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ORIGINAL PAPER

Susceptibility of corkwing wrasse *Symphodus melops*, goldsinny wrasse *Ctenolabrus rupestis*, and Atlantic salmon *Salmo salar* smolt, to experimental challenge with *Vibrio tapetis* and *Vibrio splendidus* isolated from corkwing wrasse

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Abstract The virulence of two *Vibrio* strains, previously isolated from diseased corkwing wrasse *Symphodus melops* and identified as *V. tapetis* and *V. splendidus*, to corkwing and goldsinny wrasse *Ctenolabrus rupestris* and to Atlantic salmon *Salmo salar*, was studied under laboratory conditions. Both bacteria were shown to be opportunistically pathogenic to corkwing wrasse, causing significantly higher mortality in the challenged groups than in the controls. Bacterial cultivation of kidney samples and re-isolation of *V. tapetis* and *V. splendidus* from most mortalities confirmed the two strains as the probable cause of mortality in the challenged groups. The control group also suffered relatively high mortality, but no specific pathogens that were suspected to be the main cause of death were isolated, other than a mixture of *Vibrio* spp. and, in the case of one individual, atypical *Aeromonas salmonicida*. Following injection challenge with both bacterial strains, no mortality was recorded in Atlantic salmon. In bath challenge trials with goldsinny wrasse, only slight mortality was observed in the challenged groups and the unchallenged control group. Bacterial examination showed that atypical *Aeromonas salmonicida* was the probable cause of death in both bath challenged and control groups of goldsinny wrasse, and no indication of infection by any *Vibrio* sp. was found.

Keywords *Vibrio tapetis* · *Vibrio splendidus* · Wrasse · Salmon · Challenge

Introduction

Several species of wrasse (*Labridae*) are used as an alternative to chemotherapeutic agents in controlling sea lice, particularly *Lepeophtheirus salmonis* Krøyer and *Caligus elongatus* Normann on farmed Atlantic salmon *Salmo salar* (Bjordal 1990;

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Costello 1996; Deady et al. 1995; Kvenseth 1996; Treasurer 1996; Young 1996). A popular strategy is to use a mixed population of wrasse in the salmon cages. Two commonly used species in salmon culture are goldsinny wrasse *Ctenolabrus rupestis* and corkwing wrasse *Symphodus melops*. The occurrence of pathogens in wrasse could be a drawback to this technique, both in terms of mortality among the wrasse themselves and because of the possibility of transferring diseases to the salmon.

Both typical (Hjeltnes et al. 1995) and atypical strains of *Aeromonas salmonicida* have been isolated from wrasse, the atypical strains having been found to be responsible for high mortality in goldsinny wrasse in salmon cages and in laboratory studies (Treasurer and Laidler 1994; Laidler et al. 1999, Samuelsen et al. 2002, 2003). However, evidence of transfer of a pathogenic agent between salmon and wrasse is scarce. Attempts to infect Atlantic salmon with a strain of atypical *A. salmonicida* isolated from wrasse have failed (Frerichs et al. 1992). During a furunculosis epizootic in Atlantic salmon in 1990 typical *A. salmonicida* was isolated from a kidney sample of one dead corkwing wrasse (Hjeltnes et al. 1995). Recently, a *Vibrio* sp. was isolated from captured corkwing wrasse that were suffering high mortality (Samuelsen et al. 2000). This strain has later been classified into the species *V. splendidus*, while a different strain, classified into *V. tapetis*, was subsequently isolated from another group of captured corkwing wrasse that were also experiencing high mortality (Jensen et al. 2003). Whereas *V. splendidus* is a commonly found opportunistic fish pathogen, *V. tapetis* had previously been found in only bivalves, being the causative agent of Brown Ring Disease in several clam species, particularly the Manila clam, *Ruditapes philippinarum* (reviewed by Paillard 2004). Recently, a strain of *V. tapetis* was isolated from Atlantic halibut suffering vibriosis (Reid et al. 2003). Thus, with both *V. tapetis* and *V. splendidus*, it seems possible that pathogenic strains might be transferred to new hosts.

