed with fish vaccines (e.g. Nakai et al. 1989). What is the nature of these cross protecting antigens? From capsular antigens (Rasmussen and Larsen 1987), attention focused on the OMP (Chart and Trust 1984) – a porin of ~40 kDa molecular weight, which is now regarded as a common antigen (Simón et al. 1996). This porin, Om1, was examined by immunoblotting, ELISA and dot blot; antiserum to Om1 of serogroup 01 cross reacted with *Vibrio* spp., but not with other genera – except *Plesiomonas shigelloides* (Simón et al. 1998). Detailed investigation described a 38 kDa OMP, which was considered to be involved in environmental adaptation and resistance to bile (Wang et al. 2003).

Debate has centred over the possible interaction of exotoxins and/or endotoxins (Bullock and Conroy 1971; Abe 1972; Umbreit and Ordal 1972; Grischkowsky 1973; Inamura et al. 1984; De La Cruz and Muroga 1989). Umbreit and Ordal (1972) reported mortalities in goldfish following injection of filter-sterilised supernatant derived from 24 h broth cultures. Thus, the debilitating effect of bacterial ECP was suggested (but not proven!) at this stage; but later held responsible for virulence [a neurotoxic acetylcholinestase has been isolated from the ECP; Peréz et al. 1998] (Lamas et al. 1994a, b). This experiment was repeated, and resulted in >70 % mortalities among a group of goldfish (Umbreit and Tripp 1975). Unfortunately, the 3-year interval between publications did not achieve any significant improvement in knowledge. A similar basic theme was used by Abe (1972), who injected 'endotoxins' into chinook salmon and recorded the presence of haemorrhaging lesions at the point of injection. A significant development stems from the work of Wolke (1975) and Roberts (1976), who suggested that 'haemolytic toxins' might be responsible for the anaemic response in infected fish. Subsequent efforts by Munn (1978, 1980) demonstrated conclusively that haemolysins were involved. These were described as thermolabile enzymes (activity was lost by heating to 50 °C for 10 min) with optimum pH of 7.2–7.4, which were inactivated by gangliosides. The molecular weight of one of these enzymes was estimated as 191 kDa (Munn 1980). In the ensuing experiments, haemolytic activity was first detectable in cultures (filtrates) after 19 h incubation at 20 °C. Production peaked at 39 h, and then declined. One explanation for this result is that haemolysin production only occurs during the stationary phase of growth. Another possibility is that production of the enzyme occurs intracellularly, with release into the environment taking place at a later time, perhaps during autolysis of the bacterial cells. Munn postulated that inactive haemolysins may also be secreted by the bacteria. These enzymes could then be re-activated by as-yet unknown phenomena, at a later period. A haemolysin gene, vah1, has been identified, but does not account for the haemolytic activity. Subsequently, a rtx operon was studied, and concluded to represent a second haemolysin gene cluster. Both vah1 and the RtxA activator have cytotoxic activity to Atlantic salmon kidney cells, with the former causing cell vacuolation, and the later causing rounding of the cells (Li et al. 2008). Proteases of 36 kDa molecular weight have also been implicated with virulence (Inamura et al. 1984, 1985; Kodama et al.

1984; Kanemori et al. 1987). In particular, a zinc metalloprotease has been associated with invasion processes (Norquist et al. 1990). Crude ECP has led to the development of an inflammatory response, including leucopenia, in rainbow trout (Lamas et al. 1994a, b). No doubt the debate will continue unabated for some considerable period, but the ultimate result should be a better understanding of the pathogenicity mechanisms. Basically at present, the scenario involves uptake and penetration of the host tissues, scavenging for iron as a result of a plasmid/chromosomal mediated trait, and damage to the fish by means of haemolysins and proteases. It is relevant to note that an *empA* zinc metalloprotease, which is secreted as a ~46 kDa proenzyme followed by extracellular activation involving removal of ~10 kDa peptide (Varina et al. 2008), was detected during the stationary phase in strains which had been incubated in Atlantic salmon gastro intestinal mucus (Denkin and Nelson 2004; Staroscik et al. 2005). Is there a role for quorum sensing in controlling the expression of virulence genes? Homologs of four quorum sensing genes, coined *VanT* (this was expressed at low cell density)*, VanMN, VanPQ* and *VanOU,* have been identified in *V. anguillarum,* and influence expression of the *empA* metalloprotease (Croxatto et al. 2004). A conclusion is that *V. anguillarum* involves quorum sensing to regulate stress responses necessary for survival, particularly in aquatic habitats (Weber et al. 2011).

Suppression subtractive hybridization (SSH) led to the recognition of a virulence-related gene fragment, which was sequenced, and determined to encode a 547 amino acid putative membrane-bound lytic murein transglycosylase D (MltD) with 34 % homology to an equivalent in *Esch. coli.* A *mltD* mutant with reduced protease and not haemolytic activity was constructed, which demonstrated enhanced virulence over the wild type strain following i.p. injection in a zebra fish model with the resulting LD_{50} dose of 1.01×10^2 CFU/fish and 3.92×10^3 CFU/fish, respectively. *mltD* was cloned, and the recombinant MltD protein possessed diastrase, gelatinase, haemolytic and phospholipase activities (Xu et al. 2011).

In a rather elegant set of experiments, Harbell et al. (1979) catalogued the precise changes in the blood following infection with the pathogen. As anaemia is one of the signs of vibriosis, it seems hardly surprising that the haemoglobin level decreases concomitant with an increase in the erythrocyte osmotic fragility, and a decline in the number of leucocytes. Reductions were also recorded in osmolarity, and in the amounts of plasma protein, albumin, chloride, sodium and alkaline phosphate. In contrast, levels of plasma glucose, lactate dehydrogenase, and glutamic oxaloacetic transaminase increased.

Disease Control

Management Techniques Kocylowski (1963) recommended transfer of eels to cold, well-aerated water to alleviate problems with vibriosis.

Disease Resistant Fish There is contradictory evidence about the role of genetically resistant fish strains at conferring resistance to vibriosis. Winter et al. (1979) determined that there was no variation in resistance to vibriosis among different transferrin genotypes of coho salmon and steelhead trout. We support this observation, with regards to rainbow trout. However in contrast, Pratschner (1978) reported the presence of differential resistance to vibriosis between transferrin genotypes of coho salmon. More recently, an association has been made between polymorphism in the major histocompatibility complex (MHC) 11α genes in Japanese flounder (*Paralichthys olivaceus*) and resistance to *V. anguillarum* whereby three alleles, *Paol-DAA*1301*, *Paol-DAA*1401* and *Paol-DAA*2201*, were associated significantly with resistance (Xu et al. 2010). Furthermore, a relationship has been mooted between alleles of MHC class II A genes and disease resistance in half smooth tongue sole (Li et al. 2012).

Vaccine Development *V. anguillarum* has been one of the few successful candidates for vaccine development. Commercial formalin-inactivated whole cell vaccines are available, which have gained widespread use in mariculture. The benefit of these products is attested by their success in Atlantic halibut (Bricknell et al. 2000; Bowden et al. 2002), African catfish (Vervarcke et al. 2004) and sea bass (Angelidis et al. 2006) when after application by bathing and following challenge, complete protection was recorded (RPS = 100 %). An immersion product developed for cod led to an RPS of >80 % against *V. anguillarum* O2a and O2b (Mikkelsen et al. 2007). Ironically, the reasons for the success of these products are often obscure, although there is evidence that one commercial formalin-inactivated whole cells vaccine induces the Mx gene (these are inducible by Type I interferons and have a role in antiviral activity) expression in Atlantic salmon after administration intraperitoneally (Acosta et al. 2004). Moreover, there is some evidence to suggest that vaccinated fish generally fare much better, i.e. they exhibit better all-round health and growth characteristics, than the unvaccinated counterparts. Moreover, immunostimulants, e.g. levamisole, further enhance protection (Kajita et al. 1990).

The immunogenicity appears to be a reflection on the presence of heat-stable (to 100–121 °C) LPS in the cell wall (Salati et al. 1989b; Kawai and Kusuda 1995), which may be released in the culture supernatant (Chart and Trust 1984; Evelyn 1984), and OMP (Boesen et al. 1997). It has been postulated that a probable mechanism of protection concerns the inhibition of bacterial attachment by unknown factors in the skin mucus (Kawai and Kusuda 1995). That supernatants are also among the most immunogenic parts of *V. anguillarum* vaccines was verified following the anal uptake of different vaccine fractions in carp and rainbow trout (Joosten et al. 1996). The large molecular weight LPS, i.e. 100 kDa (Evelyn and Ketcheson 1980), are considered to confer protection to the recipient host. Moreover, the compounds are able to withstand severe extraction methods. Also, Chart and Trust (1984) isolated, from the outer membrane, two minor proteins with molecular weights of 49–51 kDa, which were potent antigens. A weakly antigenic protein, with a molecular weight of ~40 kDa was also present. Perhaps, these are heat-labile, and explain the reasons for the greater protection achieved with formalin-inactivated vaccines

compared to heat-killed products (Kusuda et al. 1978b; Itami and Kusuda 1980). The potential of LPS as an immunogen was clearly demonstrated by Salati et al. (1989b). These workers injected i.p. crude LPS (0.05–0.5 mg) into ayu. Following challenge, mortalities among the vaccinates and controls were 0 % and 86.7 %, respectively. Similarly, O-antigen preparations induced an immune response following injection in a wide range of fish species, including ayu, carp, Japanese eel, Japanese flounder, rainbow trout and red sea bream (Nakamura et al. 1990). Incorporating purified 43 kDa OMP of *Aer. hydrophila* in FCA and a booster 3 weeks later (without FCA) led to a demonstrable immune response and protection against challenge by *V. anguillarum* in blue gourami *(Trichogaster trichopterus)* (Fang et al. 2000). The *ompK* gene was cloned and sequenced, and recombinant protein was overexpressed, purified, and purified protein, ompK, administered to Indian major carp (*Labeo rohita*), which were protected from challenge (Hamod et al. 2012). Development of live attenuated vaccines have been tried, with some success (Norquist et al. 1989; 1994). A field trial with an attenuated live *V. anguillarum* vaccine [VAN1000] involved bathing 10 g rainbow trout in a dose equivalent to 1×10^6 cells/ml for 60 min at 9 °C in brackish water. Following a natural challenge, 68 % of the unvaccinated controls succumbed compared to 14 % of the vaccinates (Norquist et al. 1994). Interestingly, these workers considered that the live vaccine protected against both furunculosis and vibriosis. However, there may be problems with regulatory authorities regarding licensing for fisheries use.

To date, most of the vaccine development programmes have concentrated on bivalent products, containing cells of *V. anguillarum a*nd *V. ordalii* (e.g. Nakai et al. 1989). At various times, these have been applied to fish by injection (of dubious practicality for masses of fish), on food (oral administration), by bathing/immersion, by spraying, and by anal and oral intubation. The evidence has shown that oral application, perhaps the most convenient method, fares least successfully. Indeed, comparative vaccine trials have produced a wealth of information. For example, Baudin-Laurençin and Tangtrongpiros (1980) reported cumulative percentage mortalities among experimental groups of fish, as follows:

unvaccinated controls 33.8 %
oral-vaccinated fish 31.7 %
immersion vaccinated group 2.1 %
group vaccinated by injection 1.4 %

Similar findings, although generally more favourable for orally administered vaccines, were published by Amend and Johnson (1981) and Horne et al. (1982). Thus, Amend and Johnson (1981) revealed the following mortalities in vaccinated salmonids:

unvaccinated controls 52 %
oral-vaccinated fish 27 %
immersion vaccinated group 4 %
spray vaccinated fish 1 %
group vaccinated by injection 0 %

This compares with the work of Horne et al. (1982), who reported mortalities of:

unvaccinated controls 100 %
oral-vaccinated fish 94 %
immersion vaccinated group 53 %
group vaccinated by injection 7 %

In a detailed examination of the effects of oral administration of formalin-inactivated vaccines in chinook salmon, Fryer et al. (1978) noted that maximal protection followed the feeding of 2 mg of dried vaccine/g of food for 15 days at temperatures even as low as 3.9 °C. An important corollary was the observation that longer feeding regimes did not result in enhanced protection. This should be considered if prolonged durations of vaccination, via the oral route, are advocated. The reason for the apparently discouraging results with oral vaccination regimes may reflect the breakdown of vaccine inside the digestive tract (Johnson and Amend 1983). To resolve this problem, Johnson and Amend (1983) incorporated a vaccine into gelatin, and applied it orally and anally in attempts to overcome digestion in the stomach and intestine. Encouraging results were obtained, particularly in regard to anal application, in which mortalities following challenge were:

unvaccinated controls 97 %
vaccine (minus gelatin) applied orally 35 %
vaccine (with gelatin) applied orally 69 %
vaccine (minus gelatin) applied anally 37 %
vaccine (with gelatin) applied anally 7 %

Similar encouraging data were published by Dec et al. (1990). These workers used a commercial vaccine (produced by Rhône-Merieux), which was administered orally to turbot and sea bass. Following challenge 28 days later, the following mortalities were reported:

oral-vaccinated sea bass 11.3 %
unvaccinated sea bass 40.9 %
oral vaccinated turbot 19.2 %
unvaccinated turbot 65.4 %

Incorporation of vaccine with natural food, i.e. plankton, has shown promise with ayu (Kawai et al. 1989). Thus in one set of experiments, 7.6 % of the vaccinates died, compared to 35.8 % of the controls.

Noting that a pJM1-plasmid-free culture was comparatively attenuated, Shao et al. (2005) used a plasmid-free culture, coined MVAV6201, as a live vaccine to deliver two recombinant proteins, GFP-HlyAs [HlyA = *Esch. coli* α-haemolysin] and AngE-HlyAs, which were fused with the α-haemolysin secretion signal and expressed from the secretion vector pMOhlyl. Almost 70 % and ~300 μg/l of GFP-HlyAs and AngE-HlyAs were secreted into the culture supernatant, respectively (Shao et al. 2005).

Bypassing the potential deleterious effects of the stomach and upper regions of the gastro-intestinal tract enables effective vaccination to proceed. This suggests that micro-encapsulation techniques may be important for the development of successful oral vaccines. In this respect, the use of alginate microparticles has given promising results with an orally administered *V. anguillarum* vaccine (Joosten et al. 1997). An interesting point is the implication that the posterior region of the gastro-intestinal tract is involved with the correct functioning of oral vaccines. This region has also been determined to be one of the initial sites of attachment of the pathogen. Therefore, it may be inferred that the best protection stems from methods paralleling those of the natural infection cycle.

In contrast to oral methods, injection has proved to be excellent as a means of vaccinating fish against vibriosis, with the development of high levels of immunity (Antipa 1976; Antipa and Amend 1977; Sawyer and Strout 1977; Harrell 1978; Evelyn and Ketcheson 1980). Evidence suggests that 24 h and up to 14 days (but not 21 days) after i.p. injection with formalin-killed whole cells, the bacteria migrate to the spleen (particularly around small blood vessels when applied in FCA), heart, kidney and peritoneum of Atlantic cod (Arnesen et al. 2002). Unfortunately, the injection technique is slow, and seems feasible only for large and/or valuable fish. Nevertheless, several types of preparations, including heat-killed and formalised vaccines, have been evaluated by injection. In addition, passive immunisation (by injection) has demonstrated the transfer of immunity between fish. In one comparison, it was clearly demonstrated that heat-killed preparations were more successful than products treated with formalin, when administered by injection. Reference is made to the work of Antipa (1976), who injected chinook salmon with vaccines and, following challenge with the pathogen, reported cumulative mortalities of:

unvaccinated controls 85.4 %
formalised vaccine 37.8 %
heat-killed vaccine 22.3 %

Sonicated heat-killed vaccines, administered in adjuvant, also stimulate elevated levels of antibody in the skin and mucus (Harrell et al. 1976; Evelyn 1984). At least these studies indicate the presence of heat-stable antigen, which features significantly in the establishment of protective immunity.

Anal intubation, but not i.p. injection, of African catfish *(Clarias gariepinus)* with a whole cell vaccine of *V. anguillarum* O2 led to increased antibody levels after 14 days in the bile and skin mucus as detected by ELISA (Vervarcke et al. 2005). Antibodies in a group vaccinated by oral intubation were lower, but still higher than the i.p. vaccinated group (Vervarcke et al. 2005).

Immersion techniques are most suited for the vaccination of animals in the fish farm environment. Formerly, considerable attention was focused on hyperosmotic infiltration, involving use of a strong salt solution prior to immersion in a vaccine suspension (Croy and Amend 1977; Aoki and Kitao 1978; Nakajima and Chikahata 1979; Antipa et al. 1980; Giorgetti et al. 1981). However, it is now appreciated that the technique is extremely stressful to fish (Busch et al. 1978), and the level of protection achieved is only comparable to the much simpler direct immersion method

(Antipa et al. 1980), which is consequently favoured. Indeed, many articles have been published about the benefit of immersion vaccination (Håstein et al. 1980; Amend and Johnson 1981; Giorgetti et al. 1981; Horne et al. 1982; Johnson et al. 1982a, b; Kawai and Kusuda 1995) and the longer, i.e. 2 h, 'bath' technique (Egidius and Andersen 1979).

A further refinement involves use of low-pressure sprays, which are easy to use, and apparently economic in the quantity of vaccine administered (Gould et al. 1978). The success was illustrated by 0 % mortalities in a group of fish spray-vaccinated compared to 80 % mortalities among unvaccinated controls after challenge (Gould et al. 1978).

All of the aforementioned methods enable fish to develop an immune response to the pathogen. This aspect has been discussed comprehensively, as regards chinook salmon, by Fryer et al. (1972). It is thought that the maximum agglutination titre is in the region of 1:8192, depending on the fish species used (Groberg 1982). The development of immunity is clearly a function of water temperature, and generally humoral antibodies are formed more rapidly at high rather than low temperatures. For example, in coho salmon, humoral antibodies appeared in 25 days and 10 days at water temperatures of 6 °C and 18 °C, respectively (Groberg 1982). The poor relative performance of orally administered vaccines has been partially attributed to an inability of the fish to develop humoral antibodies (Fryer et al. 1978; Gould et al. 1978; Kusuda et al. 1978b; Groberg 1982). However, the role of these antibodies in protection against disease is unclear.

Inevitably, *V. anguillarum* has been the target of modern approaches for vaccine development. For example, Japanese flounder were protected (RPS = 85.7 %) after 4- weeks against challenge with *V. anguillarum* by using a DNA vaccine comprising a mutated zinc metalloprotease gene (m-*EmpA*) applied as a 50 μg dose by i.m. injection. Protection was reduced by lowering the dose to 5 (RPS = 57.1 %) and 20 μg (RPS = 71.4 %) (Yang et al. 2009). Zhao et al. (2011) used the GAPDH gene *gapA* to express *Aer. hydrophila* GAPDH in an attenuated *V. anguillarum* strain, which was injected i.p. (10^6 CFU/fish) into turbot and challenged after 4-weeks. Challenge of cytoplasm GAPDH expressing strain AV/pUC-gapA-vaccinated fish with *V. anguillarum* led to an RPS value of 92 %. A plasmid-based antigen expression system has been developed and considered to have potential for future vaccine development programmes (Xiao et al. 2011).

The potential of flagella genes *FlaA, FlaB, FlaD*, and *FlaE* were evaluated in Japanese flounder (*Paralichthys olivaceus*). Thus, recombinant FlaA, FlaB, FlaD, and FlaE were expressed in *Esch. coli,* purified and injected intraperitoneally into Japanese flounder. The result was that recombinant FlaB led to a significantly higher RPS and serum antibody titre than the other three proteins (Jia et al. 2013).

Pseudomonas P1SW and *Vibrio* V3SW were recovered from seawater and administered live separately or together by immersion and orally (the bacteria were embedded in sodium alginate microspheres) to turbot (*Scophthalmus maximus*) at doses equivalent to 2×10^8 CFU with evidence that both organisms became distributed in the internal organs, and some protection became apparent after challenge.

Better protection was reported with both bacteria administered together (Wang et al. 2013). Is this an example of a heterologous vaccine or a probiotic?

Bystander Effect A sublethal dose of *V. anguillarum* VIB1 given intraperitoneally to rainbow trout led to the transfer of resistance by inter-animal communication of one or more unknown signals to naive fish (= 'bystander' fish) that had never been exposed to the pathogen. Thus, challenge with a lethal dose of *V. anguillarum* led to less mortalities (60 %) over a longer time span than the controls (100 % mortalities). The communication signal caused an abrupt and transient increase in intracellular calcium and a corresponding decrease in clonogenicity in a reporter assay (Mothersill et al. 2015).

Probiotics Putative *Aeromonas* and *Vibrio*, from halibut, have been found to inhibit the growth of fish pathogenic *Vibrio* (Bergh 1995). The relative incidence of microbial antagonists is indicated from a study of >400 bacterial isolates from the gastro-intestinal tract and surface of turbot and fish food and water, in which 28 % (mostly from the intestinal mucus) were inhibitory to *V. anguillarum* (Westerdahl et al. 1991). An isolate of *V. alginolyticus,* previously used as probiotic in Ecuadorian shrimp hatcheries, has been effective at controlling diseases caused by *V. anguillarum* (Austin et al. 1995b). *Vagococcus fluvialis* protected sea bass against vibriosis, achieving an RPS of 42.3 % (Sorroza et al. 2012). Similarly, *Pediococcus pentosaceus* was fed to orange-spotted grouper (*Epinephelus coioides)* leading to enhanced growth, immunostimulation and protection against challenge (Huang et al. 2014). Furthermore, *Lactococcus lactis* subsp. *lactis* was fed to *Artemia* nauplii, which in turn were fed to European sea bass (*Dicentrarchus labrax)* larvae for 5 consecutive days leading to significantly improved survival after challenge (survival = 81 % compared to 24 % of the controls) (Touraki et al. 2013).

Dietary Supplements/Immunostimulants The use of bovine lactoferrin, dosed orally at 100 mg/kg for 3 days enhanced the resistance of rainbow trout to subsequent challenge by *V. anguillarum* (Sakai et al. 1993). Peptidoglycan, derived from *Bifidobacterium thermophilus,* was administered in feed (fed at 3 % of body weight daily) at 0.2 and 2 mg/kg to rainbow trout of 0.12 g average weight for 56 days (Matsuo and Miyazono 1993). These doses were the equivalent of 6 or 60 μg of peptidoglycan/kg body weight of fish/day. Sub-groups of the fish were challenged on day 26 and 56 by immersion in *V. anguillarum,* with mortalities monitored over a 21 day period. At the halfway point of the feeding trial, survival following challenge with *V. anguillarum* was markedly higher than among the controls. Yet at day 56, there was not any apparent difference in survival between the experimental groups and controls. So, it would appear that the benefits of this approach were short-lived, and in the long term were not beneficial (Matsuo and Miyazono 1993). Feeding 4 g of mannan oligosaccharides/kg of feed to European sea bass (*Dicentrarchus labrax)* juveniles for 8-weeks before challenge led to a reduction in mortalities from 66 % to 12.5 % (Torrecillas et al. 2012).

Inhibitors of Quorum Sensing *V. anguillarum* produces quorum sensing molecules, which may well be involved with the regulation of virulence. If these molecules could be blocked using quorum sensing inhibitors, then virulence could be reduced or even annulled. Funanone C-30, dosed at 0.01 or 0.1 μM. was determined to be beneficial at reducing mortalities caused by cohabitation with *V. anguillarum* infected rainbow trout from 80–100 % in the controls to 4–40 % in treated groups (Rasch et al. 2004).

Bacteriophage Therapy Two bacteriophage preparations (10^8 PFU/ml) were applied to tank water, and controlled *V. anguillarum* infection in zebra fish (Silva et al. 2014).

Antimicrobial Compounds Antimicrobial compounds, including florfenicol (Seljestokken et al. 2006) have proved to be very useful in controlling vibriosis. It is perhaps ironic that emphasis has been placed on using drugs as food additives, because vibriosis is typified by inappetance. Consequently, antimicrobial compounds need to be administered (by food) very early in the disease cycle, if success is to be achieved. Workers have indicated the value of many compounds, including chloramphenicol, flumequine (Vik-Mo et al. 2005), furanace, nitrofurazone, oxolinic acid, oxytetracycline and sulphamerazine. As a general comment, we advise upon caution when contemplating the need for pharmaceuticals, particularly antibiotics, because of the potential risk of resistance which may be attributed to plasmids, i.e. R factors (Aoki 1988). Aoki et al. (1974) reported that 65/68 *V. anguillarum* isolates carried R factors, conveying resistance to chloramphenicol, streptomycin, sulphonamides and tetracycline. Therefore if R factors abound, it is unlikely that the common antibiotics will do much to retard the disease cycle.

Another approach has involved the use of antimicrobial peptides, namely a cecropin-melittin hybrid peptide and pleurocidin amide, which is a C-terminally amidated form of a natural flounder peptide. These were applied continuously at a rate of 200 μg/day and 250 μg/for for cecropin-melittin hybrid peptide and pleurocidin amide respectively by miniosmotic pumps installed in the peritoneal cavity of coho salmon with the result that less mortalities occurred compared to the controls. In the case of pleurocidin amide, 5 % mortalities were recorded compared to 67–75 % of the controls (Jia et al. 2000).

Vibrio cholerae (non-O1)

Characteristics of the Disease

During the summer of 1977, an epizootic occurred in a wild population of ayu in the River Amano, Japan. From diseased animals, an organism conforming to the description of *V. cholerae* was isolated (Muroga et al. 1979; Kiiyukia et al. 1992). Subsequently, *V. cholerae* was associated with a disease of goldfish in Australia

(Reddacliff et al. 1993). The organism has been linked with ascites disease in cultured Mandarin fish (*Siniperca chuatsi*) (Cao et al. 2013). *V. cholerae* non-O1/non-O139 was isolated from Cardinal tetra (*Paracheirodon axelrodi*) fry and adult Raphael catfish (*Platydoras costatus*), which were from aquaria in the Czech Republic. Also, disease was recorded in a range of wild fish (Rehulka et al. 2015).

Petechial haemorrhages developed on the body surface. Internally, there was congestion of the organs (Muroga et al. 1979; Kiiyukia et al. 1992). Reddacliff et al. (1993) reported that septicaemia developed in infected goldfish. Diseased common nase and chub revealed haemorrhages, erythema and hyperaemia in the abdominal region, within the mouth and at the base of the finage (Rehulka et al. 2015).

Isolation

This may be achieved by inoculating kidney material (from swabs) onto the surface of nutrient agar plates, with incubation at 25 °C for an undisclosed period (presumably 2–5 days) (Muroga et al. 1979; Kiiyukia et al. 1992).

Characteristics of the Pathogen

Box 10.9: *Vibrio cholerae*
Cultures comprise small (1.5–3.0 × 0.7–1.0 μm in size) Gram-negative, fermentative rods, which are motile by single polar flagella. Growth occurs in 0–6 % (w/v) sodium chloride, at 10–42 °C, and at pH 7–10. Catalase, ß-galactosidase, indole, lysine decarboxylase and oxidase are produced, but not arginine dihydrolase, H_2S, ornithine decarboxylase or phenylalanine deaminase. Aesculin, blood (haemolysis) chitin, gelatin, lipids and starch, but not urea, are degraded. The methyl red test and Voges Proskauer reaction are positive. Nitrates are reduced. Citrate, fructose, galactose, glucose, maltose, sucrose and tartrate are utilised, but not adonitol, arabinose, cellobiose, dulcitol, inositol, inulin, malonate, mannose, melezitose, melibiose, raffinose, rhamnose, salicin, sorbitol or xylose. Acid is produced from sucrose, but not lactose. Growth occurs at 37 °C and in 0 % but not 7 % (w/v) sodium chloride. Sensitivity is recorded to the vibriostatic agent, O/129. The G + C ratio of the DNA is 47–49 moles % (Muroga et al. 1979; Yamanoi et al. 1980; Kiiyukia et al. 1992).

The phenotypic traits published by Muroga et al. (1979) and Kiiyukia et al. (1992) were in close agreement with the description of *V. cholerae* (Farmer et al. 2005). Indeed, there were only two discrepancies with the characteristics of the ‘El

Tor' biotype, i.e. production of ornithine decarboxylase and utilisation of mannose (these were reported as negative for the fish pathogenic isolates). However, the fish isolates did not react, by slide agglutination, with antisera to *V. cholerae*. Therefore, the conclusion was that the fish pathogenic isolates comprised *V. cholerae* non-O1. It should be emphasised, however, that the fish isolates demonstrated 86% DNA:DNA homology with a reference strain of *V. cholerae*. This coincided well with the intraspecific homology values of *V. cholerae* (Citarella and Colwell 1970). Again, there was reasonably close agreement between *V. cholerae* and the isolates studied by Reddacliff et al. (1993). Yet, a detailed taxonomic study by Austin et al. 1997) reported heterogeneity among 6 isolates received as *V. cholerae,* insofar as they were recovered as single member ribotype clusters, and displayed diverse BIOLOG-GN fingerprints and API 20E profiles. However, 5 cultures corresponded with *V. cholerae* serogroups, namely O8, O9, O23, O32 and O63.

Epizootiology

V. cholerae survives in the aquatic environment. For example at 25 °C, strain PS-7701 survived for 32 days in fresh water, saline Ringers buffer, and normal strength and diluted seawater. Survival was considerably reduced at 2 °C (Yamanoi et al. 1980); an observation which coincides with the findings of Singleton et al. (1982a, b). It is likely that infection occurs via the water borne route, insofar as *V. cholerae* appears to inhabit the aquatic environment (Lee et al. 1982; West and Lee 1982).

Pathogenicity

The evidence suggests that *V. cholerae* is a highly virulent fish pathogen, insofar as ayu and eels may be infected following immersion in only 1.26×10^4 cells/ml and 1.26×10^2 cells/ml, respectively. Yamanoi's team noted that with ayu, mortalities began in 2 to 7 days at water temperatures of 21 and 26 °C, but no deaths occurred if the water temperature was at 16 °C. In comparison, an eel population suffered 10% mortalities within 5 days at a water temperature of 21 °C, and 30% deaths in 3–7 days at 26 °C. Clearly, this information suggests that *V. cholerae* is likely to be troublesome only in higher water temperatures.

Vibrio furnissii

There has been an indication that *V. furnissii* may be associated with eel disease in Spain (Esteve 1995). However, isolates were recovered from water rather than diseased eels. Therefore, the association with fish pathology is dubious.

Vibrio harveyi (= V. carchariae = V. trachuri)

Characteristics of the Diseases

A comparative newcomer to the growing list of vibrio fish pathogens, *V. carchariae* was originally isolated from a dead sandbar shark (*Carcharhinus plumbeus)* which died at the National Aquarium in Baltimore, USA, in 1982 (Grimes et al. 1984a). Subsequently, a similar organism was recovered from lemon sharks (*Negraprion brevirostris)* (Colwell and Grimes 1984). Grimes et al. (1984b) and Colwell and Grimes (1984) described the disease as a 'vasculitis'. Infected animals became lethargic, stopped feeding, appeared disorientated, and developed necrotic subdermal cysts. On postmortem examination, encephalitis, meningitis, kidney necrosis, vasculitis, and unspecified liver and spleen damage were noted. Evidence has been forthcoming that the pathogen is more serious in compromised than healthy hosts (Grimes et al. 1985). A similar organism has been isolated from a chronic skin ulcer on a shark (Bertone et al. 1996). *V. carchariae* was associated with gastro-enteritis leading to heavy mortalities among cultured groupers *(Epinephelus coioides)* during 1993 in Taiwan (Yii et al. 1997). One isolate, EmI82KL, was noted to be motile, did not auto-agglutinate, but was haemolytic to grouper, rabbit, sheep and tilapia blood.

A parallel development involved game fish, namely common snook *(Centropomus undecimalis)*. These were found to suffer with opaque white corneas within 24 h of capture in Florida, USA (Kraxberger-Beatty et al. 1990). From such damaged specimens, *V. harveyi* was recovered. Common snoop developed opaque white corneas within 24 h of capture. In the absence of treatment, blindness resulted. With a second species, jack crevalle (*Caranx hippos),* deep dermal lesions were noted in wild specimens, which were also captured in Florida. Internal abnormalities were not recorded (Kraxberger-Beatty et al. 1990). In another example from an aquarium in Barcelona, eye lesions in the short sunfish *(Mola mola)* due to biting by other fish – were colonised by *V. harveyi* (Hispano et al. 1997). Other diseases include:

- Flounder infectious necrotizing enteritis, which is characterised by distended abdomens filed with opaque fluid, enteritis, necrosis of the posterior intestine (in extreme cases, this was detached from the anus and exiting via the vent), reddening in the vicinity of the anus, lethargy and inappetance has been documented in farmed summer flounder in Rhode Island, USA (Soffientino et al. 1999; Gauger et al. 2006).
- Skin ulcers and haemorrhaging in the vicinity of the mouth and fins has been reported in sole *(Solea senegalensis)* in Spain (Zorrilla et al. 2003).
- Infectious gastro-enteritis, which has been reported in cultured red drum *(Sciaenops ocelatus)* from Taiwan (Liu et al. 2003). Disease signs included swollen intestine containing yellow fluid.
- Nodular lesions in the branchial chamber and operculum, and granuloma in tiger puffer (*Takifugu rubripes*) in Japan (Mohi et al. 2010).

A disease, resembling vibriosis and equated to a new species *V. trachurii,* has been long associated with Japanese horse mackerel *(Trachurus japonicus)* especially during summer when the seawater temperature exceeds 25 °C (Iwamoto et al. 1995). Infected fish displayed erratic swimming, darkened in colour, developed pronounced bilateral exophthalmia, and developed haemorrhages on the internal organs. However, the organism is now recognised as synonym of *V. harveyi* (Thompson et al. 2002).

The organism has been recovered in mixed communities with *V. anguillarum* and *V. chagassi* from sand melts with skin haemorrhages (Fabbro et al. 2011).

There has been a gradual awareness of the increasing significance of *V. harveyi* as a killer of marine fish and penaeids. Saeed (1995) blamed *V. harveyi* with causing mortalities in cultured brown spotted grouper *(Epinephelus tauvina)* and silvery black porgy *(Acanthopagrus cuvieri)* in Kuwait.

Isolation

Samples of kidney and liver were inoculated into thioglycollate broth (Appendix in Chap. 12), followed by single colony isolation on plates of TSA supplemented with 1 % (w/v) sodium chloride (Grimes et al. 1984a). Yii et al. (1997) used TSA supplemented with 2 % (w/v) sodium chloride and TCBS with intestinal fluid from diseased grouper. Generally, the use of TSA, MacConkey agar, TCBS and cytophaga agar were advocated, with incubation at 25–35 °C for 24–48 h. Thus according to Kraxberger-Beatty et al. (1990), the eyes were removed and homogenised in sterile saline; loopfuls of the material being streaked onto the agar-containing media. It should be emphasised that attempts were not made to surface sterilise the eyes. Therefore, the resulting homogenate would have also contained aquatic (saprophytic) bacteria, as well as any potential pathogen.

Characteristics of the Pathogen

Twelve cultures were recovered from corneas, and were identified as *V. harveyi* as a result of biochemical tests and DNA:DNA hybridisation (90–94 % DNA homology with the type strain of *V. harveyi).* Certainly, the characteristics of the isolates were in good accord with the species description of *V. harveyi* (Farmer et al. 2005). A result of DNA:DNA hybridisation, Ishimaru and Muroga (1997) determined that pathogenic vibrios recovered from milkfish in Japan were indeed *V. harveyi.*

The synonymy of *V. harveyi* and *V. carchariae* was realised by Pedersen et al. (1998) as a result of phenotypic and genotypic studies, and by Gauger and Gómez-Chiarri (2002) from 16S rDNA sequencing. In terms of taxonomic standing, *harveyi* has precedence, and therefore this name will be used in preference to *carchariae.*

Box 10.10: *Vibrio harveyi*
V. harveyi shows similarities to *V. alginolyticus,* principally because of the presence of swarming on agar medium. Essentially, cultures comprise pleomorphic fermentative Gram-negative rods (1.0–1.6 × 0.5–0.7 μm in size), which are motile by polar and/or lateral flagella. Catalase, indole, lysine and ornithine decarboxylase and oxidase are produced, but not arginine dihydrolase. The Voges Proskauer reaction is negative. Nitrates are reduced. Alginate, blood, DNA, gelatin and lecithin are degraded, but not aesculin, casein, cellulose, pectin or starch. Many compounds are utilised, including arabinose, aminobutyrate, cellobiose, ethanol, glucose, glycine, α-ketoglutarate, propanol, sucrose and trehalose but not from inositol or lactose. Growth occurs in 3–8 % but not 0 % or 10 % (w/v) sodium chloride, and at 11–40 °C. Sensitivity is demonstrated to 150 μg but not to 10 μg of the vibriostatic agent, O/129. The G + C ratio of the DNA is 45–47 moles%. The only differences with the description of *V. carchariae* centre on the degradation of starch *(V. carchariae* = +), and growth in 7 % (w/v) sodium chloride *(V. carchariae* = −). The G + C ratio of the DNA of *V. carchariae* is 47 moles%.

Results of DNA:DNA homology experiments showed a high degree of homology with *Ph. damselae,* at 88 % re-association. Eight RAPD and 13 ribotypes were recognised by Pujalte et al. (2003). Phenotypic and genotypic traits point to relatedness between *V. harveyi* and *V. campbellii* (Gomez-Gil et al. 2004). Moreover, these authors considered that some cultures of *V. harveyi* should more correctly have been identified as *V. campbellii.* This raises the question about the role of *V. campbellii* in fish pathology. This general theme about the accuracy of identification to vibrios within the Harveyi clade has been voiced by others (Hoffmann et al. 2012).

Diagnosis

Molecular methods. Problems with the accuracy of phenotypic-based identification of pathogens, such as *V. harveyi,* may be overcome by 16S rDNA sequencing (Ransangan and Mustafa 2009). A high level of specificity and sensitivity (detection limit = 4.0×10^3 cells/ml) involved the use of *toxR* gene for the detection of *V. harveyi;* the PCR of which took <5 h to enact and selectively recognised 20 authentic representatives of the taxon (including cells in diseased fish tissues) but not representatives of other vibrios (Pang et al. 2006). In another development, multilocus sequence analysis was described as a reliable and rapid means of identification (Cano-Gomez et al. 2011).

Pathogenicity

Infected (i.p. injection) dogfish (*Squalus acanthias)* died within 18 h. Lemon sharks were more resistant to infection, although internal damage followed the injection of 5×10^7 cells. Yii et al. (1997) reported an LD_{50} dose for grouper of 2.53×10^7 colony forming units (CFU)/g of body weight, with disease signs reminiscent of the natural infection, i.e. swollen intestine full of a yellowish fluid. This was higher than the LD_{50} value of 1.5×10^5 to 1.6×10^6 CFU/fish reported for sea bass (Pujalte et al. 2003). For comparison, Won and Park (2008) determined the LD_{50} dose to olive flounder (*Paralichthys olivaceus*) and black rockfish (*Sebastes schlegeli*) as $2.48 \times \sim 10^5$ – $8.76 \times \sim 10^7$ and $2.0 \times \sim 10^4$ – $2.52 \times \sim 10^6$ CFU/g of fish, respectively. Although the pathogenic mechanism remains to be fully elucidated, it is relevant to note that the organism is slightly cytotoxic and produces ECPs (Zhang and Austin 2000; Zorrilla et al. 2003; Won and Park 2008) containing haemolysins (Zhang and Austin 2000; Zhu et al. 2006; Li et al. 2011), caseinase, gelatinase, lipase and phospholipase (Zhang and Austin 2000). A possible relationship between bioluminescence, quorum sensing and pathogenicity is unclear (Defoirdt et al. 2008). Of relevance, Zhang et al. (2001) correlated virulence to salmonids with the possession of duplicate haemolysin genes, termed *vhhA and vhhB*. The VHH haemolysin protein demonstrated homology to the lecithinase of other vibrios, namely *V. mimicus* and *V. cholerae. vhhA* was overexpressed in *Esch. coli,* and the purified protein was characterized, and determined to be cytotoxic to flounder gill cells and lethal to flounder with an LD_{50} dose of 18.4 μg of protein/fish (Zhong et al. 2006). Three strains, VJ1, VJ2 and VJ3, recovered from diseased Japanese flounder in China demonstrated significantly increased resistance to the bactericidal effects of host serum and induced greater levels of respiratory burst activity in head kidney macrophages although there was an inability to replicate in the macrophages. In cultured cells, apoptosis was induced as observed by DNA fragmentation, apoptotic bodies and elevated caspase 3 activity (Li et al. 2011). Using black sea bream *(Mylio macrocephalus)* and silver sea bream *(Sparus sarba)* fibrobast cell lines, recombinant VHH induced apoptosis involving a decrease in mitochondrial membrane potential, and thence an increase in caspase 3 activity within 2–8 h, although there was not observed effect on ROS. However, HSP70 levels were increased followed by 6 h of recovery. The reduction in mitochondrial membrane potential was suppressed when cells had a 6 h heat shock recovery period. Moreover, the protective effect of HSP70 was annulled if cells were exposed to the HSP70 inhibitor, quercetin. Thus, HSP70 may be attributed with an anti-apoptotic role. Overall, the research highlighted that haemolysin leads to cell death by induction of apoptosis (Deane et al. 2012).

