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Haemagglutinating activity of *Aeromonas* spp. from different sources; attempted use as a typing system

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SUMMARY

The haemagglutinating ability of 141 isolates of *Aeromonas* spp. for human, horse and guinea-pig erythrocytes was examined. Although the majority of isolates (136/141) agglutinated human group O erythrocytes, all the eight possible patterns of agglutination were observed. Haemagglutination of human group O erythrocytes, but not horse or guinea-pig, was associated with the ability to agglutinate yeast cells (*Saccharomyces*) and with aggregation in a low concentration of ammonium sulphate. Haemagglutinating ability was further characterized by reactions in the presence of mannose, galactose or fucose. All the possible patterns of inhibition with individual sugars were observed, but haemagglutination of human group O erythrocytes not inhibited by mannose, galactose or fucose was more common among isolates from patients with diarrhoea, and isolates producing a Vero cell cytotoxin than would be expected by chance. This might represent a virulence factor.

When used as a typing system haemagglutination patterns did not correspond with the clustering of isolates expected on epidemiological grounds. Repeated subculturing resulted in a loss of agglutinating ability, an increase in the number of sugars inhibiting haemagglutination and an increase in the salt concentration required for aggregation. Haemagglutination did not seem to be useful as a means of typing aeromonads.

INTRODUCTION

There is increasing evidence that *Aeromonas* species cause diarrhoea, although not all strains may be pathogenic (Gracey, Burke & Robinson, 1982; Daily *et al.* 1981). Aeromonads are common in freshwater, including treated drinking water of an otherwise acceptable bacteriological standard (Burke *et al.* 1984*b*; LeChevallier *et al.* 1982). It is therefore important to identify virulence factors if pathogenic strains are to be recognized in the laboratory. Although various toxins have been described which might be responsible for diarrhoea (Potomski *et al.* 1987*a*, *b*) little work has been published on the role of other putative virulence factors.

A study by Burke and co-workers (1984*a*) in Australia showed that *Aeromonas* spp. isolates from children with diarrhoea exhibited fucose, galactose and mannose resistant agglutination of human group O erythrocytes significantly more often

than those from controls or from water supplies. This is a relatively simple test which might be of value in clinical laboratories to assist the identification of virulent strains. We therefore decided to examine strains isolated from a variety of human and environmental sources in the UK for haemagglutination patterns related to diarrhoeal disease. We also tested our strains by the salt aggregation test (SAT) (Wadstrom, Faris & Lindahl, 1984). In other Gram-negative species 'salting out' at low concentrations of ammonium sulphate is related to high hydrophobicity, as measured by hydrophobic interaction chromatography, and to the presence and number of fimbriae (Faris, Wadstrom & Freer, 1981; Faris *et al.* 1983).

Haemagglutination patterns have also been suggested as a means of typing aeromonads (Adams, Atkinson & Woods, 1983). Serological typing is difficult to apply; the heterogeneity of *Aeromonas* species means that many sera are required (Sakazaki & Shimada, 1984) and a high proportion of strains may be untypable (Leblanc *et al.* 1981). Typing by haemagglutination pattern might be particularly useful for preliminary screening in clinical laboratories. We therefore examined haemagglutination patterns of isolates from the same and different sources in an attempt to determine the likely usefulness of this as a means of typing.

MATERIALS AND METHODS

Bacteria

One hundred and fifty-two isolates of *Aeromonas* spp., including 31 *A. hydrophila*, 91 *A. caviae*, 19 *A. sobria* and 11 not belonging to any of these species were examined. Sixty-seven isolates were from human faeces, 66 from water samples and 19 from other environmental samples.

Isolates were characterized as described elsewhere (Barer, Millership & Tabaqchali, 1986). In brief, they were identified to genus level using API 20E trays (Appareils et Procédés d'Identification, La Balme les Grottes – 38390, Montalieu, France), the oxidase test and sensitivity to a 150 μg disk of vibriostatic agent O/129. Isolates were then tested for growth at 42 °C, β-haemolysis and in conventional media for fermentation of arabinose and salicin, gas production from glucose, acetoin production, breakdown of aesculin and elastase, oxidation of gluconate, and decarboxylation of lysine to assign them to species. They were also tested for the production of cytotoxin active in Vero cell monolayers.

