Investigation of Aquatic Pathogens and Diversity Analysis of *Aeromonas* Isolates

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1 Introduction

China is a country in the production and consumption of a large amount of aquatic products, accounting for about two-thirds of the total amount in the world's total. In recent years, aquaculture constantly tended to operate in the ways with high density, industrialization and intensification, for increasing the output of aquatic products. However, the water rich in nutrients led to pathogenic bacteria blooming in aquaculture. Annual losses from aquaculture diseases accounts for about 30% of production, while pathogenic bacterial infection diseases accounts for about half.

The major aquatic bacterial pathogens mainly comprise multiple genera of both Gram-negative and Gram-positive bacteria, such as *Vibrio*, *Aeromonas*, *Escherichia coli*, *Streptococcus iniae*, *Salmonella*, *Edwardsiella tarda*, *Pleisionomas*, *Flavobacterium*, *Acinetobacter*, *Pasteurella*, *Pseudomonas*, *Staphylococcus aureus*, *Mycobacterium*, *Clostridium* and others [1]. *Aeromonas*, including 25 different species, is the important pathogen of fish, shrimp and other animals [2, 3], causing serious economic losses [4].

In this work, fish of Tianjin and Xiamen, Fujian, China, *Penaeus vannamei* shrimp seeds of Hangu, Tianjin, China, and seawater samples of Jiyun River and Bohai Bay were collected in Tianjin. The epidemiology of aquatic pathogens was isolated with the selective media. The isolates were identified with the 16S rRNA

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sequencing analysis [5]. Since 16S rRNA gene sequence is highly homologous among most *Aeromonas* species, the *Aeromonas* isolates in this study were also analyzed with the multilocus sequence typing (MLST) method for further discrimination of the *Aeromonas* isolates [6].

2 Materials and Methods

2.1 Samples, Media and Bacteria Isolation

Freshwater fishes were obtained from five local markets and two fish farms in Tianjin, China from March through May 2012. The fishes belonged to 11 different species, including *Hypophthalmichthys molitrix* (4), *Ctenopharyngodon idella* (4), *Carassius auratus* (4), *Hypophthalmichthys nobilis* (4), *Megalobrama amblycephala* (2) and *Ephippus orbis* (2). *Penaeus vannamei* shrimp seeds (2) and Saltwater fish *Epinephelus awoara* (12) were respectively obtained from Hangu, Tianjin, and Xiamen, Fujian, China, during March 2012. Seawater samples (4) were obtained from in Tianjin, China from March through May 2012.

The gastrointestinal tract was removed from fish and the content was collected and stored at 4 °C. The content sample was serially diluted in phosphate buffered saline and spread on three types of agar plates, including SS (*Salmonella Shigella* agar), TCBS (thiosulfate citrate bile salts sucrose agar) [7, 8], MAC (MacConkey agar) [9], CIN-1 (Cepulodin Irgasan Novobiocin Agar). L-agar plates containing ampicillin (20 μg/ml), erythromycin (20 μg/ml), and kanamycin (20 μg/ml) were also used for screening the possible bacterial pathogens in the gut samples.

2.2 The Identification of Isolated Strains

The genomic DNA was extracted from the isolated strains. The DNA was used as template for PCR amplification of the 16S rRNA gene with primers described previously [10]. The PCR product was subjected to sequencing directly. The obtained sequences were analyzed by the BLAST program on the NCBI website. MEGA 5 was used to construct the phylogenetic trees [11].

2.3 MLST Analysis

Multilocus sequence typing (MLST) was proposed as a universal method to characterize bacteria based on sequence polymorphisms within internal fragments of housekeeping genes. The primers for amplifying a set of six housekeeping genes,

namely, *gyrB*, *groL*, *gltA*, *metG*, *ppsA*, and *recA*, was synthesized for MLST of the *Aeromonas* isolates [12]. The sequences of distinct alleles were deposited in the *Aeromonas* MLST database (http://pubmlst.org/aeromonas). Allele number and sequence type (ST) number were determined by described previously [12].

