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Characterization of *Aeromonas hydrophila* from hemorrhagic diseased freshwater fishes in Anhui Province, China

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Abstract

Aeromonas hydrophila currently has the status of a foodborne pathogen causing zoonotic diseases spreading from animals to humans. Sixty of typically hemorrhagic diseased freshwater fishes were collected from twelve aquafarms in Anhui Province. Twenty of *A. hydrophila* isolates were isolated and characterized by RAPD-PCR, antibiotics susceptibility testing and determination of virulence factors. RAPD-PCR fingerprinting revealed the complex diversity and genetic polymorphism (I-XIV RAPD types) with *D* of 0.958 on 90% patterns similarity and eight resistance patterns were observed by antibiotics susceptibility testing with *D* of 0.747. Furthermore, the virulence genes were present in 85% (*aer*), 40% (*epr*), 75% (*ast*), 35% (*ahyB*), 35% (*act*) and 80% (*alt*) of the strains, respectively. The result indicated that the same characterization (I RAPD type, resistance pattern and virulence factors) was found in *A. hydrophila* isolates from A aquafram, showing their close genetic relationship or origins.

Introduction

Anhui province is the second largest region of freshwater culture area in Chinese Mainland. Freshwater aquaculture was an important industry, which has been developing rapidly. *A. hydrophila* is an important pathogen causing freshwater fishes hemorrhagic diseases, widely distributed in the food, drinking water and environment (Daskalov, 2006). More importantly, a fact is that *A. hydrophila* is also the cause of zoonotic diseases or food borne infections (Kirov, 1993; Krovacek *et al*., 1995; Daskalov, 2006).

Generally, phenotypic analyses and molecular typing methods are powerful tools for determining whether isolates recovered from different hosts or environments are related, providing evidence for a common source of transmission or infections (Beaz-Hidalgo *et al*., 2010). Several typing methods such as RAPD-PCR, ERIC-PCR and resistance patterns were used to determine genetic diversity and relationship between *Aeromonas* strains from different samples (Beaz-Hidalgo *et al*., 2006; Maiti *et al*., 2009; Beaz-Hidalgo *et al*., 2010). In China, hemorrhagic diseases due to *A. hydrophila* infections in aquaculture of freshwater fishes had caused huge economic losses every year (Beaz-Hidalgo *et al*., 2006; Maiti *et al*., 2009; Beaz-Hidalgo *et al.*, 2010). So, molecular and phenotypic characterization of *A. hydrophila* isolates from diseased fishes will be helpful for determining sources of pathogens to control the spread of diseases outbreaks. In this study, twenty of *A. hydrophila* isolates from sixty diseased freshwater fishes were characterized by antibiotics susceptibility testing, RAPD-PCR fingerprinting and detection of virulence factors.

Materials and Methods

Collection of fish samples with hemorrhagic diseases

Sixty of hemorrhagic diseases samples including 25 of *Hypophthalmichthys molitrix*, 25 of *Carassius aumtus*, and 10 of *Parabramis pekinensis* were collected from the 12 aquafarms (A-E from Hefei city, F-H from Bengbu city, I-L from Anqing city) in Anhui province of China during June to September 2010. The main symptoms of diseased freshwater fishes contained limosis, operculum bleeding, muscle hemorrhage and hemorrhage ascites.

Isolation of A. hydrophila *isolates*

Isolation protocol of *A. hydrophila* from these samples was described by Vivekanandhana *et al.* with little modification (2005). In brief, all the specimens were rinsed with sterile water to remove

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the adhering particles. The focus of infected body of the fish was swabbed with sterile cotton swab. Then, swabs were transferred to alkaline peptone-water (APW, Haibo, Qingdao) and incubated at 28 ^{o}C for 24 h. After incubation, a loopful of the APW culture was streaked on Aeromonas hydrophila medium (Haibo, Qingdao, China) and incubated at 37^{o}C for 18–24 h. The purple or black colonies were considered as presumptive positive for *A. hydrophila.* The presumptive isolates were confirmed as *A. hydrophila* based on the following reactions: motile, Gram-negative, cytochrome oxidase positive, glucose positive, arginine dihydrolase positive, ornithine decarboxylase negative, ONPG positive, esculin positive, sucrose positive, l-arabinose utilization and fermentation of salicin (Deng *et al*., 2009).