Parvathi et al. (2009) pointed to the heterogenicity of the *vhh* gene in Indian isolates, albeit from shrimp. Sun et al. (2001) reported that the loss of a single residue change in haemolysin resulted in loss of haemolytic and phospholipase activities and thus pathogenicity to turbot. Also, siderophores are produced (Owens et al. 1996). More recently, research has highlighted a role for bacteriophage (Oakey and

Owens 2000; Austin et al. 2003) and bacteriocin-like substances (BLIS; Prasad et al. 2005). The *Vibrio harveyi* myovirus like (VHML) phage enhanced virulence of *V. harveyi* to Atlantic salmon and enhanced haemolytic activity (Austin et al. 2003). Using a culture that was pathogenic for salmonids, it was revealed that BLIS was inhibitory to four other isolates of the same taxon and to representatives of other vibrios, including *V. parahaemolyticus*. The BLIS was extracted from cell-free supernatants, and determined to be an unique protein of ~32 kDa molecular weight (Prasad et al. 2005).

Comparatively high doses of the organism labeled as *V. trachuri* were required to cause disease in Japanese horse mackerel. Thus using 36.8 g fish at a water temperature of 26 °C, 1.1×10^8 cells/fish caused 100 % mortalities within 24 h of i.p. injection. A dose of 1.1×10^7 cells/ml led to 50 % mortalities within 4 days. By immersion in 3.6×10^7 cells/ml for 2 min, 100 % mortalities ensued within 3 days (Iwamoto et al. 1995). The disease signs mimicked those on naturally infected fish, i.e. erratic swimming and melanosis. Incidentally, the organism failed to infect red sea bream.

The question about the host response to infection by *V. harveyi* was addressed in turbot (*Psetta maxima*) model whereby SSH identified immune gene expression (to the infection) by a major histocompatibility complex class 1a gene, and a heat shock protein 70 gene. This may be an important component in the immune system insofar as a rapid transcriptional upregulation after infection may be important to fish survival (Zhang et al. 2011c). Also, some signaling molecules were found in cDNA libraries, and included src-family tyrosine kinase SCK, sgk-1 serine-threonine protein kinase and amyloid precursor-like protein 2 (Wang et al. 2008).

Disease Control

Vaccine Development Vaccine development programmes have not been especially successful in the past, although this situation appears to be slowly changing (Xu et al. 2009) with the realisation that subcellular components, e.g. ECPs and OMPs, may elicit an immune response (Arijo et al. 2008). A whole cell preparation, which was applied to barramundi *(Lates calcarifer)* by i.p. injection, anal intubation and immersion, led to antibody production thereby demonstrating that fish could respond to vaccination (Crosbie and Nowak 2004). By expressing the *HL*1 gene, which encodes the haemolysin from *V. harveyi,* in yeast *(Saccharomyces cerevisiae)*, the protein (= haemolysin) was expressed on the cell surface and was active against flounder erythrocytes. Moreover, serum from flounder that had received the live modified yeast cells by i.p. injection revealed haemolytic activity. Challenge experiments demonstrated that flounder and turbot were protected soon after administration of yeast and then exposure to a virulent culture of *V. harveyi* (Zhu et al. 2006). VhhP2, which is an OMP, has been used successfully as a sub-unit vaccine suspended in *Bacillus* sp. B187 as adjuvant with success when injected i.p. in 100 µl amounts in Japanese flounder followed by a booster 20 days later, and challenge after a further 14 days. A live recombinant vaccine, which presents VhhP2, was

administered i.p. (RPS = 92.3 %) and orally (RPS = 61.2 %) (Sun et al. 2009). Pang et al. (2010) used OmpN mixed with FCA to vaccinate via i.p. (100 µl/fish), estuary cod (*Epinephelus coioides*), that survived challenge after 28-days with a virulent culture (RPS = 60 and 70 % depending on the nature of the challenge strain). Meanwhile, Zhang et al. (2011a) cloned, sequenced and characterised two OMP genes, *OmpK* and *GADPH,* and expressed the recombinant proteins in the prokaryotic expression vector pET-30a(+), which were purified and used to vaccinate intraperitoneally (100 µg/fish) large yellow croaker (*Pseudosciaena crocea*) with booster doses after 3-weeks. The outcome was that r-OmpK and r-GADPH led to RPS values of 37.7 % and 40 %, respectively, after challenge. OmpK was expressed in yeast, *Pichia pastoris,* and fed for 5 days to sea bass (*Lateolabrax japonicus*) on alginate microspheres with a resultant production of antibodies, and protection against challenge (RPS = 61.5 %) (Mao et al. 2011). A denatured inactive cytotoxic 530 amino acid recombinant secreted protease, Vhp1, was recovered from a pathogenic isolate was determined to be an effective subunit vaccine (RPS = 70 %) with improved performance when expressed in *Esch. coli* as a live vaccine (RPS = 90 %) (Cheng et al. 2010). In a further study, Wang et al. (2011) used the purified 35 kDa outer membrane protein OmpU (50 µg dose) and a DNA vaccine involving the insertion of the *ompU* gene into pEGFP-N1 plasmid (10 µg) injected i.m. in turbot. Use of the purified OmpU led to complete protection after challenge 5 weeks later (RPS = 100 %) whereas lesser protection resulted with the DNA vaccine (RPS = 51.4 %). Two potentially protective immunogens, DegQ and Vhp1, have been accommodated in DNA vaccines and used to vaccine Japanese flounder with promising results for pDV after challenge (RPS = 84.6 %) (Hu and Sun 2011). Hu et al. (2011) continued the work by developing a recombinant product that expressed *V. harveyi* DegQ as a soluble antigen that elicited significant protection against both *Edw. tarda* and *V. harveyi* in laboratory-based experiments with turbot when administered by i.p. (RPS = 90.9 %) orally (RPS = 60.5 %) or immersion (RPS = 47.1 %) or a combination of oral plus immersion (RPS = 77.8 % after one month in a mock field trial; RPS = 81.8 % after two months). A serine protease has been reported as a protective antigen, and enabling fish to resist challenge (Zhang et al. 2008).

The value of whole cell preparations was enhanced by entrapment within liposomes with the outcome being increased protection and a demonstrable stimulation of immune parameters (Harikrishnan et al. 2012b).

A bivalent vaccine (with *Ph. damselae* subsp. *piscicida)* based on formalised cells and ECP administered to sole by immersion with booster or by i.p. injection led to high levels of protection (RPS = ~88 %) for 4 months after which the benefit declined (Arijo et al. 2005). A combination vaccine with inactivated cells of *V. alginolyticus*, *V. harveyi*, *V. vulnificus* and infectious spleen and kidney necrosis virus was evaluated in orange-spotted grouper (*Epinephelus coioides)* with an overall RPS of 80 % (Huang et al. 2012).

Probiotics

V. harveyi has been a target for probiotics with a number of micro-organisms demonstrated to be successful at prophylaxis if administered as feed additives (e.g. Geng et al. 2012).

Dietary Supplements

Chaga Mushroom An ethanol extract of Chaga mushroom (*Inonotus obliquus*) fed at 1.0 and 2.0 % to kelp grouper (*Epinephelus bruneus*) for 30 days led to weight gain, enhanced lysozyme, respiratory burst, anti-protease and serum bacteriocidal activity and increased total protein albumin, globulin, erythrocyte, leukocyte, lymphocyte, monocyte, haemoglobin, and haematocrit levels, and less mortalities after challenge with *V. harveyi* (Harikrishnan et al. 2012a).

Japanese Pepper Tree The benefit of using Japanese pepper tree *(Zanthoxylum piperitum)* as a dietary supplement dosed at 1.0 and 2.0 % to kelp grouper was advocated with results revealing immunostimulation and reduction in mortalities after experimental challenge (Harikrishnan et al. 2012b).

Neem Neem (*Azadirachta indica*) leaf was fed to Asian sea bass (*Lates calcarifer*) fingerlings at 1–5 g/kg of feed for 2-weeks leading to improved growth, immunostimulation (increased anti-protease, bacteriocidal, lysozyme and phagocytic activity, and superoxide anion production), and protection against experimental challenge (Talpur and Ikhwanuddin 2013).

Kudzu The dietary administration of 1.0–2.0 % kudzu (*Pueraria thunbergiana*) extract for 1–4 weeks to kelp grouper led o weight gain, immunostimulation (erythrocyte and leucocyte counts, phagocytic ratio/index, and enhanced anti-protease bacteriocidal, lysozyme and superoxide anion activities) and protection from challenge (Harikrishnan et al. 2012a, b, c, d, e).

Chitooligosaccharides Dietary chitooligosaccharides dosed at 4 g/kg of feed for 8 weeks enhanced growth and immune function (leukocyte count, and lysozyme, respiratory burst and superoxide dismutase activities) protected ovate pompano (*Trachinotus ovatus)* against infection with *V. harveyi* (Lindell et al. 2012).

Garlic The value of garlic (*Allium sativum*) was reinforced by its use to control infection in Asian sea bass (*Lates calcarifer*). In this study, garlic was added to diets at 10 g/kg of feed, and fed to fish daily for two weeks, with the outcome that the better growth occurred, and survival after challenge was improved (survival of the experimental group = 83.35 % compared with 33.3 % of the controls). Also, immune

parameters were enhanced, and included the numbers of erythrocytes and leucocytes, haematocrit, haemoglobin, albumin, globulin and serum protein, and anti-protease, bacteriocidal, lysozyme, phagocytic and respiratory burst activities. (Talpur and Ikhwanuddin 2012).

Ginger Ginger (*Zingiber officinale*) was fed to Asian sea bass (*Lates calcarifer*) at 5 and 10 g of ginger/kg of feed for 15 days leading to improved growth, immunostimulation (increased numbers of erythrocytes and leucocytes, and enhanced anti-protease, bacteriocidal, lysozyme, phagocytic and respiratory burst activities) and survival after challenge (86.6 %) compared to the control (26.7 %).

Peppermint Peppermint (*Mentha piperita*) was fed at 1–5 g/kg to Asian sea bass (*Lates calarifer*) for 4-weeks leading to improved growth, immunostimulation (increased erythrocyte and leucocyte counts, and enhanced haematocrit haemoglobin, serum protein and globulin, and anti-protease, bacteriocidal, lysozyme, phagocytic activity and respiratory burst, activities) and survival from challenge (Talpur 2014).

Inhibitors of Quorum Sensing

V. harveyi produces quorum sensing molecules, which may well be involved with the regulation of virulence. If these molecules could be blocked using quorum sensing inhibitors, then virulence could be reduced or even annulled. The 28 kDa AiiA protein of *B. thuringiensis* disrupted quorum sensing in *V. harveyi* (Bai et al. 2008).

Antimicrobial Compounds

Kraxberger-Beatty et al. (1990) reported success with Prefuran (Argent) dosed at 0.1 mg/l for an unspecified period. Saeed (1995) found success with oxytetracycline as a food additive. Yii et al. (1997) determined susceptibility to a wide range of inhibitory compounds, including chloramphenicol, doxycycline, nalidixic acid, oxolinic acid, oxytetracycline and sulphonamide, but not ampicillin or penicillin G.

Vibrio ichthyoenteri

Characteristics of the Disease

Since, 1971, opaque intestines and intestinal necrosis, i.e. enteritis, accompanied by high mortalities have been reported in Japanese and Korean hatcheries rearing Japanese flounder (Ishimaru et al. 1996; Kim et al. 2004) and olive flounder (Lee et al. 2012).

Characteristics of the Pathogen

V. ichthyoenteri was described as a result of an examination of 7 isolates from flounder larvae (Ishimaru et al. 1996).

Box 10.11: *Vibrio ichthyoenteri*
Cultures are non pigmented on marine 2216E agar, but produce yellow colonies on TCBS, which contain Gram-negative fermentative rods, which are motile by single polar flagella. Catalase and oxidase are produced, but not arginine dihydrolase, ß-galactosidase, H_2S, indole or ornithine decarboxylase. Nitrates are reduced. Neither agar, chitin, gelatin, lipids nor starch are degraded. Polyhydroxybutyrate is not accumulated intracellularly. Growth occurs at 15–30 °C but not at 4 or 35 °C, and in 1–6 % but not 0 or 8 % (w/v) sodium chloride. Acid is produced from fructose, D-glucose, maltose, D-mannose, sucrose and trehalose, but not from adonitol, L-arabinose, D-cellobiose, dulcitol, erythritol, D-galactose, glycerol, inulin, inositol, lactose, D-mannitol, melibiose, raffinose, L-rhamnose, salicin or D-sucrose. Neither D-cellobiose, citrate, D-gluconate, L-leucine not D-xylose are utilised. Sensitivity is recorded to the vibriostatic agent, O/129. The G+C ratio of the DNA is 43–45 mol %.

Control

Susceptibility was recorded to potentiated sulphonamides, namely trimethoprim-sulphamethoxazole (Lee et al. 2012).

Vibrio mimicus

Characteristics of the Disease

V. mimicus has been mentioned a cause of ascites disease in fish (Cen et al. 2013), notably grass carp (*Ctenopharyngodon idella*) (Zhang et al. 2014b). In cultured yellow catfish (*Pelteobagrus fulvidraco*) in China, there was an association with skin ulcers. The fish became lethargic, inappetant, leading to 70–100 % mortalities. Internally, there was extensive hemorrhaging in the abdominal wall, and enlargement of the gall bladder, kidney, liver and spleen (Zhang et al. 2014b).

Isolation

Muscle from below the diseased skin was inoculated onto 5 % (v/v) sheep blood in TSA and TCBS with incubation at 28 °C for 48 h (Zhang et al. 2014b).

Characteristics of the Pathogen

Box 10.12: *Vibrio mimicus*
Colonies are green on TCBS, and white on blood agar (ß-haemolytic). Cultures contain Gram-negative curved rods, which grow in 0–6 % (w/v) sodium chloride. Lysine decarboxylase and indole are produced, but not arginine dihydrolase or H_2S. The Voges-Prokauer reaction is negative. Urea is not attacked. Sequencing the 16S rRNA gene reveals 99.71 % homology with the type strain of *V. mimicus* (and 99.42 % homology with the type strain of *V. cholerae)* (Zhang et al. 2014b).

Pathogenicity

Experimental challenge with yellow catfish confirmed, pathogenicity with an LD_{50} dose of 3.39×10^6 CFU/fish by i.p. injection when skin ulceration developed (Zhang et al. 2014b).

Disease Control

Vaccine OmpU has been recognized to be an important antigen, and upon i.p. injection led to specific antibody production and protection against challenge. OmpU must, therefore, be considered as possible candidate for an effective vaccine (Cen et al. 2013).

Vibrio ordalii

Characteristics of the Disease

Disease caused by *V. ordalii* has been documented in Japan (e.g. Muroga et al. 1986) and the Pacific Northwest, USA. Essentially, the disease may be categorised as a haemorrhagic septicaemia. However, there are subtle differences in the pathologies of the diseases caused by *V. anguillarum* and *V. ordalii*. In the case of *V. ordalii* in

Pacific salmon, there is a tendency for the formation of micro-colonies in the skeletal and heart muscle, gill tissue, and in both the anterior and posterior regions of the gastro-intestinal tract (Ransom 1978; Ransom et al. 1984). Moreover, bacteraemia developed much later in the disease cycle than with *V. anguillarum.* Perhaps, this accounted for the lower numbers of bacterial cells in the blood. A further difference concerned the marked decrease in the numbers of leucocytes in the blood, i.e. leucopenia (Ransom 1978; Harbell et al. 1979; Ransom et al. 1984).

Isolation

As with *V. anguillarum,* isolation involves use of seawater agar and TCBS with incubation at 15–25 °C for up to 7 days (Ransom 1978; Ransom et al. 1984).

Characteristics of the Pathogen

The establishment of a new species to accommodate strains previously classified as *V. anguillarum* biotype II, i.e. *V. ordalii* (Schiewe 1981; Schiewe et al. 1981), generated an awareness that vibriosis could be caused by more than one bacterial taxon.

Box 10.13: *Vibrio ordalii*
Cultures comprise fermentative Gram-negative curved rods of 2.5–3.0 × 1.0 µm in size, motile by means of single polar flagella. Growth occurs quite slowly, insofar as 4–6 days incubation at 22 °C are required for the production of off-white, circular, convex colonies of 1–2 mm in diameter on seawater agar. *V. ordalii* is, however, is not especially active. Catalase and oxidase are produced, but not arginine dihydrolase, ß-galactosidase, H_2S, indole, lysine or ornithine decarboxylase, or phenylalanine deaminase. DNA, chitin (by some isolates) and gelatin are degraded, but not aesculin, lipids, pectate, starch or urea. Nitrates are reduced by some isolates. The methyl red test and Voges Proskauer reaction are negative. Tartrate is utilised. Only a few carbohydrates, e.g. galactose (variable result), maltose, mannitol and sucrose are attacked with the production of acid. Negative responses are recorded for adonitol, arabinose, cellobiose, dulcitol, erythritol, glycerol, inositol, lactose, melibiose, raffinose, rhamnose, salicin, sorbitol, trehalose and xylose. Growth occurs at 15–22 °C but not at 37 °C and in 0.5–3.0 % but not 0 % or 7 % (w/v) sodium chloride. Sensitivity is recorded to the vibriostatic agent, O/129. The G + C ratio of the DNA is 43–44 moles % (Schiewe 1981; Schiewe et al. 1981; Austin et al. 1997).

V. ordalii was homogeneous by plasmid profiling, ribotyping and serogrouping, accommodated two LPS groups, and were heterogeneous by BIOLOG-GN fingerprints and API 20E profiles (Austin et al. 1997).

Results of DNA:DNA hybridisation studies have confirmed the homogeneity and validity of *V. ordalii,* with intraspecific homologies of approximately 80 %. There is only a 58–59 % association with *V. anguillarum* (Schiewe et al. 1981). *V. ordalii* contains plasmids (Tiainen et al. 1995), but the profile is inevitably different to *V. anguillarum.* In one study, Schiewe and Crosa (1981) determined that 11 isolates of *V. ordalii* contained a common plasmid type (pMJ101) with a molecular weight of 20 mDa. Indeed, this plasmid (= ~32 kb) is common to all *V. ordalii* isolates (Pedersen et al. 1996a).

There is serological (antigenic) cross-reactivity between *V. ordalii* and *V. anguillarum* serogroup 02 (Chart and Trust 1984).

Diagnosis

Phenotypic methods Kent (1982) reported that *V. ordalii* produces a characteristic profile in API 20E, i.e.

−−−−−−−−−−++−−−+−−−+

Molecular Methods PCR and RT-PCR used primers designed to amplify a 112 bp fragment flanking the gene responsible for haemolysin production, *vohB*, enabling specific and sensitive (detected 103 copies of the *vohB* gene) levels of detection. The RT-PCR, which was one hour quicker that the PRC, detected 5.27×10^2 to 4.13×10^3 CFU/ml, i.e. 62–145 copies of the *vohB* gene in Atlantic salmon kidney, liver, spleen and muscle that had been seeded with the pathogen. The RT-PCR detected *V. ordalii* in 5 out of 8 farmed Atlantic salmon whereas culturing was achieved with only one fish (Avendaño-Herrera et al. 2014).

Epizootiology

V. ordalii appears to have a more restricted niche than *V. harveyi* and may be considered as a common water borne organism. It has been postulated that infection (colonisation) begins in the rectum and posterior gastro-intestinal tract. Alternatively, its presence on skin suggests that entry may proceed by direct invasion of the integument (Ransom 1978).

Pathogenicity

The virulence plasmid, pJM1, has not been detected in *V. ordalii* (Crosa 1980). However, a 30 kb cryptic plasmid, designated pMJ101 and which replicates in the absence of DNA polymerase I without generating single stranded intermediates, has been found in all isolates of *V. ordalii* (Bidinost et al. 1994, 1999). Moreover, haemolysins and proteases have not been found (Kodama et al. 1984).

Disease Control

Vaccine Development The methods discussed for *V. anguillarum* apply. Likewise with *V. anguillarum,* the immunogenicity of LPS has been demonstrated (Velji et al. 1990, 1991, 1992).

Probiotics An isolate of *V. alginolyticus,* previously used as probiotic in Ecuadorian shrimp hatcheries, has been effective at controlling diseases caused *V. ordalii* (Austin et al. 1995b).

Vibrio parahaemolyticus

Whereas *V. parahaemolyticus* is recognized as a pathogen of shellfish, notably penaeids, and as a cause of human food-borne infections, particularly resulting from the consumption of contaminated seafood, its role as a fish pathogen is less clear. However, the organism has been mentioned in terms of fish pathogenicity (Harikrishnan et al. 2012a, b, c, d, e; Wang et al. 2015).

Disease Control

Vaccination OmpK was fused with the protein transduction domain of the transactivating transcriptional factor in *Esch. coli,* and the fusion protein vaccinated by immersion marbled eels leading to protection after challenge (Wang et al. 2015).

Dietary Supplements An extract of the Chinese medicinal herb *Siegesbeckia glabrescens* was fed at 1 % and 2 % to kelp grouper (*Epinephelus bruneus*) for 1–4 weeks with evidence of immunostimulation (enhanced alternative complement, myeloperoxidase, reactive oxygen species, and serum lysozyme activity) and protection against challenge with a virulent culture (Harikrishnan et al. 2012a, b, c, d, e).

Vibrio pelagius

Characteristics of the Disease

An epizootic of juvenile farmed turbot in Northwest Spain occurred during January and February 1991 when the water temperature was 12–15 °C, with fish displaying eroded dorsal fins and tail, haemorrhages at the base if the fins, haemorrhages on the internal organs, and intestines full of mucus liquid (Angulo et al. 1992). The total losses amounted to 3 % of the turbot population. Subsequently, larval turbot were described with swollen and necrotic secondary gill lamellae, sloughing-off of the intestinal mucosa, and necrosis of the haematopoietic tissues of the kidney (Villamil et al. 2003a).

Isolation

Samples from diseased tissues were inoculated onto TSA supplemented with 2 % (w/v) sodium chloride, marine 2216E agar and TCBS with incubation at 25 °C for 48 h (Angulo et al. 1992).

Characteristics of the Pathogen

Four isolates were obtained in pure culture, and described as (Angulo et al. 1992):

Box 10.14: *Vibrio pelagius*
The cultures comprise Gram-negative motile fermentative rods, that produce catalase, ß-galactosidase (variable), indole (variable) and oxidase, but not arginine dihydrolase, H_2S, or lysine or ornithine decarboxylase, reduce nitrates, degrade alginate (variable), gelatin, starch, Tween 80 and urea (variable), produce acid from mannitol, mannose, trehalose and sucrose (variable) but not L-arabinose, arbutin, inositol, salicin or sorbitol, and are sensitive to the vibriostatic agent O/129, ampicillin and novobiocin. The methyl red test is generally positive. The Voges Proskauer reaction gives a variable response. Growth occurs in 6 % (w/v) but not 0 % or 10 % (w/v) sodium chloride, at 35 °C but not 4 or 42 °C. However, the isolates do not agglutinate with O antigens of *V. pelagius* ATCC 25916.

It is noteworthy that the fish isolates differed from the reference culture of *V. pelagius* ATCC 25916 in indole production, urea degradation and whole cell agglutination, which must cast some doubt on the validity of the original identification.

Epizootiology

This was not considered by Angulo et al. (1992).

Pathogenicity

Infectivity experiments with rainbow trout (10 g) and turbot (5 g in size) confirmed virulence, and an LD_{50} of 1.9×10^5 cells/fish and 9.5×10^4 cells/fish, respectively (Angulo et al. 1992). Subsequently, Villamil et al. (2003a) published the LD_{50} for larval and post-larval turbot as <5 bacterial/ml and 3.9×10^5 bacteria/ml, respectively. The profound virulence for larvae is clearly demonstrated. The administration of bacterial cells to head kidney macrophages resulted in a marked inhibition of the chemoluminescence response when compared with controls, i.e. untreated macrophages, but an increase in the nitric oxide production. Additionally, in turbot larvae, the application of live cells of the pathogen via i.p. injection led to a dramatic inhibition of the chemoluminescence response in one day (Villamil et al. 2003b).

Disease Control

Antimicrobial Compounds Treatment with oxytetracycline was effective at stopping mortalities (Angulo et al. 1992). Also, it was considered that potentiated sulphonamides and flumequine would be successful.

Vibrio ponticus

Characteristics of the Disease

The organism was associated with an ulcerative condition, considered to resemble vibriosis, in Japanese sea bass (*Lateolabrax japonicus*) culture in China during 2004 when water temperatures increased, and the mortalities exceeded 80 % (Xie et al. 2007). Disease signs were described as including irregular haemorrhagic blots on the skin, that turned white, before ulcerating (Xie et al. 2007).

Isolation

Marine agar and TCBS were used with incubation at 25 °C for 48 h (Xie et al. 2007).

Characteristics of the Pathogen

A total of 6 cultures were recovered from livers of diseased Japanese sea bass:

Box 10.15: *Vibrio ponticus*
Cultures on marine agar are white, and comprise Gram-negative motile, curved rods that produce oxidase, arginine dihydrolase and indole but not lysine or ornithine decarboxylase. The Voges Proskauer reaction is negative. Growth occurs at 25 and 37 °C but not at 4 or 40 °C, and in 2–8 % but not 0 % or 10 % (w/v) sodium chloride. Acid is produced from amygdalin, arabinose, glucose and sucrose. 3-hydroxybutyrate is utilised. Nitrates are reduced to nitrite (Xie et al. 2007).

Generally, the phenotypic characteristics were in common with the description of Macián et al. (2004). Sequencing of the 16S rDNA gene revealed the highest homology (99.3 %) to *V. ponticus* (Xie et al. 2007).

Pathogenicity

Cultures were pathogenic to Japanese sea bass following challenge by i.p. and i.m. injection with the LD_{50} doses reported as $2.5–3.2 \times 10^3$ CFU/fish (Xie et al. 2007). Dead and moribund fish displayed ulceration and subcutaneous bleeding (Xie et al. 2007).

Vibrio scophthalmi

In 2005, A massive mortality occurred in farmed olive flounder (*Paralichthys olivaceus*) in Korea during 2005, from which five isolates were recovered from diseased fish (Qiao et al. 2012).

Characteristics of the Pathogen

Box 10.16: *Vibrio scophthalmi*
The isolates degrade aesculin and urea, and reduce nitrate, produce alkaline phosphatase, esterase lipase, leucine arylamidase, and naphthol-AS-BI-phosphohydrolase, but do not produce lysine or ornithine decarboxylase or *o*-nitrophenyl-ß-galactoside. The isolates produce acid from adipate, fructose, glucose, and maltose (Qiao et al. 2012).

Sequencing of the 16S rRNA gene revealed 98–100 % identity with *V. scophthalmi* and *V. ichthyoenteri*. However, the *dnaJ* gene sequences showed a higher homology with *V. scophthalmi* (Qiao et al. 2012).

Pathogenicity

Pathogenicity was confirmed in challenges involving olive flounder, with LD_{50} reported as 10^6–10^8 CFU/g of fish. The symptoms included darkening of the skin, haemorrhaging in the intestine and liver, ascites, and distended abdomen (Qiao et al. 2012).

Vibrio splendidus

Characteristics of the Disease

During 1987, a disease occurred in cultured turbot in northwest Spain. During the outbreak, there was continuous low level mortality amounting to 4 % of the total stock. Infected fish contained a virus, deemed to be a reovirus, and a bacterium, which was considered to resemble *V. splendidus* (Lupiani et al. 1989). Interestingly, a similar organism has been recovered from diseased Atlantic salmon in Scotland (B. Austin, unpublished data), turbot [including larval turbot in Spain; Thomson et al. 2005] and sea bass in Norway (Myhr et al. 1991) and gilt-head sea bream in Spain (Balebona et al. 1998). Diseased turbot displayed swollen abdomen and haemorrhaging in the mouth, at the anus and base of the fins. The swimming behaviour was not unusual. Internally, the stomach and intestine were swollen, and filled with a mucoid liquid. Haemorrhaging was apparent on the walls of the peritoneal cavity, which also contained a reddish liquid. The liver was pale (Lupiani et al. 1989; Angulo et al. 1994).

V. splendidus 1 and V. *campbellii*-like organisms have been implicated with acute mortalities in turbot *(Colistium nudipinnis)* and brill *(C. guntheri)* in New Zealand (Diggles et al. 2000). Here, the disease signs included inappetance, erratic swimming, distended abdomen distended stomach and intestine, which contained clear fluid, haemorrhaging around and at the base of the fins, necrosis and sloughing-off of the mucosa from the stomach and intestine, haemorrhaging and necrosis in the kidney and liver, and some vacuolation in the brain and spinal cord. A suggested link was made to adverse water quality and inadequate diet (Diggles et al. 2000).

V. splendidus was recovered from the kidney of dead and moribund corkwing wrasse in Norway. The disease signs centred on inappetance, reduced swimming activity, and in some cases surface ulceration (Jensen et al. 2003). Subsequently, *V. splendidus*-like bacteria were recovered from wrasses and lumpsucker *(Cyclopterus lumpus)* in Norway (Gulla et al. 2015).

Isolation

Kidney (anterior), liver, spleen and fluid from within the peritoneal cavity contained bacterial populations, which grew on TSA supplemented with 2 % (w/v) sodium chloride and on TCBS with incubation at 22 °C for 48 h (Lupiani et al. 1989). Jensen et al. (2003) used nutrient agar supplemented with 5 % (v/v) sheep blood and 1.5 % (w/v) NaCl, which was inoculated with kidney tissue and incubated aerobically at 15 °C for up to 7 days.

Characteristics of the Pathogen

Box 10.17: *Vibrio splendidus*
Cultures contain motile fermentative Gram-negative rods, which produce arginine dihydrolase (some isolates), catalase, ß-galactosidase, lysine decarboxylase and oxidase, but not H_2S, indole, ornithine decarboxylase or phenylalanine deaminase, degrade blood (ß-haemolysis) chitin, gelatin, lipids and starch, but not aesculin or urea, reduce nitrates, demonstrate positivity for the methyl red test but not the Voges Proskauer reaction, are sensitive to the vibriostatic agent, O/129, grow at 37 °C and in 3 % (w/v) but not 0 or 7 % (w/v) sodium chloride, utilise citrate, ß-gentiobiose, glucose, ribose and sorbitol but not malonate or sucrose, and produce acid from glucose (no gas), maltose and mannose but not from arabinose, inositol, mannitol, sorbitol or sucrose. The G+C ratio of the DNA is 45.7–47.8 moles % (Jensen et al. 2003).

A detailed taxonomic study by Austin et al. (1997) of 22 isolates revealed that fish pathogenic isolates (these were non-pathogenic in laboratory-based infectivity experiments with Atlantic salmon and rainbow trout) were markedly heterogeneous, being recovered in 7 ribotype clusters, many API 20E profiles and 6 BIOLOG-GN groups (the type strain was recovered as a single member cluster). However, only two serogroups (there were some cross reactions with *V. pelagius* antiserum) and one LPS profile was recognised. With such heterogeneity, it is difficult to decide whether or not any fish-isolates actually constitute *bona fide V. splendidus.*

Agglutination was recorded with antisera prepared against whole cells of *V. splendidus* and strongly to O antigen of *V. splendidus* and *V. tubiashii.* Weak agglutination was recorded against O antigen of *V. anguillarum* and *V. ordalii* (Lupiani et al. 1989). It is considered that the pathogen showed similarities to both *V. anguillarum* (including *V. ordalii)* and *V. splendidus.* Differences to *V. splendidus* (biotype II) included arginine dihydrolase, ß-galactosidase and indole production, the Voges Proskauer reaction, degradation of gelatin and urea, and acid production from arabinose, mannitol and sorbitol (Baumann et al. 1984). Consequently, an identification as *V. splendidus* can only be regarded as tentative, pending further investigation. However, the isolate recovered from corkwing wrasse by Jensen et al. (2003) was equated with *V. splendidus* on the basis of phylogenetic and DNA:DNA hybridisation (78.4 % with the type strain) relationships.

Epizootiology

The organism survives in seawater (Prol-Garćia et al. 2010), where it is likely that the organism is a component of the normal aquatic bacterial microflora, with survival of >114 days recorded (Lopez and Angulo 1995). Recovery has also been achieved from the digestive tract of larval cod (Reid et al. 2009).

Pathogenicity

The bacterium was pathogenic to rainbow trout and turbot, with an LD_{50} of 2.2×10^4 cells and 1.2×10^4 cells, respectively (Angulo et al. 1994). An aerolysin-like enterotoxin has been found, and linked to damage of the intestinal tract and mortalities in cod and turbot larvae (Macpherson et al. 2012).

Disease control

Use of Antimicrobial Compounds Susceptibility was recorded to chloramphenicol, flumequine, nitrofurantoin, nifurpirinol, oxolinic acid and potentiated sulphonamide, but not to ampicillin, oxytetracycline or streptomycin. Treatment with

oxolinic acid was partially successful. However, the presence of virus undoubtedly complicated the chemotherapeutic regime (Lupiani et al. 1989). In a subsequent study, Angulo et al. (1994) reported success with flumequine as a feed additive.

Vibrio tapetis

Characteristics of the Disease

Although *V. tapetis* is primarily the cause of brown ring disease in bivalves, the organism was recovered from the kidney of dead and moribund corkwing wrasse in Norway (Jensen et al. 2003) and wild-caught Dover sole with vesicular skin lesions (Declercq et al. 2015). The disease signs centred on inappetance, reduced swimming activity, and in some cases surface ulceration on corkwing wrasse (Jensen et al. 2003), circular grey-white skin discolouration leading to ulceration in Dover sole (Declercq et al. 2015).

Isolation

Jensen et al. (2003) used nutrient agar supplemented with 5 % (v/v) sheep blood and 1.5 % (w/v) NaCl, which was inoculated with kidney tissue and incubated aerobically at 15 °C for up to 7 days.

Characteristics of the Pathogen

A culture was obtained from diseased corkwing wrasse in Norway. The characteristics were, as follows:

Box 10.18: *Vibrio tapetis*
The culture comprises motile, Gram-negative fermentative rods that produce oxidase but not arginine dihydrolase, and require NaCl. The Voges Proskauer reaction is negative. Growth does not occur at 37 °C. Blood is not degraded. Glucose, ribose and sucrose are utilised, but not mannitol or sorbitol. The G+C ratio of the DNA is 43.8 mols% (Jensen et al. 2003).

There was 78.4 % DNA: DNA re-association with a named reference culture of *V. tapetis* (Jensen et al. 2003).

Disease Control

Use of Antimicrobial Compounds Sensitivity was recorded to flumequine, oxolinic acid and oxytetracycline (Jensen et al. 2003).

Vibrio vulnificus

Characteristics of the Disease

Between 1975 and 1977 in Japan, there were serious outbreaks of disease among cultured eels from six separate localities (Muroga et al. 1976a, b; Nishibuchi and Muroga 1977, 1980; Nishibuchi et al. 1979, 1980). The disease has certainly spread to Europe, with incidences in Spain (Biosca et al. 1991; Amaro et al. 1992), and The Netherlands (Haenen et al. 2014). This is a haemorrhagic condition characterised by redness on the body, notably flank and/or tail. In advanced cases, pathological changes may be observed in the gastro-intestinal tract, gills, heart, liver and spleen (Miyazaki et al. 1977). Superficially, the disease resembles classical vibriosis. During 2005, the pathogen was recognized as the cause of high levels of mortality in farmed ovate pompano *(Trachinotus ovatus)* in PRC, with disease signs including external haemorrhaging and ulcers, and haemorrhaging gills, intestine and liver (Li et al. 2006). A new serogroup, termed *V. vulnificus* biotype 2 serovar A was recognised in Spain during 2000 and in Denmark by 2004, and affected eels of 5–10 g in weight producing severe disease signs including extensive haemorrhaging and necrosis (Fouz et al. 2006).

Isolation

Use of standard bacteriological media, such as seawater agar and TSA supplemented with sodium chloride, and incubation for up to 7 days at 20–25 °C is sufficient to obtain cultures of the pathogen (Muroga et al. 1976a, b; Nishibuchi and Muroga 1977, 1980; Nishibuchi et al. 1979, 1980).

Characteristics of the Pathogen

Box 10.19: *Vibrio vulnificus*
Isolates comprise short Gram-negative, fermentative rods, motile by means of single polar flagella. Arginine dihydrolase, catalase and oxidase are produced, but not ß-galactosidase, indole (indole producing cultures have been recovered from eels in Denmark; Dalsgaard et al. 1999), or lysine or ornithine decarboxylase. Casein, blood, lecithin and Tween 80 are degraded, but not gelatin or urea. Nitrates are reduced. The Voges Proskauer reaction is positive. Acid is produced from a wide range of compounds including D-amygdalin, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, glycogen, maltose, mannose, melibiose, starch and trehalose, but not from adonitol, arabinose, dulcitol, inositol, inulin, mannitol, raffinose, L-rhamnose, D-sorbitol, sucrose (cultures from PRC were positive for sucrose) or D-xylose. Tartrate is utilised. Growth occurs at 20 and 37 °C, but not at 5 or 42 °C, and in 0.5–5.0 % but not 0 % (w/v) sodium chloride. Sensitivity is recorded to the vibriostatic agent, O/129. The G+C ratio of the DNA is 45.7–47.8 moles % (Tison et al. 1982; Amaro et al. 1992).

A group of bacteria was recovered, which appeared to belong to the same taxon. The closest neighbour was thought to be *V. anguillarum* type B (= *V. anguillarum forma anguillicida),* according to the description of Nybelin (1935). However, this group lacks taxonomic meaning, and consequently it was agreed to resurrect the name of *V. anguillicida* (after Bruun and Heiberg 1935) to accommodate the organisms (Muroga et al. 1976a, b; Nishibuchi et al. 1979). Some excellent detective work by Tison et al. (1982) led to the realisation that the organisms, in fact, approximated *V. vulnificus* (= lactose-fermenting vibrios). A new biotype, i.e. biogroup 2, was duly established. However, the biotype concept was considered no longer appropriate (Arias et al. 1997a, b), and it was subsequently deemed preferable to refer to the organisms as a serovar rather than biotype (Biosca et al. 1997a). Nevertheless, *V. vulnificus* biotype 2 serovar A was subsequently described (Fouz et al. 2006). A third biotype accommodates human isolates (Fouz et al. 2007). Serotype A infects only fish whereas serotype E is virulent to both fish and humans (Fouz et al. 2010). A complication is that analysis of 7 housekeeping and virulence-related genes has revealed that the taxon comprises three phylogenetic lineages, which do not match the current biotypes. Moreover, biotype 2 is polyphyletic, which supports a notion that these pathogenic strains should be regarded as a pathovar (Sanjuán et al. 2011). From an examination of 80 cultures, eel isolates were separated from others by ribotyping (Arias et al. 1997a, b). Moreover, AFLP was very discriminatory (Arias et al. 1997a, b).

There are some differences between the species description of *V. vulnificus* and the fish isolates. For example, the latter do not produce indole, ornithine

decarboxylase, acid from mannitol or sorbitol, or grow at 42 °C. These differences add weight to assigning the fish pathogens to a new biogroup. However, the results of DNA:DNA hybridisation, which show 90 % homology between *V. vulnificus* and the group of fish pathogens (Tison et al. 1982), confirm taxonomic (species) relatedness.

Results of serology have clearly demonstrated that isolates of *V. vulnificus* contain heterologous surface antigens. It must be emphasised, however, that the fish isolates are distinctly homogeneous (Nishibuchi and Muroga 1980). There are common soluble intracellular antigens, which are homologous in all isolates. In particular, there is a *V. vulnificus* specific antigen, which may have value in the development of rapid diagnostic tests.

Diagnosis

Serology An ELISA was developed for *V. vulnificus* and field tested, the results of which indicated a sensitivity of 10^4–10^5 cells/well, and an ability to detect non culturable cells (Biosca et al. 1997b).

Molecular Methods González et al. (2004) used a multiplex PCR and DNA microarray, and achieved the simultaneous and differential diagnosis of *Aer. salmonicida, Ph. damselae* subsp. *damselae, V. anguillarum, V. parahaemolyticus* and *V. vulnificus,* with a minimum detection limit of <20 fg per reaction, which equates to 4–5 bacterial cells. A multiplex PCR was developed that detected biotype 2 with differentiation from the zoonotic serovar E (Sanjuán and Amaro 2007).

Epizootiology

V. vulnificus is ubiquitous in the coastal marine and estuarine environment, where it occurs routinely in low numbers (Oliver et al. 1983), although serovar E (biotype 2) is regarded as being rare in natural waters but extended survival occurs in sterile microcosms (Marco-Noales et al. 2004). Populations of the pathogen are almost certainly controlled by grazing and microbial antagonism (Marco-Noales et al. 2004). However, the reservoir is almost certainly the aquatic, especially seawater, environment (Høi et al. 1998), with spread occurring through water (Fouz et al. 2010). It has been documented to survive in brackish water and on the surfaces of eels for 14 days (Amaro et al. 1995). It is feasible that fish are constantly exposed to the potential vagaries of this organism. Serotype A and E enter mostly via the anus and gills, respectively, with pathogenicity recorded for a range of species, including rainbow trout, sea bass and tilapia (Fouz et al. 2010).

Pathogenicity

The source of infection would seem to be water, with gills as a principle portal of entry into the eel (Marco-Noales et al. 2001). In other work, intramuscular injection of eels with large numbers of bacterial cells, i.e. 4.85×10^8, resulted in 80 % mortality in the population within 7 days, at a water temperature of 25 °C. This confirms the pathogenicity, albeit weak, of *V. vulnificus*. The pathogenicity mechanisms, contained in ECP, that are lethal to fish (Lee et al. 1997) included haemolysins, lipases, phospholipases and proteases (Amaro et al. 1992). Evidence has been presented which attributed virulence to the LPS O side chain (Amaro et al. 1997). Interestingly, cultures from diseased European eel produced opaque, translucent colonies, and the cells possessed a capsule, which was not essential for the development of disease (Biosca et al. 1993). Virulence is related to the presence of plasmids (Lee et al. 2013). In particular, the plasmid in biotype 2 contains a *rtxA* gene that encodes a multifunctional, autoprocessing repeats-in-toxin (MARTX), which is involved in the lysis of eukaryotic cells, including epithelial cells and phagocytes, and may act in fish during the onset of septic shock (Lee et al. 2013).