Faecal isolates were also classified, according to the symptoms of the patient from whom they were obtained, into those from patients with diarrhoea, those from patients with diarrhoea who had other bowel disease or pathogens and those from persons without gastroenteritis, as previously described (Millership, Barer & Tabaqchali, 1986).

Culture conditions

Isolates were kept on nutrient agar stabs or slopes for up to 3 months (18 months for reproducibility studies) or in glycerol broth at −70 °C. For testing agglutinating or hydrophobic properties isolates were grown on horse blood agar plates incubated overnight at 37 °C.

Cell suspensions

Human group O erythrocytes were used within 24 h of collection. Defibrinated horse blood (Tissue Culture Services) and guinea-pig blood in Alsever's solution (Gibco) were stored at 4 °C. All erythrocytes were washed three times in phosphate buffered saline (PBS), pH 7·4, and a 3% v/v suspension made. Dried baker's yeast reconstituted and washed three times in PBS (pH 6.8) was used as a source of *Saccharomyces* in a 3% v/v suspension.

Yeast agglutination and haemagglutination tests

Yeast and haemagglutination (HA) tests were performed as described by Atkinson & Trust (1980). In brief, a fresh suspension of bacteria was made in PBS, pH 7·4, such that a 1 in 30 dilution corresponded to a McFarland standard tube 6, a viable count of approximately 5×10^{10}. Equal volumes (20 μl) of bacterial and yeast or red cell suspensions were mixed on a glass microscope slide, rotated for 5 min and examined macroscopically and microscopically for clumping. Appropriate controls were included.

Haemagglutination inhibition tests

Isolates which agglutinated erythrocytes were tested for haemagglutination inhibition (HAI) by L-fucose, D-galactose or D-mannose (Sigma). Bacterial suspension were made in a 1% w/v solution of each sugar in PBS, pH 7·4. The method was otherwise as described for HA tests. Inhibition was defined as a 50% or greater reduction in clumping compared to that of the corresponding HA test.

Salt aggregation tests

Bacterial suspensions were made in PBS, pH 7·4, and 20 μl added to 20 μl of 2, 1 or 0·5 M ammonium sulphate on a glass slide. After 5 min rotation the mixture was observed for clumping.

Use as a typing system

Isolates were typed using a combination of HA and HAI patterns. Isolates were first grouped into one of eight erythrocyte agglutination patterns 1–8 (Table 1). All those agglutinating human red cells were then further subdivided on the basis of haemagglutination inhibition patterns a–h (Table 2). Isolates within each species with identical HA and HAI patterns were considered to be indistinguishable.

The reproducibility of the technique was examined by retesting at least 10% of isolates from the same plate culture. Isolates were retested after 6 months and 18 months on nutrient agar stabs with repeated subculturing.

Isolates were also clustered according to their source. Those from members of the same family, from one water sampling point on different days or from different sampling points served by a common water source were considered likely to be the same strain.

Table 1. *Relationship of haemagglutination patterns to species of the genus* Aeromonas

Pattern no.	Agglutination patterns: Human	Horse	Guinea pig	No. of isolates in each species: s	h	c	o	No. of strains toxin: +ve	−ve	No. of strains from†: A	B	C	D	E
1	+	+	+	9	9	31	5	12	42	11	6	7	24	6
2	+	−	+	2	2	6	3	6	7	3	5	0	5	0
3	+	+	−	4	13	21	2	10	30	1	8	4	17	10
4	−	+	+	1	1	4	0	0	6	3	0	0	2	1
5	−	−	+	1	0	1	0	0	2	0	0	1	1	0
6	−	+	−	1	1	5	1	0	8	0	1	2	5	0
7	+	−	−	0	4	12	0	2	14	1	4	5	6	0
8	−	−	−	0	1	1	0	0	2	0	1	0	1	0
	Autoagglutinating			1	0	10	0	1	10	3	1	0	5	2

* s, sobria; h, hydrophila; c, caviae; o, other strains.

