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# A multiplex PCR for detection of haemolytic aeromonas hydrophila from vegetable sources in Karnataka, India

**Puttalingamma Venkataiah, Niveditha Sundar Poojary and Batra Harshvardhan**

Defence Food Research Laboratory, Siddarthanagar, Mysore - 570 011, India

**Abstract**

*Aeromonas hydrophila* and other aeromonads are ubiquitous organisms commonly found in majority of food matrices intended for human consumption. They cause diarrhea, septicemia and extra-intestinal infections in normal and immunocompromised patients. The aim of the present study, to develop a multiplex PCR based strategy for the detection of pathogenic *Aeromonas* spp. by targeting virulence-associated genes including aerolysin *(aerA),* cytotonic enterotoxins *(alt),* serine protease (*Ahe 2*) gene, along with a 16S rRNA gene specific to genus *Aeromonas.* A competitive internal amplification control (IAC) was introduced into mPCR assay to negotiate false negative results during PCR reaction. The results showed that, developed method was useful for specific detection and differentiation of pathogenic and non pathogenic strains of *Aeromonas* spp. mPCR method was successfully evaluated onto several spiked as well as naturally contaminated food samples originated form Karnataka, India. The sensitivity of developed method was determined as $5x10^{-5}$ $CFUml^{-1}$ from experimentally spiked food and water samples. To check the practical usefulness of developed mPCR method a total of 120 vegetable samples were evaluated. Out of 120 samples, eight samples were stayed as positive for *A. hydrophila* contamination. Multiplex PCR results were well correlated with that off conventional culture based methods. In conclusion developed mPCR method could provide a powerful tool for detection and differentiation of pathogenic *A. hydrophila* from food and environmental samples. And also it could be a better alternative for time consuming conventional culture based methods for routine food analysis laboratories.

**Keywords:** *Aeromonas* species, mPCR, IAC, Vegetables and Virulence factors.

## INTRODUCTION

*Aeromonas hydrophila* is commonly involved in causing human infections such as septicemia, gastroenteritis and cellulitis, wound sepsis with necrosis, gangrene, as well as pneumonia and traveler's diarrhea normally resulting from handling and consumption of contaminated food [1&2]. *A. hydrophila* secretes several extracellular proteins including enterotoxin, hemolysin and aerolysin that are associated with the bacterial virulence factors [3,4,5 & 6].

Worldwide, this species has been commonly isolated from clinical and environmental samples i.e., wide range of aquatic systems including lakes, rivers and drinking water as well as a variety of foods [7, 8, 9 & 10]. Isolation of A. *hydrophila* from water and food sources, and the increasing resistance of this organism to antibiotics and chlorination in water, presents a significant threat to public health [11]. Toxins produced by this organism are generally active on the cell surface, causing pore formation and disruption of plasma membrane that subsequently leads to cell lysis [12 & 13].

The conventional detection of these bacteria in food is laborious and time consuming that may take up to 3–5 days for the results. Virulence associated genes have been targeted for the detection of potentially pathogenic *A. hydrophila* by PCR assays that include either cytolysin or hemolysin or both of these genes. [11, 14 & 15]. Yousr, *et al.,* and Fadl, *et al.,* [16 & 17] reported a rapid mPCR which can simultaneously detect pathogenic *A. hydrophila* from fish targeting aerolysin and 16S rDNA genes. Several studies revealed that these organisms can produce exotoxins, including α and β - hemolysins and cytolytic enterotoxin [18 & 9]. Broad methods have been reported, including, tissue cell culture assays, immunoassays, DNA probes and animal models that detect identification of presence of virulence factors in pathogenic *A. hydrophila* [19 &14].

In this study, we describe a robust and specific mPCR assay for the detection of three important virulence-associated genes of *Aeromonas* spp. that include *aerA,* aerolysin gene of *A. hydrophila, altA* a cytotonic enterotoxin gene and extracellular serine protease (*ahe*2) and combined with a genus-specific 16S rRNA gene. The overall aim of this study, therefore was to standardize a mPCR and then to assess its suitability of direct application onto food samples which could be achieved following one step enrichment in alkaline peptone water supplemented with 10 µg/ml ampicillin (APW-A).

