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Haemagglutinating activity, serum sensitivity and enterotoxigenicity of *Aeromonas* spp.

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Summary. Of 97 isolates of *Aeromonas* spp. that were examined for haemagglutination (HA) and enterotoxigenicity, 35 were from clinical and 62 from environmental sources; 66 of them were also screened for sensitivity to normal human serum (NHS). HA was caused by 44 isolates (45%); it was unrelated to the source of the strain, but it was caused by a higher proportion of the isolates of *A. hydrophila* than of *A. sobria* or *A. caviae*. Of the haemagglutinating strains, 82% were enterotoxigenic, whereas most of the non-haemagglutinating strains were non-toxigenic when tested initially. All the latter became enterotoxin producers after serial passage through rabbit ileal loops, but without change in HA. Most (64%) of the isolates, including 68% of *A. caviae* (72% of clinical and 65% of environmental), were resistant to the bactericidal action of NHS. Most (92%) of the serum-sensitive strains were killed by activation of both the classical and alternate pathways of complement, the others only by the alternate pathway. Most (74%) of the serum-resistant strains caused fluid accumulation in the initial tests in ileal loops, regardless of species or source. Haemagglutinating and serum-resistant strains caused significantly more accumulation of fluid ($p < 0.05$) than non-haemagglutinating and serum-sensitive strains. This study shows partial correlation between HA or serum sensitivity and enterotoxigenicity, but the properties are probably not genetically linked.

Introduction

Aeromonas spp. have been implicated in extra-intestinal infections and diarrhoea in man,[1,2] the strains often originating from water. *A. hydrophila* and *A. sobria*, but rarely *A. caviae* isolates, produced enterotoxin[3–6] and also showed resistance to the bactericidal action of normal human serum (NHS).[7,8] Moreover, enterotoxigenic diarrhoeal isolates of *A. hydrophila* showed haemagglutination (HA) which was not sensitive to mannose and fucose; but *Aeromonas* strains showing HA sensitive to mannose and fucose, or no haemagglutination (NHA) were non-toxigenic strains of *A. caviae*, commonly from non-diarrhoeal infection or the environment.[9]

In this study, we have explored the HA and serum-sensitivity patterns of clinical and environmental isolates of *Aeromonas* spp. and their correlations with enterotoxin production, species and source.

Materials and methods

Bacterial strains

Strains of *Aeromonas* from cases of acute diarrhoea in children and adults (35) and from environmental sources (62) were tested for HA and enterotoxin production (table I). Twenty-nine clinical and 37 environmental strains were also examined for susceptibility to NHS (table II). By the criteria of Popoff,[10] strains were classified into three species (*A. hydrophila*, *A. sobria*, *A. caviae*), according to their ability to hydrolyse aesculin, to ferment salicin, and to produce gas, acetoin and H_2S. The strains were maintained in peptone agar stab cultures at room temperature and did not undergo more than three subcultures before being tested.

Haemagglutination

The method of Atkinson and Trust was used.[11] Human group O erythrocytes were collected by venepuncture and stored in Alsever's solution at 4°C. Before use, they were washed three times in phosphate-buffered saline (PBS; 0·04M, pH 7·4) and then a 3% suspension was prepared in PBS.

Colonies of overnight cultures of *Aeromonas* strains on nutrient agar plates were incubated in Brain Heart Infusion Broth (BHIB, Difco), and incubated for 18 h at 37°C to yield *c.* 10^9 bacteria/ml. These cultures were centrifuged and washed twice in PBS.

HA tests were performed at room temperature by mixing 20 µl of erythrocyte suspension with 20 µl of bacterial suspension on a slide alongside a control suspension of erythrocytes and PBS, and gently

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Table I. Haemagglutinating activity and enterotoxigenicity of *Aeromonas* isolates