† A, Patients with diarrhoea; B, patients with other gastrointestinal disease; C, asymptomatic patients; D, water; E, other.

Statistics

All statistical analyses were by the χ^2 test.

RESULTS

Salt aggregation tests

Eleven isolates were autoagglutinable. One hundred and twenty-three isolates aggregated at an ammonium salt concentration of 0·5 M. Eight isolates did not aggregate even in 2 M salt.

Yeast agglutination

The majority of isolates, 135/141 (96%), agglutinated yeast cells. The remaining six isolates were among those that did not aggregate even in 2 M salt (Fig. 1).

Haemagglutination tests

The majority of isolates, 136/141 (96%) agglutinated human group O erythrocytes. Those that did not also failed to aggregate in low salt concentrations. Agglutination results for yeast and human red cells were identical except for one isolate which agglutinated yeast but not human red cells. Agglutination of horse and guinea-pig red cells was more variable and there was no relation between salt aggregation and agglutinating ability (Fig. 1).

Eight patterns of haemagglutination were observed (Table 1). There was no statistically significant association between haemagglutination pattern and bacterial species, toxin production or source of the isolate ($P > 0{\cdot}05$ in each case).

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Table 2. *Relationship of haemagglutination inhibition pattern to species of* Aeromonas, *Vero cell cytotoxin production and source*

Pattern designation	HAI pattern*			No of isolates in each species†				No. of isolates toxin		No. of isolates from‡				
	Mann	Fuc	Gal	s	h	c	o	+ve	−ve	A	B	C	D	E
a	−	−	−	13	12	20	3	18	30	14	13	0	12	9
b	+	−	−	3	3	10	1	3	14	0	1	3	9	4
c	−	+	−	2	3	9	2	2	14	1	5	4	4	2
d	−	−	+	0	2	17	0	0	19	2	4	3	9	1
e	−	+	+	0	4	8	1	4	9	1	1	2	9	0
f	+	+	−	0	3	5	1	2	7	0	1	4	4	0
g	+	−	+	0	3	4	2	0	9	0	0	3	5	1
h	+	+	+	0	1	8	1	1	9	1	0	0	9	0
	Autoagglutinating			1	0	10	0	1	10	3	1	0	5	2

* '−', no inhibition; Mann, D-mannose; Fuc, L-fuctose; Gal, D-galactose.

† s, sobria; h, hydrophila; c, caviae; o, other strains.

‡ A, patients with diarrhoea; B, patients with other gastrointestinal disease; C, asymptomatic patients; D, water; E, other sources.

Haemagglutination inhibition patterns

The inhibition of haemagglutination of 23 isolates reacting with more than one red cell species was tested. In all but 3 (87%) of the 23 isolates reacting with more than one red cell species the HAI pattern was identical whichever red cell was used in the test. Therefore further testing was performed using human group O erythrocytes alone.

Eight patterns of inhibition of agglutination of human group O erythrocytes were observed (Table 2). There was no association between HAI pattern and bacterial species ($0{\cdot}1 > P > 0{\cdot}05$). However, isolates showing haemagglutination not inhibited by mannose, fucose or galactose were recovered from 14/22 (64%) of patients with diarrhoea, and 13/26 (50%) of patients with other gastrointestinal disease. In contrast none of 19 isolates from patients without symptoms and only 12/66 (18%) of water isolates exhibited this HAI pattern ($P < 0{\cdot}001$). This HAI pattern was also significantly more common among isolates producing a Vero cell cytotoxin ($0{\cdot}01 > P > 0{\cdot}001$).

Eleven of 14 (79%) toxin producing isolates from all patients with diarrhoea showed haemagglutination resistant to mannose, fucose and galactose. In contrast only 4 of 20 (25%) toxin producing isolates from asymptomatic patients or water samples had the same HAI pattern.

Use of a typing system

Within each species at least nine types were identified by HA and HAI typing. Type 1a was the most common among isolates of *A. sobria* (37%) and *A. caviae* (8%), whereas 3a was most common in *A. hydrophila* (16%).

