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Biochemical characteristics & secretory activity of *Aeromonas* species isolated from children with gastroenteritis in Chennai

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An attempt was made to delineate the phenotypic markers for the detection of enterotoxigenic strains of *Aeromonas*. Eighteen *Aeromonas* species comprising one isolate of *A. hydrophila*, six isolates of *A. sobria* and 11 isolates of *A. caviae* were obtained from 379 children suffering from acute diarrhoea in Chennai. Nine of these isolates inclusive of three *A. sobria* and six *A. caviae* were found to produce secretory response *in vitro* in the rabbit intestinal mucosa mounted in the Ussing chambers as revealed by significant increases in the short circuit current. Eleven strains hydrolysed aesculin, 8 fermented arabinose, 6 produced acetyl methyl carbinol, 14 produced lysine decarboxylase, 3 fermented salicin, 9 produced β-haemolysin, 9 produced CAMP-like factor and only two isolates took up congo red dye. None of these phenotypic traits were found to correlate with the *in vitro* secretory activity.

Key words *Aeromonas* - diarrhoea - secretory response

Aeromonas species have been implicated in diarrhoea the world over[1]. This species produces a variety of virulence factors such as haemolysins, cytotoxins, siderophores, enterotoxins *etc*[2]. Some studies have reported that enterotoxigenicity is related to species, particularly to *A. hydrophila* and *A. sobria*[3] and that only the β-haemolytic strains were enterotoxigenic[4]. Some others have suggested certain phenotypic characteristics such as production of acetyl methyl carbinol (VP), lysine decarboxylase, haemolysis on sheep blood agar, CAMP-like factor, inability to hydrolyse arabinose, susceptibility to cephalothin as potential virulence markers and that these tests would identify up to 97 per cent of enterotoxigenic isolates[5-8]. *A. caviae*, because of their VP negativity and non-haemolytic nature would be excluded if these criteria are employed. But *A. caviae* have been also recognised an important diarrhoeagenic aeromonad in children and aged individuals[9] based on its enterotoxigenicity in suckling mice and cytotoxic response in HEp-2 cells *in vitro*. The present study was conducted to delineate important phenotypic marker(s), if any, to identify enterotoxigenic strains in a routine diagnostic laboratory. The *Aeromonas* isolates obtained from children suffering from acute diarrhoea in Chennai were biochemically characterised and the enterotoxigenicity of these isolates determined by their ability to induce secretory response in Ussing chambers. The phenotypic characteristics were examined for correlation with secretory activity.

Material & Methods

Isolation and identification: The study was conducted from February 1997 to April 1998 at Chennai. Rectal swabs were collected in duplicate from 379 children under 5 yr of age suffering from acute diarrhoea, admitted to the Institute of Child Health and Hospital for Children, Chennai, and transported to the laboratory in Cary Blair medium. One swab was inoculated onto Inositol Brilliant green bile salts (IBB) agar (Himedia, Mumbai) and the other was inoculated into alkaline peptone water (APW) for enrichment and incubated at 37°C for 18-24 h. A loopful from the APW was inoculated onto IBB and 5 per cent sheep blood agar (SBA). The non-inositol fermenting colonies on IBB were subcultured on nutrient agar and incubated at 37°C overnight. The colonies on nutrient agar and SBA were tested for oxidase and positive isolates were identified as described earlier[10]. The biochemical tests, esculin hydrolysis, production of acetyl methyl carbinol (VP), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), arginine dihydrolase (ADH), fermentation of arabinose, sucrose and salicin, and detection of extracellular enzyme activities for protease, lipase and amylase were carried out by conventional methods[11].

In vitro secretory activity: Cell free culture filtrates were prepared by growing the isolates in brain heart infusion (BHI) broth at 37°C for 18 h and the cultures were centrifuged at 7000 *g* at 4°C and the supernatants were filtered through 0.22 µm syringe filters (Millipore Inc, USA). These culture supernatants were tested for secretory activity in the intestinal mucosa mounted in the Ussing chambers (World Precision Instruments, USA) as earlier described[12]. Briefly, the rabbit ileal tissue cut in a rectangular shape, obtained from a freshly sacrificed animal was mounted in the Ussing chambers, the tissue was bathed in Ringers solution on both serosal and mucosal sides and aerated with 5 per cent CO_2 and 95 per cent O_2 mixture. The instrument was balanced and the readings of short circuit current (Isc) and potential difference (PD) were recorded. 400 µl of culture filtrate was added on both serosal and mucosal sides and PD and Isc were measured every 15 min for 90 min. An enterotoxigenic strain of *Vibrio cholerae* was used as a positive control. The cell free culture filtrates of isolates showing increases in the Isc were regarded as positive for *in vitro* secretory activity[13].

