

# Direct detection of diarrheagenic *Aeromonas* from faeces by polymerase chain reaction (PCR) targeting aerolysin toxin gene

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**Abstract.** – Detection of diarrheagenic *Aeromonas* specific aerolysin toxin (*Aer*) gene by PCR based assay and isolation, identification of diarrhea causing *Aeromonas* from faeces by culture methods were carried out in the Division of Active Surveillance, National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India for a period of 12 months. Out of 602 faecal samples collected from patients with acute diarrhea admitted in Infectious Diseases (ID) Hospital, Kolkata, 68 (11.29%) samples were found to be possessing *Aer* gene by PCR technique. The conventional culture methods using selective media yielded only 64 (10.6%) *Aeromonas* strains from the same faecal samples. The different *Aeromonas* species possessing *Aer* gene identified by PCR based technique include *A. hydrophila* (55.8%), *A. caviae* (17.6%), *A. veronii* (10.2%), *A. schubertii* (4.4%), *A. jandaei* (2.9%) and *A. trota* (8.8%). The isolation and identification of *Aeromonas* by routine culture did not detect enterotoxigenic *A. trota* present in four diarrheal faecal samples. The failure of the growth of enterotoxigenic *A. trota* on selective media may be attributed to the ampicillin susceptibility of those strains. The quality control studies revealed that PCR method for the direct detection of *Aer* gene from the faeces has the sensitivity of 100% and specificity of 98%.

*Key Words:*

*Aeromonas*, *Aeromonads*, *Aer*-gene, Diarrhea, Enterotoxigenicity, Aerolysin and PCR-assay.

## Introduction

The significance of *Aeromonas* species as a causative agent of human diarrhea has recently been established<sup>1</sup>. Several water and food borne diarrheal outbreaks due to these

bacterial strains have been reported especially in the developing countries<sup>2-4</sup>. *Aeromonads* have been found to cause a variety of extra-intestinal infections in the normal as well as severe infections in the immune-compromised patients<sup>5</sup>. Six different species of *Aeromonas* viz., *A. hydrophila*, *A. caviae*, *A. veronii* (biotype *Sobria* and *Veronii*), *A. schubertii*, *A. jandaei* and *A. trota* have been associated with human diarrhea<sup>6</sup>. *Aeromonas* produces several virulence factors like aerolysin (enterotoxin), haemolysin, cytotoxin, proteases, adhesins (pilins) etc.<sup>7-9</sup>. Many studies on molecular biology of the virulence genes of diarrheagenic *Aeromonas* revealed that these strains harbouring aerolysin toxin gene (*Aer*) are potential diarrheagenic in nature<sup>10-12</sup>. Present study attempted to establish a method to utilize this diarrhea specific toxin gene marker as a tool for the diagnostic detection of diarrhea causing *Aeromonas* directly from faeces by PCR based technique.

## Materials and Methods

### *Isolation of Aeromonas by culture methods*

A total of 602 faecal samples from the diarrheal patients with the age group ranging between 1 and 62 years were tested for the presence of enterotoxigenic *Aeromonas*. The faecal samples were transported to the laboratory in the Cary-Blair transport medium. All the samples were enriched in Alkaline Peptone water (APW) at 37° C for 18 hours. The enriched inoculums from APW was streaked on Ampicillin Sheep Blood agar (ASBA) and incubated at 37° C for 24

hours<sup>13</sup>. Both haemolytic and non-haemolytic colonies were tested for oxidase test. Strains were stocked on nutrient agar slabs at room temperature for further studies.

#### **Differentiation of *Aeromonas* species**

The oxidase positive colonies were further confirmed by the biochemical tests and species differentiation was performed by the method described by Aerokey II group of tests for the identification of *Aeromonas* species<sup>14</sup>.

#### **Identification of enterotoxigenic *Aeromonas***

From all of the *Aeromonas* isolates, cell free fluid (CFF) were prepared as per the procedure elaborated by Gray et al (1990). The enterotoxigenicity of the isolates was measured by the Mouse Paw Oedema test (MPOT)<sup>16</sup>.

#### **Pretreatment of the faeces for PCR-assay**

Pretreatment of the faeces was required to remove the PCR-inhibitors present in it<sup>17</sup>. About a gram of stool sample was emulsified in 10 ml of normal saline. This suspension was heated at 94° C for 5 minutes followed by centrifuge at 1700 g for 3 minutes. The supernate was examined by PCR.

