

# INCIDENCE AND PATHOGENICITY OF AEROMONAS SPP. IN RAW, PASTEURISED AND UHT MILK IN GREATER HYDERABAD MUNICIPAL CORPORATION, INDIA

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## ABSTRACT

*125 samples (50 raw milk, 50 pasteurized milk and 25 UHT milk) were screened for Aeromonas spp. by both isolation and PCR assay. Conventional method had identified 32% in raw milk, 8% in pasteurised milk and none in UHT milk, whereas PCR assay detected 36% in raw milk, 10% in pasteurised milk and none in UHT milk suggesting that PCR was a better and sensitive method than the former method. Aerolysin (aer) was identified in 22.2%, 20% and no samples of raw milk, pasteurised milk and UHT milk, respectively. Thermostable cytotoxic enterotoxin (ast) was observed in 33.3% samples of raw milk, 20% samples of pasteurised milk and none among UHT milk. Presence of Aeromonas spp. in pasteurised milk emphasizes the need for better hygiene to avoid post processing contamination. Presence of toxins of Aeromonas spp. is of major public health concern in case of food borne outbreaks.*

**Key Words:** Aeromonas spp., milk, toxins, public health, PCR.

## INTRODUCTION

The Aeromonads are Gram-negative, motile, rod-shaped (0.3–1.0µm by 1.0–3.5µm), facultative anaerobic, catalase-positive, motile by polar flagella (except *A.salmonicida* which is non-motile), nonspore forming bacteria that are autochthonous and widely distributed in aquatic environments (Daskalov, 2006). Owing to their ubiquitous nature and ability to form biofilm and resist chlorination, *A.hydrophila* was listed in the Contaminant Candidate List of potential water borne pathogens by U. S. environmental protection agency (Borchardt et al., 2003).

Initially, Aeromonads were recognized only as causatives for systemic illnesses in poikilotherms. The genus *Aeromonas* at present is regarded as an important pathogen of fish and other

cold blooded species and also as an etiological agent causing a broad spectrum of human and animal illnesses (Janda and Abbott, 1998). There is increasing concern over *Aeromonas* spp., because of their ability to grow at refrigeration temperature and produce heat stable extracellular enzymes such as proteases, lipases and amylases leading to quality deterioration of dairy products (Ahmed et al., 2014). Milk is an excellent medium for the growth of microorganisms. Hence, any contamination during production, processing and distribution may lead to food borne outbreaks. Aerolysin (aer), a hemolytic enterotoxin and Thermostable cytotoxic enterotoxin (ast) are of major importance as they are able to cause gastroenteritis and diarrhoea in human beings (Hasan, 2006). The current study was aimed to 1) determine the incidence of *Aeromonas* spp. in milk and milk products by both

conventional and PCR assay, 2) characterize the isolates with respect to potential virulence factors.

## MATERIALS AND METHODS

A total of 125 samples (50 from raw milk, 50 from pasteurised milk and 25 from UHT milk) were collected from local markets, milk vendors, government and private dairies in and around Greater Hyderabad Municipal Corporation. All samples were collected in aseptic manner and transferred to laboratory at the earliest possible.

### Conventional method

#### Enrichment of samples

Most strains of *Aeromonas* spp. are resistant to Ampicillin, which is useful in isolation in presence of competitive microflora. 10 ml of sample was inoculated in to 90 ml of alkaline peptone water with ampicillin 10 mg/L (APW-A), homogenised and incubated at 37°C for 18hrs under aerobic conditions (Venkataiah et al., 2013)

#### Isolation and identification

Inoculum from enriched broths was streaked on *Aeromonas* isolation medium® (Himedia Product code- M884) and Ampicillin dextrin agar (ADA) (Himedia). Inoculated media was incubated at 37°C for 24hrs under aerobic conditions. Presumptive colonies were streaked on Nutrient agar for biochemical characterization (Carnahan et al., 1991; Rose and Okrend, 1998; Yucel et al., 2005). All the tests were compared with standard *Aeromonas* spp. cultures obtained from MTCC viz. *A. hydrophila* (MTCC 1739) and *A. sobria* (MTCC 3613) to confirm the presence of *Aeromonas* spp. in milk and milk products.

