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Occurrence of haemolytic & cytotoxic *Aeromonas* species in domestic water supplies in Chennai

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A study on the occurrence of *Aeromonas* species in the domestic water supplies in Chennai showed that as much as 37.9 per cent of the water samples analyzed from various sources harbored *Aeromonas* spp. Majority of the isolates belonged to *Aeromonas sobria* (13.7%), *A. caviae* (11.6%) and *A. hydrophila* (9.5%). Among the 37 metropolitan water samples analyzed, 11 samples yielded *Aeromonas* spp. inclusive of three isolates of *A. hydrophila*, four of *A. sobria* and two isolates each of *A. caviae* and *A. jandaei*. From a total of 28 bore well water samples analyzed, *Aeromonas* spp. were recovered from 15 samples, comprising five isolates of *A. hydrophila*, six of *A. sobria* and four isolates of *A. caviae*. *Aeromonas* spp. inclusive of one isolate of *A. hydrophila*, five of *A. caviae*, three of *A. sobria* and one isolate of *A. veronii* were isolated from 10 of the 30 water packets of various commercial brands sold in Chennai. Of a total of 36 isolates obtained, 32 (89%) produced β-haemolysin with the titres ranging from 2-32 and 20 isolates (56%) were cytotxic to vero cell monolayers. All the *Aeromonas* isolates were resistant to ampicillin and polymyxin B. All *A. hydrophila* and *A. caviae* isolates were also resistant to cephalothin and erythromycin and 83.3 per cent of *Aeromonas* isolates were resistant to erythromycin. Aeromonads resistant to tetracycline, gentamycin, co-trimoxazole and nalidixic acid appear to be emerging. The study revealed that *Aeromonas* spp. occur in the potable and domestic water supplies and even in the chlorinated water supplies in Chennai city, which are potentially enteropathogenic and hence may be hazardous to public health. In view of these findings drinking and domestic water quality standards need to be re-evaluated.

Key words *Aeromonas* - β-haemolysin - cytotoxin - domestic water supply

During the last couple of decades, *Aeromonas* species have received increased attention the world over owing to their role in gastroenteritis and a variety of new syndromes such as haemolytic uremic syndrome, burn associated sepsis, different kinds of respiratory infections and various forms of wound infections[1,2]. *Aeromonas* species are reported to be ubiquitous waterborne organisms and can be readily isolated from fresh and saline waters, soil and foodstuffs[3-5]. Most of the mesophilic *Aeromonas* species are potential human pathogens. Reports on wound infections associated with *Aeromonas* species often follow injuries sustained from crabs, fish bones, aquaria, *etc*[6,7]. Some studies have indicated the association of gastroenteritis and consumption of untreated water[8]. *Aeromonas* associated diarrhoea has been reported from several cities in India[9-12]. Recently it was reported that 58 per cent of the drinking water samples in Vellore harbored *Aeromonas*[13]. The present study was conducted to find out the carriage rate of potable and domestic water supplies in Chennai city for *Aeromonas* species and examine their potential enteropathogenicity indicated

by certain virulence characteristics such as ability to produce haemolysin, extracellular enzymes and cytotoxin, and also understand their drug resistance patterns.

Material & Methods

Isolation of Aeromonas species from water samples: A total of 95 samples were collected from three different water sources namely metropolitan water supply (37 samples), bore well water (28 samples) from different parts of Chennai and packets of water (for drinking) of various commercial brands (30 samples). Metro water and bore well water samples were collected in sterile 500 ml screw capped bottles. Water samples were processed by the following methods.

Enrichment method – *Aeromonas* spp. were isolated from various water samples by modification of the method of Joseph *et al*[14]. 10 ml of water samples were inoculated into 10 ml of alkaline peptone water and incubated at 37°C for 16-18 h. The enriched samples were then plated onto a selective medium, *viz*, Inositol Brilliant Green Bile salts (IBB) agar (Himedia, Mumbai). After overnight incubation at 37°C, the non-inositol fermenting colonies were sub-cultured onto nutrient agar for testing oxidase. Motile, Gram negative bacilli, which showed oxidase positivity, glucose fermentation by oxidation-fermentation (O-F) test and resistance to 150µg of 0/129 (2,4-diamino 6, 7 diisopropyl pteridine), were identified as belonging to the genus *Aeromonas*.