To date, 28 plasmid profiles have been identified among 112 strains with biotype I lacking high molecular weight plasmids with the exception of a 48 kb putative conjugative plasmids that was present in just under half of isolates. Biotype 2 possessed the 68–70 kb virulence plasmid, with the majority also possessing a 52–56 kb putative conjugative plasmid. Biotype 3 strains possessed a 48 kb putative conjugative plasmid. Gene *vep07* is regarded as essential for virulence (Roig and Amaro 2009).

Disease Control

Vaccine Development A vaccine, coined Vulnivaccine which contains capsular antigens and toxoids (being the best of several alternatives; Collado et al. 2000) of serovar E that was administered by immersion for 1 h in three doses at 12 day intervals, has been evaluated in eels with a results that protection (RPS = 60–90 %) was correlated with serum and local (mucus) antibody levels (Esteve-Gassent et al. 2003) with the eels responding to 70–80 kDa OMP, protease and LPS (Esteve-Gassent and Amaro 2004). During field trials by prolonged immersion and boostering after 14 and 24–28 days of 9.5 million glass eels in Spain and parallel experiments in Denmark, Vulnivaccine achieved RPS of 62–86 % (Fouz et al. 2001). With the appearance of a second serotype, i.e. A, a bivalent vaccine was constructed, and verified to be effective in terms of protection and humoral and local immunity following application orally, by anal and oral intubation and by i.p. injection (RPS = 80–100 %) (Esteve-Gassent et al. 2004). A combination vaccine with inacti-

vated cells of *V. alginolyticus*, *V. harveyi*, *V. vulnificus* and infectious spleen and kidney necrosis virus was evaluated in orange-spotted grouper (*Epinephelus coioides)* with an overall RPS of 80 % (Huang et al. 2012). Recombinant epinecidin-1 was administered orally for 30 days to grouper (*Epinephelus coioides*) and zebra fish (*Danio rerio*) leading to reduced mortalities after challenge (Pan et al. 2012). An inactivated bivalent vaccine containing cells of *Str. iniae* and *V. vulnificus* was administered intraperitoneally to sex reversed hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) leading to protection against both pathogens after challenge with RPS values of 69–100 % and 79–89 %, respectively (Shoemaker et al. 2012).

A recombinant bivalent expressed OMP of *V. vulnificus* and *Aer. hydrophila* was used to vaccinate via the i.p. route American eel (*Anguilla rostrata*) with challenge after 28-days leading to RPS values of 50 % for both pathogens (Guo et al. 2015b).

Immunostimulants/Feed Additives Recombinant antimicrobial peptide, epinecidin-1 (from grouper, *Epinephelus coioides),* was fed to grouper and zebra fish for 30 days resulting in increased expression of immune-related genes, notably tumor necrosis factor 1 in grouper, and interleukin-1ß, nitric oxide synthase 2, nuclear factor kB and Toll-like receptor 4 in zebra fish, and a reduction in mortalities after experimental challenge with *V. vulnificus* (Pan et al. 2012). Live cells of *Bac. coagulans* was fed at 10^4 to 10^{10} CFU/ 50 ml of media, and mixed with 50 g of eel powder for 30-days to grouper (*Epinephelus coioides*) and zebra fish (*Danio rerio*) leading to resistance to challenge, and enhanced expression of immune-related genes, i.e. interleukin (IL)-1 ß, myeloid differentiation factor (MyD)88, tumor necrosis factor (TNF)-2, and IL-1 ß in grouper, and Toll-like receptor (TLR) 4, TNF-α, TRAM 1, and nuclear factor (NF)-kappa B in zebrafish (Pan et al. 2013). *Bac subtilis* at 1.05×10^7 or 5.62×10^7 CFU/g of diet, and 0.2 % or 0.4 % of the prebiotic fructooligosaccharide was fed for 8-weeks to juvenile ovate pompano (*Trachinotus ovatus*) before challenge (Zhang et al. 2014a). The data revealed that the higher bacterial dose and the lower amount of prebiotic led to improved growth and resistance to challenge (Zhang et al. 2014a). Liposome-encapsulated cinnamaldehyde, which is from cinnamon, improved survival of zebra fish against challenge with *V. vulnificus* (Faikoh et al. 2014).

Use of Antimicrobial Compounds Although this aspect has not been addressed, it seems likely that infections will respond to broad-spectrum antimicrobial compounds, such as flumequine, oxolinic acid, oxytetracycline and potentiated sulphonamides (Muroga et al. 1976a, b; Nishibuchi and Muroga 1977, 1980; Nishibuchi et al. 1979, 1980).

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Chapter 11
Miscellaneous Pathogens

Abstract *Pseudoalteromonas piscicida, Pseudoalteromonas undina, Shewanella putrefaciens, Arcobacter cryaerophilus, Halomonas (=Deleya) cupida, Acinetobacter* sp., *Moraxella* sp., *Moritella marina, Moritella viscosa, Mycoplasma mobile, Myxococcus piscicola, Aquaspirillum* sp., *Janthinobacterium lividum, Pasteurella skyensis, Piscirickettsia salmonis,* Rickettsia-like organisms, *Streptobacillus,* '*Candidatus* Arthromitus', '*Candidatus* Branchiomonas cysticola', '*Candidatus* Clavochlamydia salmonicola', '*Candidatus* Piscichlamydia salmonis' and '*Candidatus* Renichlamydia lutjani' have been associated with fish diseases. *Moritella viscosa* has been recovered from winter ulcer disease (= skin lesions) in Atlantic salmon with pathogenicity mechanisms reflecting the presence of extracellular products. Protection has been achieved with an adjuvanted formalin inactivated whole cell vaccine. *Piscirickettsia salmonis* is an obligate parasite, which has been associated with coho salmon syndrome, Huito disease and salmonid rickettsial septicaemia. Good protection was recorded by use of a formalised whole cell suspension. '*Candidatus*' are uncultured organisms, which may be visualised in pathological material.

Keywords Epitheliocystis • Winter ulcer disease • Piscirickettsiosis • Red egg disease • Epizootic ulcerative syndrome

Alteromonadaceae Representatives

Pseudoalteromonas piscicida

Characteristics of the Disease

The organism was associated with whitening of the egg cases, followed by mortalities within 24 h among eggs of damselfish, *Amphiprion clarkii* and *Amblyglyphidodon curacao* (Nelson and Ghiorse 1999).

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Isolation

Individual diseased eggs were placed on marine agar with incubation at 28 °C for 2 days (Nelson and Ghiorse 1999).

Characteristics of the Pathogen

An isolate, coined Cura-d, from *Amblyglyphidodon curacao* eggs was identified by 16S rDNA sequencing as *Pseudoalteromonas piscicida.*

Box 11.1: *Pseudoalteromonas piscicida*
Colonies on marine 2216E agar are 3–6 mm in diameter and orange to dark orange in colour (the centres are often white) with the pigment diffusing into the agar. Cells comprise oxidative Gram-negative polarly flagellated rods, which utilise fructose, maltose, mannose and sucrose but not L-threonine, and contain intracellular granules but not poly ß-hydroxybutyrate. Growth occurs at 40 °C (Nelson and Ghiorse 1999).

Pathogenicity

Damsel fish eggs challenged with a culture resulted in enhanced mortalities, compared to those of uninfected controls (Nelson and Ghiorse 1999).

Pseudoalteromonas undina

Characteristics of the Disease

The organism has been recovered from sea bass and sea bream with or without any clinical signs of disease in Spain (Pujalte et al. 2007).

Isolation

Cultures were achieved using inocula from head kidney or liver by use of marine agar with incubation at 20–25 °C for up to 10 days (Pujalte et al. 2007).

Characteristics of the Pathogen

One strain, U58, isolated from diseased sea bass was considered to be pathogenic to fish albeit in high doses, and equated with *Pseudoalteromonas undina* because of the 16S rDNA sequence homology (Pujalte et al. 2007).

Box 11.2: *Pseudoalteromonas undina*
Colonies comprise Gram-negative motile, strictly oxidative rods that produce oxidase but not arginine dihydrolase, indole or lysine or ornithine decarboxylase. The Voges Proskauer reaction is negative. Growth occurs between 4 and 37 °C but not at 40 °C. Growth does not occur in the absence of sodium chloride. Casein, DNA, starch and Tween 80 are degraded, but not alginate or lecithin. Acetate, N-acetyl-D-glucosamine, L-arginine, aspartate, L-citrulline, fumarate, D-glucose, glutamate, glycine, L-histidine, 3-hydroxybutyrate, L-leucine, malate, maltose, L-ornithine, propionate, L-serine, succinate, sucrose, L-threonine, D-trehalose and L-tyrosine are used as the sole source of carbon, but not t-aconitate, 4-aminobutyrate, amygdalin, L-arabinose, citrate, D-cellobiose, D-fructose, D-galactose, D-galacturonate, D-gluconate, D-glucuronate, glycerol, p-hydroxybenzoate, m-inositol, 2-ketoglutarate, lactate, lactose, D-mannitol, D-mannose, melibiose, putrescine, L-rhamnose, D-ribose, salicin, sarcosine, D-sorbitol or D-xylose (Pujalte et al. 2007).

Pathogenicity

One culture, U58, was weakly virulent, killing sea bass but not gilthead sea bream following intracoelomic injection at a dose of 10^7 CFU/fish [$LD_{50} = 1.3 \times 10^7$ CFU/fish] (Pujalte et al. 2007). Dead fish displayed distended abdomen, reddened anus and haemorrhaging in the viscera.

Shewanella putrefaciens

Characteristics of the Disease

During Spring of 1985, a disease occurred which resulted in high mortalities in rabbitfish, *Siganus rivulatus,* farmed in sea cages in the Red Sea. From diseased animals, a Gram-negative bacterium was recovered, which was capable of re-infecting healthy fish (Saeed et al. 1987). To date, the disease has not been described in any other fish species, or, for that matter, elsewhere.

Disease signs included lethargy, discoloration, exophthalmia, haemorrhaging and necroses on the body and mouth, and fin damage. Internal damage was not reported (Saeed et al. 1987). In Poland during 2007–2012, the organism was associated with mortalities in which the disease signs were necrotized gills and necrotic skin lesions/ulcers, particularly in common carp and rainbow trout. Internally, there was haemorrhaging, oedematous kidney and enlarged spleens (Pekala et al. 2015).

Isolation

Bacteria were isolated from the kidney, liver and spleen following inoculation onto BHIA supplemented with 3 % (w/v) sodium chloride, with incubation at an unspecified temperature (presumed to be ≤37 °C) for an undetermined period (Saeed et al. 1987).

Characteristics of the Pathogen

Box 11.3: *Shewanella putrefaciens*
Cultures comprise motile Gram-negative rods, which are neither fermentative nor oxidative for glucose, but which grow at 15–42 °C, in 0.85–9.0 % (w/v) sodium chloride, at pH6.2–9.6, and on MacConkey agar. Catalase, H_2S, ornithine decarboxylase and oxidase are produced, but not arginine dihydrolase, ß-galactosidase, indole, lysine decarboxylase, or phenylalanine or tryptophan deaminase. Gelatin but not urea is attacked. Nitrates are reduced, and citrate is utilised. The Voges Proskauer reaction is negative. Acid is not produced from amygdalin, arabinose, glucose, inositol, mannitol, melibiose, rhamnose, sorbitol or sucrose. Growth occurs at 37 °C, and in 7 % but not 0 % (w/v) sodium chloride (Saeed et al. 1987).

From the results of the API 20E rapid identification system, it was considered that the pathogen was *Ps. (=Alteromonas) putrefaciens,* a taxon which has been subsequently re-classified as *Shewanella putrefaciens* (MacDonell et al. 1986). The Polish isolates were confirmed as *She. putrefaciens* by sequencing (Pekala et al. 2015).

Epizootiology

It is assumed that the organism was derived from the coastal marine environment. Possibly, the organism comprises part of the normal microflora of fish (see Lee et al. 1977; Gillespie 1981).

Pathogenicity

Following i.p. injection, fish (average weight = 50 g) developed clinical disease, with 80 % mortalities within 48 h. The organism was recovered from the kidney, liver and spleen of dead fish. Infection was not achieved following immersion in a dense suspension of the organism (Saeed et al. 1987).

Disease Control

Vaccine Development A formalin killed suspension showed promise at controlling mortalities when applied (twice) by i.p. injection (Saeed et al. 1987). Thus, two injections resulted in 40 % less mortality than the unvaccinated controls. Vaccination by immersion was unsuccessful.

Campylobacteriaceae Representative

Arcobacter cryaerophilus

Characteristics of the Disease

Diseased rainbow trout were recovered from three fish farms in Turkey during 1997 and 1998. The reported disease signs included deformation of the upper jaw, darkened or alternatively pale pigment, fin rot, pale gills, haemorrhaging in the musculature, haemorrhaging and bloody fluid in the intestine, skin ulcerations, damaged spleens and swollen kidney (Aydin et al. 2000, 2002).

Isolation

Growth occurred on *Campylobacter*-selective agar and enriched TSA with incubation at 25 °C for 1–7 days (Aydin et al. 2002).

Characteristics of the Pathogen

According to the authors (Aydin et al. 2002), the cultures were identified according to the results in Bergey's Manual of Systematic Bacteriology, and there does appear to be a close agreement in the characteristics.

Box 11.4: ***Arcobacter cryaerophilus***
The small white colonies comprise aerobic Gram-negative motile rods that produce catalase and oxidase but not alkaline phosphatase, arginine dihydrolase, H_2S, indole or urease, reduce nitrates to nitrites, do not attack aesculin, gelatin, starch or Tween 20 or 80, grow at 14–42 °C and in potassium cyanide, but not in 3 % (w/v) sodium chloride, do not produce acid from adonitol, arabinose, dulcitol, erythritol, fructose, galactose, glucose, glycogen, inositol, inulin, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sorbose, sucrose, trehalose or xylose, or utilise citrate (Aydin et al. 2002).

Pathogenicity

Infections were achieved following i.m. injection with 5×10^5 cells/fish for 7–21 days (Aydin et al. 2002).

Disease Control

Antimicrobial Compounds Sensitivity was recorded to formalin and enrofloxacin, with bathing in the former followed by oral application of the latter controlling natural infection (Aydin et al. 2000).

Comamonadaceae Representative

Delftia acidivorans

Ps. anguilliseptica and *Delftia acidivorans* was recovered from a consignment of glass eels in Barcelona Airport, Spain. The eels presented with lethargy and disorientation, open skin lesions, sloughing off of skin behind the gills, vent and caudal fin, enlarged kidney and spleen. A few of the animals swam rapidly and erratically in a circular pattern, reminiscent of whirling disease. Material from diseased tissues was inoculated onto TSA, BHIA and TCBS with incubation at 22 °C, and after 7-days there was evidence of *Ps. anguilliseptica* and *Delftia acidivorans* as confirmed by sequencing of the 16S rDNA gene, and comparison of the data with the GenBank data base. *Delftia* produced greyish-beige slightly glossy colonies with irregular edges, and which comprised Gram-negative catalase- and oxidase-positive organisms. Treatment was achieved with baths of formalin and hydrogen peroxide, and bathing and then oral application of gentamycin with sulphadiazine-trimethoprim (Andree et al. 2013). Unfortunately, pathogenicity of the isolates was not confirmed in laboratory-based infectivity experiments.

Hahellaceae Representative

Hahella chejuensis

This organism has been associated with red egg disease of tilapia hatcheries in Thailand (Senapin et al. 2016).

Characteristics of the Disease

A novel disease of red tilapia and Nile tilapia eggs, coined red egg disease/syndrome, was first recognized in tilapia hatcheries in Thailand during 2000. The condition was characterized by the egg's changing colour from yellowish to red during incubation, leading to a failure to hatch. The cumulative loss in fry production was recorded to be 10 %, increasing to 50 % during very cold weather. As many as 50 % of the eggs were lost because of the condition during December 2013 to March 2014 (Senapin et al. 2016).

Isolation

Egg homogenates were inoculated onto TSA plates with or without 1.5 % (w/v) sodium chloride with incubation at 30 °C for 24–48 h whereupon red pigmented colonies were observed, and purified (Senapin et al. 2016).

Characteristics of the Pathogen

Box 11.5: *Hahella chejuensis*
Cultures are red-pigmented, and comprise Gram-negative rods, that produce catalase and oxidase. Sequencing of the 16S rRNA gene reveals 99.5–99.7 % homology with the reference culture of *Hahella chejuensis* in the GenBank data base (Senapin et al. 2016).

Diagnosis

PCR was effective at recognizing the presence of *Hahella chejuensis* in samples (Senapin et al. 2016).

Epizootiology

The source of the infection was not determined.

Pathogenicity

Experimental challenge of eggs with 2.7×10^4 CFU/ml revealed that the organism reproduced the signs of red egg disease with 7 % and 18 % of the egg developing a red colour by day 2 and 3, respectively, after challenge, and adversely affecting hatching (Senapin et al. 2016).

Halomonadaceae Representative

Halomonas (=Deleya) cupida

Characteristics of the Disease

During April to June 1984, heavy mortalities occurred in black sea bream, *Acanthopagrus schlegeli,* fry (up to 14 days after hatching) in hatcheries in western Japan. Generally, the fish were too small to discern disease signs (Kusuda et al. 1986).

Isolation

Although the wisdom of using homogenates of whole fish for bacteriological examination may be debated, it is probably an acceptable compromise with fry. Nevertheless, care needs to be taken when interpreting results insofar as bacteria may be derived from the normal surface/intestinal microflora as well as from diseased tissues. In this investigation, cultures were obtained from homogenates following inoculation of BHIA with incubation at 25 °C for 24 h (Kusuda et al. 1986).

Characteristics of the Pathogen

Homogenates of black sea bream revealed the presence of *Halomonas (=Deleya) cupida (=Alcaligenes cupidus), V. alginolyticus* and *V. nereis* (Kusuda et al. 1986).

Box 11.6: *Halomonas cupida*
Cultures comprise motile Gram-negative salt-requiring rods, which grow at 10–25 °C but not at 4 °C and 30 °C, and are unreactive in the oxidative-fermentative test. Arginine dihydrolase, catalase and lysine and ornithine decarboxylase are produced, but not H_2S, indole or oxidase. Nitrates are reduced, but the methyl red test and Voges Proskauer reaction are negative. Haemolysis is recorded to eel erythrocytes, but not those of sheep or yellowtail. Sensitivity is recorded to the vibriostatic agent, O/129. Acid is produced from adonitol, galactose, lactose, maltose, mannitol, L rhamnose, D sorbitol, salicin, sucrose (weak) and trehalose, but not from fructose or inulin.

From these characteristics, a close relationship to *Alcaligenes cupidus* was noted (see Kersters and De Ley 1984). The only discrepancy concerned acid production from fructose. However, this taxon was re-classified as *Deleya cupida* (Baumann

et al. 1983), and by inference to *Halomonas cupida* on the basis of 16S rRNA sequencing (Dobson and Franzmann 1996).

Epizootiology

H. cupida is a waterborne organism (Baumann et al. 1972). Therefore, the source of infection was most likely the marine environment.

Pathogenicity

Pathogenicity was confirmed in laboratory-based experiments involving waterborne challenge with 10^3–10^5 cells/ml. Approximately 75 % mortalities were recorded within 4 days Kusuda et al. (1986).

Moraxellaceae Representatives

Acinetobacter sp.

Characteristics of the Disease

During Autumn 1978 when the water temperature was between 8 and 11 °C, an outbreak of disease occurred in a group of 60 sexually mature Atlantic salmon. The fish, each of 5–12 kg in weight, were wild stock from the River Surma, Norway, and were held in brackish water during the occurrence of disease. During the 5-week period of disease, the cumulative mortalities amounted to 92 % of the population. However, only about 40 % of the animals displayed clinical signs of disease, namely hyperaemia of the dermal blood vessels, and haemorrhaging in the scale packets, with severe oedema extending into the lower epidermis in the vicinity of the base of the fins. Ulceration developed. Lesions appeared in the kidney, liver and spleen, and small haemorrhages occurred in the air bladder and on the visceral peritoneal surfaces (Roald and Hastein 1980).

Isolation

Pure cultures were recovered on 5 % (w/v) blood agar supplemented with 0.5 % (w/v) sodium chloride, following incubation at 22 °C for 48 h. Thus, blood, kidney, liver, spleen and ulcers in the muscle appeared to contain dense bacterial populations. Pure cultures were obtained, which were capable of reproducing the infection during laboratory-based studies (Roald and Hastein 1980).

Characteristics of the Pathogen

Box 11.7: *Acinetobacter* sp.
Cultures comprise round, raised, translucent, mucoid colonies of 1.5 mm in diameter within 48 h incubation at 22 °C. Colonies contain fairly unreactive short, plump, facultatively anaerobic, non-motile Gram-negative rods of 1.6–1.8 × 0.8–1.2 μm in size. Catalase and oxidase are produced, but not arginine dihydrolase, ß-galactosidase, H_2S, indole, lysine or ornithine decarboxylase or tryptophan deaminase. Blood (haemolysis) is degraded, but not gelatin or urea. Nitrates are not reduced. The methyl red test and Voges Proskauer reaction are negative. Sodium citrate is not utilised. Acid is produced from galactose, maltose and mannose, but not from adonitol, amygdalin, arabinose, cellobiose, glucose, inositol, lactose, mannitol, melibiose, raffinose, rhamnose, saccharose, salicin, sorbitol or xylose. Unfortunately, the G+C ratio of the DNA was not determined.

From the available information, Roald and Hastein (1980) considered that the pathogen corresponded to an as yet unnamed species of *Acinetobacter.* Although this appears to be a sound decision, *bona fide Acinetobacter* spp. should not produce oxidase (Juni 2005). In fact, this ability belongs to the morphologically similar *Moraxella* (Juni and Bøvre 2005) and *Neisseria* (Vedros 1984). There is some degree of resemblance between the fish pathogen and *Mor. atlantae* and *Mor. osloensis,* although one of the general traits of moraxellae is an inability to produce acid from carbohydrates. Thus as a compromise solution, it would appear that the fish pathogen should be classified in the area loosely bounded by *Acinetobacter, Moraxella* and *Neisseria.*

Epizootiology

Unreactive Gram-negative rods that are difficult to identify and which resemble *Acinetobacter* are common inhabitants of fresh water (Allen et al. 1983) and marine ecosystems (Austin 1982). In particular, the organisms populate the skin and gills (Horsley 1973) and digestive tract (Shewan 1961; Trust and Sparrow 1974; Roald 1977) of salmonids. Therefore, a ready inoculum of cells is likely to be in continual contact with fish. Conceivably any break in the integument of the host may lead to colonisation of the nutrient-rich tissues by components of the water-borne or, indeed, fish microflora. This may lead to the start of a disease cycle.

Pathogenicity

Nothing is known about the pathogenic mechanisms of this organism. Due to the comparative inactivity of cultures, it seems unlikely that exotoxins are implicated. This leaves a possible role for endotoxins. Nevertheless, it has been established that pure cultures will reproduce the disease condition. Thus, peptone water cultures, administered by i.m. injection into Atlantic salmon fingerlings, each of 15 g average weight, resulted in total mortalities of the population within 72 h at a water temperature of 12 °C (Roald and Hastein 1980).

Disease Control

Antimicrobial Compounds Oxytetracycline, at a single dose of 100 mg/fish, appeared to be effective for chemotherapy when administered by intramuscular injection.

Acinetobacter johnsonii and *Acinetobacter lwoffii*

Eight isolates were recovered from diseased farmed rainbow trout and common carp in Poland. Identification was by use of the API 20 NE rapid identification system and by sequencing of the 16S rDNA gene with the result that the isolates were equated with *Acinetobacter calcoaceticus, Acinetobacter johnsonii, Acinetobacter junii/johnsonii, Acinetobacter lwoffii* and *Acinetobacter* spp. (Kozinska et al. 2014). In laboratory-based infectivity experiments, *Acinetobacter johnsonii* and *Acinetobacter lwoffii* were found to be pathogenic, with the disease signs resembling those in aquaculture (Kozinska et al. 2014).

Moraxella sp.

Characteristics of the Disease

During winter 1987, mortalities were recorded among juvenile striped bass, *Morone saxatilis,* in the Potomac River, Maryland, USA. Gills of diseased fish were affected with the parasites *Trichodina* and *Ergasilus.* In addition, a reo-like virus and a bacterium was recovered from some individuals. Large haemorrhagic lesions and missing scales occurred on the dorsal surface of the 11 affected fish. Haemorrhages were apparent in the swim bladder. The liver was enlarged, pale and mottled in appearance. Membranous material appeared to connect the liver with the body wall (Baya et al. 1990).

Isolation

Pure culture growth of round, raised, translucent, mucoid colonies developed in 48 h of incubation at 22 °C of kidney, liver and pancreas tissue on TSA (Baya et al. 1990).

Characteristics of the Pathogen

A bacterium was recovered as pure culture growth, and equated with *Moraxella* (Baya et al. 1990).

Box 11.8: *Moraxella* sp.
Cultures comprise non-motile, short (0.8–1.0 × 1.3 μm in size) non-fermentative paired rods with pronounced bipolar staining. Catalase and oxidase are produced, but not so arginine dihydrolase, ß-galactosidase, H_2S, indole or lysine or ornithine decarboxylase. Sheep's blood is degraded, but not gelatin or urea. Citrate utilisation, nitrate reduction and the Voges Proskauer reaction are negative. Acid is produced weakly from galactose and mannose, but not from amygdalin, arabinose, glucose, inositol, lactose, maltose, mannitol, rhamnose, salicin, sorbitol or sucrose.

Whereas similarities to *Moraxella* were noted (Juni and Bøvre 2005), the organism strongly resembled the *Acinetobacter* described by Roald and Hastein (1980). Indeed, the major differences concerned acid production from maltose. Clearly, the precise taxonomic position of both organisms must await further study.

Epizootiology

It may be assumed that the organism constitutes part of the aquatic microflora.

Pathogenicity

Laboratory-based experiments revealed that the organism was pathogenic to rainbow trout and striped bass, with an LD_{50} dose of 10^5–10^6 cells (Baya et al. 1990).

Disease Control

Antimicrobial Compounds The pathogen was susceptible to chloramphenicol, nitrofurantoin, oxolinic acid, penicillin and tetracycline (Baya et al. 1990).

Moritellaceae Representatives

Moritella marina (= V. marinus)

Characteristics of the Disease

The organism was associated with shallow skin lesions of Atlantic salmon farmed in Iceland at low temperatures, i.e. ~10 °C (Benediktsdóttir et al. 1998).

Isolation

Benediktsdóttir et al. (1998) used 5 % (v/v) horse blood agar supplemented with 1.5 % (w/v) sodium chloride with incubation at 15 °C for 7 days.

Characteristics of the Pathogen

Nineteen Icelandic and one Norwegian isolate, and the type strain of *V. marinus* NCIMB 1144 were identified as *V. marinus* after an examination of phenotypic data and analyses by numerical taxonomy (Benediktsdóttir et al. 1998). On the basis of 16S rRNA sequencing the taxon was transferred to a newly established genus, as *Moritella marina* (Urakawa et al. 1998). However, apart from emphasising a relationship to *Shewanella,* the authors did not make any comment about family membership.

Box 11.9: *Moritella marina*
Cultures produce lysine decarboxylase and oxidase but not arginine dihydrolase or ornithine decarboxylase, reduce nitrates, are positive for the methyl red test but not the Voges Proskauer test, degrade blood (ß-haemolysis), chitin, DNA, gelatin, lipids and starch, do not produce acid from carbohydrates except N-acetyl glucosamine, maltose, mannitol, mannose and ribose, is resistant to the vibriostatic agent, O/129, and grow at 4–20 °C but not at 25 °C.

Pathogenicity

The LD_{50} dose to Atlantic salmon was $<3.5 \times 10^3$ cells (Benediktsdóttir et al. 1998).

Moritella viscosa (= V. viscosus)

Characteristics of the Disease

Ulcers, of indeterminate cause, appeared on the flanks of Atlantic salmon in seawater during winter (= winter ulcer disease), principally in Iceland and Norway (Salte et al. 1994; Lunder et al. 1995; Benediktsdóttir et al. 1998), and more recently in Scotland. Since its first recognition, a view has emerged that two new vibrios, *V. wodanis* and *Moritella viscosa,* are responsible (Benediktsdóttir et al. 2000). *Moritella viscosa* was subsequently recovered from two diseased (with skin lesions) farmed Atlantic cod in Norway (Colquhoun et al. 2004).

Characteristics of the Pathogen

Two groups of psychrotrophic bacteria were recovered; one homogeneous group was determined to be closest to *Moritella marina* (43 % re-association by DNA: DNA hybridisation), and hence was initially named as *V. viscosus* (Lunder et al. 2000). However, by 16S rDNA sequencing, the closest match was *Moritella* HAR 08 and HAR 013 (Lunder et al. 2000) and *Moritella marina* (99.1 % sequence homology. Hence, the organism was re-classified to *Moritella,* as *Moritella viscosa* (Benediktsdóttir et al. 2000). Phenotypic and genotypic variation has been reported among isolates (Grove et al. 2010).

Box 11.10: ***Moritella viscosa***
Colonies on bovine blood agar containing 2 % (w/v) NaCl of up to 1 mm in diameter after 24 h at 15 °C or 22 °C are viscous (and adhere to the medium), translucent and grey in colour that contain motile, fermentative Gram-negative rods that produce alkaline phosphatase, caprylate esterase, catalase, lysine decarboxylase and oxidase but not arginine dihydrolase, chemotrypsinase, α-fucosidase, indole, α- or ß-, α-mannosidase or ornithine decarboxylase, degrade bovine blood (ß-haemolysis), casein, DNA, gelatin, lecithin, starch, Tween 80 and urea but not aesculin, alginate, produce acid from galactose and glucose but not L-arabinose, cellobiose, glycerol, inositol, lactose, mannitol, mannose, melibiose, raffinose, L-rhamnose or xylose, grow at 15 °C (and also survive freezing) in 1–4 % (w/v) NaCl, are sensitive to the vibriostatic agent, O/129, and are negative for the methyl red test and Voges Proskauer reaction. All isolates examined harbour one or more plasmids. The G+C ratio of the DNA is 42.5 mol% (Lunder et al. 2000).

Diagnosis

Molecular Methods A PCR has been successful for the detection of *Moritella viscosa* with a detection limit of 6.09×10^{-14} g of DNA, which is equivalent to 10 bacterial genomes (Grove et al. 2008).

Pathogenicity

The pathogen demonstrated the ability to adhere to mucus from Atlantic salmon epithelial surfaces, i.e. foregut, gills, hindgut, pyloric caeca and skin (Knudsen et al. 1999). Epidermal keratocytes have been shown to remove the pathogen from surfaces, but the engulfment capacity was considered to be inefficient. Immersion challenge revealed a significant relationship between the area of exposure and mortality. Moreover, better invasion capability and mortality resulted with exposure of the head and gill region rather then elsewhere on the body surface. The development of ulceration corresponded to the area of the surface exposed to the pathogen, which suggested that the formation of ulcers resulted principally from skin surface colonization (Karlsen et al. 2014b).

Experimental infections were achieved in juvenile cod (minimum lethal dose = 4.0×10^4 CFU/fish by i.m. or i.p.) and to a lesser extent in halibut (minimum lethal dose = 6.5×10^4 CFU/fish by i.m. or i.p). Cod was infected by immersion challenge in 2×10^7 CFU/ml for two separate periods of 1 h with 37.5 % mortalities ensuing within 22 days at 9 ± 1 °C (Gudmundsdóttir et al. 2006). ECPs, which contain varying levels of esterase, metallopeptide, cytotoxic and haemolytic activities, and a lethal toxic factor, have been associated with virulence (Bjornsdottir et al. 2011). Three putative toxins have been identified as increasing their transcription over time during the infection process, and include a cytotoxic necrotizing factor (*cnf*), a haemolysin and a putative repeat in toxin gene (*rtxA*) with the outcome being rounding of fish cells (Tunsjø et al. 2011). Also, there was an upregulation of putative lateral flagellin and a protease in Atlantic salmon ulcer tissues, which may reflect their involvement in colonization and subsequent tissue damage (Tunsjø et al. 2011). A type VI secretion system has now been described. This system, which is important in virulence, is involved in the transportation of bacterial proteins from the cell into the host cell or into the surrounding environment (Bjornsdottir et al. 2012).

Two major genetic clades have been recognized, one of which is mostly restricted to Atlantic salmon. Highly virulent isolates from Norwegian Atlantic salmon were less pathogenic to rainbow trout. Other less typical isolates were somewhat pathogenic to both salmonid species (Karlsen et al. 2014a, b). All isolates revealed DNA sequences encoding aerolysin, bacterioferritins, cytotoxic necrotizing factor (mostly), haemolysin coregulated protein, invasion, lectin, MARTX, Type VI secretion ATPase, and phospholipase. A putative insecticidal toxin complex was detected mostly in Atlantic salmon isolates (Karlsen et al. 2014a, b).

Disease Control

Vaccine Development Atlantic salmon, which were vaccinated i.p. with an adjuvanted whole cell, formalised suspension containing *Moritella viscosa,* were protected against subsequent challenge, achieving an RPS of 97% (Greger and Goodrich 1999). A multivalent (containing antigens to 5 pathogens) oil-adjuvanted vaccine, which contained *Moritella viscosa* antigens, did not induce protection in turbot, but did lead to some intra-abdominal adhesions (Björnsdóttir et al. 2004). Subsequent work demonstrated that the protective antigens included lipooligosaccharides and a ~17–19 kDa outer membrane antigen, which induced antibody response in vaccinated fish (Heidarsdöttir et al. 2008). A ~20 kDa OMP (= MvOMP1) has been also regarded as the major protective antigen, raising the possibility of its use in a future subunit vaccine (Björnsson et al. 2011).

Antimicrobial Compounds Florfenicol should work, with *in vitro* and *in vivo* experiments being seemingly successful. Plasma concentration of 3.0 ± 1.8 mg/ml were recorded in Atlantic salmon after the administration of suitably medicated feed. However, mortality patterns after infection were less convincing (Coyne et al. 2006).

Mycoplasmataceae Representative

Mycoplasma mobile

Cell wall defective/deficient bacteria, i.e. L-forms and mycoplasmas, have been recently associated with fish diseases. Since the initial recovery of a motile mycoplasma from fish (Kirchhoff and Rosengarten 1984), a new species, i.e. *Mycoplasma mobile,* has been described (Kirchhoff et al. 1987). In addition, there is an increasing awareness of L-forms in fish diseases. To date, L-forms have been described for *Aer. salmonicida* and *Y. ruckeri* (McIntosh and Austin 1990). Both L-forms and mycoplasmas are osmotically fragile organisms without cell walls, requiring specialized procedures for their recovery and growth.

Characteristics of the Disease

The mycoplasma was associated with “red disease”, a condition in the gills of tench (*Tinca tinca)* (Kirchhoff et al. 1987).

Characteristics of the Pathogen

Box 11.11: *Mycoplasma mobile*
Cultures produce "fried-egg" colonies of 10–500 μm diameter on Hayflick medium, which contains horse or bovine serum, after incubation at an unstated temperature for 2–6 days. Colonies contain filterable (through the pores of 0.45 μm pore size filters) Gram-negative conical or flask shaped wall-less cells with distinctive terminal structures. The cells demonstrate marked ability to adhere to and glide on glass, plastic, erythrocytes and tissue culture cells. Growth occurs at 4–30 °C but not at 37 °C. Catalase (weak), oxidase (weak) and phosphatase (weak) are produced. Blood is degraded, but not arginine, casein, gelatin or urea. Acid is produced fermentatively from arabinose, fructose, galactose, glucose, lactose, maltose and mannose. 2,3,5-triphenyltetrazolium chloride and potassium tellurite (weakly) are reduced, but not methylene blue. Gluconate is not oxidised, nor is phenylalanine deaminated. The G + C ratio of the DNA is 22.4–24.6 moles % (Kirchhoff et al. 1987).

Serologically, the tench isolate was distinct from all other validly described species of *Acholeplasma* and *Mycoplasma.* From the traits, listed above, it was deemed that the organism formed a new species, for which the name of *Mycoplasma mobile* was coined (Kirchhoff et al. 1987).

Epizootiology

Mycoplasma-like bacteria occur on fish (Kirchhoff and Rosengarten 1984), from which infection probably occurs.

Myxococcaceae Representative

Myxococcus piscicola

There has been one report of gill disease (in 1972) caused by a supposed new species of the fruiting organism *Myxococcus,* for which the name of *Myxococcus piscicola* was suggested (Xu 1975). This organism was associated with an epizootic in grass carp (*Ctenopharyngodon idelluls)* fingerlings held in ponds in Wuhan, China. Conical fruiting bodies were produced on agar. These fruiting bodies were surrounded by a thin membrane, without peduncle or branches (Xu 1975).

Neisseriaceae Representative

Aquaspirillum sp.

There has been a report of putative *Aquaspirilllum* sp., along with *Aer. hydrophila, Pseudomonas* sp. and *Streptococcus* sp., being associated with a disease, termed epizootic ulcerative syndrome, in snakeheads and catfish obtained from two fish farms in Thailand (Lio-Po et al. 1998). However, the evidence for the involvement of *Aquaspirillum* is not convincing.

Slight lesions were reported to occur after 24 h of infection in experimental fish (Lio-Po et al. 1998). This limited pathology casts doubt on the role of the organism as a fish pathogen. Perhaps, synergism with *Aer. hydrophila, Pseudomonas* sp. and *Streptococcus* may occur. Alternatively, *Aquaspirillum* may be an opportunistic invader or saprophyte living on diseased tissue.

Lio-Po et al. (1998) used TSA supplemented with 10 % (v/v) horse serum and cytophaga agar at an unspecified temperature and duration to recover *Aquaspirillum, Aer. hydrophila* and *Streptococcus* from diseased animals.

Oxalobacteraceae Representative

Janthinobacterium lividum

Characteristics of the Disease

During 1991, purple-pigmented Gram-negative rod-shaped bacteria were associated with mortalities at two fish farms. At one site in Scotland, moribund rainbow trout (size range = 0.5–1.0 g) were diagnosed with RTFS. The second site in Northern Ireland also experienced high mortalities (~35 % of the stock) in rainbow trout fry of 0.2–0.5 g in size, 2–3 weeks after the introduction of feeding. At this site, the rise in mortalities coincided with a change from the use of spring to river water (Austin et al. 1992). In addition during January 1992, we found similar purple-pigmented bacteria to be associated with skin lesions, on larger rainbow trout (100–200 g in weight), which were otherwise debilitated with ERM. It is relevant to note that this fish population had received prolonged and varied chemotherapy.

Small fish, considered to be displaying RTFS, became lethargic, displayed exophthalmia, pale gills, enhanced skin pigmentation, swollen abdomen and (sometimes) skin lesions. Internally, the kidney was swollen, the spleen was pale and elongated, and some ascitic fluid was present in the peritoneal cavity (Austin et al. 1992). On the larger rainbow trout, the organism was associated with surface lesions. In particular, the skin was sloughed-off along the entire flank of the animals, from operculum to tail, exposing the underlying (necrotic) muscle.

Isolation

Homogenates of whole fish (prepared in quarter strength Ringer's buffer; Oxoid) and, where possible, loopfuls of kidney, liver, spleen, ascitic fluid and material from surface lesions were spread over the surface of a variety of media, including blood agar (5 % v/v bovine blood in Gibco blood agar base), cytophaga agar, KDM2, L-F medium (Appendix in Chap. 12) and TSA, with incubation aerobically at 22 °C for up to 14 days. Purple-pigmented colonies were apparent after 3 days.

Characteristics of the Pathogen

Pure cultures have been recovered and characterised:

Box 11.12: *Janthinobacterium lividum*
Cultures comprise purple-pigmented motile Gram-negative strictly aerobic rods, which produce arginine dihydrolase, catalase and oxidase, but not ß-galactosidase, indole or tryptophan deaminase. Nitrates are reduced, and the Voges Proskauer reaction is positive. Gelatin, but not urea, is degraded. Growth occurs at 4–30 °C but not 37 °C, and in 0–2 % but not 3 % (w/v) sodium chloride. Caprate, citrate, malate, maltose, mannitol, mannose and phenylacetate are utilised, but not N-acetyl glucosamine or adipate.

From these traits, the organisms were identified as typical (Table 11.1; from Northern Ireland) and atypical (Table 11.1; from Scotland) *Janthinobacterium lividum* (Sneath 1984; Logan 1989).

Epizootiology

Typical and atypical forms of *J. lividum* are regarded as part of the normal microflora of fresh water (Sneath 1984) and soil (Moss and Ryall 1980). Therefore, there would be a ready inoculum of the pathogen in the environment around fish.

Pathogenicity

Injection of 5×10^2 cells/fish i.m. and i.p. resulted in 100 % mortalities within 14 days. Generally, infected rainbow trout fry and fingerlings became lethargic within 2 days. Moribund and newly dead fish displayed pale (almost white) gills, elongated spleen, pale liver, swollen watery kidney, internal haemorrhaging, pronounced gastro-enteritis, and slight amounts of ascitic fluid in the peritoneal cavity (Austin et al. 1992).

