A study on the prevalence of Aeromonas spp. and its enterotoxin genes in samples of well water, tap water, and bottled water

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Abstract

Aim: The aim of this work was to study the prevalence of Aeromonas spp. and its enterotoxin genes in various water sources.

Materials and Methods: 125 samples (50 from well water, 50 from tap water, and 25 from bottled water) were collected from various sources in and around Greater Hyderabad Municipal Corporation and examined for the presence of aeromonads by both cultural and polymerase chain reaction (PCR) assay. Alkaline peptone water with ampicillin was used as enrichment. Aeromonas isolation medium and ampicillin dextrin agar were used as selective media. The boiling and snap chilling method was used for DNA extraction. Primers targeted against 16S rRNA, aer, and ast were used to identify aeromonads and its enterotoxins.

Results: 48%, 18%, and 12% of well water, tap water, and bottled water samples were found positive by cultural assay with an overall prevalence of 28.8%. Aeromonads were detected in 32% (52% in well water, 20% in tap water, and 16% in bottled water) of samples by PCR assay. Aerolysin (aer) gene was noticed in 34.6%, 20%, and 0% of well water, tap water, and bottled water samples, respectively, with an overall prevalence of 27.5%. Thermostable cytotoxic enterotoxin (ast) was observed in 37.5% (42.3% in well water, 30% in tap water, and 25% in bottled mineral water) of samples.

Conclusions: Presence of aeromonads and its toxin genes in various sources of water is of public health concern and emphasizes the need for necessary preventive measures to tackle the problem.

Keywords: Aeromonas spp., enterotoxins, polymerase chain reaction, prevalence, water.

Introduction

Aeromonas spp. are ubiquitous in aquatic environments and was reported to be isolated from ground, surface, marine, drinking, and waste waters [1]. Some species of aeromonads were reported to be the cause of various diseases in aquatic animals, livestock, and humans. In 1968, Von Graevenitz and Mensch reported the importance of Aeromonas spp. as a human pathogen and suggested aeromonads may be associated with gastrointestinal disease [2]. Today aeromonads were reported to be the cause of community acquired infection, nosocomial infection, and travelers’ diarrhea and infections associated with hurricanes, tsunamis, and earthquakes [3].

The ability of aeromonads to colonize drinking water systems, produce biofilms and resist chlorination is of public health significance, as the organisms are able to elaborate toxins and cause various disease manifestations ranging from gastroenteritis to septicemia. Hence, considering its importance Aeromonas hydrophila was listed in the contaminant candidate list [4] and the Environmental Protection Agency Method 1605 was validated for its detection and enumeration in drinking water system. In 1986, health authorities in the Netherlands introduced “indicative maximum values” for Aeromonas densities in drinking-water [5]. Most important hemolysin produced by Aeromonas spp. is aerolysin (also called as cytotoxic enterotoxin, asao toxin and cholera toxin cross-reactive cytolytic enterotoxin), which possess both hemolytic and enterotoxigenic properties [4]. Environmental strains containing aerolysin are potentially enterotoxigenic when passed from host to host, but environmentally adapted strains are not pathogenic when acquired directly from the environment [4]. The gene ast is a thermostable cytotoxic enterotoxin, which causes fluid accumulation in ligated ileal loops in animal models and probably has an undescribed role in causing diarrhea in humans [6]. Aeromonads are able to grow in water with a wide variation in temperature ranging from 0°C to 45°C, with an optimum of 22-32°C. Most of the infections caused by Aeromonas spp. are acquired via contact with contaminated water.
sources or through ingestion of foods in various “farm to table” operations [7]. Khajanchi et al. [8] reported water and clinical isolates were found to have the same virulence signature, suggesting transmission of *Aeromonas* spp. from water to humans. In this regard, a study was conducted to investigate the prevalence of emerging pathogen - *Aeromonas* spp. and its enterotoxin genes in various water sources.

**Materials and Methods**

**Ethical approval**

Live animals were not used in this study, so ethical approval was not necessary. Water samples were collected from various water sources.

**Sample collection**

125 samples (50 from well water, 50 from tap water, and 25 from bottled water) were collected under aseptic conditions and transferred back to the laboratory at the earliest possible. Well water samples were collected from various artesian wells, dug wells, and bore wells. Tap water samples were collected from domestic water supply lines. Bottled water samples were collected from water tins supplied to households and bottled water from various brands available in the market. All the samples were collected in and around Greater Hyderabad Municipal Corporation, Telangana, India.

**Conventional method**

10 ml of sample was inoculated into 90 ml of alkaline peptone water with ampicillin (APW-A) 10 mg/L and incubated at 37°C for 18 h. The enriched inoculum from APW-A was streaked on to *Aeromonas* isolation medium (Figure-1) and ampicillin dextrin agar (ADA) (Figure-2) and incubated at 37°C for 24 h. The presumptive colonies were streaked on nutrient agar and subjected to biochemical tests for confirmation [9] (Table-1). All the media were obtained from Himedia® labs, India.