Direct plating onto *Aeromonas* selective media – Water samples were directly inoculated onto IBB medium and after 18-24 h of incubation at 37°C, the non-inositol fermenting colonies were subcultured onto nutrient agar and identified as *Aeromonas* as described above.

Membrane filtration and enrichment method – 500 ml of water sample was filtered through 0.22 µm millipore filter (Millipore Inc, USA) and the filter was placed in 10 ml of alkaline peptone water and incubated at 37°C overnight for enrichment. Subsequent processing was as described earlier under the enrichment method.

The *Aeromonas* isolates obtained were identified to species level with the help of Aerokey II scheme[15] using seven key tests, esculin hydrolysis, gas from glucose in TSI, fermentation of arabinose and sucrose, production of indole, Voges Prosauker reaction and susceptibility to cephalothin. In addition to these biochemical characteristics, the isolates were also subjected to lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase tests also. Identification of the isolates was confirmed by Dr Toshio Shimada, National Institute of Infectious Diseases, Japan.

Detection of extracellular enzymes: The ability of *Aeromonas* isolates to elaborate extracellular enzymes such as protease, lipase and amylase was evaluated as described by Smibert and Krieg[16].

Haemolysin assay: The isolates were examined for haemolysin production by plating them onto 5 per cent sheep blood agar and incubating at 37°C for 24 h and observing the plates for appearance of a clear zone of β-haemolysis around the colonies. The haemolysin titres were determined by the method of Burke *et al*[17]. *Aeromonas* isolates were inoculated onto brain heart infusion broth and incubated overnight at 37°C. The broth cultures were then centrifuged at 10,000 rpm at 4°C and the culture supernatants were collected. Doubling dilutions of cell-free bacterial preparations in phosphate buffered saline *p*H 7.4 (PBS) were prepared in 96 well microtitre plates. Equal volume (50 µl) of 1 per cent sheep RBC suspension prepared by three washes in PBS was added. Haemolysis was recorded after incubation at 37°C for 1h, then at 4°C for 1h and at 4°C overnight. Haemolysis of at least 50 per cent erythrocytes was taken as positive result.

Vero cell cytotoxicity: The *Aeromonas* isolates were assayed for cytotoxicity to Vero cell monolayers grown in microtitre plates[18]. Briefly, 5-6 colonies of fresh cultures from each strain were inoculated into 5 ml of nutrient broth and incubated at 37°C for 6 h. This was then transferred to 10 ml of BHIB and incubated at 37°C for 18 h. The broth was then centrifuged at 10,000 rpm at 4°C, the supernatants collected, filtered through 0.22 µm filters (Millipore Inc, USA) and preserved at -20°C. These culture supernatants were tested for cytotoxicity to Vero cell monolayers cultured in 96 well (Corning, USA) tissue culture plates in Eagle's minimal essential medium (Sigma, USA), supplemented with 10 per cent foetal calf serum (FCS), penicillin and streptomycin for 48 h. Sterile BHIB was used as negative control,

uninoculated cells as cell control and *Shigella dysenteriae* type I as positive control. Positive cytotoxin activity was recorded as cell rounding, detachment followed by cell death which was determined microscopically after 18 h of incubation at 37°C.

Results

Isolation and identification of Aeromonas species: From the 95 water samples analyzed, 36 (37.9%) yielded *Aeromonas* spp. (Table I). The rate of isolation of aeromonads from the bore well samples (53.6%) was found to be higher compared to a relatively lower percentage recovery from the water packets (33%) and metropolitan water samples (32.4%). Most of the *Aeromonas* isolates belonged to the three species generally reported from environmental and clinical sources. Nine isolates were identified as *A. hydrophila*, 13 as *A. sobria* and 11 as *A. caviae*. Two isolates of *A. jandaei* and one isolate of *A. veronii* were recovered from metropolitan water supply and water packet respectively (Table I).

Biochemical characteristics: Among the 16 physiological and biochemical traits examined, 58.3 per cent of the isolates (*A. hydrophila* and *A. caviae*) hydrolyzed esculin, 33.3 per cent fermented arabinose (mainly *A. caviae*), 94.4 per cent fermented sucrose, 69.4 per cent produced lysine decarboxylase, and 66.7 per cent produced acetyl methyl carbinol. All the *A. caviae* isolates did not produce lysine decarboxylase and acetyl methyl carbinol.

