



## Prevalence of *Aeromonas spp.* and its Virulence Genes in Samples of Carabeef and Mutton

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### ABSTRACT

A survey was conducted to ascertain the distribution and virulence of *Aeromonas spp.* (species) in carabeef and mutton samples in Greater Hyderabad Municipal Corporation, India. Conventional method using *Aeromonas* isolation medium and Ampicillin Dextrin agar, PCR targeting 16S rRNA were used to identify *Aeromonas spp.* in meat samples. Conventional cultural method revealed 28% and 40% positives in mutton and carabeef, respectively whereas PCR assay detected 32% (mutton) and 44% (carabeef) positives suggesting that PCR has a better sensitivity than the cultural method. PCR positives were examined for the presence of toxin genes. Both aerolysin (37.5% in mutton and carabeef) and thermostable cytotoxic enterotoxin (36.36% in mutton and 27.27% in carabeef) were detected, indicating the potential pathogenicity of *Aeromonas spp.* in meat products.

**Keywords:** *Aeromonas spp.*, carabeef, mutton, PCR, prevalence

Long since the identification of Aeromonads more than a decade ago, there has been increasing incidence of these bacteria in human diseases (Janda and Abbott, 2010). The taxonomy of this genus undergone many modifications with the introduction of hybridization groups and was called “a sea of change” (Carnahan 1993). The current taxonomy of the genus *Aeromonas* is based upon DNA-DNA hybridization and 16S ribosomal DNA relatedness studies (Hasan 2006). Many virulent factors have been reported to be produced from Aeromonads including toxins, proteases, hemolysins, lipases, adhesions, agglutinins, enzymes. Among the exotoxins, aerolysin (*aer*), a hemolytic enterotoxin and thermo stable cytotoxic enterotoxin (*ast*), a heat stable (56°C for 10 to 20 minutes) toxin reported to have the ability to cause diarrhea and seems to play a major role in disease manifestation (Didugu *et al.* 2015).

Initially, Aeromonads were recognized only as causing systemic illnesses in poikilothermic animals. The genus *Aeromonas* at present is regarded as an important disease-causing pathogen of fish and other cold blooded species and also as the etiological agent causing a broad spectrum of human and animal illnesses (Janda and Abbott, 2010).

Aeromonads are ubiquitous and are isolated from a wide range of sources like untreated and chlorinated water, milk, vegetables, ice cream and various meats like chicken meat, mutton, carabeef and pork. They are able to resist chlorination, form biofilms, adopt to diverse nutrient environments, survive and grow even in refrigeration temperature (Igbinsosa *et al.* 2012). Wide range of clinical manifestations may be noticed in humans like diarrhea, meningitis, endocarditis and septicemia when they are exposed to water and foods contaminated with Aeromonads in ‘farm to table’ operations (Janda and Abbott, 2010).

Meat holds the top position among the various meats consumed in India. Use of untreated water and improper hygiene was common while processing and preparation. Changing lifestyles and consumer preference towards packed and refrigerated meat products improve the chances of contamination of *Aeromonas spp.* There is dearth of literature regarding incidence of *Aeromonas spp.* in carabeef. In this juncture, the current study was aimed to detect *Aeromonas spp.* and its virulence genes in carabeef and mutton, as the trend towards consumption of these meats is increasing day by day among Indian consumers.

## MATERIALS AND METHODS

### Sample collection

A total of 100 samples (50 each from carabeef and mutton) were collected from local markets and Government slaughter houses in and around Greater Hyderabad Municipal Corporation of Telangana state, India. All samples were collected in aseptic manner and transferred to laboratory at the earliest possible.

