Detection of hemolytic strains of *Aeromonas hydrophila* and *A. sobria* along with other *Aeromonas* spp. from fish and fishery products by multiplex PCR

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Abstract Hemolytic strains of *Aeromonas* spp. from fish and fishery products were detected by multiplex PCR. The selected primers for the amplification of segments of *ahh1*, *asa1* and *16S rRNA* gene yielded products with the size of 130 bp, 249 bp and 356 bp, respectively. This assay was found to be highly sensitive, as it could detect 7 and 9 cells of *Aeromonas hydrophila* and *A. sobria* with a detection limit of 1 pg of pure genomic DNA. The assay, when screened for 73 commercial fish and fishery product samples consisting of freshwater, marine fish and shellfish, showed 56 % positive for *Aeromonas* spp., 16 % for *Aeromonas hydrophila* and 13 % for *A. sobria*. This assay provides specific and reliable results and can be a powerful tool for the simultaneous detection of hemolytic strains of *A. hydrophila*, *A. sobria* and other *Aeromonas* spp. from fish and fishery products.

Keywords *Aeromonas hydrophila* · Species-specific detection · Hemolytic strains · Fish and fishery products · Multiplex PCR

Introduction

Aeromonads are a group of mesophilic motile and psychrophilic non-motile, gram-negative rod shaped, oxidase-positive, facultative anaerobic microorganisms belonging to the family Aeromonadaceae (Monfort and Baleux 1990). *Aeromonas* currently has the status of an important emerging

food-borne pathogen, primarily because of its ability to grow at cold temperatures (Kirov 2001). Varieties of foods such as raw, cooked and processed foods, also those held at refrigeration temperature, under modified atmosphere and under modified growing conditions including seafood have been shown to harbour motile Aeromonads (Pin et al. 1996; Devlieghere et al. 2000). Aeromonads have been detected in dairy products (4 %), vegetables (26–41 %), meats and poultry (3–70 %), with the largest numbers recorded from shellfish (31 %) and fish (72 %) (Janda and Abbott 2010). *Aeromonas hydrophila* (DNA hybridization group, HG1), and *Aeromonas veronii* biovar *sobria* (HG8) (otherwise known as *A. sobria*) are the most common species belonging to this genus, often encountered in fish and fishery products (Rahman et al. 2002; Vivekanandhan et al. 2005). *Aeromonas* spp. grow rapidly during iced storage of freshwater fishes, with more than a third of the population belonging to the species *A. hydrophila* and *A. sobria* (Gonzalez et al. 2001). The distribution of *Aeromonas hydrophila* in marine ecosystem including marine fish and retail seafood outlets is also well known and this organism is found to be normal flora in a variety of fish (Yogananth et al. 2009).

A. hydrophila and *A. sobria* are often found in association with hemorrhagic septicemia in cold-blooded animals including fish, reptiles, and amphibians (Janda and Abbott 2010). However, these organisms have also been implicated as primary pathogens in cases of acute diarrheal disease in immune-competent humans of all age groups (Aggar et al. 1985; Chopra and Houston 1999). A number of epidemiological surveys have shown that *A. hydrophila* (HG1), *A. caviae* (HG4) and *A. sobria* (HG8) are the most common species associated with human intestinal diseases (Borrell et al. 1997). Hemolytic uremic syndrome, a life-threatening condition normally associated with infections due to *Escherichia coli* O157: H7, has also been associated with *A. hydrophila*