Production of haemolysin: Most of the isolates produced clear zones of β-haemolysis when grown on 5 per cent sheep blood agar. Thirty two of the 36 *Aeromonas* isolates including 8 *A. caviae* were β-haemolytic (Table II). The haemolysin titres produced by these isolates ranged from 2 in *A. caviae* to 32 in *A. hydrophila*.

Production of cytotoxin: Among the 36 isolates, 20 were found to produce Vero cell rounding factor (Table II).

Antibiotic susceptibility: All the *Aeromonas* species were resistant to ampicillin and polymyxin B (Table III). All *A. hydrophila* and *A. caviae* isolates were also resistant to cephalothin, and erythromycin. Five of 13 (38.5%) *A. sobria* isolates were resistant to cephalothin. 83.3 per cent of *Aeromonas* isolates were resistant to erythromycin. While 2.8 to 25 per cent of the *Aeromonas* isolates showed resistance to other antibiotics such as tetracycline, gentamycin, co-trimoxazole, nalidixic acid

Table I. Isolation of *Aeromonas* spp. from various water sources

Source	No. of samples analyzed	*Aeromonas* species isolated					Total
		A. hydrophila	*A. caviae*	*A. sobria*	*A. jandaei*	*A. veronii*	
Metropolitan water supply	37	3	2	4	2	-	11 (29.7)
Bore well water samples	28	5	4	6	-	-	15 (53.6)
Water packets	30	1	5	3	-	1	10 (33.3)
Total	95	9	11	13	2	1	36 (37.9)
		(9.5)	(11.6)	13.7)			

Figures in parentheses are the percentage values

Table II. Production of haemolysin and cytotoxin by *Aeromonas* isolates

Virulence factor	Isolates					Total
	A. hydrophila (n=9)	*A. sobria* (n=13)	*A. caviae* (n=11)	*A. jandaei* (n=2)	*A. veronii* (n=1)	
Haemolysin	9 (4-32)	12 (2-16)	8 (2-16)	2 (4)	1 (4)	32
Cytotoxin	7	9	3	1	0	20

n, number of isolates tested; figures in parentheses are haemolysin titres

Table III. Antibiotic resistance of *Aeromonas* isolates

Antibiotic (μg/disc)	*A.hydrophila* (n=9)	*A. caviae* (n=11)	*A. sobria* (n=13)	*A. jandaei* (n=2)	*A. veronii* (n=1)	Total (%)
Streptomycin (10)	0	0	0	0	0	0
Norfloxacin (10)	0	0	0	0	0	0
Nitrofurantoin (300)	0	0	0	0	0	0
Tetracycline (30)	0	0	1	0	0	2.8
Nalidixic acid (30)	1	0	1	0	0	5.6
Gentamycin (10)	0	1	3	0	0	11.1
Co-trimoxazole (25)	0	0	2	0	0	5.6
Neomycin (30)	3	2	4	0	0	25.0
Cephalothin (30)	9	11	5	2	1	77.8
Erythromycin (15)	9	11	9	1	0	83.3
Ampicillin (10)	9	11	13	2	1	100
Polymyxin B (300 IU)	9	11	13	2	1	100

and neomycin. All the isolates were susceptible to streptomycin, norfloxacin and nitrofurantoin.

Discussion

The study has indicated that a significant percentage of water samples (38%) examined (36/95) were contaminated with *Aeromonas* spp. As much as 53 per cent of the bore well water samples were found to harbor *Aeromonas* spp., indicating possible colonization of aeromonads in the bore well. A matter of concern is, especially the recovery of aeromonads from 33per cent of the water sachets of various well known commercial brands sold in Chennai. Similar isolation rates have been reported in domestic water supplies in Western Australia, where, 34 per cent of the water samples were found to harbor *Aeromonas* spp.[19] and their recovery was independent of seasonal temperature, with isolations from the reservoirs reaching the peak during the winter.

Isolation of aeromonads from the water samples of the city water supply possibly indicates inadequacy of chlorination of the water supplies or resistance of aeromonads to chlorination. Occurrence of *Aeromonas* spp. in chlorinated water supplies has been earlier recorded at Calcutta[20], Canada[21] and in the United States[4].

