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Occurrence, molecular characterization, and antimicrobial susceptibility of *Aeromonas* spp. in marine species of shrimps cultured at inland low salinity ponds

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ABSTRACT

We aimed to document the risk of *Aeromonas* spp. in marine shrimp species cultured in inland low salinity ponds in Thailand. In 14 of 18 shrimp samples retrieved from inland grow-up ponds, *Aeromonas* spp. were detected at ranges from 4667 to 1,500,000 CFU/g body weight. The phylogenetic tree constructed with the *gyrB* and cpn60 concatenated sequences indicated that the 87 isolates consisted of *Aeromonas veronii* (70%), *Aeromonas aquariorum* (18%), *Aeromonas caviae* (7%), *Aeromonas jandaei* (2%), and *Aeromonas schubertii* (2%). The potential virulence of the isolates was examined by phenotypic and PCR assays. Hemolytic activity and the extracellular activity of lipase, DNase, and gelatinase were observed in most isolates (94–99%). PCR revealed the presence of 9 genes related to virulence in the 87 isolates: *act* (75%), *aer* (74%), *alt* (30%), *ast* (1%), *ascV* (34%), *aexT* (24%), *fla* (92%), *ela* (34%), and *lip* (24%). The susceptibility profiles to 14 antimicrobial agents of isolates were typical for the genus, but resistance to cefotaxime, a third-generation cephalosporin, and imipenem were found in two *A. aquariorum* and in three *A. veronii* isolates, respectively. These resistances were confirmed by determining minimum inhibitory concentrations. Our results indicate that the microbiological risk posed by *Aeromonas* should be considered for marine shrimp species that are cultured in low-salinity ponds. These shrimps may also be a vehicle for the transfer of different genotypes of *Aeromonas* and antibiotic-resistant determinants to regions worldwide through trade.

1. Introduction

Aeromonas spp. are ubiquitous, Gram-negative microbes commonly found in fresh water and estuary environments (Janda and Abbott, 2010). They can be isolated from water, fish, invertebrates, soil, and food. Members of this genus have been the focus of increased attention because of their potential to act as pathogens in humans and aquatic animals. In humans, they are responsible for intestinal and extra intestinal diseases ranging from relatively mild illnesses such as gastroenteritis and wound infections to life-threatening conditions such as septicemia and necrotizing fasciitis (Janda and Abbott, 2010). These infections are thought to occur through contact or consumption of water and food contaminated with the bacteria.

Shrimp are a popular seafood worldwide. The increased demand for shrimp has encouraged expansion of the aquaculture industry in South Asia and Latin America. The production of marine shrimps from aquaculture facilities was close to 3.8 million tons in 2010 (FAO, 2012). The major microbiological hazard posed by shrimps to human health is thought to be species of halophilic bacteria from the genus *Vibrio* (Dalsgaard et al., 1995; Gopal et al., 2005), primarily because shrimp farming began in coastal areas. As the

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industry has expanded, marine shrimp are now cultured in both coastal and inland areas (Roy et al., 2010). Introduction of the white leg shrimp *Litopenaeus vannamei*, which is native to Latin America, has resulted in further increases in the production of shrimp in inland culture facilities in most regions, including South Asian countries (Szuster, 2006). This shift into inland environments has been associated with a decrease in the salinity of pond waters. This change has likely affected the bacterial community normally associated with shrimp and may have resulted in an increase in bacteria that are typically associated with inland water environments, such as aeromonads. There is, however, little information on *Aeromonas* spp. associated with cultured shrimps.

Aeromonas spp. currently includes 24 published species. Among these, *Aeromonas hydrophila, Aeromonas veronii* bv sobria, and *Aeromonas caviae* are responsible for the majority of human infections and clinical isolations. Some other species such as *A. veronii* biover veronii, *Aeromonas jandaei*, and *Aeromonas media* are infrequently implicated in human diseases. Identification of these species is difficult using conventional biochemical methods alone due to the taxonomical complexity (Janda and Abbott, 2010). The 16S rDNA sequence used for bacterial identification exhibits high similarity among *Aeromonas* species. Recently, the sequencing of housekeeping genes (*gyrB* and *rpoD*) has proved to be useful for phylogenetic analysis and species identification of *Aeromonas* spp. (Yáñez et al., 2003; Soler et al., 2004).

The pathogenesis of *Aeromonas* species is a function of a variety of virulence factors, including cytotoxic enterotoxin, hemolysin, cytotonic toxins, the secretory system, and flagella (Janda and Abbott, 2010). Additionally, *Aeromonas* produce a variety of extracellular hydrolytic enzymes such as lipases, proteases, and DNase, which are involved in the degradation of host cell components. Documentation of phenotypic and genetic traits of potential virulence factors is an important step in determining the potential pathogenicity of aeromonads.