Source and species of isolate	Number of strains tested	HA-positive strains								NHA strains	
		Number of strains showing							Mean (SD) volume of fluid in initial tests (ml/cm of RIL)	Number of strains	Mean (SD) volume of fluid in initial tests (ml/cm of RIL)
		MS	MFS	MFGS	MGS	FGS	MFGR	Any HA			
Clinical											
A. hydrophila	5	1(1)	2(2)		1(0)			4(3)	0·71 (0·05)	1(0)	
A. sobria	15	3(3)		2(2)				5(5)	0·69 (0·11)	10(2)	0·43 (0·03)
A. caviae	15	5(4)				1(1)		6(5)	0·60 (0·08)	9(2)	0·41 (0·01)
Environmental											
A. hydrophila	9		3(2)	2(2)	2(0)			7(4)	1·17 (0·44)	2(0)	
A. sobria	16	5(5)	1(1)	2(2)				8(8)	0·79 (0·15)	8(2)	0·46 (0·02)
A. caviae	37	2(2)	5(4)	5(4)	1(0)		1(1)	14(11)	0·61 (0·09)	23(10)	0·48 (0·04)
Positive control*									1·20 (0·20)		
Negative control†									0·00 (0·00)		
Total	97	16(15)	11(9)	11(10)	4(0)	1(1)	1(1)	44(36)		53(16)	

Figures in parenthesis indicate number of enterotoxigenic strains in the initial tests before passage in RILs.
* BHIB culture of *V. cholerae* strain 569B.
† BHIB.

rocking by hand. Strains were considered HA-negative if agglutination did not occur within 5 min.[9]

Sensitivity of HA to sugars was studied in a similar three-volume test with 20 μl of erythrocyte suspension 3%, 20 μl of sugar 1% in PBS, and 20 μl of bacterial suspension. Reactions in the presence of D-mannose (M), L-fucose (F) or D-galactose (G) were compared with a positive control (erythrocytes, bacteria and PBS) and a negative control (20 μl of erythrocytes and 40 μl of PBS). The reaction was recorded as sensitive (S), if a previously positive result became negative in the presence of sugar, and resistant (R) if it remained positive.

Susceptibility to NHS

Group O blood was obtained by venepuncture from healthy individuals with no history of aeromonas infection; pooled sera were separated and used immediately or stored at −70°C. Fresh or freshly thawed NHS was used unaltered or after addition of 10 mM $MgCl_2$ ethylene glycol tetra-acetic acid (MgEGTA), prepared by the method of Fine *et al.*,[12] giving a final concentration of 10 mM MgEGTA. Serum-sensitive *Escherichia coli* strain K12 served as a control for each experiment.

Bactericidal activity and complement activity were determined by the method of Carruthers and Kabat.[13] Briefly, the bacterial inoculum (*c.* 10^7 cfu) in 0·3 ml of PBS was mixed with 0·7 ml of NHS with and without MgEGTA; 0·1 ml of the mixture was withdrawn for an initial viable count, and the remainder was incubated at 37°C for 30, 60 and 120 min. Serial 10-fold dilutions in PBS were inoculated in duplicate on to nutrient agar plates and incubated overnight at 37°C; bacterial colonies were then counted. Strains showing < 10% survival (i.e., > 1 $\log_{10}$ reduction in cfu) at 60 and 120 min were designated as showing prompt and delayed serum-sensitivity, respectively.

Enterotoxin assay

Live cells and culture filtrates of all the *Aeromonas* strains were tested for enterotoxigenicity in the adult rabbit ileal loop (RIL) model of De and Chatterjee[14] as adopted by Annapurna and Sanyal.[3] Those strains that caused little or no accumulation of fluid in the initial tests were subjected to successive passage through RILs until they caused fluid accumulation similar to that of the positive control strain 569B of *Vibrio cholerae*.[15]

Results

Of the 97 isolates of *Aeromonas* spp., 44 (45%) were HA-positive (table I); these included 79% of the *A. hydrophila*, 42% of the *A. sobria* and 38% of the *A. caviae* isolates. The haemagglutinating strains of *A. hydrophila* and *A. caviae* were almost equally distributed between clinical and environmental sources, but there were proportionately more environmental strains of *A. sobria*, although the difference was not significant.