The widespread practice of polyculture of the wrasse species and Atlantic salmon in sea-cages emphasises the need to study the susceptibility of these species to the pathogens commonly associated with the other species in the polycultures. The aim of the present study was to examine the virulence of the strains of *V. tapetis* and *V. splendidus*, isolated from corkwing wrasse, to Atlantic salmon and goldsinny and corkwing wrasse, by means of challenge experiments.

Materials and methods

Experimental fish

Corkwing and goldsinny wrasse were captured by weir outside Bergen in September 2000 by a local fisherman and were transported to the Institute of Marine Research in Bergen. The fish were stocked in flow-through storage tanks (1 m deep × 2.5 m diameter) with a flow rate of 12 l min^{-1} at a temperature of 9 ± 0.5°C. The salinity was 33 p.p.t. in all tanks used in the present study. The fish were offered plastic tubing and artificial seaweed made from black plastic bags as hiding places. Mean weights with standard deviations were approximately 30 ± 6 g for goldsinny and 45 ± 9 g for corkwing wrasse. The fish were fed krill to satiation every day. No mortality and no signs of disease were recorded in the fish prior to the challenge experiments.

Unvaccinated Atlantic salmon from Jakta Fiskeoppdrett AS, Fotlandsvåg, Norway, with a mean weight of 65 ± 9 g were stocked in flow-through storage tanks

(1 m deep × 2.5 m diameter) with a flow rate of 12 l min^{-1} and a seawater temperature of 11.0 ± 0.5°C. The fish were fed a ration of 1% body weight per day of dry pellets (T. Skretting A/S, Stavanger, Norway).

Bacterial strains

The strain LP1 was isolated from diseased corkwing wrasse in September 1998, as described by Samuelsen et al. (2000). A different *Vibrio* sp., LP2, was isolated from diseased corkwing wrasse in the laboratories of the Institute of Marine Research in September 1999 (Jensen et al. 2003). From analyses of a series of genetic, biochemical, serological and morphological properties, the strains had earlier been assigned to the species *V. splendidus* (LP1) and *V. tapetis* (LP2) (Jensen et al. 2003).

Bath challenge experiments

The bacteria were grown in Difco 2216 Marine Broth in shaking culture at 20°C. The optical density in the cultures at 600 nm was measured by a Hitachi U-1100 Spectrophotometer to 1.421 (challenges with LP1), 1.732 and 1.792 (challenges with LP2, of goldsinny wrasse and corkwing wrasse, respectively) The numbers of colony-forming units (CFU) per millilitre in the bacterial cultures were assessed by a dilution series in sterile 25 p.p.t. seawater and plating out on Difco 2216 Marine agar Petri dishes, which were incubated at 20°C. Colonies were counted after 4 days. The estimates of CFU in the bath challenge tanks, calculated from the measurements of the CFU per millilitre in the bacterial cultures were 3.2×10^6 CFU ml^{-1} (challenges with LP1), 3.8×10^5 CFU ml^{-1} and 2.1×10^5 CFU ml^{-1} (challenges with LP2, of goldsinny wrasse and corkwing wrasse, respectively).

Two bath challenge experiments were performed, one with corkwing wrasse and one with goldsinny wrasse. In both these experiments the fish were starved for 48 h before challenge. Fish were randomly distributed to five groups of 60 fish and transferred from the storage tank to five circular (0.8 m depth and 1 m inside diameter) flow-through seawater tanks, which were maintained at 9.0 ± 0.5°C. The water flow in these tanks was maintained at 10 l min^{-1} The water supply to the tanks was turned off immediately before the bacterial suspension was added, and the tanks were oxygenated. Following addition of the bacterial suspension the fish were kept in the bath for 1 h, after which the water supply was restored. In both bath challenge experiments two groups of fish were exposed to the LP1 strain, and two groups to the LP2 strain, while a fifth group served as unchallenged controls. Two days after challenge, the fish were offered food. Mortalities were recorded, and dead fish were removed from the tanks once a day. Kidney samples from the newly dead fish were examined for the presence of bacteria, as described below. Mortality data were statistically analysed by chi-square analysis. The experiments were terminated 12 days after challenge (corkwing wrasse) and 14 days after challenge (goldsinny wrasse). Five survivors from each tank were killed, and kidney samples were examined as described below. The remaining fish were anaesthetised and killed.