Table 11.1 Differential characteristics of *J. lividum* recovered from moribund and dead rainbow trout fry

Character		*Chromobacterium violaceum*[b]	*Iodobacter fluviatile*[b]	*J. lividum*[b]	Atypical *J. lividum*[b]	Isolates from Ireland	Isolates from Scotland
Gelatinous colonies		–	–	v	+	+	+
Oxidative (O)-fermentative (F)							
Metabolism of glucose		F	F	O	O	O	O
Growth at:	4 °C	–	+	+	+	+	+
	37 °C	+	–	–	–	–	–
Degradation of:							
Aesculin		–	–	+	–	+	–
Arginine		+	–	–	–	–	–
Production of acid from:							
L-arabinose		–	–	+	+	+	+
Gluconate		+	+	–	–	+	+
Glycerol		+	–	+	–	+	–

A comparison has been made to taxa which accommodate purple-pigmented bacteria[a]
v variable result
[a]From Austin et al. (1992)
[b]From Logan (1989)

Disease Control

Antimicrobial Compounds Cultures were sensitive to furazolidone, oxolinic acid, oxytetracycline and potentiated sulphonamides (Austin et al. 1992b). Therefore, it is surmised that one or more of these compounds would be useful in chemotherapy.

Pasteurellaceae Representative

Pasteurella skyensis

Characteristics of the Disease

The organism was recovered from four separate incidences of disease among farmed Atlantic salmon in Scotland during summer over a 4-year period from 1995 to 1998. The fish displayed inappetance (Jones and Cox 1999).

Isolation

Isolation from kidney was on TSA supplemented with 1.5 % (w/v) sea salts and 5 % (v/v) defibrinated horse blood aerobically at 20 °C for 48 h when small grey colonies resulted. After initial isolation, culturing was possibly on TSA (or Columbia agar) supplemented with 1.5 % (w/v) sodium chloride and 10 % (v/v) citrated sheep or horse blood (Birkbeck et al. 2002).

Characteristics of the Pathogen

Four isolates were obtained (Birkbeck et al. 2002):

Box 11.13: *Pasteurella skyensis*
Cultures on blood-containing medium comprise non motile, facultatively anaerobic pleomorphic Gram-negative catalase-negative rods that produce esterase (lipase), indole, leucine arylamidase, lysine and ornithine decarboxylase, oxidase (weakly), acid and alkaline phosphatase and naphthol-ASBI-phosphohydrolase but do not produce arginine dihydrolase, ß-galactosidase, urease, the Voges Proskauer reaction or reduce nitrate. Acid is produced from glucose, lactose, maltose, mannitol, mannose and trehalose, but not from adonitol, arbutin, dulcitol, galactose, inositol, inulin, melibiose, raffinose, rhamnose, salicin, sucrose, sorbitol or xylose. Haemolytic activity is at best weak. There is a requirement for blood and 1.5 % (w/v) for growth. Growth occurs at 14–32 °C but not at 37 °C. The G + C ratio of the DNA is 39–41 mol% (Birkbeck et al. 2002).

The phenotypic tests were used to link the pathogen to the Pasteurellaceae, and the results of 16S rRNA sequencing confirmed the association to the family with *Pa. phocoenarum* (97.1 % homology) being regarded as closest neighbour phylogenetically.

Epizootiology

The source of the pathogen may well have been fish, e.g. mackerel (Birkbeck et al. 2002).

Pathogenicity

I.p. injection of $1–4 \times 10^6$ cells led to some mortalities among experimental groups of Atlantic salmon within 4 weeks at 15 °C (Birkbeck et al. 2002).

Piscirickettsiaceae Representative

Piscirickettsia salmonis

Characteristics of the Disease

Degenerate or obligately parasitic bacteria, i.e. chlamydias and rickettsias, have been long established as pathogens of invertebrates, and sporadically mentioned in connection with fish diseases (Wolf 1981). Yet, firm evidence of their role in fish pathology has not been forthcoming until an upsurge of interest in Chile. Thus since 1989, a disease coined "coho salmon syndrome", Huito disease (Schäfer et al. 1990) or salmonid rickettsial septicaemia (Cvitanich et al. 1991) has been observed in coho salmon, chinook salmon, Atlantic salmon and rainbow trout, with a spread to Atlantic salmon in Norway (Olsen et al. 1997) and white sea bass in California, USA (Arkush et al. 2005). Losses fluctuated between 3 and 7 % of stock per week; the cumulative mortalities reaching 90 %. The organism was formally recognised as a new taxon, for which the name of *Piscirickettsia salmonis* was proposed (Fryer et al. 1992), and is a representative of the *Gammaproteobacteria*. Although the pathogen was initially associated with salmon, there has been a spread to other groups, including sea bass (McCarthy et al. 2005; Arkush et al. 2005). Also, the geographical range has spread from Chile to North America and Europe, including Greece (McCarthy et al. 2005) and Scotland (Birkbeck et al. 2004). Infected fish gathered at the surface of cages, became sluggish and were inappetant. External signs included melanosis, epidermal indurations, and paleness of the gills, which was indicative of anaemia. The haemacrits fell to $\leq$27 %. Internally, haemorrhages were evident on the abdominal walls, visceral fat and on the air bladder. A mottled liver and swollen spleen was apparent in heavily infected animals. The kidney was inflammed and swollen. The intestine was full of yellowish mucoid material (Schäfer et al. 1990; Branson and Diaz-Munoz 1991). Initially, the disease was not considered to occur during the freshwater stage of fish culture, Instead, mortalities have been noted to begin 6–12 weeks after transfer of fish from fresh to seawater (Fryer et al. 1992). However in a later development, the pathogen was recovered from the freshwater stage of coho salmon and rainbow trout (Bravo 1994; Gaggero et al. 1995).

Isolation

Isolation of the pathogen from the kidney of infected fish was possible in the cytoplasm of salmon cell lines (including CH5E-214) with incubation at 12–21 °C (optimally at 15–18 °C), whereupon a cytopathic effect was demonstrated in 5–6 days (Fryer et al. 1990). The cell sheet was completely lysed in 14 days. Originally, growth did not occur on bacteriological media, including BHIA, blood agar, mycoplasma medium, charcoal yeast extract agar, Loeffler medium (Appendix in

Chap. 12) or Mueller-Hinton agar. However, a marine-based broth medium with L-cysteine has been reported to enable the successful culture of *Pis. salmonis* from infected fish with incubation at 18 °C for 6 days (Yañez et al. 2012). A blood and serum-free medium was described leading to growth of *Piscirickettsia salmonis* at 17–19 °C (optimally at 25 °C), in which the cell mass retained pathogenicity (Henriquez et al. 2013).

The problem of purifying the bacteria from tissue culture cells was addressed by use of 30 % percoll in which bacteriophage like particles were observed by TEM (Yuksel et al. 2001) and resolved by use of iodixanol (= Optiprep) as substrate for differential centrifugation gradients which together with DNase1 digestion led to sufficient pure, i.e. 99 %, bacteria for DNA work (Henríquez et al. 2003).

Characteristics of the Pathogen

Box 11.14: ***Piscirickettsia salmonis***
The pathogen is a pleomorphic, non-motile Gram-negative predominantly coccoid (and ring forms) organism of variable size (0.5 × 1.5–2.0 μm), occurring intracellularly as individuals, pairs or groups. Electron microscopy reveals that each organism is bound by two membrane layers; a characteristic trait of the Rickettsiales, and possibly the tribe Erlichiae.

A single isolate, designated LF-89, was studied in detail by Fryer et al. (1992). The 16S rRNA conformed to the gamma subdivision of the Proteobacteria. Moreover, LF-89 did not show any specific relationship to any of 450 bacterial 16S rRNA sequences held on file. Nevertheless, similarities were apparent with *Wolbachia persica* (similarity = 86.3 %) and *Coxiella burnetii* (similarity = 87.5 %) than to representatives of *Ehrlichia, Rickettsia* or *Rochalimaea.* In short, it was deemed that the salmonid pathogen was sufficiently novel to warrant description in a new genus of the family Rickettsiaceae. The organism recovered from white sea bass was reported to have a 96.3–98.7 % 16S rDNA homology with *Pis. salmonis* (Arkush et al. 2005), which is low for a confirmed identity.

Analysis of 16S rRNA revealed that Irish isolates formed two groupings whereas Canadian, Norwegian and Scottish cultures clustered together (Reid et al. 2004). The possibility of genetic differences between isolates was examined with a view to explaining reasons for differences in virulence and mortality rates. By electrophoretic analysis of the internal transcribed spacer region of 11 Chilean isolates, two groupings were recognised (Casanova et al. 2003).

Diagnosis

Serology Isolation in cell culture or detection in acridine orange stained smears or by iFAT has been advocated (Lannan and Fryer 1991; Lannan et al. 1991). A monoclonal based ELISA was successful and specific in detecting *Piscirickettsia* in infected fish tissues (Aguayo et al. 2002).

Molecular Methods PCR technology offers promise for the detection of *Piscirickettsia salmonis* (e.g. Heath et al. 2000; Mauel and Fryer 2001; Venegas et al. 2004), and systems has already been designed which is capable of detecting 1 tissue culture infectious dose (Mauel et al. 1996) and 1–10 genome equivalents (competitive PCR; Heath et al. 2000). Subsequently, a PCR was described that was effective with a few millilitres of serum (Marshall et al. 1998). The benefit of this system is that the test could be carried on live fish, such as valuable broodstock. A *Taq*Man PCR was specific and sensitive (0.5 $TCID_{50}$/ml) (Corbeil et al. 2003). Real-time PCR has been successful at specifically detecting the pathogen in paraffin sections of fish tissue (Karatas et al. 2008). A multiplex PCR was developed for the simultaneous detection *Aer. salmonicida, Pis. salmonis, Str. phocae* and *V. anguillarum*. The detection limit using purified total bacterial DNA was 500 fg/µl for *Pis. salmonis*. The limits of detection using spiked tissues, i.e. kidney, liver, muscle or spleen, were 100 cells (Tapia-Cammas et al. 2011).

Use of Microwave Radiation The use of microwave radiation [700 w energy from a domestic microwave] has been suggested for *Pis. salmonis* (Larenas et al. 1996).

Epizootiology

It is presumed that the pathogen is passed directly between fish, or via invertebrate vectors. Data have shown that physical contact may be necessary for horizontal transmission between salmonids (Almendras et al. 1997). Certainly, the pathogen appears to survive extracellularly (Lannan and Fryer 1994), and 16S rDNA product has been recovered from bacterioplankton DNA obtained from the coastal environment in the USA (Mauel and Fryer 2001). Using competitive PCR, Heath et al. (2000) reported $3–4 \times 10^3$ cells or their DNA in surface seawater in a net pen in southern Chile.

Pathogenicity

Coho salmon, Atlantic salmon and white sea bass were infected and clinical disease with mortalities resulted after i.p. injection with cell lines of the rickettsia (Garcés et al. 1991; Arkush et al. 2005). Some differences have been detected in the comparative virulence of isolates to coho salmon from British Columbia, Chile and Norway (House et al. 1999). For example, the LD_{50} dose for a Scottish isolate was

calculated as $<2 \times 10^3$ cells (Birkbeck et al. 2004). The nature of the antigens to which animals respond has been addressed by Kuzuk et al. (1996), who used rickettsias purified from CSE cells by differential and Percoll density gradient centrifugation and rabbit antiserum. The conclusion was that the rabbit antiserum reacted with four protein and two carbohydrate (core region of the LPS) surface expressed antigens of 65, 60, 54, 51, 16 and ~11 kDa. However, a complication arises as a report of a subsequent study by Barnes et al. (1998), who concluded that the major antigens were of 56, 30 and 20 kDa. Nevertheless by using rickettsial suspensions, attempts were made to infect coho salmon via the gills, intestine (by anal intubation) and skin with the data revealing that use of all sites led to infection. However, intact skin (injury facilitated invasion) and gills were found to be the most effective entry sites, followed by intestine (Smith et al. 1999, 2004). Isolations have been made from the brain (of coho salmon), and it is reasoned that this may well be an important location of the pathogen in the host (Skarmeta et al. 2000). Experimental evidence has supported the possibility of vertical transmission when after the examination of male and female broodstock, the pathogen was detected by immunofluorescence in milt and the coelomic fluid in 14/15 of the fish (Larenas et al. 2003). Subsequently, the pathogen was detected in the resultant fry, albeit without evidence of clinical disease. After *in vitro* infection of ova, the organism was seen by SEM to be attached to the surface (of the ovum) by apparent membrane extensions; these were reasoned to allow the later entry into the ovum (Larenas et al. 2003).

Evidence from the use of cell cultures points to the pathogen replicating within the cytoplasmic vesicles of cells although the mechanisms for survival and replication are unclear. However, Isla et al. (2014) determined that during the infection process involving the SHK-1 cell line, *Pis. salmonis* increased significantly the expression of ClpB and BipA protein, which were recognised as heat shock and virulence proteins, respectively. These would permit *Pis. salmonis* to survive the hostile conditions of the macrophages, thus evading cellular degradation, and enabling intracellular replication (Isla et al. 2014). The pathogen has been recognized to possess several mechanisms for acquiring iron, which points to the ability of the organism to obtain ferrous and ferric iron from various sources (Pulgar et al. 2015). Clathrin has been determined to be required for internalization, and actin cytoskeleton have a role throughout the infective process (Ramirez et al. 2015). Cytotoxicity by thermostable ECPs has been described (Rojas et al. 2013).

Disease Control

Vaccine Development Attempts have been made to develop vaccines. In one study, formalised cells ($10^{6.7}$ $TCID_{50}$/ml) administered by i.p. led to the development of good protection in a field trial with coho salmon (Smith et al. 1995). Heat inactivated (100 °C for 30 min) and formalised whole cell suspensions containing 10^9 cells/ml gave commendable protection with RPS of 71 and 50 %, respectively, when applied intraperitoneally in adjuvant to Atlantic salmon (Birkbeck et al. 2004). An approach using oral vaccination by feeding every 3 days for 30 days at 6 mg of

whole cell vaccine/fish/day [containing 1×10^{10} cells/g of feed] incorporated in a polysaccharide matrix, termed MicroMatrix™, has been described and demonstrated promise (Tobar et al. 2011). Research has highlighted a relationship between antibody titre and protection. Consequently, there was a need for several rounds of oral vaccination to maintain high antibody tire, and thus protection (Tobar et al. 2015).

Antimicrobial Compounds Sensitivity was recorded to clarithromycin, chloramphenicol, erythromycin, gentamicin, oxytetracycline, sarofloxacin, streptomycin and tetracycline, but not to penicillin or spectinomycin (Cvitanich et al. 1991).

Rickettsia-Like Organisms

Characteristics of the Disease

An increasing number of publications have described rickettsia-like organisms (RLO) as causal agents of disease (e.g. Rodger and Drinan 1993; Chen et al. 1994; Khoo et al. 1995; Palmer et al. 1997; Jones et al. 1998; Corbeil et al. 2005; Timur et al. 2013). Whether or not these organisms correspond with *Piscirickettsia salmonis* has not been always established. For example, Chern and Chao (1994) reported that the RLO caused mass mortalities in tilapia from Taiwan during October 1992 to February 1993, with disease signs including the presence of white nodules and microscopic granulomas on all the organs, and an enlarged spleen. Subsequently, Comps et al. 1996) described a small coccoid organism from the brain of juvenile sea bass obtained from the South of France. These were derived from a population which suffered 20 % mortalities. Also, an RLO has been identified among grouper in Taiwan (Chen et al. 2000b), tilapia (Mauel et al. 2003) and white sea bass in the USA (Chen et al. 2000a) and farmed Atlantic salmon in Tasmania and Canada (Cusack et al. 2002). Moribund grouper displayed dark lesions, splenomegaly (with white nodules), necrosis in the liver, kidney and spleen, and the blood was thin (Chen et al. 2000b). However, it is difficult from the literature to determine the significance of the Tasmanian RLO to fish (Corbeil et al. 2005). RLOs were associated with systemic granulomas in sea bass, with infected fish displaying anorexia and lethargy leading to high mortalities (Timur et al. 2013).

An intracellular RLO has been linked with red-mark syndrome (Fig. 11.1) in the UK and strawberry disease [an inflammatory skin condition] in the USA as result of immunohistochemistry using polyclonal antibodies to *Piscirickettsia salmonis,* a quantitative PCR and 16S rRNA sequencing (Lloyd et al. 2008, 2011; Metselaar et al. 2010). However, intact cells of the pathogen have not been observed nor has culturing been achieved, to date. Therefore, a question surrounds the interpretation of the serological data. However, the disease appears to be spreading, and has now bee reported to occur in farmed rainbow trout in Turkey (Kubilay et al. 2014).

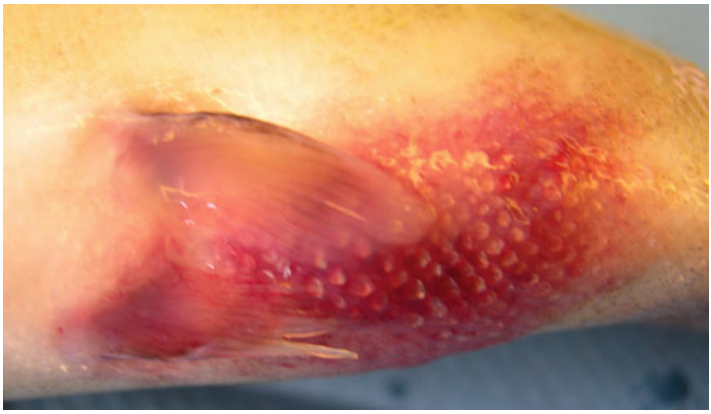

Fig. 11.1 The *reddened* area associated with *red mark* disease syndrome (= winter strawberry disease) in >500 g rainbow trout

Characteristics of the Pathogens

In one case, the pathogen was described as a Gram-negative rod of 0.86 +/− 0.32×0.63 +/− 0.24 μm in size, and thought likely to be a representative of the Rickettsiaceae (Chern and Chao 1994).

A Tasmanian isolate from Atlantic salmon was distinct from *Piscirickettsia* in terms of sequence alignment of the 16S rRNA, and for the present regarded as an RLO (Corbeil et al. 2005).

Disease Control

Antimicrobial Compounds Treatment with oxytetracycline was reported to be successful in one case (Chern and Chao 1994).

Streptobacillus

A possibly unique organism has been recovered from seawater farmed Atlantic salmon in Ireland. The organism, which occurred intracellularly in tissues, was considered to be related to *Streptobacillus moniliformis* and the fusobacteria on the basis of 16S rRNA analyses (Maher et al. (1995). BHI supplemented with 10 % (v/v) foetal calf serum and 1 % (w/v) sodium chloride with incubation for 10 days at 22 °C was used to recover the organism (Maher et al. 1995).

'*Candidatus*'

'*Candidatus*' are uncultured chlamydia-like organisms, which have been associated with epitheliocystis in numerous fish species (Fig. 11.2).

'*Candidatus* Actinochlamydia clariae'

Juvenile African sharptooth catfish (*clarias gariepinus*), which were farmed in Uganda developed epitheliocystis (Steigen et al. 2013). The fish were observed to be swimming at the surface, sometimes upside down, and showing respiratory distress. Histology of gill tissues revealed large numbers of epitheliocysts, and also the presence of some *Ichthyobodo* sp. and *Trichodina* sp. Sequencing of the 16S rRNA gene of the bacteria from the epitheliocysts revealed 86.3 % homology to '*Candidatus* Piscichlamydia salmonis'. TEM revealed similarity to clamydias. A unique feature was the radiation of tubules/channels (= actinae) from the inclusion membrane (Steigen et al. 2013).

'*Candidatus* Arthromitus'

The condition, which affects rainbow trout during summer (water temperature = >15 °C), has been recognized in Europe, i.e. Croatia, France, Italy, Spain and the UK, (Del-Pozo et al. 2009), and may result from climatic and stress conditions. It is

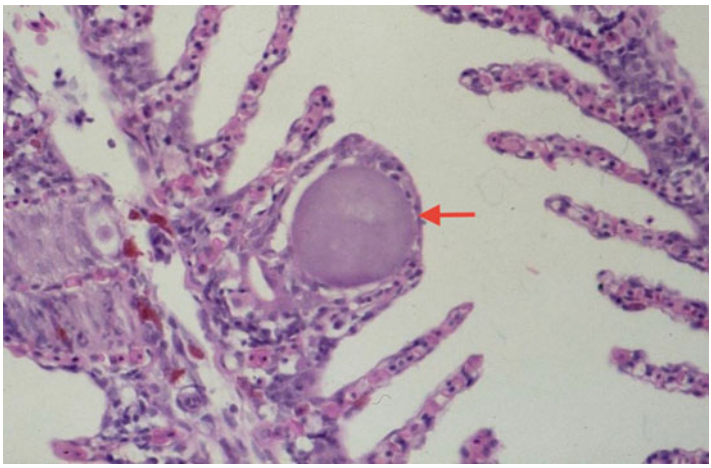

Fig. 11.2 Epitheliocystis (*see arrow*) in *Sparus aurata.* The photograph, courtesy of Professor J.F. Turnbull, was taken at a magnification of × 400

characterized by the huge populations of segmented, filamentous bacteria in the digestive tract, and daily losses of 0.5–1 % (Michel et al. 2002). Affected fish are lethargic and inappetant, and yellow, mucoid faeces may extend from the vent. The digestive tract is haemorrhagic and odematous, and filled with mucoid material (Michel et al. 2002). Isolation of bacteria with the morphological characteristics of '*Candidatus* Arthromitus' was not achieved (Michel et al. 2002). However, the pathogen could be detected using a nested PCR, bot blotting and Southern blotting material from the digestive tract of rainbow trout (Del-Pozo et al. 2010; Cecchini et al. 2012). By the nested PCR, positivity was recorded in the distal intestine and pyloric caeca of rainbow trout displaying signs of gastro-enteritis (Del-Pozo et al. 2010). Possibly, the organism comprises part of the normal microflora of the digestive tract (Michel et al. 2002a). Alternatively, it is feasible that the organism could be introduced via feed. Pathogenicity may involve an unspecified toxin (Michel et al. 2002).

'Candidatus Branchiomonas cysticola'

Atlantic salmon, which were farmed in Norway, displayed gill epitheliocystis, from which '*Candidatus* Piscichlamydia salmonis' was recognized by PCR together with another organism (Toenshoff et al. 2012). The new bacterium, which was regarded as responsible for the formation of the cysts, was recognized by 16S rRNA methods as a new ß-proteobacterial representative, and, coined '*Candidatus* Branchiomonas cysticola'. Reticulate and intermediate bodies but not the elementary bodies typical of chlamydia were observed in the cysts as described previously from salmon (Toenshoff et al. 2012). Thus, the authors regarded that this form of epitheliocystis was caused by a novel agent phylogenetically distinct from the Chlamydiae.

Since its original description, the pathogen has been recognized in farmed Atlantic salmon in Ireland (Mitchell et al. 2013). To aid diagnosis, a highly sensitive and specific RT-PCR was developed, and as a result of its use, the dominance of the organism in epitheliocystis in Ireland and Norway was recognized (Mitchell et al. 2013).

'Candidatus Clavochlamydia salmonicola'

Chlamydia are obligate intracellular parasites, and one group in particular has been linked to a disease of Norwegian salmonids from freshwater involving the formation of unicellular cysts in epithelial tissues, i.e. epitheliocystis. Cells have been observed, and described as are pleomorphic of up to 2 µm in length, and have a developmental cycle similar to that of the *Chlamydia* (Karlsen et al. 2008). Based on sequencing of the partial 16S rRNA gene, the causal agent has been linked the Chlamydiaceae, and the name '*Candidatus* Clavochlamydia salmonicola' proposed (Karlsen et al. 2008).

'Candidatus Piscichlamydia salmonis'

This organism has been proposed for the causal agent of epitheliocystis in seawater reared Atlantic salmon in Ireland and Norway (Draghi et al. 2004). Furthermore, the organism has been identified by RT-DGGE in the majority (16/21) of Atlantic salmon displaying proliferative gill inflammation in Norway (Steinum et al. 2009) and in epitheliocystis Arctic charr in Canada and the USA (Draghi et al. 2010).

'Candidatus Renichlamydia lutjani'

Chlamydia-like Gram-negative organisms were seen intracellularly in basophilic granular inclusions [epitheliocyst-like] in the kidney and spleen of blue-striped snapper (*Lutjanus kasmira*). Molecular evidence pointed to a new chlamydia, which is interesting because this is the first demonstration of the group associated with a disease of the internal organs (Corsaro and Work 2012).

'Candidatus Similichlamydia latridicola'

The gills of striped trumpeter (*Latris lineata*), which were farmed in Tasmania, Australia together with wild specimens, were examined histologically, and revealed the presence of basophilic epitheliocystis-like inclusions. 16S rRNA sequencing recognized the presence of a novel chlamydia-like organism, for which the name of '*Candidatus* Similichlamydia latridicola' was proposed (Stride et al. 2013).

'Candidatus Syngnamydia venezia'

Broad nosed pipefish (*Syngnathus typhle*) with epitheliocystis were recognized to have a new obligately intracellular chlamydia-like pathogen for which name of '*Candidatus* Syngnamydia venezia' was proposed (Fehr et al. 2013).

Chlamydiales Representative

A previously unknown chlamydia has been associated with epitheliocystis in a leopard sharp (*Triakis semifasciata*) from an aquarium in Switzerland (Polkinghorne et al. 2010).

Unidentified Gram-Negative Bacteria

There is an increasing awareness of diseases caused by apparently unique bacteria. For example, Sorimachi et al. (1993) and Iida and Sorimachi (1994) described jaundice in yellowtail, attributed to an unknown filamentous bacterium of 4–6 μm in length, which grew only in L15 medium and Eagles MEM medium each supplemented with 10 % (v/v) foetal calf serum at 23–26 °C.

The human and animal pathology literature abounds with references to hard-to-identify or unidentified pathogens. An example from fish pathology concerns a hitherto unknown intracellular bacterial pathogen of farmed Atlantic salmon in Ireland (Palmer et al. 1994). During 1992–1993 when the water temperature was 8–9 and 15–16 °C, fish became lethargic, swam close to the water surface, and displayed loss of balance. Apart from an infestation of salmon lice, the fish revealed the presence of petechia or haemorrhagic areas on the abdominal walls, petechia on the pyloric caeca and swim bladder, congestion of the kidney and spleen, splenomegaly, and kidney swelling. The fore and hind guts contained white mucus. Some fish had pale friable livers, pale spleens, visceral adhesions, and false membranes in the peritoneum (Palmer et al. 1994). The intracellular Gram-negative cocco-bacilli required serum or blood for growth, which was accomplished on 7 % (v/v) horse blood agar and 10 % (v/v) foetal calf serum medium after 4–14 days incubation at 15 or 22 °C (Palmer et al. 1994).

Box 11.15: Unknown Gram-Negative Organism of Palmer et al. (1994)
Cultures produce small colonies of 0.3 mm in diameter after incubation aerobically for 10–14 days. Anaerobic incubation results in larger colonies of 0.6 mm in diameter. Improved growth results by the addition of 0.5 mg of L-cysteine hydrochloride/l. Colonies are off-white, convex and granular, and contain non-motile, Gram-negative ß-haemolytic cocco-bacilli. Alkaline phosphatase (weakly positive/variable), arginine dihydrolase, H_2S (weakly positive/variable), indole (weakly positive/variable) and lipases are produced, but not α-glucosidase, N-acetyl glucosamine, catalase, ß-galactosidase, ß-glucuronidase, lysine decarboxylase or oxidase. Nitrates are not reduced. The Voges Proskauer reaction is negative. Neither aesculin, gelatin, Tween 80 nor urea is attacked. Citrate is not utilised. Fructose, glucose and maltose are fermented, but not glycogen, lactose, mannitol, mannose, ribose, sucrose or xylose. Growth occurs at 10 °C but not at 37 °C, and in 1–4 but not 5 % (w/v) sodium chloride.

The organism was linked tenuously to the Neisseriaceae and Pasteurellaceae., although the rickettsias may be an appropriate home. According to Palmer et al. (1994) infectivity was achieved by injecting cells into Atlantic salmon, which became moribund after 6–10 days, and died within 30 days. Moribund fish dis-

played haemorrhagic areas in the jaw, cranium and at the base of the fins. Control may be possible by means of antibiotic therapy as the pathogen was susceptible to amoxicillin and penicillin G, but resistant to cotrimoxazole and oxytetracycline (Palmer et al. 1994).

Another unidentified Gram-negative organism was linked to a previously undescribed condition, coined *Varracalbmi* (= bloody eye), in Norwegian farmed Atlantic salmon. The disease, which occurred in northern Norway during 1989–1992, was described as a haemorrhagic, necrotizing, pyogranulomatous inflammation of the eye, being termed panophthalmitis. Lethargy, deep ulcers, necrosis, and haemorrhagic, granulomatous, pyogenic visceral organs occurred (Valheim et al. 2000). Mortalities of only 2.5 % were reported (Valheim et al. 2000). The source of the infection was unknown, but may well have been another cold water marine fish (Valheim et al. 2000). The unnamed organism associated with *Varracalbmi* grew at 22 °C on 5 % (v/v) citrated bovine blood agar supplemented with 2 % (w/v) NaCl from inocula obtained from dermal lesions, kidney and liver (Valheim et al. 2000). The organism was challenged i.p. into groups of Atlantic salmon smolts (average weight = ~37 g) with the outcome that total mortality resulted with a dose of 4×10^7 cells (51 % mortality with a dose of 4×10^4 cells) (Valheim et al. 2000).

Box 11.16: Causal Agent of *Varracalbmi*

The small, i.e. 1 mm in diameter, colonies, which grow anaerobically into the agar medium, comprise Gram-negative non-motile slender fermentative rods sometimes arranged as short chains (when grown in broth), which produce arginine dihydrolase, oxidase but not catalase, indole or lysine or ornithine decarboxylase, attack starch (weakly) and lecithin (weakly) but not aesculin, casein, chitin, gelatin, Tween 20 or 80 or urea, and are non-haemolytic (α-haemolysis is recorded after a week), require NaCl, and grow at 4–22 °C, do not reduce nitrates, but are sensitive to the vibriostatic agent, O/129. Growth occurs with galactose, glucose, glycerol, lactose, maltose, mannitol, mannose and sorbitol, but not arabinose, cellobiose, erythritol, melibiose, raffinose or rhamnose (Valheim et al. 2000).

The authors considered that the organism is linked to the Pasteurellaceae or Vibrionaceae (Valheim et al. 2000), and this seems appropriate.

An unusual Gram-negative bacterial culture was linked to ulceration (singular circular ulcers of 10–15 mm in diameter on the flank) in Scottish farmed rainbow trout (Fig. 11.3) (Austin et al. 2003a). The unnamed organism was linked to *Ultramicrobacterium* by 16S rRNA sequencing (homology = 95 %) (Austin et al. 2003a).

Gram-negative bacteria were associated with a mass mortality of cultured rockfish *(Sebastes schlegeli)* in Japan during the spring of 2001, with the disease signs reflecting aneurysms, hyperplasia, lamellar fusion, haemorrhaging and necrosis of

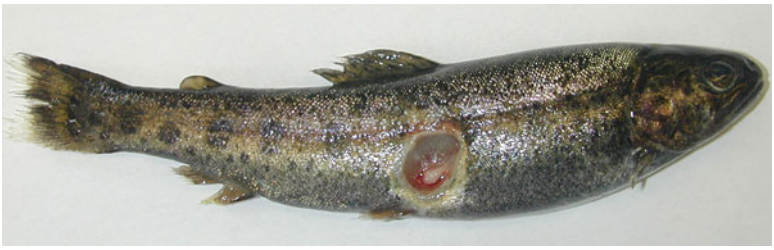

Fig. 11.3 Limited tail erosion and an ulcer on the flank of rainbow trout. The casual agent was considered to be linked to ultramicrobacteria

the gills. Some fish revealed bacterial invasion of the kidney, myocardium and spleen. Yet, cultures were not recovered for further work (Kobayashi et al. 2005).

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Chapter 12
Isolation/Detection

Abstract There is no single technique suitable for the recovery of all known bacterial fish pathogens. Scientists need to use a combination of methods and incubation conditions to achieve pure cultures. Even so, not all taxa are culturable: for example *Candidatus* have not been cultured *in vitro*.

Keywords Media recipes • Isolation • Marine 2216E agar • TSA • BHIA

To an extent, the range of media to be used is governed by personal choice and experience (Table 12.1). The formulae of commonly used media is included in an appendix (Appendix "Appendix – Media used for the isolation and growth of bacterial fish pathogens") at the end of the chapter. However, the incubation regimes adopted in many laboratories needs some attention as 30 or 37 °C for 24–48 h is not sufficient to recover anything apart from the fastest growing organisms, which may mean that the real pathogens are missed.

- For marine fish, it is advisable to include media prepared with seawater, e.g. seawater/marine 2216E agar (for example, as supplied by Difco). Alternatively, some media such as TSA may be supplemented with 1–2 % (w/v) sodium chloride, which is suitable for the isolation and growth of many heterotrophic marine bacterial fish pathogens.
- For marine fish with damaged gills, low nutrient agar, such as cytophaga agar (see Appendix "Appendix – Media used for the isolation and growth of bacterial fish pathogens") prepared in seawater, should be used.
- For freshwater fish, the routine use of TSA and/or BHIA (as supplied by Difco or Oxoid) is recommended. Nutrient agar, which may be used, is more suited for medical rather than fish bacteria.

Although most of the pathogens are aerobic, it is worthwhile remembering that *Cl. botulinum* and *Eu. tarantellae* are anaerobic. For the former, Robertson's meat broth (Appendix "Appendix – Media used for the isolation and growth of bacterial fish pathogens") should be used for isolation, whereas with the latter, BHIA is satisfactory. For some aerobic organisms, i.e. *Acinetobacter*, atypical *Aer. salmonicida*

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Table 12.1 Methods of isolation for bacterial fish pathogens

Medium[a]	Temperature of incubation (°C)	Pathogen
Aeromonas selective medium	?	*Aer. veronii* biovar sobria
AUSTRAL-SRS broth + L-cysteine	18 °C	*Piscirickettsia salmonis*
BHIA	20–37 °C	*Aer. schubertii, Aer. sobria, Cit. freundii, Cor. aquaticum, Delftia acidivorans, Edw. ictaluri, Edw. tarda, Haf. alvei, Halomonas cupida, Lactococcus garvieae, Lactococcus piscium, Planococcus* sp., *Ps. alcaligenes, Sal. enterica* subsp. *arizonae, Ser. liquefaciens, Ser. marcescens, Sta. aureus, Sta. epidermidis, Streptococcus* spp., *Y. ruckeri*
BHIA	22–24 °C (anerobically)	*Eu. tarantellae*
BHIA supplemented with 5–10 % (v/v) blood	20–25 °C	*Aerococcus viridans, Cit. freundii, Haf. alvei, Ph. damselae, Salmonella enterica* subsp. *arizonae*
BHIA supplemented with 3 % (w/v) NaCl	15–25 °C	*Shewanella putrefaciens*
BHIA supplemented with 10 % (v/v) foetal calf serum and 1 % (w/v) NaCl	22 °C	*Streptobacillus*
Blood agar	15–37 °C	streptococci, *Vag. salmoninarum, Y. intermedia*
Blood agar	15, 22 or 25 °C	*Aer. caviae, Bacillus mycoides, Edw. piscicida, Erysipelothrix rhusiopathiae Myc. neoaurum, Rhodococcus erythropolis, Rhodococcus qingshengii, Weissella* sp.
Blood agar supplemented with 1.5 % (w/v) NaCl	37 °C	*Weissella ceti*
	15 °C	*V. splendidus, V. tapetis*
Blood agar supplemented with 0.5–1.5 % (w/v) NaCl	22–25 °C	*Acinetobacter* sp., *Ph. damselae* subsp. *piscicida, Ali. logei, Moritella marina, Pasteurella skyensis*
Campylobacter-selective agar	25 °C	*Arcobacter cryaerophilus*
CBB	15–25 °C	*Aer. salmonicida*
Cresol red thallium acetate sucrose inulin agar	22 °C	*Carnobacterium maltaromaticum*-like organ
Cystine heart agar supplemented with 1 % (w/v) haemoglobin	?	*Francisella* sp.

(continued)

Table 12.1 (continued)

Medium[a]	Temperature of incubation (°C)	Pathogen
Cytophaga agar	18–20 °C	*Fla. branchiophilum, Fla columnare, Fla. hydatis, Fla. johnsoniae, Fla. ovolyticus, Fla. psychrophilus*
Cytophaga agar prepared in sea water	20 °C	*Chryseobacterium* spp., *Tenacibaculum* spp., *Myxococcus piscicola, Sporocytophaga* sp. (possibly)
Cytophaga agar supplemented with 5 mg of neomycin/ml and 200 IU of polymyxin B/ml	30 °C	*Flectobacillus roseus*
Fle. maritimus medium	20 °C	*Tenacibaculum* spp., *Ps. baetica*
Glucose asparagine agar	25–37 °C	*Noc. salmonicida*
Hayflick medium	room temperature	*Mycoplasma mobile*
Hsu-Shotts medium	22 °C	*Fla. spartansii*
Hsu-Shotts medium supplemented with 4 mg of neomycin/ml	22 °C	*Chryseobacterium* spp.
KDM2/SKDM	15 °C	*Ren. salmoninarum*
LG agar	25–30 °C	*Lactococcus garvieae*
Löwenstein-Jensen medium/Dorset egg medium	15–22 °C	*Mycobacterium* spp., *Nocardia* spp.
Marine 2216E agar	20–30 °C	*Myroides odoratimimus, Pseudoalteromonas piscicida,.*
Middlebrook 7H10 agar	30 °C	*Mycobacterium* spp.
Middlebrook medium	25 °C	*Myc. montefiorense*
Mueller Hinton agar supplemented with 5 % (v/v) sheep blood	26 °C	*Lactococcus lactis* subsp. *lactis*
Nutrient agar	20–25 °C	*Haf. alvei, Ps. anguilliseptica, Ps. chlororaphis, V. cholerae*
Nutrient agar	37 °C	*Bacillus* spp.
Nutrient agar supplemented with 0.05 % (w/v) activated charcoal	20 °C	*Fla. psychrophilum*
1 % Ogawa-egg medium	30 °C	*Myc. gordonae*
0.1 % (w/v) peptone and agar prepared in sea water	20–25 °C	*Fla. piscicida*

(continued)

Table 12.1 (continued)

Medium[a]	Temperature of incubation (°C)	Pathogen
Robertson's meat broth	30 °C (anaerobically)	*Cl. botulinum*
Seawater agar (marine 2216E agar)	15–25 °C	*Ph. damselae* subsp. *piscicida, Ali. fischeri, Vibrio* spp., *Pseudoalteromonas undina*
Skimmed milk agar	15–25 °C	*Janthinobacterium* sp., *Micrococcus luteus, Planococcus* sp.
TCBS	15–25 °C	*Delftia acidivorans, Ph. damselae, Ali. fischeri V. alginolyticus, V. anguillarum,, V. harveyi, V. ordalii, V. pelagius* and *V. splendidus*
Tissue culture (salmonid cell line)	12–21 °C	*Francisella* sp., *Piscirickettsia salmonis*
Todd-Hewitt agar supplemented with 30 µg/ml of Congo red	37 °C for 30 h	*Str. dysgalactiae*
TSA [possibly supplemented with 1–2 % (w/v) NaCl	15–25 °C	*Aer. allosaccharophila, Aer. caviae, Aer. dhakensis, Aer. hydrophila, Aer. piscicola. Aer. salmonicida, Aer. sobria, Bacillus* sp., *Car. piscicola, Cit. freundii, Delftia acidivorans, Edw. piscicida, Enterobacter cloacae, En. faecalis* subsp. *liquefaciens, Esch. vulneris, Pantoea agglomerans, Providencia vermicola, Flavobacterium* sp., *Hahella chejuensis, J. lividum, Klebsiella pneumoniae, Lactobacillus* spp., *Lactococcus lactis* subsp. *lactis, Micrococcus luteus, Moraxella* sp., *Planococcus* sp., *Plesiomonas shigelloides, Pr. rettgeri, Pseudomonas* spp., *Rhodococcus* sp., *Rhodococcus qingshengii, Ser. marcescens, Ser. plymuthica, Sta. warneri, Ali. salmonicida, V. harveyi, V. pelagius, V. splendidus, V. vulnificus, Y. ruckeri*
TSA supplemented with 10 % (v/v) horse serum	unspecified	*Aquaspirillum*
TSA supplemented with 5 % (v/v) defibrinated sheep blood	30 °C	*Str. ictaluri*
Yeast extract glucose agar	25 °C	*Vag. salmoninarum*

[a]Most of these media may be obtained from Difco and/or Oxoid

and *Ph. damselae* subsp. *piscicida*, the use of media supplemented with blood aids isolation.

However, the recovery of organisms on solid or in liquid media does not infer the isolation of the pathogen causing the disease. A minimum requirement is that dense growth should develop on the isolation plates. One, two or even three colonies types should not be ignored as this situation could suggest infection by more than organism. It should be emphasised that the organisms recovered on bacteriological media do not necessarily mean the instigator of the disease has been recovered. A microbial species succession could well occur with one organism instigating the infection, which may then be exacerbated by another. The possibility of secondary invaders or contaminants/saprophytes should not be ignored. Yet, scant growth of a diverse range of colony types does not suggest recovery of the actual pathogen.