Confirmation of A. hydrophila by 16S rRNA

Genomic DNA of *A. hydrophila* was extracted by a universal extraction kit (Sangon, Shanghai) and was stored at -20^{o}C for further use. The universal primers:5'-AGGAGGTGATCCAACCGCA-3'and 5'-AGAGTTTGATCATGGCTCAG-3' were used to amplify the full 16S rRNA gene which was sequenced (Sangon, China) for homology by Blast in NCBI (*http://blast.ncbi.nlm.nih.gov/Blast*). The PCR mixture (25 µl) consisted of 0.5 µM of primers for each, 2.5 µl of 10× buffers, 200 µm dNTPs, 2.5 mM $MgCl_2$, and 3.0U of *Taq* DNA polymerase (Sangon, Shanghai). The PCR reaction was performed: one cycle at 95^{o}C for 2 min, followed by 30 cycles of 1.0 min at 94^{o}C, 45 min at 5^{o}C, and 1.0 min at 72^{o}C; and the final extension at 72^{o}C for 8 min. PCR products were detected by electrophoresison 1.0% agarose gel with EB staining (0.008%, v/v).

RAPD-PCR fingerprinting of A. hydrophila isolates

For RAPD-PCR fingerprinting, primers CRA26 (5'-GTGGATGCGA-3') and CRA25 (5'-AACGCGCAAC-3') (Neilan, 1995), were used. PCR mixture (25 µl) consists of 1 µM of primer for each, 2.5 µl of 10x PCR buffer, 200 µM dNTPs, 2.5 mM $MgCl_2$, and 3.0U of *Taq* DNA polymerase and 50 ng of DNA template. The PCR conditions are as following: one cycle of 95^{o}C for 5 mins, followed by 30 cycles of 94^{o}C for 1 min, at 52^{o}C for 1 min, and at 72^{o}C for 4 min; and the last extension at 72^{o}C for 8 min. The RAPD-PCR patterns of *A. hydrophyla* isolates were analyzed using average linkage and rescaled distance by software SPSS 17.0. Similarity of RAPD-PCR patterns over 90% was considered to have the same RAPD type.

Resistant patterns of A. hydrophila isolates

Nine antibiotics of different chemical types including Penicillin G (10 unit), Cephalothin (30 µg), Chloramphenicol (30 µg), Tetracycline (30 µg), Streptomycin (10 µg), Vancomycin (30 µg), Norfloxacin (10 µg), Nitrofurantoin (300 µg), Sulphamethoxazole/trimethoprim (19:1, 25 µg) were used to reveal the resistance patterns. The antibiotics susceptibility testing was performed and the results were explained according to the guideline of CLSI (2011).

Detection of virulence genes in A. hydrophila *isolates*

The virulence genes (*act*, *ast*, *aer*, *alt*, *ahyB*, *epr*) in *A. hydrophila* were detected by PCR. The primers were as following:
ast F: 5'-TCTCCATGCTTCCCTTCCACT-3';R:5'-GTGTAGGGATTGAAGAAGCCG-3',
epr F:5'-GCTCGACGCCCAGCTCACC-3', R: 5'-GGCTCACCGCATTGGATTCG-3',
act F: 5'-AGAAGGTGACCACCAAGAACA-3', R: 5'-AACTGACATCGGCCTTGAACTC-3',
ahyB F: 5'-GTTCGTGATGCAGGATG-3', R: 5'-CGCCGTGTTGGTACCAGTT-3');
aer F: 5'-CGCCTTGTCCTTGTA-3'; R: 5'-AACCGAACTCTCCAT-3', and
alt F: 5'-TGACCCAGTCCTGGCACGGC-3'; R: 5'-GGTGATCGATCACCACCAGC-3'.
The PCR application and electrophoresis were described by Sen and Rodgers (2004) and Jiang *et al*. (2010). The positive fragments (331 bp, 387 bp, 232 bp, 421 bp, 301 bp, 442 bp in size) from different virulent genes were selected for sequencing and aligned by Blast in GenBank of NCBI (*http://blast.ncbi.nlm.nih.gov/Blast*).