## MATERIALS AND METHODS

### Bacterial strains used in the study

The bacterial strains used in this study are listed in Table 1. *Aeromonas hydrophila* (ATCC-35654) strain used as standard in the present study was procured from MicroBiologics, USA. Additionally eight isolates (A- 5,A- 10, A- 34,A- 35, A-36,A- 42, A- 48,A- 68) recovered from different sources in our laboratory were used in the present study for evaluation of the multiplex PCR method.

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*Corresponding Author

Puttalingamma Venkataiah
Defence Food Research Laboratory, Siddarthanagar, Mysore - 570 011, India

Email: puttu_v2005@yahoo.com

**Collection of Samples and isolation of *A.hydrophila***

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).Yellow colonies were counted and confirmed by biochemical key tests. Samples were collected from different sources including main vegetable market, extension vegetable shops and supermarkets located in different parts of the Mysore city, Karnataka (Table-1). *A. hydrophila* were identified biochemically to species level by using 15 tests namely motility, Arginine, Lysine, Indole, Gas from Glucose, sugar fermentation test , Voges-Proskauer test, Urease, 7.5% Nacl, citrate, ampicillin, catalase, indole, methyl red test and $H_2S$ production. Strains which matched biochemical profile of *A. hydrophila* were confirmed as *A. hydrophila.*

**DNA Extraction and Standardization of mPCR**

DNA was extracted by boiling method from the overnight cultures. Multiplex PCR was carried out in 50 µl reaction containing 0.4 µmol $l^{-1}$ *of aerA*-F and *aer*A-R, 0.4 µmol $1^{-1}$ of *alt*-F and *alt*-R, 0.4 µmol $1^{-1}$ *ahe2*-F and *ahe*2-R and 0.2 mol $1^{-1}$ of 16s r RNA F and R primers, 0.2 m mol $l^{-1}$ of each dNTPs, 1.2 unit of Taq polymerase, 2.0 mM MgCl2 in 1XPCR buffer (MBI Fermentas) with 10 ng of template DNA. Amplification consisted of initial denaturation at $94^0$C for 5 min followed by 30 cycles of denaturation at $94^o$C for 1 min, primer annealing at $58^0$C for 2 min and extension at $72^0$C for 2 min and a final extension at $72^0$C for 10 min followed. The PCR products were analyzed on a 2% (wt/vol) Agarose gel. (Sigma, India).

**Primers**

Four sets of primers were designed to detect four genes, *aerA*, *alt*, *ahe*2 and *16s r*RNA using the Gene Bank database sequences (Table 3). Conserved regions were selected and primers were designed with Gene runner software (USA). All primers were procured from Eurofins Genomics Pvt Ltd. Bangalore. PCR products ranged from 221 bp to 841 bp in length.

**Spiking Studies**

In order to validate the mPCR method for detection of *A. hydrophila*, water and food samples were collected and artificially inoculated. Samples of rice based dishes (vegetable pulav) were procured from the local market and water collected from tap water. Vegetable pulav (ten gram) and water sample (10ml) were inoculated with 100µl of *A.hydrophila* (ATCC 35659) culture with cell concentration ranging from $10^{12}$ to $10^1$ CFU ml-1. Each inoculated food sample was later tenfold diluted with peptone water, mixed well and incubated overnight (18 h) at $37^0$C. One milliliter from each sample was taken at the end of incubation period and processed for DNA extraction by boiling method. The DNA (1.5 µl) was used as template in PCR assay.