Production of haemolysin: Production of haemolysin by the *Aeromonas* isolates was tested as described earlier[5]. 50 µl of cell free culture filtrates of the *Aeromonas* isolates prepared as described above were mixed with 50 µl of 2 per cent suspension of sheep RBCs in PBS in a round bottomed 96 well microtitre plate and examined for haemolysis after incubation at 37°C for 1 h and a further incubation at 4°C for 18 h.

CAMP-like factor: The ability of *Aeromonas* strains to act synergistically with a β-haemolytic *Staphylococcus aureus* to produce more potent haemolysis indicated by arrowhead shaped zones of haemolysis was determined by the methods described earlier[14].

Congo red uptake: *Aeromonas* isolates were inoculated onto trypticase soy agar containing 50 µg of congo red dye per ml of the medium and incubated for 18 h at 37°C. The plates were examined for isolates showing various shades of orange under obliquely reflected light on a black background which indicated uptake of the dye by the organisms[15].

Statistical analysis: The relationship between various biochemical characteristics singly and in various combinations with secretory activity was examined by simple correlation analysis using SPSS computer software.

Results & Discussion

In the present study we were able to recover *Aeromonas* species from 18 paediatric patients suffering from diarrhoea. The 18 isolates were identified to genus level by the criteria of Aerokey II[16]. *A. caviae* was recovered from 11 patients, while *A. sobria* was isolated from six patients and *A. hydrophila* from one, indicating the predominance of *A. caviae* in paediatric patients as reported by earlier workers[17].

Out of 18 isolates, 6 produced VP, 11 hydrolysed aesculin, 8 fermented arabinose, 3 fermented salicin, 14 produced lysine decarboxylase, 13 produced arginine dihydrolase, 9 produced β-haemolysin, 9 exhibited CAMP-like factor, 2 took up congo red dye, 8 produced lipase, 13 produced protease, 14 produced amylase and 3 isolates showed susceptibility to cephalothin (Table).

Table. Phenotypic characteristics of *Aeromonas* isolates from children with acute diarrhoea

Test	Isolates																		Total		
	A.h 8143	A.s 38a	A.s 374	A.s 49	A.s 6143	A.s 811	A.s 1262	A.c 359	A.c 544	A.c 649	A.c 96	A.c 106	A.c 113	A.c 1154	A.c 1233	A.c 1235	A.c 1264	A.c 121	A.h (n=1)	A.s (n=6)	A.c (n=11)
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1	6	11
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1	6	11
TSI	K/A,g	K/A,g	K/A,g	A/A	K/A	K/A,g	A/A	A/A	A/A	A/A	A/A	A/A	A/A	K/A	A/A	K/A	A/A	A/A			
Indole	+	+	+	+	-	+	-	+	+	+	-	+	+	-	+	+	-	+	1	4	8
VP	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	1	5	0
Aesculin hydrolysis	+	-	-	-	+	-	-	+	+	+	+	+	+	+	+	-	-	+	1	1	9
Res to O/129-150 μg	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1	6	11
Inositol fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0
Sucrose fermentation	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	1	6	10
Arabinose fermentation	+	-	-	-	+	-	-	-	+	+	+	+	-	-	-	+	-	+	1	1	6
Mannitol fermentation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1	6	11
Glucose fermentation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1	6	11
Lactose fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0
Salicin fermentation	-	-	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	0	1	2
Lysine decarboxylase	+	+	+	-	+	+	+	+	-	+	+	-	+	+	+	-	+	+	1	5	8
Arginine dihydrolase	+	-	-	+	-	+	+	+	+	+	+	+	+	-	+	-	+	+	1	3	9
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0
NO_3 reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1	6	11
Cephalothin sensitivity	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	1	1	1
CAMP-factor	+	+	+	-	+	+	+	-	+(w)	-	-	-	+(w)	+(w)	-	-	-	-	1	5	3
Congo red uptake	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	0	2	0
Lipase	+	-	-	+	-	+	-	-	+	-	-	+	+	-	+	-	-	+	1	2	5
Protease	+	+	+	+	+	+	-	-	+	-	+	+	-	+	+	+	+	-	1	5	7
Amylase	+	+	+	+	+	-	+	+	+	-	+	+	+	-	-	+	+	+	1	5	8
Haemolysin	+	+	+	-	+	+	+	-	+	-	-	-	+	+	-	-	-	-	1	5	3
Secretory activity	-	-	+	+	-	+	-	-	+	+	+	-	+	-	-	-	+	+	0	3	6