Results and discussion

Isolation of A. hydrophila *strains*

Previous studies have indicated that *A. hydrophila* has been isolated from the different fish species (Daskalov, 2006; Deng *et al*., 2009). In present study, 20 of *A. hydrophila* strains were isolated from sixty diseased fish samples with hemorrhagic diseases showing 33.3% infection by *A. hydrophila*. Full 16S rRNA gene sequencing indicated that *A. hydrophila* isolates have 98-99% sequences homology with A. hydrophila strains (accession no. GU013470.1, GU205191.1, FJ940823.1) in NCBI by Blast. *A. hydrophila* was mainly isolated from *Carassius aumtus* (8 samples) and *Hypophthalmichthys molitrix* (11 samples). Only one tenth of *Parabramis pekinensis* (1 sample) with hemorrhagic diseases was infected by *A. hydrophila*. From our samples tested in this time, *A. hydrophila* isolates might not

be major pathogen causing *Parabramis pekinensis* hemorrhagic diseases. In addition, 16S rRNA sequencing also showed that other pathogens such as *Aeromonas veronii*, *Klebsiella pneumoniae* were also isolated from these samples (data not shown).

RAPD-PCR patterns of A. hydrophila *isolates*

RAPD-PCR fingerprinting revealed genetic polymorphism and good discriminatory power as shown in Fig1. Fourteen RAPD-PCR types are observed with *D* of 0.958 on the index of discriminatory ability (*D*) (Hunter and Gaston, 1988). Isolates AH1, AH2 and AH3 from aquafarm A had the same RAPD type (I type), which was also observed in isolates AH8 and AH9 (XIII type) showing closely genetic characterization or related origins, while AH17 and AH18 from aquafarm K have the same resistance pattern, but showing II and V RAPD types respectively.

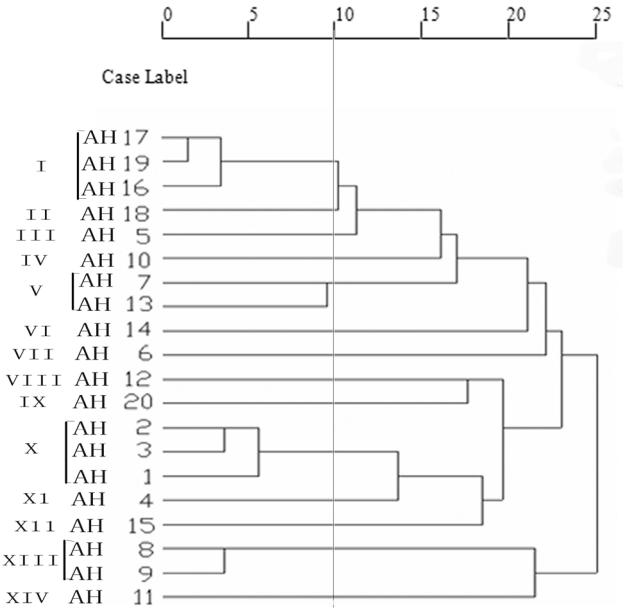

Figure 1. Cluster analysis of RAPD-PCR patterns of *Aeromonas hydrophila* isolates by SPSS15.0 using Average Linkage and Rescaled Distance.

Antibiotic resistance patterns of A. hydrophila *isolates*

Eight antibiotic resistance patterns were observed with *D* of 0.747 as seen in Table 1. All the strains were resistant to Penicillin G which was consistent to previous reports (Joseph *et al.*, 1979; Deng *et al.*, 2009). We also found that all strains are sensitive to streptomycin, which was contrary to the description by Deng *et al*. (2009). This study revealed a frequent occurrence of resistance to cephalothin, penicillin G and vancomycin in association with resistance to other antimicrobial agents. Such high level of multiple resistances may arise from selective pressure due to the unreasonable use of antibiotics. Despite the fact that it is not clear to what extent antibiotics are being used in the study area, their overuse may not be excluded as a major factor (Son *et al.*, 2003).