**Standardization of Multiplex PCR for the Detection of Selected Genes**

The reaction conditions for the multiplex PCR assay were optimized to ensure that all of the target gene sequences were satisfactorily amplified. The primers were designed with care to avoid areas of homology with other organisms. The primers had almost equal annealing temperature, which reduced the possibility of nonspecific amplification. The annealing temperature of $58^0$C was finally selected based on nearly equal intensity of PCR products. Figure 1and 2.shows the presence of amplified products after agarose gel electrophoresis. Reliable amplification of four bands of *aerA, alt, Ahe2 and 16sr* RNA, was obtained in standard *A.hydrophila* strain.

**Robustness**

There was no significant loss in the visibility of bands at less (10%, 20%) or more (10%, 20%) concentrations of PCR reagent. The most prominent change of all concentrations was at 20% less concentrations. Temperature variation of ± $2^0$C also did not make any appreciable change in the profile.

**RESULTS**

**Isolation and Characterization**

Ninty seven Ampicilin resistant colonies were streaked on Ampicillin Dextrin Agar (ADA containing 10 µg/ml Ampicilin) were isolated from different vegetable samples (n-120). Of these, 8 samples were suspected to confirmed *A. hydrophila* by biochemical tests (Table 2). These isolates along with the reference strains were further subjected to monoplex and multiplex PCR assay for the detection of virulence associated genes namely aerolysin, cytotonic enterotoxic, serine protease genes and 16s rRNA. PCR amplification of DNA from *A.hydrophila* 8 isolate using specific primers for aerolysin and cytotonic entertoxin genes resulted in the expected PCR products of 221 bp and 841 bp sizes respectively (Fig-1 & 2). The sequence information of these genes were confirmed with the Genbank database of the National Center for Biotechnology Information (NCBI) by using the BLASTN program (http://www.ncbi.nlm.nih.gov/

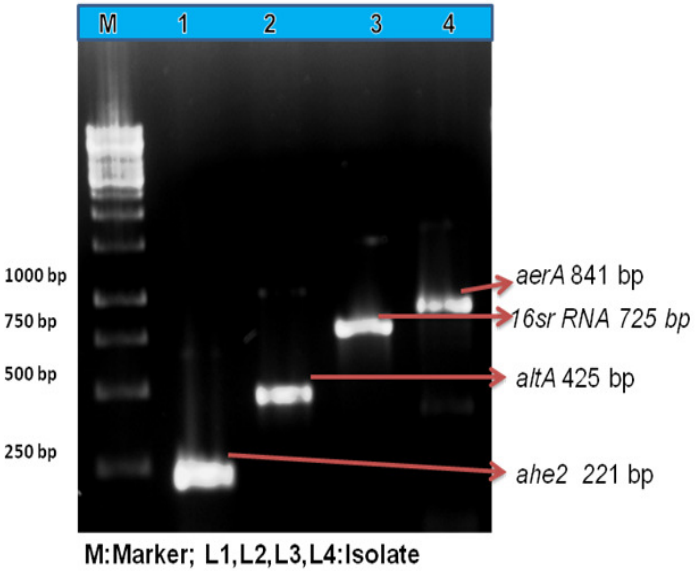
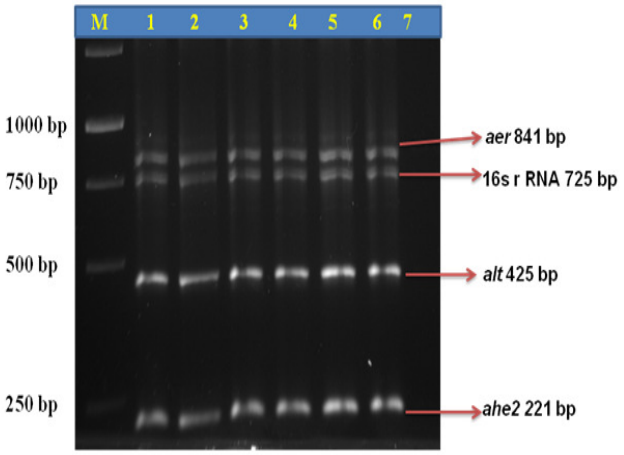



