Abstract. – Objective: The aim of the present study was to isolate and identify Aeromonas (A.) species and assess their toxin producing ability in foods of animal origin from North East India.

Materials and Methods: A total of 332 animal food samples (fish, poultry meat, pork and chevon) of which 38 (11.44%) isolates were identified by the 16S rRNA technique were included in the study. The enterotoxigenicity of the isolates was measured by the Mouse Paw Oedema Test (MPOT), rabbit ileal loop (RIL) and vascular permeability reaction (VPR) test.

Results: Maximum positivity was shown by the samples from fish (13.13%), followed by poultry meat (11.5%), pork (9.85%) and chevon (2.5%). A. hydrophila was the predominant species (92.10%) followed by A. sobria (5.26%) and A. caviae (2.63%).

Conclusions: All the Aeromonas strains isolated by culture methods expressed enterotoxigenicity by MPOT, RIL assay and VPR test.

Key Words: Aeromonas, Enterotoxicity, Food animals.

Introduction

Aeromonas (A.) species are Gram-negative, facultative anaerobic bacteria, with a wide distribution in nature¹¹,¹²,¹⁴. These bacteria can grow²³ and produce enterotoxin and hemolysin at refrigeration temperatures of 4°C¹⁷. Aeromonas species are significant potential agents in foodborne illnesses since they have been isolated from fresh and raw foods¹⁴. Various species of Aeromonas have been associated with gastroenteritis, septicemia and wound, soft tissue, skin and blood infections in human beings. An increasing number of reports have implicated Aeromonas species as the etiologic agent in acute diarrhea and in wound and skin injuries, indicating that these organisms are more important than previously thought¹,⁶,⁵,¹⁰,²³. Aeromonas species produce a variety of virulence factors, including hemolysins, enterotoxins, cytotoxins and adhesins, that have been implicated in the pathogenicity of these bacteria⁴,⁷,⁹,¹⁰,¹⁶,¹⁸,²¹. A cytotoxic enterotoxin also known as “aerolysin”, which has enterotoxic, cytotoxic and hemolytic activities, has been described as the most powerful virulence factor associated with Aeromonas mediated gastrointestinal illnesses⁴,²³. Despite its etiological importance, relatively few studies have investigated the production of enterotoxins, cytotoxins and hemolysins in foods of animal origin²⁴,²⁰,²¹. In the present study, we report the isolation and identification of Aeromonas species and toxins produced by them in foods of animal origin from North East India.

Materials and Methods

Bacterial Isolates

A total of 332 animal food samples of which 38 isolates were identified by the 16S rRNA technique were included in the study. Animal food samples obtained from meat were enriched in alkaline peptone water (APW) at 37°C for 18 hours. The enriched inoculums from APW were streaked on ampicillin sheep blood agar (SBA) and incubated at 37°C for 24 hours. Both haemolytic and non-haemolytic colonies were tested for oxidase test. Strains were stocked on nutrient agar stabs at room temperature for further studies. Of the 38 isolates, 35 (92.10%), 2 (5.26%) and 1 (2.63%) were recognized as A. hydrophila, A. sobria and A. caviae respectively. All the strains were dominantly environmental isolates in our collection.
Differentiation of Aeromonas Species
The oxidase positive colonies were further confirmed by the biochemical tests and species differentiation was performed by the method described by Aerokey II group of tests for the identification of Aeromonas species. Identification of the genus Aeromonas was done using standard tests including motility, Gram staining, D-glucose fermentation (37°C/24 h) (+), catalase (+). The species were identified by specific assays, such as esculin hydrolysis, arabinose utilization, gas production from glucose and indole.

Preparation of Bacterial Culture Supernatants
Aeromonas were cultured in 20 ml of trypti-case soy broth (Difco Laboratories, Detroit, MI, USA) by shaking (100 rpm) (New Brunswick Scientific Co. U.S.A) for 24 h at 37°C. Culture supernatants were collected by centrifugation (10,000 µg for 30 min at 4°C) and filtered through a 0.22-µm membrane filter (Millipore Corp., Bedford, MA, USA). The filtrates were stored at 37°C until assayed.

