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SHORT COMMUNICATION

Distribution and Virulence Gene Comparison of *Aeromonas* Strains Isolated from Diseased Fish and Water Environment

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Abstract

A total of 71 *Aeromonas* strains were isolated in the south of Jiangsu Province China in order to analyze the difference of *Aeromonas* spp. distribution between diseased fish and water environment. The sequence of 16S *rDNA* and *gyrB* demonstrated that the 71 *Aeromonas* isolates could be divided into 4 species, including *A. veronii* (55), *A. hydrophila* (11), *A. salmonicida* (3) and *A. media* (2). *A. veronii* was the most common species isolated from fish and water environment. All *Aeromonas* isolates were screened for three putative virulence genes, *aer*, *hly* and *alt*. *hly* was the most common gene among three virulence genes.

Key words: *Aeromonas*, diseased fish, *gyrB*, 16S *rDNA*, virulence gene, water environment

Species of *Aeromonas* are common inhabitants of aquatic environments and have been described in connection with fish and human diseases (Saavedra *et al.*, 2004; Figueras, 2005). At present the genus comprises 19 species: *A. hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. caviae* (synonym *A. punctata*), *A. media, A. eucrenophila*, *A. sobria*, *A. veronii* (synonyms are *A. ichthiosmia* and *A. culicicola*), *A. jandaei*, *A. schubertii, A. trota* (synonym *A. enteropelogenes*), *A. allosaccharophila*, *A. encheleia*, *A. popoffii*, *A. simiae*, *A. molluscorum*, *A. bivalvium*, *A. aquariorum*, and *A. tecta*, as well as two DNA homology groups without a species name, *Aeromonas* sp. HG11 (proposed to be the synonym of *A. encheleia*) and HG13 (Enteric group 501) (Demarta *et al.*, 2008; Harf-Monteil *et al.*, 2004; Martin-Carnahan and Joseph, 2005; Martinez-Murcia *et al.*, 2008; Minana-Galbis *et al.*, 2004; Minana-Galbis *et al.*, 2007; Pidiyar *et al.*, 2002; Saavedra *et al.*, 2006).

Direct sequencing of the 16S *rDNA* gene is generally accepted as a stable and specific marker for bacterial identification (Marchandin *et al.*, 2003). Although 16S *rDNA* gene sequencing has contributed notably to the elucidation of the phylogenetic interrelationships between *Aeromonas* species, the resolution of this molecular 'clock' has now been superseded by those of some protein-encoding housekeeping genes such as *gyrB* and *rpoD* (Yanez *et al.*, 2003; Soler *et al.*, 2004; Martin-Carnahan and Joseph, 2005; Saavedra *et al.*, 2006). The *gyrB* gene that encodes the B subunit protein of DNA gyrase (topoisomerase type II) is a single copy gene and is essential for DNA replication. This gene has been extensively used for studying phylogenetic relationship with various bacterial genera and its comparison with DNA-DNA hybridization results (Kasai *et al.*, 2000).

Aeromonas are native of aquatic environments, and are frequently found in foods, including meat, fish, vegetables, fresh and sea water (Castro-Escarpulli *et al.*, 2003). Species, such as *A. hydrophila*, *A. bestiarum*, *A. veronii biovar sobria* and *A. sobria,* have been associated with infections in kinds of fish species (Kozinska, 2007). Some strains of motile and non-motile *Aeromonads* are involved in different fish diseases, such as septicemia, ulcerative disease, and furunculosis (Aberoum and Jooyandeh, 2010; Cristi *et al.*, 2007). The mechanism of pathogenesis is complex and unclear (Janda and Abbott, 2010; Parker and Shaw, 2011). All genes that encode for virulence associated factors that allow the pathogen to establish infection in the host are defined as virulence genes. Virulence of *aeromonads* is considered to be multifactorial including cytotonic heat-labile (*alt*), and cytotonic heat-stable enterotoxins (*ast*), cytotoxic heat-labile enterotoxin (*act*), aerolysin (*aer*), flagella A and flagella B (*fla*),

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lipase (*lip*), elastase (*ela*), serine protease (*ser*), ADP-ribosyltransferase toxin (*aexT*), and DNases (*exu*) (Sha *et al.*, 2002; Chacon *et al.*, 2003; Sen and Rodgers, 2004; Nam and Joh, 2007; Vilches *et al.*, 2008; Nawaz *et al.*, 2010). It is not clear whether there is a different virulent subset of *Aeromonas* species isolated from the diseased fish and the environment.

At the present study, in order to know the distribution difference of the *Aeromonas* between diseased fish and water environment, samples were collected form the diseased fish and pond water in the south of Jiangsu Province, China. All strains were sequenced with 16S *rDNA* and *gyrB* gene to identify the species. Meanwhile three virulence genes, *aer, hly* and *alt*, were screened to compare the difference between host and environment.

Presumptive *Aeromonas* strains were isolated from host (diseased fish) and water environment in the south of Jiangsu Province China. The diseased fish showed heavy dark, hemorrhaging in the eye and mouth, in the vicinity of the opercula, around the vent and the base of the fins, and on the surface of the body. Some of them even showed abdominal swelling with lots of ascetic fluid. Bacteria isolated from niches of diseased fish, such as fish body, gill, liver, intestine, vent, ascetic fluid *etc.*, grew on plates of Rimler-Shotts Medium. Pond water were coated on plates of Rimler-Shotts Medium too. The isolates were presumptively identified as *Aeromonas* species by Gram-staining, cytochrome oxidase, catalase and oxidative/fermentative acid production from glucose (Hugh and Leifson, 1953). The putative *Aeromonas* strains (n = 71, 43 from diseased fish and 28 from water) were used in further analysis. DNA was extracted by using the UNIQ-10 Column Bacterial Genomic DNA Isolation Kit (Sangon Biotech (Shanghai) Co., Ltd, China). Strains were identified by the sequence of 16S *rDNA* and *gyrB*. Primers 16S *rDNA* and *gyrB* were in table I. Results were compared in a BLAST homology search with *Aeromonas* gene sequences deposited in the GenBank database.