#### PCR assay

Boiling and Snap chilling method (Yours et al., 2007) was used to extract DNA. About 1.5 ml of alkaline peptone water broth culture of sample

was taken in a micro centrifuge tube. The tube was spun at 10000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended with 1 ml of sterile distilled water and centrifuged at 10000 rpm for 5 min. The pellet was resuspended in 1ml of sterile distilled water and boiled for 10 min at 99°C to allow cell lysis and release of DNA. Later the tube was immediately placed in ice for 10 min. The cell lysates were centrifuged and the clear supernatant was transferred to a new tube. 2 µl of the bacterial lysate was used as template. The primers used were custom synthesized by SR life solutions, Hyderabad and were tabulated in Table 1.

#### Cycling conditions

PCR amplification of 16S rRNA, *aer* & *ast* gene fragments was set up in 20 µl reactions. The optimum reaction mixture contained 2 µl of 10X Taq polymerase buffer, 1.2 µl of MgCl<sub>2</sub>, 0.8µl of dNTP mix, 2µl each of Primer-F and Primer-R, 1 µl of Taq DNA polymerase, 2 µl Bacterial lysate and 9 µl of Millipore water. Cycling conditions used for various primers were tabulated in Table 2. The samples were analyzed in 1.5% agarose gel electrophoresis.

## RESULTS AND DISCUSSION

Results obtained for various samples using different primers were tabulated in detail in Table 3. In India, *Aeromonads* were isolated from a wide range of food like milk, milk products, various meats, fish and sea foods (Agarwal et al., 2000). Current standard culture procedures for detection of *Aeromonas* spp. takes approximately 3-5 days (Venkataiah et al., 2013), laborious and time consuming. PCR relies on the detection of specific gene fragments and so can be applied in mixed microbial cultures, avoiding problems which are obvious with biochemical and morphological tests (Balcázar et al., 2007). 16S rRNA gene was reported to be successful in detecting the presence of *Aeromonas* spp. in milk, chicken

and fish samples (Arora et al., 2006; Kingombe et al., 2010). Boiling and snap chilling was the template preparation method (Prasahnt et al., 2013) employed in this study, as it is simple, rapid, reliable and has very less chances of contamination of DNA (Deepasurendran, 2003).

In this study both isolation and PCR assay were used to identify *Aeromonas* spp. and cultural method gave 87% results compared to PCR, proving higher sensitivity of PCR than conventional cultural methods (Fig 1 and 2). The results obtained were in agreement with Gugliandolo et al., (2011) and Liu (2011), who concluded that PCR assays were more sensitive and faster than conventional procedures. Even though Balakrishna et al., (2010) and Venkataiah et al., (2013) reported similar results by both conventional and PCR assay, they suggested PCR as a better alternative.

Out of 50 raw milk samples, 16 (32 %) were positive for *Aeromonas* screened by cultural method and 18 (36 %) by PCR method (Fig 3). The incidence (32 %) of *Aeromonads* by cultural method in the present study was in agreement with Ahmed et al., (2014). Low levels of incidence (17.14 %, 22.2 % and 27 %) were reported by Subashkumar et al., (2006), Elmanama and Ferwana (2011), Ibrahim and McRae (1991) respectively, whereas higher incidences (58%, 60 %, 85%) were reported by Eid et al., (2013), Kirov et al., (1993), Igbinsa et al., (2006), respectively. Lower incidences of 2 % by Arora et al., (2006), 4 % by Balakrishna et al., (2010) and Porteen et al., (2006) than reported in the present study (36%) by PCR. *Aeromonas* is commonly present in farms, in feeding stuff, water, faeces and soil (El-Shenawy and Marth, 1990) and enter the food chain. Korashy (2006) reported that the organisms can invade the udder tissues; multiply in mammary tissues and subsequently get discharged in to milk. Contaminated water used for washing milking equipment is also considered as a significant source of contamination as Eid et al., (2013) reported an isolation rate of 68% from water of udder wash.

