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Distribution of Putative Virulence Genes in *Aeromonas hydrophila* and *Aeromonas salmonicida* Isolated from Marketed Fish Samples

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Abstract: The study sought a prevalence of virulent *A. salmonicida* and *A. hydrophila* in marketed fish samples by the molecular methods. A significant score of incidences (31.25 and 15.6% of *A. salmonicida* and *A. hydrophila*, respectively) were recorded and more diversification among the isolates based on their availability. It is important threat to the people who are consuming contaminated fish and other seafoods. Existence of putative virulence genes *hlyA* and *aerA* provides evidence for mutifactorial activities, which is encoded by the virulence factors like haemolysin and aerolysin and thus has the potential pathogenic. It was apparent that representatives of the four genotypes (*hlyA*$^+$ *aerA*$^+$, *hlyA*$^-$ *aerA*$^+$, *hlyA*$^-$ *aerA*$^-$ and *hlyA*$^+$ *aerA*$^-$) were detected. The role in assessing *Aeromonas* influences on adverse public health is warranted

Key words: Seafoods, *Aeromonas hydrophila*, haemolysin, aerolysin

INTRODUCTION

Aeromonas sp. are ubiquitous inhabitants of aquatic ecosytems such as freshwater, coastal water and sewage (Havelaar *et al*., 1992; Massa *et al*., 2001). These bacteria are usually microbiota as well as primary or secondary pathogens of fish, amphibians and some motile species (mainly *Aeromonas caviae*, *Aeromonas hydrophila* and *Aeromonas veronii* bv. *sobria*) are opportunistic pathogens of humans (Cahill, 1990; Janda and Abbott, 1998). The genome consists of psychrophiles and mesophiles from aquatic and soil environments. Among the species belonging to *Aeromonas* genus, one of the most important is *A. salmonicida*, a fish pathogen which causes a common disease among salmonids, named furunculosis (or) ulcerative furunculosis. *A. hydrophila* has been widely studied, being responsible for a variety of fish pathological conditions, altogether named aeromonosis, which occur in nature or in artificial environment. *Aeromonas* has also been recognized as a potential cause of food-associated gastroenteritis outbreaks. It causes wide spectrum of infections including soft tissue (wound) infections, occular infections, tonsillitis, endocarditis, pneumonia, urinary track infections, osteomyelitis, meningitis and septicaemia in human (Ljungh and Wadstrom, 1983; Lakshmanaperumalsamy *et al*., 2005). These infections often occur in immunocompromised hosts (Subashkumar *et al*., 2006).

Aeromonas species are enteropathogens. Such strains possess virulence properties, such as the ability to produce enterotoxins, cytotoxins, haemolysins and or ability to invade epithelial cells (Janda, 1991; Tsai and Chen, 1996; Soler *et al*., 2002). The main virulence factors of *Aeromonas* sp. that can be associated with gastroenteritis. The multiplicity of extracellular products of motile *Aeromonas* has led to difficulty in characterizing these factors and to disagreement about their properties, as well as about their direct involvement in the enteropathogenicity of *Aeromonas* sp. This has resulted in a great deal of controversy and confusion.

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The primary toxin haemolysins are produced, of which the most significant is aerolysin, a heat-labile β-haemolysin, expressed by many strains of *A. hydrophila* (Chopra *et al*., 1991; Janda, 1991; Gosling, 1996; Howard *et al*., 1996). It was reported as a pore forming cytolysin, able to cause damage to the cell membrane bilayer causing leakage of cytoplasmic contents. An interesting approach for the direct detection of potential pathogenic *A. hydrophila* isolates is the use of virulence determinants as genetic markers. In addition it has been suggested that variation in the distribution of potential virulence genes amongst *Aeromonas* might contribute to their degree of virulence factors including haemolytic toxin (*aerA* and *hlyA*), heat labile cytotoxic enterotoxin (*Act*), heat stable cytotoxic enterotoxin (*Ast*), flagellin (*fla*) and elastase (*ahyB*). PCR technique (Howard and Buckley, 1986). In addition it has been suggested that variation in the distribution of potential virulence genes amongst *A. salmonicida* and *A. hydrophila* isolates might contribute to their degree of virulence factors including haemolytic toxin (*aerA* and *hlyA*), heat stable cytotoxic enterotoxin (*Ast*) (Howard and Buckley, 1986). Attention has been given to find out the presence of virulence gene in the bacterium isolated from marketed fish sources. Despite the number of studies on the incidence of *A. salmonicida* and *A. hydrophila* were undertaken in this region. Hence the study was undertaken to find the detection of virulence factor intended for human consumption in Chennai, India.

