Prevalence of Hemolytic and Enterotoxigenic Aeromonas spp. in Healthy and Diseased Freshwater Food Fishes as Assessed by Multiplex PCR

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Abstract

A total of 103 representative colonies from 53 healthy and diseased food fish samples were tested for the incidence of Aeromonas spp. Of which 57% of colonies were positive for Aeromonas spp. by the multiplex PCR (MPCR). On an average, the prevalence was found to be 66% with highest incidence in diseased fishes (76%). About 48% of healthy fish samples were observed to contain Aeromonas. The biomolecular identification revealed that the selected virulent hemolytic and enterotoxigenic genes appeared in four different patterns viz. alt; act/hlyA/aer; alt and act/hlyA/aer; ast, alt and act/hlyA/aer, among the MPCR positive isolates of Aeromonas spp. The overall prevalence of the toxin genes alt, act/hlyA/aer and ast among the MPCR positive Aeromonas isolates was found to be 89.8%, 72.9% and 20%, respectively. The biochemical confirmation of the MPCR positive Aeromonas isolates revealed different species in the tested samples belonging to A. hydrophila, A. sobria, A. caviae, A. jandaei, A. veronii, A. schubertii and A. trota and the dominant species was found to be A. hydrophila (48%).

Keywords: Prevalence; Aeromonas spp.; Freshwater fishes; virulent genes; Multiplex PCR

1. Introduction

The genus Aeromonas is a group of gram-negative rod shaped, oxidase-positive, facultative anaerobic microorganism belonging to the family Aeromonadaceae. It currently comprises 17 hybridization groups, 24 described species, 12 subspecies and 2 biovars and is found worldwide in aquatic environments, including groundwater, surface water, estuarine and marine water, drinking water and wastewater (Janda and Abbott, 2010). Aeromonas is considered as an emerging foodborne pathogen, primarily because of its ability to grow at cold temperatures (Kirov et al., 2001).

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A number of *Aeromonas* spp. are able to grow in raw, cooked, and processed foods, at refrigeration temperature, under modified atmosphere and under modified growing conditions (Devlieghere et al., 2000; Gonzalez-Rodriguez et al., 2002). Aeromonads have been detected in dairy products (4%), vegetables (26–41%), meats and poultry (3–70%), with the largest numbers recorded from fish (72%) and shellfish (31%) (Janda and Abbott, 2010).

Members of the genus *Aeromonas* have received increasing attention as opportunistic as well as primary pathogens in humans, and aquatic and terrestrial animals. Human infections include gastrointestinal tract syndromes, wound and soft tissue infections, blood-borne dyscrasias and various other infections (Janda and Abbott 2010). Among the established species, *A. hydrophila*, *A. caviae*, and *A. veronii* biovar sobria (otherwise known as *A. sobria*) are the most commonly known to cause intestinal and extraintestinal human infections, while *A. veronii* biovar veronii, *A. jandaei*, *A. trota*, *A. schubertii*, *A. popoffii*, *A. bestiarum*, *A. salmonicida*, *A. media*, and *A. eucrenophila* are infrequently or rarely involved in gastroenteritis (Janda and Abbott, 2010).

Virulence of *Aeromonas* spp. is multifactorial and incompletely understood (Pablos et al., 2009). A number of virulence factors such as extracellular enzymes, siderophores, cytotoxic and cytotoxic enterotoxins, haemolysins, endotoxins and cell associated factors such as S-layers, pili, polar and lateral flagella, outer membrane proteins and plasmids were associated with pathogenicity of *Aeromonas* spp. (Snowden et al., 2006; Pablos et al., 2009). Exotoxins are major virulence factors of *Aeromonas* spp. that include a cytotoxic heat-labile enterotoxin (Act), also known as aerolysin/hemolysin (Chopra et al., 1993); a cytotoxic heat-labile enterotoxin (Alt), also known as lipase, extracellular lipase, or phospholipase (Chopra et al., 1996); and a cytotoxic heat-stable enterotoxin (Ast) (Sha et al., 2002). These toxins are encoded by the genes act, alt and ast, respectively (Sen and Rodgers, 2004).