Fig 1. Monoplex PCR for the detection of A. hydrophila




LM; ATCC 35659 :L1; L2-Isolate 1;L3-Isolate 2; L4-Isolate 3; L5-Isolate 4; L6-Isolate; L7-Control

Fig 2. Multiplex PCR for the detection of A. hydrophila

## Standardization of Multiplex PCR for the Detection of Selected Genes

The reaction conditions for the multiplex PCR assay were optimized to ensure that all of the target gene sequences were satisfactorily amplified. The primers were designed with care to avoid areas of homology with other organisms. The primers had almost equal annealing temperature, which reduced the possibility of non-specific amplification. The annealing temperature of 58°C was finally selected based on nearly equal intensity of PCR products. Figure 1&2, shows the presence of amplified products after agarose gel electrophoresis. Reliable amplification of four bands of *aerA, alt, Ahe2* and *16s r* RNA was obtained in standard *A. hydrophila E. coli* as a negative control.

## Robustness

There was no significant loss in the visibility of bands at less (10%, 20%) or more (10%, 20%) concentrations of PCR reagent. The most prominent change of all concentrations was at 20% less concentrations. Temperature variation of ± 2°C also did not make any appreciable change in the profile.

## Analysis of artificially contaminated food samples

*A.hydrophila* could be detected in artificially inoculated vegetable pulav and water samples after overnight enrichment in alkaline peptone water supplemented with 10 µg/ml ampicillin (APW-A). Detection limit was found to be as low as $10^3$ CFU/g for vegetable pulav and $10^2$ CFU/ml from water samples tested. Spiking studies revealed that the developed mPCR method is sensitive enough to detect cells as low as $10^3$ CFU/ml or gram of the overnight-enriched food samples viz., water and rice based vegetable pulav.

Table 1. Isolation of *Aeromonas spp.* from different vegetable samples

| | Samples | No. of samples Screened | Confirmed *Aeromonas* spp. obtained (main market) |
|---|---|---|---|
| 1 | Radish | 15 | 2 |
| 2 | Carrot | 15 | 1 |
| 3 | Coriander root, | 15 | 3 |
| 4 | Cabbage | 15 | 1 |
| 5 | Tomato | 15 | 1 |
| 6 | Capsicum | 15 | 0 |
| 7 | Ridge gourd | 15 | 0 |
| 8 | Leafy vegetable | 15 | 1 |

Table 2. Biochemical and Molecular characterization of *A. hydrophila*

| | Biochemical test | | | | | | | | | | | | PCR results | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Motility | Arginine | Lysine | Indole | Gas from Glucose | VP test | Lactosel | Urease | 7.5% Nacl | Citrate | Sucrose | Ampicillien | *aerA* | *altA* | *ahe2* | *16sr PNA* |
| ATCC *35654* | + Ve | +Ve | + Ve | +Ve | + Ve | +Ve | -Ve | -Ve | +Ve | -Ve | +Ve | R | +Ve | +Ve | +Ve | +Ve |
| A-5 | +Ve | +Ve | +Ve | +Ve | +Ve | +Ve | -Ve | -Ve | +Ve | -Ve | +Ve | R | +Ve | +Ve | +Ve | +Ve |
| A-10 | + Ve | +Ve | + Ve | +Ve | + Ve | +Ve | -Ve | -Ve | +Ve | -Ve | +Ve | R | +Ve | +Ve | +Ve | +Ve |
| A-34 | +Ve | +Ve | +Ve | +Ve | +Ve | +Ve | -Ve | -Ve | +Ve | -Ve | +Ve | R | +Ve | +Ve | +Ve | +Ve |
| A-35 | +Ve | +Ve | +Ve | +Ve | +Ve | +Ve | -Ve | -Ve | +Ve | -Ve | +Ve | R | +Ve | +Ve | +Ve | +Ve |
| A-36 | -Ve | -Ve | -Ve | -Ve | -Ve | -Ve | -Ve | -Ve | +Ve | -Ve | +Ve | R | +Ve | +Ve | +Ve | +Ve |
| A-42 | +Ve | +Ve | +Ve | +Ve | +Ve | +Ve | -Ve | -Ve | -Ve | -Ve | -Ve | R | +Ve | +Ve | +Ve | +Ve |
| A-48 | +Ve | +Ve | +Ve | +Ve | +Ve | +Ve | -Ve | -Ve | +Ve | -Ve | +Ve | R | +Ve | +Ve | +Ve | +Ve |
| A-68 | +Ve | +Ve | +Ve | +Ve | +Ve | +Ve | -Ve | -Ve | +Ve | -Ve | +Ve | R | +Ve | +Ve | +Ve | +Ve |

