INTERNATIONAL JOURNAL OF SCIENCE AND NATURE

Www.scienceandnature.org

PLASMID PROFILING OF *AEROMONAS* SPP. ISOLATED FROM FOODS OF ANIMAL SPECIES IN NORTH-EAST INDIA

[1]Indu Sharma, [2]Pramanik, A. K. and [3]Kumar, A.
Department of Microbiology, Assam University, Silchar-788011. India
2. Department of Veterinary Public Health, West Bengal University of Animal and Fishery Sciences, Kolkata-700037, India.
3. Department of Veterinary Public Health, IVRI, Izat nagar, India.

ABSTRACT
A total of 332 samples of animal food origin comprising 104 poultry, 137 fish, 51 pork and 40 goat (chevon) were screened. The alkali-lyses method of plasmid DNA isolation resulted in high yield of plasmids and the plasmids detected among the 33 isolates were diverse and exhibited different sizes with differing intensities. Most of the strains tested contained various sizes ranging from 23.13 Kb to 2.0 Kb.

KEYWORDS:, *Aeromonas*, Plasmid profiling, Food animals

INTRODUCTION

In the recent times, there have been reports of many cases of diseases caused by pathogenic zoonotic bacteria in India, especially due to *Aeromonas*. *Aeromonas* spp. have 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).

The spectrum of infectious diseases caused by *Aeromonas* species includes gastrointestinal infections as well as extra intestinal infections such as cellulites, wound infections, septicemia, urinary tract infection and hepatobiliary and ear infections (Vila *et al*., 2003). 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 (Wadstrom *et al*., 1976) 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.

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.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 332 samples of which 38 isolates were identified by the 16S rRNA technique were included in the study. The isolates were grown on ADA (Ampicillin Dextrin Agar (HI-MEDIA Laboratories, Mumbai, India) at 37°C for 18-24 hours. Of the 38 isolates, 35 (92.10%), 2 (5.26%) and 1 (2.63%) were recognized as *A. hydrophila*, *A. sobria* and *A. caviae*. All the strains were dominantly environmental isolates in our collection. The details of the samples collected have been summarized in the following Table1.

Table. 1: Details of food samples procured from retail shops of Meghalaya and Assam

Sl.No.	Type of food Animals	Materials Collected	No. Of samples Collected
1.	Poultry	Intestine	104
2.	Fish	Gills, intestine meat	137
3.	Pig	Meat	51
4.	Goat	Meat	40
Total			332

BIOCHEMICAL STUDIES

The following biochemical tests were done in all strains according to conventional protocols: esculin hydrolysis, citrate utilization, motility, indole production, and acid production from rhamnose, sorbitol, lactose, D-sucrose, and salicin. Test samples were incubated under the same conditions as used for bacterial growth. All tests were carried out in duplicate, and appropriate positive and negative controls were included.

PLASMID ISOLATION

Bacteria were screened for plasmid DNA by the alkaline lysis method (Birnboim and Doly (1979). A single colony of bacteria was transferred into 3 ml of Luria broth (LB) and ampicillin was added at the concentration of 50ul/ml. The culture was incubated at 37°C for 17-18 hrs. with vigorous shaking. A 1.5 ml of the culture was poured into a microfuge tube and centrifuged at 8000 rpm for 2 min. at 4°C. The medium from the microfuge tube was removed and the bacterial pellet was left dry. This bacterial pellet was then resuspended in 150 µl of Solution 1 by vortexing and 150 µl of Solution 2 was added and mixed gently by inverting the tubes 4-6 times. Incubation for 5 min. at room temperature was done and 150 µl of ice cold solution 3 was added and mixed gently by inverting the tubes 4-6 times. Re-incubation on ice for 5 min. was done again and the microfuge tube was centrifuged at 15,000 rpm for 10 min. at 4°C. The supernatant was then transferred into a fresh microfuge tube and an equal volume of phenol/chloroform was added. The tube with sample was vortexed for 1 min. and centrifuged at 10,000 rpm for 5 min. The upper, aqueous phase was transferred to a fresh microfuge tube and an equal volume of chloroform isoamyl alcohol (24:1, V/V) was added. This was vortexed for 1 min. and centrifuged again at 1000 rpm for 5 min. The upper, aqueous phase was then transferred into a fresh microfuge tube and 0.6 volume of isopropanol was added and this was kept at room temperature for 5 min. and then centrifuged at 15,000 rpm for 10 min. The supernatant was removed and the plasmid was washed in pre-chilled 70% ethanol, and centrifuged at 15,000 rpm for 10 min. After centrifugation the supernatant was again removed and the pellet was dried under vacuum. The plasmid was then dissolved in 10 µl of TE buffer (pH 8.0) and the extracted DNA was then stored at -20°C. The purity of the plasmid DNA was checked by optical density (O.D) measurement at 260/280 nm and finally analyzed in agarose gel (0.75%).