A.h, *A.hydrophila;* A.s, *A.sobria;* A.c, *A.caviae;* TSI, triple sugar iron agar; K/A, alkaline slant, acid butt; A/A, acid slant, acid butt; g, gas; VP, Voges Proscaur's test; (w), weak reaction

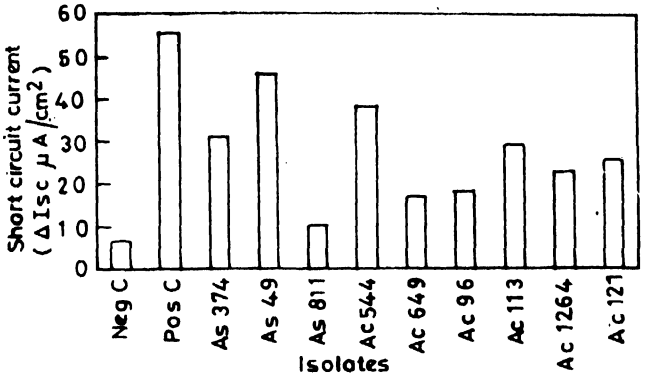

Fig. Secretory activity of *Aeromonas* isolates in Ussing chambers.

Nine of these isolates (3 of *A. sobria* and 6 of *A. caviae)* induced increase in the short circuit current (ΔIsc) of the ileal tissue mounted in the Ussing chambers, the average ΔIsc ranging from 10.5 μA to 46μA/ cm^2 (Fig.).

Attempts to correlate some of the phenotypic characteristics such as production of VP, LDC, ADH, CAMP factor, haemolysin *etc.*, with the *in vitro* secretory response, showed no correlation between any of these parameters with secretory activity. Some earlier studies[6,8] had reported that the enterotoxigenic strains are VP positive, arabinose negative, lysine decarboxylase positive and produce haemolysin[5]. In the present case, such a correlation was not observed either singly or in combination to be predictive of enterotoxigenicity. Singh and Sanyal[18] also did not observe correlation between any of the biochemical characteristics such as VP, lysine decarboxylase, non-fermentation of arabinose or haemolysin production based on their study of 147 isolates inclusive of 54 isolates from patients of diarrhoea.

Congo red uptake has been reported as a virulence marker for several pathogenic bacteria such as *Shigella*[19], *Yersinia enterocolitica*[20] and *V. cholerae*[21]. Earlier studies on the congo red uptake by *Aeromonas* spp showed no correlation with VP or lysine decarboxylase[15]; correlation between congo red uptake and enterotoxigenicity was not attempted. In the present study, only two isolates showed ability to take up congo red dye and its relationship with secretory activity could not be established.

Production of CAMP - like factor was suggested for differentiation of *Aeromonas* species[14]. *A. hydrophila* produced CAMP - like factor both aerobically and anaerobically and *A. sobria* produced CAMP factor only aerobically, whereas *A. caviae* did not produce CAMP factor [14]. In the former two species, which are more often reported to be associated with human disease, production of CAMP factor was considered as a virulence factor. Three of our *A. caviae* isolates which were α-haemolytic showed mild CAMP-like factor upon further incubation of the plates overnight at 4°C. Hence this scheme of differentiation of *Aeromonas* is unlikely to be useful in view of the fact that *A. caviae* isolates can be haemolytic[22].

Susceptibility to cephalothin was reported to be associated with *A. sobria*[7], the species that is considered to be invasive among the aeromonads[23]. In the present study, susceptibility to cephalothin was uncommon among *A. sobria* isolates and there was no relationship with secretory response of the isolates.

Production of several extracellular virulence factors such as lipase, protease and haemolysin was reported to be variable in *Aeromonas* isolates obtained from faecal samples of patients suffering from gastroenteritis and, it has been suggested that high protease and low lipase activity along with the autoagglutinating character be considered as virulence marker[24]. In the present study, although 13 isolates showed protease activity and 8 isolates lipase activity, both these parameters also did not show any correlation with the secretory activity. Production of enterotoxin *in vitro* varies with the conditions of culture and the method of assay[25] and probably many other factors. Cahill[26] suggested that the cytotoxins, haemolysins, enterotoxins *etc.*, are manifestations of a single factor. In view of our observations and the lack of correlation of the phenotypic parameters with the *in vitro* secretory activity it is difficult to suggest any of the phenotypic characters as markers of virulence or indicators of diarrhoeagenic potential in *Aeromonas* species.

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