The determination of the pathogenicity among *Aeromonas* food isolates is mostly based on biological assays assessing the cytotoxicity or adherence to human or animal cell lines (Illanchezian et al., 2010; Singh et al., 2010). Although not linked to a biological assay, a positive reaction in a PCR-based detection method targeting virulent gene can be considered as an indication of the potential pathogenicity of the *Aeromonas* isolate under study (Bin Kingombe et al., 2004). In recent years several molecular methods, particularly polymerase chain-reaction-(PCR)-based methods, have been developed for routine identification of the species of *Aeromonas* most frequently involved in human disease (Sen 2005) and for genetically detecting putative virulence genes (Chang et al., 2008; Kannan et al., 2001).

The prevalence of *Aeromonas* spp. in food fish samples has been well documented in India during the past decades (Vivekanandhan et al., 2005; Yogananth et al., 2009; Nagar et al., 2011). But, few studies have been performed to determine the potential pathogenicity of the isolates, and none to compare the genotypic and phenotypic virulence properties of *Aeromonas* from food fish samples. Therefore, in this study, an investigation on the distribution of genes encoding the most important enterotoxins (cytotoxic and hemolytic) in *Aeromonas* strains isolated from freshwater food fishes was undertaken.
2. Materials and methods

2.1 Sampling
A total of 53 samples of freshwater food fishes including 36 healthy and 17 diseased fishes were randomly procured from fish markets of Tuticorin and Playakayal regions of Tamilnadu, India at one week interval for a period of ten months (August 2011- May 2012). Healthy fishes included 14 common carps (Cyprinus carpio), 15 catfishes (Clarias batrachus), 4 tilapias (Oreochromis mossambicus) and 3 barbs (Puntius tetrazona). Diseased fishes included 9 catfishes (Clarias batrachus) and 8 murrels (Channa striatus). The diseased fishes were characterized by septicemia and lesions with accompanying hemorrhages at the bases of fins, and melanosis, to a subacute to chronic variety in older fish, and hemorrhages in muscle (Austin and Adam, 1996). The collected samples were packed in insulated boxes with flake ice and gel ice packs during transportation from the sampling site to the laboratory and used for analysis within 3 h, after reaching the laboratory.

2.2 Bacterial strains, media and reagents
Type cultures of Aeromonas used in this study included Aeromonas hydrophila (ATCC 7966), Aeromonas sobria (MTCC 1608), A. caviae (MTCC 7725) and Aeromonas liquefaciens (MTCC 2654). Other bacterial pathogens such as Salmonella typhi (ATCC 122235), S. paratyphi A (MTCC 735), Vibrio cholerae (NICED 16582), and some isolates of Vibrio spp. viz. V. parahaemolyticus, V. vulnificus and V. alginolyticus obtained from our pathology laboratory were also used. Media and their ingredients were purchased from Hi-Media Laboratories Pvt. Ltd, Mumbai, India. Molecular grade water (Sartorius Stedim Biotech, Gottingen, Germany) was used in the preparation of buffers and reagents.

2.3 Isolation of Aeromonas spp.
About 25 g of muscle tissue from each fish sample was aseptically removed and homogenized using a pestle and mortar. In the case of diseased fishes, the infected muscle tissue was taken for the analysis. The homogenate was enriched in 225 ml buffered peptone water (pH 7.0) for 18 to 24 h at 37°C. The enriched sample was then serially diluted, employing 1 ml of the enrichment broth and 9 ml of saline, and spread plated onto Aeromonas Starch DNA Agar Base (ASDAB) plates and incubated at 37°C for 24h to obtain yellow colonies. Oxidase-positive yellow colonies from each plate were taken to be representative colonies of Aeromonas spp. and were further used for biomolecular identification.

2.4 Biomolecular identification of the isolates
The picked up oxidase-positive yellow colonies were purified, grown on tryptcase soy broth (TSB) and incubated at 37°C for 18 h to obtain young culture prior to the extraction of genomic DNA.

2.4.1. Bacterial DNA extraction
The genomic DNA from bacterial cells was extracted following guanidine hydrochloride method according to Haldar et al. (2005) with slight modifications. Briefly, one ml of the young cell suspension was centrifuged at 10,000 x g for 10 min at 4°C and the cell pellet was mixed with 600 μl of guanidine hydrochloride buffer (pH 8.0), incubated at room temperature for 30 min and again centrifuged at 10,000 x g for 10 min at 4°C. From that, 500 μl of the supernatant was transferred to another tube and mixed with 100% ice cold ethanol and centrifuged at 13000 x g for 20 min at 4°C. The supernatant was discarded and the pellet was washed with 95% and 90% ethanol, respectively.
followed by centrifugation at 10,000 x g for 10 min at 4°C. The pellet was then re-suspended in 50 μl of molecular grade water, quantified in a Biophotometer (Eppendorf AG, Hamburg, Germany) and then stored at -20°C to be further used as PCR template.

