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Isolation and Molecular Characterization of *Aeromonas* from Food Animals by PCR method

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A total of 332 samples of animal food origin comprising 104 poultry, 137 fish, 51 pork and 40 goat (chevon) were screened. Molecular typing of *Aeromonas* isolates revealed that all the 38 isolates belonged to *Aeromonas* spp. and were positive for 16S rRNA (100%), *ahh1* (60.52%), *asa1* (42.10%), *A. hydrophila aerA* (13.15%) and AHCYTOEA/*aerA* (5.26%). It was noted that aerolysin producing toxin genes were the most prevalent irrespective of their geographical locations and the most common single gene carried among all the isolates examined was *ahh 1* (60.52%).

Key words:, *Aeromonas*, PCR, Food animals.

Since the recent times, *Aeromonas* spp. has become increasingly recognized as enteric pathogens. These organisms cause acute diarrhoea in children (Agger *et al*., 1985; Bottarelli and Ossaprandi, 1999) and adults (Gracey *et al*., 1982) and sporadic diarrhoea or dysentery in those older than 60 years, which can be severe and even life threatening (Champsaur *et al*., 1982; Echeverria *et al*., 1981). However, today, these are also responsible for causing gastroenteritis outbreaks in humans and traveler's diarrhoea (Yamada *et al*., 1997).

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Aeromonas has been recognized as a potential cause of food associated gastroenteritis outbreaks but its actual involvement in large food borne outbreaks is unknown. Its first reports linking it to diarrhoeic conditions in man emanated only in early 1980(s). *Aeromonas* associated gastroenteritis is probably under diagnosed due to the lack of recognition of its significance, confusion over its taxonomy and the difficulty for a laboratory to routinely identify isolates with virulence-associated properties, such as enterotoxin production and entero-invassiveness (Janda *et al*., 1994). According to the International Commission on Microbiological specifications for Food 1996, many classical procedures for the detection of *Aeromonas* spp. were found to be laborious and time consuming or not allowing quantitative assessment of these organisms, thus indicating the need for a reliable, universal and standard method.

Today a variety of fresh foods and food products are known to harbor *Aeromonas* spp. (Palumbo *et al.,* 1989) as the organisms psychrotropic nature allows them to grow well at

temperatures ranging from 4-7°C and this may be considered as one of the important cause that plays a role in the food spoilage and as potential food poisoning agents (Kirov and Brodribb, 1993). The organisms have also been isolated from a variety of food sources such as seafood's, meat, dairy products, poultry, sausages and grocery store produce (Rose and Okrend, 1998; Burke *et al*., 1984). Another potential source is asymptomatic and immunocompressed individuals particularly food handling workers and majority of the human isolates of *Aeromonas* spp. associated with these are haemolytic, cytolytic and proteolytic (Janda *et al*., 1994).

Virulence of *Aeromonas* spp. is multifactorial and incompletely understood. Factors contributing to virulence include toxins, proteases, hemolysins, lipases, adhesins, agglutinins, and various hydrolytic enzymes (Janda and Abbott, 1996). These virulence factors are useful in distinguishing between potentially pathogenic and non-pathogenic strains. Some investigators observed that *Aeromonas* induced gastroenteritis is due to an enterotoxin which is cytotoxic in nature, but Stelma *et al.,* (1986) reported aerolysin to be the main virulence factor involved in intestinal disorders. About 6.5% of diarrhoeal cases in the southern part of India have been attributed to *Aeromonas* (Komathi *et al*., 1998), which indicates an urgent need for information on the casual role of this pathogen in other parts of the country.

MATERIAL AND METHODS

The materials for the present study were collected basically from food animals that is pig, goat, poultry and fish which are commonly consumed by the people of North-Eastern region and also fulfill their local consumption requirement.

The samples were procured from retail shops of Meghalaya and Assam states. A total of 332 samples (Chicken intestine 104, Fish 137, Pork 51 and Goat meat (chevon) 30) were collected usually in the early morning hours immediately after slaughter of animals. The period of collection ranged from the month of March 2007 to December 2007. The collected samples were brought to the laboratory aseptically in chilled state and processed immediately for their bacteriological profile of public health significance.

The details of the samples collected have been summarized in the following Table 1.

Table 1. Details of samples procured from retail shops of Meghalaya and Assam processed for bacteriological contamination

S. No.	Type of Food Animals	Materials Collected	No. of Samples Collected
1.	Poultry	Intestine	104
2.	Fish	Gills, Intestinemeat	137
3.	Pieg	Meat	51
4.	Goat	Meat	30
	Total		332

Collection of materials

The techniques employed for collection of materials during the study were made as per the lines of procedures suggested by Cruickshank *et al*. (1975) and Gracey, (1985).

