METHODS FOR THE DETECTION OF HAEMAGGLUTININS IN *AEROMONAS*

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SUMMARY. When grown in specified conditions and tested by a rocked-tile method, 40 of 41 isolates of two species of *Aeromonas* formed simultaneously at least two haemagglutinins among which were: (i) a mannose-sensitive haemagglutinin with strongest activity for guinea-pig or fowl red cells, formed by all of 31 isolates of *A. hydrophila* and 9 of 10 isolates of *A. punctata* ss. *caviae*; (ii) a haemagglutinin, sensitive to L-fucose or D-mannose, that reacted with human red cells and which was formed by all 41 isolates; and (iii) a mannose-resistant 'tanned red cell' haemagglutinin formed by 29 isolates of *A. hydrophila* and one isolate of *A. punctata* ss. *caviae*. Results emphasise that for the fullest possible identification of haemagglutinins produced by *Aeromonas* spp., strains should be cultured in a variety of conditions and tested with a wide range of red-cell species.

INTRODUCTION

Aeromonads may cause infections in compromised patients (Trust and Chipman, 1979), but their rôle as primary intestinal pathogens of man is still in question, a situation that is of considerable interest because of the sizeable proportion of acute intestinal infections with no known association with recognised enteropathogens. The search for mechanisms of pathogenicity in *Aeromonas* has revealed that certain strains of *A. hydrophila* produce an enterotoxin that causes diarrhoea in experimental animals (Burke *et al.*, 1981) and adhesive factors (Atkinson and Trust, 1980) which may promote colonisation of intestinal epithelium, an essential step in the infective process. The ability to cause haemagglutination is a convenient and effective means for determining the presence of bacterial adhesins (Duguid and Old, 1980). However, no systematic study of the adhesive mechanisms of *aeromonads* has yet been made. This report presents the results of a preliminary study made with a view to determining the prevalence and complexity of the haemagglutinins produced by two species of *Aeromonas*.

MATERIALS AND METHODS

Bacteria. The 41 isolates of *Aeromonas* examined (table 1) were received from Ms J. Robinson, Princess Margaret Children's Medical Research Foundation, Perth, Western Australia. Isolates had been classified according to Schubert (1974) and the donor supplied information about source and enterotoxin-producing ability. They were stored at ambient temperature on semi-solid maintenance medium (Burke *et al.*, 1981).

Media. Nutrient Broth (No. 2), Tryptone Soya Broth (TS broth) and Nutrient Agar were commercial products from Oxoid Ltd, Basingstoke, Hampshire. Blood agar was Blood Agar Base (Oxoid) with the addition of defibrinated horse blood (Difco, West Molesey, Surrey) 5% v/v. Liquid media were used in 10-ml volumes in cotton wool-stoppered test tubes (150 × 15 mm); agar media were poured in *c.* 20-ml amounts in petri dishes (85-mm diameter). Phosphate-buffered saline (PBS, pH 7.3) contained NaCl 8.0g, K₂HPO₄ 1.2g and KH₂PO₄ 0.3g/L of deionised water.

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TABLE I
Isolates of Aeromonas examined

Species*	Number of isolates		Source†	
	tested	producing enterotoxin	faeces	water
<i>A. hydrophila</i>	23	23	18	5
	8	0	0	8
<i>A. punctata</i> ss. <i>caviae</i>	10	0	10	0

* According to Schubert (1974).

† Strains were isolated in W. Australia by the donor.

Culture methods. For haemagglutinin production, bacteria were grown in different broth and agar media in various cultural conditions. All isolates were grown on blood agar incubated at 20°C for 48 h or at 30°C for 24 h, and in TS broth incubated at 20°C for 6 days (primary culture methods). In addition, some isolates were cultured on nutrient agar or in nutrient broth: (i) at 37°C for 24 h; (ii) at 30°C for four serial subcultures at 24-h intervals (nutrient agar) or six serial subcultures at 48-h intervals (nutrient broth). Broth-grown cultures were centrifuged (2000*g* for 10 min) and suspended in PBS to a concentration of *c.* 10¹⁰ bacteria/ml. Agar-grown bacteria were harvested directly in PBS to a concentration of *c.* 10¹¹ bacteria/ml.

Red blood cells. The red blood cells of domesticated fowls (hens), guinea pigs, horses, man (group O only), pigs, sheep and oxen were separated by centrifugation from citrated, heparinised or defibrinated blood, washed twice in PBS and resuspended to 3% v/v in fresh PBS. For the preparation of 'tanned' cells, equal volumes (5 ml) of ox cells 3% v/v and tannic acid (BDH Chemicals, Poole, Dorset) 0.01% w/v, both in physiological saline (NaCl 0.85% w/v in deionised water), were mixed and incubated at 37°C for 15 min; the red cells were then washed thrice in PBS and resuspended in fresh PBS to 3% v/v.