2.4.2 Primers
Four sets of primers belonging to genus Aeromonas were selected for the study based on the earlier reports. The primer A16S1 amplifies a 953 bp fragment of 16S rRNA gene of Aeromonas spp. (Pinto et al., 2012) and primers AHS and AHL amplify a 536 bp and 361 bp fragment of the heat-stable enterotoxin (ast) gene and the heat-labile enterotoxin (alt) gene, respectively for Aeromonas spp. (Bin Kingombe et al., 2010). The primer act/hlyA/aer complex amplifies 400 bp fragment of cytotoxic enterotoxin, aerolysin and hemolysin gene of Aeromonas spp. (Balsalobre et al., 2009). All the primer sequences were synthesized by Ocimum Biosolutions Inc., Indianapolis, USA. The oligonucleotide primer sequences, target genes and their product sizes are given in Table 1.

### Table 1 Primers used in the MPCR assay for Aeromonas

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Primer sequences (5’ to 3’)</th>
<th>Target genes</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A16S1</td>
<td>F- CTACTTTTGCCGCGAGCGG</td>
<td>16S rRNA</td>
<td>953</td>
<td>Pinto et al., 2012</td>
</tr>
<tr>
<td></td>
<td>R- TGAATCCGAAGCAGTCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHS</td>
<td>F- GACTTCAATCGCTTCCTCAACG</td>
<td>ast</td>
<td>536</td>
<td>Bin Kingombe et al., 2010</td>
</tr>
<tr>
<td></td>
<td>R- GCATCGAAGCTACTGGTGAGCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>act/hlyA/aer complex</td>
<td>F- AGAAGGTGACYACCAAGAACAC</td>
<td>act : cytotoxic enterotoxin beta-hemolysin</td>
<td>400</td>
<td>Balsalobre et al., 2009</td>
</tr>
<tr>
<td></td>
<td>R- CCACCTTCAACCTCACCAG</td>
<td>hlyA: beta-hemolysin aer: aerolysin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHL</td>
<td>F- TGCTGGCGCTGCTTGCCGAGT</td>
<td>alt</td>
<td>361</td>
<td>Bin Kingombe et al., 2010</td>
</tr>
<tr>
<td></td>
<td>R- AGGAATCGTGTAGCAGAACCAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.4.3 Multiplex PCR (MPCR) assay
The amplification was done with 50 μl of reaction mixture containing template DNA of Aeromonas, 10X PCR buffer (100 mM Tris (pH 9.0), 500 mM KCl, 15 mM MgCl₂, and 0.1% gelatin), 10 mM MgCl₂, 100 mM of each dNTP, 25 pmol of each forward and reverse primers and 1.25 U of Taq DNA polymerase. The MPCR conditions consisted of initial denaturation at 94°C for 3 min, followed by denaturation at 94°C for 60 sec, extension at 72°C for 90 sec for 45 cycles; and final extension at 72°C for 3 min. Only in the case of annealing temperature, it was optimized by performing gradient PCR (Eppendorf AG, Germany) at different temperatures ranging from 63°C to 67°C for 60 sec. The DNA template of Salmonella typhi (ATCC 122235), S. paratyphi A (MTCC 735), Vibrio cholerae (NICED 16582), and other Vibrio spp. viz. V. para-haemolyticus, V. vulnificus and V. alginolyticus were
used as negative control for the assay and template DNA of *Aeromonas hydrophila* (ATCC 7966), *Aeromonas sobria* (MTCC 1608), *A. caviae* (MTCC 7725) and *Aeromonas liquefaciens* (MTCC 2654) were used as positive control. The MPCR products were run on 2% agarose gel stained with ethidium bromide (10mg/ml) in Tris Acetate EDTA buffer (TAE, pH 8.4) using Submarine Electrophoresis System (GE HealthCare Bio-Science Ltd., HongKong) and observed under UV Transilluminator using Gel Documentation System (Alpha Innotech, California, USA). The products were identified in comparison with the 100bp DNA ladder (Real Biotech Corporation, Ohio, USA).

