Detection of toxigenic strains of *Aeromonas* species in foods by a multiplex PCR assay

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Abstract *Aeromonas hydrophila* and other aeromonads are ubiquitous organisms found in meat, vegetables, drinking water and various other food items. They cause diarrhea and extra-intestinal infections in normal and immunocompromised patients. The aim of the study was to develop a multiplex PCR assay for the detection of virulence-associated genes of *Aeromonas* including hemolysin (*hlyA*), aerolysin (*aerA*), glycerophospholipid-cholesterol acyl transferase (*GCAT*) along with a 16S rRNA gene. Internal amplification control (IAC), which was coamplified with *aerA* primers, was also included in this study. The results showed that all cultures of *Aeromonas* were accurately identified by the assay without showing non-specificity. *A. hydrophila* could be detected at a range of 10–50 CFU ml⁻¹ from experimentally spiked fish, chicken and milk samples following overnight enrichment in alkaline peptone water supplemented with 10 µg/ml ampicillin (APW-A) by this multiplex PCR (mPCR). When evaluated on a total of 74 naturally occurring food samples, four samples were identified to contain *Aeromonas* by mPCR. All these results were compared to the conventional culture, isolation and biochemical identification procedures. The high throughput and cost-effective mPCR method developed in this study could provide a powerful tool for detection of pathogenic

Aeromonas spp. from food and environmental samples and in addition, the method has advantages in terms of specificity, sensitivity and ease of use compared to other reported PCR methods and DNA hybridization assays.

Keywords *Aeromonas* · PCR · Water · Fish · Chicken · Milk

Introduction

Aeromonas hydrophila and other aeromonads are ubiquitous organisms found in meat, fish, vegetables, drinking water and various other food items. They cause diarrhea and extra-intestinal infections in normal and immunocompromised patients [1]. In addition, these microorganisms are also considered important fish pathogens [2]. *Aeromonas* spp. exhibit a number of putative virulence factors including enzymes (lipases, esterases, DNases), hemolysin/aerolysin, enterotoxins, agglutinins, adhesions, pili and outer membrane proteins [3, 4]. A number of *Aeromonas* spp. are able to survive and multiply in a variety of food products including raw, cooked and processed foods at low temperatures [5–9] and can produce virulence factors even at these low temperatures.

The laboratory procedures for detection and identification of *Aeromonas* from foods by conventional procedures are laborious and time-consuming taking 3–5 days [10]. Several virulence-associated genes have been targeted for the detection of potentially pathogenic aeromonads by PCR assays that include either cytotoxin or hemolysin or both of these genes [11–15]. Recently Chu and Lu [14] reported a multiplex PCR (mPCR) to rapidly and specifically detect

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pathogenic *A. hydrophila* from symptomatic and asymptomatic fish targeting aerolysin and 16S rDNA genes. Wang and his coworkers [15] also standardized a mPCR for the detection of hemolysin genes in *A. hydrophila* and *A. sobria* from clinical samples. These reported mPCRs did not include an internal amplification control (IAC), which has become almost mandatory in diagnostic PCRs and could detect simultaneously only two virulence-associated genes of *Aeromonas* spp.

In the present work, a multiplex PCR assay is being reported for the detection of three virulence-associated genes in *Aeromonas* spp. that include *aerA*, aerolysin gene of *A. hydrophila*; *HlyA*, an extracellular hemolysin gene of *A. hydrophila*; *GCAT*, glycerophospholipid-cholesterol acyl transferase gene, a weak hemolysin gene (found in *A. hydrophila* and other aeromonads) combined with a genus-specific 16S rRNA gene for detecting all species of *Aeromonas* alongwith an IAC designed to check the inhibitors in PCR in order to avoid false negative reactions. The mPCR was evaluated on different *Aeromonas* spp. and could detect pathogenic *Aeromonas* spp. both from experimentally spiked and naturally contaminated food samples. The results obtained were compared to isolation and identification of the organism undertaken using classical conventional biochemical tests.

Materials and methods

Bacterial strains

The bacterial strains used in this study are listed in Table 1. Reference strains *A. hydrophila* ATCC 35654 and *A. salmonicida* ATCC 33658 were obtained from Hi-Media laboratories, Mumbai. The other cultures were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, National Collection of Industrial Microorganisms (NCIM), Pune and Institute of Life Sciences (ILS), Bhubaneswar. These cultures were grown in tryptone soya broth (TSB) (Hi-Media, Mumbai) at 35°C in shaking conditions. Template DNA from each bacterial strain was extracted by boiling method [17] or by DNA extraction kit (MBI Fermentas).