Identification of Enterotoxigenic Aeromonas
From all of the Aeromonas isolates, cell free fluid (CFF) was prepared as per the procedure elaborated by Gray et al.12. The enterotoxigenicity of the isolates was measured by the Mouse Paw Oedema Test (MPOT). The test was performed following the method of Kumar et al.14 with slight modifications. The healthy albino mice of average size were used. The mean thickness of each paw (fore and hind separated) was determined by Vernier Caliper. The test materials were inoculated intradermally in 0.1 ml quantity in each paw. Brain-heart infusion (BHI) was inoculated as negative control. The mice was observed for 18 hrs and increased in thickness (oedema) was measured. A preparation yielding a RT % of the order of 121.0 ± 3.8 and above was taken as positive for enterotoxigenity. Each preparation was tested thrice in separate mice.

Rabbit Ileal Loop (RIL) Assay
The enterotoxigenicity of Aeromonas isolates was tested in the RIL, following the procedure of Singh and Sanyal.26 Briefly, six samples were tested in eight separate loops each with an interloop between two consecutive loops of an adult rabbit ileum (New Zealand white variety weighing 1.2-1.9 kg). One ml of CFF was injected into each loop of a rabbit ileum in duplicate rabbits following laparotomy. In the first loop, 1 ml CFF of Escherichia coli was injected as a positive control and 1 ml sterile normal saline was injected in the eighth loop as a negative control. In the remaining loops between positive and negative controls, 1 ml CFF of six test isolates was inoculated sequentially. The inoculated rabbits were killed after 16-18 h of incubation to measure the volume of fluid accumulation per cm of gut in each rabbit. Fluid accumulation was greater than 0.5 ml/cm of gut was considered a positive response in the RIL. The experiment was repeated twice.

Vascular Permeability Reaction (VPR) Assay
The assay was carried out according to the procedure followed by Kumar et al.15 with slight modifications. Adult healthy rabbits were shaved on the surface of their abdomen and the remaining hairs were removed with depilatory cream prior to skin testing. The sheared area was washed with clean warm water. The area was divided into 8-12 parts of approximately 2 sq. cm sizes with a marking pencil. The toxin preparations were injected intradermally in 0.1 ml quantity. Cell free fluid (CFF) of E. coli and BHI broth were injected as positive and negative control respectively. The animals were caged individually. After a designated time interval, that is, 1-18 hrs of inoculation of rapid permeability factor (RPF) and delayed permeability factor (DPF) was observed. The skin at the site of inoculation was examined for erythema, induration, necrosis or any other changes. The test was repeated thrice for each preparation.

Detection of Hemolysin
The hemolytic activities of the Aeromonas species was determined by sheep blood assay (SBA) plate assay. It was observed as a zone of hemolysis around the colonies on blood agar plates containing 5% (v/v) of sheep blood after 24 h incubation at 37°C.16

Statistical Analysis
The data was analysed for comparing compare the proportion of isolates in the different food animals using the Marascuillo’s procedure by the following formula:

\[ r_{ij} = \sqrt{\chi^2; \kappa - 1} \left( \frac{p_i(1-p_i)}{n_i} + \frac{p_j(1-p_j)}{n_j} \right) \text{ where } i \neq j \]
Occurrence of enterotoxigenic Aeromonas species in foods of animal origin in North East India

Results

The distribution of the 38 Aeromonas species identified (from 332 meat samples) is shown in Table I. According to the classification of Popoff (25), of the 38 isolates identified, 35 (92.10%), 2 (5.26%) and 1 (2.63%) were recognized as A. hydrophila, A. sobria and A. caviae. Maximum positivity was shown by the samples from fish (13.13%), followed by poultry meat (11.5%), pork (9.85%) and goat meat (2.63%). A. hydrophila was the predominant species (92.10%) followed by A. sobria (5.26%) and A. caviae (2.63%). All these Aeromonas strains isolated by culture methods expressed enterotoxigenicity by MPOT, RIL assay and VPR test.

