Cytotoxin testing of environmental Aeromonas spp. in Vero cell culture

V. Balaji, Mary V. Jesudason & G. Sridharan*

Departments of Clinical Microbiology & *Virology, Christian Medical College, Vellore, India

Received August 8, 2003

Background & objectives: Aeromonas spp. are known to cause a variety of infections in humans and this organism has been isolated from a variety of sources including environmental sources. The pathogenicity of the environmental isolates in and around Vellore has not been studied. This study was conducted to determine the cytotoxicity of the Aeromonas spp. isolated from water bodies, soil sediments, plankton and sewers in and around Vellore.

Methods: Aeromonas spp. isolated from environmental sources were identified by standard procedures. Representative isolates of Aeromonas spp. were tested for cell free cytotoxic factor in tissue culture system. Undiluted and diluted cell free filtrates of isolates and known toxigenic and non-toxigenic bacteria were added to Vero cell monolayer in microtitre plates. After appropriate incubation in 5 per cent CO₂ atmosphere, the microtitre plate was examined for cytopathic effect. Cell detachment and shrinkage of Vero cells were recorded as toxic changes.

Results: All 36 environmental isolates demonstrated cytopathic effect of which 41.7, 50 and 8.3 per cent belonged to A. hydrophila, A. veronii biotype sobria and A. caviae respectively.

Interpretation & conclusion: The results demonstrated the presence of potentially pathogenic environmental aeromonads in and around Vellore and they produced cytotoxin.

Key words Cytotoxicity - environmental Aeromonas spp
cytotoxicity of Aeromonas spp. isolated from environmental sources.

Material & Methods

During July-August 1996 and April 1997 to March 1998, drinking water samples were collected on a single occasion from 12 different areas including a water treatment plant of the town from an area where clinical cases of Aeromonas gastroenteritis (culture proven) were reported. Samples from environment namely plankton, soil sediment, and water were collected from a pond, lake and a moat in and around Vellore once a month throughout the study period. Samples from open sewers were collected from four areas of the town. Collection and processing of environmental samples were carried out as described previously. Isolation and identification of colonies were carried out using the standard procedures including production of hemolysis on 7 per cent sheep BA. Organisms were stored at room temperature in nutrient agar stab culture. These were sub-cultured and tested.

Preparation of culture free filtrate: Two to three colonies of the Aeromonas spp. were inoculated into 3-4 ml of nutrient broth (Beef extract, Hi-media, Mumbai; Peptone, Titan biotech Bhiwadi; Sodium chloride, NICE, Cochin) and incubated at 37°C for 2 h. The turbidity was adjusted to No. 3 Browne's opacity tube. Four to five drops of this was inoculated into 10-12ml of trypticase soy broth (TSB) (Beckton Dickinson, Sparks MD, USA) with 0.6 per cent yeast extract (Hi-media, Mumbai) in a 25 ml Ehrlenmeyer flask and incubated at 37°C for 16 h, as a shake culture (constant rotation at 3.5 g). The culture was centrifuged at 1350 g (Remi Instruments Ltd, Mumbai) for 20 min at 4°C, the supernatant was separated and filtered through a 0.22 µ pore size (Milipore, USA) syringe membrane filter unit. The filtrate was collected in a sterile screw capped tube and stored at 4°C till use (<3 days).

Cell culture assay: Toxin testing was carried out on a confluent monolayer of Vero cells in a 96 well flat bottom microtitre plate (Tarson, India). 50µl of undiluted bacteria-free filtrate which was added to the first well, 50µl of serially diluted (doubling dilution) filtrate diluted in minimum essential medium (MEM) (TPP, Zurich, Switzerland) with foetal calf serum (Life technologies, Grand island, New York, USA) in tubes were added from the 2nd to 8th well of the same row. Two rows of known positive and negative controls and one row each for TSB, and MEM control were set up. For positive control a known cytotoxic filtrate of clinical isolate of Aeromonas hydrophila was used and for negative control filtrate of a faecal Escherichia coli was used. Morphological changes in Vero cells were recorded at two hourly intervals twice and after overnight incubation at 37º C in a CO2 incubator. Cell detachment and shrinkage of Vero cells were recorded as toxic changes.

Results & Discussion

The ecology and toxigenicity of environmental Vibrios in Vellore has been extensively studied, but the toxigenicity testing of environmental Aeromonas spp. was not carried out earlier.

