Detection, quantitation and stability of the β haemolysin of *Aeromonas* spp.

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Summary. Amongst 58 isolates of motile aeromonads evaluated for the ability to produce β haemolysin, haemolytic activity was significantly associated with strains belonging to the *Aeromonas hydrophila* and *A. sobria* groups. Of erythrocytes from nine animal species tested, mouse red blood cells provided the best indicator system for detection of β -haemolysin activity. Furthermore, differences in the stability of the β haemolysins of selected *A. sobria* and *A. hydrophila* isolates at different temperatures, and in the presence of urea or dithiothreitol were observed.

Introduction

Members of the genus *Aeromonas*, common inhabitants of freshwater environments, synthesise many extracellular enzymes, including protease, lipase, chitinase and a haemolysin (Janda, 1985). Several studies have suggested that at least two distinct haemolysins are produced by motile aeromonads—an α haemolysin, which is produced optimally at 22°C and causes incomplete lysis of erythrocytes, and a β haemolysin, which lyses various species of red blood cells and is harvested in highest yield from cultures grown at 37°C (Wretland *et al.*, 1971; Ljungh *et al.*, 1981; Thelestam and Ljungh, 1981; Ljungh, 1984).

Recent attention has focused on the β haemolysin of motile *Aeromonas* spp. because of its potential diagnostic and pathogenic significance. Several groups of workers have demonstrated a significant association between extracellular β -haemolysin production by *Aeromonas* isolates *in vitro* and elaboration of enterotoxigenic-like activity as detected in suckling mouse or rabbit ileal loop assays (Burke *et al.*, 1982; Turnbull *et al.*, 1984). Furthermore, purified, or partially purified, β haemolysin has been found to be lethal for various eukaryotic cell lines (Bernheimer and Avigad, 1974; Thelestam and Ljungh, 1981; Asao *et al.*, 1984).

Most studies of the biological properties of the β haemolysin have been performed with a limited number of stock strains or clinical isolates. Since haemolysins are important virulence factors for several gram-negative species, and detection of

haemolytic activity in *Aeromonas* isolates from stool specimens may have clinical implications, we have evaluated procedures available for detection of this haemolytic property *in vitro*.

Materials and methods

Bacterial isolates and β -haemolysin production

Fifty-eight *Aeromonas* isolates of clinical origin were investigated (table I). Each was identified to the genus and species according to the criteria of Popoff and Veron as modified in our laboratory (Janda *et al.*, 1984b). Haemolytic activity was evaluated on agar and in broth. For plate assays, isolates were radially streaked on to Trypticase Soy Agar (BBL Laboratories, Cockeysville, MD, USA) containing sheep red blood cells 5%, and were incubated for 72 h at 37°C. After incubation, plates were examined daily for haemolysis, and the maximum zone of haemolysis extending from the edge of growth for each isolate was recorded. Cell-free haemolysin was produced by methods described previously (Janda *et al.*, 1985). Briefly, *Aeromonas* isolates were grown in Brain Heart Infusion Broth (BHIB) in flasks at 35°C for 18 h with shaking. At the end of the incubation period, the optical density (OD) at 425 nm of each broth was determined, and haemolysin activity was separated from cell debris by low-speed centrifugation followed by filtration of the supernate through 0.45- μ m Acrodisc Filters (Gelman, Ann Arbor, MI, USA). These supernates were immediately assayed for haemolytic activity against a suspension of guinea-pig erythrocytes 1% v/v (GPE; Flow Laboratories, McLean, VA, USA) in microtitration wells with phosphate-buffered saline (PBS; pH 7.0) as diluent. Plates were incubated for 1 h at 37°C followed by overnight refrigeration before the final readings were recorded. The haemolytic titre (HAU/ml) was defined as the highest dilution of each BHIB supernate that caused 50% haemolysis of GPE. The

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haemolytic activity for each isolate was defined as the ratio of titre to growth (HAU/OD₄₂₅). The β haemolysin of selected *Aeromonas* isolates was evaluated for its activity against erythrocytes of different animal species and for inactivation by various physical and chemical agents.

Sensitivity of erythrocytes of various animal species to β haemolysin

Cell-free supernates of cultures of 19 *Aeromonas* isolates (*A. sobria*, 10, *A. hydrophila*, 9) were titrated for β -haemolytic activity against erythrocytes of nine different species of animal. Cell-free BHIB supernates were tested in microtitration plates in serial two-fold dilutions (in PBS) against 1% v/v concentrations of the following erythrocytes: rabbit, horse, ox and goat (Cleveland Scientific, Cleveland, OH, USA), guinea-pig and monkey (Flow Laboratories, McLean, VA, USA), and mouse and human O erythrocytes. The latter two erythrocyte species were obtained in our laboratory by cardiac puncture and venepuncture respectively. Plates were incubated as described above.

