Relationship of toxin production to species in the genus *Aeromonas*

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Summary. Ninety-five strains of *Aeromonas* were divided into three species—*A. sobria*, *A. hydrophila* and *A. caviae*—on the basis of results in 13 biochemical tests. The minimum number of tests necessary to distinguish these species was determined. Culture filtrates of the strains were tested for cytotoxin and cytotoxinin, haemolysin and protease. One filtrate with high-titre cytotoxin, haemolysin and protease activities was subjected to gel filtration on Sephadex G75 and isoelectric focussing. Of the five cell lines tested, Vero cells were most sensitive to the cytotoxin; no reproducible cytotoxic effects were observed. The haemolysin effect appeared to be equivalent to cytotoxin. At least two distinct protease activities were found that might be responsible for the cytotoxic effects described. Cytotoxin production was species related; it was present in *A. sobria* and *A. hydrophila* but not in *A. caviae*.

Introduction

Reports from many parts of the world suggest that *Aeromonas* species cause an acute self-limiting diarrhoeal illness in man (Rosner, 1964; von Graevenitz and Mensch, 1968; Chatterjee and Neogy, 1972; Cumberbatch *et al.*, 1979; Gracey *et al.*, 1982; Pitarangsi *et al.*, 1982; Janda *et al.*, 1983; Holmberg and Farmer 1984). This suggestion is supported by finding various exotoxins (Ljungh *et al.*, 1981), in particular enterotoxin that can be detected by animal assays (Burke *et al.*, 1981b; Turnbull *et al.*, 1984), in culture filtrates. However, some surveys have not shown a relationship between diarrhoeal disease and isolation of *Aeromonas* (Catsaras and Buttiaux, 1965; Bhat *et al.*, 1974; Millership *et al.*, 1983) and the organism can be isolated from persons who do not have gastro-intestinal disease. In one study, 3.2% of people without signs of gastro-intestinal infection had *Aeromonas* in their faeces (von Graevenitz and Zinterhofer, 1970). These discrepant results could be explained if strains varied in their pathogenicity and it may, therefore, be important to identify pathogenic strains as quickly and easily as possible.

In a clinical laboratory the simplest way to do this is by biochemical tests. Several studies have already shown a correlation between results of biochemical

tests and production of cytotoxin or enterotoxin *in vitro* (Burke *et al.*, 1982; Janda *et al.*, 1983; Turnbull *et al.*, 1984; Agger *et al.*, 1985) although no one biochemical test is wholly predictive. Thus it would be useful to attempt to divide *Aeromonas* into species or biotypes and to relate source of isolate and in-vitro toxin production to taxonomic grouping.

The aims of this study were threefold: (1) to subdivide a large collection of wild strains of *Aeromonas* from man and from the environment into biotypes or species; (2) to establish the minimum number of tests required in a diagnostic laboratory to assign an unknown isolate reliably to a taxonomic group; and (3) to examine the effect of the extracellular products of these strains on mammalian cell lines *in vitro*.

Materials and methods

Bacterial strains

Isolates were identified as *Aeromonas* by methods previously described (Millership *et al.*, 1983) with API 20E trays and vibriostatic agent O129. One hundred and eighteen strains were examined. When epidemiologically related strains differed by fewer than two of the phenotypic characters described below, the latest isolates were excluded leaving 95 in the study; 57 were from human faeces, three from other human samples and 29 from water, of which 24 were from chlorinated mains supplies. All isolates except one came from north-east London or

the City of London. The three type strains of Popoff and Véron (1976)—ATCC 15468, CIP 7433 and NCTC 8049—and three strains kindly supplied by Maidstone Public Health Laboratory were also included.

Taxonomic study

Thirteen biochemical characters were examined on the basis of the scheme of Popoff and Véron (1976). When tests were also present on API 20E trays, the results obtained with both methods were compared.

β haemolysis was observed on two media: ready poured horse-blood agar (HBA) supplied by Oxoid or by Tissue Culture Services. Plates were incubated for 18 h at 37°C in air and cultures were scored as positive if a clear zone of *β* haemolysis, however narrow, extended beyond the diameter of single colonies.

Lactose fermentation was determined on MacConkey Agar (Oxoid) after incubation for 18 h at 37°C.

Fermentation of L-arabinose and salicin, and gas formation from glucose were determined in peptone water sugars (Southern Group) after incubation for up to 30 days at 30°C.

Aesculin breakdown was detected on plates or slopes of bile-aesculin agar (Gibco) after growth for up to 48 h at 30°C.

Growth at 42°C was tested on HBA and the test was considered to be positive only if there was no reduction in size of single colonies by visual comparison with those grown at 37°C.

Acetoin production, metabolism of gluconate and lysine decarboxylase were tested by standard methods (Cowan, 1974).