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infection (Bogdanovic et al. 1991; Janda and Kokka 1991; Robson et al. 1992) and a cytotoxin with homology to Shiga toxin 1 has been identified in both the *A. hydrophila* and *A. caviae* (Haque et al. 1996). According to Kirov (2001), the majority of aeromonads associated with gastroenteritis belong to *A. sobria* (HG 8), *A. hydrophila* (HG1 and HG3), *A. caviae* (HG4), and to a lesser extent *A. veronii* biovar *veronii* (HG 8/10), *A. trota* (HG13), and *A. jandaei* (HG9). Virulence of *Aeromonas* spp. is multifactorial and incompletely understood (Pablos et al. 2009). A number of virulence factors derived from *A. hydrophila* and *A. sobria* have been examined in an effort to explain the pathogenesis of infections due to these organisms. Toxins with hemolytic, cytotoxic and enterotoxic activities have been described in many *Aeromonas* spp. (Bin Kingombe et al. 1999; Gonzalez-Rodriguez et al. 2002; Rahman et al. 2002). Nacescu et al. (1992) postulated a correlation between the pathogenic potential and the hemolytic activity of *Aeromonas* spp. A majority of the *A. hydrophila* and *A. sobria* isolates were highly hemolytic, whereas only 11 % of the *A. caviae* isolates were capable of lysing sheep erythrocytes (Nakano et al. 1990). Two hemolytic toxins have been described in *A. hydrophila*: the *ahh1* hemolysin (Hirono and Aoki 1991) and *aer* aerolysin (Howard et al. 1987). Most *Aeromonas* hemolysins described to date are related to one of these two toxins and are reasonable predictors of human diarrhoeal disease in both the *A. hydrophila* and *A. sobria* (Heuzenroeder et al. 1999).

There are several conventional as well as rapid systems including PCR that have been tried for the detection of sea-food borne pathogens (Kumar et al. 2006; Jeyasekaran et al. 2011; Raj et al. 2011). Although *Aeromonas* spp. has recently received increasing attention as an agent of food-borne diarrhoeal diseases in healthy people (Janda and Abbott 2010), the PCR has been mostly used for the characterization of isolates either for their virulence properties or for the phylogenetic positions (Yanez et al. 2003; Bin Kingombe et al. 2010; Fontes et al. 2011). In this study, hemolytic strains of *A. hydrophila* and *A. sobria* along with other *Aeromonas* spp. from fish and fishery products were detected by a MPCR assay targeting the amplification of *ahh1*, *asa1* and *16S rRNA* genes, respectively.

Material and methods

Bacterial strains, media, and reagents

Type cultures of *Aeromonas* used in this work 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), *Salmonella 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 used to check the specificity of the assay. Media and their ingredients were purchased from Hi-Media Laboratories Pvt. Ltd, Mumbai, India. Molecular grade water (Sartorius Stedim Biotech, Gottingen, Germany) was used for the preparation of buffers and reagents.

Bacterial DNA extraction

For the extraction of DNA, bacterial type cultures were grown on trypticase soy broth (TSB) and incubated at 37 °C for 18 h to obtain young culture prior to the extraction of genomic DNA. The genomic DNA from bacterial cells was extracted following guanidine hydrochloride method according to Haldar et al. (2005) with some modifications. One milliliter of the young cell suspension was centrifuged at $10,000 \times g$ 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 $10,000 \times g$ 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 $13,000 \times g$ 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 $10,000 \times g$ for 10 min at 4 °C. The pellet was then resuspended 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.

Primers

Three sets of primers belonging to genus and species specific genes of *Aeromonas* were selected for the study. All the primer sequences were synthesized by Ocimum Biosolutions Inc., Indianapolis, USA. The oligonucleotide primer sequences, target genes, specificity and their expected product sizes are listed in Table 1.

Optimization of multiplex PCR

Multiplex PCR for the simultaneous detection of *Aeromonas* spp., *A. hydrophila* and *A. sobria* in a single reaction was optimized by performing PCR at different annealing temperatures from 57 °C to 61 °C for 60 s by targeting *16S rRNA*, *ahh1* and *asa1* genes, respectively in a Gradient Master Cycler (Eppendorf AG, Hamburg, Germany). All the PCR mixtures were handled in the Bio-Safety Cabinet Type II (Clean Air Systems, Chennai, India). The amplification was done with 50 µl of reaction mixture containing template DNA of *A. hydrophila* and *A. sobria*, 10X PCR buffer (100 mM Tris (pH 9.0), 500 mM KCl, 15 mM MgCl₂, and 0.1 % gelatin), 100 mM of each dNTP, 30 pmol of each forward

Table 1 Primers used in the multiplex PCR assay for *Aeromonas* spp

Primer pair	Primer sequences (5' to 3')	Target genes	Specificity	Product size (bp)	References
A16S	F- GGG AGT GCC TTC GGG AAT CAG A R- TCA CCG CAA CAT TCT GAT TTG	<i>16S rRNA</i>	<i>Aeromonas</i> spp.	356	Wang et al. (2003)
ASA1	F- TAA AGG GAA ATA ATG ACG GCG R- GGC TGT AGG TAT CGG TTT TCG	<i>asa 1</i>	<i>A. sobria</i>	249	Wang et al. (2003)
AHH1	F- GCC GAG CGC CCA GAA GGT GAG TT R- GAG CGG CTG GAT GCG GTT GT	<i>ahh 1</i>	<i>A. hydrophila</i>	130	Wang et al. (2003)