### Conventional method

Most strains of *Aeromonas spp.* were resistant to ampicillin, which was useful in isolation of Aeromonads in presence of competitive micro flora. Alkaline peptone water with ampicillin 10 mg/L (APW-A) was used as enrichment medium. *Aeromonas* isolation medium (M884) (Fig. 1) and Ampicillin dextrin agar (M1262) (Fig. 2), obtained from Himedia® laboratories were used as selective media. *Aeromonas* isolation medium is a modification on the formulation of Ryan, a modified XLD medium, which supports the growth of *Aeromonas*, *Pleisomonas*, *Proteus* as well as *Enterobacteriaceae*. The selectivity of the medium is increased by addition of Ampicillin (FD039). Presumptive colonies were streaked on Nutrient agar for biochemical confirmation. A string of biochemical tests were used to confirm *Aeromonas spp.* (Didugu *et al.* 2015). All the tests were compared with standard *Aeromonas spp.* viz. *A. hydrophila* (MTCC 1739) and *A. sobria* (MTCC 3613), obtained from Microbial type Culture Collection (MTCC), Chandigarh and used as a standard for cross verification of positive results.

### PCR assay

Boiling and Snap chilling method (Didugu *et al.* 2015) was used to extract DNA. 2 µl of the bacterial lysate was used as template. Oligonucleotide primers were custom synthesized by SR life solutions and their details are presented in Table I.

PCR amplification of *16S rRNA*, *aer* & *ast* gene fragments were 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>, 0.8 µl of dNTP mix, 2 µl each of Primer-F and Primer-R, 1 µl of Taq DNA polymerase, 2 µl Bacterial lysate and 9 µl of Millipore water.

Initial denaturation of 94°C for 5 min followed by 30 cycles of each of denaturation at 94°C for 1 min, annealing at 55°C for 60 sec and extension at 72°C for 1 min with a final extension period of 5 min at 72°C was found to be optimum for 16S rRNA, whereas annealing temperature of 51°C for 1 min is the only variation with *aer* gene for obtaining desired PCR product. For primers derived from *ast* gene, initial denaturation was at 95°C for 5 min followed by 25 cycles of each of denaturation at 94°C for 1 min, annealing at 54°C for 30 sec and extension at 72°C for 1 min with a final extension period of 5 min at 70°C was found to be optimum for obtaining desired PCR product of 331 bp without any spurious products. The samples were analysed in 1.5% agarose gel electrophoresis.

## RESULTS AND DISCUSSION

*Aeromonas spp.* were identified in samples of carabeef and mutton using cultural and PCR assay. Aerolysin and thermostable cytotoxic enterotoxin genes were detected

**Table 1:** Details of primers used in this study

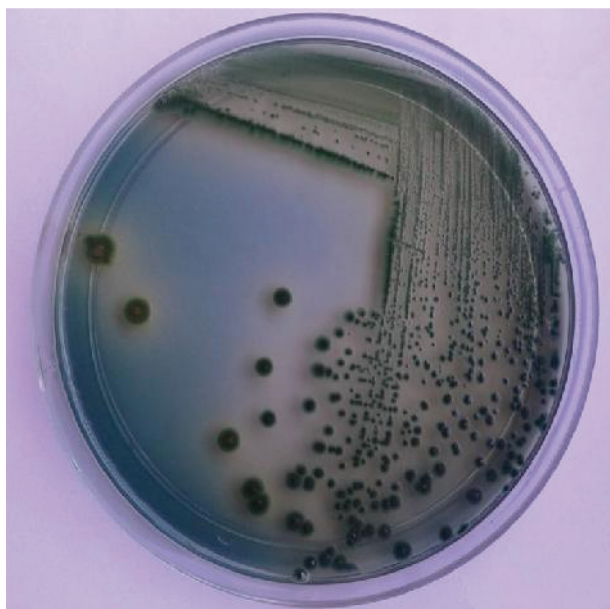
Primer	Target gene	Length		Primer sequence	Amplification product (bp)	Reference
<i>16S rRNA</i>	<i>16S rRNA</i>	21	5'F	TCA TGG CTC AGA TTG AAC GCT	599	Arora <i>et al.</i> (2006)
		24	5'R	CGG GGC TTT CAC ATC TAA CTT ATC		
Aerolysin	<i>aer</i>	18	5'F	GCA GAA CCC ATC TAT CCA	252	Porteen <i>et al.</i> (2007)
		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 CCG		