However, typing did not pick out any of the clusters identified on epidemiological grounds. The HA and HAI types for all isolates clustered according to source is shown in Table 3.

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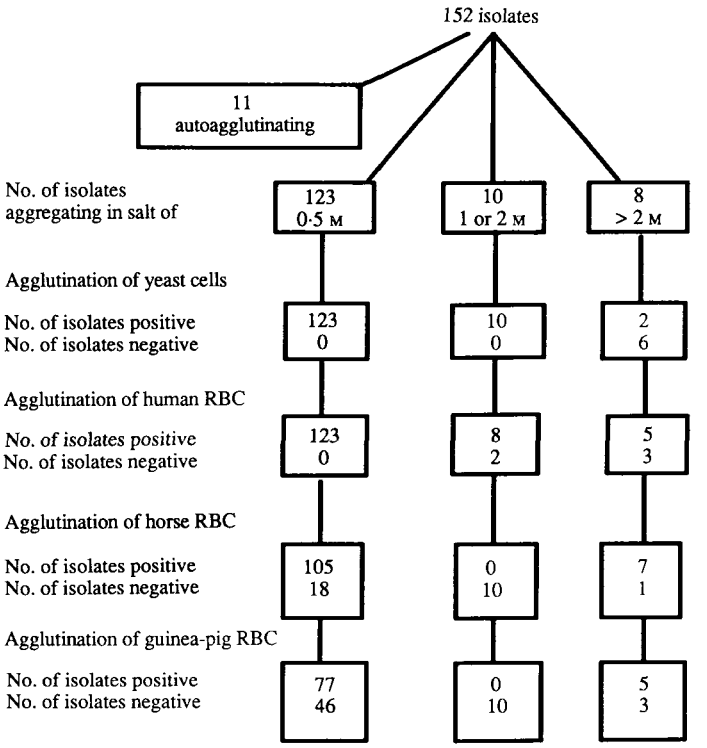

Fig. 1. Salt aggregating and agglutinating properties of *Aeromonas* spp. isolates.

Reproducibility

Seventeen isolates retested from the same plate culture all gave identical haemagglutination and haemagglutination inhibition results for all red cell species. After 6 months on nutrient agar slopes 6 of 22 strains (27%) had lost the ability to agglutinate one or more red cell species, and the haemagglutination of a further 4 isolates as inhibited by sugars to which they were previously resistant. At 18 months 36/73 isolates (49%) had lost haemagglutinating ability and a further 8 (11%) were inhibited by additional sugars. The loss of haemagglutinating ability was associated with failure to aggregate in low salt concentrations. At 6 months 9/22 (42%) and at 18 months 42/73 (58%) of strains required an increased salt concentration for aggregation.

DISCUSSION

We have shown that the majority of strains of *Aeromonas* species agglutinate yeast and human group O erythrocytes. A correspondence between ability to agglutinate yeast cells and human group O erythrocytes has been noted previously (Adams, Atkinson & Woods, 1983). These authors, like ourselves, also noted a variety of patterns of haemagglutination and haemagglutination inhibition. An *Aeromonas* strain where haemagglutination was inhibited only if two sugars were

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Table 3. *Clusters of isolates typed by haemagglutination and haemagglutination inhibition patterns*

