Molecular identification of diarrheal *Aeromonas* using immuno magnetic polymerase chain reaction (IM-PCR) technique: a comparative study with conventional culture method

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Abstract

Background: *Aeromonas* are ubiquitous bacteria causing many clinical conditions including acute diarrhea. Diarrheagenic *Aeromonas* harbors aerolysin gene secreting virulent enterotoxin, aerolysin.

Objectives: To develop a molecular and immunological based method for detection of *Aeromonas*.

Methods: Diarrheal *Aeromonas* strains were identified from stool samples using culture, enterotoxicity testing using mice model. During immune magnetic polymerase chain reaction IM-PCR protocol, aerolysin specific antibodies were bound with immuno magnetic binding. Sensitivity and specificity tests for IM-PCR were conducted.

Results: There was high detection of *Aeromonas* using IM-PCR (12.4 %) technique when compared to low isolation with culture (5.1%). Our study confirmed that some strains of enterotoxic *Aeromonas* strains were uncultivable. Enterotoxicity tests on culture isolates revealed many strains were negative. IM-PCR detected high, (62/500) rate of identification of Aeromonas with aerolysin toxin gene. *Aeromonas* species identified after IM-PCR were A. hydrophila (40.3%), A. veronii (17.7 %), A. caviae (14.5 %), A. trota (11.2 %), A. jandei (9.6 %) and *A. schuberti* (6.4%). All *A. trota* strains were undetected by cultivation.

Conclusion: High sensitivity and specificity of IM-PCR are due to preparation of aerolysin antibodies and immuno magnetic binding, prior to PCR. Since diseases due to *Aeromonas* are increasingly reported, IM-PCR is recommended for detection from clinical specimens.

Keywords: Aeromonas, IM-PCR, acute diarrhea, aerolysin, enterotoxicity.

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Introduction

The members of the genus Aeromonas are Gram negative bacilli similar to Vibrionaceae[1,2]. Importance of aeromonad diarrhea has been established by many microbiologists[3–6]. In developing nations, numerous food and water related epidemics due to *Aeromonas* were reported[7–9]. Even in developed countries variety of infections due to Aeromonas have been noted[10–13]. *Aeromonas* is also known to cause other infections ranging from

meningitis, pneumonia, wound associated sepsis to ocular disease[14–16]. It was noticed that aeromonads can cause diseases in healthy individuals and very severe infections in immune compromised patients[17,18]. *Aeromonad* diarrhea in humans is caused by six species of Aeromonas consisting of *A. hydrophila*, *A. veronii* (biotype Sobria and Veronii), *A. caviae*, *A. trota*, *A. jandei* and *A. schuberti*[19]. Many virulent determinants of this bacterium such as pili adhesins, enterotoxin (aerolysin), cytolytic toxin, hemoytic toxin, lipases,and proteases have been studied[20,21]. Various bacteriological data revealed that aerolysin toxin gene is similar to cholera toxin (CT) gene and Aeromonas strains containing aerolysin toxin gene are highly associated with diarrhea[22]. This article brings out our study based on immuno magnetic polymerase chain reaction (IM-PCR) technique to detect pathogenic *Aeromonas* strains harboring aerolysin gene from diarrheal stool samples. Current diagnostic methods in diarrheal *Aeromonas* in laboratories and research organizations include microscopy, staining, culture using selective media, biotyping and serotyping. These routinely used procedures are time consuming, inaccurate and insensitive in detection. Current proposed method is novel and unique because it uses both immunological based binding and molecular based PCR set up for the diagnosis of diarrheal *Aeromonas*.

Materials and methods

Cultivation of *Aeromonas* using conventional culture techniques

A total of 500 diarrheal stool specimens were analyzed over a period of twelve months in our Diarrheal Active Surveillance Unit (DASU), Jimma University, Ethiopia. Isolation of *Aeromonas* was performed using alkaline peptone water and ampicillin sheep blood agar (ASBA) for 24 hours at 37°C2[3].

Enterotoxic potential of isolated *Aeromonas* strains

Aeromonas cultures grown in peptone water were centrifuged at 6000 x g for 10 minutes to obtain cell free supernatant. Aerolysin present in the supernatant was furtherconcentrated and purified by sterile filtration (0.22 µm filters, Millipore, Billerica, USA). 50 µL doses of purified aerolysin toxin were injected through sub-plantar route into paw of Swiss male mice (7 week old with weight 30 – 35 g). Severity of inflammatory responses and edema were recorded starting from 1 hour up to 96 hours[24].

Immunization and preparation of aerolysin specific antibodies

Rabbits were immunized with aerolysin recombinant protein (aerA, MyBioSource, Canada) in divided, increasing doses over a period of 1, 2, 3 and 6 months.