**Polymerase chain reaction (PCR) assay**

Bacterial DNA was obtained by boiling and snap chilling protocol [10]. The sample was inoculated into APW-A, and 1.5 ml of incubated broth was taken in a micro centrifuge tube. The tube was then centrifuged at 8000 rpm for 10 min, and the supernatant was discarded. 50 μl of sterile distilled water was added to the tubes and boiled in a water bath at 90°C for 10 min and immediately transferred onto ice. Further, the tube was centrifuged at 13,000 rpm for 5 min. For PCR assay, 2 μl of the bacterial lysate was taken as a template.

The primers [11,12] used for the detection of *Aeromonas* spp. and its toxins were custom synthesized by SR life science solutions® (Table-2). Master mix was prepared by using 2 μl of the bacterial lysate, 2 μl of ×10 Taq polymerase buffer, 1.2 μl of MgCl₂, 1 μl of Taq DNA polymerase (1 U/μl), 0.8 μl of 10 mM dNTP mix, and 2 μl each of forward and reverse primer (10 pmol/μl), which was made up to 20 μl using molecular grade water. Cycling conditions followed for various primers were mentioned in Table-3. Routinely, a master mix was set up for 18 μl and distributed to the PCR tubes, to which 2 μl of the template was added. The samples were analyzed...
in 1.5% agarose gel electrophoresis with ethidium bromide. Two Aeromonas spp. viz. A. hydrophila (MTCC 1739) and Aeromonas sobria (MTCC 3613) were obtained from MTCC (microbial type culture collection), Chandigarh. The results obtained by both cultural and PCR assay were compared with the standard cultures and confirmed the presence of Aeromonas spp.

Results and Discussion

Among 125 samples investigated, 48%, 18%, and 12% samples of well water, tap water, and bottled water were found positive by the cultural method, respectively. 26 (52%) samples in well water, 10 (20%) in tap water, and 4 (16%) in bottled water were found positive by PCR assay, targeting 16S rRNA. Aerolysin was detected in well water (34.6%) and tap water (20%) with an overall prevalence of 15% among the isolates positive by PCR. 42.3%, 30%, and 25% of samples positive by PCR revealed the presence of thermostable cytotonic enterotoxin in well, tap, and bottled water samples, respectively. All the results were presented in detail in Table-4.

Prevalence of 48% and 52% reported by cultural and PCR assays, respectively (Figure-3), in well water samples in this study were in agreement with the results of 48.7% in Libya [13] and 50% in Palestina [14], whereas lower prevalence of 8.3% [15] and 22.5% [16] were reported. Contrary to the findings of this study higher prevalence of 46.2% in Palestina [14] and 96.3% in Chennai [23] were also reported indicating a high prevalence of aeromonads. The occurrence of Aeromonas spp. in chlorinated drinking water [24] and in drinking water reservoirs [25] were reported emphasizing the ability to resist chlorination.

Prevalence of 12% and 16% by cultural and PCR assay, respectively (Figure-3), were observed in bottled water samples in this study, which were similar to the findings of Scoaris et al. [15], whereas lower prevalence of 0% [26] was also reported. Biscardi et al. [27] reported the presence of aerolysin by isolating 6 strains of A. hydrophila from 61 mineral and thermal water samples. Higher prevalence of 43% in both bottled mineral water and municipal supply sources [28] and 75% in bottled water [29] were also reported. Day to day, there is increasing concern among people regarding health and hygiene, leading to profound raise in consumption of bottled water since last decade. Hence, detection of Aeromonas spp. in bottled water should not be ignored, and there is a need for strict implementation of Hazard Analysis and Critical Control Points in the production of

### Table-2: List of primers used for detection of Aeromonas spp. and its toxins.

| Primer        | Target gene | Length | Primer sequence | Amplification product (bp) | Reference |
|---------------|-------------|--------|-----------------|----------------------------|-----------|
| 16S rRNA      | 16S rRNA    | 21     | 5’F TCA TGG CTC AGA TTG AAC GCT | 599           | 11        |
|               |             | 24     | 5’R CGG GGC TTT CAC ATC TAA CTT ATC | 252           | 11        |
| Aerolysin     | aer         | 18     | 5’F GCA GAA CCC ATC TAT CCA G | 331           | 12        |
|               |             | 20     | 5’R TTT CTC CGG TAA CAG GAT TG |              |           |
| Cytotoxin     | ast         | 21     | 5’F TCT CCA TGC TTC CTT TCC ACT |              |           |
|               |             | 21     | 5’R GTG TAG GGA TTG AAG AAG CCG |              |           |

### Table-3: Thermal cycling conditions followed for various genes.

| Step               | 16S rRNA | aer | ast |
|--------------------|----------|-----|-----|
| Initial denaturation| 94°C/5 min| 94°C/5 min| 95°C/5 min |
| Final denaturation  | 94°C/1 min| 94°C/1 min| 95°C/25 s  |
| Annealing           | 55°C/1 min| 55°C/1 min| 55°C/30 s  |
| Initial extension   | 72°C/1 min| 72°C/1 min| 72°C/1 min |
| Final extension     | 72°C/5 min| 72°C/5 min| 70°C/5 min |
| Hold                | 4°C       | 4°C  | 4°C  |

Figure-3: Polymerase chain reaction bands of Aeromonas spp. isolates form different water samples, Lane M: 100 bp DNA ladder, Lane 2, 3: Well water samples, Lane 4: Tap water samples, Lane 5: Bottled water samples.
bottled water. Variation in the results among various authors may be due to differences in the site of sampling, time of sampling, source of sample, processing method adopted, geographic, seasonal variations, and procedures adopted for isolation [30]. In this study, in agreement with Gugliandolo et al. [31] and Liu [32], it is revealed that PCR (Figure-4) was a better, sensitive, and cheap method compared to cultural assay (90% compared to PCR). Balakrishna et al. [21] and Venkataiah et al. [9] suggested PCR as a better alternative, even though similar results were obtained by both conventional and PCR assay.