Appendix – Media Used for the Isolation and Growth of Bacterial Fish Pathogens

Anderson and Conroy's Medium for Sporocytophaga *– Like Organisms*

5.0 % (w/v) enzymic digest of fish muscle
0.1 % (w/v) peptone
0.1 % (w/v) yeast extract
0.9 % (w/v) agar
pH 7.0
This medium is prepared in seawater.

Blood and Serum Free Medium for Piscirickettsia salmonis *(Henriquez et al. 2013)*

0.5 % (w/v) glutamic acid
0.2 % (w/v) yeast extract
0.8 % (w/v) peptone from meat (peptic digested)
0.132 % (w/v) $(NH_4)_2SO_4$
0.01 % (w/v) $MgSO_4.7H_2O$
0.63 % (w/v) K_2HPO_4
0.9 % (w/v) NaCl

0.008 % (w/v) $CaCl_2.2H_2O$
0.002 % (w/v) $FeSO_4.7H_2O$
pH 6.6; 121 °C/15 min – but $CaCl_2.2H_2O$ and $FeSO_4.7H_2O$ were sterilised separately to avoid precipitation; glucose was also sterilized separately to the yeast extract and peptone.

Bootsma and Clerx's Medium for Flavobacterium columnare

0.05 % (w/v) casitone
0.05 % (w/v) yeast extract
1.0 % (w/v) agar
pH 8.0

Brewer's Thioglycollate Medium

0.1 %(w/v) Lab-lemco
0.2 % (w/v) yeast extract
0.5 % (w/v) peptone
0.5 % (w/v) dextrose
0.5 % (w/v) sodium chloride
0.11 % (w/v) sodium thioglycollate
0.0002 % (w/v) methylene blue
0.1 % (w/v) agar No. 1
pH 7.2
Sterilise at 121 °C/15 min

Charcoal Agar; for the Growth of Renibacterium salmoninarum

1.0 % (w/v) peptone
0.05 % (w/v) yeast extract
0.1 % (w/v) L-cysteine hydrochloride
0.1 % (w/v) activated charcoal
1.5 % (w/v) agar
pH 6.8; sterilise at 121 °C for 15 min (the charcoal may be placed in dialysis tubing prior to sterilisation in order to obtain a clear broth medium).

Columbia Agar

2.3 % (w/v) special peptone
0.1 % (w/v) starch
0.5 % (w/v) sodium chloride
1 % (w/v) agar No. 1
pH 7.3
Sterilise at 121 °C/15 min.

Coomassie Brilliant Blue Agar (CBB)

0.01 % (w/v) coomassie brilliant blue dye [C.I. 42655]
TSA
sterilise at 121 °C/15 min.

Cysteine Blood Agar

0.3 % (w/v) beef extract
0.1 % (w/v) L-cysteine hydrochloride
20 % (v/v) human blood
0.5 % (w/v) sodium chloride
0.05 % (w/v) yeast extract
1.5 % (w/v) agar

Cytophaga Agar (Anacker and Ordal 1959)

0.05 % (w/v) tryptone
0.05 % (w/v) yeast extract
0.02 % (w/v) sodium acetate
0.02 % (w/v) beef extract
0.9 % or 1.0 % (w/v) agar
pH 7.2–7.4; sterilise at 121 °C for 15 min.

Improved Growth Medium for Fla. psychrophilum *(Daskalov et al. 1999)*

Cytophaga agar/broth supplemented with:
0.05 % (w/v) D(+) galactose
0.05 % (w/v) D(+) glucose
0.05 % (w/v) skimmed milk powder
The supplement were prepared as 10 % (w/v) solutions, and filtered separately through 0.22 μm porosity filters, and added to molten, cooled medium

LG Agar for Lactococcus garvieae *(Chang et al. 2014)*

5 % (w/v) sucrose
3 % (w/v) oxgall (Difco)
0.9 % (w/v) proteose peptone No. 3
0.6 % (w/v) pancreatic digest of casein
0.5 % (w/v) proteose peptone
0.1 % (w/v) dextrose
1.5 % (w/v) agar
The ingredients were dissolved, adjusted to pH 7.0 before sterilizing at 121 °C/15 min.
The medium was cooled to 55 °C, and 8 ml/l of 1 % (w/v) filter-sterilized tetrazolium mixture, i.e. 2,3,5-triphenyltetrazolium chloride and tetrazolium blue chloride in the ratio of 9:1, and 1 ml/l of 1 % (w/v) filter-sterilized potassium tellurite were added. When solidified, the medium was amber coloured.

Nutrient Agar Supplemented with Activated Charcoal for Fla. psychrophilum *(Álvarez and Guijarro 2007)*

Nutrient agar supplemented with 0.05 % (w/v) activated charcoal

Dorset-Egg Medium

75 % (v/v) fresh egg mixture (whites and yolks)
0.25 % (w/v) lab-lemco powder
0.25 % (w/v) peptone
0.125 % (w/v) sodium chloride

crystal violet may also be incorporated to suppress contaminants.
inspissated (~80 °C for 1 h)

Edwardsiella tarda *(ET) Medium (Lindquist 1991; Castro et al. 2011)*

4.0 % (w/v) MacConkey agar base
0.1 % (w/v) yeast extract
0.45 % (w/v) agar [in addition to that contained in the MacConkey agar]
900 ml of distilled water
Autoclave (121 °C/15 min) and cool. Then add filter-sterilised solutions [100 ml to make 1.0 l in total] of:

A
0.2 % (w/v) glucose
0.5 % (w/v) sucrose
0.5 % (w/v) xylose
1.0 % (w/v) L-lysine
0.68 % (w/v) sodium thiosulphate
0.08 % (w/v) ferric ammonium sulphate
100 ml of distilled water

B
10 ml of colistin (1 mg/ml)

Emerson Agar

0.4 % (w/v) beef extract
0.1 % (w/v) yeast extract
0.4 % (w/v) peptone
1 % (w/v) dextrose
0.25 % (w/v) sodium chloride
2 % (w/v) agar
pH 7.0
Sterilise at 121 °C/15 min.

Flavobacterium columnare *Selective Medium (Fijan 1969)*

0.02 % (w/v) beef extract
0.05 % (w/v) tryptone
0.05 % (w/v) yeast extract

0.02 % (w/v) sodium acetate
0.9 % (w/v) agar
pH 7.2–7.4
sterilise at 121 °C/15 min, allow to cool, and add filter sterilised antibiotic solutions:
5 μg/ml of neomycin sulphate and 10 IU/ml of polymyxin B.

FLP Medium (for **Fla. psychrophilum***; Cepeda et al. 2004)*

0.05 % (w/v) glucose
0.02 % (w/v) $CaCl_2.2H_2O$
0.05 % (w/v) $MgSO_4.7H_2O$
0.4 % (w/v) tryptone
0.04 % (w/v) yeast extract
1 % (w/v) agar
pH 7.2–7.4
sterilise at 121 °C/15 min

Kidney Disease Medium 2 (KDM2)

1.0 % (w/v) peptone
0.05 % (w/v) yeast extract
0.1 % (w/v) L-cysteine hydrochloride
1.5 % (w/v) agar
pH 6.5; sterilise at 121 °C for 15 min, cool to 45 °C, and add 20 % (v/v) sterile foetal calf serum.

L-F Medium

- 1.0 % (w/v) brain heart infusion
- 10.0 % (w/v) sucrose
- 0.5 % (w/v) yeast extract
- 1.0 % (w/v) agar No. 3
- Sterilise at 115 °C/20 min

10 % (v/v) horse serum, inactivated by heating at 60 °C/30 min.
Cool the medium and warm the horse serum to ~50 °C, mix, and pour as plates.

Loeffler (Serum) Medium

75 % (v/v) horse serum
0.25 % (w/v) Lab-lemco powder
0.25 % (w/v) peptone
0.25 % (w/v) dextrose
0.125 % (w/v) sodium chloride
distilled water to 1 l.

Löwenstein-Jensen Medium

0.15 % (w/v) potassium hydrogen phosphate
o.15 % (w/v) magnesium sulphate hydrated
0.037 % (w/v) magnesium citrate
0.22 % (w/v) asparagine
0.73 % (v/v) glycerol
1.83 % (w/v) potato starch
60.97 % (v/v) fresh egg mixture (whites and yolks)
0.024 % (w/v) malachite green
inspissated (~80 °C for 1 h)

MacConkey Agar

2 % (w/v) peptone
1 % (w/v) lactose
0.5 % (w/v) bile salts
0.5 % (w/v) sodium chloride
0.0075 % (w/v) neutral red
1.3 % (w/v) agar
pH 7.4
Sterilise at 121 °C/15 min.

Medium K (Mudarris and Austin 1988)

0.5 % (w/v) beef extract
0.6 % (w/v) casein
0.2 % (w/v) tryptone
0.1 % (w/v) yeast extract

0.1 % (w/v) calcium chloride
1.5 % (w/v) agar
pH 7.0; sterilise at 121 °C/15 min

Middlebrook 7H10 Agar

0.05 % (w/v) ammonium sulphate
0.15 % (w/v) potassium phosphate
0.15 % (w/v) sodium phosphate
0.04 % (w/v) sodium citrate
0.0025 % (w/v) magnesium sulphate
0.00005 % (w/v) calcium chloride hydrated
0.0001 % (w/v) zinc sulphate
0.0001 % (w/v) cupric sulphate
0.05 % (w/v) L-glutamic acid
0.5 % (v/v) glycerol
0.04 % (w/v) ferric ammonium citrate
0.0001 % (w/v) pyridozine
0.00005 % (w/v) biotin
0.000025 % (w/v) malachite green
1.5 % (w/v) agar
pH 6.6; sterilise at 121 ° C for 15 min, cool to 50–55 °C and add supplements of either bovine albumin – fraction V, glucose and beef catalase (to 0.5 % w/v, 0.2 % w/v and 0.0003 % w/v, respectively); oleic acid, bovine albumin – fraction V, glucose, beef catalase and sodium chloride (to 0.005 % v/v, 0.05 % w/v, 0.2 % w/v, 0.0004 % w/v and 0.085 % w/v, respectively); or oleic acid, bovine albumin – fraction V, glucose, beef catalase, sodium chloride and Triton (to 0.005 % v/v, 0.05 % w/v, 0.02 % w/v, 0.0004 % w/v, 0.085 % w/v and 0.025 % v/v, respectively).

Myxobacterium Selective Medium (Hsu et al. 1983)

0.3 % (w/v) casein
0.2 % (w/v) tryptone
0.05 % (w/v) yeast extract
0.03 % (w/v) calcium chloride
1.0 % (w/v) agar
pH 7.0
sterilise at 121 °C/15 min, allow to cool to ~50 °C, and add filter sterilised antibiotic solution:

10 µg/ml of erythromycin, 10 µg/ml of neomycin sulphate or 256 IU/ml of polymyxin sulphate (this may be replaced by colistin sulphate).

Peptone Beef Extract Glycogen Agar (PBG)

1 % (w/v) beef extract
0.5 % (w/v) glucose
1 % (w/v) peptone
0.5 % (w/v) sodium chloride
0.004 % (w/v) bromothymol blue
1.5 % (w/v agar)
2 % (w/v agar) for overlay.
Sterilise separately at 121 °C/15 min. The basal medium is used for pour plates, after which the water agar is used as an overlay.

Petragnani Medium

900 ml whole milk
36 g potato flour
500 g potato
1200 ml whole egg (whites and yolks)
70 ml glycerol
1.2 g malachite green
pH 7.2

Photobacterium damselae ***subsp.*** **piscicida** ***Medium (Hashimoto et al. 1989)***

1 % (w/v) casamino acids/polypeptone
0.5 % (w/v) yeast extract
0.2 % (w/v) galactose
1 % (w/v) sodium glutamate
0.5 % (w/v) magnesium acetate
Agar may be added to solidify the medium.

Pseudomonas *F Agar*

1 % (w/v) tryptone
1 % (w/v) proteose peptone
0.15 % (w/v) dipotassium phosphate
0.15 % (w/v) magnesium sulphate
1 % (w/v) agar
1 % (v/v) glycerol
pH 7.0
Sterilise at 121 °C/15 min.

Ribose Ornithine Deoxycholate Medium for the Isolation of Yersinia ruckeri *(Rodgers 1992)*

0.3 % (w/v) yeast extract
0.1 % (w/v) sodium deoxycholate
0.5 % (w/v) sodium chloride
0.68 % (w/v) sodium thiosulphate
0.08 % (w/v) ferric ammonium citrate
0.75 % (w/v) maltose
0.375 % (w/v) ribose
0.375 % (w/v) ornithine hydrochloride
0.1 % (w/v) sodium dodecyl sulphate
0.008 % (w/v) phenol red
1.25 % (w/v) agar
pH 7.4; 10 ml of a filtered (0.22 µm) solution containing 0.5 g sucrose/ml should be added after the basal medium has been autoclaved (121 °C/15 min) and cooled to 50 °C.

Rimler-Shotts Medium (Shotts and Rimler 1973)

0.05 % (w/v) L-lysine hydrochloride
0.65 % (w/v) L-ornithine hydrochloride
0.35 % (w/v) maltose
0.68 % (w/v) sodium thiosulphate
0.03 % (w/v) L-cysteine hydrochloride
0.003 % (w/v) bromothymol blue
0.08 % (w/v) ferric ammonium citrate
0.1 % (w/v) sodium deoxycholate
0.0005 % (w/v) novobiocin

0.3 % (w/v) yeast extract
0.5 % (w/v) sodium chloride
1.35 % (w/v) agar
pH 7.0; after boiling to dissolve the ingredients, the medium is not sterilised further.

Robertsons Meat Broth (= Cooked Meat Medium)

45.4 % (w/v) heart muscle
1 % (w/v) peptone
1 % (w/v) Lab-lemco powder
0.2 % 9 w/v) dextrose
0.5 % (w/v) sodium chloride
pH 7.2
Sterilise at 121 °C/15 min.

Selective Flexibacter Medium (Bullock et al. 1986)

0.2 % (w/v) tryptone
0.05 % (w/v) yeast extract
0.3 % (w/v) gelatin
1.5 % (w/v) agar
sterilise at 121 °C for 15 min, cool to 45 °C and add filter sterilised neomycin sulphate (0.0004 % w/v)

Selective Kidney Disease Medium (SKDM)

1.0 % (w/v) tryptone
0.05 % (w/v) yeast extract
0.005 % (w/v) cycloheximide
1.0 % (w/v) agar
pH 6.8; sterilise at 121 °C for 15 min, cool to 50 °C, add sterile foetal calf serum to 10 % (v/v), and filter sterilised solutions of L-cysteine hydrochloride (0.1 % w/v), D-cycloserine (0.00125 % w/v), polymyxin B sulphate (0.0025 % w/v), and oxolinic acid (0.00025 % w/v).

Semi Defined Medium for Renibacterium salmoninarum

Amount/1 l	Ingredient	Preparation
10 g	Tryptone	
10 ml	Mineral salts solution	Contains per 200 ml: EDTA dihydrate (disodium salt), 100 mg; $MgCl_2$, 4 g; $CaCl_2.2H_2O$, 1.4 g; $FeCl_2.6H_2O$, 100 mg; $ZnSO_4.7H_2O$, 100 mg; $MnSO_4.4H_2O$, 100 mg; $CuSO_4.5H_2O$, 50 mg; $CoCl_2.6H_2O$, 50 mg; $Na_2MoO_4.2H_2O$, 50 mg
10 ml	Nitrogen compounds	Contains per 100 ml: uracil, 50 mg; guanine, 50 mg; adenine, 50 mg; xanthine, 50 mg
1 mg each	Vitamins	Nicotinic acid, riboflavin, thiamine, calcium pantothenate
2 mg each	Vitamins	Pyridoxal HCl, pyridoxine HCl (prepared after Rogosa et al. 1961)
20 ml	Buffer (pH 6.8)	Contains per 100 ml: K_2HPO_4, 15 g; KH_2PO_4, 15 g; sterilised at 121 °C/15 min
8 ml	Cysteine HCl	Prepared immediately prior to addition as a solution (12.5 % [w/v]) in N NaOH to give a pH of 6.8; filter sterilised
2 ml	Glucose	Prepared as a solution (50 % [w/v]) in distilled water; sterilised at 115 °C/20 min

Note: tryptone, mineral salts solution and nitrogen compounds are added to distilled water, the pH adjusted to 6.8 with N NaOH, and the medium dispensed in 190 ml amounts in 800 ml capacity Erlenmeyer flasks. Sterilisation is a 121 °C/15 min. After cooling to ~50 °C, the pre-sterilised buffer, glucose, cysteine HCl and vitamin solutions are added to a final volume of 200 ml. Agar to 1 % (w/v) may be incorporated, as necessary.

Shotts and Waltman Medium for the Isolation of Edwardsiella ictaluri *(Shotts and Waltman 1990)*

1 % (w/v) tryptone
1 % (w/v) yeast extract
0.125 % (w/v) phenylalanine
0.12 % (w/v) ferric ammonium citrate
0.0003 % (w/v) bromothymol blue
0.1 % (w/v) bile salts
1.5 % (w/v) agar
980 ml of distilled water
dissolve by boiling, cool to 50 °C and adjust pH to 7.0, sterilise at 121 °C for 15 min, cool to 50 °C and add mannitol (filter sterilised) to 0.35 % (v/v) and colistin sulphate to 10 µg/ml.

Skimmed Milk Agar

0.05 % (w/v) yeast extract
1.0 % (w/v) skimmed milk powder
1.0 % (w/v) agar No. 3
pH 7.2
sterilise at 115 °C/15 min.

TCY Medium (Hikida et al. 1979)

0.1 % (w/v) casamino acids
0.1 % (w/v) tryptone
0.02 % (w/v) yeast extract
0.1 % (w/v) calcium chloride
1.08 % (w/v) magnesium chloride
0.07 % (w/v) potassium chloride
3.13 % (w/v) sodium chloride
pH 7.0–7.2

Thioglycollate Broth

0.05 % (w/v) L-cystine
0.25 % (w/v) sodium chloride
0.5 % (w/v) dextrose
0.5 % (w/v) yeast extract
1.5 % (w/v) pancreatic digest of casein
0.05 % 9 w/v) sodium thioglycollate
pH 7.1
Sterilise at 121 °C/15 min

Thiosulphate Citrate Bile Salt Sucrose Agar (TCBS)

0.5 % (w/v) yeast extract
1.0 % (w/v) peptone
1.0 % (w/v) sodium thiosulphate
1.0 % (w/v) sodium citrate
0.8 % (w/v) ox bile
2.0 % (w/v) sucrose

1.0 % (w/v) sodium chloride
0.1 % (w/v) ferric citrate
0.004 % (w/v) bromothymol blue
0.004 % (w/v) thymolblue
1.4 % (w/v) agar
pH 8.6; after boiling to dissolve the ingredients the medium will not require further sterilisation.

Todd-Hewitt Broth

1 % (w/v) infusion from 450 g of fat-free minced beef
2 % (w/v) tryptone
0.2 % (w/v) dextrose
0.2 % (w/v) sodium bicarbonate
0.2 % (w/v) sodium chloride
0.04 % (w/v) disodium phosphate, anhydrous
pH 7.8
Sterilise at 115 °C/10 min.

Vibrio alginolyticus *Agar (VAL)*

03.7 % (w/v) Brain heart infusion
8.5 % (w/v) sodium chloride
1.5 % (w/v) sucrose
0.1 % (w/v) oxgall
0.002 % (w/v) bromocresol purple
1.5 % (w/v) agar
distilled water to 1 l
Boil, cool to 55 °C, adjust to pH 7.4 ± 0.2; do not autoclave

Vibrio anguillarum *Medium (VAM)*

1.5(w/v) sorbitol
0.4 % (w/v) yeast extract
0.5 % (w/v) bile salts
3.5 % (w/v) sodium chloride
0.001 % (w/v) ampicillin
0.004 % (w/v) cresol red
0.004 % (w/v) bromothymol blue

1.5 % (w/v) agar
distilled water to 1 l
pH 8.6; heat to dissolve, do not autoclave

Waltman-Shotts Medium for the Isolation of Yersinia ruckeri *(Waltman and Shotts 1984)*

0.2 % (w/v) tryptone
0.2 % (w/v) yeast extract
1.0 % (v/v) Tween 80
0.5 % (w/v) sodium chloride
0.01 % (w/v) calcium chloride hydrated
0.0003 % (w/v) bromothymol blue
1.5 % (w/v) agar
pH 7.4; sterilise at 121 °C for 15 min, cool to 50 °C and add filter sterilised sucrose to 0.5 % (w/v).

Yeast Extract Glucose Agar (Michel et al. 1997)

2.5 % (w/v) nutrient broth No. 2[Oxoid]
0.3 % (w/v) yeast extract
0.5 % (w/v) glucose
1.5 % (w/v) agar
pH 6.8

Yersinia ruckeri *Selective Medium (Furones et al. 1993)*

TSA
1 % (w/v) SDS
100 µg/ml of coomassie brilliant blue
100 µg/ml of Congo red

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Chapter 13
Diagnosis

Abstract Historically, scientists have seemed loath to make rapid diagnoses, preferring to adopt laborious testing regimes. Yet, diagnostic techniques have their drawbacks insofar as it is not always certain that the actual pathogen is recognized. Culture-dependent methods may highlight secondary invader or contaminants. Culture-independent techniques, although often highly sensitive and specific, do not provide information about the precise location, role or activity/inactivity of the organism. Nevertheless, there have been dramatic improvements in diagnostic methods, encompassing recent developments in molecular biology.

Keywords Diagnosis • Phenotyping • Serology • Molecular methods • Rapid methods

Diagnosis has often appeared to be as much art as science, with a multitude of preferred methods adorning the notebooks of most diagnosticians. However, the rapid acceptance of culture-independent, molecular-based methods has revolutionised diagnostics in terms of accuracy and sensitivity. Yet, diagnosis may still be achieved on purely histological material, with little effort made identify the pathogens. This is especially true of some of the acid-fast bacterial fish pathogens. When identification of the pathogen is attempted, it is a common fault that diagnosis proceeds with emphasis on traditional biochemical tests designed originally for bacteria important to human medicine. Consequently, a superficial glance at many laboratories would suggest that diagnostic techniques for fish pathogens need to be brought up to date. The question to be resolved is which is best – culture-dependent or culture independent approaches? Culture-independent methods will provide answers as to what is present in the diseased animal, but the data do not necessarily reflect the role or relevance of any micro-organism in the host. It is to be hoped that any astute diagnostician considers all available information concerning a disease situation before reaching a judgment. Useful information includes:

- Gross clinical signs of disease on individual fish;
- Internal abnormalities apparent during post-mortem examination;
- Histopathological examination of diseased tissues;

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- Microbiological examination of tissues (this requires special dexterity to avoid contamination by the normal microflora present on the surface and in the intestinal tract of fish, and in water; special contamination problems may be encountered with the examination of small fish, such as fry, and for surface samples, e.g. rotted fins/tail).

Gross Clinical Signs of Disease

The appearance of every sick fish tells a story, which fits into the proverbial jigsaw pattern of disease diagnosis. Good observation uncovers many useful clues. To an extent, the external appearance of the animal may be ignored by the eager diagnostician in the rush to attack the specimen with scalpel and swabs. Formalin and microbiological media may be the order of the day. Of course, the same is not true elsewhere in veterinary and human medicine where diagnosis is often achieved by apparently cursory glances at the diseased individual.

Fish may display many behavioural and physical changes, some of which give valuable clues as to the nature of the disease. It should be emphasised, however, that many symptoms are common to a multitude of bacterial diseases. Consequently, in the following discussion symptoms have been categorised in clear groups rather than splitting them into a plethora of esoteric detail. Thus, many external signs of disease are recognised, and include:

Sluggish behaviour; Twirling, spiral or erratic movement; Faded pigment; Darkened pigment/melanosis; Eye damage – exophthalmia (Figs. 13.1 and 13.2) ('pop-eye')/corneal opacity/rupture; Haemorrhaging in the eye; Haemorrhaging in the mouth; Erosion of the jaws/mouth; Haemorrhaging in the opercula region/gills; Gill damage; White nodules on the gills/skin; White spots on the head; Fin rot/damage; Haemorrhaging at the base of fins; Haemorrhaging on the fins; Tail rot/erosion; Saddle-like lesions on the dorsal surface (columnaris, saddleback disease); Distended abdomen developing into a distinct bloating (Fig. 13.3); Haemorrhaging on the surface and in the muscle; Necrotising dermatitis; Ulcers (Fig. 13.4); External abscesses; Furuncles (or boils); Blood-filled blisters on the flank; Protruded anus/vent; Haemorrhaging around the vent; Necrotic lesions on the caudal peduncle; Emaciation (this should not be confused with starvation); Inappetance; Stunted growth; Sloughing off of skin/external surface lesions; Dorsal rigidity

Internal abnormalities relevant to bacterial fish pathogens apparent during post-mortem examination, include:

Skeletal deformities; Gas-filled hollows in the muscle; Opaqueness in the muscle; Ascitic fluid in the abdominal cavity; Peritonitis; Petechial (pin-prick)

Fig. 13.1 Exophthalmia and surface haemorrhaging on a carp that was suspected to have been infected with *Aer. hydrophila* (Photograph courtesy of Dr. P. Orozova)

Fig. 13.2 Pronounced bilateral exophthalmia on a trout (Photograph courtesy of Dr. S.M. Sharifuzzaman)

haemorrhages on the muscle wall; Haemorrhaging in the air bladder; Liquid in the air bladder; White nodules (granulomas) on/in the internal organs; Yellowish nodules on the internal organs; Nodules in the muscle; Swollen and/or watery kidney; False membrane over the heart and/or kidney; Haemorrhaging/bloody exudate in the peritoneum; Swollen intestine, possibly containing yellow or bloody fluid/gastro-enteritis; Intestinal necrosis and

Fig. 13.3 Distended abdomen/dropsy on a fathead minnow for which the causal agent was considered to be *Aeromonas* (Photograph courtesy of Professor C. Mothersill)

Fig. 13.4 Surface haemorrhaging and ulceration on an eel (Photograph courtesy of Professor V. Jencic)

opaqueness; Hyperaemic stomach; Haemorrhaging in/on the internal organs; Brain damage; Blood in the cranium; Emaciation; Pale, elongated/swollen spleen; Pale (possibly mottled/discoloured) liver; Yellowish liver (with hyperaemic areas); Swollen liver; Generalised liquefaction; The presence of tumours

Histopathological Examination of Diseased Tissues

Although many histological procedures may be routinely used, it is important for the bacteriologist that Gram-stained sections should be prepared. Thus, the presence of any offending bacterial pathogen will quickly be recognised. Microscopic examination of Gram-stained material will enable the determination of the basic staining

reaction and micromorphology of the pathogen. Possibilities include the presence of Gram-positive or Gram-negative rods (spore-bearing or asporogenous), cocci and mycelium. For Gram-positive organisms, the acid-fast stain will help in the recognition of *Mycobacterium, Nocardia*, and possibly *Rhodococcus.*

Bacteriological Examination

Tissues to be Sampled

Generally, examination should be made of any damaged tissues. Experience has taught us that it is always advisable to include a kidney sample, which often permits the most satisfactory isolation of the pathogen. Quite simply it is adequate to sample the material by means of swabbing, while minimizing the changes of contamination. The swabs should then quickly be used to inoculate the bacteriological media.

Identification of Bacterial Isolates

The most common shortcomings in diagnosis of fish diseases concern the identification of bacterial isolates. There are three schools of thought, namely those that rely on serology, molecular techniques, ***and*** those relying on more conventional phenotypic tests.

Serology

We will preface further discussion by wholeheartedly endorsing a view that reliable diagnoses occur only with monospecific antisera to assure the homologous reaction between antigen and antibody. The development of monoclonal antibodies has improved diagnoses by standardising serological tests, i.e. by means of defined reagents (Goerlich et al. 1984), and enhanced the reliability of ELISA, iFAT and immunohistology for the detection of pathogens, such as *Mycobacterium* spp. and *Ren. salmoninarum* (Adams et al. 1995). In contrast, it may be argued that the more conventional polyclonal antibodies have generated contradictory results. Also, the extent of any cross-reactions with polyclonal antibodies has often not been adequately determined. Goerlich et al. (1984) noted that a monoclonal antibody raised against a typical virulent isolate of *Aer. salmonicida* (i.e. the strain possessed an A-layer) reacted only with virulent cultures, but not with avirulent cells (i.e. those lacking an A-layer).

Nevertheless, tentative diagnoses, especially of asymptomatic infections (Busch and Lingg 1975; Hansen and Lingg 1976; Johnson et al. 1974) may result from use of polyclonal antibodies in any of a multiplicity of serological procedures, including FAT, whole-cell (slide) agglutination, precipitin reactions, complement fixation, immunodiffusion, antibody-coated latex particles (this is akin to human pregnancy testing), co-agglutination using antibody-coated staphylococcal cells, passive haemagglutination, immuno-India ink technique (Geck) or ELISA (e.g. Saeed and Plumb 1987). The serological techniques are discussed below:

- Fluorescent antibody technique (FAT)

There are two variations to this test, namely the direct and indirect methods. FAT has found use for the diagnosis of many fish diseases, especially in laboratories (Eurell et al. 1978), for which it is regarded as a highly effective procedure (Kawahara and Kusuda 1987). For example, α- and ß-haemolytic streptococcal isolates may be readily differentiated by FAT (Kawahara and Kusuda 1987).

For the direct method (see Bullock et al. 1980 and Smibert and Krieg 1981 for further details), fluorescein isothiocyanate is conjugated with whole or with the IgG fraction of the antiserum. Twofold dilutions (1:5 and 1:8) are prepared in PBS (0.1236 % (w/v) Na_2HPO_4, anhydrous; 0.018 % (w/v) $NaH_2PO_4.H_20$; 0.85 % (w/v) NaCl; pH 7.6) and used to standardise the 'conjugate'. Thus, a bacterial suspension (containing *ca*. 10^8 cells/ml in PBS) is pipetted onto grease-free microscope slides, air-dried and fixed at 60 °C for 2 min (or fixed in 95 % ethanol for 1 min., and then air-dried). The 'conjugate' is pipetted onto the slide and left in a moist chamber for 5 min. to react (room temperature, i.e. 15–20 °C, is adequate). Subsequently, the excess antiserum is removed by draining, before the slide is thoroughly rinsed in PBS (for *ca*. 10 min). The slide is air-dried, and the smear covered with a drop of buffered glycerol (Difco; at pH 9.0) before overlayering with a coverslip. This should be quickly examined with a fluorescence microscope. The optimum dilution of conjugate is determined, from the doubling dilutions, by rating the degree of fluorescence from 0 to 4+ (after Jones et al. 1978). The 'use' dilution of the conjugated antiserum is 50 % of the highest dilution, which gives maximum fluorescence. With this information, the diagnosis may proceed for unknown cultures. However, it is always important to include positive and negative controls.

For iFAT (see Laidler 1980), bacterial smears are prepared and fixed (as above). Doubling dilutions of rabbit antiserum are prepared, and 20 µl aliquots added to the bacterial smears. These are placed in a moist chamber, left for 30 min to react, and washed for 30 min in two changes of PBS. After air-drying, the smears are covered with a suitable dilution of fluorescein-labelled sheep anti-rabbit globulin, incubated for 30 min in the moist chamber, rinsed thoroughly in PBS, air-dried and mounted in buffered glycerol. Examination, with a fluorescence microscope should proceed as quickly as possible.

- Whole-cell agglutination

This is a quick and easy technique, which provides much useful data. Whole cell agglutination is used widely as a diagnostic tool, for example with the fish patho-

genic streptococci (Kitao et al. 1979; Kitao 1982) and *Edw. tarda* (Amandi et al. 1982). Essentially, a drop of bacterial suspension (*ca.* 10^8 cells/ml, prepared in 0.85 % (w/v) saline) is added to a microscope slide. This is followed by adding a drop of antiserum (use a range of dilutions), with gentle mixing for 2 min. A positive response is indicated by clumping of the cells. The reaction may also be carried out in microtitre wells, using serial dilutions of antisera (Toranzo et al. 1987). Of course, it must be emphasised that the reliability reflects the specificity of the antiserum.

- Precipitin reactions and immunodiffusion

The value of these tests for diagnosis has been overshadowed by other techniques, such as FAT and whole cell agglutination. For detailed discussion, the reader should consult Kimura et al. (1978) and Smibert and Krieg (1981).

- Complement fixation

This technique probably has greater value for fish virology than for fish bacteriology; it has been described by Ahne (1981).

- Antibody-coated latex particles

The so-called latex agglutination test has found widespread use for diagnosis of ERM (e.g. Hansen and Lingg 1976), furunculosis and vibriosis (including Hitra disease). Commercial kits have found success, despite some cross reactions (Romalde et al. 1995). However, the technique may be readily adapted for most bacterial fish pathogens. As originally described for *Aer. salmonicida* by McCarthy (1975a, b), the test involves the use of globulins from hyper-immune serum (titre = >1:5000) and sensitised latex. The globulins are precipitated by the addition of saturated ammonium sulphate to the antiserum, and the precipitated proteins are sedimented by centrifugation. They are subsequently re-dissolved in 0.9 % (w/v) saline, dialysed overnight at 4 °C against three changes of saline, and, after centrifugation, the supernatant, which contains the globulins, is stored at – 20 °C until required. The latex particles (0.81 μm diameter; Difco) are sensitised in globulin solution at 37 °C for 2 h. For the test, 200 μl of the antigen (bacterial suspension in glycine-buffered saline, i.e. 0.73 % (w/v) glycine and 1 % (w/v) NaCl; pH 8.2; supplemented with 1 % (w/v) Tween 80) is mixed for 2 min with an equal volume of sensitised latex on a clean glass plate. A positive result is indicated by clumping of the latex. The technique may be used for pure or mixed cultures and tissue. Thus, positive diagnoses may ensue from tissues unsuitable for culturing, e.g. fish stored at –20 °C for 14 days, 5 °C for 7 days, or from formalin-fixed material (McCarthy 1975a, b). As before, positive and negative controls are necessary.

- Co-agglutination with antibody-sensitised staphylococci

Reported for *Aer. salmonicida* and *Ren. salmoninarum*, this technique is similar to the latex test (Kimura and Yoshimizu 1981, 1983, 1984). Essentially, *Sta. aureus* (ATCC 12598) is suspended in 0.5 % formalin-PBS for 3 h at 25 °C to inactivate the cells, washed three times in fresh PBS. The cells are mixed with antiserum in the

ratio of 10:1 and incubated at 25 °C for 3 h. An equal volume of a boiled bacterial suspension and the sensitised staphylococci are mixed on a glass slide. Following incubation in a moist chamber at room temperature for up to 2 h, a positive response is indicated by clumping of the cells. The advantages of this technique concern its simplicity and reliability. Moreover, it was considered suitable for deployment in field conditions.

- Passive agglutination

For rough colonies of *Aer. salmonicida*, which were unsuitable for use with whole cell agglutination [because of auto-agglutination], McCarthy and Rawle (1975) recommended the mini-passive agglutination test. This technique involves the use of sheep erythrocytes sensitised with *Aer. salmonicida* O-antigen (extracted with hot physiological saline). This reacts with dilute anti-*Aer. salmonicida* immune serum, assuming that the antigen is present. The obvious advantages of this method concern its application to the detection of both rough and smooth colonies. However, McCarthy and Rawle (1975) cautioned that false negative results may sometimes be obtained with cultures that have been maintained in laboratory conditions for prolonged periods. Hence, old cultures may not be suitable for use in serological studies (or, for that matter, vaccine production!).

- Immuno-India Ink technique (Geck)

Another rapid technique, which allows diagnosis within 15 min, is the India ink immunostaining reaction as developed initially by Geck (1971). This is a microscopic technique, in which the precise mode of action is unknown, although Geck suggested that it could be regarded as an immuno-adsorption method. The technique has been described only for use with *Aer. salmonicida* (McCarthy and Whitehead 1977). A drop of bacterial suspension is smeared into a clean (de-fatted) microscope slide, air-dried, and heat-fixed. The smear is covered with a 1:1 mixture of India ink and antiserum, before incubation in a moist chamber for 10 min at room temperature. Subsequently, the mixture is removed by washing with ferric chloride (0.00001 % w/v), and the slide air-dried prior to microscopic examination. A positive result is indicated by the presence of cells, clearly outlined with India ink.

- Enzyme-linked immunosorbent assay (ELISA)

This is a technique which is becoming widely adopted for the detection and diagnosis of bacterial fish pathogens, some commercial kits having been developed. This is a useful technique, which has already gained widespread use in human and veterinary medicine. Essentially, there is a requirement for a specific antiserum, an enzyme, e.g. alkaline phosphatase or horseradish peroxidase, and a substrate, e.g. *o*-phenylenediamine (for use with alkaline phosphatase) (See Austin et al. 1986). A positive result is indicated by a colour change, which may be recorded quantitatively with a specially designed reader. Variations of the technique have been published, and include indirect ELISA, indirect blocking ELISA and competitive ELISA (e.g. Swain and Nayak 2003).

- Dot blotting

Monospecific antibodies have been in used in dot blotting techniques to record the presence of specific pathogens. Dot blotting involved spotting 1 µl quantities of bacterial samples onto nitrocellulose membranes, baking at 60 °C for 10 min, incubating in a 1:200 solution of 5 % blotto (5 % non fat dry milk, 0.1 % Triton X-100 in phosphate buffered saline [PBS]) for 5 h, washing in blotto, incubating in a 1:500 dilution of horseradish peroxidase goat anti-mouse gamma immunoglobulin for 3 h, re-washing in 0.5 % blotto, and incubation for 5 min in substrate (0.03 % diaminobenzidine, 0.006 % hydrogen peroxide, and 0.05 % cobalt chloride in PBS) (Longyant et al. 2010).

Immunohistochemistry

Immunohistochemistry, based on avidin-biotin complexes, have identified *V. salmonicida* in fixed tissues (Evensen et al. 1991). Also, a peroxidase-antiperoxidase immunohistochemical technique appears to be useful for differentiating *Y. ruckeri* (Jansson et al. 1991). Complications with the interpretation of slides by interference with melanin and/or melanomacrophages resulted in the adoption of melanin-bleaching with 3.0 g/l potassium permanganate for 20 min followed by 1 % (w/v) oxalic acid for 1.5 min before immunostaining, which removed some of the problems of interpreting the presence/absence of *Ren. salmoninarum* (Morris et al. 2002).

- Immunomagnetic separation of antigens

A comparatively novel approach concerns the recovery of *Aer. salmonicida* cells with immunomagnetic beads coated with monoclonal antibodies to LPS coupled with culturing (Nese and Enger 1993). Thus, the cells are recovered by serological procedures for culturing on routine bacteriological media.

Molecular Techniques

Molecular methods have become the norm in many diagnostic laboratories, and have gained the reputation for sensitivity and specificity. Thus, PCRs have been used with increasing regularity for the detection of bacterial pathogens both to determine the presence of single organisms and to recognize and discriminate between multiple taxa within the same genus, and across different genera. An example of the recognition of three species from within the same genus is the triplex loop-mediated isothermal amplification method that detected *V. alginolyticus, V. anguillarum* and *V. harveyi* specifically and sensitively in the tissues of

experimentally infected fish (Yu et al. 2013). Other systems have crossed the genus boundary by recognizing pathogens from two or more genera. For example:

- Del Cerro et al. (2002a, b) detected simultaneously *Aer. salmonicida, Fla. psychrophilum* and *Y. ruckeri* in fish tissues, recognising the equivalent of 6, 0.6 and 27 CFU, respectively, using multiplex PCR.
- Matsuyama et al. (2006) developed a low-density oligonucleotide DNA array for the detection and discrimination of multiple *Photobacterium* and *Vibrio* spp. within a day, albeit with some cross-hybridisation reported. These workers designed a low-density oligonucleotide DNA array between the 16S and 23S ribosomal DNA leading to the development of three oligonucleotide probes, which were immobilized on nylon membranes. The low-density oligonucleotide DNA arrays were amplified by PCR, hybridised, and the specific signals were produced with alkaline phosphatase-conjugated anti- digoxigenin labelled PCR products (Matsuyama et al. 2006).
- González et al. (2004) used a multiplex PCR and DNA microarray, and achieved the simultaneous and differential diagnosis of *Aer. salmonicida, Ph. damselae* subsp. *damselae, V. anguillarum, V. parahaemolyticus* and *V. vulnificus,* with a minimum detection limit of <20 fg per reaction, which equates to 4–5 bacterial cells.
- A multiplex PCR has reportedly been developed, and successfully recognised from culture and fish tissues the fish pathogenic lactococci-streptococci, i.e. *Lactococcus garvieae, Str. difficilis, Str. iniae* and *Str. parauberis* with a sensitivity for the purified DNA of 30 pg, 12.5 pg, 25 pg and 50 pg, respectively (Mata et al. 2004).
- A PCR based on a reverse line blot and involving a set of primers for the specific amplification of the 16S rRNA gene resulted in amplification products of 241 bp, and rapidly detected and differentiated *Lactococcus garvieae, Str. agalactiae, Str. iniae* and *Str. parauberis* (Soltani et al. 2012).
- A multiplex PCR detected and differentiated *Lactococcus garvieae, Str. agalactiae* and *Str. iniae* with sensitivities of 9.76–39.06 pg of DNA (Itsaro et al. 2013).
- A highly specific multiplex PCR protocol was developed to simultaneously detect *Edw. tarda, Str. iniae* and *Str. parauberis*, with sensitivities of 0.01 ng, 1.0 ng and 0.1 ng of genomic DNA, respectively (Park et al. 2014).
- A LAMP was developed that permitted the recognition and differentiation of for pathogens of relevance to ornamental fish, i.e. *Aer. hydrophila, Str. agalactiae,* Koi herpes virus and Iridovirus, within 65 min with a detection limit of 65 copies (Chang et al. 2013).
- DNA microarrays have been used to detect fish pathogens, including *Aer. hydrophila, Noc. seriolae, Str. iniae, V. alginolyticus, V. anguillarum* and *V. harveyi*, and demonstrated congruence with other methods, i.e. culturing and 16S rRNA gene sequencing (Shi et al. 2012).
- Lukkana et al. (2014) differentiated simultaneously *Aeromonas* from *Streptococcus* using a multiplex-PCR, and distinguished the pathogens from other waterborne bacteria. DNA fragments. were amplified using genus specific

primers, which generated PCR of 207 bp and 953 bp, respectively, and the resultant assay detected bacterial genomic DNA in brain and posterior kidney of infected Nile tilapia (Lukkana et al. 2014).