F forward; R reverse

and reverse primers and 1.25 U of Taq DNA polymerase. The optimization of MPCR conditions consisted of initial denaturation at 94 °C for 3 min., followed by denaturation at 94 °C for 60 s; annealing temperatures ranging from 57 °C to 60 °C for 60 s; extension at 72 °C for 90 s for 45 cycles; and final extension at 72 °C for 3 min.

Gel electrophoresis

The PCR products were run on 2 % agarose gel stained with ethidium bromide (10 mg/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 100 bp DNA ladder (Real Biotech Corporation, Ohio, USA).

Specificity of multiplex PCR

To test for the specificity of this assay towards *Aeromonas*, the multiplex PCR was also performed with the non-*Aeromonas* species such as *Salmonella typhi*, *Salmonella paratyphi* A, *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus*.

Sensitivity of multiplex PCR

Sensitivity of the multiplex PCR was performed using *A. hydrophila* and *A. sobria* to evaluate the minimal cells as well as genomic DNA required for their detection. To determine the minimal cell detection limit, fish tissue homogenate, 100 g, was pasteurized at 100 °C for 3 min and then divided into four lots of 25 g each in sterile petriplates. Aliquots of 100 µl of tenfold serial dilution (10^{-6} to 10^{-8}) of overnight grown cultures of *A. hydrophila* and *A. sobria* were inoculated into first three lots of the homogenate. The fourth uninoculated lot homogenate was treated as control. The initial count of cells was determined by plating, 100 µl of aliquot from each dilution on *Aeromonas* starch DNA agar

base (ASDAB) plates and incubating at 37 °C for 24 h. The homogenate containing inocula of *A. hydrophila* and *A. sobria* was enriched with 225 ml buffered peptone water and incubated at 37 °C. Immediately after inoculation and after 2, 4, 6, 8, 10 and 12 h of enrichment, 1.5 ml enriched sample was used to extract the genomic DNA and MPCR was performed as described earlier. To evaluate the genomic DNA sensitivity, DNA of *A. hydrophila* and *A. sobria* were extracted using guanidine hydrochloride and diluted with DNA-free water to a concentration ranging from 1,000 pg to 10 fg and subjected to same MPCR assay as described earlier.

Validation of multiplex PCR

Seventy three samples of fresh fish including 23 freshwater fish, 20 marine fish, 16 fresh shrimps and 14 fresh squids were screened for the presence of *Aeromonas* spp. by the developed multiplex PCR assay. The samples were procured from the local fish markets, Tuticorin, India and brought to the laboratory in iced condition and used for analysis within 3 h. For the isolation of *Aeromonas* spp., 25 g of each sample was enriched in 225 ml of buffered peptone water (pH 7.0) for 18 to 24 h at 37 °C. Enrichment was done to recover even single *Aeromonas* spp. present in the sample. The enriched sample was then serially diluted, spread plated onto ASDAB plates and incubated at 37 °C for 18 to 24 h to obtain yellow colored colonies. A total of 162 representative colonies from the ASDAB plates were picked up, purified and used for the analysis by MPCR. The isolates of *Aeromonas*, which showed PCR positive, were also confirmed to the species level by Aerokey II (Carnahan et al. 1991).

Results and discussion

In the developed multiplex PCR assay, the *16S rRNA* and *asa1* genes were amplified only in *A. sobria* and *16S rRNA* and *ahh1* genes were amplified only in *A. hydrophila* and *16S rRNA* gene was alone amplified in *A. liquefaciens* (Fig. 1). The specificity of the multiplex PCR assay tested by using the

Fig. 1 Detection of *A. hydrophila*, *A. sobria* and other *Aeromonas* spp by uniplex and multiplex PCR. Lane 1- *A. hydrophila*; Lane 2- *A. sobria*; Lane 3- *Aeromonas* spp.; Lane 4- multiplex for *A. hydrophila*, *A. sobria* and other *Aeromonas* spp.; Lane M-100 bp DNA marker

