Indian Journal of Medical Microbiology, (2003) 21 (4):233-238

Special Article

BIOCHEMICAL CHARACTERISTICS, SEROGROUPS, AND VIRULENCE FACTORS OF *AEROMONAS* SPECIES ISOLATED FROM CASES OF DIARRHOEA AND DOMESTIC WATER SAMPLES IN CHENNAI

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Abstract

Purpose: The objective of the present study was to delineate the differences between the clinical and environmental *Aeromonas* species with respect to their biochemical characteristics, serogrouping and virulence factors, in order to find a phenotypic marker of enteropathogenicity. **Methods:** A total of 55 *Aeromonas* spp. inclusive of 19 isolates from cases of diarrhoea, and 36 from water samples comprising, 10 isolates of *A. hydrophila*, 21 isolates each of *A. sobria*, and *A. caviae*, two isolates of *A. jandaei* and one isolate of *A. veronii* were subjected to analysis of their biochemical characteristics, serogrouping, and virulence factors. **Results:** Among the differences recorded in the biochemical characteristics in the three major species, the most striking characteristic was fermentation of lactose, which was observed in all the 11 *A. caviae* isolates recovered from water samples. None of the 10 clinical isolates of *A. caviae* tested fermented lactose. The clinical *Aeromonas* isolates belonged to seven typable serogroups, O:13, O:14, O:16, O:21, O:27, O:32 and O:35. The environmental isolates belonged to eight different serogroups, such as, O:3, O:11, O:14, O:16, O:18, O:28, O:64 and O:78 and were predominated by serotypes O:18 and O:64. Among the virulence factors tested, 89% of the environmental isolates produced b haemolysin, while only 62.3% of clinical isolates were able to do so. There was no significant difference between the clinical and environmental aeromonads with respect to their enterotoxigenicity in suckling mice *in vivo*, cytotoxicity *in vitro* in Vero cell monolayers, and ability to produce siderophores. **Conclusion:** Efforts to delineate the differences between the clinical and environmental *Aeromonas* spp. did not reveal significant difference between them. However, difference was observed with respect to their ability to produce b haemolysin, wherein, higher percentage of environmental isolates was haemolytic. The results also suggest that all the haemolytic environmental isolates need not be enteropathogenic. Further, serogroups O:18 and O:64 may not be involved in aeromonal diarrhoea in children in this geographic region.

Key words: *Aeromonas, diarrhoea, biochemical characteristics, serogroups, virulence factors*

The motile mesophilic *Aeromonas* species are primarily organisms of aquatic environment and are present in fresh water, estuarine and coastal water bodies and even in chlorinated water.[1,2] Aeromonads have been also reported to be associated with human disease, especially in diarrhoea in children, aged individuals and immunocompromised patients.[3,4] Incidence of *Aeromonas* associated gastroenteritis has been reported from world over and its incidence in the developed countries has been reported to be relatively low compared to that in the developing countries.[5] In India, *Aeromonas* associated diarrhoea has been reported from Bombay,[6] Calcutta,[7] Goa,[8] Vellore,[9] Pondicherry[10] and Chennai,[11,12] and the incidence in these areas was reported to range between less than 1% to about 13%.

Several molecular typing techniques have been employed for typing of *Aeromonas* spp. and these studies have indicated that the clinical and environmental aeromonads are different. *Aeromonas* strains from human diarrhoeal stools and drinking water samples were reported to be dissimilar by biotyping in conjunction with gas liquid chromatography of cell wall fatty acid methyl esters.[13] In a similar study, aeromonads isolated from public water supply in Iowa city were reported to be unrelated to those isolated from patients with gastroenteritis by ribotyping.[14] Our studies using RAPD fingerprinting also indicated that there were differences between the clinical and environmental strains of *Aeromonas* spp., and that only some strains occurring in water are potentially enteropathogenic.[15] What makes some of these strains pathogenic is not known. In order to find out the differences between

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Received : 17-08-2002
Accepted : 25-10-2002

clinical and environmental *Aeromonas* spp. we analysed the biochemical characteristics, serogroups, and virulence factors.