In developing countries, *Aeromonas* spp. appears to be common in milk and milk products including pasteurised milk. *Aeromonas* spp. was isolated in 4(8%) samples by conventional method and 5(10%) samples by PCR assay (Fig 3) among 50 pasteurised milk samples and none were positive among 25 UHT milk samples examined. The results obtained were in accordance with Korashy et al., (2006), who reported 9.7% in Pasteurised milk samples. Melas et al., (1999), Ahmed et al., (2014) and Elmanama and Ferwana (2011) did not detect *Aeromonas* spp., while Yucel and Citak (2003) discovered a strongly haemolytic *A. hydrophila* strain in 16% of samples, Freites et al., (1993) observed 29% positives and Eid et al., (2013) reported 26% positives in samples of pasteurised milk. Presence of motile *Aeromonas* strains in pasteurised milk indicates post processing contamination because of unhygienic conditions during manufacturing. The processing temperature for UHT milk was way higher than inactivation temperature of *Aeromonas* spp. (62<sup>o</sup> C or more), which might be the reason for absence of *Aeromonas* spp. in UHT milk. Difference in incidence of *Aeromonas* in milk samples investigated and among various authors might be due to geographic, seasonal variations and procedures adopted for isolation (Agarwal et al., 2000).

Traditional methods for the detection of the virulent properties in *Aeromonas* spp. are based on biological assays in vitro and in vivo, using cell lines and animal models, respectively (Heuzenroeder et al., 1999; Kirov, 1997). In recent years several molecular methods, particularly polymerase chain reaction (PCR) based methods, have been developed to genetically detect putative virulence genes (Chang et al., 2008; Kannan et al., 2001). The major hemolysin produced by *Aeromonads* was termed 'aerolysin', by Bernheimer et al., (1975), it possesses both hemolytic and enterotoxic activity. An incidence of 22.2 % of aerolysin in raw milk and 20 % in

pasteurised milk were observed in the present study (Fig 4), whereas lower levels of incidence (2 % and 4 %) were reported by Arora et al., (2006) and Porteen et al., (2006), respectively in raw milk. Robson et al., (1992) reported cases of Hemorrhagic Uremic Syndrome due to *Aeromonas* spp., enhancing emphasis on aerolysin.

The gene *ast* encoding cytotoxic enterotoxin is an attractive target as it is a heat-stable (56°C, 10 to 20 min) enterotoxin and it occurs widely in clinical and in environmental isolates of *Aeromonas* spp. In the event of food poisoning, an *Aeromonas* isolate harboring any of these genes may be a hazard, hence its detection is essential. Incidence of 33.3 % and 20 % of the gene *ast* in raw and pasteurised milk observed (Fig 5), respectively in the present study were nearer to the incidence of 30 % reported by Albert et al., (2000) and Sen and Rodgers (2004) in various samples. Low prevalence (2%) of the *ast* gene in food-borne *Aeromonas* isolates was reported by Kingombe

et al., (2010), whereas high incidence of 74.2 % was reported in food and waterborne isolates by Granum et al., (1998). The variation in results of *aer* and *ast* genes in the present study may be due to influence of environmental conditions in the expression of the putative virulence properties (Ottaviani et al., 2011) and geographic difference in virulence gene carriage (Kingombe et al., 2010). It is concluded from this study that PCR was faster and has better sensitivity in identification of *Aeromonas* spp. in milk and milk products. Detection of *Aeromonas* spp. and its toxins from raw milk and pasteurised milk is of public health concern and require necessary hygienic measures to control the occurrence of *Aeromonas* spp., an emerging pathogen.

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**Table 1**

#### Details of primers used in this study

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	Graf (1999); Arora et al., (2006)
		24	5'R	CGG GGC TTT CAC ATC TAA CTT ATC		
Aerolysin	<i>aer</i>	18	5'F	GCA GAA CCC ATC TAT CCA	252	Santos (1996), Porteen et al., (2006)
		20	5'R	TTT CTC CGG TAA CAG GAT TG		
Cytotoxic enterotoxin	<i>ast</i>	21	5'F	TCT CCA TGC TTC CCT TCC ACT	331	Sen and Rodgers (2004)
		21	5'R	GTG TAG GGA TTG AAG AAG CCG		