Immuno magnetic binding of aerolysin immunoglobulins 50 µL of aerolysin specific immunoglobulins were allowed to bind with anti-immunoglobulin coated on magnetic chromium oxide for 30 minutes at 25°C in a shaker.

Immune magnetic binding of aerolysin secreting *Aeromonas* in stool

Stool specimens were reacted with magnetic particles containing aerolysin specific immunoglobulins at 25°C for 1 hour. Magnetic particles were centrifuged at 1000 g for 5 minutes. Clear supernatant was subjected to PCR.

Molecular detection using Immuno magnetic PCR technique (IM-PCR)

Using AeroFr –CCAAGGGGTCTGTGGCGACA- (forward primer) and AeroRv–TTTCACCGGTAACAG-GATTG- (reverse primer) (Toyoba, Japan) a 209 bp part of aerolysin gene was amplified using thermal cycler (Perkin Elmer, US). Denatured DNA sample was obtained by heating the supernatant for 15 minutes and treating it with ice. Reaction mixture contained 2.0 µl of 2 mM dATP, dGTP, and dCTP, 15 nM of denatured DNA and 2.0 µl of 10 X amplification buffer. PCR thermal conditions were set as 32 cycles at 93º C for 1 minute, 54ºC for 1minute and 74ºC for 10 minutes.

Specificity and sensitivity of Immuno magnetic PCR (IM-PCR)

An overall number of 150 stool specimens from diarrheal cases were included for assessment of the specificity and sensitivity of Immuno magnetic PCR. Specimens from various sources were included. Statistical analysis were conducted using IBM – SPSS version 21.0.

Results

Comparison of *Aeromonas* isolates with other established pathogens using conventional culture techniques

Our results showed that the isolation rate of *Aeromonas* using culture was low when compared with established pathogens. (Figure 1). *A. trota* was not isolated by culture method. (Table 1).

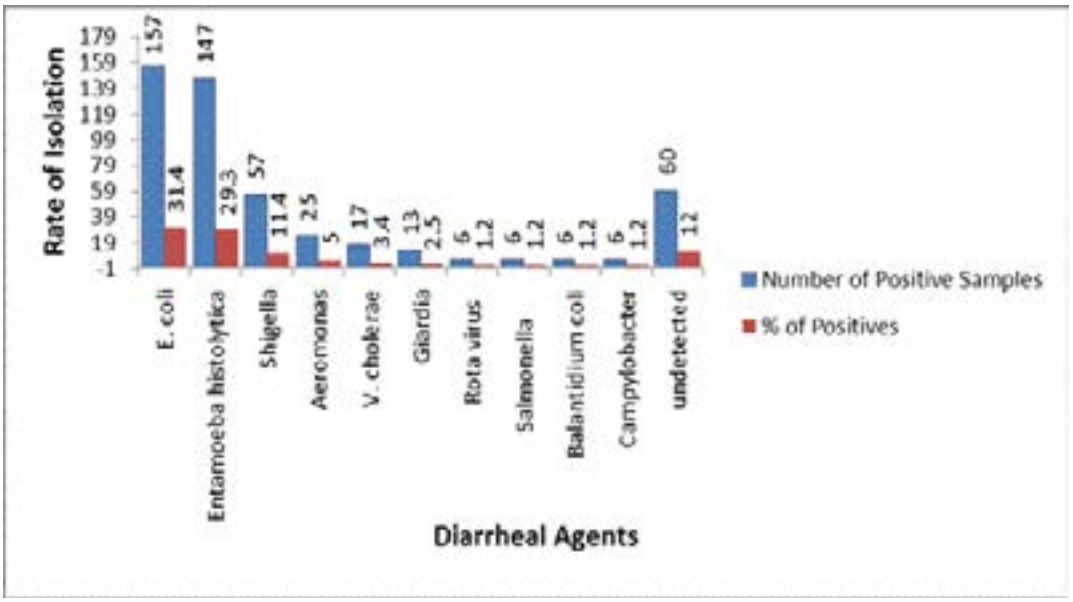

Figure.1. Isolation rates of different diarrheal agents using conventional methods (n=500).

Table.1. Identification of *Aeromonas* species using culture and immune magnetic polymerase chain reaction (IM-PCR) and comparison with the enterotoxicity of isolates.