Intraperitoneal injection challenge

Challenge by intraperitoneal (i.p.) injection was performed on unvaccinated Atlantic salmon smolts. The fish were held in tanks of the same size and water flow-rates as

described for the bath challenge experiments with wrasse, but at a temperature of 11 ± 0.5°C. Two groups of five fish were injected intraperitoneally with 0.2 ml of an O.D. 2.0 (measured at 600 nm) culture of either *V. splendidus* LP1 or *V. tapetis* LP2. The fish were observed daily for 22 days. At the end of the experiment, the fish were killed and examined for pathological changes, and kidney samples were cultured for bacteria as described below.

Bacterial re-isolation and classification

Before necropsy the fish were surface-disinfected with 70% ethanol. Kidney samples from dead fish and five fish from the survivors of each group at the end of the experiments were inoculated on nutrient agar (Oxoid, Hampshire, UK) supplemented with 5% sheep blood and 1.5% NaCl and on brain heart broth agar (BHB) (Merck, Darmstadt, Germany) and incubated aerobically at 18°C for 4–5 days. The bacteria were identified according to their morphology, sensitivity to the vibriostatic agent 2,4-diamidino-6,7-diisopropylpteridine phosphate (O/129; 10 μl of a saturated solution per disc) (Merck) and key biochemical characteristics measured by the commercial test kit API 20 E (Bio Mérieux, Marcy l'Etoile, France).

Results

Bath challenge of corkwing wrasse

Mortalities began on day 3 following challenge in the groups challenged with strain LP1, on day 6 in the groups challenged with strain LP2 and on day 7 in the unchallenged control group. Cumulative mortality rose rapidly in the challenged groups, and the mortality had reached 92% and 78% in the groups challenged with LP1 and 62% and 90% in the groups challenged with LP2 on day 12 after challenge (Fig. 1) when the experiment was terminated. Examination of kidney samples from all dead fish in the challenged groups, subsequent testing of the key biochemical characteristics (O/129 and API 20E profile), and comparison with the LP1 and LP2 profiles, confirmed the two bacterial strains to be re-isolated. However, a relatively high cumulative mortality (33% on day 12) was also observed in the control group. In contrast to that in the challenged groups, no specific pathogen that could be suspected to be the main cause of death was isolated from the dead fish in the control group. Sparse growth of a mixture of several different *Vibrio* spp., none of which were identical to LP1 or LP2 according to their API 20E profiles, was present in most fish examined. In the sample from one individual, sparse growth of a bacterium with an API 20E profile identical to that of the atypical *Aeromonas salmonicida* from goldsinny wrasse was found. An almost identical mortality pattern was found in the fish kept in the storage tank under otherwise identical conditions in the same period, where mortality was 36% (data not shown). The final cumulative mortalities in the challenged groups were significantly higher than in the control group, with $P < 0.025$ for LP1 and $P < 0.05$ for LP2.