**Table 2****Cycling conditions followed for various primers (Porteen et al., 2006; Sen and Rodgers, 2004)**

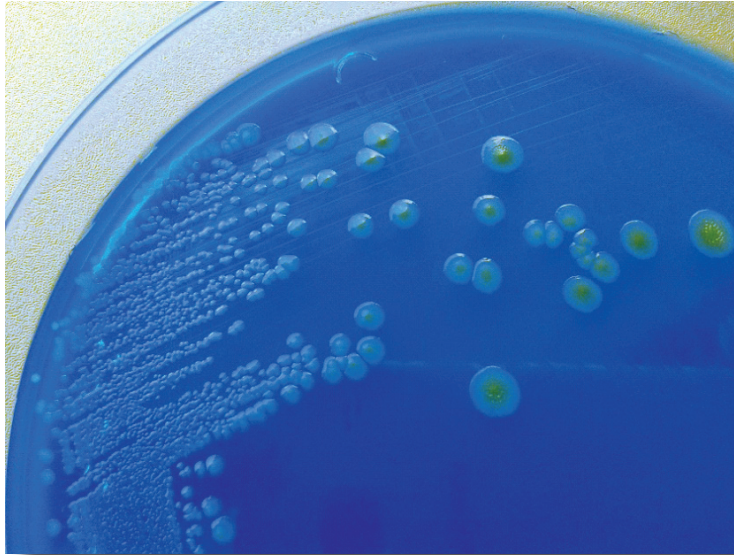
S.No.	Step	16S rRNA	<i>aer</i>	<i>ast</i>
1	Initial denaturation	94°C/5min	94°C/5min	95°C/5min
2	Denaturation	94°C/1min	94°C/1min	95°C/30sec
3	Annealing	55°C/1min	51°C/1min	54°C/30sec
4	Initial extension	72°C/1min	72°C/1min	72°C/1min
	Cycles	30 cycles	30 cycles	30 cycles
5	Final extension	72°C/5min	72°C/5min	70°C/5min
6	Hold	4°C	4°C	4°C

**Table 3****Incidence of *Aeromonas* spp. and its toxins in milk and milk products, obtained using conventional and PCR methods**

Type of sample	No. of samples	Positive result for <i>Aeromonas</i> spp.				Isolation per cent compared to PCR	Distribution of Toxins among isolates positive by PCR			
		Isolation		PCR			<i>aer</i>		<i>ast</i>	
		No	%	No	%		No	%	No	%
Milk	50	16	32	18	36	88.9	4	22.2	6	33.3
Pasteurised milk	50	4	8	5	10	80.0	1	20.0	1	20.0
UHT milk	25	0	0	0	0	0.0	0	0.0	0	0.0
Total	125	20	16	23	18.4	87.0	5	21.7	7	30.4

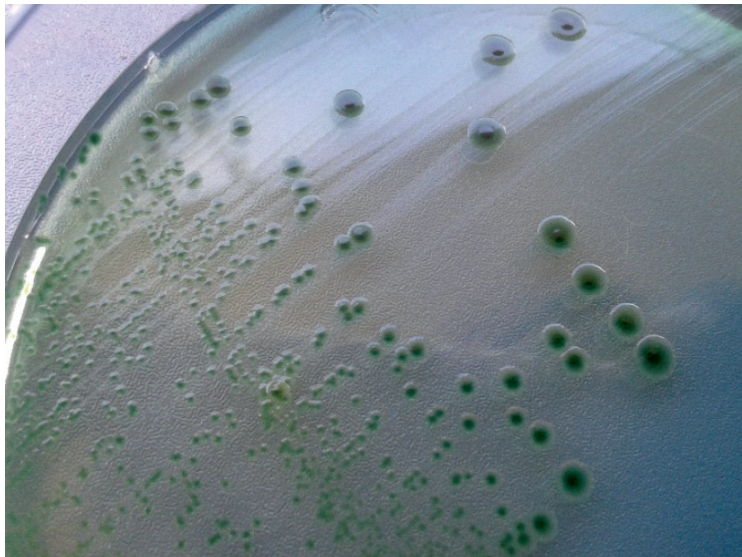
*aer*: Aerolysin*ast*: Thermostable cytotoxic enterotoxin