Species	Site*	Date of isolation	Source	HA/HAI type
A. caviae	HA		Water tank 1	1h
	HA		Water tank 2	1e
	HA		Water tank 3	1c
	HA		Water tank 4	1e
	HA	15. viii. 83	Water tank 5	1h
	HA		Water tank 6	6
	HA		Water tank 7	6
	HA		Water tank 8	3f
	HA		Water tank 9	3f
	HA	22. viii. 83	Water tank 10	1b
	HA		Water tank 11	1a
A. hydrophila	HA		Water tank 12	3e
	HA		Water tank 1	3h
	HA	15. viii. 83	Water tank 7	8
	HA		Water tank 9	†
	HA		Water tank 10	1b
A. caviae	HB	15. viii. 83	Water tank 1	1h
	HB		Water tank 2	1h
	HB		tap staff quarters	1d
	HB	22. viii. 83	tap main kitchen	†
	HB		tap main kitchen	7e
	HB	12. ix. 83	tap ward 9	3d
A. caviae	HC		Water tank 1	1g
	HC	22. viii. 83	Water tank 2	7d
	HC		Water tank 3	1d
	HC		Water tank 4	7g
A. caviae	HX	17. vi. 82	Faeces pt 11‡	3a
	HX	7. vii. 82	Faeces pt 2	2d
	HX	8. vii. 82	Faeces pt 3	1e
	HX	17. viii. 82	Faeces pt 4	†
	HX	12. v. 83	Faeces pt 5	1a
	HX	8. ix. 83	Throat swab pt 6	3a
	HX	12. ix. 83	Water tank 1	2e
A. caviae (LF)§	ITU HX	29. ix. 83	Intragastric tube feed	3b
	ITU HX	3. x. 83	Kitchen tap	1e
	ITU HX		Kitchen drain	3b
A. hydrophila	HX	10. viii. 83	Tap 2	1d
	HX	15. viii. 83	Water tank 1	6
A. hydrophila	ITU HX	14. ix. 83	Intragastric tube feed	7a
	ITU HX	29. ix. 83	Intragastric tube feed	3g
	ITU HX	17. xi. 83	Nasogastric aspiration apparatus	3a
A. sobria	HX	21. vii. 83	Faeces pt 7	1a
	HX	10. i. 84	Water tank 2	1a
A. caviae	FA	2. vi. 83	Faeces pt 1	7d
	FA	7. vi. 83	Faeces pt 2	3g
A. caviae	FB	2. vi. 82	Faeces pt 1	3f
	FB		Faeces pt 2	3e
A. hydrophila	Rs	29. viii. 83	Fast flowing stream	3g
	Rs		Fast flowing stream	3a
	Rs		Lake at edge of water	3a

* H, hospital; thus HA is hospital A; F, family; ITU, intensive therapy unit; Rs, Rhinog Hills.

† autoagglutinating isolate.

‡ pt, patient.

§ LF, lactose-fermenting strains.

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used together has been described (Atkinson & Trust, 1980). This suggests that strains may express more than one agglutinin at once.

Failure to aggregate in a low salt concentration was associated with failure to agglutinate yeast cells and human erythrocytes, but not horse or guinea-pig red cells. In other Gram-negative species ability to aggregate in low concentrations of salt is associated with strong hydrophobicity and the presence and number of fimbriae (Faris, Wadstrom & Freer, 1981; Faris *et al.* 1983). Our results suggest that investigation of the hydrophobicity of *Aeromonas* isolates and the occurrence of fimbriae in relation to yeast and human O erythrocyte agglutinins might be fruitful.

We have found that haemagglutination of human group O erythrocytes resistant to mannose, fucose and galactose is more common among isolates of *Aeromonas* spp. from patients (both adults and children) with diarrhoea and among strains producing a Vero cell cytotoxin than would be expected by chance. An association with isolates from children with diarrhoea has been noted previously (Burke *et al.* 1984*a*). It is also interesting that this HAI pattern is three times more frequent among toxin-producing isolates from patients with diarrhoea than among toxin-producing isolates from other sources. This test might be useful in clinical laboratories when screening potentially virulent strains.

It has been suggested that HA and HAI patterns could be used as a typing system (Adams, Atkinson & Woods, 1983). We found little correlation between the clusters identified by this method and those expected on epidemiological grounds. Multiple strains at a single site have been described previously (Picard & Goullet, 1984; Cookson, Houang & Lee, 1984) but the degree of variation we have found is rather surprising. Adams, Atkinson & Woods (1983) noted that patterns were stable when strains were stored at −70 °C or after passage in mice. However, we have found that agglutinating ability is lost on repeated subculture, which would limit the usefulness of the technique for typing purposes. The tendency to lose agglutinating ability on subculture may explain some of the variation observed within clusters of apparently related strains; it may also explain why not all aeromonads from patients with diarrhoea have the HA and HAI pattern associated with virulence.

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