**Table 2:** Results of *Aeromonas spp.* and its virulence genes isolated from carabeef and mutton

Type of sample	No. of samples	Positive result for <i>Aeromonas spp.</i>					Distribution of toxins among isolates positive by PCR			
		Cultural method		PCR assay		% of cultural method compared to PCR	<i>aer</i>		<i>ast</i>	
		No	%	No	%		No	%	No	%
Mutton	50	14	28	16	32	87.5	6	37.5	6	37.5
Carabeef	50	20	40	22	44	90.9	8	36.36	6	27.27
Total	100	34	34	38	38	89.5	14	36.8	12	31.6

among isolates positive by PCR. All the results were presented in detail in Table 2.

Meat is a rich source of protein. Indian meat production is increased from 1.9 MT in 1998-99 to 5.9 MT in 2002-03 with a growth rate of 7.8% (DAH, 2013). Globalization, trending scenario towards new tastes and rapidly emerging middle class adopting to diets with higher meat content points to increased meat consumption that is expected to reach 18kg per capita in 2020 (Rosegrant *et al.* 2001). Aeromonads, being ubiquitous in water and now treated as emerging pathogens (Igbinsa *et al.* 2012), need to be studied for their incidence and pathogenicity in Indian scenario.



**Fig. 1:** *Aeromonas spp.* colonies on Aeromonas isolation medium

Aeromonads were detected by cultural (28%) and PCR assay (32%) in 50 samples of mutton (Fig. 3). The incidence of 32% observed in the present study was in

agreement with findings of Dallal *et al.* (2012), whereas lower incidence of 6.5 % was also reported (Osman *et al.* 2012).



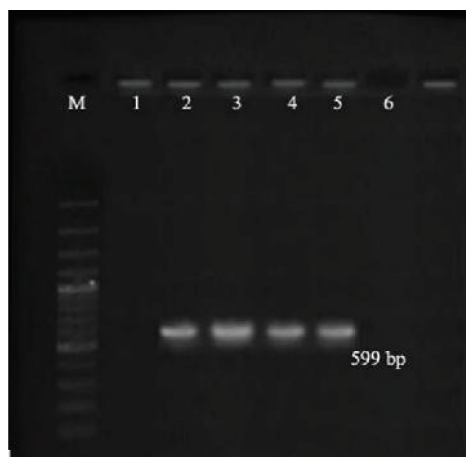
**Fig. 2:** *Aeromonas spp.* colonies on Ampicillin dextrin agar (ADA)

Incidence of 28% in chicken meat was reported by other authors (Elmanama and Ferwana 2011, Nagar *et al.* 2011), which was in conformity with our results. Retail shop meat contain higher microbial load because of the large amount of exposed surface area, more readily available water, nutrient and greater oxygen penetration (Bhandari *et al.* 2013).

Beef consumption was a religious taboo in most of the states in India. Carabeef is a near and cheap alternative and its importance as lean and red meat is enhancing day by day. 50carabeef samples were screened and it was found that 40% and 44 % were positive by cultural and PCR method (Fig. 3), respectively. Elmanama and Ferwana (2011) reported an incidence of 35%, nearer

to the findings of the current study. On contrary lower incidence of 12.3% in various meats was reported (Osman *et al.* 2012) by cultural methods.

The variation in results may be due to water contamination during slaughter, method of slaughter followed, contaminated equipment and prolonged exposure of meat in local markets to environment and geographical variations (Ghenghesh *et al.* 2008). Complex composition of food matrices may lead to false negative results in PCR (Liu, 2011). This can be overcome by inclusion of enrichment step with alkaline peptone water in this study as recommended by many workers (Venkataiah *et al.* 2013, Das *et al.* 2012). Didugu *et al.* (2015) suggested PCR as a less time consuming and better sensitive method compared to cultural method. 89.5% of samples were found positive by cultural method compared to PCR in this study, in accordance with the findings of these authors.