DNA extracted from non-*Aeromonas* species such as *Salmonella typhi*, *Salmonella paratyphi A*, *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus* showed that the assay did not amplify *16S rRNA*, *asa1* and *ahh1* genes in any of the non-*Aeromonas* species tested (Fig. 2). The development of multiplex PCR assay for *Aeromonas* spp. has been mainly focused by the earlier authors to identify the *Aeromonas* spp. alone by the presence of different genes as well as to identify *A. hydrophila* using the set of genes unique for *A. hydrophila*. Wang et al. (2003) developed a MPCR using *aerA* and *ahh1* gene specific for *A. hydrophila* and *asa1* specific for *A. sobria* from clinical isolates. Bin Kingombe et al. (2010) reported a MPCR assay to detect the presence of three enterotoxin genes in *Aeromonas* spp. which include cytotoxic (*act*), heat-labile (*alt*) and heat-stable enterotoxin gene (*ast*), for the detection of *Aeromonas* spp in food. Choresca et al. (2010) developed a MPCR assay to detect *A. hydrophila* by amplification of 130 bp fragment of hemolysin and 309 bp fragment of aerolysin gene from diseased *Clarius* spp. Balakrishna et al. (2010) reported another MPCR assay for the detection of *A. hydrophila* by amplification of hemolysin (*hlyA*), aerolysin (*aerA*), glycerophospholipid-

Fig. 2 Specificity of MPCR assay for the detection of *A. hydrophila*, *A. sobria* and other *Aeromonas* spp. Lane 1- *A. hydrophila*; Lane 2- *A. sobria*; Lane 3- *A. caviae*; Lane 4- *A. liquefaciens*; Lane 5- *A. hydrophila* and *A. sobria*; Lane 6- *Salmonella typhi*; Lane 7- *S. paratyphi A*; Lane 8- *Vibrio cholerae*; Lane 9- *V. parahaemolyticus*; Lane 10- *V. vulnificus*; Lane 11- *V. alginolyticus*; Lane M-100 bp DNA marker

cholesterol acyl transferase (*GCAT*) along with *16S rRNA* gene. Pinto et al. (2012) developed a MPCR method for *Aeromonas* spp using *hlyA*, *aerA*, *alt* and *ast* genes encoding hemolysin A, aerolysin, aeromonas labile temperature cytotoxic enterotoxin and aeromonas stable temperature cytotoxic enterotoxin, respectively. As most of the developed MPCR assays focused on the detection of either *Aeromonas* spp. or *A. hydrophila*, the present assay was targeted for the amplification of three sets of genes for simultaneous detection of *A. hydrophila* and *A. sobria* along with other *Aeromonas* spp. (Fig. 1). The 16S RNA primer was used to amplify a 356 bp fragment of the *16S rRNA* gene which is conserved for the genus *Aeromonas* for confirming the presence of *Aeromonas* spp. Similarly, for the specific detection of *A. hydrophila* and *A. sobria*, the primers were selected for the virulence associated genes, *ahh1* and *asa1*, respectively having hemolytic properties.

The serially diluted overnight grown culture of the *Aeromonas* spp. contained >250 cells/100 μ l in 10^{-6} dilution; 75 and 96 cells/100 μ l of *A. hydrophila* and *A. sobria* in 10^{-7} dilution; 7 and 9 cells/100 μ l of *A. hydrophila* and *A. sobria* in 10^{-8} dilution. The multiplex PCR assay performed for testing the cell sensitivity with the DNA extracted from the serially diluted cells showed all the three genes got amplified within 4 h of enrichment in buffered peptone water, when the cell counts were >250; within 8 h when the counts were 75 cells and 96 cells of *A. hydrophila* and *A. sobria*, respectively; and within 12 h when contained 7 cells and 9 cells of the young culture of *A. hydrophila* and *A. sobria*, respectively (Fig. 3). The yields of the products were significantly high and distinguishable even when lower counts of cells were amplified by the multiplex PCR assay.