Table 3. Genes targeted and primer sequences

| Gene | Primer sequence(5` to 3`) | Product size(bp) | Accession number |
|---|---|---|---|
| *aerA F* | TTA CTC GAG AGG AAG CCC A | 841 | Hq4256.1 |
| *aerA R* | TAG GGA TAG GAG ATG TCA G | | |
| *altA* F | TCA ACA CCA TCA CCG ACG T | 452 | L77573.1 |
| *altA* R | ATC GAA CTT GAA CAG GGC A | | |
| 16S RNA F | ATG GCT GGG TAA TAA TGG G | 745 | NC-008570.1 |
| 16S RNA R | GCC TTG TAG AGC TCG ATC | | |
| F *ahe*2 | ACG GAG TGC GTT CTT CCT ACT CCA G | 221 | X 67043 |
| R *ahe*2 | CCG TTC ATC ACA CCG TTG TAG TCG | | |

## DISCUSSION

*A hydrophila* is a causative agent of a number of human infections. Such strains possess virulence properties and produce types of enterotoxins they are required to be surveyed particularly when the strain is involved in disease outbreaks to assess the response strategies for containment. Many methods, both the conventional as well as rapid detection methods including PCRs have been tried for the detection of Foodborne pathogens [14, 11, 15, 16 & 17]. However, in the case of *Aeromonas*, the PCR has mostly been used for the characterization of isolates either for their multifactorial virulence factors or for the phylogenetic position [9 & 15]. The present assay targeted the amplification of four genes, of which three are virulence-associated genes and mainly responsible for hemolytic toxins. These included aerolysin (*aerA*), cytotonic enterotoxins gene *(alt)*, serine protease (*ahe*2) along with a 16S rRNA gene.

The increasing presence of *A. hydrophila* in fish may become a potential human health hazard [20 & 21]. 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. For the mPCR reported here, all the primers were designed with the view to have a common annealing temperature to get preferable amplification at a single temperature and care was also taken to maintain at least 100 bps differences between product sizes for good resolution during agarose gel electrophoresis. Another important criterion for a diagnostic PCR is robustness. Spiking studies revealed that the developed mPCR method is sensitive enough to detect cells as low as $10^3$ CFU/gram of the overnight-enriched food samples and $10^2$ CFU/ml water. Spiking studies revealed that the developed mPCR method is sensitive enough to detect cells as low as $10^3$ CFU/ml or gram of the overnight-enriched food samples viz., milk and rice based vegetable pulav.

Classical pathogen identification methods take five to seven days including inoculating into a medium, culturing, staining, and confirming pathogen existence via biochemical or serological tests still being followed in many laboratories. Polymerase chain reaction (PCR), however, provided rapid and inexpensive detection of pathogens and could significantly reduce the time required to identify *A. hydrophila* strains. Like other observations we could also find utility of a single step enrichment of samples in APW-A for the mPCR. Ampicillin present in APW-A suppresses most of the other microflora that can interfere with PCR.

When evaluated on restricted naturally occurring food samples (total 120 samples), the mPCR detected eight samples positive for *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. The presently described mPCR that takes care of important virulent components of *A. hydrophila* strains can definitely be helpful in providing early information onto the killing potential of such strains during the initial phase of outbreaks and can also help in molecular epidemiological investigations even to trace the source of the outbreak.

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