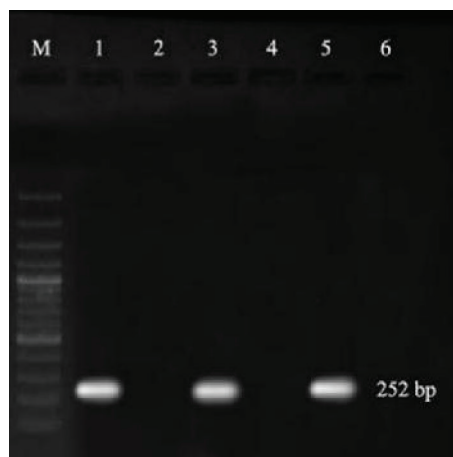


**Fig. 3:** PCR bands of *Aeromonas spp.* from Mutton and Carabeef samples

Lane M: 100bp DNA ladder  
Lane 2 & 3: Mutton samples showing positive results  
Lane 4 & 5: Carabeef samples showing positive results

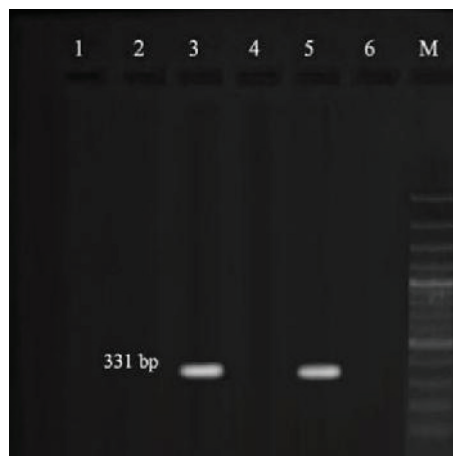
All toxins are not produced by all strains of *Aeromonas spp.*, although they carry genes. Growth conditions and environmental temperatures (Igbinsosa *et al.* 2012) influence the toxin elaboration of strains. The incidence of 36.36 % (carabeef) and 37.5 % (mutton) of aerolysin (*aer*) observed in this study (Fig. 4) was almost similar with the results of 35 % in various foods (Das *et al.* 2012). On contrary higher incidence of 80.64 % in fish samples (Aguilera- Arreola *et al.* 2005) and 88.93 % in shellfish samples (Yours *et al.* 2007) was reported. Lower

Incidence than the present study *viz.* 17.64% in various meats (Osman *et al.* 2012), 22.7% in retail foods (Nagar *et al.* 2011), Zero per cent (Pinto *et al.* 2012) were also reported.



**Fig. 4:** PCR bands of *aer* gene from Mutton and Carabeef samples

Lane M: 100 bp DNA ladder  
Lane 1, 3: Mutton samples showing positive results  
Lane 5: Carabeef sample showing positive results



**Fig. 5:** PCR bands of *ast* gene from Mutton and Carabeef samples

Lane M: 100 bp DNA ladder  
Lane 3: Mutton sample showing positive results  
Lane 5: Carabeef sample showing positive results

Incidence of 27.27% (carabeef) of the gene *ast* in the present study (Fig. 5) was proximate to the incidence of

30% reported (Albert *et al.* 2000) in various samples. An incidence of 39% reported (Aravena-Román *et al.* 2014) in clinical and environmental samples was comparable with findings of 37.5% (mutton) observed in this study. In contrast, higher level of incidence was reported (Aguilera Arreola *et al.* 2005) in variety of food and environmental samples.

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). From this study, it can be concluded that presence of *Aeromonas spp.* and its toxins in meat products indicate the possibility of occurrence food borne outbreaks and it emphasizes the need for better hygiene, regular screening and strict regulations to avoid public health hazards.

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