Inactivation studies of aeromonas β haemolysin

The supernates of cultures of four isolates of *Aeromonas* were subjected to various physical and chemical treat-

ments to study the effects of these agents on the activity of β haemolysin. One-ml samples of cell-free supernate of each isolate were incubated at different temperatures (4, 25, 37, 56 and 100°C) or exposed for 30 min to urea (4M), dithiothreitol (20 mM), trypan blue (25 and 50 μ g/ml), pronase (Type XIV from *Streptomyces griseus*), proteinase K (Type XI from *Tritirachium album*), α chymotrypsin (Type II), or trypsin (Type I) from bovine pancreas. All proteases were used at a final concentration of 100 μ g/ml unless otherwise specified. After incubation, supernates, including control samples (t=0 min), were titrated against GPE 1% as described previously, and the percentage residual activity was calculated.

Results

Haemolysin production and activity

By plate assay, 79% of all isolates screened were haemolytic whereas only 52% produced a detectable cell-free haemolysin in BHIB (≥ 4 HAU/ml; table I). Haemolytic activity was associated particularly with two of the three *Aeromonas* spp. All 24 *A. hydrophila* isolates and all 18 *A. sobria* isolates were haemolytic whereas 12 out of 16 *A. caviae* strains were not. Amongst *A. hydrophila* and *A. sobria* isolates, the β haemolysin was detected in 76% and

Table I. Haemolysin production by *Aeromonas* spp.

Species and source		Haemolytic activity in						
		Plate assay* at			Broth assay			
		24 h	48 h	72 h	Number positive at 24 h	Titre†		Activity§
						range	mean‡	
<i>A. hydrophila</i>								
wound	10	6	8	10	8	0-128	43.2	44.6
stool	6	6	6	6	6	8-128	56.0	59.1
blood	5	4	5	5	4	0-32	14.4	16.6
other	3	1	3	3	2	0-32	21.3	20.6
<i>A. sobria</i>								
wound	2	2	2	2	1	0-4	2.0	2.2
stool	6	5	6	6	3	0-16	5.3	5.5
blood	5	3	5	5	4	0-32	17.6	15.2
other	5	5	5	5	2	0-8	3.2	4.1
<i>A. caviae</i> (all sources)¶	16	0	2	4	0	...	...	...
TOTAL	58	32	42	46	30	0-128	18.0	18.6

* Number positive at indicated time.

† HAU₅₀/ml.

‡ Mean haemolytic titres for each source were calculated by dividing the sum of the average haemolytic titres for each strain by the total number of isolates tested in each group.

§ HAU₅₀/OD₄₂₅ (mean values).

¶ Sources of *A. caviae* isolates: stool (7), blood (5), wound (4).

95% of the isolates in agar-plate assays after 24 h and 48 h incubation respectively. No *A. caviae* isolate was found to elaborate a cell-free haemolysin in broth assays.

While not apparent in plate assays, *A. hydrophila* isolates produced more β haemolysin when grown in BHIB than did *A. sobria* isolates ($p < 0.005$), although both species grew equally well in this medium (data not shown). A similar phenomenon was observed when control strains were tested; *A. hydrophila* ATCC 7965 produced a titre of 64 HAU/ml against GPE whereas *A. sobria* strains ATCC 9071 and CIP 7433 produced titres of 16 and 4 HAU/ml respectively. Within the *A. hydrophila* group, the highest levels of haemolysin were produced by stool isolates; isolates from blood cultures expressed the lowest activity. The converse situation occurred amongst *A. sobria* strains.

Erythrocyte sensitivity to β haemolysin

The cell-free supernates of cultures of 19 *Aeromonas* isolates were tested against erythrocytes of nine different animal species and the results are given in table II. Mouse and monkey erythrocytes were most sensitive to the β haemolysin of *Aeromonas* and were lysed by all 19 isolates. However, titres with mouse erythrocytes were twice as high as those with monkey cells. Human O, goat, and sheep erythrocytes were insensitive to the activity of β haemolysin; only four of the 10 *A. sobria* isolates tested were haemolytic for one or more of

these erythrocyte species. Two *A. sobria* isolates that were non-haemolytic when tested against GPE produced low titres (4–8 HAU/ml) against both mouse and monkey erythrocytes. Several additional isolates, including *A. caviae* ATCC 15468, that were non-haemolytic in preliminary broth assays, failed to produce detectable haemolytic activity against any of the nine erythrocyte species tested.