Prevalence of 34.6%, 20%, and 0% of aerolysin (aer) were observed in well water, tap water, and bottled water, respectively, with an overall prevalence of 27.5% in this study (Figure-5), whereas higher prevalence of 66.6% [33], 80.6% [16], and 88.9% [10] were reported in various environmental samples. Ormen and Ostensvik [17] reported that 79% of isolates carry aerolysin gene in ambient water and drinking water in Norway. 38.5% of environmental samples carried aer gene and 44.8% contained at least one of the putative virulence properties [34]. The occurrence of aerolysin in mineral and thermal waters was also reported [27]. Even though all species of aeromonads may not contain toxin genes, high levels of prevalence noticed in the present study indicate the wide presence of aerolysin gene among the isolates in the studied area.

An overall prevalence of 37.5% for ast gene (42.3%, 30%, and 25% from well water, tap water, and bottled water, respectively) was observed in this study (Figure-6), which was comparable to the results of various authors [12,34]. On contrary higher levels of prevalence (96.7% [16] and 97.6% [35]) were reported in variety of food and environmental samples, whereas Bhowmik et al. [26] reported that none among the surface waters examined were positive for ast gene. The degree of variation observed may be due to

![Figure-4: Comparison between amplicon products obtained from genes 16S rRNA, aer, and ast of Aeromonas spp. Lane M: 100 bp DNA ladder, Lane 1: Amplicon product of 16S rRNA, Lane 2: Amplicon product of aer., Lane 3: Amplicon product of ast.](image)

![Figure-5: Polymerase chain reaction bands of aer gene from different water samples, Lane M: 100 bp DNA ladder, Lane 5: Well water samples showing positive results, Lane 6: Tap water samples showing positive results.](image)

![Figure-6: Polymerase chain reaction bands of ast gene from different water samples, Lane M: 100 bp DNA ladder, Lane 2: Well water samples showing positive results, Lane 3: Bottled water samples showing positive results.](image)

**Table-4:** Results of *Aeromonas* spp. and its toxins obtained from well, tap, and bottled water sources.

| Type of sample | No. of samples |
|---------------|----------------|
| Well water    | 50             |
| Tap water     | 50             |
| Bottled water | 25             |
| Total         | 125            |

| Positive result for *Aeromonas* spp. (N (%) | % of cultural method compared to PCR | Distribution of toxins among isolates positive by PCR (N (%)) |
|---------------------------------------------|-------------------------------------|-------------------------------------------------------------|
| Cultural method                             | PCR assay                           | aer                          | ast                     |
| Well water 24 (48)                         | 26 (52)                             | 92.3                         | 9 (34.6)               | 11 (42.3)               |
| Tap water 9 (18)                           | 10 (20)                             | 90.0                         | 2 (20.0)               | 3 (30.0)               |
| Bottled water 3 (12)                       | 4 (16)                              | 75.0                         | 0 (0.0)                | 1 (25.0)               |
| Total 36 (28.8)                            | 40 (32)                             | 90.0                         | 11 (27.5)              | 15 (37.5)              |

PCR=Polymerase chain reaction
differences in the expression of genes associated with environmental conditions [34].

A wide range of disease manifestations caused by *Aeromonas* spp. such as skin and soft tissue infection following Tsunami in Thailand, hurricane Katrina; cellulitis, myositis and septicemia in humans working in aquatic environment; gastrointestinal infection and acute renal failure from aquarium water; respiratory infections and pneumonia in accidental drowning were reported [4,7]. The presence of significant percentage of aeromonads and its enterotoxin genes in various samples of water in this study may lead to any of the above conditions, causing various human health complications. Hence, the versatility of aeromonads in varied ecosystems is of emphasis and the sobriquet “Jack of all trades” [36], by which aeromonads are called, cannot be ignored.

**Conclusion**

From this study, it is concluded that aeromonads are prevalent in various water sources. Presence of toxin gene markers in isolates revealed the pathogenic potential of *Aeromonas* spp., emphasizing the importance of hygiene and proper monitoring at various stages of water treatment to reduce or to eliminate the risk of water borne Aeromoniasis.

**Authors’ Contributions**

MT and KN designed and planned the research experiments. KRK suggested and supervised. HD performed the research work and drafted manuscript. BSNK and AP helped in conducting research. GVP helped in PCR standardization.

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**Competing Interests**

Authors declare that they have no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in the manuscript.

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