**Fig 1: Aeromonas spp. on Ampicillin Dextrin Agar**

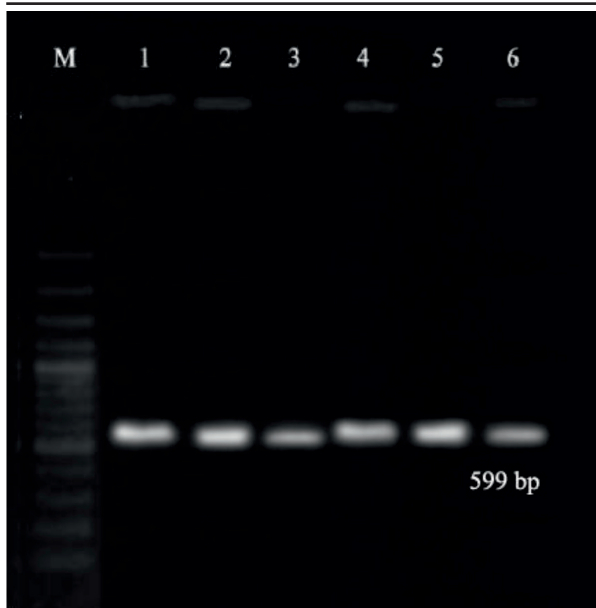


**Large convex colonies with yellow tinge indicating Aeromonas spp.**

**Fig 2: Aeromonas spp. on Aeromonas isolation medium**



**Dark green opaque colonies with dark centre indicating Aeromonas spp.**



**Fig 3**  
**Result of milk samples for *Aeromonas* spp**

Lane M : 100bp DNA ladder

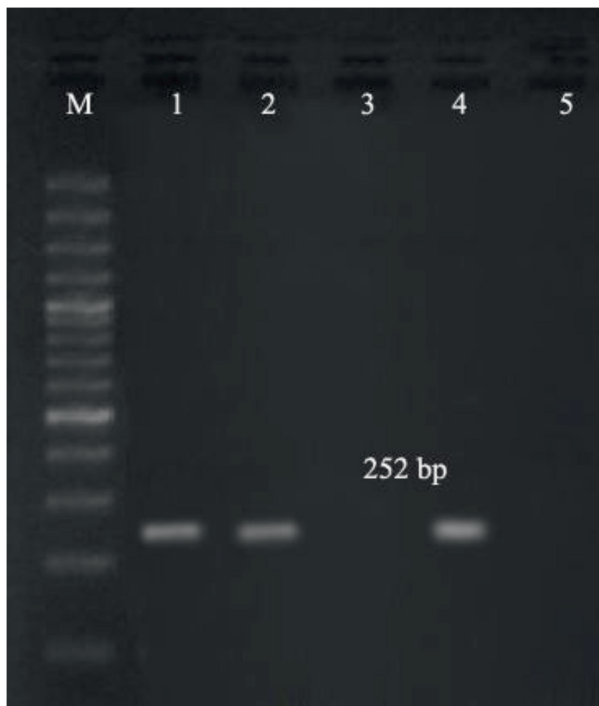
Lane 1-3: Raw milk samples showing positive results

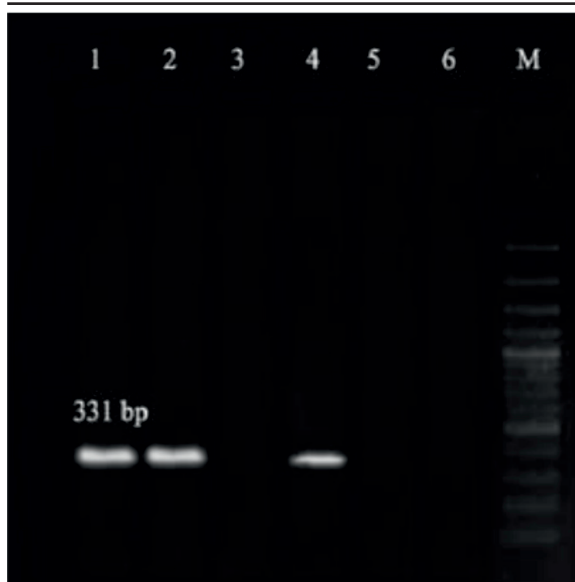
Lane 4-6: Pasteurised milk samples showing positive results

**Fig 4**  
**Result of milk samples for *aer***

Lane M : 100 bp DNA ladder

Lane 1,2&4: Milk samples showing positive results





**Fig 5**  
**Result of milk samples for ast**

Lane M : 100 bp DNA ladder

Lane 1, 2&4 : milk samples showing positive results

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