Cultural method

For isolation and identification of *Aeromonas* from food and water samples, 18 h enrichment in alkaline peptone water supplemented with 10 µg/ml ampicillin (APW-A) (Hi-Media, Mumbai) was done and growth plated on ampicillin dextrin agar (ADA) (Hi-Media, Mumbai). After

Table 1 Bacterial strains used for the evaluation of specificity of PCR primers

Bacterial strain	<i>aerA</i>	<i>hlyA</i>	<i>gcat</i>	16S rRNA	IAC
<i>Aeromonas hydrophila</i>					
ATCC 35654	+	+	+	+	+
ATCC 49140	+	+	+	+	+
MTCC 646	+	+	+	+	+
NICED Ah F 2002	+	+	+	+	+
NICED Ah F 12044	+	+	+	+	+
NICED Ah F 15309	+	+	+	+	+
NICED Ah F 22162	+	-	+	+	+
<i>Aeromonas salmonicida</i>					
ATCC 33658	+	+	+	+	+
MTCC 1945	-	-	+	+	+
<i>Aeromonas sobria</i>					
MTCC 1608	-	+	-	+	+
ILS PAS1	-	-	-	+	+
<i>Aeromonas culicicola</i> , NCIM 5147	-	+	-	+	+
<i>Vibrio cholerae</i> (lab strain)	-	-	-	-	+
<i>Escherichia coli</i> MTCC 730	-	-	-	-	+
<i>Salmonella typhimurium</i> (lab strain)	-	-	-	-	+
<i>Proteus vulgaris</i> (lab strain)	-	-	-	-	+
<i>Staphylococcus aureus</i> NCIM 2492	-	-	-	-	+

20 h incubation at 35°C the colonies resembling yellow color were biochemically characterized.

Primers and internal amplification control

Four pairs of primer sets for genes *aerA*, *hlyA*, *GCAT* and 16S rRNA were designed using the GenBank database sequences. All primers used in this study were synthesized from Sigma-Aldrich Company, Bangalore. An IAC was constructed for which both the forward and reverse primers had 5' overhanging ends of *AerA* primers used in mPCR specific for *aerA* gene, whereas, the 3' ends of both primers were joined by complementary sequence of pUC 18 DNA. All the primer sequences and IAC sequences are listed in Table 2.

The PCR reaction mixture for generation of IAC DNA contained 300 nM of each primer, 200 µM of each dNTP (Sigma), 0.5 units of *Taq* polymerase, 2 mM MgCl₂ in 1X PCR buffer (Sigma) and 250 pg of template DNA. PCR was carried out through 30 cycles in eppendorf master cycler gradient thermal cycler (Hamburg, Germany) under the following conditions: denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 1 min in each cycle. The DNA was denatured initially for 5 min in the beginning and extended at 72°C for 10 min. PCR product was purified using the PCR purification kit (Qiagen). The concentration of IAC DNA was determined spectrophotometrically at 260 nm and was stored in DDW. The following equation was used to calculate the copy number of the PCR product concentration: $\text{weight of PCR fragment (in g } \mu\text{l}^{-1}) \times (6.023 \times 10^{23}) / (660 \text{ g mol}^{-1} \times \text{number of base pairs of PCR fragment}) = \text{the number of genomic copy per microlitre [16]}$.

PCR amplification

Multiplex PCR was carried out in 30 µl reaction containing 300 nM each of *aerA* F and *aerA* R primers, 300 nM each

of *hlyA* F and *hlyA* R primers, 50 nM each of 16S rRNA F and 16S rRNA R primers and 200 nM each of GCAT F and GCAT R primers, 200 µM of each dNTP, 7000 copies of IAC DNA, 1 unit of *Taq* polymerase, 2 mM MgCl₂ in 1X PCR buffer with 1 µl of template DNA. PCR was carried out through 35 cycles in eppendorf master cycler gradient thermal cycler at 94°C for 1 min denaturation, 58°C for 1.5 min annealing and 72°C for 1.5 min extension. The DNA was denatured for 5 min in the beginning and finally extended for 10 min at 72°C. PCR products were analyzed in 2% agarose gel.

Determination of sensitivity of mPCR

To assess the minimum amount of *A. hydrophila* DNA detectable by mPCR, 10-fold serial dilutions of overnight growth of *A. hydrophila* ATCC 35654 was serially diluted at a concentration of 10⁶–10⁰ CFU ml⁻¹. DNA was prepared by boiling method as described earlier. A 2.0 µl aliquot of each dilution was added to five separate PCR tubes in the presence of 7000 copies of IAC DNA. *Escherichia coli* genomic DNA was used as a negative control.

Determination of specificity of mPCR

The specificity of the mPCR primers was checked against different organisms as shown in Table 1 by taking 5 µl of template DNA from each of the organism (approx. 10⁷ CFU). The procedure for PCR was essentially the same as described above.