One unexpected finding was the difference noted in the relative sensitivity of several erythrocyte species to the β haemolysin produced by *A. hydrophila* and *A. sobria* isolates (table II). For instance, rabbit and guinea-pig erythrocytes were equally efficient in detecting the β haemolysin of *A. hydrophila* but the latter were significantly more sensitive in their ability to detect the haemolysin of *A. sobria* isolates ($p < 0.05$). Even within each species (*hydrophila*, *sobria*), the relative sensitivity of each species of erythrocyte tested varied considerably between isolates; the β -haemolysin of one *A. sobria* isolate was most active against horse erythrocytes (64 HAU/ml), an unusual finding for the group as a whole.

Although sheep erythrocytes were relatively insensitive for the detection of haemolytic activity, the β haemolysin of *A. hydrophila* isolates was more active against this species than the haemolysin of *A. sobria*. However, when agar plate assays were performed and all 58 isolates were tested (table II), *A. sobria* strains produced substantially larger zones of haemolysis on plates containing sheep cells than did *A. hydrophila* strains (data not shown, $p < 0.001$). Furthermore, plate assays correlated

Table II. Sensitivity of various erythrocyte species to the β haemolysin of *Aeromonas*

Erythrocyte species	Sensitivity*	Haemolytic titres† of supernates of				
		<i>A. hydrophila</i>		<i>A. sobria</i>		Total
		Range	Mean‡	Range	Mean‡	
Mouse	100	16–256	74.7	4–64	22.4	47.4
Monkey	100	16–64	30.2	4–32	13.6	21.5
Guinea Pig	89	16–64	39.1	0–32	17.2	27.6
Rabbit	79	16–64	33.8	0–32	9.6	20.2
Horse	68	8–64	29.3	0–64	8.8	18.5
Ox	68	16–64	24.9	0–16	4.4	15.0
Human O	68	4–32	12.8	0–16	3.2	7.8
Goat	42	0–32	12.4	0–4	0.4	6.1
Sheep	37	0–16	5.3	0–4	0.4	2.7

* Number of isolates that gave positive results/number tested.

† HAU₅₀/ml.

‡ Mean haemolytic titres for *A. hydrophila* and *A. sobria* were determined by dividing the sum of the average haemolytic titres for each isolate tested against individual erythrocyte species by the total number of isolates tested per group (*A. hydrophila*, 9; *A. sobria*, 10).

poorly with expression of cell-free haemolysin as detected by GPE or any other erythrocytes.

Physical and chemical inactivation of β haemolysin

The β haemolysins of four isolates were stable for 30 min when incubated at temperatures between 4 and 37°C; haemolytic activity was lost after boiling (table III). Haemolytic activity in supernates containing cell-free haemolysin was resistant to challenge by various proteases and trypan blue. For example, the haemolysin of *A. hydrophila* AH-3 was incubated with concentrations of pronase up to 2000 µg/ml for 4 h without any significant reduction in haemolytic activity. In other tests, a more variable response was observed which appeared to be strain-dependent. The haemolysins of isolates AH-3 and AH-6 (both *A. hydrophila*) were sensitive to heat inactivation at 56°C and were completely or partially denatured by exposure to urea, but not dithiothreitol (DTT); the haemolysin of *A. sobria* ATCC 9071 was partially inactivated by incubation at 56°C, but was resistant to urea, as was isolate AS-8. The haemolysin of the latter isolate was not affected by incubation at 56°C or exposure to urea, but did show a 75% reduction in haemolytic activity in the presence of DTT. The stability on storage of individual haemolysins differed also. The haemolysin of strain AH-3 was completely stable when stored at -20°C for 5 days but strain AS-8 lost all activity in similar conditions.

Discussion

The results of this study show that the majority of isolates currently placed in the *A. hydrophila* and *A. sobria* groups produce a β haemolysin but the anaerogenic aeromonads, designated *A. caviae*, fail to elaborate this factor. This activity, along with previous differences noted in exoenzyme production, serum sensitivity, and decreased virulence potential, clearly separates *A. caviae* from *A. hydrophila* and *A. sobria* (Janda *et al.*, 1984a; Janda, 1985; Janda *et al.*, 1985). Because haemolytic activity has previously been shown by several groups to be a marker for enterotoxigenic strains, the results of this study suggest that *A. hydrophila* and *A. sobria* are primarily enterotoxigenic but *A. caviae* lacks this activity. This finding supports the hypothesis that a species-associated disease spectrum may exist within the motile *Aeromonas* group in relation to human infections.