MATERIALS AND METHODS

Isolation and Identification of Bacterial Isolates

Presumptive *Aeromonas* sp. cultures were isolated from fish samples gathered from various vendors of fish markets situated in Vadapalani, Chennai, India during August 2006 to February 2007. The edible flesh portion of the fishes (10 g) were macerated and transferred into 10 mL sterile distilled water and kept it for shaking incubation at 37°C for 15 min. After incubation, 1 mL of suspension was transferred to Alkaline Peptone Water (APW) and incubated at 37°C for 18 h. Bacterial colonies were grown on Starch-Ampicillin (SA) agar plates (Palumbo *et al*., 1985) and Gram-negative, oxidase positive and catalase-positive isolates were plated out onto selective Rimler Shotts (RS) agar medium (HiMedia, India) and yellow-to honey-coloured colonies were taken for confirmation analysis by using Kaper's multitest medium. Appearance of alkaline surface and acid butt after 24 h at 37°C demonstrated the presence of *A. hydrophila*, whereas black butt exhibited the presence of *A. salmonicida*. The presumptive identification was performed by oxidase reaction, fermentation of glucose, presence of lysine, arginine decarboxylation and absence of ornithine decarboxylase, proposed by Cowan and Steel (1970).

Detection of Virulence Genes in *A. salmonicida* and *A. hydrophila*

Primers

Unique primers were designed for the amplification of the genes coding for the cytotoxic virulence factor of *Aeromonas* isolated from fish, shrimps and water sources. All the available partial and full-length gene sequences for a given virulence factor was determined according to Sen and Rodgers (2004). *hlyA* gene primer F1 (5'-GGC CGG TGG CCC GAA GAT GCA GG-3') and R1 (5'-GGC GGC GCC GGA CGA GAC GGG-3') to amplify a 597 bp; *aerA* gene primer F1(5'-GCC TGA GCG AGA AGG T-3') and R1 (5'-CAG TCC CAC CCA CTT C-3') to amplify a 416 bp. Bin-Kingombe *et al*. (1999) also suggested the above primers.

PCR Analysis

Reactions were performed in 25 μL volumes in 0-2 mL optical-grade PCR tubes (Tarsons, India). Each 25 μL of reaction mix contained 1 μM of each primer, 12.5 μL of Taq PCR Master mix (2X) containing, $MgCl_2$, Taq DNA polymerase and dNTPs (Genei, India). The amount of template used was 80 ng in 5 μL volume. Cycling conditions consisted of an initial single cycle at 95°C for 5 min,

followed by 25 cycles of melting for 25 sec at 9524°C, annealing for 30 s at 55°C, elongation for 1 min at 72°C and a final single cycle at 70°C for 5 min. PCR was performed in PCR thermalcycler (MJ Research Model PTC 150 MiniCycler, Watertown, Mass, USA). Each DNA extract was first evaluated with a single primer set. The assay was then made multiplex by including the second primer set. Before performing PCR on the water isolates, each primer set was tested with MTCC control strains to confirm the production of an amplicon of predicted size.

Post-PCR Analysis

The PCR products were detected by subjecting a sample from each reaction tube to 2% agarose gel electrophoresis stained with ethidium bromide (1.6 mg mL^{-1}) at 11 V cm 1 for 90 min in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA (pH 8). Representative PCR amplicons from each primer set were purified by QIAquick PCR purification kit (Qiagen). The sequences of the amplicons were determined in order to confirm the successful amplification of the target genes. Sizes of the amplified products were determined by comparison with DNA ladder (3000-100 bp, Genei, India).

RESULTS

Incidence of *A. salmonicida* and *A. hydrophila*

In this study, occurrence of *A. salmonicida* and *A. hydrophila* in fish were procured from marketed fish specimen. A total of 32 fish samples, 46.87% of fishes were contaminated with *A. salmonicida* and *A. hydrophila* (Table 1). It was the significant level of incidence in the sampling

Table 1: Incidence of *A. salmonicida* and *A. hydrophila* from fish samples

	A. salmonicida		*A. hydrophila*		
Total No. of fishes	Positive	(%)	Positive	(%)	Total (%)
32	10	31.25	5	15.6	46.87

Table 2: Biochemical identification of *A. salmonicida* and *A. hydrophila*

Test	*A. salmonicida*	*A. hydrophila*
Colour of the colony		
Rimler-Shotts medium	Green	Yellow
Kaper multitest medium	Black	Yellow butt
Gram-stain	-	-
Cytochrome oxidase	+	+
Catalase	+	+
Motility	-	+
O/F F	F	
Methyl red	-	+
VP +	+	
Hydrogen sulfide (H_2S)	+	-
Indole	-	+
Citrate: Simmons	+	-
ONPG	+	+
Nitrate-Nitrite	+	+
Acid (-A) and Gas (-G) from		
Arabinose	AG	A
Cellobiose	A	A
Fructose	A	AG
Galactose	--	A
Glucose	AG	AG
Maltose	A	AG
Mannitol	AG	A
Mannose	---	AG
Raffinose	AG	--
Sorbitol	AG	--
Trehalose	A	AG

+: Positive; -: Negative; F: Fermentative; A: Acid; G: Gas

site. About 31.2 and 15.6% of fishes were found to be *A. salmonicida* and *A. hydrophila*, respectively. The fish isolates were identified by using selective identification tests and biochemical tests. The results were presented in Table 2. *A. salmonicida* and *A. hydrophila* has been differentiated easily by Kaper's multitest medium.