The extracted plasmids were electrophoresed for two hours at 70 mA on a 0.75% agarose gel in TAE buffer as described by Meyer's *et al*. (1976). After the gels were stained with ethidium bromide (1.5 mg/l for 10 mins), they were photographed under U.V illuminator using a Gel Logic 100 Imaging System, Biostep.

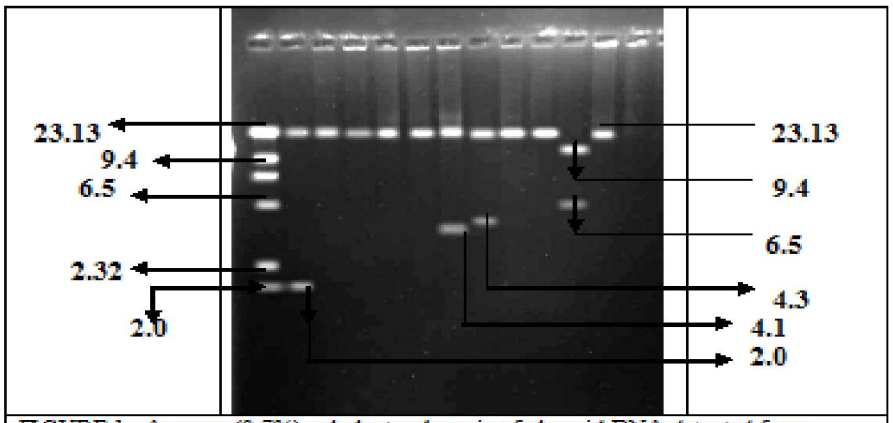

FIGURE 1. Agarose (0.7%) gel electrophoresis of plasmid DNA detected from *Aeromonas* isolates. Lane 1: Lamda Hind III DNA marker, Lane 2-12 test organisms.

RESULTS

The alkali-lyses method of plasmid DNA isolation resulted in high yield of plasmids. Of the 38 *Aeromonas* isolates, 33 contained plasmid DNA (all isolates were screened at least three times). Most of the strains tested contained various sizes ranging from 23.13 Kb to 2.0 Kb (Fig. 1). The plasmids detected among the 33 isolates were diverse and exhibited different sizes with differing intensities. However, in this study, relationship between plasmid and antibiotics were not employed so proper detection of the plasmid mediated antibiotic resistance could not be studied.

DISCUSSION AND CONCLUSIONS

Since the identification of *Aeromonas* spp. often requires the use of non-conventional biochemical assays that are time consuming and often require long incubation period before the final results can be recorded, attempts have been made to find out the minimal identifying characteristics for the use in clinical laboratories. Based on observations recorded in the present investigation and that of reported positivity of different characteristics (Joseph *et al*., 1987; Abbott *et al*., 1992), the use of three tests for the identification of *Aeromonas* spp. with about 90% accuracy has been recommended. These tests include aesculin hydrolysis, Voges-Proskauer and gas from glucose. Strains which are aesculin negative, VP positive and gas from glucose positive can be identified as *A. hydrophila*, where as *A. caviae* are negative for gas from glucose and aesculin hydrolysis and do not ferment glucose but positive for VP. In contrast *A. sobria* ferments glucose and hydrolysis aesculin and maybe VP negative or positive. But in

contrary to this, Martínez-Murcia *et al*., 2005; reported that none of the biochemical tests evaluated in their present study were able to separate two species

Plasmids are autonomous self-replicating structures possessing genes that directly or indirectly confer some unique properties to their host bacterium. The genetic determinants of antibiotic resistance are located extra-chromosomally and transfer of drug resistance is attributed to the presence of R-plasmids (Davis *et al*., 1978). In this study, the alkali-lysis method resulted in yield of zero to 6 numbers of plasmids of molecular weights ranging from 2.0 Kb to 23.13 Kb. The present investigation revealed that, plasmid mediated antibiotic resistance could not be studied as conjugal experiments were not employed. However, earlier reports indicated that R plasmid encoding antibiotic resistance in *Aeromonas* spp. varied in sizes from 85.6 to >150 Kb (Chang and Bolton, 1987). Majumdar (2006), reported that 21 Kb plasmid appeared to play an important role in the virulence factors of *Aeromonas* spp. Kruse and Sorum (1994) reported that the transfer of R-plasmids is a phenomenon that belongs to the environment and can occur between bacterial strains of humans, animals and fish origins that are unrelated. They also stated that combination of the environment with bacterial pathogens resistant to antimicrobial agents was a real threat not only as a source of disease but also as a source from which R-plasmids can easily spread to other pathogens of diverse origins. Hanninen and Hirvela-Koski (1997) found that plasmid profile analysis was a more sensitive method for differentiation of strains of *A. salmonicida* in fish farms. Based on their observations, they suggested that unique plasmids could be used as epidemiological markers to study the transmission of infection from one fish farm to another. The alkali-lyses method of plasmid DNA isolation resulted in high yield of plasmids. Of the 38 *Aeromonas* isolates, 33 contained plasmid DNA of varying sizes ranging from 23.13 to 2.0 Kb.