A total of 36 representative environmental Aeromonas isolates were tested for cytotoxicity in Vero cell culture. These include 15 isolates from water bodies (13 from natural water bodies and two from drinking water taps), 10 from plankton, 6 from soil sediments and 5 from Moore swabs. All the 36 isolates demonstrated cell detachment and cell shrinkage as cytotoxic changes. The changes were observed within 2 h of inoculation and maximum cytopathic effect was observed by 18 h. Of these, 41.7, 50 and 8.3 per cent belonged to A. hydrohila, A. veronii biotype sobria and A. caviae respectively. Other studies reported similar findings; 83 and 100 per cent of the isolates from water source, 73 per cent of the food isolates, 89 and 90 per cent of the clinical isolates produced cytotoxicity in the Vero cell culture. Schiavano et al demonstrated that cytotoxin production was the most common virulence factor compared to adhesive and invasive ability.

Among the Aeromonas spp. tested in this study, cell detachment and shrinkage were observed as cytopathic change. In our earlier study with clinical isolates, vacuolation was also observed in addition to these changes. Martins et al reported similar finding for food and clinical isolates of A.veronii biotype sobria alone.

All 36 cytotoxic environmental isolates produced ≥ haemolysis on sheep blood agar. This finding was similar
to water isolates studied by others\textsuperscript{18,21} and our earlier report on clinical isolates\textsuperscript{20}.

Interestingly, no \textit{Aeromonas} spp. were isolated from the samples taken from the water treatment plant, while water tested from street pipes (municipal water supply) grew \textit{Aeromonas} spp. suggesting that contamination was not occurring at source but at a point in the pipe supply to the town. These pipes were in close proximity to open sewerage (positive for \textit{Aeromonas} spp.) channels giving the potential for cross contamination.

The water bodies sampled were extensively used for washing clothes and utensils, bathing, and a source of drinking water for the domestic animals. The moat, in addition is a source of fish (culture positive for \textit{Aeromonas} and \textit{Vibrio} spp. reported by us earlier\textsuperscript{13}) sold in the local market. Probably these might be the source of infection.

The other interesting finding was that higher numbers of \textit{Aeromonas} spp. were isolated from environment samples during the summer months namely April, May and June. This is in accordance with the observation that more frequent clinical cases of \textit{Aeromonas} gastroenteritis occurred in summer months\textsuperscript{22}. The three most common \textit{Aeromonas} species isolated from cases of gastroenteritis namely, \textit{A. hydrophila}, \textit{A. veronii biotype sobria} and \textit{A. caviae}\textsuperscript{1} were found in environmental samples. This suggested the widespread presence of potentially pathogenic \textit{Aeromonas} spp. in the environment.

Though different enterotoxins have been demonstrated in \textit{Aeromonas} spp.\textsuperscript{5,6}, probably the cytotoxic enterotoxin, as well as haemolysins provide a mechanism by which these bacteria might cause intestinal disease.

\textbf{Acknowledgment}

Authors thank the Christian Medical College and Hospital, Vellore, for the financial assistance provided by the Institutional Fluid Research Grant.

\textbf{References}

\begin{enumerate}
  \item Michael J. Recent advances in the study of the taxonomy, pathogenicity and infectious syndromes associated with the genus \textit{Aeromonas}. \textit{Clin Microbiol Rev} 1991; 4 : 397-409.
  \item Singh DV, Sanyal SC. Haemolysin and enterotoxin production in \textit{Aeromonas caviae} isolated from diarrhoeal patients, fish and environment. \textit{J Diarrh Dis Res} 1992; 10 : 16-20.
  \item Thomson CJ, Jesudason MV, Balaji V, Malathi B, Uma M, Amyes SGB. Prevalence of \textit{Vibrio} spp. in drinking water and environmental samples in Vellore, south India. \textit{Epidemiol Infect} 1998; 121 : 67-76.
  \item Ormen O, Ostensvik O. \textit{Aeromonas} spp. and their aerolysin status in Norwegian natural water sources. \textit{J Appl Microbiol} 2001; 90 : 797-802.
\end{enumerate}


Reprint requests: Dr Mary V. Jesudason, Professor & Head, Department of Clinical Microbiology
Christian Medical College & Hospital, Vellore 632004, India
e-mail: micro@cmcvellore.ac.in