The genomic DNA sensitivity of the multiplex PCR assay performed with the serial dilution of genomic DNA of *A. hydrophila* and *A. sobria* showed amplification of genes even at 1 pg concentration of template DNA (Fig. 4). With regard to

Fig. 3 Sensitivity of the MPCR assay for the detection of cells of *A. hydrophila*, *A. sobria* and other *Aeromonas* spp. after 12 h of enrichment. Lane 1- > 250 cells/ml in 10^{-6} dilution; Lane 2-75 cells/ml of *A. hydrophila* and 96 cells/ml of *A. sobria* in 10^{-7} dilution; Lane 3-7 cells/ml of *A. hydrophila* and 9 cells/ml of *A. sobria* in 10^{-8} dilution; Lane 4- Positive control; Lane M- 100 bp DNA marker

Fig. 4 Sensitivity of the MPCR assay for the detection of genomic DNA of *A. hydrophila*, *A. sobria* and other *Aeromonas* spp. Lane 1–1,000 pg; Lane 2–100 pg; Lane 3–10 pg; Lane 4–1 pg; Lane 5–100 fg; Lane 6–10 fg; Lane M, 100 bp DNA marker

the sensitivity of the developed multiplex PCR assay, 7 and 9 cells of *A. hydrophila* and *A. sobria* could be detected in fish within 12 h of enrichment in buffered peptone water (Fig. 3) with the detection limit of 1 pg of DNA (Fig. 4). The sensitivity of the bacterial cells as well as genomic DNA reported for the MPCR or PCR assay vary widely. Pollard et al. (1990) reported a detection limit of 1 ng of the total DNA for the amplification of the aerolysin gene in *A. hydrophila*. On the other hand the MPCR assay developed by Balakrishna et al. (2010) could detect 50 CFU/g of *A. hydrophila* in fish samples based on aerolysin and hemolysin genes with overnight incubation in alkaline peptone water (APW-A) broth and the detection limit of the assay was 5 pg with pure genomic DNA. As the amplification of bacterial DNA directly from food complexes by PCR often give poor yield, enrichment of the samples prior to PCR has been recommended by many workers (Merino et al. 1995). Thus, the multiplex PCR assay developed had high sensitivity in terms of cells and genomic DNA content compared to that reported by other workers for *Aeromonas* spp.

The developed multiplex PCR assay performed for the representative colonies (162 Nos.) isolated from 73 commercial fresh fish samples comprising freshwater and marine fish and shellfish showed that 91 isolates were positive for *Aeromonas* spp. (Table 2). More specifically, of the 53

representative isolates from freshwater fishes, 33 were positive for *Aeromonas* spp., which consisted of 8 *A. hydrophila*, 6 *A. sobria* and 19 were other *Aeromonas* spp. The biochemical confirmation of the PCR positive isolates showed a different trend with 12 *A. hydrophila*, 6 *A. sobria*, 5 *A. trota*, 4 *A. schubertii*, 5 *A. caviae*, and 1 *A. jandaei*. There existed a difference in the number of positive isolates of *A. hydrophila* and *A. sobria* confirming by multiplex PCR and biochemical reactions. Multiplex PCR seemed to provide less positive results for *A. hydrophila* as four isolates biochemically confirmed as *A. hydrophila* were other *Aeromonas* spp. in the multiplex PCR assay.

On the other hand, of the 46 representative colonies isolated from marine fishes, 26 were positive for *Aeromonas* spp., in which 8 were *A. hydrophila*, 8 *A. sobria* and 10 other *Aeromonas* spp. (Table 2). From the fresh shrimps, 17 were positive for *Aeromonas* spp. out of 33 representative colonies isolated. Among which, 6 were *A. hydrophila*, 2 *A. sobria* and 9 other *Aeromonas* spp. In the fresh squids, 15 out of 30 representative colonies were positive for *Aeromonas* spp. Of which 5 as *A. hydrophila*, 6 as *A. sobria* and 4 as other *Aeromonas* spp. were recorded. There was no misidentification of *A. hydrophila* or *A. sobria* in the above samples in the case of marine fishes, which is in little variance with the results obtained from freshwater fishes. The biochemical confirmation showed concurrence with the results of the developed multiplex PCR assay in the case of marine fishes. The other aeromonads identified by biochemical reaction belonged to different species such as *A. caviae*, *A. trota*, *A. schubertii*, *A. veronii* and *A. jandaei*. On an average, 91 representative isolates were positive for *Aeromonas* spp. corresponding to 55 Nos. of samples. The presence of *A. hydrophila* and *A. sobria* was 16 % and 13 % respectively, in the commercial fish samples examined. None of the samples contained both the *A. hydrophila* and *A. sobria* together.