- A highly specific microarray was constructed based on coupling a 16S rDNA PCR with DNA hybridization that permitted the simultaneous detection and differentiation of *Aer. hydrophila, Edw. tarda, Fla. columnare, Lactococcus garvieae, Ph. damselae, Ps. anguilliseptica, Str. iniae* and *V. anguillarum* (Chang et al. 2012). Targets on the probes were reacted with streptavidin-conjugated alkaline phosphatase and nitro blue tetrazolium/5-bromo-4-chloro-3′-indolylphosphate, *p*-toluidine salt resulting in the formation of easily visible blue spots. The sensitivity was 1 pg for genomic DNA and 10^3 CFU/ml for pure cultures (Chang et al. 2012).
- The simultaneous recognition of five bacterial fish pathogens, i.e. *Ph. damselae, Ps. baetica, T. maritimum, T. soleae* and *V. harveyi,* was achieved by reverse line blot hybridization. Species-specific probes targeted the 16S-23S intergenic spacer region (ISR) or the 23S rRNA gene leading to correct identifications with sensitivity of the assay ranging from 1- 100 pg of genomic DNA of the pure culture. (Lopez et al. 2012).
- A multiple PCR RNA polymerase using the ß subunit (*rpoB*) gene differentiated *Ph. damselae, V. harveyi* and *V. ichthyoenteri* in olive flounder with detection limits in kidney of 2.5×10^6 CFU/g, 2.5×10^4 CFU/g and 2.5×10^5 CFU/g, respectively (Kim et al. 2014a).
- A real-time fluorogenic loop-mediated isothermal amplification assay integrated on a microfluidic disc chip was capable of simultaneously detecting 10 pathogens, i.e., *Noc. seriolae, Ps. putida, Str. iniae, V. alginolyticus, V. anguillarum, V. fluvialis, V. harveyi, V. parahaemolyticus, V. rotiferianus* and *V. vulnificus*. The assay was well automated with only one pipetting operation per chip for dispensing the sample. The assay was reproducible and specific, and the limits of detection were from 0.40 to 6.42 pg/1.414 µl with a reaction time of <30 min (Zhou et al. 2014).

The ability to discriminate reliably between multiple pathogens is a major improvement on previous approaches. The extreme sensitivity should be a bonus, but could give positive readings in the absence of clinical disease leading to control measures, which might not always be needed.

Phenotypic Tests

For many pathogens emphasis has been placed on conventional phenotypic tests for diagnosis, although in many laboratories there has been a rapid move towards molecular methods. Nevertheless, phenotypic methods are still used extensively. Caution is advocated should consideration be given to using commercially available diagnostic kits. The API 20E and API-ZYM, API 20NE API 50CH, API 50 L,

Biolog-GN, Enterotubes and RapidID 32 systems have made an inroad into routine diagnostic laboratories. Whereas some systems, e.g. Biolog-GN, are clearly adaptable to- and useful for environmental isolates, others have been developed specifically for a given use, usually medically important bacteria. The consequence is that the supporting identification schemes may be inappropriate for fish pathogens. Many workers have used API 20E, which was developed for medically important enteric bacteria that grew overnight at 35–37 °C, for the identification of fish pathogenic bacteria, but the system weighs heavily on the use of sugar fermentation reactions, which may be influenced by the presence of plasmids. Of course, it is necessary to modify the API 20E protocol for use with marine bacteria by suspending cultures in 2–3 % (w/v) saline rather than distilled water, and the inoculated test strips were incubated at 25 °C for up to 48 h. There may be some issues, for example, the API 20E profile(s) for *T. maritimum* and *Ps. anguilliseptica* may be indistinguishable. Also, *Y. ruckeri* may be confused with *Haf. alvei.* Nevertheless, it is accepted that the API 20E and API 20NE systems have a role in the diagnosis of bacterial fish pathogens, and the manufacturer's computer-based identification matrices are regularly updated to account for new information. In addition, API-ZYM is very useful for diagnosing *Ren. salmoninarum* (See Table 13.1), which gives a characteristic profile:

$$-+-+-+--+-++---+--+/--$$

Biolog-GP has been regarded as effective for the identification of some Gram-positive bacterial fish pathogens when compared with the results of 16S rRNA gene sequencing, correctly identifying 18 *Lac. garvieae* and 10 *Str. iniae* isolates but not *Lac. piscium* (the type strain did not grow on the recommended medium), *V. salmoninarum* (misidentified as *V. fluvialis*) and *Str. parauberis* (misidentified as *Ent. faecalis*) (Verner-Jeffreys et al. 2011).

Diagnoses may also be achieved also by means of diagnostic schemes based on reactions in conventional phenotypic tests. The procedure may be automated, as with the Abbott Quantum II system (Teska et al. 1989), and involves spectrophotometric readings at 492.6 nm, with a sample cartridge containing 20 inoculated biochemical chambers. Alternatively, specially constructed diagnostic tables may be used, such as presented in Tables 13.1, 13.2 and 13.3.

- Colony morphology and pigmentation

This should be recorded from 'young' colonies, i.e. shortly after growth is initially detected. The presence of aerial hyphae may be assessed with a stereomicroscope. The presence of pigment should be assessed from basal medium supplemented with 5–10 % (w/v) skimmed milked powder (Oxoid).

- The Gram-staining reaction

With smears from young cultures, this reaction serves also to determine the presence of rods, cocci, mycelia, microcysts and endospores. For convenience, we recommend the use of commercially available staining and decolorising solutions,

Table 13.1 Distinguishing profiles of Gram-positive bacteria as obtained with API ZYM[a]

API ZYM test no.																					
Taxon (and source of strains)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Actinomyces viscosus (ATCC 15987)	−	−	−	+	−	+	+	−	−	−	+	+	+	+	−	−	+	−	−	+
Aerococcus viridans subsp. *homari* (NCIMB 1119)[b]	−	+	+	+	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Arthrobacter aurescens (NCIMB 8912)	−	−	−	+	−	+	−	+	+	−	+	+	−	+	+	+	+	−	+	−
Arthrobacter crystallopoietes (ATCC 15481)	−	−	−	+	−	+	+	−	+	−	−	+	−	−	−	−	−	−	−	−
Arthrobacter globiformis (NCIMB 8907)	−	−	−	+	−	+	+	+	+	−	+	+	−	+	−	+	+	−	+	−
Arthrobacter nicotianae (NCIMB 9458)	−	−	−	+	−	+	+	−	+	−	−	+	−	−	−	+	−	+	+	−
Bacillus cereus (CCM 2010)	−	+	+	+	−	+	+	−	+	+	+	+	−	−	−	+	−	−	−	−
Bacillus licheniformis (ATCC 9945)	−	+	+	+	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	−
Bacillus megaterium (CCM 2007)	−	+	+	+	−	+	−	−	−	+	+	+	+	+	−	−	−	−	−	−
Bacillus polymyxa (ATCC 12321)	−	+	+	+	−	+	−	−	−	+	−	+	+	+	−	−	−	−	−	−
Bacillus sphaericus (ATCC 10208)	−	+	+	+	−	+	−	−	−	+	+	+	−	−	−	−	−	−	−	−
Brevibacterium flavum (ATCC 13826)	−	+	−	+	−	+	+	−	+	−	+	+	−	−	+	−	+	−	−	−
Cor. acnes (NCTC 737)	−	−	−	+	−	−	−	−	(+)	−	−	+	−	−	−	−	−	−	−	−
Cor. pyogenes (NCTC 5224)	−	+	−	+	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−
Cor. xerosis (NCTC 7929)[b]	−	+	+	+	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
En. faecalis (CCM 1875)	−	−	−	+	−	+	−	−	−	+	+	+	−	+	−	+	−	−	−	−
En. faecium (CCM 2801)[b]	−	+	+	+	−	+	−	−	(+)	−	−	+	−	−	−	−	−	−	−	−

(continued)

Table 13.1 (continued)

API ZYM test no.																					
Taxon (and source of strains)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Kurthia zopfii (CCM 3478)	−	−	−	(+)	−	+	−	−	−	+	+	+	−	−	−	−	−	−	−	−
Lactobacillus casei (CCM 1753)	−	−	+	+	−	+	+	−	−	−	+	+	−	+	−	−	+	−	−	−
Lactobacillus curvatus (NCIMB 9710)	−	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−
Lactobacillus brevis (NCDO 1749)	−	−	(+)	+	−	+	+	−	−	−	+	+	+	+	−	+	+	−	−	−
Lactobacillus sp. (pseudokidney disease, 3 isolates)	−	−	(+)	+	−	+	(+)	−	−	(+)	+	−	(+)	−	−	+	+	−	−	−
Listeria denitrificans (ATCC 14870)	−	−	−	+	−	+	−	−	−	−	−	+	+	+	+	+	+	−	−	−
Listeria grayi (CCM) 5887)	−	−	(+)	+	−	(+)	(+)	−	−	−	−	+	−	−	−	−	−	−	−	−
Listeria murrayi (CCM 5990)	−	−	+	+	−	+	−	−	−	(+)	−	+	−	−	−	−	−	−	−	−
Microbacterium lacticum (NCIMB 8450)	−	−	−	+	−	+	+	−	−	−	+	+	−	+	−	−	−	−	−	−
Micrococcus luteus (NCIMB 9278)	−	+	−	+	−	+	−	−	−	+	+	+	−	−	−	+	−	−	−	−
Myc. aquae (Körmendy)	−	+	+	+	+	+	−	−	−	−	+	+	−	−	−	−	−	−	−	−
Myc. fortuitum (Körmendy)	−	+	+	+	−	+	+	−	+	−	+	+	−	−	−	−	−	−	−	−
Myc. marinum (Körmendy)	−	+	+	+	−	+	−	−	−	−	+	+	−	−	−	−	−	−	−	−
Myc. smegmatis (Körmendy)	−	+	+	+	−	+	−	−	−	−	+	+	−	−	−	−	−	−	−	−
Mycobacterium sp. (Ashburner, SC 744)	−	+	+	+	−	+	−	−	−	−	+	+	−	−	−	+	−	−	−	−
Noc. asteroides (ATCC 14759)	−	+	+	+	−	−	+	+	−	−	+	+	−	−	−	−	−	−	−	−
Noc. corallina (ATCC 4273)	−	+	+	+	−	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−
Planococcus citreus (NCIMB 1493)	−	−	−	+	−	+	+	+	(+)	(+)	−	+	−	+	−	+	−	−	−	−

(continued)

Table 13.1 (continued)

API ZYM test no.																					
Taxon (and source of strains)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Propionibacterium acnes (CCM 3343)	–	–	–	(+)	–	+	–	–	–	–	–	+	–	+	–	–	–	–	+	–
Ren. salmoninarum (48 isolates)	–	+	–	+	–	+	–	–	+	–	+	+	–	–	–	+	–	–	(+)	–
Rothia dentocariosa (ATCC 17931)	–	–	–	+	–	+	+	–	+	–	–	+	–	–	–	+	–	–	–	–
Sta. epidermidis (NCIMB 2699)	–	+	(+)	+	–	–	–	–	–	–	+	+	–	–	–	+	–	–	–	–
Str. agalactiae (CCM 6187)	–	+	–	+	–	–	–	–	–	–	+	+	–	–	–	(+)	–	–	–	–
Str. dysgalactiae	–	+	–	–	–	+	(–)	–	–	–	+	(+)	–	–	+	+	–	–	–	–
Streptomyces griseus (ATCC 23345)	–	+	–	+	–	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–
Presumptive coryneform (laboratory isolate 198)	–	–	+	+	–	+	–	–	–	–	+	+	–	–	–	+	–	–	–	–

1 control, *2* alkaline phosphatase, *3* esterase (butyrate), *4* esterase (caprylate), *5* lipase (myristate), *6* leucine arylamidase, *7* valine arylamidase, *8* cystine arylamidase, *9* trypsin, *10* chemotrypsin, *11* acid phosphatase, *12* phosphoamidase, *13* α-galactosidase, *14* β-galactosidase, *15* β -glucuronidase, *16* α-glucosidase, *17* β -glucosidase, *18* N-acetyl- β -glucosaminidase, *19* α -mannosidase, *20* α -fucosidase

Sources: *ATCC* American Type Culture Collection, *CCM* Czechoslovak Collection of Microorganisms, *NCDO* National Collection of Dairy Organisms

NCIMB National Collection of Industrial and Marine Bacteria, *NCTC* National Collection of Type Cultures; Dr. L.D. Ashburner, Freshwater Fisheries

Research Station, Victoria, Australia; Dr. B. Körmendy, Central Veterinary Institute, Hungary

[a]15 ° C/18 h

[b]Distinguish by results of the Gram-staining reaction

such as those marketed by Difco. Heat-fixed smears should be stained for 1 min with crystal violet, washed in tap water, covered with Grams-iodine for 1 min, rewashed, decolorised by a few seconds in acetone-alcohol, and counterstained for 30 s in safranin. The smears are washed thoroughly, and gently blotted dry, prior to microscopic examination preferably at a magnification of ×1000.

- The acid-fast staining reaction

This reaction highlights the presence of *Mycobacterium*, *Nocardia* and possibly *Rhodococcus.* Heat-fixed smears may be flooded with carbol fuchsin, and heated until the steam rises by means of wafting a source of heat (from a Bunsen burner or cotton wool plug soaked with alcohol) underneath the slide. After 5 min, the stain is washed away with tap water, and the smear decolorised with acid-alcohol until only

Table 13.2 Diagnostic traits of the Gram-positive bacterial fish pathogens

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Aerococcus viridans	w	C[f]	−	−	−	−	−	+	?	−	−	−	−	?	?	+ α	?	−	?	?	?	+	?	−
Bacillus spp.	w	r	−	+	−	−	−	+	F	?	?	?	?	−	?	−	?	?	?	+	?	?	?	?
Bacillus mycoides	w	r	−	+	−	−	−	+	?	?	?	?	?	−	?	+	?	?	?	+	?	?	?	?
Car. piscicola	w	r	−	−	−	−	−	+	F	−	?	?	−	−	−	−	?	−	+	+	+	+	?	−
Cl. botulinum[b]	w[a]	r	−	+	−	+	−	−	−	−	?	?	−	−	−	+	?	−	?	+	?	?	?	?
Cor. aquaticum	y	r	−	−	−	−	−	+	−	+	?	+	−	−	−	+ (β)	?	−	+	+	+	?	?	−
Erysipelothrix rhusiopathiae	w	r	−	−	−	?	−	−	?	−	?	?	−	−	+	+ (α/ß)	?	−	?	?	?	?	?	?
Eu. tarantellae	w	r	−	−	−	−	−	−	−	−	?	?	?	−	−	+	?	?	?	+	?	?	?	?
Lactococcus garvieae	w	c	−	−	−	−	−	+	F	−	?	?	−	−	−	+	?	?	+	+	+	+	?	+
Lactococcus lactis subsp. *lactis*	w	c	−	−	−	−	−	+	F	−	?	?	−	−	−	+ (α)	?	−	?	+	?	+	?	−
Lactococcus piscium	w	c	−	−	−	−	−	+	F	−	?	?	?	−	−	?	+	?	+	−	?	?	?	−
Micrococcus luteus	y	c[f]	−	−	−	−	−	+	O	+	−	−	+	−	−	?	?	?	?	?	+	?	?	?
Myc. abscessus	−	r	−	−	+	−	−	+	O	+	?	?	?	−	?	?	?	+	?	?	?	+	?	?
Myc. chelonei subsp. *piscarium*	w	r	−	−	+	−	−	+	O	+	?	?	−	−	?	?	?	+	+	−	?	−	+	−
Myc. fortuitum	w	r	−	−	+	−	−	+	O	+	?	?	−	−	?	?	?	+	?	+	?	?	+	?
Myc. marinum	w	r	−	−	+	−	−	+	O	−	?	?	−	−	?	?	?	+	?	v	−	_	−	?
Myc. montefiorense	w	c/r	−	−	+	−	−	+	?	−	?	?	?	−	−	?	?	−	?	−	?	−	?	?
Myc. neoaurum	y	r	−	−	+	−	−	+	O	?	?	?	?	−	?	?	?	?	?	−	+	−	?	?
Myc. pseudoshottsii	y	c/r	−	−	+	−	−	+	?	(+)	?	+	?	−	?	?	?	+	?	−	?	?	?	?
Myc. shottsii	w	c/r	−	−	+	−	−	+	?	(−)	−	−	?	−	?	?	?	+	?	−	?	?	?	?
Noc. asteroides	w	c/r	(+)	−	+	−	−	+	O	+	?	?	−	−	−	?	+	−	?	+	?	?	−	−
Noc. seriolae	w	c/r	−	−	+	−	−	+	O	+	?	?	−	−	+	?	−	−	?	?	?	?	?	?

Planococcus sp.	y	c	−	−	−	+	−	+	O	+	?	+	+	−	−	?	?	?	?	+	+	+	?	?
Ren. salmoninarum[c]	w	r	−	−	−	−	+	+	−	+	−	−	−	−	−	?	−	?	+	−	+	−	?	−
Rhodococcus sp.	re	r	−	−	+	−	−	+	F	−	?	?	−	−	?	?	?	+	?	−	+	?	?	+
Rhodococcus erythropolis	w	r	−	−	?	−	−	+	O	+	?	?	−	−	−	−	?	?	?	−	+	−	?	?
Rhodococcus qingshengii	w	r	−	−	?	−	−	+	O	+	?	?	−	−	?	?	?	−	?	+	+	−	?	?
Sta. aureus	y	c	−	−	−	−	−	+	F	+	?	?	?	+	?	+ (β)	?	?	?	+	−	?	?	?
Sta. epidermidis	w	c	−	−	−	−	−	+	F	+	?	+	−	−	−	+ (β)	−	+	?	+	+	+	?	?
Sta. warneri	(y)	c	−	−	−	−	−	+	F	+	?	?	−	−	?	?	?	+	?	?	?	?	?	?
Str. agalactiae	w	c	−	−	−	−	−	+	F	−	−	−	?	−	?	−	−	?	−	−	?	−	?	−
[d]*Str. dysgalactiae*	w	c	−	−	−	−	−	+	F	−	−	−	?	−	?	+ (α/ß)	?	?	−	+	+	−	?	−
Str. ictaluri	w	c	−	−	−	−	−	+	?	−	?	−	?	?	?	α	?	−	?	+	?	−	?	−
[d]*Str. iniae*	w	c	−	−	−	−	−	+	F	−	−	−	?	−	?	+ (α)	+	?	−	+	?	−	?	−
Str. milleri	w	c	−	−	−	−	−	+	F	−	−	+	−	−	−	+ (β)	−	−	?	?	+	?	?	−
Str. parauberis	w	c	−	−	−	−	−	+	?	−	+	?	?	−	?	+ (α)	?	?	+	+	?	−	?	?
Str. phocae	w	c	−	−	−	−	−	+	F	−	?	?	?	?	?	ß	−	?	−	+	?	−	?	−
Noc. salmonicida	o/r	m	+	−	−	+	−	+	O	+	?	?	?	−	−	?	−	+	?	−	−	−	−	−
Vag. salmoninarum	w	c/r	−	−	−	−	−	+	F	−	?	?	?	−	+	+ (α)	?	−	+	−	?	?	?	−
[e]*Weissella ceti*	w	c/r	−	−	−	−	−	+	F	−	−	−	−	−	?	+ (α)	?	−	−	+	?	+	?	−

1 colony pigmentation, *2* rods-cocci, *3* presence of aerial hyphae, *4* presence of endospores, *5* acid-fast staining reaction, *6* motility, *7* requirement for L-cysteine hydrochloride, *8* growth in air, *9* oxidative-fermentative metabolism of glucose, *10* catalase production, *11* α-galactosidase production, *12* β-galactosidase production, *13* oxidase production, *14* coagulase production, *15* H_2S production, *16* blood degradation (haemolytic activity), *17* starch degradation, *18* urea degradation, *19* growth at 10 °C, *20* growth at 37 °C, *21* growth in 0 % (w/v) sodium chloride, *22* growth in 6.5 % (w/v) sodium chloride, *23* growth in 0.001 % (w/v) crystal violet, *24* acid production from sorbitol

(continued)

Table 13.2 (continued)

(+) weakly positive result, *v* variable results, *?* not stated, *c* cocci, *r* rods, *m* mycelium, *O* oxidative metabolism, *F* fermentative metabolism
[a]Colony pigmentation: w, y, re and o correspond to off-white/white, yellow/golden, red and orange, respectively
[b]Confirm by presence of toxin
[c]Confirmatory profile with API-ZYM
[d]Differentiate serologically or by sequencing of the 16S rRNA gene
[e]Differentiate rby sequencing of the 16S rRNA gene
[f]May be in pairs, tetrads or small clusters
Sta. epidermidis and *Sta. warneri* may be distinguished by the lack of the former to produce acid from lactose and mannose
Str. ictaluri and *Str. iniae* and *Str. parauberis* may be distinguished by the lack of the first mentioned to degrade aesculin

Table 13.3 Diagnostic traits of the Gram-negative bacterial fish pathogens

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Acinetobacter sp.	w	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	?	−	?	?	?
Aer. allosaccharophila	w	−	−	−	−	+	F	?	+	+	+	+	?	−	?	+	+	+	+	+	+
Aer. caviae	w	−	−	−	−	+	F	+	+	+	+	−	+	−	+	+	?	+	+	+	+
Aer. dhakensis	w	−	−	−	−	+	F	+	+	+	−	+	?	+	+	+	+	+	+	+	+
Aer. hydrophila	w	−	−	−	−	+	F	v	+	+	+	−	−	v	+	+	+	+	+	+	+
Aer. jandaei	w	−	−	−	−	+	F	+	?	+	+	+	?	+	?	+	+	?	+	+	+
Aer. piscicola																					
Aer. salmonicida subsp. *achromogenes*	w	−	−	−	−	−	F	+	+	+	+	−	−	−	−	+	+	+	−	−	+
Aer. salmonicida subsp. *masoucida*	w	−	−	−	−	−	F	+	+	+	+	+	−	+	+	+	+	+	+	−	+
Aer. salmonicida subsp. *salmonicida*	b	−	−	−	−	−	F	+	+	−	+	−	−	−	+	+	+	+	+	−	+
Aer. salmonicida subsp. *smithia*	b	−	−	−	−	−	F	−	+	−	+	−	−	−	−	+	+	−	−	−	+
Aer. schubertii	w	−	−	−	−	+	F	+	?	−	+	+	+	−	?	+	+	+	?	+	+
Aer. sobria	w	−	−	−	−	+	F	+	+	+	+	+	?	+	+	+	+	?	+	+	+
Arc. cryaerophilus	W	−	−	−	−	+	−	−	?	−	+	?	−	−	?	−	−	−	+	+	−
Chrys. aahli	y	−	−	−	−	−	?	−	−	−	+	−	?	−	?	+	−	−	+	−	+
Chrys. balustinum	y	−	−	−	−	−	O	−	−	+	+	+	?	?	?	+	−	?	+	+	?
Chrys. piscicola	y	−	?	−	−	−	−	−	−	−	+	−	−	−	α	+	−	?	?	−	+
Chrys. scophthalmum	o	−	−	−	+	−	F	?	?	−	+	−	?	−	?	+	−	?	?	?	?
Cit. freundii	w	−	−	−	−	+	F	−	+	−	−	−	?	−	?	−	?	?	+	+	+
Edw. anguillarum	w	−	−	−	−	+	F	−	−	+	−	?	?	?	?	−	?	?	+	+	+
Edw. ictaluri	w	−	−	−	−	+	F	−	−	−	−	+	+	−	+	−	?	+	+	+	+

(continued)

Table 13.3 (continued)

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Edw. piscicida	w	–	–	–	–	+	F	–	–	+	–	+	+	–	+	–	?	?	+	+	+
Edw. tarda	w	–	–	–	–	+	F	–	–	+	–	+	+	–	(+)	–	?	+	+	+	+
Ent. cloacae	w	–	–	–	–	+	F	+	?	?	–	–	?	?	?	?	?	?	+	+	?
Esch. vulneris	w	–	–	–	–	+	F	+	?	–	–	+	+	–	+	–	–	+	+	+	?
Fla. branchiophilum	y	–	–	–	–	–	O	?	?	–	+	?	?	?	?	+	+	?	+	–	+
Fla. columnare	y	–	–	–	+	–	O	?	?	–	+	–	–	–	?	+	–	?	+	(+)	+
Fla. hydatis	y	–	–	–	+	–	O	?	?	–	–	+	–	–	+	+	+	?	+	+	+
Fla. johnsoniae	y	–	–	–	+	–	?	?	+	–	+	?	?	?	?	+	+	?	+	–	–
Fla. oncorhynchi	y	–	–	–	–	–	?	?	+	–	+	?	?	?	–	–	+	–	+	–	+
Fla. psychrophilum	y	–	–	–	+	–	–	?	?	–	–	–	?	–	?	+	–	?	–	–	+
Fla. spartansii	y	–	–	–	+	–	?	–	+	–	+	–	?	v	?	+	+	+	+	–	+
T. dicentrarchi	*y*	–	–	–	+	–	O	?	?	–	+	?	?	–	?	+	–	–	+	–	–
T. dicolor	y	–	–	–	+	–	O	?	?	–	+	?	?	–	?	+	–	–	+	+	–
T. gallaicum	y	–	–	–	+	–	O	?	?	–	+	?	?	–	?	+	–	–	+	+	–
T. maritimum	y	–	–	–	+	–	O	?	?	–	+	?	?	–	?	+	–	?	+	–	–
T. ovolyticum	y	–	–	–	+	–	O	–	–	–	+	–	?	?	?	–	–		–	–	–
T. soleae	y	–	?	–	+	–	O	?	–	–	+	?	?	–	?	+	–	–	–	–	–
Haf. alvei	w	–	–	–	–	+	F	–	+	–	–	+	+	+	?	–	?	?	+	+	+
Halomonas cupida	w	–	–	–	–	+	–	+	?	–	–	+	–	–	+	?	?		–	–	–
J. lividum	p	–	–	–	–	+	O	+	–	–	+	?	?	+	?	+	?	+	+	–	+
Klebsiella pneumoniae	w	–	–	–	–	+	F	–	?	–	–	–	?	?	?	–	?	+	+	+	+
Fr. noatunensis subsp. orientalis	w	–	–	–	–	–	–	?	?	?	–	?	?	?	?	?	?	–	+	+	?
Fr. noatunensis	w	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	(+)	–?	?
Moraxella sp.	w	–	–	–	–	–	O/–	–	–	–	+	–	?	–	+	–	?	+	?	?	?
Moritella marina	w	–	–	–	–	+	F	–	?	?	+	?	+	–	+	+	+		–	–	–

Moritella viscosa	–	–	–	–	–	+	F	–	?	–	+	+	–	–	+	+	+	?	–	–	–vvv
Mycoplasma mobile	?	–	+	–	+	–	?	?	?	?	+	?	?	?	+	–	?	+	+	–	?
Myxococcus piscicola	y	+	–	–	+	–	?	?	?	?	?	?	?	?	?	?	?	?	?	?	+
Pantoea agglomerans	y	–	–	–	–	+	F	–	+	–	–	–	+	+	–	+	–	?	+	+	?
Ph. damselae	w	–	–	–	–	+	F	+	–	+	+	–	+	+	?	–	+	+	+	+	–
Ph. damselae subsp. *piscicida*	w	–	–	–	–	–	F	–	–	–	+	+	+	+	–	–	–		+	–	–
Piscirickettsia salmonis	?	–	–	+	–	–	–	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Plesiomonas shigelloides	w	–	–	–	–	+	F	+	+	+	+	+	?	–	?	–	?	?	+	+	+
Pr. rettgeri	w	–	–	–	–	+	F	–	–	+	–	–	+	–	?	–	–	–	+	+	+
Pasteurella skyensis	?	–	–	–	–	–	F	–	–	+	(+)	+	?	–	(+)	?	?	+	+	–	–
Pseudoalteromonas piscicida	o	–	–	–	–	+	O	?	?	?	?	?	?	?	?	?	?	?	+	+	–
Pseudoalteromonas undina	?	–	–	–	–	+	O	–	?	–	+	–	?	–	?	?	+	+?	+	–	–
Ps. anguilliseptica	w	–	–	–	–	+	O	–	–	–	–	–	?	?	–	+	–		+	–	+
Ps. baetica	w/fl	–	–	–	–	+	O	+	–	?	+	–	?	–	+	+	–	?	+	–	+
Ps. chlororaphis	g/fl	–	–	–	–	+	O	+	?	?	+	?	?	?	?	+	?	?	+	?	+
Ps. fluorescens	w/fl	–	–	–	–	+	O	v	–	–	+	–	?	–	?	+	_		+	+	+
Ps. koreensis	y/g	–	–	–	–	+	O	?	–	–	+	?	?	?	+	–	–	?	+	?	+
Ps. luteola	–	–	–	–	–	+	O	+	+	–	+	–	?	–	?	+	?	?	?	?	?
Ps. plecoglossicida	w/fl	–	–	–	–	+	O	+	+	?	+	–	?	?	+	–	–	?	+	?	+
Ps. pseudoalcaligenes	w	–	–	–	–	+	O (alk)	+	–	–	+	–	?	–	?	+	–	–	+	–	+
Sal. enterica subsp. *arizonae*	w	–	–	–	–	+	F	?	+	–	–	+	+	–	–	–	–	+	+	+	+

(continued)

Table 13.3 (continued)

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Ser. liquefaciens	w	−	−	−	−	+	F	−	+	−	(−)	+	−	−	+	+	−	+	+	+	+
Ser. marcescens	r	−	−	−	−	+	F	−	+	−	−	+	−	+	+	+	+	+	+	+	+
Ser. plymuthica	r	−	−	−	−	+	F	−	+	−	−	−	−	+	−	+	?	+	+	+	+
Shewanella putrefaciens	w	−	−	−	−	+	−	−	−	−	+	−	?	−	?	−	?	−	+	+	−
Sporocytophaga sp.	y	+	−	−	+	−	O	?	?	?	?	?	?	?	?	?	?		?	?	−
Stentrophomonas maltophilia	w	−	−	−	−	−	O	?	−	−	−	−	?	?	+	?	−	?	?	?	?[a]
V. aestuarianus	w	−	−	−	−	+	F	+	+	+	+	?	+	−	+	−	?	+	+	+	−
V. alginolyticus	w	−	−	−	−	+	F	−	+	+	+	+	+	+	+	+	+	+	+	+	−
V. anguillarum	w	−	−	−	−	+	F	−	+	v	+	−	−	+	?	+	+	+	+	+	−
V. cholerae	w	−	−	−	−	+	F	−	+	+	+	+	+	+	+	+	+	?	+	+	+
V. harveyi	w	−	−	−	−	+	F	−	−	+	+	+	?	−	+	+	−	−	+	+	−
V. ichthyoenteri	w	−	−	−	−	+	F	−	−	−	+	?	?	?	?	−	−	+	+	−	+
Ali. logei	w	−	−	−	−	+	F	−	?	−	+	−	?	−	+	−	−	+	−	−	−
V. ordalii	w	−	−	−	−	+	F	−	−	−	+	−	−	−	?	+	−	+	(+)	−	−
V. pelagius	w	−	−	−	−	+	F	−	v	v	+	−	+	v	?	+	+	?	+	+	?
V. ponticus	w	−	−	−	−	+	F	+	?	+	+	−	?	−	?	−	?	−	+	+	−
Ali. salmonicida	w	−	−	−	−	+	F	−	−	−	+	?	?	−	−	−	?		−	−	+
V. splendidus	w	−	−	−	−	+	F	+	−	−	+	−	+	+	+	−	+	+	+	+	−
V. tapetis	W	−	−	−	−	+	F	−	?	?	+	?	?	−	−	−	?	?	?	−	−
V. vulnificus	w	−	−	−	−	+	F	−	−	−	+	+	+	−	+	+	+	+	+	+	−
Ali. wodanis	y	−	−	−	−	+	F	−	?	+	+	−	+	−	(+)	+	+	?	−	−	−
Y. intermedia	w	−	−	−	−	+	F	−	+	+	−	−	+	+	?	?	?	?	+	+	?
Y. ruckeri	w	−	−	−	−	+	F	−	+	−	−	+	+	−	?	+	?	+	+	+	+

With the widespread use of sequencing of the 16S rRNA gene as a primary method of identification/characterisation, phenotypic characters have been downplayed. Consequently, it is not possible to include the data for all the bacterial fish pathogens. Also, there are similarities in the profiles of some organisms; discrimination needing 16S rRNA sequencing data

1 colony pigmentation, *2* presence of microcysts, *3* filterability through 0.45 μm pore filters, *4* growth only occurs in tissue culture, *5* gliding, *6* motility by flagella, *7* oxidative- fermentative metabolism of glucose, *8* arginine dihydrolase production, *9* β-galactosidase production, *10* indole production, *11* oxidase production, *12* lysine decarboxylase production, *13* methyl red test, *14* Voges Proskauer reaction, *15* degradation of blood, *16* gelatin degradation, *17* starch degradation, *18* acid production from maltose, *19* growth at 30 °C, *20* growth at 37 °C, *21* growth in 0 % (w/v) NaCl

b, fl, g, p, r, w and y correspond to brown, fluorescent, green, purple, red/pink, cream/white and yellow pigment, respectively

+, −, v, ?, (+) and (−) correspond to positive (≥80 % of positive responses), negative (≤20 % of positive responses), variable (21–79 % of positive responses), unstated, weakly positive, and weakly negative result, respectively

O, F, Alc = oxidative or fermentative metabolism, or production of alkali, respectively

Ps. baetica and *Ps. fluorescens* by the latters ability to utilise a wide range of compounds including D-alanine whereas the former does not

It is difficult to distinguish *T. dicentrarchi* and *T. maritimum* phenotypically. Reliance may need to be placed on sequencing of the 16S rRNA gene

From the original description, it is difficult to distinguish *T. dicolor* from *T. gallaicum* phenotypically. Reliance may need to be placed on sequencing of the 16S rRNA gene

[a]T

a faint pinkish tint remains. The slide is re-washed, before applying a methylene blue counterstain for 30 s. Following re-washing with tap water, the slide is gently blotted dry, and examined by oil-immersion (Doetsch 1981).

- Motility

In our experience, wet-preparations prepared from barely turbid suspensions are most satisfactory when viewed by phase contrast microscopy at ×400 magnification.

- Gliding motility

This may be assessed from the development of spreading growth on low-nutrient (cytophaga) agar. It should be differentiated from locomotion by means of flagella.

- Filterability through the pores of 0.45 μm pore size porosity filters

The ability of cells to pass through the pores of 0.45 μm pore size porosity filters is indicative of the presence of L-forms and mycoplasmas. Thus, the bacterial suspension is filtered, and the filtrate applied to a suitable growth medium. Growth within 7 days is indicative of filterability.

- The ability to grow only in fish cell cultures

Viruses and rickettsias are only capable of growth in suitable cell cultures.

- Aerobic or anaerobic requirements for growth

These are apparent after incubating inoculated media aerobically and anaerobically.

- Catalase production

This is recorded by effervescence within 1 min from 3 % (v/v) hydrogen peroxide following application of a bacterial colony. Quite simply, the 'young' colony may be scraped with a thin glass rod and transferred to a drop of hydrogen peroxide on a glass slide.

- Fluorescent (fluorescein) pigment production

This is assessed by the presence of a fluorescent green pigment seen under ultraviolet light, after 7 days incubation on the medium of King et al. (1954).

- Growth at 10, 30 and 37 °C

Growth at 30 and 37 °C should be recorded within 72 h incubation on basal medium. At 10 °C, the media should be retained for up to 14 days.

- Growth on 0 % and 6.5 % (w/v) sodium chloride and on 0.001 % (w/v) crystal violet

This is reported after 7 and 14 days incubation on suitably modified basal medium.

- Requirement for 0.1 % (w/v) L-cysteine hydrochloride

This is essentially a requirement for the growth of *Ren. salmoninarum.* Inoculated media should be incubated at 15 °C, and examined at weekly intervals for up to 16 weeks.

- Oxidation-fermentation test

This involves the measurement of acid production from glucose metabolism under aerobic and/or anaerobic conditions in the basal medium of Hugh and Leifson (1953). The production of an alkaline reaction is indicated by a deep blue colour which develops, usually in the open tube. For marine organisms, it is necessary to use the modified medium of Leifson (1963). The presence of acid, indicated by a colour change to yellow, should be recorded after incubation for 1, 2 and 7 days.

- Indole production

This is recorded after 7 days incubation in 1 % (w/v) peptone water. For marine organisms, this should be prepared MSS (2.4 % (w/v) NaCl; 0.7 % (w/v) $MgSO_4.7H_20$; 0.075 % (w/v) KCl; after Austin et al. 1979). A positive response is indicated by a red coloration following the addition of a few drops of Kovacs reagent.

- α-galactosidase production

One of the most reproducible methods is to record α-galactosidase production from the API zym system after incubation for 48 h at 15 or 25 °C.

- β-galactosidase production

This involves use of the medium of Lowe (1962). Inoculated medium is incubated for 7 days, whereupon a positive response is indicated by a yellow coloration. For marine organisms, the medium should be prepared in MSS.

- Production of arginine dihydrolase and lysine decarboxylase

We recommend use of the medium described by Møller (1955). Essentially inoculated medium is incubated for 7 days, when a positive reaction is indicated by a purple coloration. With marine organisms, the medium should be prepared in MSS.

- Urease production

Using the medium of Stuart et al. (1945), a positive response develops as a reddish coloration, within 28 days. For marine organisms, it is suggested that the medium is supplemented with 2.4 % (w/v) sodium chloride.

- Methyl red test and Voges-Proskauer reaction

These may be recorded after 7 days incubation in MRVP broth (Difco). Following the addition of a few drops of methyl red, a bright red coloration indicates a positive methyl red test. The Voges Proskauer reaction is recorded after use of commercially available reagents. A positive reaction is indicated by a red coloration which

develops within 18 h (usually within 1 h) after the addition of the reagents. As before with marine organisms, the medium may be prepared in MSS.

- Degradation of blood

This should be recorded within 7 days as zones of clearing around colonies on basal medium supplemented with 5 % (v/v) defibrinated sheep's blood.

- Degradation of gelatin

This is detected after 7 days incubation by the addition of saturated ammonium sulphate solution to the medium of Smith and Goodner (1958). A positive result is indicated by zones of clearing around the bacterial growth. For marine organisms, the medium should be supplemented with MSS.

- Degradation of starch

Basal medium supplemented with 1 % (w/v) soluble starch is streaked, and incubated at 15–25 °C. After 7 days, the starch plates are flooded with an iodine solution (e.g. Difco Gram's iodine). The degradation of starch is indicated by a clear area surrounded by a blue/black background.

- Acid production from maltose and sorbitol

The use of Andrade or phenyl red-peptone water supplemented with maltose or sorbitol is advocated (see Cowan 1974). This medium contains 1 % (w/v) bacteriological peptone 0.5 % (w/v) sodium chloride (for marine organisms this amount should be increased to 2 %), 1 % (w/v) maltose and Andrade or phenyl red indicator. The filter sterilised (0.22 μm pore size porosity filter) maltose solution should be added to the basal medium after autoclaving, and the completed medium dispensed into test tubes. The production of acid is indicated by the development of a pink colour within 48 h at 25–37 °C.

- Production of hydrogen sulphide

Many methods have been developed to detect the production of hydrogen sulphide. We have found success with triple sugar iron agar (Oxoid), which should be prepared as slopes in test tubes. Following incubation of the inoculated media at 15–25 °C for up to 7 days, the production of hydrogen sulphide is indicated by blackening of the agar.

- Coagulase test

We recommend a simple test using citrated plasma (of rabbit, sheep, donkey or ox). The bacterial culture should be emulsified [to form a dense suspension of $\sim 5 \times 10^8$ cells/ml] in a drop of 0.9 % (w/v) saline on a clean grease-free microscope slide. This suspension is then carefully mixed with one drop of citrated plasma. A positive result, which is indicated by clumping of the bacterial cells, is apparent within 2–3 min.

Most of the above-mentioned phenotypic tests have been derived from medical microbiology. Nevertheless, careful attention to detail will generate useful data

about bacterial fish pathogens. Undoubtedly, more modern methods will eventually enter the realms of fish microbiology. These methods may include the development of highly reliable rapid techniques, such as offered by high-pressure liquid chromatography and mass-spectrometry. Moreover, lipid analyses could be adapted further for fisheries work. Serological techniques, such as those involving ELISA and monoclonal antibodies, are steadily entering the domain of the fish disease diagnosticians. In addition, molecular genetic techniques, notably gene probe technology, are under evaluation in several laboratories.