2.5 Species wise identification of MPCR positive *Aeromonas* spp.
All the MPCR positive isolates of *Aeromonas* spp. were further biochemically identified to the species level following Aerokey II (Carnahan et al., 1991), to check the different virulent gene distribution patterns among the various isolates of *Aeromonas* spp.

3. Results

3.1. Isolation of *Aeromonas* spp.
A total of 103 representative colonies from 53 freshwater food fish samples was picked up from ASDAB plates and were tested for the incidence of *Aeromonas* spp. Of which, 59 colonies pertaining to 35 samples were positive for *Aeromonas* spp. by multiplex PCR (MPCR). Of the 35 positive samples, 20 belonged to healthy fishes and 15 belonged to diseased fishes. More specifically, in the case of healthy fishes, 8 out of 14 carps, 9 out of 15 catfishes, 1 out of 4 tilapias and 2 out of 3 barbs samples showed the presence of *Aeromonas* spp. In the case of diseased fishes, all the 9 samples of catfishes and 6 out of 8 murrels tested showed the presence of *Aeromonas* spp. (Table 2). In the case of healthy fish samples, the prevalence of *Aeromonas* spp. was found to be highest in barbs (67%), followed by catfishes (60%), carps (57%) and tilapias (25%). Among the diseased fishes, the prevalence of *Aeromonas* spp. was found to be highest in catfishes (100%). Overall, 66% of the tested samples were found to be positive for *Aeromonas* spp.

3.2. Bacterial DNA extraction
The extraction carried out using the guanidine hydrochloride method gave good DNA yield within 2h. The extracted genomic DNA varied from 60 to 80µg/ml for the different bacterial type cultures of *Aeromonas* spp. tested.

3.3. Multiplex PCR (MPCR) assay
The optimum annealing temperature for the MPCR of the selected four sets of primers was found to be 65°C for 60 sec (Fig. 1). The MPCR produced an intense band of the expected product for these primers. Repeated PCR amplification of these genes gave reproducible results. The MPCR assay when performed by using the DNA extracted from *Salmonella typhi*, *S. paratyphi* A, *Vibrio cholera*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus* did not amplify with any of the selected genes (Fig. 2).
Fig 1. Ethidium bromide stained 2% agarose gel showing results of optimization of MPCR for hemolytic and enterotoxigenic strains of *Aeromonas* spp. in healthy and diseased freshwater food fishes. Lane 1: 63°C; 2: 64°C; 3: 65°C; 4: 66°C; 5: 67°C; M: 100 bp DNA marker

When the MPCR was performed for *Aeromonas hydrophila*, *A. sobria*, *A. caviae* and *A. liquefaciens*, it showed the amplification of these genes in different patterns (Fig. 3). This indicated that each of the selected oligonucleotide primers for the respective targeted gene segments were specific for the detection of *Aeromonas* in genus level.

Fig 2. Ethidium bromide stained 2% agarose gel showing results on specificity of the MPCR for hemolytic and enterotoxigenic *Aeromonas* spp. in healthy and diseased freshwater food fishes. Lane 1: *A. hydrophila*; M: 100 bp DNA marker; 2: *Salmonella typhi*; 3: *S. paratyphi* A; 4: *Vibrio cholerae*; 5: *V. parahaemolyticus*; 6: *V. vulnificus*; 7: *V. alginolyticus*
The biomolecular identification of the isolates from healthy and diseased fish samples by MPCR showed that the 16S rRNA gene was amplified for all the positive isolates of Aeromonas spp. The other selected virulent hemolytic and enterotoxigenic genes appeared in four different patterns viz. alt; act//hlyA/aer; alt and act//hlyA/aer; ast, alt and act//hlyA/aer among the MPCR positive Aeromonas spp. All the Aeromonas isolates were positive for at least one of the selected virulent genes.

The alt gene was found to be dominant among the positive isolates of Aeromonas spp. from healthy fish samples, followed by combined pattern of alt and act/hlyA/aer genes (Table 2). Of the 11 positive isolates of Aeromonas spp. from 8 carps samples, 5 isolates were positive for alt gene, 3 for alt and act/hlyA/aer genes and 3 for ast, alt and act/hlyA/aer gene pattern. Of the 17 positive Aeromonas spp. isolated from 9 positive catfishes samples, 8 were positive for alt gene, 1 for act/hlyA/aer gene, 6 for alt and act/hlyA/aer genes and 2 for ast, alt and act/hlyA/aer genes. All the positive Aeromonas spp. isolated from tilapia samples was positive for alt and act/hlyA/aer genes. All the isolates of Aeromonas spp. from barbs samples were positive for alt gene.