#### **PCR-assay**

A 480 bp section of the aerolysin (enterotoxin) gene was amplified with *Aer-1* and *Aer-2* primer pairs with the sequences 5'-ATGACCCAGTCCTGGCACGG-3' and 5'-GCCGCTCAGGGCGAAGCCGC-3' respectively. These primers were supplied by Toyoba, Osaka, Japan. The denatured DNA template for the PCR was made available by boiling the supernate for 10 minutes and immediately kept in ice. The reaction mixture consists of 2.5 µl of 10 X amplification buffer (500 mM KCl, 100 mM Tris HCl, 15 mM MgCl<sub>2</sub> pH 8.3), 8 µl of 25 µM MgCl<sub>2</sub>, and 2.5 µl each of 2 mM dATP, dGTP, and dCTP, 10 nM each of the denatured DNA template. PCR reactions were carried out in a Perkin-Elmer - CA, USA, Thermal Cycler with the following programme: 30 cycles at 95° C for 1 minute, 55° C for 1 minute and 72° C for 8 minutes. The PCR amplicons were visualized by ethidium bromide staining after electrophoresis using 1.5% agarose gel.

#### **Identification of *Aeromonas* species after PCR-assay**

The faecal samples that were showing *Aeromonas* negative by culture methods but were positive by PCR assay were again enriched in APW at 37° C for 18 hours. The enriched inoculums from APW was streaked on blood agar plates and incubated at 37° C for 24 hours. In order to obtain a better isolation, both haemolytic and non-haemolytic colonies were again subcultured on blood agar plates. Each bacterial colony was tested for oxidase test. The oxidase positive colonies were further confirmed by the biochemical tests as described in Aerokey II group of tests. These *Aeromonas* strains were further tested for enterotoxigenicity by MPOT.

#### **Sensitivity and specificity of PCR assay**

A total of 160 faecal samples from the diarrheal patients of which 30 were from aeromonad diarrhea, 50 were from cholera cases, 10 were due to enterotoxigenic *E. coli* (ETEC) diarrhea. 10 were from Shigella dysentery, 50 were from healthy volunteers and 10 samples were environmental *Aeromonas* isolates, were used for this study. All the samples were screened for the presence of enterotoxigenic *Aeromonas* by routine culture methods followed by MPOT and PCR. The percentage of sensitivity and specificity were calculated as per the formula derived by Cochrane et al (1971).

## **Results**

Of the 602 faecal samples that were tested by culture methods, 64 (10.6%) showed the presence of *Aeromonas*. The different isolated and identified *Aeromonas* species were *A. hydrophila* 38 (59.3%), *A. caviae* 12 (18.7%), *A. veronii* 7 (10.9%), *A. schubertii* 3 (4.6%), *A. jandaei* 2 (3.1%) and *A. trota* 2 (3.1%). All these *Aeromonas* strains isolated by culture methods expressed enterotoxigenicity by MPOT. A total of 68 (11.29%) faecal samples with *Aeromonas* harbouring aerolysin toxin (*Aer*) gene were directly detected by PCR assay (Table I). All the four faecal samples that were positive only by PCR revealed the presence of enterotoxigenic *A. trota* strains. A clear, sin-

Table I. Identification of enterotoxigenic *Aeromonas* by culture methods and PCR.

Isolated <i>Aeromonas</i> species	No. of strains positive by culture method	No. of strains positive by PCR	Enterotoxigenicity testing by MPOT
<i>A. hydrophila</i>	38 (59.3)	38 (55.8)	Enterotoxigenic
<i>A. caviae</i>	12 (18.7)	12 (17.6)	Enterotoxigenic
<i>A. veronii</i>	7 (10.9)	7 (10.2)	Enterotoxigenic
<i>A. schubertii</i>	3 (4.6)	3 (4.4)	Enterotoxigenic
<i>A. jandaei</i>	2 (3.1)	2 (2.9)	Enterotoxigenic
<i>A. trota</i>	2 (3.1)	6 (8.8)	Enterotoxigenic
Total	64 (10.6)	68 (11.29)	

Figures in parentheses are percentages.

gle and discrete band representing 480 bp section of the aerolysin toxin (*Aer*) gene harboured by different enterotoxigenic *Aeromonas* species present in faeces was visualized after PCR-assay (Figure 1). The results of sensitivity and specificity testing of PCR-assay are given in the Table II. This method has showed sensitivity of 100% and specificity of 98%.