Exposure to water contaminated with *Aeromonas* spp. has been reported to precede *Aeromonas* infections[8,22]. The incidence of gastroenteritis associated with *Aeromonas* spp. has been reported to parallel the isolation of aeromonads from the drinking water distribution systems in Perth[19]. *Aeromonas* spp. contaminated domestic water supplies may also act as an important source of non-gastrointestinal infections in immunologically compromised patients[23]

The aeromonads isolated in the present study mainly belonged to three species *viz., A. hydrophila, A. sobria* and *A. caviae* which are often reported to be associated with human disease. In addition, *A. jandaei* and *A. veronii* were also recovered from three water samples. Among the various phenotypic traits examined, esculin hydrolysis was usually found associated with *A. hydrophila* and *A. caviae,* and, production of acetyl methyl carbinol and lysine decarboxylase with *A. hydrophila* and *A. sobria.* In the present study 33.3 per cent of the *Aeromonas* isolates (mainly *A. caviae*) fermented arabinose, while earlier studies on the clinical and environmental aeromonads had shown the occurrence of a relatively higher percentage (58.5%) of aeromonads fermenting arabinose[24]. Possession of three or more characters such as ability to produce acetyl methyl carbinol, lysine decarboxylase, high haemolysin titre, inability to ferment arabinose are reported to be associatd with enterotoxin production[25].

As reported in an earlier study[26], a majority of *A. caviae* isolated in the present study were also

Table IV. Occurrence of haemolytic and cytotoxic *Aeromonas* spp. in different sources of water

Water source (no. analysed)	No. of isolates	No. of haemolytic isolates (%)	No. of cytotoxic isolates(%)
Metropolitan water supply (37)	11	10 (90.9)	7 (63.6)
Bore well water samples (28)	15	13 (86.7)	9 (60.0)
Drinking water packets (30)	10	9 (90)	4 (40)

β-haemolytic. The haemolysin titres of the *Aeromonas* isolates recovered in this study appear to be relatively lower than the titres reported by Singh and Sanyal[26]. Fifty six per cent of the isolates obtained in this study produced Vero cell cytotoxicity. Earlier studies had reported cytotoxicity in about 73 per cent of the *Aeromonas* isolates while the haemolysin production was observed in a relatively lower percentage of isolates compared to the results obtained in this study[27,28].

Most of the *Aeromonas* isolates (>90%) recovered had the ability to produce protease, amylase and lipase. The ability of aeromonads to elaborate extracellular enzymes such as proteases, lipase, amylase, β-lactamases, haemolytic enterotoxins and nucleases plays a major role in infection and also provides them additional biological diversity[2,29].

Streptomycin, nitrofurantoin and norfloxacin were found to be effective against *Aeromonas* spp. recovered in the present study. Resistance to β-lactams such as ampicillin and polymyxin B was as expected since aeromonads have innate ability to produce β-lactamases. However, it appears that resistant forms of aeromonads to tetracycline, gentamycin, cotrimoxazole and nalidixic acid appear to be emerging. However, the situation does not appear to be alarming as reported from Bangladesh on the aeromonads recovered from the aquatic environments[30] where resistance to chloramphenicol, streptomycin and tetracycline was found in 72 per cent of aeromonads. In another study, resistance to streptomycin, tetracycline and erythromycin was recorded in 57, 48 and 43 per cent of the isolates respectively[31]. It appears that the environmental areas of heavy human activity are possibly associated with a higher incidence of antibiotic resistance in aeromonads as suggested earlier[30].

Occurrence of *Aeromonas* spp. with more than 86 per cent of the isolates being able to produce β-haemolysin and more than 40 per cent of the isolates elaborating cytotoxins in water (Table IV) meant for human use appears to be a matter of serious concern to the public health in the city. In view of the fact that the *Aeromonas* spp. are potential enteric and non-enteric pathogens particularly for immuno compromised hosts, water for domestic use must be free from these organisms. The permissible counts of *Aeromonas* spp. in water need to be worked out and the microbiological quality standards should consider inclusion of monitoring *Aeromonas* counts in order to provide quality water supply to the public. The rcommended national and international standards do not include the permissible count of aeromonads in the domestic water supplies meant for human consumption and hence this aspect needs to be addressed.

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