In the initial tests in RILs, live cells and culture filtrates of the majority (82%) of the haemagglutinating strains caused fluid accumulation; these included 64% of the *A. hydrophila*, 100% of the *A. sobria* and 80% of the *A. caviae* strains. Most of these enterotoxigenic isolates showed MS–HA, MFS–HA or MFGS–HA. Although 30% of the non-haemagglutinating strains also showed a secretory response, the haemagglutinating strains caused significantly

Table II. Serum-sensitivity and enterotoxigenicity of *Aeromonas* isolates

Source and species of isolate	Number of strains tested	Serum-sensitive strains				Serum-resistant strains	
		Number of strains in which sensitivity was		Total number of strains	Mean (SD) volume of fluid in initial tests (ml/cm of RIL)	Number of strains	Mean (SD) volume of fluid in initial tests (ml/cm of RIL)
		prompt	delayed				
Clinical							
A. hydrophila	5	2(0)		2(0)		3(3)	0·71 (0·05)
A. sobria	10	3(1)	2(1)	5(2)	0·50 (0·01)	5(5)	0·72 (0·13)
A. caviae	14	4(2)		4(2)	0·45 (0·05)	10(6)	0·65 (0·11)
Environmental							
A. hydrophila	6	3(1)		3(1)	0·70 (0·01)	3(3)	1·43 (0·18)
A. sobria	11	3(1)		3(1)	0·50 (0·03)	8(5)	0·80 (0·20)
A. caviae	20	7(1)		7(1)	0·45 (0·02)	13(9)	0·66 (0·10)
Positive control*					1·20 (0·20)		
Negative control†					0·00 (0·00)		
Total	66	22(6)	2(1)	24(7)		42(31)	

Figures in parenthesis indicate number of enterotoxigenic strains in the initial tests before passage in RILs.
* BHIB culture of *V. cholerae* strain 569B.
† BHIB.

Table III. Serum-sensitivity of 18 representative strains of *Aeromonas* incubated in NHS and in NHS with MgEGTA

Species of isolate	Strain no.	Percentage survival of inoculum* after incubation with					
		NHS + PBS for			NHS + MgEGTA for		
		30 min	60 min	120 min	30 min	60 min	120 min
Serum sensitive (prompt)							
A. hydrophila	C-96011	38	< 1·0	< 0·01	103	162	< 1·0
	E-6	< 1	< 0·1	< 0·01	36	18	< 0·1
A. sobria	C-21	< 1	< 0·1	< 0·01	93	80	8·0
	E-PDG1	< 1	< 0·1	< 0·01	163	158	< 0·1
A. caviae	C-62	114	< 0·1	< 0·01	274	941	< 0·1
	C-12	29	4·0	< 1·0	34	3	< 0·1
	C-230	57	7·0	< 1·0	33	6	< 1·0
	E-31	< 1	< 0·1	< 0·01	227	43	< 0·1
	E-29	< 1	< 0·1	< 0·01	198	52	< 0·1
Serum sensitive (delayed)							
A. sobria	C-5	123	85	9·0	233	133	5·0
	C-10	200	98	5·0	152	87	6·0
Serum-resistant							
A. hydrophila	C-40	115	230	565	205	321	281
	E-HG1	100	169	18	100	428	564
	E-BD1	116	177	352	134	206	367
A. sobria	C-24	133	178	400	793	547	433
	E-3	213	240	141	120	201	568
A. caviae	C-421	170	229	374	72	147	390
	E-SG4T	253	540	1330	165	176	243

C, clinical; E, environmental.
* Mean of three determinations with cultures grown on different days and incubated with the same batch of NHS.

more fluid accumulation ($p < 0·05$, Student's t test) than those showing NHA (table I). The majority of the strains showing NHA or MGS–HA were non-toxigenic irrespective of species or source, but became toxigenic without change in HA properties after successive passage through RILs (table I).

The majority (64%) of the isolates (55% of *A. hydrophila*, 62% of *A. sobria* and 68% of *A. caviae*) were resistant to the bactericidal action of NHS. Most (92%) of the serum-sensitive strains were killed promptly, but two of the clinical strains (*A. sobria*) showed delayed sensitivity (table II). There was no significant correlation between serum sensitivity and species or source of the isolates. Most of the strains that showed prompt sensitivity in NHS (significant decrease in viable counts within 60 min) showed only delayed sensitivity (within 120 min) in NHS with MgEGTA. The two strains with delayed sensitivity in NHS also showed delayed sensitivity in NHS with MgEGTA (table III).