The specificity of the PCR assay for the *Aeromonas* spp. has been checked by few authors. Pollard et al. (1990) faced some difficulties with the non-specific amplification of the *aer* gene specific for *A. hydrophila* with some *Streptococcus* spp.

Table 2 Detection of *Aeromonas* spp. from different fish and fishery products by multiplex PCR assay and conventional methods

Samples	No. of samples	No. of representative colonies tested	Number positive by MPCR with % in parenthesis			Number positive by biochemical tests with % in parenthesis					
			<i>16S rRNA</i>	<i>ahh1</i>	<i>asa1</i>	<i>A. hydrophila</i>	<i>A. sobria</i>	<i>A. caviae</i>	<i>A. jandaei</i>	<i>A. trota</i>	<i>A. schubertii</i>
Freshwater fishes	23	53	33 (62)	8 (24)	6 (18)	12 (36)	6 (15)	5 (18)	1 (3)	5 (15)	4 (12)
Marine fishes	20	46	26 (57)	8 (31)	8 (31)	8 (31)	8 (31)	5 (19)	3 (12)	2 (7)	0
Fresh shrimps	16	33	17 (52)	6 (35)	2 (12)	6 (35)	2 (12)	0	2 (12)	3 (18)	4 (23)
Fresh squids	14	30	15 (50)	5 (33)	6 (40)	5 (33)	6 (40)	1 (7)	0	3 (20)	0
Total	73	162	91 (56)	27 (16)	22 (13)	31 (19)	22 (13)	11 (7)	6 (4)	13 (8)	8 (5)

Value within the parenthesis denotes percentage

The developed MPCR assay was also checked for specificity with non-*Aeromonas* species mainly *Salmonella typhi*, *S. paratyphi* A, *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus* and it did not detect them (Fig. 2). The multiplex PCR assays developed by Nam and Joh (2007) and Balakrishna et al. (2010) were specific for *Aeromonas*, as they did not amplify the selected virulent genes in *E. coli*, *Vibrio cholerae*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Proteus vulgaris*. Wang et al. (2003) reported cross reaction for the *asa1* gene that was not detected in 5 of 21 *A. sobria* isolates. In this assay, such cross reactions were not noticed and no isolate other than *A. hydrophila* and *A. sobria* showed the presence of *ahh1* and *asa1* genes, respectively, rendering it to be specific.

Using the developed MPCR assay, it was confirmed that 56 % of the commercial fresh fish samples contained *Aeromonas* spp. based on the presence of *16S rRNA* gene. Bin Kingombe et al. (2004) found 51 isolates as MPCR positive for *Aeromonas* spp. out of 65 isolates contributing to about 78 %, which is more or less similar to our present findings. The few contradictory results obtained by conventional biochemical tests and multiplex PCR assay in respect of *A. hydrophila* were analyzed in detail. There were reports that biochemical confirmation at times lacks specificity for some strains of bacterial pathogens (Park et al. 2003; Beaz-Hidalgo et al. 2010) and it need not be the same for all species. In this work, four isolates of *A. hydrophila* biochemically confirmed did not show the presence of hemolysin gene, *ahh1* in the multiplex PCR assay. It has been earlier reported that the production of hemolytic cytotoxins has been regarded as strong evidence of pathogenic potential in *Aeromonas* spp. (Wang et al. 2003).

Conclusion

In conclusion the microbiological limit for *Aeromonas* spp. in foods including seafood in Commission Regulation EC No 1441/2007 is still lacking, but considering the worldwide increasing incidence of *A. hydrophila* and *A. sobria* infection in immuno-compromised individuals (Sanchez et al. 2005; Tsai et al. 2006) and its wide spread distribution in marine and freshwater environment, there is an immediate need to establish specific microbiological criteria in foods, especially seafood and an intensive monitoring on the occurrence of these species is strongly recommended to assess the human health risk arising from contaminated seafood consumption. As the conventional method for the isolation of *Aeromonas* spp. is a laborious process and further biochemical confirmation lacks specificity, multiplex PCR assay could be a powerful tool for the rapid and simultaneous detection of *A. hydrophila*, *A. sobria* and other *Aeromonas* spp. from the samples of fish and fishery products with more specificity and sensitivity.

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