Aeromonas species identified	Rate of isolation by culture, %	Enterotoxicity test of Aeromonas isolates after culture	Rate of detection after IM-PCR[a], %	Number of strains showing enterotoxicity after IM-PCR[a]
A. hydrophila	12, (38.7)	10/12	25, (40.3)	25/25
A. caviae	8, (25.8)	7/8	9, (14.5)	9/9
A. veronii	5, (16.1)	5/5	11, (17.7)	11/11
A. schuberti	3, (9.7)	2/3	4, (6.4)	4/4
A. Jandei	3, (9.7)	1/3	6, (9.6)	6/6
A. trota	ND[b]	ND[b]	7, (11.2)	7/7

Key to abbreviations:
a: Immune magnetic polymerase chain reaction.
b: Not detected.

Enterotoxicity test using paw of mice

Enterotoxicity tests using paw of mice on *Aeromonas* isolates exhibited that not all strains were enterotoxigenic. Positive results of out of the total *aeromonad* culture isolates were *A. hydrophila* (10/12), A. caviae (7/8), A. veronii (5/5), *A. schubertii* (2/3) and A. jandaei (1/3) (Table 1).

Immune magnetic binding of aerolysin secreting *Aeromonas* in stool

Immune diffusion method showed specific antigen- antibody reaction suggesting antibodies produced in rabbits was highly specific. This recommended the application of aerolysin specific antibodies in immune magnetic binding. Molecular detection of all aerolysin genes containing *aeromonads* in stool were detected using IM-PCR technique.

Identification of pathogenic *Aeromonas* by IM-PCR

The total number of *Aeromonas* strains identified using this technique was 62/500 (12.4%) (Figure 2). In addition, species of *Aeromonas* detected after IM-PCR were, *A.*

hydrophila (40.3%), *A. veronii* (17.7%), A. caviae (14.5%), *A. trota* (11.2%), *A. jandei* (9.6%) and *A. schuberti* (6.4%) (Table 1).

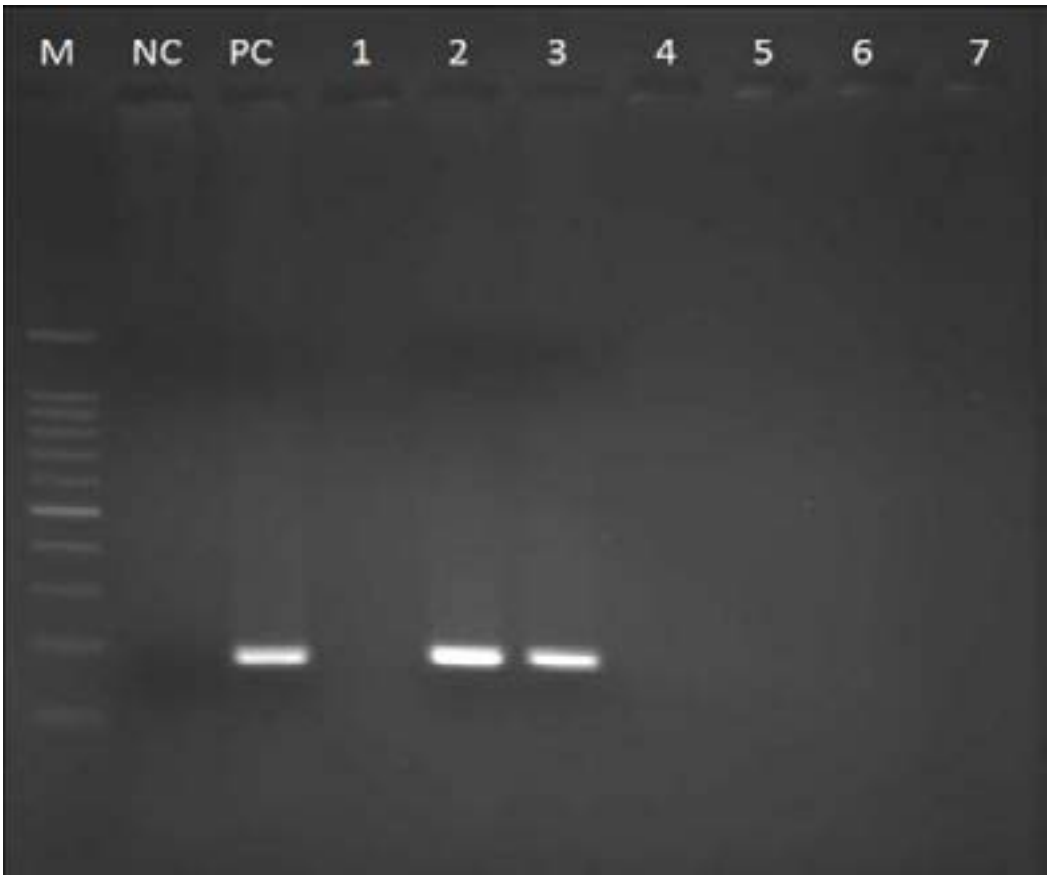

Figure 2. Agarose gel electrophoresis (2% agarose) after IM -PCR. M- Molecular marker (100 bp DNA ladder), NC- Negative control, PC- Positive control, Lane 1-. negative sample, Lane 2 & 3 - *Aeromonas* isolates with aerolysin gene amplicons. Lane 4 to 7 – negative samples.

