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Isolation of pathogenic bacteria from the skin ulcerous symptomatic gourami (*Colisa lalia*) through 16S rDNA analysis

Mosharrof Hossain
Division of Food Science and Aqualife Medicine, Chonnam National University, Chonnam-Yeosu-550-749, Korea.

Abstract: *Aeromonas* spp infections are probably the most common bacterial disease diagnosed in cultured warm water fish. In the present study, six strains of *Aeromonas* spp bacteria were isolated from the gourami (*Colisa lalia*) by 16S rDNA sequencing analyses that are pathogenic to freshwater fish. Among them, three were under *Aeromonas veronii* species, two were *Aeromonas* sp ATCC and one was *Aeromonas hydrophila*. *Colisa lalia* usually imported in Korea from the South and South-east Asian countries for recreational purposes. However, they are playing important role as a disease vector or carriers. The infected fish of this study frequently have hemorrhages at the base of the fins or on the skin, and gross ulcerative lesions. Internal signs include, fluid in the abdomen, swollen liver and spleen, and the intestine was distended and fluid-filled. In this study, the utility of 16S rDNA sequencing was employed to isolate *Aeromonas* bacteria from freshwater imported fish are important to environment, veterinary, and clinical purposes.

Key word: Bacteria, *Colisa lalia*, 16S rDNA, *Aeromonas*

Introduction

New molecular techniques, such as the 16S rDNA polymerase chain reaction (PCR) assay have recently been applied to bacteria screening. This assay relies on the amplification of the gene coding for ribosomal RNA (16S rRNA), which is present in almost all bacteria (Lee *et al.*, 2002; Vernon *et al.*, 2002). This assay has several advantages over traditional microbiological methods. It can detect infections by uncultivable pathogens where routine microbiological techniques have failed to detect the presence of bacteria in the clinical samples. In addition, when combined with DNA sequencing the assay provides a definite identification of the infectious agents. The 16S rRNA contains conserved and highly divergent regions. Conserved regions permit the design of broad range PCR primers that will find its target in most bacteria. The genus *Aeromonas* belonging to the family Aeromonadaceae is found in a diversity of habitats, including soil, water, and are pathogens of warm and cold-blooded animals (Palu *et al*., 2005). Water environments and food animals are thus important potential sources for the transmission of *Aeromonas* spp. resulting in human infections (Daskalov, 2006; Kuhn *et al.,* 1997a, b). *Aeromonas* infections caused by bacteria which are present in the water all of the time. Usually, when fish get sick with an *Aeromonas* infection, something happened to make them susceptible to bacterial invasion. There are several species of *Aeromonas* bacteria which can infect fish. The first is *Aeromonas salmonicida*, which causes a disease called furunculosis in salmon and trout. The two species of *Aeromonas* which cause disease in warm water fishes are *Aeromonas hydrophila* and *Aeromonas sobria* (GcGarey *et al.*, (1991). *Aeromonas* infections are probably the most common bacterial disease diagnosed in cultured warm water fish. Generally, mortality rates are low and losses may occur over a period of time. In these instances, some factor; usually stress has caused the fish to become more susceptible to the bacteria. Common sources of stress are poor water quality, over crowding, or rough handling. Some strains of *Aeromonas* spp are more virulent, which means that they possess special properties which enable them to cause more serious disease outbreaks. If these more damaging strains become endemic in a population of fish, it became difficult to introduce new fish into the water body without major losses of newly-stocked fish. Many species have been implicated in fish disease, including *A. hydrophila, A. veronii biovar sobria*, *A. allosaccharophila*, and *A. salmonicida*. Among these, *A. hydrophila*, *A. veronii biovar sobria*, *A. jandaei*, *A. schubertii* and *A. caviae* are most commonly implicated in human intestinal infections (Janda & Abbott, 1998). These species account for about 85% of the clinical isolates of this genus and considered major pathogens (Sen & Rodgers, 2004). *Colisa lalia* is a most common freshwater species recently imported in Korea for the aquarium fish as for recreation. However, recently lymphocystis disease virus was detected in these imported fish make great concern (Hossain *et al*., 2008). Thus, in the present study 16S rDNA was used to investigation of bacterial pathogen of this imported freshwater gourami *Colisa lalia*.