Identification of Virulence Gene of *A. hydrophila*

The results of the present study haemolysin was encoded by the *hlyA* and *aerA* genes which were observed in the fish isolates of *A. hydrophila* and *A. salmonicida*. However, this study targeted and aligned the gene of interest was *hlyA* and *aerA* genes from fish isolates. Most of the *A. hydrophila* found conserved region for *hlyA* gene. From these conserved sequences, the respective primers were designed and directed the amplification of 597 bp fragment. Of the 15 samples, presence of *hlyA* gene in 73.3% of the haemolysin producers (Fig. 1). However, *hlyA* gene was not available in whereas strain *As* 5, *As* 8, *Ah* 2 and *Ah* 5. These might have not produce the haemolysin on blood agar plate and 416 bp responsible for the production of aerolysin (Fig. 2). In all other samples has to be homology among the strains except the strain *As* 8 and *Ah* 2. This was demonstrated by a tendency for isolates carrying variable *hlyA* and *aerA* genotypes ($hlyA^+$ $aerA^+$, $hlyA^-$ $aerA^+$, $hlyA^-$ $aerA^-$ and $hlyA^+$ $aerA^-$) available in all the isolates.

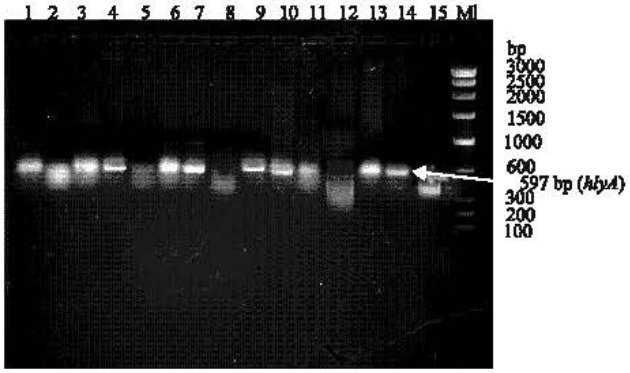

Fig. 1: Haemolysin (*hlyA*) gene of *A. salmonicida* and *A. hydrophila isolated* from fish; Lane 1-15: Strain *As* 1-10, Lane 11-15: Strains *Ah* 1-5, Lane M. DNA Marker (3000-100 bp)

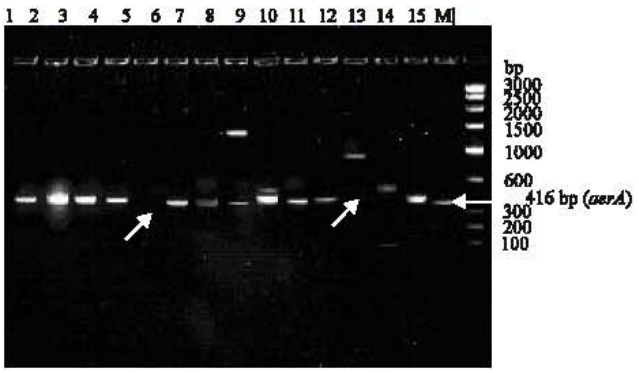

Fig. 2: Aerolysin (*aerA*) gene of *A. salmonicida* and *A. hydrophila* from fish; Lane 1-15: Strain *As* 1-10, Lane 11-15: Strains *As* 1-5, Lane M: DNA Marker (3000-100 bp)

DISCUSSION

Incidence of *A. salmonicida* and *A. hydrophila* in Fish

Fishery products, which are of great importance for human nutrition worldwide and provide clear health benefits (Darlington and Stone, 2001), can also act as a source of foodborne pathogens Overall, the majority of reported seafood-associated outbreaks are caused by toxins (biotoxins and histamine) and viruses (noroviruses and hepatitis A virus) but fish and shellfish may also be a vehicle for pathogenic bacteria naturally occurring in aquatic environments, referred to as indigenous, or derived from polluted waters and/or from post-capture contamination (Huss *et al*., 2003). In the present investigation, a significant level of incidence of *A. salmonicida* and *A. hydrophila* were recorded in the fish samples. During the period of sampling we have observed higher (46.8%) contaminations in the marketed fish this may due to improper handling of fish, transportation of seafoods from the catching area. About 31.2 and 15.6% of fishes were found to be *A. salmonicida* and *A. hydrophila*, respectively.