Elastin breakdown (Scharmann, 1972), H₂S production (Veron and Gasser, 1963) and utilisation of L-arginine (Veron, 1975) were tested as in the original papers with the following modifications. For H₂S production Lab-lemco powder (Oxoid) was substituted for *maceration de viande*. The inorganic medium for L-arginine testing was not sterilised in parts and then mixed; instead the complete medium was boiled and cooled on 3 successive days before pouring. A single colony of each test organism was diluted in 5 ml of normal saline and a swab dipped in this was streaked across the agar. A filter-paper strip soaked in L-arginine solution was then added at right angles.

At least 10% of isolates were re-examined by each test as a check on reproducibility.

The results were analysed numerically by calculation of a simple matching coefficient and clustering by the method of variance (Delabre *et al.*, 1973). Species groups were defined by the "acuteness coefficient" of Véron (1974). The proportion of isolates in each species that gave positive and negative results for each test was then determined.

Assuming that every test is independent of every other, the probability of an unknown isolate from a given species having any one phenotype is the probability (*p*) of each test result multiplied together ($p_1 \times p_2 \times p_3 \times p_4$ if four tests are used). Thus the proportion of strains able to

be fully identified and the probability of misidentifying an isolate can be calculated when using fewer than 13 tests.

Toxin tests

Six colonies of each *Aeromonas* strain to be tested were subcultured into 10 ml of Nutrient Broth (Oxoid No. 2). This was incubated for 4–6 h at 37°C then added to flasks of Tryptone Soy Broth (Oxoid) with yeast extract (Oxoid) 0.6%. After incubation at 37°C for 16–18 h with shaking at 100–200 rpm, the broths were centrifuged and filtered through a 0.45-μm membrane filter (Millipore). In later experiments the filtration step was omitted because it made no difference to the results.

Haemolysin activity was assayed with 1% rabbit red cells (Burke *et al.*, 1981a) taking 100% lysis as the endpoint.

Caseinolytic activity was determined by placing 50-μl samples of culture filtrate in flat-bottomed microtitration trays; 100 μl of sterile skimmed milk diluted 1 in 7 v/v in phosphate buffered saline (PBS), pH 7.3, were then added and the trays incubated at 37°C. Absorbance at 630 nm was read in a Dynatech MR600 ELISA reader after 0, 4 and 18 h. A reduction in absorbance was taken as a semiquantitative estimate of caseinolysis.

Gelatin hydrolysis was estimated with agar (Oxoid No. 1) containing gelatin 1% in Tris HCl buffer, pH 7.3. Plates were poured 2 mm deep and 4-mm wells were cut. After addition of culture filtrate to each well and incubation for 18 h at 37°C, zones of hydrolysis were delineated with 80% ammonium sulphate. The experiment was repeated with stab inocula of viable cultures of each strain tested.

Cytotoxic and cytotoxic effects were examined in five cell lines—Vero, Chimp liver, Int 407, adrenal Y1 and chinese hamster ovary (CHO) cells. Monolayers were prepared in microtitration plates (Guigliano *et al.*, 1982) on day 1. On day 2 serial dilutions of culture filtrate (before and after heating at 56°C for 20 min) were prepared in the appropriate maintenance medium and added to the cell monolayers. In early experiments, changes in the cell lines were noted after incubation at 37°C for 4, 18, 48 and 72 h. In later experiments, observations were continued for 18 h only. Several toxin-containing strains were also tested on CHO cells after lysis of a fresh overnight culture by polymyxin at a concentration of 50 μg/ml before preparation of the filtrate.

The filtrate from a strain identified as *A. sobria* isolated from a patient with diarrhoea and producing toxin in high titre was extracted in 70% ammonium sulphate and separated on a Sephadex G75 column. The filtrate was also subjected to isoelectric focussing. Fractions from both procedures were assayed for cytotoxic, haemolytic and cytotoxic effects as before. Protease activity was determined on the chromatography column eluate by measuring casein hydrolysis and on the isoelectric focussing fractions by the gelatin agar method.

Results

Fig. 1 shows the dendrogram produced from the

Fig. 1. Dendrogram constructed from the taxonomic study of 95 strains of *Aeromonas* spp.

taxonomic study. The genus *Aeromonas* divides into the three species previously named—*A. hydrophila*, *A. caviae* (or *A. hydrophila* ssp. *anaerogenes*) and *A. sobria*. There are also a few strains in a fourth intermediate group. Table I shows the number of strains in each species that gave positive results in each test. If only four tests (β haemolysis, aesculin breakdown, gas from glucose and growth at 42°C) are considered, the probability of assigning an unknown isolate correctly to any species is approximately 75% (table II). All four tests may be read after 18 h; only three otherwise identifiable strains took longer than this either to split aesculin or to produce gas from glucose. The majority of the remaining strains did not fall into any ideal phenotype and further tests will be required. At worst there is only a 1% likelihood of assigning an isolate

to a completely “wrong” species with these four tests. If a further three tests—acetoin production, arabinose and salicin fermentation, which may take up to 5 days to become positive—are used, one “wrong” result would still allow identification. At least 80% of isolates could be identified with a risk of misidentification of no more than 1 in 2500.