Table 1. Antibiotics resistance patterns of *A. hydrophila* isolates

A. hydrophila isolates (aqarfarm)	Antibiotics								
	Ce	Te	S/T	Va	Pe	Ni	Ch	St	No
AH1(A)	I	S	R	R	R	I	S	S	S
AH2(A)	I	S	R	R	R	I	S	S	S
AH3(A)	I	S	R	R	R	I	S	S	S
AH4(B)	R	S	R	R	R	I	S	S	S
AH5(C)	I	S	R	R	R	S	S	S	S
AH6(C)	I	S	R	R	R	I	S	S	S
AH7(D)	I	S	I	R	R	S	S	S	S
AH8(E)	I	S	I	R	R	S	S	S	S
AH9(E)	I	S	R	R	R	I	S	S	S
AH10(F)	I	S	I	R	R	S	S	S	S
AH11(F)	I	S	R	R	R	I	S	S	S
AH12(G)	I	S	I	R	R	S	S	S	S
AH13(G)	I	S	I	I	R	I	S	S	S
AH14(H)	R	S	I	R	R	I	S	S	S
AH15(I)	I	S	I	R	R	S	S	S	S
AH16(J)	I	S	S	R	R	S	S	S	S
AH17(K)	I	S	I	R	R	S	S	S	S
AH18(K)	I	S	I	R	R	S	S	S	S
AH19(L)	I	S	I	R	R	S	S	S	S
AH20(L)	I	S	I	R	R	S	S	S	S

Cephalothin (Ce); Tetracycline (Te); Sulphamethoxazole/trimethoprim (S/T); Vancomycin (Va); Penicillin G (Pe); Nitrofurantoin (Ni); Chloramphenicol (Ch); Streptomycin (St); Norfloxacin (No). R: resistance, I: intermediate; S: sensitivity.

Presence of virulence factors in genomic DNA of A. hydrophila *isolates*

Detection of virulence genes indicated that the genes were present in 85% (*aer*), 40% (*epr*), 75% (*ast*), 35% (*ahyB*), 35% (*act*), 80% (*alt*) of the strains respectively as shown in Table 2. Genomic DNA of each strain comprised at least two virulence genes. The difference of virulent genes in genomic DNA may be from geographic variation.

Interestingly, isolates such as AH1, AH2 and AH3 from different pools in aquafarm A show the same RAPD type (I type), resistance patterns and virulence factors, indicating that they have the close relationship in phylogenies. These isolates might be transmitted due to the same water source and implements in aquaculture.

Table 2. Presence of virulence factors in genomic DNA of *A. hydrophila* isolates by PCR

A. Hydrophila isolates(aquafarm)	Virulence factors					
	aer	*epr*	*ast*	*ahyB*	*act*	*alt*
AH1(A)	+	-	+	-	-	+
AH2(A)	+	-	+	-	-	+
AH3(A)	+	-	+	-	-	+
AH4(B)	-	-	+	-	-	+
AH5(C)	-	+	+	+	+	-
AH6(C)	+	-	+	-	-	+
AH7(D)	+	+	-	+	+	+
AH8(E)	+	+	+	-	-	+
AH9(E)	+	-	+	-	+	+
AH10(F)	+	-	-	-	-	+
AH11(F)	+	+	+	+	+	-
AH12(G)	+	+	+	+	+	+
AH13(G)	+	-	+	-	+	-
AH14(H)	+	+	-	+	-	+
AH15(I)	+	+	+	+	+	-
AH16(J)	+	+	-	+	-	+
AH17(K)	+	-	+	-	-	+
AH18(K)	-	-	+	-	-	+
AH19(L)	+	-	+	-	-	+
AH20(L)	+	-	-	-	-	+

"+":positive results; "-": negative results

Conclusion

The results in this study indicated that phenotypic characterization combined with molecular characterization will be helpful to trace the origin of *A. hydrophila* isolates.

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