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Chapter 14
Control

Abstract Disease control strategies include better husbandry/management practices, consideration of the use of genetically disease resistant fish strains when available, the use of suitable diets/dietary supplements, vaccines, non specific immunostimulants, probiotics, prebiotics, natural plant products, antimicrobial compounds, water disinfection, and prevention of/restriction in the movement of infected stock.

Keywords Vaccines • Probiotics • Immunostimulants • Plant products • Antimicrobial compounds

It is worth remembering the age-old adage that 'prevention is better than cure', and certainly it is possible to devote more attention to preventing the occurrence of disease in fish. This is especially true for farmed fish, which tend to be at the mercy of all the extremes which their owners are capable of devising. Principally in the industrialised nations, farmed fish are subjected to questionable water quality and high stocking regimes. These are among the known prerequisites for the onset of disease cycles. Yet, owners are among the first to seek help if anything adverse happens to the valuable stock. Fish may be reared under ideal conditions, in which case, the stock are inevitably in excellent condition without signs of disease. Such sites, for example located in Venezuela and the former Yugoslavia, are usually supplied by fast-flowing, clear river water. Careful feeding regimes are adopted, and the stocking levels are comparatively low. The latter point would make the enterprise unacceptable in the more industrialised nations of Western Europe. Therefore, much attention has been devoted to control measures. These have been categorised in Table 14.1. Although most emphasis has been placed on aquaculture, some effort has gone towards considering disease in wild fish stocks.

Wild Fish Stocks

It is questionable what, if anything may be done to control disease in wild fish stocks. Perhaps, the first step should be to determine the precise extent of disease among wild fish populations. Surveys have been carried out with a view to assessing

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the incidence of 'abnormalities' in marine fish. Indeed, some workers have attempted to correlate the incidence of disease with pollution. Some attention has focused on archive material, collected at or before the turn of the century, and housed in some museums, e.g. in Liverpool, UK, with access usually granted to interested individuals. A detailed study would soon demonstrate whether or not 'abnormalities' in fish are a new or old phenomenon. Surely, this information could then be correlated with the changes in pollution of the aquatic environment. It is our contention that dense populations of fish have always maintained a given level of diseased individuals, regardless of whether the populations are shoals in the sea or aquacultural stocks. Therefore, it is possible that reducing pollution will not noticeably alter the health index of wild fish. Nevertheless, by using a circuitous route, it may be possible to ensure that wild fish stocks are not likely to be exposed to pathogens, and therefore be at less risk of disease. Theoretically, this could be achieved by controlling outbreaks of disease in farmed fish and thereby reducing the possibility of pathogens escaping into the environment. It should be emphasised, however, that there is a dearth of information, which suggests that disease may be transferred from farmed to wild fish stocks. At worst, there is a perceived problem, and this could easily escalate into adverse propaganda for the aquaculture industry. It is essential that consideration should be urgently given to control measures, which will reduce any possible risk of pathogens escaping into the natural environment.

Farmed Fish

There are many approaches, which need to be adopted in order to control bacterial disease in farmed fish (Table 14.1). These will be explained separately below.

Table 14.1 Methods of controlling bacterial fish diseases

Classification of fish stocks	Disease control measures	
Wild	Control of pollutants (water quality)	
Farmed	1.	Adequate husbandry/management practices
	2.	Use of genetically resistant fish strains
	3.	Suitable diets and where appropriate, use of dietary supplements
	4.	Use of vaccines
	5.	Use of nonspecific immunostimulants
	6.	Use of probiotics/biological control
	7.	Use of prebiotics/medical plant products
	8.	Use of antimicrobial compounds
	9.	Water treatments
	10.	Preventing the movement of infected stock

Husbandry/Management

It may be problem that under severe economic pressures the aquaculturist is tempted to produce the maximum yield of fish in a finite volume of water, i.e. to use very high stocking levels. Some sympathy must be directed towards the fish farmers especially when prices paid for the stock are low and profit margins are tight or inadequate. The underlying problem is that within intensive cultivation systems, the fish may be 'stressed' beyond the limit commensurate with the production of healthy specimens. Interestingly, it has been suggested that reducing the stocking density when water temperatures are high may well prevent some diseases, such as columnaris outbreaks in rainbow trout (Suomalainen et al. 2005). The outcome would be that the lower stocking level leads to less disease, and more output. Stress may be compounded by other inappropriate management practices in which aeration and water flow are insufficient, overfeeding occurs, and hygiene declines below the threshold at which disease is more likely to ensue. It may need only one diseased individual to act as a reservoir of infection to the rest of the stock. Unsatisfactory occurrences, which are readily controlled, include:

- the accumulation of organic matter, namely faecal material and uneaten fish food, within the fish holding facilities, which allow the buildup of microbial populations, some of which may cause disease. Also, such organic matter may attract vermin, which may pose other health issues including the risk of human disease, e.g. Weil's disease/leptospirosis;
- the presence of dead fish in the tanks/ponds/cage/raceways for prolonged periods (= bad sanitation). Again, the permits the increase in microbial populations as well as contributing harmful chemicals to the water;
- the accumulation of a biofouling community, i.e. algae and slime, in the fish tanks, and the problem associated with the collapse of blooms resulting in the release of toxic materials;
- the depletion of the oxygen content of the water with a concomitant increase in nitrogen levels, especially as ammonium salts;
- the lack of proper disinfection for items entering the fish holding facilities. Reference is made here to nets, protective footwear, and size grading machinery;
- lack of adequate sanitary disposal arrangements for dead fish, allowing access to birds, e.g. seagulls, and vermin, and the build-up and spread of potentially harmful micro-organisms.

Good basic hygiene (water quality) and farm husbandry practices may successfully alleviate many of the problems attributed to disease.

Disinfection/Water Treatments

Apart from the use of antibiotics and related compounds, which are actively discouraged from use in any nonmedical situation in many countries, the application of other chemicals to water as disinfectants is effective for disease control. Such chemicals include benzalkonium chloride, chloramine B and T, chlorine, formalin and iodophors.

Another approach is to alter (increase or decrease, according to the season) the temperature of water within fish holding facilities, which may be achieved by altering the water flow, providing shading, or actively heating/cooling as appropriate.

Genetically Resistant Stock

This is a topic worthy of greater attention, insofar as there are numerous observations, which point to the value of genetically resistant strains or selective breeding for reducing the problems of disease. As a word of caution, however, comparative studies need to be carefully controlled so that meaningful results are obtained. In any comparison, the age, size and relative condition of the animals need to be standardised. Nevertheless, there has been prolonged interest in breeding disease-resistant fish. It is obvious that the breeding of disease-resistant fish may be a valuable addition in the armoury of disease control in aquaculture. However, in fish farming where more than one disease is prevalent, it is not necessarily the case that a fish strain which is resistant to one disease, would similarly show resistance to others. Nevertheless, we consider that disease-resistant strains of fish have potential for areas in which diseases are enzootic. Further effort is clearly required to bring the concept to fruition. One overriding concern is the public misconception of the risks associated with GMOs, which is profound in some countries, particularly in Western Europe, but less so or non-existent in others.

Adequate Diets/Dietary Supplements/Non-Specific Immunostimulants

An area of comparatively recent interest is that of dietary influence on fish health. Could some essential nutrients be lacking, or other compounds be present in dangerous excess? A mass of published work has pointed to the benefit to health, welfare and growth of the addition of any of a wide range of natural products to fish feed. For example, Ketola (1983) highlighted a requirement for arginine and lysine by rainbow trout fry, with fin erosion resulting from a deficiency of lysine. The list

includes natural plant products, immunostimulants, vitamins and micro-organisms. The outcome of the work, which has been summarized, in earlier chapters, is that the addition of comparatively small amounts of these products to fish feed leads to statistically significant improvements in growth, including feed conversions, non-specific immunostimulation particularly of innate and cellular immune parameters, e.g. increases in phagocytic activity, and health benefits in terms of protection against challenge by specified pathogens within a fairly small timespan, i.e. positive effects may be observed within 1–2 weeks of the first application of the product. In addition, there have been indications of non-specific health benefits, such as an increase in vigour and an absence of background levels of fin/tail rot. For example, ß-glucans, which may be obtained from the yeast *Saccharomyces cerevisiae*, when administered to carp led to significantly increased leucocyte populations, enhanced proportions of neutrophils and monocytes, and elevated superoxide anion production by kidney macrophages (Selvaraj et al. 2005). Moreover, spray-dried, heterotrophically grown preparations of the unicellular alga *Tetraselmis suecica* have been accredited with antimicrobial activity and possibly immunostimulatory activity when used as dietary supplements (Austin et al. 1992a). Similarly, the yeast *Debaryomyces hansenii* was immunostimulatory when fed for 4 weeks at 10^6 CFU/g to leopard grouper (*Mycteroperca rosacea*) (Reyes-Becerril et al. 2008).

The list of immunostimulatory compounds is extensive, and includes those which have often been applied by i.p. injection, i.e. Batpamum, chitin, dimerised lysozyme, ß-1,3 glucans, killed cells of mycobacteria, laminaran, sulphated laminaran, lactoferrin, levamisole, LPS, oligosaccharides, Prolactin and synthetic peptides (Dalmo and Seljelid 1995; Yoshida et al. 1995; Ortega et al. 1996; Siwicki et al. 1998; Sakai 1999). Initially, Olivier et al. (1985a, b) observed that administration of killed cells of mycobacteria enhanced resistance in coho salmon to various bacteria. However, considerable interest has been directed towards the potential for ß-1,3 glucans, which are often included as one of the ingredients in fish feed. An extensive literature points to the success of glucans in preventing disease (Yano et al. 1989; Raa et al. 1990; Robertsen et al. 1990; Nikl et al. 1991; Matsuyama et al. 1992; Chen and Ainsworth 1992). Thus, it has been recognised that ß-glucans enhance the non-specific resistance to disease, including pasteurellosis (Couso et al. 2003), ERM, Hitra disease and vibriosis, by immunostimulation (Robertsen et al. 1990; Kumari and Sahoo 2006). Matsuyama et al. (1992) used the glucans schizophyllan and scleroglucan to protect against streptococci. Thus, 2–10 mg of glucans/kg of fish when administered by i.p. injection, enhanced resistance of yellowtail to streptococcicosis. In particular, there was an elevation of serum complement and lysozyme, and an increase in phagocytic activity of pronephros cells. Initially, success only appeared to result from injection of the glucans into fish. Yet, claims have now been made that application via food also meets with success (Onarheim 1992). Also, resistance to streptococcicosis and vibriosis has been enhanced following the oral administration of peptidoglycan from *Bifidobacterium* (Itami et al. 1996) and *Cl. butyricum* (Sakai et al. 1995), respectively.

Vaccines

The expectation during the 1970s was that vaccines would become the primary means of disease prevention. Yet in many cases the programmes of research have not resulted in many commercial products. However, there have been success stories, such as the vaccines for the control of vibriosis (caused by *V. anguillarum* and *V. ordalii*) and ERM. The research has progressed from the simplistic and often completely successful approach of using chemically-inactivated whole cell suspensions to purified subcellular antigenic components of pathogens and to live attenuated vaccines.

Historically, the first serious attempt to develop a bacterial fish vaccine may be traced to the work of Duff (1942), who used chloroform-inactivated cells to protect cutthroat trout (*Salmo clarki*) against furunculosis. Since then, vaccines have been formulated against half of the total number of bacterial fish pathogens.

Composition of Bacterial Fish Vaccines

The composition of bacterial fish vaccines may be categorised as follows:

- Chemically or heat-inactivated whole cells. These vaccines may be mono- or polyvalent. Essentially, these are the simplest, crudest and cheapest forms of fish vaccines.
- Inactivated soluble cell extracts, i.e. toxoids.
- Cell lysates.
- Attenuated live vaccines, (e.g. LaFrentz et al. 2008) possibly genetically-engineered cells. These would be unacceptable to some regulatory authorities because of the perceived risk that the vaccine strain may revert to a pathogenic mode.
- Attenuated live, heterologous vaccines. An example is Bacillus Calmette and Guèrin (BCG), which is live attenuated *Mycobacterium bovis* product, protected Japanese flounder against mycobacteriosis (Kato et al. 2010).
- Subunit vaccines, e.g. the genes product of the *tapA* gene for the control of *Aer. salmonicida* infections (Nilsson et al. 2006).
- DNA vaccines (e.g. Pasnik and Smith 2006; Jiao et al. 2009; Sun et al. 2010).
- Purified sub-cellular components, e.g. glyceraldehyde-3-phosphate dehydrogenase (GAPDH), OMP and LPS. These vaccines require a detailed understanding of microbial chemistry, aspects of which are deficient for many of the bacterial fish pathogens.
- Serum, for passive immunisation (e.g. Shelby et al. 2002). This is largely of academic interest only, insofar as it is difficult to envisage use of the technique in the fish farm environment. A possible exception is for brood stock or pet fish.
- Mixtures of the components, detailed above.

It is difficult to identify any particular type of preparation which excels in terms of protection. Unfortunately, even the best vaccines may not completely prevent the occurrence of disease, sometimes necessitating the use of costly medicines to combat low levels of infection.

A potentially exciting and relevant development has involved the realization that some molecules may have broad spectrum use as vaccine candidates by offering protection against a range of pathogens. In this connection, recombinant GADPHs offer cross protection with RPS values of >60 % (Li et al. 2011).

Methods of Vaccine Inactivation

Methods to inactivate whole cell preparations include the use of chemicals, namely 3 % (v/v) chloroform, 0.3–0.5 % (v/v) formalin and 0.5–3.0 % (v/v) phenol, heat (e.g. 56 °C or 100 °C for 30 or 60 min), sonication, pressure (600 kgf/cm^2 for 5 min), electric current (100 mA at 12v DC for 5 s) lysis with sodium hydroxide at pH 9.5 or with SDS (Austin 1984b; Hossain and Kawai 2009). Commercially, most interest has centred on use of formalin, which has given encouraging results with numerous bacterial fish pathogens, including *Aer. hydrophila, Edw. ictaluri, Ph. damselae* subsp. *piscicida, Ps. anguilliseptica, V. anguillarum, V. ordalii* and *Ali. salmonicida.* However, it is unfortunate that only a few studies have been carried out to compare different inactivated preparations.

Methods of Administering Vaccines to Fish

A number of methods of administering vaccines to fish have been tried with varying degrees of success (see Austin 1984b), and include:

- Injection, with or without the presence of adjuvant, such as FCA/FIA. This technique is slow, and will inevitably require prior anaesthesia of the animals. Injection is only feasible for valuable fish, brood stock or pet fish. Fortunately, mass injection techniques are available.
- Oral uptake, via food. This should be the method of choice insofar as fish could be fed and vaccinated simultaneously. However, there may be problems with the degradation of the vaccine in the gastro-intestinal tract, although this is being overcome by new exciting oralising compounds.
- Immersion in a solution/suspension of the vaccine. This is quick (i.e. taking 30–120 s to perform) and easy, permitting large numbers of fish to be readily vaccinated. However, there could be problems regarding disposal of the spent vaccine. Thus, it is debatable whether or not disposal should take place in the fish farm effluent.

- Bathing in a very dilute preparation of the vaccine for prolonged periods, i.e. several hours. This is obviously very economic in the use of vaccine. It is feasible that the technique could be carried out during routine periods of confinement, such as during transportation of the stock between sites. However, with immersion, careful thought needs to be given to the question of disposal.
- Spraying or showering the vaccine onto fish. This can be automated, such that fish are vaccinated on conveyor belts during routine grading.
- Hyperosmotic infiltration. This involves a brief immersion (30–60 s) in a strong salt solution, i.e. 3–8 % (w/v) sodium chloride, followed by dipping for 30–60 s into the vaccine. This method is very stressful to fish, and its use has been consequently reduced. However, *En. faecalis, Lactococcus garvieae* and *Str. iniae,* which have been identified from diseased farmed fish in Taiwan, have been the subject of vaccine development involving formalin-inactivated whole cell preparations that were administered to Nile tilapia by hyperosmotic infiltration leading during the fifth and sixth week after challenge to RPS values of 71.2–88.7 %. Boosters did not improve protection (Young et al. 2012).
- Anal/oral intubation. In particular, anal intubation offers possibilities for bypassing the deleterious effects of the stomach and intestine. The technique is, however, cumbersome and requires further development.
- Ultrasonics/ultrasound (Zhou et al. 2002; Navot et al. 2011).

It is often difficult to determine which is the most effective method of vaccine application. The method of choice often reflects the whims of the user as much as scientific reasoning. The available evidence suggests that oral administration fares least well, although new approaches of micro-encapsulation offer promise. The use of oral vaccination for booster doses, has been successful, such as with *Aer. hydrophila, Aer. salmonicida, Fla. columnare, Ph. damselae* subsp. *piscicida, V. anguillarum, V. ordalii* and *Y. ruckeri* vaccines; i.p. injection may better that oral uptake in terms of the resultant humoral antibody titre and protection, although there is not always a direct correlation between antibody-titre and protection. However, with *Edw. ictaluri, Edw. tarda, V. anguillarum* and *V. ordalii* preparations, immersion has been demonstrated as superior to injection. Similarly with the 'vibrio' vaccines, the shower method exceeds injection in terms of resulting protection. There is a question mark about the difference between vaccines – particularly oral vaccines – and immunostimulants/feed additives that also give health benefits including immunostimulation and protection against disease. The levels of protection between these alternatives are often compatible, with the natural products being cheaper and easier to adopt particularly in terms of regulatory machinery that is engrained in many countries.

Traditionally, potency testing has been by infectivity experiments whereby vaccinated fish are challenged with a virulent culture of the pathogen, and mortalities counted and used to calculate the RPS. The severity of the approach has focused attention of regulatory authorities, and a stated aim is to move towards non-lethal testing methods, notably the measurement of key immunological parameters, e.g. antibody production, which may be measured by ELISA (Romstad et al. 2012).

Antimicrobial Compounds

There is a trend away from the use of antibiotic-like antimicrobial compounds in all non-human applications with concerns about the possibility of tissue residues and the development of bacterial resistance. It is astounding that so many compounds (these have been reviewed by Snieszko 1978; Herwig 1979; Austin 1984a) have found use in aquaculture. The complete list reads like an inventory from any well-equipped pharmacy. Antibiotics, many of which are important in human medicine, appear side by side with compounds used almost exclusively in fisheries. In many instances, the introduction of a compound into fisheries use has followed closely after the initial use in human medicine. Perhaps in retrospect, it is surprising that there has not been any significant furore from the medical profession about, what could be perceived as misuse of pharmaceutical compounds.

The use of antimicrobial compounds in fisheries essentially started with the work of Gutsell (1946), who recognised the potential of sulphonamides for combating furunculosis. Indeed, it may be argued that the effectiveness of sulphonamides led to a temporary decline of interest in vaccine development. In fact, the eventual emergence of antibiotic-resistant strains of fish pathogenic bacteria led to renewed interest in vaccines. However during the years following the Second World War, sulphonamides appeared to be the mystical saviour of fish farming. Important developments included the work of Rucker et al. (1951), who identified sulphadiazine as an effective chemotherapeutant for BKD. This claim was subsequently refuted by Austin (1985). The next substantial improvement with sulphonamides resulted from potentiation, i.e. the use of mixtures of trimethoprim and sulphonamide. These have proved to be extremely useful for the treatment of furunculosis.

Following the introduction of sulphonamides, the range of antimicrobial compounds in aquaculture rapidly expanded to encompass chloramphenicol (Wold 1950), oxytetracycline (Snieszko and Griffin 1951), kanamycin (Conroy 1961), nifurprazine (Shiraki et al. 1970), oxolinic acid (Endo et al. 1973), sodium nifurstyrenate (Kashiwagi et al. 1977a, b), flumequine (Michel et al. 1980) and Baytril (Bragg and Todd 1988). Unfortunately, detailed comparative studies of the various antimicrobial compounds are rare; consequently it is often difficult to assess the value of one drug (= any medicinal compound; Sykes 1976) over another. Nevertheless, a pattern has emerged which points to the benefits of quinolines for controlling diseases caused by a wide range of Gram-negative bacteria

Whatever the range of compounds available, their effectiveness is a function of the method of administration to fish (and in the way in which it is carried out). We have listed seven basic approaches to the administration of antimicrobial compounds to fish (Table 14.2). These are the oral route via medicated food and bioencapsulation, bath, dip and flush treatments, injection and topical application. With the oral method, drugs are mixed with food, and then fed to the fish. Usually, the treatment regime leads to the administration of a unit weight of drug to a standard weight of fish per day for a pre-determined period. Examples of commonly used antimicrobial compounds have been included in Table 14.3. Fortunately, medi-

Table 14.2 Methods for application of antimicrobial compounds to fish

Method of application	Comments
Oral route (on food)	Need palatable components; minimal risk of environmental pollution
Bioencapsulation	Need palatable compounds; minimal risk of environmental pollution
Bath	Need for fairly lengthy exposure to compound, which must be soluble or capable of being adequately dispersed; problem of disposal of spent drug
Dip	Brief immersion in compound, which must be soluble or capable of being adequately dispersed; problem of disposal of dilute compound
Flush	Compound added to fish holding facility for brief exposure to fish; must be soluble or capable of being adequately dispersed; poses problem of environmental pollution
Injection	Feasible for only large and/or valuable fish; usually requires prior anaesthesia; slow; negligible risk of environmental pollution
Topical application	Feasible for treatment of ulcers on valuable/pet fish

cated food appears to be quite stable (McCracken and Fidgeon 1977). Moreover, this method is advantageous insofar as the quantities of compound fed to the fish are carefully controlled, and if sensible feeding regimes are adopted, only minimal quantities would reach the waterways. Three provisos exist, namely that:

- the fish are capable of feeding,
- the drug is palatable,
- the drug is capable of absorption intact through the gut.

An interesting approach has involved bioencapsulation, principally of quinolones (Duis et al. 1995). This theme was expanded with some excellent work which examined the potential for *Artemia* nauplii to serve as carriers to sulphamethoxazole and trimethoprim for the chemotherapy of diseased marine fish fry (Touraki et al. 1996). Both these compounds accumulated in the nauplii, with maximal levels recorded after 8 h. In a trial with sea bass larvae challenged with *V. anguillarum*, an improvement in survival followed use of the medicated nauplii (Touraki et al. 1996). Whether or not the fish will feed is largely a function of the nature and severity of the disease. Often in advanced cases of disease the fish will not feed. Therefore, it is vitally important that treatment begins as soon as possible after diagnosis has been established. The aquaculturist will need to seek specialist advice as soon as any abnormal behaviour or unhealthy condition is noted. This means that good management practices need to be routinely adopted.

Palatability of fisheries antimicrobial compounds receives only scant attention. Whereas it is accepted that little can be done to improve the palatability of the active ingredient, effort could be directed towards improving binders and bulking agents, which are commonly contained in proprietary mixes. Perhaps, consideration could be given to using chemical attractants.

Application by the water-borne route becomes necessary if the fish refuse to eat, and, therefore, would be unlikely to consume any medicated food. With these methods, the fish are exposed to solutions/suspensions of the drug for a pre-determined period. This may be only briefly, i.e. a few seconds duration (‘dip’), or for many

Table 14.3 Methods of administering some commonly used antimicrobial compounds to fish

Antimicrobial compound	Diseases controlled	Method(s) of administration
Acriflavine, neutral	Columnaris	5–10 mg/l in water for several hours to several days
Amoxicillin	Furunculosis, gill disease	60–80 mg/kg body weight of fish/day/10 days
Benzalkonium chloride	Fin rot, gill disease	1–2 mg/l of water for 1 h, 100 mg/l of water for 2 min
Chloramine B or T	Fin rot, gill disease mycobacteriosis	18–20 mg/l of water at pH 7.5–8.0, treat for 2–3 days
Enrofloxacin (=Baytril)	BKD, furunculosis	10 or 20 mg/kg body weight/day/for 10 days
Erythromycin	BKD, streptococcicosis	25–100 mg/kg of fish/day for 4–21 days
		20 mg of erythromycin/kg of broodstock as an injection
Florfenicol	Furunculosis, vibriosis	10 mg/kg body weight of fish/day for 10 days
Flumequine	Furunculosis, ERM, vibriosis	6 mg/kg of fish/day for 6 days
Furanace	Coldwater disease, columnaris, fin rot, gill disease, haemorrhagic septicaemia, vibriosis	(a) 2–4 mg/kg of fish/day for 3–5 days
		(b) 0.5–1 mg/l of water for 5–10 min, as a bath
Iodophors	Acinetobacter disease, BKD, flavobacteriosis, furunculosis, haemorrhagic septicaemia, mycobacteriosis	50–200 mg of available iodine/l of water for 10–15 min
Oxolinic acid	Columnaris, ERM, furunculosis, haemorrhagic septicaemia, vibriosis	(a) 10 mg/kg of fish/day for 10 days
		(b) 1 mg/l of water, as a bath for 24 h (recommended for columnaris)
Oxytetracycline	Acinetobacter disease, CE, coldwater disease, columnaris, edwardsiellosis, emphysematous putrefactive disease, ERM, enteric septicaemia, fin rot, furunculosis, gill disease, haemorrhagic septicaemia, redpest, salmonid blood spot, saltwater columnaris, streptococcicosis, ulcer disease	50–75 mg/kg of fish/day for 10 days (doses of 300 mg/kg of fish/day for indefinite periods are used to treat RTFS)
Potentiated sulphonamide	ERM, furunculosis, haemorrhagic septicaemia, vibriosis	30 mg/kg of fish/day for 10 days
Sodium nifurstyrenate	Streptococcicosis	50 mg/kg of fish/day for 3–5 days

minutes to several hours ('bath'). It is essential that the compounds are soluble or, if insoluble, are dispersed evenly in the water by means of surfactants or other dispersants (Austin et al. 1981). Also, seawater cations may well antagonise antimicrobial compounds in seawater (Barnes et al. 1995). One major drawback, however, concerns the disposal of the spent compound. Ideally, it should not be released into the aquatic environment, particularly if there are any abstraction points for potable water supply systems in the vicinity. Neglect of this point could lead to legal repercussions.

Flush treatments also involve the addition of drugs, albeit at high concentrations, to the water in stock-holding areas. After addition, the drug is flushed through the system by normal water flow. Flushing inevitably results in only a brief exposure to the inhibitory compound; therefore, quick acting agents are absolutely necessary. As before, the major problem is adequate disposal of the spent drug.

Injection of drug solutions is feasible for valuable stock, such as brood fish and ornamental/pet fish. However, the technique is slow and will undoubtedly require prior anaesthesia of the animals.

The topical application of antimicrobial compounds is worthy of consideration for valuable and/or pet fish. In the case of ulcers, we recommend that the animal should be gently removed from the water, and the antimicrobial compound (preferably as a powder) applied to the lesion, which is then sealed with a waterproof covering, e.g. with dental paste. The lesions tend to heal quickly, with only limited evidence of scarring.

Whatever the chosen method of application, drugs may be used for prevention, i.e. prophylaxis, or treatment, i.e. chemotherapy, of fish diseases. Certainly, it is comforting to note that there are treatments available for the majority of the bacterial fish pathogens. Providing that drugs are used prudently and correctly, they will continue to offer relief from the rigours of disease for the foreseeable future.

Preventing the Movement and/or Slaughtering of Infected Stock

Some diseases, e.g. BKD, ERM and furunculosis, are suspected to be spread through the movement of infected stock. Therefore, it is sensible to apply movement restrictions or even adopt a slaughter policy to diseased stock, as a means of disease control. This may prevent the spread of disease to both farmed and wild fish. Of course, the issue of movement restrictions is highly emotive among fish farmers. However, the procedure may be beneficial to the industry when viewed as a whole. Certainly, the concept of movement restrictions usually involves legislative machinery, of which the Diseases of Fish Act (1983) in Great Britain is a prime example. To work effectively, there is a requirement for both the efficient monitoring of all stock at risk to disease, and the dissemination of the information to all interested parties. However, we believe that in any allegedly democratic society where such measures are adopted, there should be adequate compensation to the fish farmer for loss of revenue.

Probiotics/Biological Control

There has been a great increase in the number of publications about probiotics over the last two decades. There is much evidence that members of the natural aquatic microflora, including components of the fish intestinal microflora (Fjellheim et al. 2007; Pérez-Sánchez et al. 2011), are effective at inhibiting fish pathogens, by competitive exclusion (e.g. Laloo et al. 2010) which may involve the production of antibiotics or low molecular weight inhibitors. Dopazo et al. (1988) discovered the presence in the marine environment of antibiotic-producing bacteria, which inhibited a range of bacterial fish pathogens, including *Aer. hydrophila*. These inhibitors produced low molecular weight (<10 kDa) anionic, thermolabile antibiotics. Subsequently, Chowdhury and Wakabayashi (1989); Austin and Billaud (1990) and Westerdahl et al. (1991) reported the presence of microbial inhibitors of *Fla. columnare, Ser. liquefaciens* and *V. anguillarum.* Smith and Davey (1993) identified a fluorescent pseudomonad which antagonised *Aer. salmonicida.* Apart from the lactic acid bacteria (e.g. Pérez-Sánchez et al. 2011), that are mostly linked with probiotic activity in terrestrial animals, aquaculture has utilised a wide range of Gram-positive and Gram-negative bacteria, yeast, microalgae and even bacteriophages. The use of Gram-negative bacteria from genera associated with fish disease, e.g. *Aeromonas* and *Vibrio,* is of concern because of the perceived risk of the introduction of virulence genes such as by horizontal gene transfer although this has never occurred – yet! "Good" bacteria have been described for the control of numerous diseases, and there is a tendency that the probiotic works faster than an oral vaccine (see Irianto and Austin 2002). The assumption that probiotics must be live preparations was dashed when it was demonstrated that formalised suspensions of cells were effective at controlling atypical *Aer. salmonicida* infection in goldfish (Irianto et al. 2003) and furunculosis in rainbow trout (Irianto and Austin 2003), when applied as feed additives. Furthermore, subcellular components, i.e. OMPs and ECPs, of probiotics were immuno-reactive with *V. harveyi* antiserum (Arijo et al. 2008) and were immunostimulatory and protected against challenge with *Aer. hydrophila* in rohu (Giri et al. 2015a, b). Along a similar theme, i.p. or i.p. injection of cell wall proteins, OMPs, LPS and whole cell proteins of two probiotics, *A. sobria* GC2 and *Bacillus subtilis* JB-1 protected rainbow trout against challenge with *Y. ruckeri* (Abbass et al. 2009). There is evidence that feeding probiotics enhances the resistance to disease when stocking density is high. Thus, Tapia-Paniagua *et al.* (2014) described the use of *She. putrefaciens* Pdp11 in increasing the stress tolerance of sole (*Solea senegalensis*) when farmed under high stocking levels. The fish which received Pdp11 better survived a natural outbreak of disease caused by *Vibrio* spp. and modulated the intestinal microflora, which was correlated with a high number of goblet cells (Tapia-Paniagua et al. 2014).

Apart from competitive exclusion, probiotics work by stimulation of the innate immune response (Irianto and Austin 2003; Kim and Austin 2006) in which case they could be considered as heterologous oral vaccines, and interference with adhesion to intestinal mucosal surfaces (Chabrillón et al. 2005). The beneficial effect of probiotics may be further enhanced by the use of prebiotic carbohydrates, notably

arabinoxylo-oligosaccharide, ß-glucan, glucose, inulin, oligo- fructose and xylo-oligosaccharide that promote the growth of the "good bacteria" (Rurangwa et al. 2009).

In addition to the organisms mentioned above, there is a report of the benefits for disease control of using the biopesticide, *Bacillus thuringiensis* (Meshram et al. 1998).

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Chapter 15
Conclusions

Abstract There are ongoing developments in the understanding of bacterial fish pathogens. New and emerging diseases are regularly recognised especially in aquaculture. Great emphasis is placed on better diagnoses, pathogenicity mechanisms, and disease control especially by immunoprophylaxis (vaccines, nonspecific immunostimulants, probiotics and natural plant products). There is an interaction between some pollutants and occurrence of fish diseases. Some fish pathogens may also cause disease of humans, including *Edw. tarda, Myc. fortuitum, Myc. marinum, Ph. damselae, Ps. fluorescens, Str. iniae* and *V. vulnificus.*

Keywords Zoonosis • Co-infection • Developments • Pollution • New diseases

Over the last three decades since the first edition of **Bacterial Fish Pathogens**, there has been a continual increase in the list of fish pathogens described in the scientific literature. Inevitably with improvements in taxonomy, there has been the description of new species, e.g. *Fr. noatunensis,* and a reclassification of others, e.g. *V. salmonicida* to *Ali. salmonicida.* With the widespread use of 16S rRNA sequencing these taxonomic changes should be more reliable than the previous reliance on phenotypic and serological methods. Over a similar timeframe, molecular methods have come to dominate the development of diagnostic procedures, vaccine development and the study of pathogenic mechanisms. Diagnostics has progressed from a culture-dependent mentality to a culture-independent approach, although a positive result for the presence of antigens or DNA does not necessarily infer that intact and viable bacterial cells are present in the host, and causing/contributing to the disease process. However, scientists still tend to study single isolates that differ from laboratory to laboratory, and make inter-laboratory comparisons difficult. These cultures may have been kept in the laboratory for many years, and have a dubious relationship to their counterparts in a diseased animal. Yet such cultures may be used to develop diagnostic procedures, and determine pathogenicity mechanisms. Another concern is that there has been a move away from "good" microbiology techniques as other scientific disciplines, e.g. molecular biology, occupy centre stage. Reading many publications, it is apparent that all too often scientists do not confirm the authenticity of the cultures with which they have been working. Consequently, it is often questionable whether a publication describing some biological aspect of a

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named pathogen is accurate. It would sometimes help if authors referred colloquially to a group, e.g. aeromonads, rather than a named species, such as *Aer. hydrophila.* The dominance of 16S rRNA sequencing should have helped, but evidence suggest that practitioners do not always appreciate the shortcomings of the technique. 16S rRNA sequencing is a powerful tool, but the data obtained have to be used in context, and not as the sole means of bacterial taxonomy.

The realization that not every microbial cell is capable of being cultured has come as a wakening call to science. Does this lack of culturability reflect an absence of suitable methods, damage/senescence of the bacterial cells, or the presence of unique microbial forms that are incapable of growing outside of the host? What exactly is the status of *Candidatus,* and will it ever be capable of *in vitro* growth? It is speculative how often the presence of unculturable bacteria may have been masked by secondary invaders or saprophytes that grow on whatever medium has been used for isolation. Then, there are situations, such as red mark syndrome and strawberry disease of rainbow trout, that are now considered to be infectious disease(s), but what is the causal agent? Is rickettsia really responsible or is there something else involved in the pathology? In the new Millennium, there are many more fundamental questions to be answered, and so interest in bacterial fish diseases is likely to increase rather than contract. So what developments are likely to be seen in the future:

The Recognition of New and Emerging Conditions

As aquaculture increases both in total production and in the range of species used, new diseases will continue to occur. In some cases, this will reflect the movement of micro-organisms from one host to another. This is likely to happen when a new fish species is introduced into an area for farming, and there is an exchange of organisms. It may be that the new species lacks resistance, and therefore infection develops. Also in large monoculture situations, it is easier for a potential pathogen to enter a weakened host, initiate a disease cycle, and spread to adjacent animals. New diseases will require research to develop effective control and diagnostic procedures.

Co-Infections

There is a tendency for scientists to want to associate diseases with single species of pathogens (Fig. 15.1). The possibility of two or more organisms working together or sequentially may seem like heresy. Yet, the current approaches to diagnoses would be unlikely to spot species succession, and co-infections might be mistaken for evidence of contamination. With species succession the situation could arise whereby one organism instigates an infection and others develop/exacerbates the condition. There have been personal observations of *Aer. salmonicida* instigating

Fig. 15.1 Pronounced bloat in goldfish, which revealed the presence of multiple bacterial pathogens (Photograph courtesy of Dr. A. Newaj-Fyzul)

ulceration in cyprinids, after which motile aeromonads exacerbate and may lead to the development of a systemic disease during which time the initial pathogen has effectively disappeared. Certainly, co-infection which may be attributed to more than one pathogen is likely to have been underreported. Nevertheless, there is evidence for the presence of two or more "pathogens" within some diseased fish (Loch et al. 2012). Diseased Chinook salmon (*Oncorhynchus tshawytscha*), which were returning to spawn in tributaries associated with Lake Michigan, USA, were found to be populated by multiple bacterial pathogens (Loch et al. 2012). Specific examples:

- Uncultured obligately intracellular chlamydia-like organisms associated with epitheliocystis may be associated with co-infections. Specifically, '*Candidatus* Piscichlamydia salmonis' and '*Candidatus* Clavochlamydia salmonicola' have been attributed with co-infections both in the same population and within the same individuals of wild brown trout in Switzerland (Schmidt-Posthaus et al. 2012).
- Sometimes, a second organism may be a secondary invader, such as the case with *Aer. hydrophila*, which was found in fish from lakes in Saskatchewan, Canada with columnaris (Scott and Bollinger 2014).
- Possibly, the presence of one pathogen/parasite renders the host more susceptible to another as was considered in the case of parasitism by *Ichthyophthirius multifiliis* leading to greater susceptibility to and invasion of hybrid tilapia with *Fla. columnare* (Xu et al. 2014).
- Dual infection of *Ren. salmoninarum* and *Nanophyetus salminicola* Chapin in wild juvenile Chinook salmon has been recorded (Sandell et al. 2015).
- *Neoparamoeba perurans*, which causes amoebic gill disease, and *Y. ruckeri* have been indicated to co-infect Atlantic salmon in Tasmania, Australia (Valdenegro-Vega et al. 2015).

- Loach (*Misgurnus anguillicaudatus*), which were cultured in China, were found to be harbouring a new disease condition that was associated with two bacterial taxa, i.e. *Shewanella putrefaciens* and *V. anguillarum* (Qin et al. 2014).
- *Moritella viscosa* and *Ali. wodanis* occur in salmon with winter ulcer disease (Hjerde et al. 2015), with the latter – in a cell culture model – adhering to but not invading fish cells (Karlsen et al. 2014). Culture supernatant caused the cultured cells to undergo marked changes, specifically vacuolation, retraction, rounding up and detachment from the culture vessel surface, and the actin filaments of the cytoskeleton became re-arranged. The interpretation was that *Ali. wodanis* secreted toxins, and influenced the development of infection by *Moritella viscosa* (Karlsen et al. 2014).
- In Thailand, 4.8 % of cases of streptococcosis in Nile and red tilapia during 2003–2012 were attributed to mixed infections (Jantrakajorn et al. 2014).
- Co-infection of *Edw. tarda* with aquabirnavirus led to higher mortalities in Japanese flounder (Pakingking et al. 2003).
- *Ps. anguilliseptica* was linked with *Delftia acidovorans* in European eels (Andree et al. 2013).
- The presence of the protozoan *Ichthyophthirius multifiliis* led to higher mortalities (80 % mortalities compared to 22 % when the protozoan was absent) when channel catfish were infected with *Aer. hydrophila,* with gill, kidney, liver, skin and spleen revealing higher bacterial loading than non-parasitized fish (Xu et al. 2012a, b).
- Cyprinid herpesvirus 2 has been found in association with *Aer. sobria* in crucian carp in Italy (Fichi et al. 2013).
- Co-infection of bacteria, which were equated with *Aer. veronii, Fla. columnare Plesiomonas shigelloides Str. agalactiae* and *V. cholerae*, and iridovirus have been found in Nile tilapia farms (Dong et al. 2015b). Experimental infection confirmed the pathogenicity of single cultures of *Aer. veronii* and less so *Fle. columnare* and *Str. agalactiae* but not so *Plesiomonas shigelloides*. The fish challenged with *Aer. veronii* or *Fle. columnare* developed clinical signs of disease reminiscent of the naturally infected fish. The authors suggested that *Aer. veronii* and *Fle. columnare* were the principle pathogens co-responsible for the disease observed on the Nile tilapia farms; the other bacteria were supposed to be opportunists (Dong et al. 2015a, b).
- Concurrent infections with *Edw. ictaluri* and *Fla. columnare* have been documented in striped catfish (*Pangasianodon hypophthalmus*) in Thailand with identification achieved by phenotyping and sequencing the 16S rDNA gene (Dong et al. 2015a, b). Experimental challenges by injection or immersion mimicked the natural disease, and revealed that in combination both bacteria achieved high mortalities. By immersion, 2.6×10^6 CFU of *Edw. ictaluri*/ml and 1.0×10^4 CFU of *Fla. columnare*/ml led to 96.7 % mortalities among striped catfish compared to 80 % and 3.3 % mortalities with the same doses of *Edw. ictaluri* and *Fla. columnare*, respectively, administered separately (Dong et al. 2015a, b).

We are convinced that more examples will be forthcoming as scientists realise and look for co-infections during diagnostic investigations.

Taxonomy and Diagnosis

With the current focus on biodiversity, there has been a resurgence of interest in taxonomy although the rush to name new taxa based around the study of single isolates is of questionable value to science. Nevertheless, the current reliance on molecular methods, and notably sequencing of the 16S rRNA gene is likely to continue; the approach has certainly improved the standard of disease diagnoses with bacterial identification losing some of its subjectivity. With the development of methods such as LAMP, molecular biology has become more user friendly, and may now be undertaken in routine rather than specialized laboratories. The availability of more rapid, reliable and cheaper molecular techniques will further improve diagnostic potential. In the meantime, serology offers rapid, field-based diagnostic systems. However, there remains the overriding issue about what a positive result means for an ultrasensitive test especially in the absence of overt disease signs. If a test detects background levels of a pathogen – an example would be *Aer. hydrophila* which occurs naturally in the eutrophic freshwater environment and which could easily contaminate pathological specimens – then the value for disease diagnosis is limited.