In MPOT (Table II), of the 38 isolates examined, CFF of 21 (55.26%) isolates produced significant oedema. The relative thickness (%) for enterotoxicity was observed to be higher than 121.0 ± 38 that is 283.4. Positive results in RIL exhibited fluid accumulation at the rate of around 0.2 to 0.6 ml/cm, respectively. The accumulated fluid was found to be thick and dark in nature. In VPR test, the CFF was found to produce redness, swelling and induration on intradermal injection on the abdominal region. Slight bluing of the injected site was observed.

All of Aeromonas isolates produced hemolysin when cultured in SBA plates, but when assayed in the culture supernatant hemolytic assay, only 22 (57.89%) of the isolates produced cell-free hemolysins. Haemolysin produced by the Aeromonas isolates was able to lyse the erythrocytes present on the sheep blood agar plates, thus, clear zones around the colonies was observed which confirmed the presence of haemolytic activity of Aeromonas isolates. The Marascuillo’s comparison between the isolates from different food animals differed significantly (p>5). However, the values in between the samples of different foods were not found significant except in fish samples.

Discussion

The genus Aeromonas has been implicated in food poisoning and in some human diseases. Several studies have tried to establish a relationship between toxins such as cytotoxic enterotox-
in (“aerolysin”) and Aeromonas-mediated illnesses. In this work, the *Aeromonas* species in animal food sources were identified and their profile of toxin production was determined in order to compare the virulence profiles.

All *Aeromonas* isolates produced hemolysin when cultivated on SBA plates, but in the cell-free hemolytic assay, only 57.89% showed hemolytic activity towards sheep blood. These results could be explained by the presence of different hemolysins, i.e. one associated with cell contact with erythrocytes and another associated with release into the extracellular medium. Alternatively, the hemolysin may be released in an inactive form into culture supernatants and then be activated later by proteases produced by the cell; this could explain the inhibition of this biological activity.

*A. hydrophila* produced more hemolysin than *A. sobria* and *A. caviae*. As reported earlier, a majority of *A. hydrophila* isolated in the present study were also haemolytic. Haemolytic activity is closely correlated with cytotoksin production, and majority of the cytotoksin-producing organisms such as *A. hydrophila* and two isolates of *A. sobria* also exhibited haemolytic activity.

Production of haemolytic toxins has been regarded as indication of pathogenic potential, though non-haemolytic *Aeromonas* have also been implicated as human pathogens. All *Aeromonas* isolates with haemolysin positive genotype were virulent in suckling mouse assay model. Burke et al. reported very high correlation between haemolysin and enterotoxin production as all enterotoxigenic *Aeromonas* isolates produced haemolysin. Rabbit ileal loop assay is commonly used to detect the enterotoxigenic potential of many enteropathogens including *Aeromonas* spp. In the present study, CFF of 6 isolates were tested by this method. A dilation index (DI) of 0.4 and above was considered positive. Singh and Sanyal reported that all the three species of *Aeromonas* were shown to be potentially enterotoxigenic regardless of the source and strains that showed little or no fluid accumulation in initial experiments but became enterotoxin producers after one to three passages through rabbit ileal loops. Asao et al. had also reported positive RIL test with purified haemolysin. In MPOT, CFF of all the 38 *Aeromonas* isolates was tested and of these only 21 produced oedema. This test appeared to be economical and convenient as it did not require the sacrificing of the animals and also very small quantity of the enterotoxin preparation was required for the test. In VPR test, of the 38 *Aeromonas* isolates, 10 isolates exhibited redness, swelling and induration on intradermal injection on the abdominal region along with swelling.

Data analysis revealed that the infection was highly significant in fish when compared with other food animals which may be related to its habitat, cultural and storage practices.

### References

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