Bath challenge of goldsinny wrasse

The mortality rate was low in both the challenged groups and the control group (Fig. 2). No significant differences in cumulative mortality had been found between

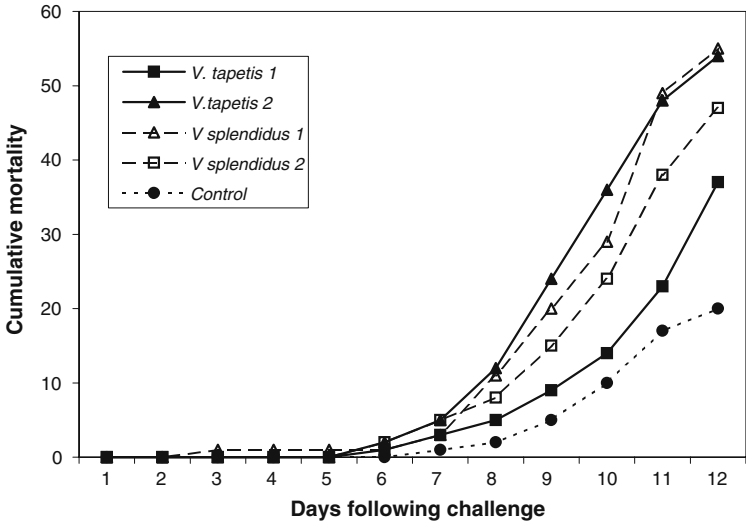

Fig. 1 Cumulative mortality (average number of dead fish per tank, total number of fish per tank = 60) in bath challenge with corkwing wrasse. The experimental groups were challenged with *Vibrio splendidus* strain LP1 and *Vibrio tapetis* strain LP2, while the third group was an unchallenged control group Two tanks with corkwing wrasse were challenged with each bacterium, whereas one tank was an unchallenged control

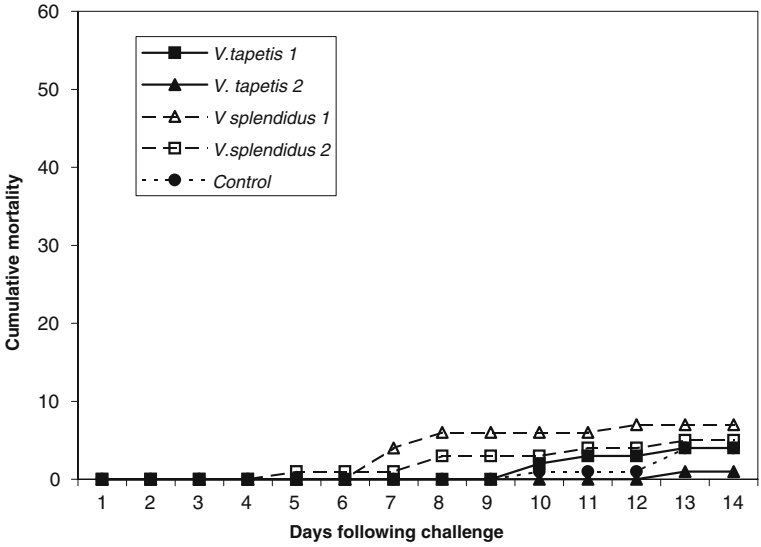

Fig. 2 Cumulative mortality (average number of dead fish per tank, total number of fish per tank = 60) in the bath challenge with goldsinny wrasse. The experimental groups were challenged with *Vibrio splendidus* strain LP1 and *Vibrio tapetis* strain LP2, while the third group was an unchallenged control group. Two tanks with goldsinny wrasse were challenged with each bacterium, whereas one tank was an unchallenged control

challenged and unchallenged groups when the experiment was terminated on day 14 after challenge. Bacterial examination of kidney samples from dead fish showed atypical *A. salmonicida* to be abundant in the dead individuals in both challenged

and unchallenged groups. The API profiles of all isolated strains were identical to the atypical *A. salmonicida* strains isolated from goldsinny wrasse in our previous studies (Samuelsen et al. 2002, 2003) and to the single *A. salmonicida* strain isolated from a corkwing wrasse in the present study. No other bacteria were found. No gross pathological changes were recorded.

Intraperitoneal injection challenge of Atlantic salmon

No mortality was observed in any of the groups during the experimental period of 22 days, and no gross pathological changes were recorded. Bacterial cultivation of the kidneys revealed no bacterial growth.