For the two tests available on API 20E, the results were not comparable with those of the conventional tests. The results for acetoin production differed in 21% of cases; in all but one the API 20E result was negative when the standard method was positive. Fermentation of arabinose was similar; results differed in 26% of tests and in 21% API 20E results were negative.

Some tests were unsatisfactory. The elastin breakdown test was reliable but it was difficult to

Table I. Phenotypes of the species of *Aeromonas*

Character	Results *(percentage of test strains) obtained in tests with											
	<i>A. sobria</i> (20)			<i>A. hydrophila</i> (18)			<i>A. caviae</i> (46)			group 4 (11)		
	+	-	ND	+	-	ND	+	-	ND	+	-	ND
Aesculin breakdown	10	90	0	94	6	0	98	2	0	36	64	0
Arabinose fermentation	10	90	0	94	6	0	98	2	0	27	73	0
Salicin fermentation	10	90	0	94	6	0	98	2	0	45	55	0
Elastin breakdown	0	95	5	61	39	0	0	100	0	0	100	0
Acetoin production	85	15	0	83	17	0	0	100	0	9	91	0
Gas from glucose	100	0	0	94	6	0	9	91	0	73	27	0
H ₂ S production	85	15	0	94	6	0	2	98	0	73	27	0
L-arginine utilisation	40	60	0	83	17	0	89	9	2	27	73	0
Growth at 42 °C	100	0	0	28	72	0	17	83	0	9	91	0
Lysine decarboxylase	60	25	15	44	33	22	7	85	9	45	27	27
Gluconate metabolism	75	25	0	0	100	0	0	98	2	0	100	0
β haemolysis	0	100	0	94	6	0	0	100	0	9	91	0
Lactose fermentation	5	95	0	0	94	6	41	57	2	55	45	0

* + = positive result; - = negative result; ND = not determined.

Table II. Probability of assigning unknown strains to a species

	Ideal phenotype of		
	<i>A. sobria</i>	<i>A. hydrophila</i>	<i>A. caviae</i>
Aesculin breakdown	-	+	+
Gas from glucose	+	+	-
Growth at 42 °C	+	-	-
β haemolysis	+	+	-
Chance of identifying correctly	80%	60%	74%
Chance of identifying wrongly	< 0.1%	< 1.5%	< 0.08%
<i>If three more tests are used</i>			
Arabinose fermentation	-	+	+
Salicin fermentation	-	+	+
Acetoin production	+	+	-
Chance of identifying correctly	90%	84%	95%
Chance of identifying wrongly	< 0.0009%	< 0.04%	< 0.0008%

keep the elastin particles in suspension while preparing the medium and positive results took at least 3 days to appear.

H₂S production was poorly reproducible and difficult to interpret. When fresh, the medium was

very sensitive and after 7 days all strains produced some blackening. The most reliable results were obtained after 3 days. When the medium had aged for 3 months sensitivity was reduced and at least 7 days were needed for blackening to occur.

Growth on L-arginine medium gave various effects from no visible growth to inhibition by high concentration or by very poor growth after 7 days. Results also depended on initial inoculum size; thus interpretation was difficult. Only 70% of tests gave unequivocally identical results on repeat testing.

All other tests were completely reproducible on repeat testing except β haemolysis; 90% of 108 isolates gave the same results on the two kinds of HBA.

Toxin production

Ten strains were tested in Vero, Int 407, Chimp liver and Y1 cell lines. Cytotoxic effects on more than 90% of the cells in a well at titres ≥ 20 were produced by four strains in all cell lines. These effects were reduced or abolished by heating at 56 °C. Vero cell cultures were the most sensitive and were used for test with the remaining 79 strains. Some titres of cytotoxin on these cells were $> 10\,000$. The toxic effect was often visible at low dilution within 4 h. At high dilutions some cytotoxic filtrates produced marked vacuolation rather than cell degeneration.

Thirty strains produced cytotoxin; 15 *A. sobria*, 13 *A. hydrophila* and 2 from group four.

Thirty culture filtrates, including 12 containing cytotoxin, were heated at 56°C and tested on Y1 cells. All produced rounding similar to that of the cholera toxin control with titres up to 96. However, in successive experiments rounding was found to be unreliable because the effect was occasionally seen in control preparations exposed to *Escherichia coli* K12 filtrate. Cytotoxic filtrates often still showed toxic effects at low titres and rounding effects at higher dilutions. Cytotoxic filtrates tended to have the highest "cytotoxin" titres.