3 Results

3.1 Strain Separation

120 bacteria strains were isolated from 37 different samples with various media. Morphology of bacterial colonies grown on the TCBS plate were mostly yellow and green, smooth, and moist; on the SS plate were mostly yellow, pink, colorless with black center; on the MAC plate mostly were pink and red, flat; on the CIN-1 plate mostly were pink, big, and smooth.

3.2 Identification of Isolated Strains

120 isolated strains were identified with the comparative sequence analysis of the 16S rRNA gene. The 16S rRNA sequences were deposited in GenBank with accession no KC210757-KC210826, KC210827- KC210872 and KC252599-KC252602. All the isolates were tentatively classified into 15 genera of seven families, including *Aeromonas* spp. (53), *Vibrio* spp. (19), *Proteus* spp. (5), *Citrobacter* spp. (9), *Hafnia* spp. (8), *Providencia* spp. (6), *Pseudomonas* spp. (3), *Kluyvera* spp. (2), *Enterobacter* spp. (2), *Bacillus* spp. (2), *Pantoea* sp. (1), *Leclercia* sp. (1), and *Acinetobacter* sp. (1). *Aeromonas* strains were not isolated from all the samples except from *Epinephelus awoara* from samples of Xiamen, Fujian, China. The *Vibrio* isolates include *V. parahaemolyticus* (2), *V. alginolyticus* (5), *V. azureus* (2), *V. anguillarum* (9), and *V. fluvialis* (1), found in the samples from Tianjin and Xiamen, Fujian, China (Table 1).

3.3 Phylogenetic Analysis

Based on the 16S rRNA gene sequences, the phylogenetic tree of all isolated strains was constructed. As shown in Fig. 1, *Aeromonas* isolates, *Enterobacteriaceae* isolates, *Vibrio* isolates, and *Shewanella* isolates were in grouped in one cluster, respectively; 9 *C. freundii* isolates were also grouped into two clades, one comprising of 4 members and the other comprising of 5 members. The phylogenetic

Table 1 Comparative analysis of 16S rRNA gene sequences of the isolated

Genus	Number	Host[a]	Medium[b]
Vibrio	19	Ea, Pv, Eo	MAC, TCBS, CIN-1
Proteus	5	Ea, Hn	TCBS, Tc^{20}
Citrobacter	9	Ea, Hm, Ma, Hn, Cau	SS, MAC, TCBS, CIN-1, Km^{20}, Em^{20}
Hafnia	8	Ea, Cc	SS, MAC, TCBS, Em^{20}
Providencia	6	Ea, Ci	CIN-1, TCBS
Kluyvera	2	Ea	CIN-1
Morganella	2	Cau, Ci	TCBS
Pantoea	1	Ci	SS
Enterobacter	2	Ea, Hn	TCBS, Km^{20}
Leclercia	1	Ea	Em^{20}
Shewanella	6	Hn, Eo, Hm, Pv	CIN-1, TCBS
Pseudomonas	3	seawater, Pv	SS
Acinetobacter	1	Pv	SS
Bacillus	2	Ea	SS, TCBS
Aeromonas	53	seawater, Hn, Eo, Cau, Hm, Pv, Cc, Ci, Ma	TCBS, MAC, SS, CIN-1

[a]Hm *Hypophthalmichthys molitrix*; Ci *Ctenopharyngodon idella*; Cau *Carassius auratus*; Hn *Hypophthalmichthys nobilis*; Ma *Megalobrama amblycephala*; Eo *Ephippus orbis*; Pv *Penaeus vannamei*; Ea *Epinephelus awoara*; Cc *Cyprinus carpio*

[b]Em^{20}, LB medium contain 20 μg/ml erythromycin; Tc^{20}, LB medium contain 20 μg/ml tetracycline; Kn^{20}, LB medium contain 20 μg/ml kanamycin

analysis result indicated that the isolated strains within the same genus or species might belong to different lineages (Fig. 1).

Since 16S rRNA gene sequence is highly homologous among most *Aeromonas* species, the *Aeromonas* isolates in this study were identified only at genus level [6].