Preparation and enrichment of experimentally spiked food/water samples

In order to validate the mPCR method for detection of *Aeromonas* spp., water and food samples were collected and experimentally inoculated with different number of the

Table 2 Primers for multiplex PCR amplification of *Aeromonas*

Primer	Sequence (5'-3')	Product size (bp)	Target gene	Accession No.
AerA F	ACTGTCTGCCCTGGATATTC	759	<i>aerA</i>	M16495
AerA R	GTTCCAGTTCGGACGGTTGT			
HlyA F	GTGGCAAATAAGCGGTCTGG	450	<i>hlyA</i>	AB021152
HlyA R	CGATCAGACTGGGTACATC			
GCAT F	GGCAAGATGTACAGCAAGATG	617	<i>GCAT</i>	X07279
GCAT R	GTAGCAGGCGTTCTCCGTGT			
16S F	CTGGACAAAGACTGACGCTC	261	<i>16S rRNA</i>	AY987754
16S R	GACATGTCAAGGCCAGGTAA			
IAC 1	ACTGTCTGCCCTGGATATTCT GGCTTAACATATGCGGCA	902	IAC	L08752
IAC 2	GTTCCAGTTCGGACGGTTGT TCGGAACAGGAGAGCGCA			

IAC primers are flanked by *AerA* primers on 5' end.

organism. Water samples (six) were collected from household supply and five sets of 100 ml from each water sample were seeded with *A. hydrophila* bacteria to achieve a concentration of 10^3 , 10^2 , 10^1 , 10^0 , and 10^{-1} CFU ml⁻¹. Each 100 ml sample was filtered through 0.45- μ m-size membrane filter. After filtration, the filter membranes were aseptically dipped in 50 ml of APW-A and incubated at 35°C overnight. One hundred microliters of each was spread plated on to ADA plates and incubated for 18 h at 35°C.

For testing the mPCR assay on foods, pasteurized 2% skim milk and freshly ground fish and chicken were purchased from a local retail shop. Before spiking experiments the food samples were checked for the presence of *Aeromonas* spp. *Aeromonas* spp. could not be detected by culture in any of these food products. These were inoculated with 10^3 – 10^{-1} CFU of *A. hydrophila* or g⁻¹, as appropriate for the sample. After inoculation, 10 ml of each milk sample was mixed with 90 ml of APW-A and 25 g of each ground meat and chicken sample was mixed with 225 ml of APW-A by use of a stomacher. An uninoculated control was prepared by seeding samples with 100 μ l APW. A 100 μ l aliquot of each APW-A sample mixture was plated on to AD agar either immediately or after incubation overnight at 35°C. One millilitre of APW growth was taken at the end of incubation period from all samples and processed for DNA extraction by boiling. The DNA (2.5 μ l) was used as template in PCR assay.

Analysis of field samples

Twelve samples each of raw fish (25 g), chicken (25 g) and 25 samples of milk (25 ml) were collected as described ear-

lier. Twenty-five potable water samples (100 ml), which included 10 groundwater samples (hand pump water) and 15 samples from municipal water supply (tap water) were collected from different locations from Mysore city in sterile containers. All of these above samples were subjected to mPCR and cultural methods after 16 h enrichment in APW-A broth.

Results

Multiplex PCR

Multiplex PCR was optimized using the PCR conditions as indicated in material and methods. The annealing temperature of 58°C was selected because at this temperature only adequate resolution of all the amplified products could be seen. Analysis by BLAST search indicated significant specificity of the primers for the desired gene sequences. All *Aeromonas* and non-*Aeromonas* cultures were identified correctly by the standardized mPCR (Fig. 1).

The concentration of primers was optimized after standardizing on individual PCRs with their respective genes. The concentration of 16S rRNA primers was used lowest because at this concentration there was no interference with the intensity of bands obtained with other amplification products.

IAC and detection limit

The IAC co-amplified with target DNA and had the amplicon size of 902 bp. Inclusion of varying concentrations of

Fig. 1 Multiplex PCR results for screening of standard strains as well as natural samples. Lanes M: 100 bp DNA ladder; Lane 1: *Aeromonas hydrophila* ATCC 35654; Lane 2: *Aeromonas hydrophila* Ah F 2002; Lanes 3, 4, 5, 6: isolates of *Aeromonas*; Lane 7: *Aeromonas sobria*; Lane 8: *Escherichia coli*; Lane 9: *Vibrio cholerae*.

IAC DNA in mPCR mix did not effect the detection limit of the assay and 7000 copies were found to be optimum. The detection limit of the assay was found to be 5 pg.