Procedure

Approximately 100g of the samples were collected from different parts of the slaughtered carcass (intestine, gills and meat) of the aforesaid food animals with the help of sterile B.P blade and B.P handle. The collected samples were brought to the laboratory aseptically in chilled state and processed immediately.

Enrichment

The samples were inoculated in Alkaline Peptone Water (APW) pH 8.4 for enrichment and incubated at 37°C for 18-24 hours.

Media for isolation of *Aeromonas* species

Ampicillin Dextrin Agar (ADA) media was used for isolating *Aeromonas* spp. from

different food animals. To avoid external contamination in the media, preventive measures were taken and for checking the sterility, media was kept under incubation at 37°C for overnight.

Primary isolation of bacteria

A loopful of inoculum from the enrichment broth was streaked on Ampicillin Dextrin Agar (ADA) plates. The inoculated plates were incubated at 37°C for 18-24 hours for the growth of distinguished colonies of *Aeromonas* species. Subcultures were made from single colony to obtain pure culture of the organism. The growth on nutrient agar slants and stabs were stored at 4°C and the cultures were maintained by sub-culturing the slants every month and the stabs every two months.

Identification of bacteria

The identification of the bacteria was made according to the scheme of Bergeys's manual of Systemic Bacteriology (Vol. I-1984, Vol. II-1986, Vol. III-1989), Wilson Miles and Parker (1983 and 1984) and Cruickshank *et al.* (1975). The isolated bacteria were identified on the basis of morphological characters, cultural characters and biochemical tests.

Morphological characters

The isolates were stained with Gram's staining method on the lines advocated by Cruickshank *et al*. (1975). Observations were particularly made on shape, size, axis, sides, ends, arrangements and motility

Cultural characters

Cultural characteristics were observed on Ampicillin Dextrin Agar plates and nutrient agar slants. On solid medium the colonies were examined for shape, size, surface, elevation, edge, colour, consistency, opacity, and emulsifiability. Colour of the isolates varied with specificity of bacterial to the media used. Finally biochemical tests were employed to categorize the bacteria in its respective genera and species.

Biochemical tests

Biochemical tests were performed according to the procedures of Cruickshank *et al*. (1975). The biochemical reagents prepared are given in the appendix. The biochemical reagents used were of HI-MEDIA Laboratories, Mumbai, India.

Polymerase chain reaction (PCR)

Bacterial cells were grown overnight on ADA plates at 37ºC. Two to three isolated, pure and typical *Aeromonas* colonies were taken in eppendorf tubes containing 100 µl autoclaved Mili-Q water. The cultures were mixed for 30 sec in a vortex at maximum speed. The cells were then boiled at 100ºC for 10 min. After boiling, the cultures were incubated on ice (hot-cold shock treatment). The supernatant containing the DNA was used as template for the polymerase chain reaction (PCR) to detect the presence of the virulence gene by using specific primers Table 2.

The PCR mixture (25 ml) included 12.5 ml of master mix (MBI Fermentas, USA) containing 2.5 U Taq DNA -polymerase, 200 mM each of dATP, dCTP, dTTP and dGTP, 1.5 mM $MgCl_2$ and PCR buffer, 5 ml (1mM) each of upper and lower primers and 2.5 ml of template DNA (bacterial cell suspension). PCR incubation standardized by the laboratory was performed in a thermocycler (iCycler, BIORAD, USA) in 30 cycles of initial denaturation (95°C for 4 min), denaturation (95°C for 30 sec.), primer annealing (59°C for 30 sec.) and primer extension (72°C for 10 min.) followed by final incubation at 72°C for 10 min.

The PCR amplicons were separated in 1.5% agarose (Promega, USA) gel. The gel was prepared by dissolving agarose in 1X Tris – acetate -(TAE) buffer (Genei, Bangalore). Same buffer was used for electrophoretic run. A total of 5 µl of each amplicons and 1 µl marker DNA (100 bp DNA ladder mix; MBI Fermentas, USA) were mixed separately with 1 µl of 6X gel loading dye (MBI Fermentas, USA) and loaded in the wells of the gel. Electrophoresis was carried out in Mini plus horizontal (GENE Mate® gel system, UK) electrophoretic apparatus at a constant voltage of 60 V for 1 hour and 20 min. or until the second dye marker had run 3/4th of the gel. The gel was then stained in ethidium bromide (Pharmacia Biotech, Sweden) @ 0.4 µg/ml in distilled water solution for 10-15 min and was visualized in gel documentation system (Gel Logic 100 Imaging System, Biostep) and photographed.