Materials and Methods

Fifty-two isolates were subjected to biochemical characterization using standard protocols.[16] Production of b haemolysin was determined by conventional blood agar inoculation. Presence of large zone of b haemolysis around the colonies and titres were determined according to protocols described earlier.[17] *Aeromonas* isolates were tested for enterotoxigenicity *in vivo* in 3-4 day old suckling mice.[18] Cytotoxic activity of the culture filtrates of *Aeromonas* isolates to Vero cell monolayers was tested as per the protocols described elsewhere.[19] Production of siderophores was determined by protocols described earlier using Chrome azurol S (CAS) agar and in cell free culture filtrates using CAS assay solution.[20] *Aeromonas* isolates were serotyped by tube agglutination method using polyvalent antisera at National Institute of Infectious Diseases (Tokyo, Japan) by Dr. Toshio Shimada.[21]

Results

Biochemical characteristics

All the *A. hydrophila* isolates and 63.6% of *A. sobria* were aerogenic. All the *A. caviae* isolates were anaerogenic (Table 1) and 39.4% of the isolates fermented lactose.

Table 1 : Salient biochemical features of *Aeromonas* species (n=52)

Test	Percentage positive							
	A.hydrophila		*A.sobria*		*A.caviae*		**Total**	
	C	E	C	E	C	E	**C**	**E**
Number of isolates tested	1	9	8	13	10	11	**19**	**33**
Gas production from TSI	1	9	3	11	0	0	**21.1**	**60.6**
Fermentation of lactose	0	0	0	2	0	11	**0**	**39.4**
Acid from arabinose	1	1	1	2	6	11	**42.1**	**42.4**
Fermentation of salicin	0	2	1	2	2	2	**15.8**	**18.2**
Production of Indole	1	9	7	12	7	11	**18.9**	**97**
Voges- Proskauer's Test	1	9	2	0	0	0	**15.8**	**27.3**
Aesculin hydrolysis	1	9	2	0	8	11	**57.9**	**60.6**
Lysine decarboxylase	1	9	6	13	7	0	**73.7**	**66.7**
Arginine dihydrolase	1	9	4	13	8	11	**68.4**	**100**
Amylase	1	9	7	12	7	8	**78.9**	**87.9**
Lipase	1	9	3	13	5	11	**47.4**	**100**
Protease	1	9	7	12	7	10	**78.9**	**93.9**
CAMP-like factor	1	9	5	12	3	8	**47.4**	**87.9**

C - clinical, E - environmental

None of the *Aeromonas* isolates from clinical sources fermented lactose. *A. caviae* were the only species which were isolated from domestic water samples and which fermented lactose. Fermentation of arabinose was observed in 42.1% of the isolates from each of the sources, and this feature was also associated with *A. caviae*. Production of acetyl methyl carbinol was observed in 15.8% of the clinical samples and 27.3% of the isolates from water, and was usually associated with *A. hydrophila* and *A. sobria*, while none of the *A. caviae* isolates produced acetyl methyl carbinol. Aesculin hydrolysis was observed in 57.9% of the clinical isolates and 60.6% of the isolates from domestic water samples, and this trait was found to be associated with *A. hydrophila* and *A. caviae*.

Serotyping

A total of 38 isolates comprising 16 from cases of diarrhoea and 22 from domestic water samples were serotyped. *Aeromonas* isolates obtained from clinical samples could be placed in seven typable groups, viz., O:13, O:14, O:16, O:21, O:27, O:32, and O:35. Being rough strains, two isolates of *A. caviae* and one isolate of *A. sobria* could not be serotyped (Table 2).

Aeromonads isolated from water samples belonged to O:3, O:11, O:14, O:16, O:18, O:28, O:64 and O:78 serogroups. Two serogroups (O:18 and O:64) occurred frequently in the water samples. Two isolates of *A.sobria* belonged to unknown 'O' serogroup and one isolate of *A. hydrophila* was rough type.

Table 2 : Serogroups of *Aeromonas* species recovered from cases of diarrhoea and domestic water samples in Chennai