Materials and methods

Sample fish: Skin ulcerative symptomous gourami (*Colisa lalia*) fish was sampled from the ornamental pet shop, Yeosu, Korea in 2008. These fishes were imported to Korea from South and South-east Asian countries, especially Bangladesh, India, Thailand, Singapore, and Hong Kong (personal communication with pet shops owner). Then fishes were acclimatized in freshwater 10 L fiber aquarium in the laboratory conditions approximately 20□±2□. Fishes were allowed to feed by pelleted commercial fish meal daily and closely monitored any death or increase ulcerative symptoms. Finally, the fishes were anaesthetized an overdose of MS 222 (Sigma, St. Louis, USA) or dead subsequently organ dissection.

Bacteria isolation and culture: Bacteria were isolated from different freshly anatomized fish organs like ulcerous lesions, gills, and intestine by a fume sterile loop method. Briefly, the loop heating it red hot and was touch in the respective ulcerative areas or anatomized organs, and then streaking on the pre-prepared 1% brain heart infusion (BHI) agar plates respectively. The agar plates were incubation at 20□ for 24 hours for appropriate colony formation. After the incubation the single colony of each plate was selected for re-isolation to a pure culture.

Genomic DNA isolation: All the bacterial isolates were cultured overnight in 5 ml of brain heart infusion agar medium (1%, NaCl, BHI, Eiken) at 20°C in a shaking incubator, and then it was centrifuged at 5000 ×*g* for 10 min. The bacterial pellet was washed and re-suspended in 0.5 □ of TE-buffer (10 mM Tris–HCl, 1 mM EDTA; pH 8.0), and lysed by 10% sodium dodecyl sulfate (SDS). Then the solution was boiled for 10 min at 60□, and the cellular debris was discarded following centrifugation at 10,000 ×*g* for 5 min. The total genomic DNA was isolated with phenol-chloroform and precipitated with iced cold 70% and 100% ethanol respectively. The resultant pellet was suspension with TE buffer and used as template DNA for PCR amplification.

PCR amplification and sequencing: The presumptive *Aeromonas* spp were identified on the basis of morphology, gram stain, KOH test, catalase and oxidase reaction and traditional biochemical methods (Blazevic *et al*., 1975). The identity of histamine-forming isolates was further confirmed by amplifying and sequencing approximately 1.5 kbp of the 16S ribosomal DNA (rDNA) from culture bacteria. Amplification of histamine-forming bacteria was performed using the universal primers fD1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and Rp2 (5′-ACG GCT ACC TTG TTA ACG ACT T-3′). PCR amplification was performed in 20 □reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 20 pmol of each primer, a 0.2 mM concentration for each of the four deoxynucleotide triphosphates, 0.5 U of Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA), and template DNA (10 ng). Amplifications were carried out for 35 cycles (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s) in a GeneAmp PCR 2400 Thermal Cycler (Applied Biosystems, USA) with an initial denaturizing at 94 °C for 5 min and a final extension at 72 °C for 7 min. Amplicons were detected by electrophoresis on a 1.2 % agarose gel, stained with ethidium bromide (Fig.2). Amplicons were cut off from gel and purified using a Wizard® PCR Purification Kit (Promega, USA) eluted in Tris–HCl (10 mM, pH 8.5) prior to sequencing. The amplified DNA was directly sequenced with the ABI Taq Dye Deoxy Terminator Cycle sequencing kit and ABI Model 377 automated DNA sequencer (Applied Biosystems, USA). The sequences were analyzed with the BLASTN (NCBI) for identification of bacteria.

Results and Discussion

In the present study, six strains of bacteria were isolated from the freshwater imported recreational fish gourami (*Colisa lalia*) in Korea (Table 1).