ACKNOWLEDGEMENT

The first author is thankful to the University authorities for providing financial assistance in the form of scholarship.

REFERENCES

Agger, W.A. (1986) Diarrhoea associated with *Aeromonas hydrophila.* Pediatr. Infect. Dis., **5** (Suppl.)**:** 105-108.

Agger, W.A.; McCormick, J.D. and Gurwith, M.J. (1985) Clinical and microbiological features of *Aeromonas hydrophila*-associated diarrhoea. J. Clini. Microbiol. **21:** 909-913.

Abbott, L.S.; Cheung, W.K.W.; Bystrom-Kroske, S.; Malekzadeh, T. and Janda, M.J. (1992). Identification of *Aeromonas* strains to the genospecies level in the clinical laboratory. J. Clin. Microbiol. **30(5):** 1262-1266.

Birnhoim, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research. **7:** 1513-1547.

Champsaur, H.; Andermont, A.; Matheiu, D.; Rottman, E. and Auzepy, P. (1982) Cholera-like illness due to *Aeromonas sobria.* J. Infec. Dis. **145:** 361-366.

Chang, J.B. and Bolton, M.S. (1986) Plasmids and resistance to antimicrobial agents in *Aeromonas sobria* and *Aeromonas hydrophila* clinical isolates. Antimicrob. Agents. Chemother. **31(8):** 1281-1282.

Cruickshank, R.; Duguid, J.P.; Marmion, B.P. and Swain, R.H.A. (1975) Med. Microbiol. 12th Edn. 2. Churchill Livingstone, Edinburg, London and New York.

Davis J, Steward S and Gilmann J. (1978) R-plasmid mediated antimicrobial resistance in *A. hydrophila, A. caviae, A. sobria*. Epidemiol and Infect, **19:** 234-239.

Echeverria, P.; Blacklow, N.R.; Sanford, L.B. and Cukor, G.C. (1981) Travellers' diarrhoea among American peace corps volunteers in rural Thailand. J. Infec. Dis. **143:** 767-771.

Gracey, M.; Burke, V. and Robinson, J. (1982) Short reports- *Aeromonas* spp. in traveller's diarrhoea. British Med. J. **289:**658.

Gracey, J.F. Thornton's meat hygiene. (1985) 7th Edn. (E.L.B.S.). William Clowes. Ltd. Beccles and London.

Hanninen, M.L. and Koski-Hirvela, V. (1997) Molecular and phenotypic methods for characterization of atypical *Aeromonas salmonicida.* Vet. Microbiol. **56:** 147-158.

Janda, J.M. and Abott, S.L. Human pathogens. In: Austin, B. *et al*., eds. (1996) *The genus Aeromonas*. London, Wiley. 151-173.

Janda, J.M., Guthertz, L.S., Kokka, R.P. and Shimada, T. (1994) *Aeromonas* species in septicemia : laboratory characteristics and clinical observations. *Clinical Infectious Disease.* **19**, 77–83.

Joseph, S.W.; Colwell, R.R. and McDonell, M.T. (1987) Taxonomy, ecology, isolation and identification: *Aeromonas* taxonomy. Experrientia., **43:** 349-550.

Komathi, A.G.; Ananthan, S. and Alavandi, S.V. (1998) Incidence and enteropathogenecity of *Aeromonmas* species associated with childhood gastroenteritis in Chennai (Madras), India. Jpn. J. Med. Sci. Biol. **51:** 1-12.

Kruse, H. and sorum, H. (1994) Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Appl. Environ. Microbiol. **60(11):** 4015-4021.

Murcia-Martinez, J.A.; Soler, L.; Saavedra, J.M.; Chacon, R.M.; Guarro, J.; Stackebrandt, E. and Figueras, J.M. (2005). Phenotypic, genotypic, and phylogenetic discrepancies to differentiate *Aeromonas salmonicida* from *A. bestiarum*. Int. Microbiol. **8:** 259-269.

Stelma, G.N.; Johnson, C.H. and Spaulding, P. (1986) Evidence for the direct involvement of ß-hemolysin in *Aeromonas hydrophila* enteropathogenecity. Curr. Microbiol. **14:** 71-77.

Vila, J.; Ruiz, J.; Gallardo, F.; Vargas, M.; Soler, L.; Figueras, M.J. and Gascon, J. (2003) *Aeromonas spp.* and traveller's diarrhoea: Clinical features and antimicrobial resistance. Emerg. Infec. Dis. 9**(5):** 552-555.

Yamada, S.; Matsushita, S.; Dejsirilert, S. and Kudoh, Y. (1997) Incidence and clinical symptoms of *Aeromonas* associated traveller's diarrhea in Tokyo. Epidemiol. Infect. **119:**121-126.