Clinical isolates		Isolates from water	
Aeromonas species	Serogroup	*Aeromonas* species	Serogroup
A. hydrophila 8143	O 21	*A. sobria* BW 9	O 78
A. sobria 38a	O 35	*A. hydrophila* BW 12	O 18
A. sobria 359	O 27	*A. hydrophila* BW 13	O 18
A. sobria 374	O 35	*A. sobria* BW 14	O 18
A. sobria 49	R	*A. caviae* MW 55	O 11
A. sobria 6143	O 32	*A. caviae* MW 59	O 3
A. sobria 811	O 13	*A. sobria* MW 60	O 78
A. caviae 544	O 14	*A. hydrophila* BW 62	O 18
A. caviae 649	O 16	*A. caviae* BW 65	O 16
A. caviae 96	R	*A. sobria* BW 66	O 18
A. caviae 113	O Uk	*A. sobria* BW 67	O 11
A. caviae 1154	R	*A. hydrophila* MW 71	O 28
A. caviae 1233	R	*A. hydrophila* WP 86	R
A. caviae 1235	O 13	*A. veronii* WP 88	O 16
A. caviae 1262	O Uk	*A. sobria* WP 94	O 14
A. caviae 121	O 14	*A. sobria* WP 97	O Uk
		A. caviae WP 101	O 64
		A. caviae WP 106	O 64
		A. caviae WP 109	O 64
		A. sobria WP 110	O Uk
		A. caviae WP 111	O 64
		A. hydrophila MW 115	O 64

R: Rough type; O Uk: Un-known 'O' serotype

Virulence factors

Majority of the clinical isolates (63.2%) produced haemolysin, while a relatively higher percentage i.e., 88.9% of aeromonads recovered from the water samples produced b - haemolysin (Table 3).

Table 3 : Virulence factors of clinical (C) and environmental (E) *Aeromonas* isolates

Species	Haemolysin		Vero cell cytotoxicity		Suckling mice	
	C	E	C	E	C	E
A.hydrophila	1(1)	9 (9)	1 (1)	7 (9)	1 (1)	5 (7)
A.sobria	7 (8)	12 (13)	6 (8)	9 (13)	6 (8)	1 (1)
A.caviae	4 (10)	8(11)	5 (10)	3 (11)	6 (10)	0 (2)
A jandaei	-	2 (2)	-	1 (2)	-	-
A.veronii	-	1 (1)	-	0 (1)	-	-
Total	**12(19)**	**32(36)**	**12 (19)**	**20 (36)**	**13 (19)**	**6 (10)**
Percentage	**63.2**	**88.9**	**63.2**	**55.6**	**68.4**	**60**

Figures in parenthesis are number of isolates tested

There appeared to be no significant difference between the clinical and environmental *Aeromonas* isolates with regard to their ability to produce cytotoxicity in Vero cells *in vitro*. The clinical isolates (63.2%) were cytotoxic to Vero cells, while a relatively less percentage (55.6%) of those of environmental origin were able to do so. The clinical isolates (68.4%) were also enterotoxigenic in the suckling mice. It is important to note here that 60% of the *Aeromonas* isolates of environmental origin were found to be enterotoxigenic by the suckling mouse assay.

Nineteen of the 21 clinical isolates and 26 of the 27 isolates from water produced siderophores indicating their ability to survive and grow in the iron deficient conditions (Table 4).

Table 4 : Siderophore production by *Aeromonas* isolates from clinical and water samples

Aeromonas species	No. of isolates producing siderophore			
	On CAS Agar		Total percentage	
	Clinical isolates	Isolates from water	Clinical isolates	Isolates from water
A.hydrophila	2(2)	7(8)	100	87.5
A.sobria	6(6)	9(9)	100	100
A.caviae	9(11)	7(7)	81.8	100
A.veronii	1(1)	1(1)	100	100
A.jandaei	1(1)	2(2)	100	100
Total	**19(21)**	**26 (27)**	**90.5**	**96.3**

Figures in parenthesis are number of isolates tested

Discussion

Biochemical characteristics

Among the 52 *Aeromonas* isolates subjected to biochemical characterization, *A. hydrophila* and *A. sobria* were aerogenic while the *A. caviae* isolates were anaerogenic in nature. Similarly, production of acetoin was confined to *A. hydrophila* and *A. sobria* only. However, several other biochemical reactions, such as fermentation of arabinose, lactose, aesculin hydrolysis, and lack of acid production from salicin were found to deviate from the ideal phenotype for each isolate. Janda *et al* have earlier reported similar deviation in the biochemical characteristics from ideal phenotypes.[22] Thirty nine per cent of the *Aeromonas* isolates obtained from domestic water samples, mainly comprising *A. caviae*, fermented lactose. This fact needs to be considered when selective isolation media are not employed for isolation of aeromonads from clinical and environmental sources. In the present study 42.1% of the *Aeromonas* isolates from each of the sources fermented arabinose, majority of which were *A. caviae*. In an earlier study from Australia, a relatively higher percentage (58.5%) of aeromonads were reported to be able to ferment arabinose.[23] Decarboxylation of lysine was associated with *A. hydrophila* (82.6%) and *A. sobria* (90.5%), while 33% of *A. caviae* were also associated with this trait in the present study. These results corroborate with those reported earlier.[22] Production of CAMP-like factor was usually found in haemolytic strains of *A. hydrophila* and *A. sobria* isolates. A large number of *A. caviae* isolates were also able to produce CAMP-like factor. Hence, the proposal of Figura and Guglielmetti, that the motile mesophilic *Aeromonas* strains can be presumptively differentiated based on this trait do not appear to be applicable.[24] The results obtained in the present study do not support their observation.