Specificity and sensitivity of IM-PCR

Specificity and sensitivity assessments revealed that all the 40 stool samples from established *Aeromonas* diarrhea were confirmed as positives by IM-PCR technique. Surprisingly we observed that 30 cholera samples subjected for *Aeromonas* testing by culture revealed negative whereas 1/30 of these *V. cholerae* containing sample shown positive for IM-PCR. Interestingly IM-PCR technique detected 2 out of 15 as positives for *Aeromonas* possessing aerolysin toxin gene from these environmental isolates (Table 2).

Table 2. Correlation of culture method and immune magnetic polymerase chain reaction (IM-PCR) in the identification of enterotoxic *Aeromonas*.

Source of sample causative agent	Number of samples analyzed	Number of positive samples for *Aeromonas* by culture	Number of positive Enterotoxicity samples for paw *Aeromonas* by IM-PCR[a]	test using of mice
Aeromonas	40	40	40	40
E. coli	15	-ve[b]	-ve	-[c]
Plesiomonas	10	-ve	-ve	-
Healthy volunteers	40	-ve	-ve	-
Vibrio cholerae	30	-ve	1/30	-
Environmental *Aeromonas* isolates	15	15	2/15	-ve

Key to abbreviations:
a: Immune magnetic polymerase chain reaction.
b: Negative.
c: Samples not tested for enterotoxicity test using paw of mice.

Discussion

Developing countries employ culture based identification strategy which is time consuming and results may delay the initiation of appropriate treatment[25]. There were some molecular based detection systems are developed for detection of *aeromonad* infections but unfortunately these protocols were found to be unaffordable to all diagnostic laboratories[26,27]. To overcome these disadvantages, our team has developed a novel and rapid PCR based method for detecting diarrhea causing *Aeromonas* directly from stool samples. Our IM-PCR based detection method is one of the first of its kind to detect Aeromonas directly from stool by coupling antigen – antibody based followed by PCR protocol.

Routine culture and identification for established pathogens along with Aeromonas resulted in high percentage of isolation rates for E. coli (31.4%), Entamoeba histolytica (29.3%) and Shigella (11.4%). It was observed that the isolation rate for Aeromonas was only 5.1%. In addition we have found that the undetected pathogens were also high with 12% (Table 1). Reasons for low isolation rate of *Aeromonas* and inability to detect pathogens may be attributed to possible presence of antibiotics in the samples, ampicillin sensitivity of some *Aeromonas* strains, significantly low number of pathogens in the samples or otherstressful factors that may influence the retardation of growth of pathogens in culture media. These issues during conventional culture methods were already faced by many microbiologists[23,28,29].

PCR based IM-PCR method detected significantly high, (12.4%) identification of *Aeromonas* from stool specimens. Culture media identification of *Aeromonas* species resulted in the detection of five species whereas IM-PCR technique identified six species with, *A. trota* (Table 1). The failure of growth of *A. trota* on selective media culture may be due to ampicillin sensitivity of this species.

Enterotoxicity test using paw of mice revealed that, 2 strains of *A. hydrophila*, 1 from *A. caviae*, 1 strain from A. schubertii and 2 strains of *A. jandei* were non-enterotoxic. IM-PCR method detected significantly high, 62/500 (12.4%) species of *Aeromonas* with aerolysin gene (Table 1).

IM-PCR method expressed sensitivity of 100% and specificity of 99%. IM-PCR detected one *V. cholerae* as positive and may be due to genetic similarity between cholera toxin (CT) gene and aerolysin gene[22]. Moreover two environmental samples (fresh water sources) have also become positives for IM-PCR and this suggests that environment is the main reservoir and source for pathogenic Aeromonas[30–33].

When compared to culture methods, IM-PCR method showed high accuracy, efficacy, quality, ease in application and rapidity. Increased sensitivity and specificity of IM-PCR method may be due to its novel procedures like, preparation of aerolysin specific antibodies and Immuno magnetic binding of aerolysin immunoglobulins, prior to actual PCR protocol.

Due to vast number of *aeromonad* cases are increasingly reported in humans, it is suggested that IM-PCR may be useful in the diagnosis.

Conflict of interest statement

We declare that we have no conflict of interest.

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