In the case of the diseased fishes, a different trend was observed. A combined pattern of genes was dominant with alt and act/hlyA/aer, followed by ast, alt and act/hlyA/aer genes. More specifically, of the 14 positive Aeromonas spp. isolated from 9 positive samples of catfishes, 5 were positive for act/hlyA/aer gene, 6 for alt and act/hlyA/aer genes and 3 for ast, alt and act/hlyA/aer genes. Of the 12 positive Aeromonas spp. isolated from 6 positive murrel samples, 8 were positive for alt and act/hlyA/aer genes and 4 for ast, alt and act/hlyA/aer genes (Table 2). The overall prevalence of the toxin genes alt, act/hlyA/aer and ast among the MPCR positive Aeromonas isolates was observed to be 89.8%, 72.9% and 20%, respectively.
Table 2 MPCR showing different gene patterns for representative colonies isolated from different healthy and diseased freshwater food fishes

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. of sample tested</th>
<th>No. of positive samples by MPCR assay</th>
<th>No. of representative colonies tested</th>
<th>Number positive representative colonies by MPCR in different gene patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy fishes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carps</td>
<td>14</td>
<td>8(57)</td>
<td>27</td>
<td>Number of positive colonies: 11(41) for 16S rRNA, 5(19) for act, and 3(11) for ast, 3(11) for alt/haA/aer</td>
</tr>
<tr>
<td>Cat fishes</td>
<td>15</td>
<td>9(60)</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Tilapi as</td>
<td>4</td>
<td>1(25)</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Barbs</td>
<td>3</td>
<td>2(67)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Disease fishes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cat fishes</td>
<td>9</td>
<td>9(100)</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Murrels</td>
<td>8</td>
<td>6(75)</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>53</strong></td>
<td><strong>35(66)</strong></td>
<td><strong>103</strong></td>
<td><strong>59(57)</strong> for 16S rRNA, <strong>16(15)</strong> for act/haA/aer, <strong>6(6)</strong> for alt/haA/aer, <strong>25(24)</strong> for ast, <strong>12(12)</strong> for alt/haA/aer</td>
</tr>
</tbody>
</table>

Value within the parenthesis denotes percentage

3.4. Species wise identification of MPCR positive Aeromonas spp.

The species wise identification of the positive Aeromonas spp. from healthy and diseased freshwater food fishes and distribution of gene patterns among the isolates tested are given in Tables 3 and 4.

The biochemical confirmation showed that the positive isolates belonged to seven species of Aeromonas such as A. hydrophila (48%), A. sobria (15%), A. caviae (15%), A. jandaei (11%), A. veronii (5%), A. schubertii (3%) and A. trota (3%). A. hydrophila was found to be dominant (48%) among the positive Aeromonas spp. isolated from both the healthy and diseased fishes with their highest incidence in diseased fishes.

Comparison of the biochemical results with that of MPCR assay showed that the ast gene was restricted to the isolates of A. hydrophila and occurred in combination with other genes alt and act/haA/aer (Table 4). Among the 28 positive isolates of A. hydrophila, 13 were positive for alt and act/haA/aer genes, 12 for ast, alt and act/haA/aer genes and 3 for act/haA/aer gene. Out of the 9 positive isolates of A. sobria, 5 were positive for alt gene, 4 for alt and act/haA/aer genes. On the other hand, out of the 9 positive isolates of A. caviae, 3 were positive for act/haA/aer gene and 6 for alt and act/haA/aer genes.
**Table 3** Different gene patterns observed among the *Aeromonas* spp. isolated from healthy and diseased freshwater food fishes by MPCR assay