Serotyping

The genus *Aeromonas* is antigenically diverse, being composed of more than 96 distinct serogroups on the basis of presence of unique somatic antigens. These serogroups are not species specific. A recent study on serotyping of *Aeromonas* isolates from diverse clinical and environmental sources along with reference strains indicated that serogroups O:11, O:16 and O:34 predominate in about 48% of the clinical samples.[25] They also reported that individual serogroups could be found in more than one species. High incidence of O:11, O:16 and O:34 serogroups has been reported by several investigators.[21, 26, 27] However in the present study, these serogroups do not seem to occur with as much frequency. While serotype O:16 was found in only one clinical isolate O:11 and O:34 were not found in the clinical samples. Three out of 16 clinical isolates of *A. caviae* and one isolate of *A. sobria* were untypable (rough types). A similar observation was made by Havelaar and co-workers,[13] who reported that about 45% of their strains, predominantly *A. caviae*, were untypable. Among the 22 aeromonads serotyped from environmental source, two isolates belonged to O:11 and two others to O:16. The environmental aeromonads recovered in the present study were predominated by O:64 and O:18 [5 each of the 22 (22.7%) isolates serotyped]. It may be construed that the serogroups O:11, O:16 and O:34 reported from other geographic regions are uncommon in this region.

Virulence factors

Among the *Aeromonas* isolates obtained in the present study, 63% of the clinical isolates were

haemolytic, while, more than 88% of those obtained from environment were able to haemolyse the erythrocytes. Some studies indicated haemolysin production in a relatively lesser percentage of isolates compared to the results obtained in this study.[28] As reported in an earlier study,[29] a majority of *A. caviae* isolated in the present study were also haemolytic. Out of 10 isolates from water samples tested for enteropathogenicity by suckling mouse assay, 6 (60%) of them showed their ability to induce enterotoxigenic response in the suckling mice. It has been reported that enteropathogenicity of environmental isolates was low (15%) compared to those from clinical source (42%) by suckling mouse assay.[30] In the present study, there appeared to be no significant difference in the ability to produce cytotoxins by aeromonads from the two sources. Cumberbatch *et al* reported higher (69%) incidence of cytotoxic strains of *A. hydrophila* from diarrhoeal source.[31] They also reported that cytotoxin was a stable property and could not be associated with any plasmid. Some authors had suggested that cytotoxin production was species specific and that only *A. hydrophila* and *A. sobria* produced enterotoxin.[32,33]

Majority of the isolates obtained in the present study produced siderophores. In spite of the iron limiting conditions in the culture medium, *Aeromonas* isolates readily grew well, indicating their ability to survive in the iron deficient conditions prevalent in the host environment. It has been reported that amonabactin is possibly an important virulence factor of *Aeromonas* species.[34] Earlier studies have indicated that ability to elaborate siderophores correlated with higher virulence in *Aeromonas* species.[35] Further, iron deficiency in the medium induces microbes to activate iron acquisition mechanisms and in turn, constitutes an important signal, which regulates expression of a number of virulence factors unrelated to iron metabolism.[36]

Analysis of *Aeromonas* isolates could not reveal any striking differences between those isolated from clinical and environmental sources. The conventional notion that the *Aeromonas* species are always non-lactose fermentors may prove wrong and hence, selective media such as Inositol Brilliant green Bile salts (IBB) agar or Cefsulodin Irgasan Novobiocin (CIN) agar must be incorporated along with the battery of enteric media for the recvovery of *Aeromonas* spp. Further, serogroups other than O:11, O: 16 and O: 34 could play a role in diarrhoea in this geographical region, and that, at least 60% of the environmental *Aeromonas* isolates could be enteropathogenic.

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