Discussion

The results from the bath challenge experiment with corkwing wrasse give strong indication that both *V. splendidus* LP1 and *V. tapetis* LP2 are opportunistically pathogenic to corkwing wrasse. Significantly higher mortality was observed in the challenged groups than in the control group, and successful re-isolation of the bacteria through bacterial examination of kidney samples was done from the dead fish in the challenged groups, suggesting that the two bacterial strains were the causative agents. Re-isolation of the pathogens confirmed Koch's postulate for both bacterial strains, as both strains had previously been isolated by necropsy from corkwing wrasse that were suffering from systemic vibriosis (Samuelsen et al. 2000; Jensen et al. 2003).

However, the high mortality observed in the control group of corkwing wrasse (33%) raises some questions. The mortality in the control group was close to that of the remaining 156 fish (counted the day the challenge experiment started) in the storage tank, which was 36% in the same period. This observation adds to our earlier experience (Samuelsen et al. 2000) that corkwing wrasse are difficult to keep healthy during long periods in captivity, despite attempts to minimise stress. For the three subsequent years we kept groups of wild-caught corkwing wrasse in captivity, and, in all cases, high mortality, typically 1–5% of the population per day, started after approximately 1 month. From two different groups of corkwing wrasse, we have been able to isolate one dominating pathogen, leading to the isolation of the opportunistically pathogenic strains LP1 and LP2 (Samuelsen et al. 2000; Jensen et al. 2003). In other groups, including the present control groups, mixed growth of several *Vibrio* spp. have been present in kidney samples of dead and moribund fish. No signs of larval infestations with parasites could be seen; however, the possible presence of undetected pathogens cannot be ignored. Few attempts have so far been made to characterise agents of disease in wrasse. Apart from our isolation of *Vibrio* spp. (Samuelsen et al. 2000; Jensen et al. 2003) and the presence of *Aeromonas salmonicida* in wrasse (Treasurer and Laidler 1994; Hjeltnes et al. 1995; Bricknell et al. 1996; Laidler et al. 1999; Samuelsen et al. 2002; 2003), only parasites have, so far, been recognised as disease factors (Costello et al. 1996; Karlsbakk et al. 1996). No search for viral pathogens in wrasse has, so far, been published. The results indicate that corkwing wrasse are highly susceptible to opportunistic bacteria when kept in captivity and that, in some cases, several *Vibrio* spp., as well as atypical

A. salmonicida, may cause septicaemia. This is the first report confirming the pathogenicity of specific *Vibrio* strains in wrasse.

The results of our bath challenge experiment with goldsinny wrasse suggest that neither of the two bacterial strains were pathogenic to this fish species. The fish survived a dose of bacteria that was lethal to corkwing wrasse held under identical experimental conditions, and none of the strains was re-isolated from kidney samples. We therefore conclude that, despite their pathogenicity to corkwing wrasse, the two strains represent no credible threat to goldsinny wrasse under normal conditions in a salmon sea-cage. The finding of atypical *A. salmonicida* in some kidney samples supports earlier observations demonstrating that this pathogen is frequently present in goldsinny wrasse and may cause disease following stress (Samuelsen et al. 2002; 2003), and it is tempting to speculate whether a carrier state might be the normal situation.

In the case of Atlantic salmon, injection challenge was preferred to bath challenge. This administration of pathogens, which circumvents the otherwise important primary infection barriers constituted by the epithelial layers of the fish, would normally lead to higher mortality than that in bath challenge. As demonstrated by the injection challenge, unvaccinated Atlantic salmon did not appear to be susceptible to injections of either strain. All the individuals tested survived injections with a relatively large number of bacteria. As no mortality occurred, we conclude that neither *V. splendidus* LP1 nor *V. tapetis* LP2 is pathogenic to Atlantic salmon. Under normal conditions in a fish farm it seems that these strains are thus unlikely to cause disease in either Atlantic salmon or goldsinny wrasse.

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