<table>
<thead>
<tr>
<th>Samples</th>
<th>Biochemically species wise identified isolates</th>
<th>No. of isolates</th>
<th>Nos. of isolates showing different gene patterns by MPCR assay</th>
<th>16S rRNA</th>
<th>alt</th>
<th>act/hlyA/aer</th>
<th>alt and act/hlyA/aer</th>
<th>ast, alt and act/hlyA/aer</th>
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<tbody>
<tr>
<td><strong>Healthy fishes</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. hydrophila</td>
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<td>5</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>A. sobria</td>
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<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A. caviae</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
<td>A. jandaei</td>
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<td>Cat fishes</td>
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<td>A. sobria</td>
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<td>Tilapias</td>
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<tr>
<td>Barbs</td>
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<td>Catfishes</td>
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</tr>
<tr>
<td>A. hydrophila</td>
<td>9</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>A. caviae</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Murrels</td>
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<tr>
<td>A. hydrophila</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. caviae</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. sobria</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>59</strong></td>
<td><strong>59</strong></td>
<td><strong>16 (27)</strong></td>
<td><strong>6 (10)</strong></td>
<td><strong>25 (43)</strong></td>
<td><strong>12 (20)</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Value within the parenthesis denotes percentage

In the case of 6 positive isolates of *A. jandaei*, 4 were positive for *alt* gene and 2 for *alt* and *act/hlyA/aer* genes and all the 3 isolates of *A. veronii*, 2 isolates of *A. schubertii*, and *A. trota* were positive for the *alt* gene.
Table 4 Incidence of different gene patterns in the positive isolates of *Aeromonas* spp. from healthy and diseased freshwater food fishes

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates tested</th>
<th>No. of isolates positive for different gene patterns with % in the parenthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>alt</td>
</tr>
<tr>
<td><em>A. hydrophila</em></td>
<td>28 (48)</td>
<td>-</td>
</tr>
<tr>
<td><em>A. sobria</em></td>
<td>9 (15)</td>
<td>5 (56)</td>
</tr>
<tr>
<td><em>A. caviae</em></td>
<td>9 (15)</td>
<td>-</td>
</tr>
<tr>
<td><em>A. jandaei</em></td>
<td>6 (11)</td>
<td>4 (67)</td>
</tr>
<tr>
<td><em>A. veronii</em></td>
<td>3 (5)</td>
<td>3 (100)</td>
</tr>
<tr>
<td><em>A. schubertii</em></td>
<td>2 (3)</td>
<td>2 (100)</td>
</tr>
<tr>
<td><em>A. trota</em></td>
<td>2 (3)</td>
<td>2 (100)</td>
</tr>
</tbody>
</table>

| Total        | 59 (100)                | 16 (27) | 6 (10) | 25 (43) | 12 (20) |

Value within the parenthesis denotes percentage

Overall, it has been observed that the dominant combined pattern of genes found among the positive isolates of *Aeromonas* spp. was *alt* and *act//hlyA/aer* (43%) followed by *ast, alt* and *act//hlyA/aer* (20%) genes. The *alt* gene alone was positive in 27% of the *Aeromonas* isolates followed by *act//hlyA/aer* (10%) gene.

4. Discussion

The present work was targeted at the direct detection of virulent *Aeromonas* spp. from the freshwater food fish samples followed by the characterization of different *Aeromonas* associated virulence factors in a single PCR amplification. The direct detection of virulent *Aeromonas* markers in food samples and characterization of virulence factors in food samples was promoted by Bin Kingombe et al. (2004). It was noted that among virulence determinants, two categories of enterotoxins cytotoxic and cytotoxic have been discovered. A cytotoxic enterotoxin named Act has been described and its structural gene termed *act* (Ferguson et al., 1997). This toxin is closely related to aerolysin (AerA), a channel-forming toxin identified 34 years ago (Bernheimer and Avigad, 1974) and encoded by a gene named *aerA*. The Act and AerA toxins have haemolytic, cytotoxic and enterotoxic activities, as well as lethality in mice (Galindo et al., 2006). Thus, to overcome this problem, a complex primer of *act//hlyA/aer* was chosen that amplifies these three virulence factors as a complex gene (Balsalobre et al., 2009).
In the present study, 57% of representative colonies picked up from ASDAB plates were positive for *Aeromonas* spp. by MPCR assay (Table 2). Bin Kingombe et al. (2004) reported that 59% of samples to be positive for *Aeromonas* spp. by PCR which supported our findings. The overall prevalence of the *Aeromonas* spp. from healthy and diseased fish samples was noted as 66%, with their highest incidence in diseased fishes (76%) followed by healthy fishes (48%). The prevalence was higher in comparison to some of the earlier reports. Bin Kingombe et al. (2004) tested 78 raw and 123 processed and ready to eat food samples and found 25.4% of the samples were positive for *Aeromonas* spp. Yogananth et al. (2009) found that 50% of the *Aeromonas* isolated from fish samples collected from local market in Chennai, India were positive for *A. hydrophila*. Balakrishna et al. (2010) detected *A. hydrophila* from 8.5% of raw fish samples procured from different location in Mysore, India using a MPCR assay targeting virulence associated genes of *Aeromonas* including *hlyA, aerA, GCAT*, along with *16S rRNA* gene. However, Bin Kingombe et al. (2004) found that 59% of raw food samples were contaminated with *Aeromonas* harbouring virulent genes and the contamination of raw foods with *Aeromonas* was proportionally high in raw shrimps (56%) and fish (75%). Pinto et al. (2012) reported the presence of *Aeromonas* spp. in 70.3% of the ready-to-eat seafood products. Hanninen et al. (1997) identified *Aeromonas* spp. in 93% of the fish samples tested. The variation in the incidence level of *Aeromonas* spp. in fish and fish products of different parts of the World can be attributed to the secondary contamination during handling, storage and transportation (Vivekanandan et al., 2005). The prevalence was more in diseased fishes, as the majority of bacterial infections in fishes are caused by *Aeromonas, Vibrio, Plesiomonas* and *Pseudomonas* (McGarey et al., 1991). *Aeromonas* spp. has been consistently isolated from diseased fishes in different parts of the World (Zhang et al., 2000; Rahman et al., 2002).