Molecular confirmation of *Aeromonas* species by PCR assay

The sizes of the amplification products obtained by the multiplex PCR were to those predicted from the design of the primers (Wang *et al*., 2003). Fig. 1 & 2, shows the occurrence of

Table 2. Primer pairs used for PCR amplification

Primer pair	Sequence	Target gene	Size of PCR amplicon (bp)	Reference
AHCF1	5' – GAG AAG GTG ACC ACC AAG ACC A – 3'	AHCYTOEN/*aer A hly*	232	Kingombe *et al*. (1999)
AHCR1	5' – AAC TGA CAT CGG CCT TGA ACT C – 3'			
H1	5' – GGC CGG TGG CCC GAA GAT ACG GG -3'		597	Gonzalez-Rodriguez *et al*. (2002)
H2	5' – GGC GGC GCC GGA CGA GAC GGG – 3'			
PF1	5' –TTT GGA ACC CAT TTC TCG TGT GGC - 3'	*A aro*	1236	Cascon *et al*. (1997)
PR2	5' –TGG AAG TAG TCC GGG AAG GTC TTG **G-3'**			
ASA 1F	5'-TAA AGG GAA ATA ATG ACG GCG-3'	*Aasa*	249	Wang *et al*. (2003)
ASA 1R	5'-GGC TGT AGG TAT CGG TTT TCG -3'			
AHH1F	5'-GCC GAG CGC CCA GAA GGT GAG TT-3'	*1ahh1*	130	Wang *et al*. (2003)
AHH1R	5'-GAG CGG CTG CAT GCG GTT GT -3'			
AH–aerAF	5'-CAA GAA CAA GTT CAA GTG GCC A-3'	*A. hydrophila aer A*	309	Wang *et al*. (2003)
AH-aerAR	5'-ACG AAG GTG TGG TTC CAG T -3'			
A16SF	5'-GGG AGT GCC TTC GGG AAT CAG A-3'	16S rRNA	356	Wang *et al*. (2003)
A16SR	5'-TCA CCG CAA CAT TCT GAT TTG -3'			

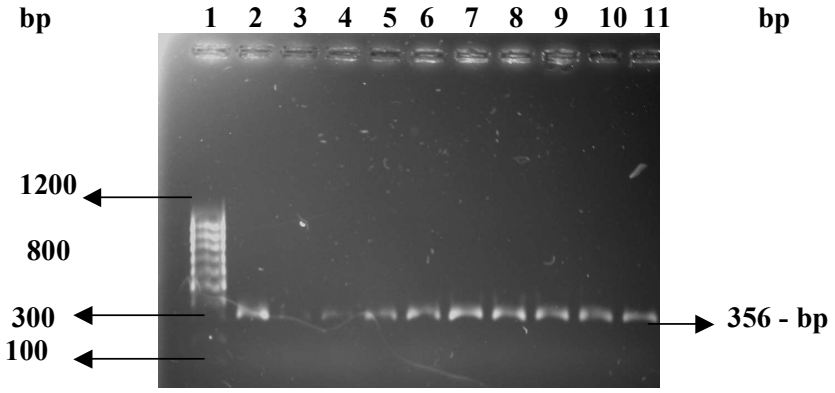
the amplified product when the 38 isolates were used as templates.

The results of molecular tying of *Aeromonas* spp. by PCR are shown in the Table 3. In PCR, all the 38 isolates of *Aeromonas* spp. amplified the primers specific for 16S rRNA gene of 356 bp. However, 7 isolates from chicken intestines; 3 isolates from fish meat; 6 isolates from fish intestines and 3 isolates of fish from gills; 3 isolates from pig meat and 1 isolate from

Table 3. Detection of toxin genes from *aeromonas* isolates by PCR

S. No.	Source	Number of isolates (%) positive for toxin genes						
		16S rRNA	*hlyA*	*ahh1*	*asa1*	*aro A*	*A. hydrophila aerA*	AHCYTOEA / *aer A*
1.	ChickenIntestine	12	-	7	5	-	2	1
2.	FishMeat	5	-	3	4	-	1	-
3.	Fish Intestine	9	-	6	3	-	1	1
4.	FishGills	4	-	3	1	-	-	-
5.	PigMeat	7	-	3	2	-	1	-
6.	GoatMeat	1	-	1	-	-	-	-

goat meat were found to be positive for *ahh1* gene of 130 bp. Five isolates from chicken intestines; 4 isolates from fish meat, 3 isolates from fish intestines and 1 isolate from fish gills and 2 isolates from pig meat were found to be positive for *asa1* gene of 249 bp. Two isolates from chicken intestines; 1 isolate from fish meat and 1 isolate from fish intestine and 1 isolate from pig meat were found to be positive for *A. hydrophila aerA* gene for 309 bp. One isolate from chicken intestine 1 isolate from fish intestine were found to be positive for AHCYTOEA / *aerA* gene of 232



Lane 1: 100 -bp DNA Marker, Lanes 2-11 test organisms.