### Discussion

Rapid and accurate detection of enterotoxigenic bacteria from the faecal sample is needed for the diagnosis of diarrhea. As the biochemical identification of bacterial isolates may take several days, a PCR based

method was developed to reduce the time required for diagnosis. The usual culture methods may not isolate uncultivable bacteria excreted through faeces from the diarrheal patients under the antibiotic therapy.

Carnahan et al (1991a) reported some ampicillin-susceptible *A. trota* strains isolated from clinical specimens. In our case, the routinely employed ampicillin containing selective media - ASBA, did not allow the growth of enterotoxigenic *A. trota* strains from the faeces. But the PCR based technique clearly identified the faeces, which were containing enterotoxigenic *A. trota*. During specificity testing of PCR, it detected only one sample containing *V. cholerae* as positive for diarrheagenic *Aeromonas*. This may be attributed to the genomic similarities between cholera toxin (CT) and aerolysin toxin (*Aer*) genes.

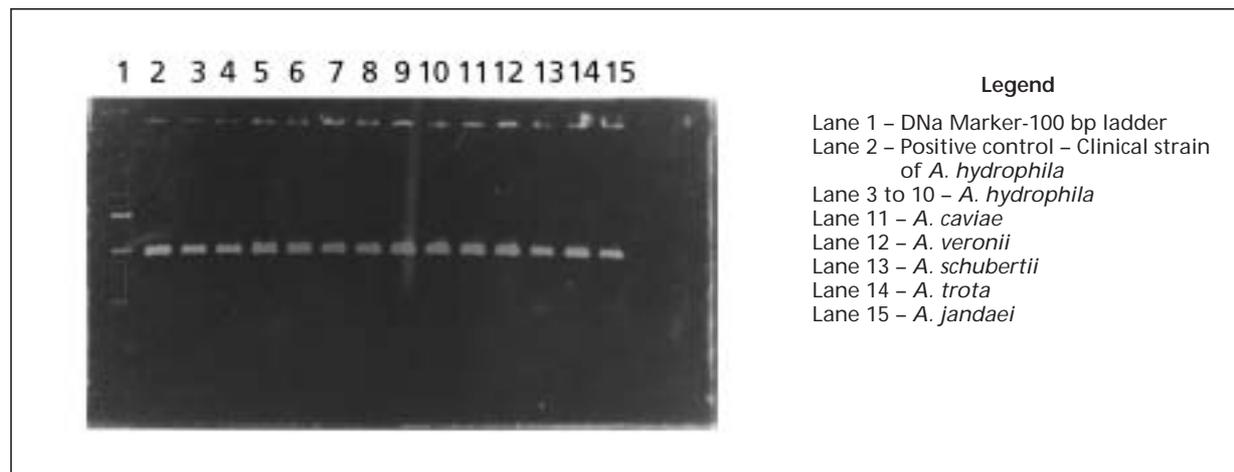


Figure 1. Results showing 480 bp amplicon of the aerolysin (enterotoxin) gene harboured by diarrhea causing *Aeromonas* spp. from faecal samples after PCR.

Table II. Correlation of culture method and PCR based method in the identification of enterotoxigenic *Aeromonas*.

S.NO.	Actual causative agent source of samples	No. of samples screened	No. of samples positive for <i>Aeromonas</i> by culture and MPOT	No. of samples <i>Aeromonas</i> by PCR
1.	<i>Aeromonas</i>	30	28	30
2.	<i>Vibrio cholerae</i>	50	-	01
3.	Enterotoxigenic <i>E. coli</i> (ETEC)	10	-	-
4.	<i>Shigella</i>	10	-	-
5.	Healthy volunteers	50	-	-
6.	Environmental <i>Aeromonas</i> isolates	10	10*	-

\**Aeromonas* isolates which were showing non-enterotoxigenic by MPOT.

Thus, the PCR method of detecting diarrheagenic *Aeromonas* from faecal samples targeting aerolysin toxin (*Aer*) gene has showed greater rapidity, reliability and accuracy than the conventional culture methods. Since the diarrhea due to *Aeromonas* is increasingly reported, this PCR based methodology may be utilized in the Disease Surveillance, Diagnostic and Molecular biology laboratories for the detection of diarrhea causing *Aeromonas*.

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