The resistance of bacteria associated with food animals to antimicrobial agents represents a potential health threat. Shrimp are intensively cultured and antimicrobials are often used as therapeutic and prophylactic agents to bacterial infections (Holmström et al., 2003). A number of studies have demonstrated resistance of bacteria to antimicrobial agents, although primarily in *Vibrio* species (Chanratchakool et al., 1995; Tendecia and de la Peña, 2001). In clinical *Aeromonas* spp., resistance to a variety of antimicrobial agents has been reported (Overman and Janda, 1999; Aravena-Román et al., 2011). *Aeromonas* spp. with resistance to multiple agents have also been isolated from the environment (Girlich et al., 2011). The shift of shrimp farming into inland areas may increase the impacts from agricultural activity around the ponds, including contamination by antimicrobials.

Thailand is one of the primary producers of cultured shrimps and is the largest exporter worldwide (Tanticharoen et al., 2008). The most commonly cultivated species include the native giant tiger shrimp, *Penaeus monodon*, and the non-native *L. vannamei*. We surveyed the prevalence of bacteria belonging to the genus *Aeromonas* in these two species of shrimps cultured in inland low salinity ponds in Thailand. We evaluated the diversity of the *Aeromonas* isolates at the species level using *gyrB* and cpn60 sequence data. We also examined the phenotypic and genetic traits related to virulence, and the susceptibility of the *Aeromonas* isolates to antimicrobial agents.

2. Materials and methods

2.1. Sampling of shrimps and isolating of Aeromonas spp.

Cultured shrimps (white-leg shrimp *L. vannamei* and black-tiger shrimp *P. monodon*) were collected from sixteen inland grow-up ponds located in Bangkok city and four provinces in the central plains region of Thailand, as described in our previous study (Yano et al., 2014). In two of the ponds, both shrimp species were cultured together. Five or more shrimps from each pond were pooled, rinsed with sterile phosphate-buffered saline solution (PBS), and weighed. Shrimp samples were homogenized with nine volumes of sterile PBS solution. Serial ten-fold dilutions of the homogenates in PBS were prepared, and 0.1 ml of the dilutions was spread in duplicate onto trypticase soy agar (TSA; Difco, USA) supplemented with 16 μg/ml ampicillin (amp-TSA). The plates were incubated for 2 d at room temperature (around 25 °C) controlled with an air conditioner, and the resultant colonies were counted. In each sample, 12 colonies from the amp-TSA and TSA plates, respectively, were randomly selected and purified on the media described above.

2.2. Identification of isolates

Isolates from the shrimp samples were identified at the genus level based on their 16S rRNA gene sequences. A single colony from fresh cultures was suspended in 500 μl TE and incubated at 98 °C for 10 min. The tube was centrifuged for 2 min at 12,000 g and the genomic DNA in the supernatant was collected for use as a template. PCR amplification and sequencing of the 16S rRNA gene were performed as described previously (Satomi et al., 2003). A total of 87 *Aeromonas* spp. isolates were further identified to the species level by analyses of housekeeping genes *gyrB* and *cpn60* (Yáñez et al., 2003; Miñana-Galbis et al., 2009). The construction of phylogenetic tree by the neighbor-joining method (Saitou and Nei, 1987) was performed using the ClustalW computer program (Thompson et al., 1994). The reference gene sequences of the following strains were obtained from NCBI: *Aeromonas aquariorum* MDC47 (FJ936120), *A. caviae (punctata)* CECT838 (AY101783), *A. hydrophila* CECT839 (EU306804), *A. jandaei* strain CECT4228 (AY101780), *Aeromonas salmonicida* CECT894 (AY101773), *A. veronii* bv. sobria CECT 4246 (AY101775), and *A. veronii* bv. veronii CECT 4257 (AY101795). Nucleotide sequences were deposited in the GenBank database with the accession numbers LC003043 to LC003129 for *gyrB* and LC003130 to LC003216 for *cpn60*. The *Aeromonas* isolates were also subjected to biochemical tests (amino acid decarboxylases, and arginine hydrolase).

2.3. Phenotypic and genetic traits of potential virulence factors

The phenotypic characteristics of the 87 *Aeromonas* isolates were evaluated as follows. Hemolysis activity was assayed on a sheep blood agar plate (Eiken, Japan) at 37 °C. Lipase activity was assayed on TSA plates supplemented with 1% of Tween 80 (v/v). Extracellular protease activity was assayed on TSA plates supplemented with 1% of casein (v/v). Gelatinase production was measured by assay of isolates in gelatin medium (3% beef extracts, 5% peptone, and 15% gelatin, pH 7.0). To test for DNA degrading activity, isolates were assayed on DNase agar plate (Eiken, Japan). All media plates were incubated at 37 °C.

The presence of 9 genes encoding virulence factors was determined using PCR in the isolates. Cytotoxic enterotoxin (*act*), cytotonic enterotoxins (*alt, ast*), flagella (*fla*), lipase (*lip*), and elastase (*ela*) were detected and characterized using the methodology described by Sen and Rodgers (2004), with a slight modifications whereby the genes were independently amplified. The genes (*alt, ast*) were also detected using the method by Aguilera-Arreola et al. (2007). Aerolysin (*aer*) was detected with primers described by Chacón et al. (2003). Type three secretion systems (TTSS) (*ascV* and *aexT*) were examined using the methodology described by Martino et al. (2011). The PCR products with predicted sizes were sequenced in 21 representative isolates for confirmation.