3.4 MLST Analysis

The fragments of the six housekeeping genes were successfully amplified and sequenced in all 53 *Aeromonas* strains. The sequence alignment results showed only 21 out of the 318 alleles was known alleles in the database, including *gyrB*22 (LSB2), *gyrB*22 (MY-1), *gyrB*97 (MJ-4), *gyrB*22 (MY), *groL*42 (MJ-1), *groL*96 (CH13), *groL*96 (SH11), *gltA*56 (MY-1), *gltA*56 (MJ-4), *gltA*51 (ML-1), *gltA*56 (MY), *gltA*56 (SJ1), *gltA*51 (SL-1), *gltA*99 (TB2), *metG*64 (TY-4), *ppsA*67 (LSJ1), *ppsA*40 (MJ-1), *ppsA*40 (SL1), *ppsA*40 (TL1), *ppsA*40 (TY-2) and *recA*42 (MJ-1). The remaining 297 sequences represented the novel alleles. Correspondingly, only 8 STs were known sequence types and 45 STs were novel. The results suggested the highly genetic diversity of the *Aeromonas* isolates in our work.

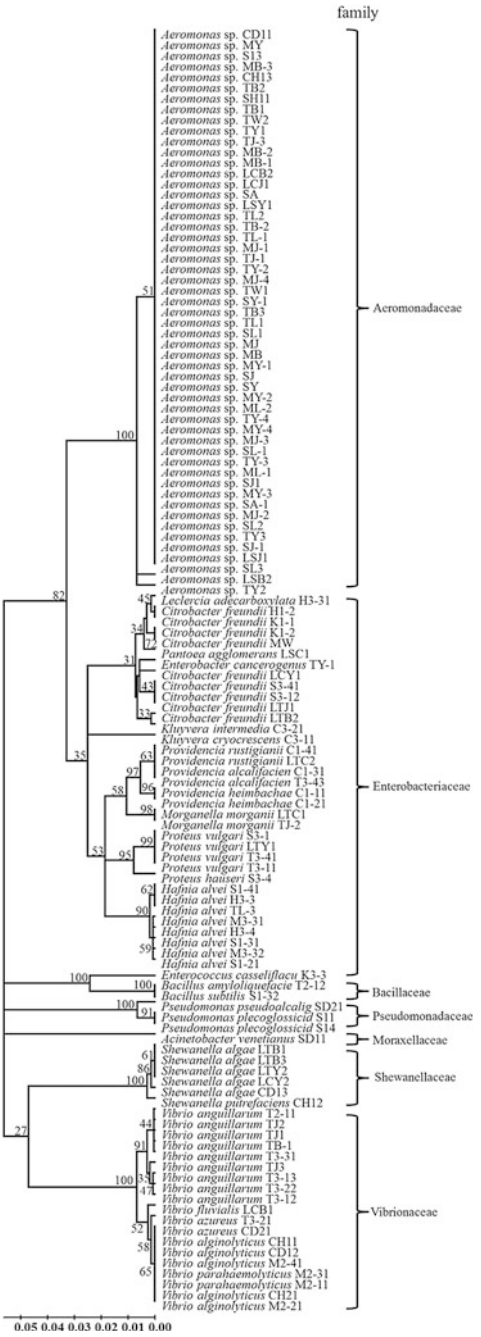

Fig. 1 Phylogenetic tree based on 16S rRNA gene sequences of the isolates

3.5 Distribution of Aeromonas in Worldwide

By analyzing the database of all 398 *Aeromonas* STs, *Aeromonas* strains geographic isolation was found. As shown in Fig. 2a, the Asia, Europe, North America and South America were respectively discovered 195, 181, 8 and 1 unique STs, only one ST is the North American and Asian shared, the ST is famous ST 251 [13].

As shown in Fig. 2b, a total of 171 unique ST were found in the Chinese region, Beijing, Tianjin, Jilin, Guangdong and Zhejiang as the main source of the data, and was found to also have geographic isolation; no duplicate ST occurs among strains isolated in different regions.