Isolation and Selective Isolation of Pathogens

With the advance of the molecular era with its culture-independent approaches, it is pertinent to enquire if culturing will continue to have a role in fish disease work. We argue that pure cultures logged in established culture collections provide an invaluable reference tool, and are invaluable for biotechnology application. However, not all organisms will grow *in vitro; Candidatus* provides a current challenge. Where culturing is a prelude to identification then clearly suitable culture systems are needed, but there are still only a few selective isolation procedures for bacterial fish pathogens.

Ecology (Epizootiology)

There has been a trend away from studying the ecology of fish pathogens, and in particular determining their role/location in the aquatic environment. Work on the NCBV state has largely ceased. This is a pity but undoubtedly reflects the lack of research funding opportunities.

Pathogenicity

There has been continual interest in the determination of pathogenicity mechanisms with current focus placed on molecular approaches and the determination of relevant, virulence genes. For the future, the data from bacterial genome sequencing may be invaluable, especially as more and more taxa are examined. The reliance on the study of single cultures continues, data from which are difficult to equate with an understanding of pathogenicity at the species level. Inter-cell communication by quorum sensing signal molecules (= acylated homoserine lactones (AHL)) in the regulation of some virulence factors is fascinating, with work revealing that AHLs are produced by some Gram-negative bacterial fish pathogens, notably *Aer. hydrophila, Aer. salmonicida, Ali. salmonicida, V. splendidus, V. vulnificus* and *Y. ruckeri* (Bruhn et al. 2005).

Control Measures

The development of effective disease control strategies is one aspect of bacterial fish disease research that has resulted in substantial progress from new approaches to vaccination [including genetic manipulation techniques], to the use of probiotics, prebiotics, nonspecific immunostimulants and plant products. It is noteworthy that much of this work is being carried out in less developed rather than western countries. These newer approaches have been matched by a downturn of interest in the use of antimicrobial compounds insofar as there is increasing concern about the development and spread of resistance and thus a reduced efficacy against human pathogens, and tissue residues. In many countries, there is evidence for a curb on the use of antimicrobial compounds in all but human applications. However, it is accepted that elsewhere antibiotics are used extensively in aquaculture, but beware of tissue residues if the product is destined for export. In the years ahead, it is to be envisaged that there will be ever-tighter regulations on the use of antimicrobial compounds in aquaculture.

The Effects of Environmental Stress Including Physical and Chemical Pollution

There is increasing concern about the possible role of pollution in disease, particularly of wild fish stocks (see also Pippy and Hare 1969; Mahoney et al. 1973; Robohm et al. 1979). As this is a politically emotive issue, and particularly as the impact of climate change is in the public arena, there is likely to be an increase in monies available and, thus, a stimulation of research interest. At present, there is considerable confusion over the precise role of pollution and fish health (Bucke

1991, 1997). Nevertheless, there are good data that long-term exposure to pollutants has adversely affected the health of fish, especially in the North Sea and Great Lakes. However, mortalities among fish populations do not necessarily imply disease. Furthermore, disease may develop long after the pollutant has been effectively removed from the aquatic environment. Much of the work attempting to correlate fish disease with aquatic pollution has resulted from surveys, many of which have been carried out in the North Sea (e.g. Dethlefsen and Watermann 1980; Dethlefsen et al. 1987, 2000; McVicar et al. 1988; Vethaak and ap Rheinallt 1992). Briefly, fish are caught with nets, and the relative incidence of disease determined. One conclusion from these surveys is that larger numbers of diseased fish occur generally in the polluted compared to clean/unpolluted locations (Dethlefsen et al. 2000). However, the distinction between polluted and clean sites is imprecise. Therefore, there would be some uncertainty as to what comprises a truly polluted or clean site. Moreover, it is uncertain from surveys how long fish might have been in a polluted environment prior to capture. Thus, the effects of fish migration on the incidence of disease needs to be considered (Vethaak et al. 1992; Bucke et al. 1992; Jacquez et al. 1994).

Pollution has been associated with some bacterial diseases, namely fin and tail rot (Vethaak 1992; Vethaak et al. 1996), gill disease/hyperplasia (Kirk and Lewis 1993) and skin disease/ulceration (Vethaak 1992; Vethaak and Jol 1996). The trigger has been attributed to stressors including contaminated diets (Landsberg 1995), heavy metals e.g. chromium (Rødsaether et al. 1977; Prabakaran et al. 2006), hydrocarbons (Khan 1987; Song et al. 2008), nitrogenous compounds, i.e. ammonia (Kirk and Lewis 1993) and nitrites (Hanson and Grizzle 1985), pesticides (e.g. Voigt 1994), polychlorinated biphenyls (Eckman et al. 2004), sewage (e.g. Austin and Stobie 1992), organic pollutants (Grawinski and Antychowicz 2001) and unspecified pollutants (e.g. Vethaak and Jol 1996). In one example, organic pollution has been attributed to the high occurrence of *Ser. plymuthica* infections in salmonid farms in Poland since 1996 (Grawinski and Antychowicz 2001). Generally, the reasons for the association between pollution and disease need to be better researched. However, proof of correlation between the occurrence of specific pollutants and disease has seldom been documented. Surveys, which have pointed to a correlation between pollution and disease, have generally not considered the nature or concentration of the pollutant(s).

An association has been made between fish diseases and unknown components of sewage dumping (Siddall et al. 1994). For example in a survey of 16 sites in the Dutch Wadden Sea, a higher incidence of skin ulcers and fin rot was noted in fish caught near fresh water drainage sluices than elsewhere (Vethaak 1992). Pollution by domestic sewage, i.e. leakage from a septic tank, was attributed to a new skin disease, which was characterised by the presence of extensive skin lesions and muscle necrosis, in rainbow trout (otherwise infected with ERM for which there might also be a link with sewage sludge; Dudley et al. 1980) in Scotland during 1992 (Austin and Stobie 1992). Interestingly, the skin lesions – but not ERM – declined substantially after the leaking septic tank was repaired.

There is accumulating evidence that contamination leads to a weakening of the immune state, i.e. immunosuppression (Klesius and Shoemaker 2003). One example

describes the increased susceptibility of chinook salmon from a contaminated estuary to *V. anguillarum* (Arkoosh et al. 1998). Undoubtedly, the future will bring further examples.

Zoonoses

It should not be ignored that some fish pathogens may also on occasion cause disease in humans. Fortunately, the incidences are low, but culprits include: *Aer. hydrophila* (causing diarrhoea and septicaemias), *Edw. tarda* (diarrhoea), *Myc. fortuitum* (mycobacteriosis; fish tank granuloma), *Myc. marinum* (mycobacteriosis; fish tank granuloma), *Ph. damselae* subsp. *damselae* (necrotising fasciitis, bacteraemia), *Ps. fluorescens* (wound infections), *Str. iniae* ("mad fish disease") and *V. vulnificus* (wound infections) (e.g. Rivas et al. 2013a, b; Gauthier 2015). Even aquarium water is not without risk as *Aer. schubertii, Aer. veronii, Aer. hydrophila, Coxiella burnetii, Legionella birminghamensis, Legionella pneumophila, Plesiomonas shigelloides V. cholerae, V. mimicus* and *V. vulnificus* were detected in Rhode Island, USA in water containing goldfish (*Carassius auratus*) and Chinese algae eaters (*Gyrinocheilus aymonieri*) by sequencing of the 16S rRNA gene (Smith et al. 2012).

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Index

A
Acetylcholinesterase, 177, 271, 504
Acholeplasma, 619
Achromobacter, 248
Acinetobacter
A. johnsonii, 14, 613
A. lwoffii, 14, 613
Acinetobacter disease, 14, 703
Actinomyces, 21, 92, 675
A. pyogenes, 92
Adhesin, 175, 199, 270, 271, 374, 409
ADP-ribosyltransferase toxin, 271
Aerococcus viridans, 7, 106–107, 644, 675, 678
Aerolysin, 177, 617
Aeromonas
A. allosaccharophila, 9, 162–164, 172, 646, 681
A. aquariorum, 162, 165
A. bestiarum, 9, 161, 164, 283
A. caviae, 9, 161, 165, 172, 644, 646, 681
A. dhakensis, 9, 161, 165–167, 646, 681
A. enteropelogenes, 161
A. formicans, 168
A. hydrophila, 9, 29, 45, 95, 161, 164–193, 218, 220, 222, 229, 231, 232, 235–239, 241, 248, 270, 293, 297, 344, 417, 443, 475, 477, 479, 539, 542, 571, 620, 646, 665, 672, 673, 681, 699, 700, 705, 712–716, 718
A. hydrophila subsp. *dhakensis,* 165
A. jandaei, 9, 161, 193–194, 681
A. liquefaciens, 9, 168, 479
A. media, 161, 220, 229
A. piscicola, 9, 161, 194, 646, 681
A. punctata, 9
A. salmonicida, 9, 215–298
A. salmonicida subsp. *achromogenes,* 9, 227–229, 231, 232, 241, 273, 294, 681
A. salmonicida subsp. *masoucida,* 218, 219, 230–232, 235, 237, 241, 681
A. salmonicida subsp. *nova,* 232
A. salmonicida subsp. *pectinolytica,* 225
A. salmonicida subsp. *salmonicida,* 216, 221, 222, 224, 226, 229, 231, 232, 234, 236, 238, 241, 258, 263, 269, 273, 274, 279, 283, 294, 681
A. salmonicida subsp. *smithia,* 220, 232, 681
A. schubertii, 9, 194–196, 644, 681, 718
A. sobria, 9, 46, 62, 161, 172, 196–198, 644, 646, 681, 705, 714
A. veronii, 198–199
A. veronii biovar sobria, 9, 161, 198–199, 644
A. veronii biovar veronii, 10, 200
A-layer, 222, 229, 265–269, 271, 276, 277, 279, 280, 283, 291, 292, 296, 667
Alcaligenes, 479
A. cupidus, 610
Aliivibrio
A. fischeri, 16, 513, 526, 646
A. logei, 16, 513–514, 519, 644, 684
A. salmonicida, 16, 514–518
Allicin, 187
Alteromonas putrefaciens, 606
Amoebic gill disease, 713

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Antimicrobial compound, 27, 31, 46, 53, 63, 66, 105, 108, 110, 112, 114, 125, 127, 132, 137, 140, 141, 167, 192–193, 257, 295–298, 325, 332, 347, 353, 355, 359, 361, 363–365, 375, 379, 406, 408, 418, 420, 421, 434, 445–446, 471, 482, 487, 492, 512–513, 518, 524, 529, 544, 554, 561, 565–567, 608, 613, 614, 618, 622, 628, 629, 694, 701–704, 716
API 50CH, 673
API 20E, 166, 172, 199, 324, 354, 361, 407, 437, 502, 507, 527, 529, 546, 558, 565, 606, 673, 674
API 20NE, 199, 354, 493, 673, 674
API ZYM, 93, 135, 405, 424, 447, 673–677, 680, 687
Approved Lists of Bacterial Names, 18, 232, 336, 397, 399, 507
Aquabirnavirus, 59, 339, 714
Aquaspirillum, 14, 620, 646
Arcobacter cryaerophilus, 10, 607–608, 644, 681
aroA, 182, 283, 293, 331, 343, 371, 376, 432
Arthrobacter
A. davidanieli, 104
A. protophormiae, 96
Ascites disease, 17, 545
Attenuated vaccine, 51, 331, 343, 345, 421, 432, 433, 539, 698
Atypical isolate, 222, 226, 229–234, 236, 240, 241, 245, 263, 266, 273, 279, 282, 292, 294

B

Bacillary necrosis, 7, 107, 108, 326
Bacillary necrosis of *Pangasius,* 326
Bacillus
B. anthracis, 110
B. cereus, 7, 108, 110, 675
B. columnaris, 411
B. devorans, 225
B. fumarioli, 108
B. mycoides, 7, 109–110, 644, 678
B. salmonicida, 225
B. subtilis, 7, 62, 110, 191, 192, 347, 443, 571, 705
B. thuringiensis, 96, 554, 706
B. truttae, 225
Bacillus Calmette Guérin, 698
Bacterial haemorrhagic ascites, 15, 489, 493
Bacterial kidney disease (BKD), 4, 7, 29, 84–89, 93–105, 112, 359, 701, 703, 704
Bacteriocin-like substances (BLIS), 551
Bacteriophage, 1, 44, 46, 50, 174, 193, 231, 235, 236, 238, 239, 246, 268, 295, 329, 338, 370, 372, 412, 419, 425, 431, 433–434, 491, 534, 544, 550, 625, 705
Bacteriophage typing, 50, 236, 238, 370
Bacteriovorax, 199
Bacterium
B. anguillarum, 524
B. salmonica, 225
B. salmonicida, 225, 226, 235, 255
B. truttae, 225
Bacteroides, 21
Bankruptcy disease, 22
B-cell, 283, 332
Bifidobacterium thermophilus, 543
Bioencapsulation, 540
Biofilm, 181, 191, 412, 416, 428, 516, 522, 523, 532
Biological control, 46, 53, 199, 491, 694, 705–706
Biopesticide, 706
Bivalent vaccine, 60, 512, 552, 570, 571
Black patch necrosis, 13, 442
BLIS. *See* Bacteriocin-like substances (BLIS)
Bloody eye, 634
Boil disease, 524
Botulinum toxin, 24
Botulism, 5, 22–24
BOX-PCR, 336
Branchionecrosis, 7, 108, 110
Brevibacterium, 89, 92, 675
Brown ring disease, 566
Brucella abortus, 350

C

Candidatus Arthromitus, 18, 630–631
Candidatus Branchiomonas cysticola, 18, 631
Candidatus Clavochlamydia salmonicola, 18, 631
Candidatus Piscichlamydia salmonis, 18, 630–632, 713
Candidatus Renichlamydia lutjani, 18, 632
Capsule, 44, 45, 51, 58, 59, 66, 174, 176, 270, 415, 430, 510, 534, 570
Carnbacterium mobile, 40
Carnobacterium divergens, 28, 40
Carnobacterium maltaromaticum-like organism, 27, 28, 644
Carnobacterium piscicola, 5, 28–31, 40, 95, 96, 646, 678
Carp erythrodermatitis (CE), 9, 217, 218
Carrier, 60, 95, 104, 221, 242, 246, 254–262, 328, 329, 359, 371, 372, 423, 702

Catenabacterium, 4, 5, 25, 26
Cecropin, 268
Cell mediated immunity, 294, 343
Cellulomonas, 92
Chemotaxis, 175, 532
Chlamydiales, 18, 632
Chlamydia psittaci, 95
Cholera, 409
Chondrococcus columnaris, 411
Chromobacterium, 222, 249, 622
  C. violaceum, 622
Chryseobacterium spp., 401, 645
  C. aahli, 12, 401–402, 681
  C. arothri, 400
  C. balustinum, 12, 398, 402–403, 681
  C. indologenes, 12, 403–404
  C. oncorhynchi, 400
  C. piscicola, 12, 404–405, 681
  C. scophthalmum, 12, 400, 405–406, 681
  C. shigense, 400
  C. tructae, 400
  C. viscerum, 400
Citrobacter, 369
  C. freundii, 10, 323–325
Clostridium
  C. botulinum, 5, 21–25, 643, 646, 678
  C. butyricum, 697
Coho salmon syndrome, 15, 624
Co-infection, 56, 59, 339, 712–715
Coldwater disease, 12, 399, 425, 429, 431, 703
Coldwater vibriosis, 16, 514, 517
Columnaris, 12, 13, 397, 400, 410–419, 664, 695, 703, 713
Complement, 44, 53, 66, 180, 182, 184, 189–191, 237, 265, 268, 270, 278, 295, 375, 445, 510, 523, 559, 668, 669, 697
Contaminated diet, 37, 717
Corynebacterial kidney disease, 7, 84
Corynebacterium, 7, 89, 92, 110–112
  C. aquaticum, 7, 95, 96, 110–112, 644, 678
  C. pyogenes, 92, 675
  C. salmoninus, 89
Coryneforms, 112
Coxiella burnetii, 625, 718
C-reactive protein, 295
Cutaneous ulcerative disease, 218
Cyprinid herpesvirus 2, 714
Cytophaga
  C. aquatilis, 12, 400, 419–420
  C. columnaris, 12, 410–419
  C. hydatis, 419
  C. johnsonae, 12, 400, 420–421
  C. marina, 398, 442–446
  C. psychrophila, 12, 399, 422–434
  C. rosea, 13, 400
Cytotoxicity, 178, 197, 280, 341, 471, 504, 627

D
Dee disease, 7, 84, 92, 101
Defensin, 268
Delayed hypersensitivity, 124
Deleya, 14, 479
  D. cupida, 610–611
Delftia acidivorans, 608, 644, 646
Dietary supplement, 45–46, 52–53, 61–63, 66, 104–105, 183–193, 294–295, 332, 345–347, 378, 418, 433, 512, 517–518, 523–524, 543, 553–554, 559, 694, 696–697
Digestarom, 332
Disease
  acute, 4, 217, 516
  cause (abiotic), 3
  cause (biotic), 3
  chronic, 4
  definition, 3
  dictionary definition, 3
  resistant, 103, 179, 281–282, 298, 331, 342, 432, 517, 538, 696
Diseases of Fish Act, 410, 704
Disinfection, 24–25, 105, 125, 295, 296, 325, 335, 418, 696
DNA probe, 241, 426, 508
DNA vaccine, 344, 433, 523, 542, 552, 698
Dormancy, 100, 250, 252
Dot blot, 96, 98, 503, 508, 536

E
Edwardsiella
  E. anguillarum, 10, 325, 681
  E. anguillimortifera, 11, 336
  E. ictaluri, 326–332, 334, 338, 340, 413, 417, 494, 644, 681, 699, 700, 714
  E. piscicida, 333–334
  E. tarda, 334–347
Edwardsiellosis, 11, 334, 339, 343, 703
Ehrlichia, 625
Emerging disease, 514
Emphysematous putrefactive disease, 11, 334, 703
Endotoxin, 27, 31, 277, 536, 613

Enteric redmouth (ERM), 12, 103, 366, 367, 369–372, 376–379, 524, 620, 669, 697, 698, 703, 704, 717
Enteric septicaemia of catfish, 10, 326
Enterobacter agglomerans, 11, 353
Enterobacter cloacae, 11, 347–348, 646, 682
Enterobacter liquefaciens, 369
Enterobactin, 534, 535
Enterococcus
E. avium, 40
E. durans, 40
E. faecalis, 35, 646, 674, 675, 700
E. faecalis subsp. *liquefaciens,* 38–39
E. faecium, 34, 35, 347, 675
E. hirae, 42
E. seriolicida, 6, 41–46
Enterotoxigenicity, 178–179
Enterovibrio nigricans, 501
Enzootic, definition, 696
Epinecidin-1, 571
Epitheliocyst, 632
Epitheliocystis, 18, 630–632, 713
Epizootic, definition, 3
Epizootic ulcerative syndrome, 9, 14, 198, 620
ERM. *See* Enteric redmouth (ERM)
Erwinia, 323
E. herbicola, 353
Erysipelothrix rhusiopathiae, 7, 83–84, 644, 678
Escherichia coli, 45, 52, 60, 182, 249, 267, 297, 344, 411, 424, 433, 443, 537, 540, 542, 550, 552, 559
Escherichia vulneris, 11, 348–349, 646, 682
Eubacterial meningitis, 5
Eubacterium
E. tarantellae, 5, 21, 25–27, 643, 678
E. tarantellus, 25, 26
Exotoxin, 31, 55, 100, 176, 179, 274, 330, 446, 481, 536, 613
External disease signs, 39, 117
Extracellular product (ECP), 176–179, 271–279, 329

F

Fish rose, 256
Fish tank granuloma, 718
Fish tuberculosis, 8, 115
Flavobacteriosis, 12, 399, 401, 703
Flavobacterium
F. araucananum, 399, 435
F. balustinum, 12, 398, 399, 402–403
F. branchiarum, 399
F. branchiicola, 399
F. branchiophilum, 12, 399, 401, 408–409, 413, 436, 444, 645, 682
F. chilense, 399
F. collinsii, 399
F. columnare, 12, 95, 107, 328, 401, 409–419, 444, 645, 648, 651–652, 673, 682, 700, 705, 713, 714
F. hydatis, 12, 400, 401, 419–420, 424, 645, 682
F. johnsoniae, 12, 401, 416, 420–421, 645, 682
F. oncorhynchi, 12, 421–422, 682
F. piscicida, 399, 434, 645
F. psychrophilum, 12, 399, 401, 409, 422–434, 444, 645, 650, 652, 672, 682
F. scophthalmum, 12, 400, 405–406
F. spartansii, 434–435, 645, 682
F. succinicans, 12, 399, 436
Flectobacillus roseus, 12, 436–437, 645
Flexibacter
F. aurantiacus, 425
F. columnaris, 411
F. elegans, 419
F. marinus, 398, 443
F. maritimus, 13, 398, 439–446, 482, 645
F. ovolyticus, 13, 398, 446–447, 645
F. psychrophilum, 422–434
Flounder infectious necrotizing enteritis, 547
Fourier transform infrared spectroscopy, 371
Francisella
F. asiatica, 469
F. noatunensis, 2, 13, 467–470, 682, 711
F. noatunensis subsp. *orientalis,* 470–471, 682
F. philomiragia, 13, 468, 469
Furunculosis, 4, 9, 216–221, 223, 225, 246, 247, 253–264, 267, 271, 272, 274–276, 278–281, 291–297, 481, 509, 524, 539, 669, 698, 701, 703–705
Fusobacterium, 21

G

Gas bubble trauma, 85
Genetic resistant fish, 538, 694
Genomovar, 412, 417
Goldfish ulcer disease, 218, 223, 293, 296
Granulomatous dermatitis, 216
Green fluorescent protein (GFP), 342
Green liver syndrome, 41
Gross clinical signs of disease, 664–666

H
Haemophilus influenzae, 239
Haemophilus piscium, 4, 9, 231, 239–298, 479, 507
Haemorrhagic septicaemia, 7, 9, 11, 31, 33, 83, 165, 167, 168, 197, 198, 218, 349, 363, 514, 524, 556, 703
Hafnia alvei, 11, 349–351
Hagerman redmouth disease, 366
Hahella chejuensis, 14, 608–609, 646
Halomonas cupida, 610, 611, 644, 682
Heat shock protein, 95, 174, 551
Hitra disease, 16, 514, 516, 517, 524, 669, 697
Housekeeping gene, 482
Husbandry, 295, 694, 695
Hyperplasia, 27, 397, 398, 405, 407, 634, 717

I
Ichthyophthiriasis, 56
Ichthyophthirius multifiliis, 61, 168, 329, 713, 714
Ichthyotoxin, 271
IgM, 180, 344, 376
IgY, 183, 291
Immunostimulant
  Baypamum, 294
  chitin, 07
  glucan, 295
  lactoferrin, 543
  levamisole, 332
  LPS, 697
  lysozyme, 61, 184–192, 518
  mycobacteria, 697
  oligosaccharide, 192, 697
  prolactin, 697
  synthetic peptide, 294, 697
Infectious dropsy, 9, 198
Infectious gastro-enteritis, 547
Infectious pancreatic necrosis, 516
Internal abnormality, 547, 663–666
Intra-abdominal adhesion, 86, 282, 618
Intraperitoneal chamber, 265
Intubation
  anal, 376, 377, 541, 551, 627, 700
  oral, 539, 541, 697, 699
Intussusception, 24
Iodobacter fluviatile, 622
Iridovirus, 672, 714
IROMP, 279, 283, 510
Iron sequestering mechanism, 510
Isolation technique, 88

J
Janthinobacterium, 14, 222, 620–622, 646
Janthinobacterium lividum, 14, 620–622

K
K88 fimbria, 267
Klebsiella pneumoniae, 11, 351–352, 646, 682
Koch's postulates, 4, 87, 101, 135

L
Lactobacillosis, 5, 6, 47
Lactobacillus
  L. acidophilus, 30, 192
  L. alimentarius, 30
  L. crispatus, 30
  L. homohiochi, 30
  L. jensenii, 30
  L. piscicola, 29
  L. salivarius, 30
  L. yamanashiensis, 30
Lactococcosis, 6, 45, 46, 53
Lactococcus
  L. *garvieae*, 6, 41–46, 53, 54, 58, 60, 181, 644, 645, 650, 672, 673, 678, 700
  L. lactis, 34, 43, 62, 64, 191
  L. lactis subsp. *lactis,* 6, 46–48, 62, 543, 645, 646, 678
  L. piscium, 6, 47–48, 644, 678
LAMP, 43, 50, 58, 96, 129, 131, 338, 371, 413, 427, 521, 672, 715
Lancefield group C, 36
Legionella pneumophila, 718
Leptospirosis, 695
L-form, 223, 224, 253–255, 283, 618, 686
Listeria, 89, 92
  L. denitrificans, 676
Listonella, 502, 527
  L. anguillarum, 17, 527
Live vaccine, 61, 104, 181, 283, 331, 417, 433, 539, 540, 552, 698

M
Mad fish disease, 718
Magainin, 268
Major histocompatibility complex, 180, 551
Meningoencephalitis, 5, 6, 27, 48, 49, 85
Metallo-caseinase, 273
Metalloendopeptidase, 271, 273, 291, 374
Metalloprotease, 103, 174, 177, 271, 273, 274, 277, 373, 374, 430, 537, 542

Microbacterium paraoxydans, 7, 113
Micrococcosis, 7, 114
Micrococcus, 92
M. luteus, 7, 96, 113–114, 646, 676, 678
Micro-encapsulation, 105, 282, 293, 432, 541, 700
Molecular techniques, 58, 67, 95–96, 240–241, 282, 371, 667, 671–673, 715
Montanide, 34, 45, 181, 292, 376, 432
Moraxella
M. atlantae, 612
M. osloensis, 612
Moritella
M. marina, 14, 615, 616, 644, 682
M. viscosa, 14, 518, 616–618, 683, 714
Morphotype, 412, 424, 431
Motile aeromonas septicaemia, 9, 161, 168, 193
Movement restriction, 704
Multiplex PCR, 43, 58, 241, 427, 503, 508, 626, 672
Mycobacteriosis, 8, 115–118, 121, 122, 124, 125, 698, 703, 718
Mycobacterium
M. abscessus, 8, 115, 117–120, 678
M. anabanti, 8, 118, 119
M. arupense, 118
M. avium, 8, 116, 119, 120, 124
M. bovis, 132, 698
M. chelonei, 8, 116, 118, 120, 121, 123, 124
M. chelonei subsp. *abscessus,* 121
M. chelonei subsp. *piscarium,* 8, 118, 120, 121, 124, 678
M. chesapeaki, 118
M. flavescens, 117
M. fortuitum, 8, 115–119, 124, 676, 678, 718
M. gordonae, 8, 116–119, 121, 124, 645
M. interjectum, 117
M. marinum, 8, 116–119, 122–125, 676, 678, 718
M. montefiorense, 8, 117, 118, 121, 645, 678
M. neoaurum, 8, 117, 119, 121, 644, 678
M. nonchromogenicum, 117
M. peregrinum, 117
M. piscium, 8, 118, 119
M. platypoecilus, 8, 118, 119
M. porcinum, 118
M. poriferae, 8, 118
M. pseudoshottsii, 8, 117, 122–124, 678
M. ranae, 8, 118
M. salmoniphilum, 8, 117–124
M. scrofulaceum, 8, 117, 118
M. septicum, 117
M. shottsii, 8, 117, 118, 122–124, 678
M. simiae, 8, 118
M. smegmatis, 8, 119, 124, 676
M. szulgai, 117, 118
M. triplex, 117, 118, 121
M. ulcerans, 8, 122
Mycolactone F, 124
Mycoplasma mobile, 14, 618–619, 645, 683
Myoviridae, 193, 295, 491
Myovirus, 551
Myroides odoratimimus, 13, 437–438, 645
Myxobacterium, 4, 397, 654–655
Myxococcus, 397, 619
M. piscicola, 14, 619, 645, 683

N
Nanoinjection, 429
NCBV, 182, 251–253, 255, 715
Necromonas
N. achromogenes, 235
N. salmonicida, 235
Necrotising fasciitis, 718
Neisseria, 612
Neoparamoeba perurans, 713
Nephrocalcinosis, 263
Nested PCR, 50, 58, 67, 96, 97, 241, 371, 426–428, 444, 631
Nisin Z, 62
Nocardia
N. asteroides, 8, 126, 127, 130–131, 676, 678
N. caviae, 129–131
N. fluminea, 132
N. kampachi, 126, 129–131
N. salmonicida, 8, 127–131, 645, 679
N. seriolae, 8, 123, 125, 126, 129–132, 672, 673, 678
N. soli, 132
N. uniformis, 132
Nocardiosis, 8, 125, 126

O
Ocular lesion, 117, 119, 132
Oedema, 8, 22, 117, 132, 341, 611
Optiprep, 625

P
p57, 95–97, 102–104, 266
Pantoea agglomerans, 352–353, 646, 683
Panzootic, definition, 3
Paracolobactrum anguillimortiferum, 11, 334–347

Pasteurella
P. pestis, 507
P. phocoenarum, 47
P. piscicida, 16, 504–513
P. seriola, 507
P. skyensis, 2, 15, 622–623, 644, 683
Pasteurellosis, 16, 502, 505, 506, 508, 697
Pathogenicity, capsule, 44
pEIB1, 535
Peptostreptococcus, 21
PFGE, 58, 229, 373, 411, 414, 479, 528
Phagocytosis, 46, 59, 62, 124, 176, 178, 191, 263, 265, 266, 332, 339, 343, 481, 510, 518, 534
Phospholipase, 111, 271, 275, 363, 370, 374, 504, 537, 550, 570, 617
Photobacterium
P. damsela, 502
P. damselae, 16, 479, 501–504, 507, 508, 549, 644, 646, 673, 683
P. damselae subsp. *damselae,* 16, 501–504, 530, 569, 672, 718
P. damselae subsp. *piscicida,* 16, 479, 502, 504–513, 552, 644, 646, 647, 655, 683, 699, 700
P. histaminum, 16, 502, 508
P. swingsii, 501
Piscirickettsia salmonis, 15, 624–628, 644, 646–648, 683
pJM1 plasmid, 540
Planococcus, 8, 136–137, 644, 646, 679
P. citreus, 676
Plasmid profile, 234, 424, 516
Plesiomonas shigelloides, 11, 354–355, 536, 646, 683, 714, 718
pMJ101 plasmid, 558
Podoviridae, 491
Pollution, 2, 3, 19, 363, 364, 694, 702, 716–718
Porin, 291, 536
Probiotic, 192, 293, 295, 543, 559, 705
Promicromonospora, 92
Proteus
P. rettgeri, 11
P. shigelloides, 355
Providencia
P. rettgeri, 355–356
P. vermicola, 11, 357, 646
Pseudoalteromonas piscicida, 10, 603–604, 645, 683
Pseudokidney disease, 5, 6, 676
Pseudomonas
P. aeruginosa, 15, 191, 270, 475
P. alcaligenes, 15, 476–477, 479, 644
P. anguilliseptica, 15, 477–482, 608, 645, 673, 674, 683, 699, 714
P. baetica, 15, 482–483, 645, 673, 683, 685
P. chlororaphis, 15, 198, 483–484, 645, 683
P. fluorescens, 15, 29, 96, 238, 365, 479, 484–487, 683, 685, 718
P. koreensis, 15, 487, 683
P. luteola, 15, 488, 683
P. mosselii, 15, 489
P. plecoglossicida, 15, 489–491, 683
P. pseudoalcaligenes, 16, 491–492, 683
P. putida, 16, 479, 489, 493, 673
P. putrefaciens, 606
Pseudotuberculosis, 16, 505, 506
Pulsotype, 370, 373
Pyroptosis, 341

Q
qPCR, 96, 123, 330
Quercetin, 550
Quorum sensing, 179, 191, 371, 516, 523, 537, 544, 550, 554, 716

R
Random genome sequencing, 534
Rapid ID32, 56
Real time PCR, 96, 123, 241, 328, 371, 444, 469, 626
Red disease, 14, 618
Redmouth, 12, 366
Red pest, 11, 524, 703
Red spot, 15, 477, 520
Renibacterium salmoninarum, 7, 84–141, 648, 658
Renogen, 104
REP-PCR, repetitive extragenic palindromic-PCR, 33, 336
Respiratory burst, 46, 62, 66, 102, 124, 182–185, 187, 190–192, 347, 432, 469, 523, 550, 553, 554
Rhodococcus, 8, 132–133, 136, 646, 667, 677, 679
R. erythropolis, 8, 123, 133–136, 644, 679
Ribotype, 233, 369, 424, 429, 507, 527, 528, 546, 549, 565
Rickettsia, 4, 89, 624–627, 633, 686, 712
Rickettsia-like organism (RLO), 15, 470, 628–632
Rochalimaea, 625
RTFS, 103, 113, 114, 137, 399, 426, 429, 431, 434, 620, 703

S
Saddleback disease, 12, 664
Salmolysin, 276
Salmonella
S. arizonae, 358–359
S. choleraesuis subsp. *arizonae,* 11, 358
S. enterica subsp. *arizonae,* 11, 358–359, 369, 644, 683
S. enteritidis serovar Typhimurium, 52
S. typhimurium, 52
Salmonid blood spot, 366, 368, 379, 703
Salmonid kidney disease, 84
Salmonid rickettsial septicaemia, 15, 624
Saltwater furunculosis, 524
Saprolegnia ferax, 260
Sekiten-byo, 15, 477, 479–481
Serine-protease, 177, 272–274, 277, 279, 552
Serology, 2, 36, 58, 94, 95, 97, 98, 172–173, 236–238, 328, 336, 338, 370, 408, 409, 413, 426, 430, 444, 479, 502, 508, 528, 529, 569, 626, 667–671, 715
Serratia
S. liquefaciens, 11, 359–361, 369, 644, 684, 705
S. marcescens, 11, 361–363, 369, 644, 646, 684
S. marcescens subsp. *kiliensis,* 369
S. plymuthica, 11, 363–365, 491, 646, 684, 717
S. rubidaea, 364
Serum killing, 432, 471
Shewanella, 615
S. putrefaciens, 10, 605–607, 644, 684, 714
Siderophore, 67, 178, 279, 342, 343, 374, 445, 534, 535, 550
Signal molecule, 191, 532, 716
S-layer, 174, 175, 182, 270, 430
Slime layer, 340
Sonophoresis, 293
Sporadic disease, definition, 3
Sporocytophaga, 13, 397, 400, 448, 645, 684
Staphylococcus
S. aureus, 8, 138, 644, 669, 679
S. epidermidis, 4, 8, 139–140, 644, 677, 679, 680
S. intermedius, 138
S. warneri, 9, 140–141, 646, 679, 680
Starvation-survival, 531
Stenotrophomonas maltophilia, 16, 493–494
Stomatococcus, 92
Streptobacillus, 18, 629, 644
S. moniliformis, 629
Streptococcicosis, 6, 34, 35, 37, 38, 42, 62, 697, 703
Streptococcosis, 6, 34, 42, 62, 64, 714
Streptococcus
S. agalactiae, 6, 34, 35, 43, 48–53, 58, 672, 677, 679, 714
S. casseliflavus, 35
S. difficile, 49
S. difficilis, 6, 34, 43, 48–53, 58, 672
S. dysgalactiae, 6, 34, 54, 55, 646, 677, 679
S. equi, 34
S. equinus, 35
S. equisimilis, 34
S. faecalis subsp. *liquefaciens,* 5, 38–39, 646
S. faecium, 34
S. ictaluri, 6, 34, 55–56, 646, 679, 680
S. iniae, 6, 34, 43, 45, 49, 50, 54–63, 66, 342, 571, 672–674, 679, 680, 700, 718
S. lactis, 35
S. milleri, 6, 34, 63–64, 679
S. parauberis, 6, 34, 43, 55, 58, 60, 64–66, 342, 672, 674, 679, 680
S. phocae, 6, 34, 36, 66–67, 241, 530, 626, 679
S. pyogenes, 34, 55
S. shiloi, 6, 49, 56–58
S. uberis, 55, 65
S. zooepidemicus, 34
Streptomyces
S. salmonicida, 127–129
S. salmonis, 127–129
Streptomycoccis, 128
Streptoverticillium salmonicida, 128
Subunit vaccine, 52, 60, 344, 378, 551, 552, 618, 698
Synthetic oligodeoxynucleotide, 104

T
Tagging devise, 85
Tenacibaculum
T. dicentrarchi, 13, 439, 682, 685
T. discolor, 13, 440–441
T. gallaicum, 13, 438, 441–442, 682, 685
T. maritimum, 13, 398, 409, 413, 426, 438, 442–447, 479, 673, 674, 682, 685
T. ovolyticum, 13, 398, 438, 446–447, 682
T. soleae, 13, 447–448, 673, 682
Terrabacter, 92
Tetrahymena pyriformis, 173, 247
Tetraselmis suecica, 697
Type I collagen, 430
Type II collagen, 374, 430
Type IV collagen, 374, 430
Type I interferon, 538
Type I pilus, 269

Type IV pilin, 270
Type III secretion, 265, 269, 341
Type VI secretion system, 341, 617

U
Ulcerative dermatitis, 216, 501
Ulcerative disease syndrome, 174
Ulcer disease, 9, 14, 16, 167, 218–220, 223, 239, 267, 281, 283, 291, 293, 296, 507, 518, 524, 616, 703, 714, 717
Ultramicrobacterium, 634
Unnamed bacteria, 634

V
Vaccine
  administration, 134, 342, 432, 618
  composition, 511
  inactivation, 181, 699
Vagococcus fluvialis, 543
Vagococcus salmoninarum, 5, 39–40
Varracalbmi, 18, 634
Vasculitis, 17, 547
Vertical transmission, 99, 106, 242, 257, 260, 627
Vibrio
  V. aestuarianus, 16, 499, 519–520, 684
  V. alginolyticus, 16, 295, 499, 520–524, 543, 549, 552, 559, 571, 610, 646, 660, 671–673, 684
  V. anguillarum, 17, 67, 95, 96, 182, 183, 238, 241, 282, 374, 400, 443, 479, 480, 500, 501, 516, 521, 524–539, 541–544, 548, 556–559, 565, 568, 569, 626, 646, 671–673, 684, 698–700, 702, 705, 714, 718
  V. anguillarum forma anguillicida, 526, 568
  V. anguillarum forma opthalmica, 526
  V. anguillarum forma typica, 526
  V. anguillicida, 526, 568
  V. brasiliensis, 500
  V. campbellii, 499, 549, 564
  V. carchariae, 17, 547–554
  V. chagassi, 499, 525, 548
  V. cholerae, 17, 250, 251, 544–546, 550, 556, 684, 714, 718
  V. coralliilyticus, 500
  V. damsela, 502
  V. ezurae, 501
  V. fischeri, 253, 513
  V. fortis, 501
  V. furnissii, 17, 546
  V. harveyi, 17, 345, 500, 501, 512, 513, 520, 523, 525, 547–554, 558, 571, 646, 671–673, 684, 705
  V. ichthyodermis, 526
  V. ichthyoenteri, 17, 554–555, 563, 673, 684
  V. kanaloaei, 501
  385
  V. logei, 514
  V. marinus, 14, 615
  V. mimicus, 17, 550, 555–556, 718
  V. nereis, 499, 610
  V. ordalii, 17, 95, 96, 501, 516, 526, 539, 556–559, 565, 646, 684, 698–700
  V. owensii, 501
  V. parahaemolyticus, 17, 499, 516, 521, 526, 530, 551, 559, 569, 672, 673
  V. pelagius, 17, 560–561, 565, 646, 684
  V. piscium var. *japonicus,* 526
  V. ponticus, 17, 561–562, 684
  V. rotiferianus, 501, 673
  V. salmonicida, 283, 291, 515, 671, 711
  V. scophthalmi, 17, 563
  V. splendidus, 18, 563–566, 644, 646, 684, 716
  V. tapetis, 18, 501, 566–567, 644, 684
  V. toranzoniae, 501
  V. trachuri, 17, 547–554
  V. tubiashi, 499, 501, 565
  V. viscosus, 616–618
  V. vulnificus, 18, 60, 180, 270, 523, 530, 552, 567–571, 646, 672, 673, 684, 716, 718
  V. wodanis, 616
Vibriosis, 16–18, 477, 500, 514, 517, 524, 530, 532, 537–539, 541, 543, 544, 548, 557, 561, 567, 669, 697, 698, 703
Virulence array protein, 266
Virulence plasmid, 504, 534, 570
Visceral toxicosis, 5, 24
Vulnivaccine, 570

W
Water treatment, 431, 694, 696
Weil's disease, 695
Weissella ceti, 33–34, 679
Weissella spp., 7, 31–32, 644
Weissellosis, 7, 31, 33
Whirling disease, 608
Wild fish stocks, 19, 505, 693–694, 716
Winter disease, 15, 477
Winter ulcer disease, 14, 16, 518, 616, 714
Wire-tagging, 85

Wolbachia persica, 625
Wound infection, 718

Y
Yersinia
Y. enterocolitica, 369
Y. intermedia, 11, 365–366, 644, 684
Y. pestis, 469, 507
Y. philomiragia, 469
Y. pseudotuberculosis, 369
Y. ruckeri, 12, 95, 96, 222, 350, 358, 366–379, 427, 491, 524, 618, 644, 646, 656, 661, 671, 672, 674, 684, 700, 705, 713, 716

Z
Zoonoses, 718