The DNA extraction was performed using guanidine hydrochloride as it gave good yield within a short time. The average DNA yield of 70μg/ml obtained for the *Aeromonas* spp. was almost comparable with that obtained by Panigrahy et al. (2011) for *Aeromonas* spp. The optimum annealing temperature for the MPCR of the selected four sets of primers was found to be 65°C for 60 sec (Fig. 1). Wang et al. (1996) also found an optimum annealing temperature of 65°C for 1 min in developing a PCR method to detect the hemolysin gene in *A. caviae*.

In this study, the *16S rRNA* gene was amplified in all the positive isolates of *Aeromonas* spp. (Fig. 3), which is very well conserved in *Aeromonas* spp. (Martinez-Murcia 1999) and reported to be positive for all isolates of *Aeromonas* (Wang et al., 2003). The selected toxin genes appeared in four different patterns *viz.* alt; act/*hlyA/aer; alt and act/*hlyA/aer; ast, alt and act/*hlyA/aer, among the MPCR positive *Aeromonas* spp. (Table 3 and Fig. 3). The heterogeneity in the distribution of the toxin genes among the *Aeromonas* spp. have earlier been reported by several workers (Albert et al., 2000; Sechi et al., 2002; Ottaviani et al., 2011) which are in agreement with our findings. All the MPCR positive *Aeromonas* isolates were positive for at least one toxin gene. Ottaviani et al. (2011) also reported that 96.4% of the food-borne *Aeromonas* isolates were positive for at least one toxin genes. However, Pablos et al. (2009) reported that all the *Aeromonas* isolates tested in their study were positive for *alt* gene, which is at par with our findings. The overall prevalence of the *alt, act/hlyA/aer* and *ast* genes was recorded to be 89.8%, 72.9% and 20%, respectively. In disagreement with some of the earlier reports (Sen and Rodger, 2004; Balsalobre et al., 2009) but in accordance with the others (Pablos et al., 2009; Bin Kingombe et al., 2010; Ottaviani et al., 2011; Pinto et al., 2012), the *alt* gene was found to be dominant among the MPCR positive isolates of *Aeromonas* spp. Balsalobre et al. (2009) reported that 70.7%, 97.6% and 26.8% of the *A. hydrophila*
strains tested were positive for act/hlyA/aer, ast and alt genes, respectively, and 2.4% and 36.6% of the A. jandaei strains were positive for act/hlyA/aer complex and alt genes. Bin Kingombe et al. (2010) reported that 36 % of isolates of food borne Aeromonas spp. as A. caviae complex, 38% as A. hydrophila complex and 24% as A. sobria complex, and 23% as other aeromonads by a multiplex PCR targeting ast, alt and act enterotoxin genes. However, there existed a difference in the prevalence level of the toxin genes with the earlier reports (Ottaviani et al., 2011; Pinto et al., 2012). The difference in the prevalence of the toxin genes among the Aeromonas spp. may be related to the geographical distribution of the strains as postulated by Albert et al. (2000). It was also noted that the ast gene never occurred singly among the MPCR positive Aeromonas spp. and always in combination with alt and act/hlyA/aer genes. Bin Kingombe et al. (2010) reported the same for the food borne isolates of Aeromonads.