The incidence of *A. salmonicida* and *A. hydrophila* in fish sold in retail outlets of various parts of the world has been well documented, but distribution of this pathogen in various fishes from retail markets was not well elaborated so far. The result of the present study expressed that the contamination of seafoods with *Aeromonas* in vadapalani market was found to be considerably higher than the previous reports. Although the source of the organisms may be ambient environment, secondary contamination due to catching, transportation, handling and etc. may also contribute for its distribution. Moreover, days of storage and processing of seafood in retail outlets may cause variations in the level of incidence of *A. hydrophila*. This may be the possible reason for the increased prevalence of *Aeromonas* observed in fish markets though all the fishes available in the market has the same source of origin. Because of obvious differences in sampling period, geographical location, origin of the samples and methodology for analysis it is difficult to compare the level of incidence.

Virulence Gene of *A. salmonicida* and *A. hydrophila*

Nucleic acid amplification methods targeting virulence genes are used for detection of pathogenic bacteria and to differentiate pathogenic from non-pathogenic strains (Bin-Kingombe *et al*., 1999; Sen and Rodgers, 2004). Wang *et al*. (2003) developed a multiplex PCR method for detection of hemolysin and aerolysin genes in *A. hydrophila* and *A. sobria* and used it to screen 121 clinical and 7 reference strains for *ahh1*, *asa1* and *aerA* genes. Several authors detected the aerolysin gene coding for the β-hemolysin in *A. salmonicida* and *A. hydrophila* (Pollard *et al*., 1990). Castro-Escarpulli *et al*. (2003) reported that 96% of strains were positive for aerolysin/haemolysin genes and the strains were also found to the hemolytic. Various putative virulence fishes have been described to *A. hydrophila* to explain the gene responsible for their pathogenicity. A number of studies have shown that haemolytic factors are involved in the virulence of aeromonads. Two haemolytic toxins have been described in *A. hydrophila*: the AHH1 haemolysin (Hirono and Aoki, 1991) and aerolysin (Howard *et al*., 1987). These haemolytic toxins have only 18% homology and are distinct (Hirono and Aoki, 1991). Aerolysin is the best studied haemolysins (Howard *et al*., 1987), but *Aeromonas* strains can produce more than one haemolytic toxin with virulence properties (Chopra *et al*., 1991; Wong *et al*., 1998). Most of the *A. hydrophila* found conserved region for *hlyA* and *aerA* gene responsible for the production of haemolysin. From these conserved sequences, the respective primers (*hlyA* and *aerA*) were designed and directed the amplification of 597 and 416 bp fragments.

The *aerA* gene was wide spread in *A. veronii* biotype *sobria* and *A. hydrophila* but less so in *A. caviae*. In contrast, the studies of Pollard *et al*. (1990), showed that the *aerA* was only detected in hemolytic, cytotoxic and entereotoxic strains of *A. hydrophila* but not in *A. veronii* biotype *sobria* and *A. caviae*. Since previous studies (Wong *et al*., 1998), have suggested that the combined effect of aerolysin (*aerA*) and *Vibrio cholerae*. HlyA-like haemolysin (*hlyA*) contributes to virulence

is *A. hydrophila* a different approach for the identification of potentially pathogenic *Aeromonas* isolates is the PCR detection of the genes for the haemolysins (*aerA* and *hlyA*) (Pollard *et al*., 1990; Heuzenroeder *et al*., 1999).

In this study haemolysin (*hlyA*) and aerolysin (*aerA*) were encoded by the *hlyA* and *aerA* gene, respectively, included multifactorial activities. However, this study targeted and aligned the gene of interest was *hlyA* and *aerA* genes from *A. hydrophila* isolated from fishes. However, this study targeted and aligned the gene of interest was *hlyA* genes from *A. hydrophila* and *A. salmonicida* isolated from fishes. Aerolysin is synthesized as a high molecular weight precursor, which crosses the inner bacterial membrane as a preprotoxin containing a signal sequence which is removed cotranslationally (Howard and Buckley, 1985). Most of the *A. hydrophila* found conserved region for *hly A* gene. From these conserved sequences, the respective primers (*hlyA*) were designed and directed the amplification of 597 bp fragment. The range of virulence of aeromonads is thought to result from the variety of genotypes ($hlyA^{+}$ $aerA^{+}$, $hlyA^{-}$ $aerA^{+}$, $hlyA^{-}$ $aerA^{-}$ and $hlyA^{+}$ $aerA^{-}$) present in the environment. Both phenotypic and genotypic heterogeneity are common among aeromonads.

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