Table 1. Identification of bacteria using 16S rDNA analysis, using PCR, and BLASTN data sequencing from gourami, *Colisa lalia.*

Organ source	Isolates No.	Highest BLASTN match with bacteria	Similarity (%)	Genebank accession number
Ulcerative lesions	1	*Aeromonas veronii*	100	EF631963
	2	*Aeromonas veronii*	100	EF631962
Gills	3	*Aeromonas* sp. ATCC	100	AB235949
	4	*Aeromonas* sp.ATCC	100	AB235954
Intestine	5	*Aeromonas veronii*	100	EF669480
	6	*Aeromonas hydroplila*	100	EF645799

According to the 16S rDNA analyses, all the isolates showed 100% similarity in the genus *Aeromonas*. Among them, three were under *Aeromonas veronii*, one was *Aeromonas hydrophila* and other two were *Aeromonas* spp. of American type culture collection (ATCC). There is no single physical or behavioral sign specific for *Aeromonas* infections. However, infected fish of this study frequently have hemorrhages at the base of the fins or on the skin, and gross ulcerative lesions (Fig.1A). Internal signs include, fluid in the abdomen, swollen liver and spleen, and the intestine was distended and fluid-filled (Fig.1 B).

Anytime an *Aeromonas* spp infection persists as a chronic problem, therefore, it is important to make an effort to determine if an underlying stress factor is causing the fish to have insufficient immune protection from the bacteria. The genus *Aeromonas* is a complex group of gram negative ubiquitous bacteria commonly isolated from clinical, environmental, and drinking water samples (Kuhn *et al.,* 1997a). *A. veronii* has been reported as a food borne pathogen causing infection in fish, food producing animals, and humans (Austin & Austin, 1993; Isonhood & Drake, 2002; Janda & Abbott, 1996). *Aeromonas* spp. has previously been isolated from ulcerative diseased fish in the Indo-Pakistan region by Iqbal *et al*. (1998). They found 27% *Aeromonas* isolates from fish with ulcerative symptom in Malaysia, Thailand, and Bangladesh belonged to *A. veronii biovar sobria*, and among those 6 of the 11 isolates from Bangladeshi fish. From the above author's description, this paper identified *A. veronii*, indicating that this *Aeromonas* species may constitute an important causative agent of epizootic ulcerative disease in Korea.

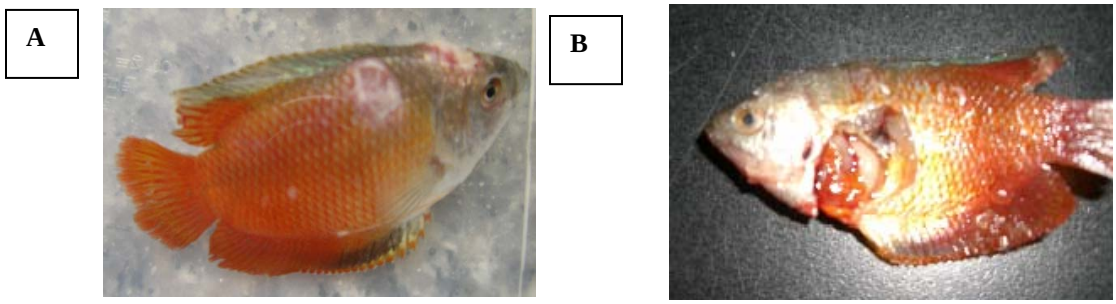

Fig. 1. Skin ulcerous symptomatic gourami,(*Colisa lalia*). A, live fish (arrows showing ulcerative lesions), B, dissected fish (arrows showing hemorrhage and fluid filled intestine).