The biochemical confirmation of the MPCR positive Aeromonas isolates revealed different Aeromonas spp. complexes in the tested samples which belonged to seven species such as A. hydrophila, A. sobria, A. caviae, A. jandaei, A. veronii, A. schubertii and A. trota and the dominant species was found to be A. hydrophila (48%), followed by A. sobria (15%) and A. caviae (15%) (Tables 3 and 4). In agreement with this, Bin Kingombe et al. (2010) also reported that the majority of the food-borne isolates belonged to A. caviae complex, A. hydrophila complex and A. sobria complex. Ottaviani et al. (2011) also reported that A. hydrophila and A. sobria have been frequently isolated from food and environmental samples, which supported our findings. Although the prevalence of other Aeromonas isolates such as A. jandaei, A. veronii, A. schubertii have been observed in this study the frequency of occurrence was less as reported earlier by Janda and Abott (2010). It has been noted that 3% of the Aeromonas isolated in this study belonged to A. trota. Bin Kingombe et al. (1999) reported that the detection of the ampicilin-sensitive A. trota is relatively low in food samples. Moreover, most often these strains are conventionally identified correctly to the genus level, but incorrectly to the species level, due to lack of biochemical tests in distinguishing these species (Abbott et al., 2003) or the difference in the prevalence are related to geographical distribution. However, the prevalence of one or more toxin gene, as observed in this study, among these isolates cannot be underestimated, as harbouring any of these toxin genes may be hazardous (Bin Kingombe et al., 2010).

It has been observed in this study that the occurrence of the ast gene was restricted to the strains of A. hydrophila which were in accordance with the earlier reports (Balsalobre et al., 2009; Bin Kingombe 2010). Bin Kingombe et al. (2010) speculated that an Aeromonas isolate harbouring the molecular enterotoxin profiles of control strains of A. hydrophila (ATCC 7966), an ast-positive strain, may induce not only an infection but also intoxication due to the presence of heat stable enterotoxin even if the contaminated food vehicle was heat treated. They also suggested that the ast gene might be specific for A. hydrophila (HG1) and might represent a potential taxonomic tool for the identification of A. hydrophila (HG1), which supported the findings of the present study. This showed that the presence of A. hydrophila possessing ast gene in healthy and diseased freshwater food fishes in the present study is considered to be highly pathogenic, as even the normal cooking temperatures may not destroy that enterotoxin, due to its heat stability. Moreover, 43% of the isolates of A. hydrophila had all the toxin genes which confirm that this species possesses a more extensive array of virulent genes than the other Aeromonas spp. (Aguilera-Arreola et al., 2007). A correlation between the higher number of toxin genes harboured in an isolate of Aeromonas and its potential to induce diarrhea have been earlier reported (Sha et al., 2002; Chang et al., 2008) and
their detection in food products is very essential. The results of the present study indicated that the
detection and identification of toxigenic *Aeromonas* spp. from healthy and diseased freshwater food
fishes by the MPCR assay developed are specific, faster, reliable and easier to perform than the
conventional phenotypic methods and also outweigh the advantage of uniplex PCR in the detection
of pathogenic strains of *Aeromonas* spp. in a single reaction targeting different virulent genes.

In conclusion, the results of the present study indicated that there is an immediate need to establish
specific microbiological criteria in foods, especially seafood and an intensive monitoring on the
occurrence of hemolytic and enterotoxigenic *Aeromonas* spp. is strongly recommended to assess
the human health risk arising from contaminated seafood consumption. Moreover, there is a need
of a series of prescribed guidelines for an ideal fish market which is to be strictly followed in order
to avoid secondary contamination and growth of virulent *Aeromonas* spp. in food fish products. As
the conventional method for the isolation of *Aeromonas* spp. is a laborious process and further
biochemical confirmation lacks specificity, multiplex PCR assay developed in this study could be a
powerful tool for assessing the prevalence of hemolytic and enterotoxigenic *Aeromonas* spp.

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