Twenty of these strains were also tested in CHO cells. No consistent cytotoxic effects that could be clearly discriminated from cytotoxin were observed even after heating the filtrates. Use of polymyxin to lyse cultures did not influence these results.

The same strains were also tested for gelatinase activity; 19 produced a zone of clearing around stab inocula. When culture filtrates were tested, all eight cytotoxin-producing strains gave positive results but only four of the remainder gave positive results. Six filtrates had heat labile activity of which two were cytotoxin positive.

Of 88 strains tested for rabbit-cell haemolysin, 28 were positive. All of these also produced cytotoxin. One strain with a low cytotoxin titre of 96 had no detectable haemolysin.

Gel filtration of one culture filtrate (fig. 2) showed cytotoxin peaks in fractions corresponding to mol.

wt of *c.* 55 000 and *c.* 41 000. Haemolysin activity co-purified with the cytotoxin. On isoelectric focussing, cytotoxin and haemolysin activities again coincided in a broad band at *p*15.01–6.2. There were also at least two protease peaks detected by both gelatin hydrolysis and casein breakdown. One peak coincided with cytotoxin activity at mol wt 41 000 and at *p*15.01–6.2. There was minor loss of activity of the latter after heating at 56°C. A further peak showing relatively greater gelatinase than caseinase activity had a mol. wt of 9000 on gel filtration and a *pI* of 8.9 on isoelectric focussing.

Discussion

In common with others (Popoff and Veron, 1976; Janda *et al.*, 1984), we have found that *Aeromonas* can be divided into at least three species—*A. hydrophila*, *A. sobria* and *A. caviae* (or *A. hydrophila* ssp. *anaerogenes*). We have identified a possible fourth group, although there were too few strains for a detailed analysis. DNA homology studies clearly distinguish the three accepted species but suggest that each represents at least two species or subspecies not so far distinguishable phenotypically (Popoff *et al.*, 1981). Our fourth group may be one of the unnamed species or subspecies.

It is also clear that four simple and rapid tests (β haemolysis, growth at 42°C, production of gas from

Fig. 2. Cytotoxin and haemolysin activities in the fractions from a Sephadex G75 gel filtration of an *Aeromonas* culture filtrate. One cytotoxic unit = the amount of toxin required to destroy 50% of a Vero monolayer in a microtitration plate. Vo = fraction number 34.

glucose, and breakdown of aesculin) would correctly identify 73% of all isolates. This might be useful in screening large numbers of isolates or for preliminary identification in diagnostic laboratories. *A. sobria* is more likely than the other two species to be pathogenic (Daily *et al.*, 1981; Watson *et al.*, 1985); thus would make rapid identification more important. With seven readily available tests (acetoin production and fermentation of salicin and arabinose as well as the above), 80% of isolates could be identified with minimal risk of misidentification.

Many authors have noted cytotoxin production (Wadstrom *et al.*, 1976; Cumberbatch *et al.*, 1979; Burke *et al.*, 1981a; Johnson and Lior, 1981; Jiwa, 1983; Asao *et al.*, 1984). The tendency for this to be confined to *A. sobria* and *A. hydrophila* has been previously observed (Turnbull *et al.*, 1984). From our gel filtration results there is a clear peak of cytotoxin activity at mol. wt 41 000 which would correspond to the β haemolysin of Ljungh *et al.* (1981). The initial peaks are probably protein aggregates. On isoelectric focussing the broad peak at pI 5.01–6.2 is similar to that described by Ljungh *et al.* (1981) and is probably the β haemolysin. Detection of the α haemolysin requires different incubation conditions (Ljungh *et al.*, 1981) and we have not attempted to demonstrate it.

Haemolysin activity closely follows cytotoxic activity on both gel filtration and immunoelectro-

phoresis (IEP), but is in general of lower titre. It would appear that β haemolysin and cytotoxin are identical. Occasional reports of haemolysin in the absence of cytotoxin might be explained if activation is required as it is for the haemolysin of *A. salmonicida*. Unwashed red blood cells can supply an activating factor (Titball *et al.*, 1985).

In the absence of a reliable assay for cytotoxic activity gel filtration of the IEP band in the region of pI 5.3 to separate enterotoxin was not attempted. There were however two protease peaks on both gel filtration and isoelectric focussing coinciding with β haemolysin. Possibly this is part of a complex molecule with haemolytic, cytotoxic and protease activity. The minor loss of activity after heating isoelectric-focussing fractions might be explained if the temperature was at the toxin's critical point.

The protease peak with a mol. wt c. 9000 is close to the enterotoxin of mol. wt 15 000 described by Ljungh *et al.* (1981) and proteases may have effects in Y1 cells. However, the pI would appear to be 8.9 whereas that of the enterotoxin is 5.2, unless this represents a third molecule.

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