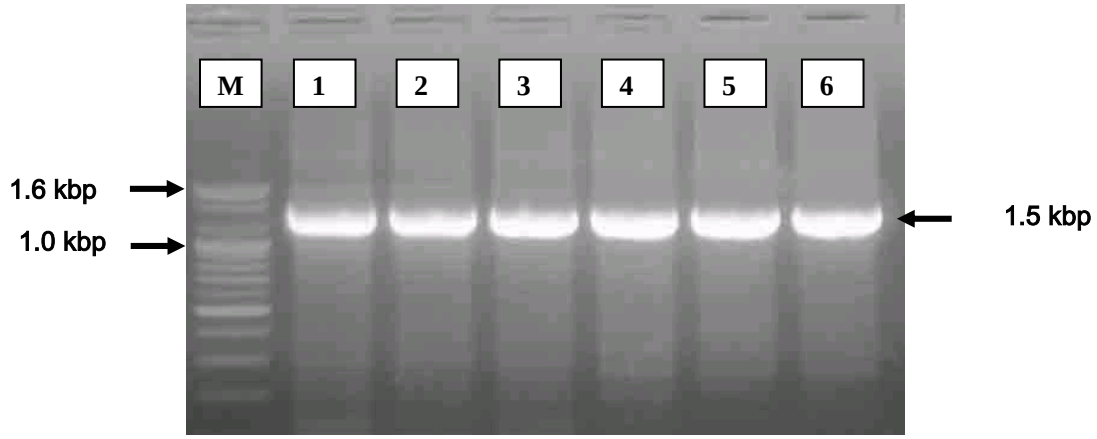

Fig. 2. PCR detection of *Aeromonas* spp. culture bacterial DNA was used for 16S rDNA analysis. M, molecular marker (100bp DNA ladder, Bioneer, Korea); 1&2, ulcerative lesions isolates; 3&4, gill isolates; 5&6, intestine isolates.(kbp, Kilo base pairs).

Although this gourami is an exotic fish in Korea but the same genus *Colisa* spp widely found in the freshwater in South Asian country like Bangladesh (Banu & Bhakta, 1985). *A. veronii* is also the causative agent of the bacterial hemorrhagic septicemia of fish, which, like *A. salmonicida* and *A. hydrophila*, are increasingly considered a major economic problem for the fish-farming industry (Ishiguro *et al.,* 1981; Wiklund & Dalsgaard, 1998).

GcGarey *et al.* (1991) described, a common fish disease is epizootic ulcerative symptom characterized by the presence of severe, open dermal ulcers on the head, on the middle of the body, and on the dorsal regions of the fish. Epizootic ulcer disease has been characterized as an epizootic disease of freshwater fish in the Indo-Pacific region since 1980 (FAO, 1986), and in Bangladesh by Barua *et al.* (1991). They stated that the disease generally develops with ulcers on the fish bodies, and the fish may die within a week of being infected. This ulcerative symptom observed in the present study in Korea was completely similar like Barua *et al*. (1991) in Bangladesh. The disease has caused substantial economic loss to fish farmers and the pet and ornamental fisheries sector. Ulcerative syndrome disease is still unknown; however, organisms belonging to the potentially fish-pathogenic genera *Aeromonas*, *Vibrio*, *Plesiomonas*, and *Pseudomonas* were often isolated from the lesions and blood samples of infected fish (Rahman *et al.*, 2002). Therefore, this study conveys important information to the *Colisa lalia* ulcerative disease in Korea as well as freshwater gourami of other Asian countries. However, representatives of *Aeromonas hydrophila* and *Aeromonas sobria* were recovered most frequently, followed by *Vibrio* and *Plesiomonas* spp. (McGarey *et al*., 1991).

Some bacteria are difficult to identify with phenotypic identification schemes commonly used outside reference laboratories. 16S ribosomal DNA (rDNA)-based identification of bacteria potentially offers a useful alternative when phenotypic characterization methods fail are been used many researcher (Drancourt *et al.*, 2000; Nakatsu *et al.*, 2000; Lee *et al.*, 2002). However, as yet, the usefulness of 16S rDNA sequence analysis in the identification of conventionally unidentifiable isolates has not been evaluated with a large collection of isolates. In this study, the utility of 16S rDNA sequencing as a means to identify *Aeromonas* species obtained from freshwater imported fish are important to environment, veterinary, and clinical purposes. The isolated *Aeromonas* spp are pathogenic itself and also the fish playing an important role as a vector for bacterial disease dispenses. Unlike phenotypic

identification, which can be modified by the variability of expression of characters, 16S rDNA sequencing provides unambiguous data even for rare isolates, which are reproducible in and between laboratories. These pathogenic *Aeromonas* spp bacteria isolated from imported gourami *Colisa lalia* in Korea may be important information to the fish hygiene and quarantine. In conclusion, the new 16S rDNA sequences data analysis pathogenic *Aeromonas* spp bacterial strains in this study are furtherer needed to check pathogenicity in the other fish species or cellular level respectively.

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