3.6 Aeromonas Relationship Between the STs

Relationships among the STs deposited in the *Aeromonas* MLST database were analyzed by using the eBURST algorithm [9]. The eBURST analysis revealed that 42 irrelevant clonal complexes (CCs) and 81 singletons were identified. Figure 3 is a schematic diagram of eBURST analysis, the identified 45 STs distributed in the 8 of 42 clonal complexes, and the low correlation between strains, indicating a high degree of biological diversity of *Aeromonas* strains. Additionally, in this study only one allele number difference was identified between ST127 (MY-1) and ST 140 (MY).

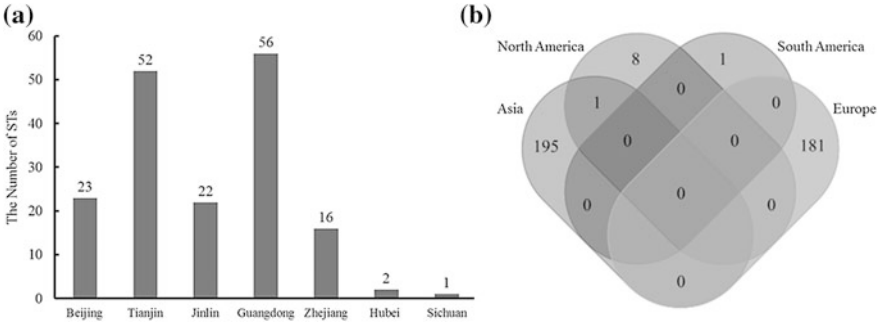

Fig. 2 Distributions of *Aermonas* STs across China (**a**) and the world (**b**)

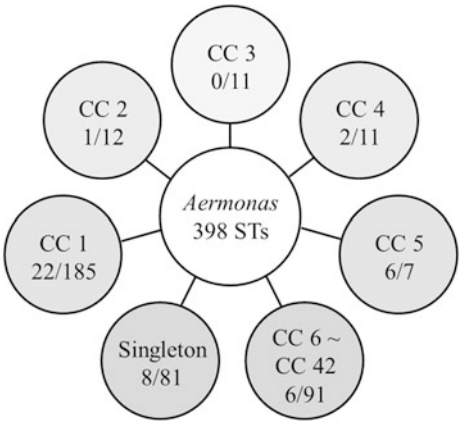

Fig. 3 eBURST algorithm analysis of 398 *Aermonas* STs. *CC* clonal complexes; Transparent circle is 398 *Aermonas* STs, *grey circle* is grouped by eBURST algorithm analysis 398 *Aermonas* STs; Fraction = (Number of *Aermonas* CCs in this study and CCs)/(Number of *Aermonas* STs in this CCs)

4 Discussion

Aquatic pathogenic bacteria can cause fish diseases, leading to the death of aquatic animals, bringing economic losses to farmers. Deng et al. [14] collected samples from a variety of aquatic animals from 64 aquaculture farms in Guangdong, China, and isolated 112 *Aeromonas* strains with multidrug-resistant phenotype and Class I integrons. Adesoji et al. [15] isolated 108 tetracycline resistant strains from water distribution systems in southwestern Nigeria, including *Aermonas* spp., *Alcaligenes* spp., *Bacillus* spp., *Klebsiella* spp., *Leucobacter* spp., *Morganella* spp., and *Proteus* spp..

MLST was performed to further discriminate the 53 *Aeromonas* isolates. Totally 297 new alleles and 45 novel STs were identified in our study. The data indicated the strains of the *Aeromonas* genus from different sources were highly genetically diversified [16, 17]. By eBURST algorithm analysis 398 *Aeromonas* STs were found belonging to 42 CCs, further suggesting the diversity of all *Aeromonas* isolates across the world. That may be due to the small number of *Aeromonas* isolates in the MLST database, and more deposited strains will better unveil the distribution patterns of *Aeromonas* strains.

This study has identified a number of novel alleles and STs of the *Aeromonas* spp. The relevant information has been deposited in the *Aermonas* MLST database. The data obtained in our study will provide more support for the study of the *Aeromonas* epidemiology.

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