Analysis of experimentally spiked food/water samples

A. hydrophila could be detected in all experimentally spiked water and food samples after overnight enrichment in APW-A. The minimum number of 10^0 bacteria (100 CFU/100ml) was detected in water samples. In fish, chicken and milk samples the detection limit of 50 CFU/g, 10 CFU/g and 50 CFU/ml respectively was observed.

Analysis of field samples

Out of 12 raw fish samples tested, one was identified to contain *A. hydrophila* by mPCR method. The same sample also yielded *A. hydrophila* organism following culture and conventional biochemical identification. Out of 12 chicken samples tested, one sample was identified to contain *Aeromonas* spp. as it produced amplification with 16S rDNA. Later on the same sample was confirmed as *A. caviae* following detailed biochemical tests as well as with API 20 system. Similarly, out of 25 raw milk samples and 25 water samples tested, one sample from each was tested positive to contain *Aeromonas* by mPCR and later on both these samples were identified to be *Aeromonas* other than *A. hydrophila* by conventional biochemical tests and API 20 system.

Discussion

Numerous methods, both the conventional as well as rapid systems including PCRs have been tried for the detection of foodborne pathogens [13, 16, 18]. However, in the case of *Aeromonas*, the PCR has mostly been used for the characterization of isolates either for their virulence properties or for the phylogenetic position [11, 23].

The present assay targeted the amplification of four genes, of which three are virulence-associated genes and their proteins show mainly hemolytic properties. These include aerolysin (*aerA*), hemolysin (*hlyA*), glycerophospholipid-cholesterol acyl transferase (*GCAT*). Hemolysin genes did not appear to be randomly distributed among the different *Aeromonas* species. The protein AerA is a heat-labile β -hemolysin, which exhibits phospholipase A and C activity. It is also a pore-forming cytolysin able to insert into the cell membrane bilayer causing leakage of cytoplasmic contents. The *aerA* is a major virulence factor of *Aeromonas* [20, 21]. A weak hemolysin, GCAT has been characterized from *A. hydrophila* and other aeromonads. It

is an unusual extracellular lipase released by members of the genus *Vibrio* and *Aeromonas*. The enzyme is considered one of the potential virulence factors that can contribute to the pathogenesis of *Aeromonas* in fish. A genus specific 16S rDNA gene was also included in the mPCR to detect all species of *Aeromonas*.

To examine the possible cross reactions, selected primers were tested both by homology search using BLAST search and screening of *Aeromonas* and non-*Aeromonas* strains. No false positives or false negatives were recorded. IAC was included as the integral component of the assay in order to make the mPCR assay acceptable to the present norms of diagnostic tool [16]. The IAC incorporated was pUC 18 plasmid DNA flanked by *aerA* primers. Optimum number of copies of IAC DNA was selected to give a good visible band in order to avoid competition between target DNA and IAC DNA for *aerA* primers.

The mPCR detected 10^4 CFU ml⁻¹ (500 CFU per reaction) of *A. hydrophila* cell suspension at a probability of 100 percent. The detection limit was 5 pg with pure genomic DNA. Detection limit of 100 CFU/100 ml (1 CFU ml⁻¹) was observed in water samples following overnight enrichment in APW-A. But with fish, chicken and milk samples a higher concentration of the organisms (50 CFU g⁻¹, 10 CFU g⁻¹, 50 CFU ml⁻¹, respectively) was needed. This may be because of the presence of large number of competing microflora and fat globules present in the milk and meat samples that might be restricting the growth of *A. hydrophila*. The conventional detection of these bacteria in foods is laborious and time-consuming that may take up to 3–5 days for the results. Direct application of PCR to food samples often gives a poor amplification signal due to the presence of complex composition of foods that may be inhibitory to PCR reaction. A single step enrichment of samples in APW-A has been recommended by many workers [8]. We also found usefulness of overnight APW-A enrichment for the mPCR reported here. Ampicillin present in APW-A suppresses most of the background microflora that can interfere with PCR but it can also inhibit the ampicillin sensitive *Aeromonas* species, which is *A. trota*. But this bacterium appears to be unusual in milk products.

When evaluated on restricted naturally occurring food and environmental samples (total 74 samples), the mPCR detected three samples positive for *Aeromonas* spp. of which, one was indicative of *A. hydrophila*. The identical results obtained following the conventional culture, isolation and biochemical identification procedures strengthen the claim of the reported mPCR as a reliable alternative for *Aeromonas* detection purposes. Though the samples tested in the present study are low in number, still the results obtained are highly encouraging. Therefore, the high throughput and cost-effective multiplex PCR method

developed in this study could provide a powerful tool for detection of pathogenic *Aeromonas* spp. from food and environmental samples and in addition, the method has advantages in terms of specificity, sensitivity and ease of use compared to other reported PCR methods and DNA hybridization assays.

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