Fig. 1. PCR Amplicon of 16S rRNA (356 -bp) of *Aeromonas* spp

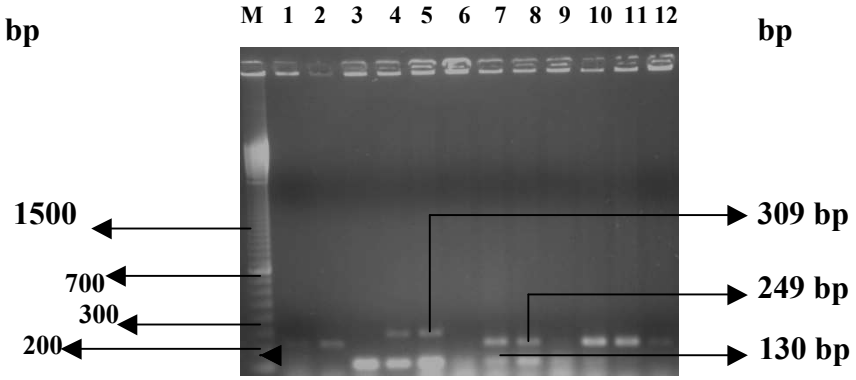


Fig. 2. PCR detection of *aer A* (309bp), *ahh1* (130bp), *asa1* (249bp) genes in *Aeromonas* isolates. M- marker; Lane (1-12)- tes

bp. The other toxin specific primers of *hlyA* and *aroA* gene were found to be negative.

Thus, the PCR assay used for molecular tying of all the 38 isolates revealed that all the isolates belonged to *Aeromonas* spp. of 38 (100%) were positive for 16S rRNA gene, 23 (60.52%) for *ahh1* gene, 16 (42.10%) for *asa1* gene, 5 (13.15%) for *A. hydrophila aerA* gene and 2 (5.26%) for AHCYTOEA / *aerA* gene.

DISCUSSION

Mesophillic *Aeromonas* spp. has emerged as an important human and animal pathogen, with worldwide distribution (Merino *et al*., 1995). Voluminous literature bears the testimony that aeromonads are involved in the etiology of gastrointestinal diseases in human beings (Burke *et al*., 1983; Gracey *et al*., 1982) with illness ranging from mild to dysentery-like diarrhoea (Champsaur *et al*., 1982). Acute self limiting gastroenteritis seems to be more common in children, particularly those below 2 years and adults above 50 years (Burke *et al*., 1983; Agger, 1986). *Aeromonas* spp. also causes extra intestinal infections that may become fatal especially among immunocomprised patients.

In this study, the use of PCR assay for molecular typing revealed that all the 38 isolates belonged to *Aeromonas* spp. and were positive for 16S rRNA (100%), *ahh1* (60.52%), *asa1* (42.10%), *A. hydrophila aerA* (13.15%) and AHCYTOEA/*aerA* (5.26%). In the present investigation it was noted that aerolysin producing toxin genes were the most prevalent irrespective of their geographical locations and the most common single gene carried among all the isolates examined was *ahh 1* (60.52%). The results recorded in this study are in agreement with those reported by Wang *et al*. (2003) in the multiplex PCR.

The PCR was species specific in that the amplification was observed only with the template from haemolytic *A. hydrophila*. Haemolytic *A. sobria* and *A. caviae* that produced enterotoxigenic factors were negative in PCR indicating that the presence or absence of these toxins did not affect the detection of aerolysin genes (Pollard *et al*., 1990). Borrell *et al*. (1997) suggested that molecular methods tend to be more accurate and the vast majority of them being focused on species identification by either using the 16S rRNA or virulence associated genes such as aerolysin. Kingombe *et al*. (1999) reported high sequence similarities between the AHCYTOEA/ *aerA* gene and other aerolysin genes, thus confirming the high level of DNA relatedness of *Aeromonas* spp. virulence factors.

The present study suggested that PCR is a reliable DNA based technique and useful tool for tying and subtyping the *Aeromonas* isolates (Khan and Cerniglia, 1997). The presence of aerolysin gene in *Aeromonas* isolates irrespective of geographical location suggested that the primers specific to this PCR assay could be useful for the rapid identification of these isolates. The high rates of positivity of *ahh 1* gene followed by *asa 1* gene in foods of animal origin has a crucial impact on public health by entering into the food chain of humans. Since *Aeromonas* is an opportunistic bacterial pathogen, good management practices, awareness regarding the diseases caused by this should be in the first priority.

CONCLUSIONS

Molecular typing of *Aeromonas* isolates by multiplex PCR assay exhibited various sizes of amplification products as predicted from the design of the primers. The assay used for the molecular typing of all the 38 isolates revealed that all the isolates belonged to *Aeromonas* spp. and all the 38 (100%) isolates were positive for 16S rRNA gene, 23 (60.52%) for *ahh1* gene, 16 (42.10%) for *asa1* gene, 5 (13.15%) for *A. hydrophila aer A* gene and 2 (5.26%) for AHCYTOEA/*aerA* gene.

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