All *Aeromonas* isolates could be divided into 4 species (table II), including *A. veronii* (55), *A. hydrophila* (11), *A. salmonicida* (3) and *A. media* (2). There were 43 strains isolated from the diseased fish, among which 30 were *A. veronii*, 10 were *A. hydrophila* and 3 were *A. salmonicida*. 28 strains were isolated from water environment, including *A. veronii* (25), *A. hydrophila* (1) and *A. media* (2). *A. veronii* was the most popular species whether *Aeromonas* strains were isolated from diseased fish (30) or from water (25). This was in agreement with previous studies (Figueras 2005; Ottaviani *et al.*, 2011; Wuming Yang, 2010; Hu *et al.*, 2012).

However, in a total of 11 *A. hydrophila* strains 10 were isolated from diseased fish and only 1 was from water environment. To compare the appearance ratio of *Aeromonas* species between the diseased fish and pond water using SPSS 11.0 Nonparametric Tests, it

Table I
Primers and annealing temperatures of 16S *rDNA*, *gyrB* and virulence genes

Genes	Primers	Tm (°C)
16S *rDNA*	F: 5'-CAC GGA TCC AGA GTT TGA T(C/T) (A/C) TGG CTC AG-3' R: 5'-GTG AAG CTTACG G (C/T)T ACC TTG TTA CGA CTT-3'	52
gyrB	F: 5'-TCC GGC GGT CTG CAC GGC GT-3' R: 5'-TTG TCC GGG TTG TAC TCG TC-3'	59
aer	F: 5'-GCTGAACCCATCTATCCTG-3' R: 5'-TTTCTCCGGTAACAGGATTG-3'	50
hly	F: 5'- GGCCGGTGGCCCGAAGATACGGG-3' R: 5'-GGCGGCGCCGGACGAGACGGG-3'	65
Alt	F: 5'-TGACCCAGTCCTGGCACGGC-3' R: 5'- GGTGATCGATCACCACCAGC-3'	60

Table II
Aeromonas isolates obtained from diseased fish and water environment (%)

Diseased fish	Crucian Carp	Black Carp	Grass Carp	Subtotal	Water Environment	Bluntnose Black Bream	Total
Aeromonas veronii	7	8	2	13	30	25	55
Aeromonas hydrophila	1	/	5	4	10	1	11
Aeromonas salmonicida	/	/	3	0	3	/	3
Aeromonas media	/	/	/	/	/	2	2
Subtotal	8	8	10	17	43	28	71

Table III
Virulence genes positive ratio of *A. veronii* and *A. hydrophila* isolated from the diseased fish and water environment (%)

Species	Isolated from	*aer*	*hly*	*alt*
A. veronii	Diseased fish	23 (7/30)	41 (12/30)	33 (10/30)
	Pond water	24 (6/25)	28 (7/25)	50 (15/30)
A. hydrophilla	Diseased fish	20 (2/10)	80 (8/10)	20 (2/10)
	Pond water	100 (1/1)	100 (1/1)	0 (0/1)

could found that there was no difference of *A. veronii* between diseased fish and water environment, 69.77% and 89.29% respectively, but *A. hydrophila* was significantly different ($P < 0.05$). *A. hydrophila* isolates were significantly more frequent from diseased fish than from water. A similar result was found by Nielsen *et al.* (2001).

All *Aeromonas* isolates were screened for three putative virulence genes, *aer, hly* and *alt*. The primers are shown in table I. Of 71 strains analyzed, 50 (70.43%) were positive for at least one of the virulence genes examined. Three virulence genes were present in 4/71 (5.63%) of isolates, among which 3 strains were isolated from diseased fish and the other was from water environment. While 17 (23.94%) isolates contained two virulence genes, among which 10 were isolated from diseased fish including 5 *A. veronii*, 4 *A. hydrophila* and 1 *A. salmonicida*, and 7 were isolated from water environment including 6 *A. veronii* and 4 *A. hydrophila*. The number of *Aeromonas* isolates containing one virulent gene was the highest. 31(43.66%) isolates, and included 18 from diseased fish and 13 from water environment.

The positive rate of virulence gene of *A. veronii* and *A. hydrophila* was compared between isolates from diseased fish and water environment (table III). In *A. veronii*, *hly* was present in nearly half of the strains from diseased fish while *alt* was present in half of the isolates from water environment. In *A. hydrophila*, *hly* was positive in most of the isolates not only diseased fish but also water environment. The positive rate *hly* was highest among three virulence genes. Several recent studies reported the involved virulence factors in fish infections (Boyd *et al.*, 2008; Dacanay *et al.*, 2010; Li *et al.*, 2011). Janda and Abbott (2010) found that only a small subset of strains containing genes for potential virulence factors seems to cause infection. It is not clear whether there is a virulent subset of *Aeromonas* species prevalent in clinical isolates with the ability to cause freshwater fish infections.

Our results showed that the species distribution in *Aeromonas* isolates from diseased fish and water environment in Jiangsu Province of China was similar without significant differences. They indicated that *Aeromonas* species in aquatic environments are varied and have considerable virulence potential.

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