Quantitatively, *A. hydrophila* isolates were more active in β haemolysin production than were *A. sobria* isolates, but minor variations in methods

Table III. Effect of various treatments on the activity of the β haemolysin of *Aeromonas* spp.

Treatment	Residual activity (%) of strains			
	AH-3	AH-6	9071	AS-8
4°C	100	100	100	100
25°C	100	100	50	100
37°C	100	100	100	100
56°C	<1	<1	38	100
100°C	<1	<1	<3	<6
Urea, 4M	<3	38	100	100
DTT, 20 mM	100	100	100	25
Trypsin	100	100	100	100
Chymotrypsin	100	100	100	100
Pronase	100	100	100	100
Proteinase K	100	100	100	100
Trypan blue:				
25 µg	100	100	100	100
50 µg	100	100	50	100

employed to detect this activity might have influenced the results, although we did not observe this. This quantitative difference was also noted in the control and type strains tested, and in a recent report attributing cholera-like diarrhoea to *A. sobria* (Champsaur *et al.*, 1982) the investigators found that that cell-free supernate of their strain had a haemolytic titre of 8 against ox and rabbit erythrocytes, but was unreactive when tested with horse and sheep erythrocytes.

Whether or not the quantitative differences in haemolysin production between these two species are clinically or biologically relevant, it does appear that this enzyme alone is not the major determinant of virulence. First, unlike the observations of others of a higher frequency of haemolytic *Escherichia coli* found in extra-intestinal disease (Minshe *et al.*, 1978; Welch *et al.*, 1981), we did not observe a similar distribution amongst *A. hydrophila* strains analyzed by source of isolation (table I), even though these isolates produced greater amounts of the haemolysin than did *A. sobria*. Second, in mouse lethality assays, both isolates belonging in phenotype to the *A. hydrophila* and *A. sobria* groups were virulent, although virulence did not strictly correlate with the quantity of β haemolysin elaborated (Janda *et al.*, 1985). As previously noted (Janda *et al.*, 1985), we have identified some haemolysin- and cytotoxin-negative strains that, when injected intraperitoneally, are able to kill mice. Finally, in co-infection experiments with two avirulent strains, one cytotoxin- and haemolysin-positive, the other cytotoxin- and haemolysin-negative, we have seen

no enhancement or agumentation of virulence (unpublished observation).

The most unusual finding of our study concerns the possibility that distinct haemolysins may be produced by individual members of the *A. hydrophila* and *A. sobria* group. Although we used unpurified haemolysin preparations, the relative sensitivity of various erythrocyte species and the insensitivity of the haemolysins to inactivation by different agents, particularly proteases, agree with the previous findings of Bernheimer *et al.* (1974). While the inactivation of β haemolysin from some isolates paralleled the findings of Ljungh *et al.* (1981), we did notice distinct differences from published reports in two isolates tested. These differences, primarily in sensitivity to urea and DTT, and inactivation at 56°C, along with the previous discrepancies noted between individual isolates (their relative activity against different erythrocytic species, stability at low temperatures, and production of haemolysin in agar-plate and broth assays) supports the possibility that different haemolysins may be produced within each group.

Lallier *et al.* (1984) have come to similar conclusions, based upon the production of β haemolysin by *A. hydrophila* (but not by *A. sobria*) at 10°C and the failure of antibody raised against the haemolysin of one *A. hydrophila* isolate to neutralise the haemolysin of another strain (Olivier *et al.*, 1981; Lallier *et al.*, 1984). Because only a few strains were evaluated in this final assay and inactivation experiments were performed on cell-free supernates containing various extracellular factors, the similarity (or differences) between β haemolysins of different *Aeromonas* strains must await the results of analytical studies with purified haemolysin preparations. Finally, recent DNA-DNA homology studies indicate that at least 10 distinct hybridization groups (species) exist within the currently-recognised species (*hydrophila*, *sobria*, *caviae*), and differences noted by various investigators in exoenzymic properties of *Aeromonas* and its pathogenicity may be related in part to the genetic complexity of this group (Popoff *et al.*, 1981; Fanning *et al.*, 1985). Further investigations are required to confirm these results.

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