Accumulation of fluid in RILs was caused by the majority (74%) of the serum-resistant isolates when

tested initially, but by only a minority (29%) of serum-sensitive isolates (table II). These enterotoxigenic serum-resistant strains comprised 100% of the *A. hydrophila*, 77% of the *A. sobria* and 65% of the *A. caviae* isolates, and they caused significantly more accumulation of fluid ($p < 0.05$, Student's t test) than the serum-sensitive strains (table II).

Discussion

In the present study, less than half of the isolates caused HA. Burke *et al.*[9] reported that the majority of diarrhoeal isolates of *A. hydrophila* showed HA patterns other than MFS–HA, and that those of *A. caviae*, mostly from non-diarrhoeal or water sources, showed either NHA or MFS–HA. However, our study of three species of *Aeromonas* showed various HA patterns including MFS–HA, independent of the source; it suggests that HA patterns may not be correlated with either species or source, although a higher proportion of *A. hydrophila* strains may cause HA.

Haemagglutinating strains of the three species, showing HA patterns including that of MFS–HA, caused fluid accumulation in RILs. Most of the strains showing NHA or MGS–HA were non-toxigenic, apart from some environmental isolates of *A. caviae*, so that there was moderate correlation of HA with enterotoxigenicity. Burke *et al.*[9] made similar observations, the only exception being that strains showing MFS–HA in their study were non-toxigenic, whereas almost all such strains in our study produced enterotoxin. The present data are at variance with those of Crichton and Walker,[16] who found that all strains of *Aeromonas* spp., whether toxigenic or non-toxigenic, caused HA.

On successive passage through RILs, all of our non-toxigenic strains became enterotoxin producers, suggesting a repression–derepression phenomenon influencing the toxin gene. However, none of the non-haemagglutinating strains caused HA, even after RIL passage, although they became enterotoxigenic; this suggests that repression–derepression may not apply to the haemagglutinin gene. It seems that there is partial correlation between HA and enterotoxigenicity of *Aeromonas* spp. but they are probably not genetically linked.

In intestinal and extra-intestinal infections, *A. hydrophila* and *A. sobria* have been reported to be more virulent and serum-resistant than *A. caviae*;[1,2,17,18] but *A. caviae* strains have been increasingly implicated in intestinal[2,15] and extra-intestinal infections including septicaemia and bacteraemia.[19] Most *A. caviae* isolates (clinical and environmental) in our study were serum-resistant, suggesting their potential virulence in these diseases. Furthermore, most of our *Aeromonas* strains were serum-resistant, regardless of species; and this suggests similar pathogenic potential.

Most of our environmental isolates were resistant to NHS (table II); this may explain the recent reports that extra-intestinal infections by *Aeromonas* spp. are almost always water-related.[1,2,20] Our data also suggest that serum resistance is shown almost equally by clinical and environmental isolates, regardless of species. These observations may indicate versatility in the role of *Aeromonas* spp. in various diseases.

Of the *Aeromonas* strains studied, 36% were serum-sensitive. Gram-negative bacteria are killed by functional components of both the classical and alternate pathways, by damaging the bacterial cell membrane activity.[21] EGTA and MgEGTA have recently been used to distinguish these two complement pathways. Serum chelated with EGTA causes disintegration of the C_1 complex and blocks the consumption of C_2; this prevents activation of the classical pathway and permits activation of the alternate (properdin) pathway, but at an apparently suboptimal concentration of Mg^{2+}. This could be achieved by addition of $MgCl_2$ in equimolar quantity to a solution of EGTA to form MgEGTA, restoring the Mg^{2+} concentration while moderately reducing the efficiency of Ca^{2+} chelation. Our results corroborate the earlier observation that strains of *Aeromonas* spp. with prompt serum-sensitivity were killed by activation of both pathways.[17] They also indicate that a few strains with delayed sensitivity were killed by activation of the alternate pathway, as was observed with *Serratia marcescens*.[22] Thus, *Aeromonas* strains interact with complement in diverse ways, and this may reflect strain-specific rather than species-related pathogenic potential.

The observation that most serum-resistant strains were enterotoxigenic suggests a possible relationship between these two properties; and the higher enterotoxigenic potential of haemagglutinating and serum-resistant strains suggests that these two properties may enhance the virulence of these organisms. However, no correlation was observed between HA or serum sensitivity and species or source of the organism.

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