Haemagglutination tests were made with the seven species of untreated and tanned red cells. One drop of bacterial suspension was added to one drop of red-cell suspension and one drop of PBS in the circular depression of a porcelain tile, which was rocked at ambient temperature (*c.* 20°C) on a mechanical rotating table for 10 min. After that time, haemagglutination reactions were recorded as: weakly (+), moderately (+ +), or strongly (+ + +) positive; or (−) negative. Absence of haemagglutination in parallel tests in which a drop of 2% w/v L-fucose, D-galactose or D-mannose (all from Sigma) was substituted for the drop of PBS in the mixture, showed that the haemagglutinating activity was sensitive to one or more of those carbohydrates. Tiles were rocked for a further 15 min at 4°C and, after the results had been recorded, were warmed gently to *c.* 50°C in an attempt to demonstrate elution of the bacteria from the red cells.

RESULTS

All but one of the 41 cultures of *Aeromonas*, grown in the cultural conditions described, produced two or more haemagglutinins (table II).

Mannose-sensitive haemagglutinins. All of the 31 isolates of *A. hydrophila* formed a mannose-sensitive haemagglutinin (MS-HA) which was detected with either fowl or guinea-pig red cells, or both. When that MS-haemagglutinating activity was present in large amounts (+ + or + + +), other species of red cells such as those of horse, man, pig and sheep were often agglutinated also but fresh or tanned ox red cells were not. The MS-HA was generally formed in both agar- and broth-grown cultures, that activity usually being strongest in blood-agar cultures incubated at 30°C; the only strain that did not produce MS-HA at 30°C did so when grown on blood agar at 37°C. On the other hand, when isolates of *A. punctata* ss. *caviae* were cultured by these same primary methods, MS-HA activity was weak or absent, although it was produced by these strains when cultured serially on nutrient agar.

TABLE II
Haemagglutinin production by 41 isolates of *Aeromonas*

Species* (number and enterotoxin-producing status)	Number of isolates producing HAs in the following combinations		
	FMS-HA only	MS-HA and FMS-HA	MS-HA, FMS-HA and MR(TO)HA
<i>A. hydrophila</i> (23 Ent ⁺)	0	2	21
(8 Ent ⁻)	0	0	8
<i>A. punctata</i> ss. <i>caviae</i> (10 Ent ⁻)	1	8	1

* According to Schubert (1974).

Ent⁺ = enterotoxin-producing; Ent⁻ = enterotoxin non-producing. MS-HA activity was detected with either fowl or guinea-pig red cells, or both, in the absence but not in the presence of D-mannose; FMS-HA activity was detected with human red cells in the absence but not in the presence of L-fucose or D-mannose; MR(TO)-HA activity was detected with tanned (but not fresh) ox red cells in the presence of D-mannose.

After culture on one or more of the broth or agar media used for primary testing, all 41 isolates of *Aeromonas* formed a second mannose-sensitive haemagglutinin which was demonstrated in tests with human red cells. Unlike the above-described MS-HA, however, this latter haemagglutinin was sensitive also to the presence of L-fucose in the reaction mixture. The fucose-mannose-sensitive haemagglutinin will be designated FMS-HA.

Mannose-resistant haemagglutinins. A tanned-ox-cell haemagglutinin (MR(TO)-HA), detected when bacteria agglutinated tanned, but not fresh, ox red cells in the presence of D-mannose, was formed by 19 of the 31 isolates of *A. hydrophila*, but by only one of the 10 isolates of *A. punctata* ss. *caviae*, when grown on the primary agar or liquid media at 20 or 30°C. A further 10 *A. hydrophila* isolates produced detectable amounts of MR(TO)-HA after two to four serial cultures in nutrient broth at 30°C.

In addition, there was evidence that four isolates of *A. hydrophila* and one isolate of *A. punctata* ss. *caviae*, when grown on blood agar at 30°C, formed a second type of mannose-resistant haemagglutinin (MR(F)-HA) that reacted with fowl red cells.

Haemagglutination tests were performed at 4°C and higher temperatures up to 50°C to test for the presence of mannose-resistant and eluting haemagglutinins (Duguid, Clegg and Wilson, 1979). None was detected in any culture examined.

DISCUSSION

In the primary growth conditions employed in this study, all of the 41 isolates of *Aeromonas* tested, regardless of source, were haemagglutinating and usually formed more than one type of haemagglutinin in the same culture.

The mannose-sensitive haemagglutinin (MS-HA) was detected more readily in agar- than in broth-grown cultures, although that observation may be attributed in part to the test methods used and in particular to the larger number of bacteria harvested from agar plates and, hence, available for haemagglutination tests. Conversely, production of 'tanned ox' haemagglutinating activity, MR(TO)-HA, not previously described in strains of *Aeromonas*, was detected more frequently in broth- than in agar-grown cultures and at lower than at higher incubation temperatures. There was no particular growth medium that favoured the optimal production of the fucose-mannose-sensitive haemagglutinin (FMS-HA); some isolates showed strong FMS-HA activity when grown on blood agar at 30°C whereas others did so only in TS broth incubated at 20°C.

It was of interest that the patterns of haemagglutination obtained for strains in tests with the seven red-cell species and with tanned cells were not reproducible when isolates were tested after serial subculture on different occasions, even when they had been grown under identical conditions. That kind of observation might be explained by phase variation of the different types

of haemagglutinins involved, though as yet little information is available on phase variation of haemagglutinins in *Aeromonas*. It should be emphasised, therefore, that haemagglutination patterns, however determined, should not be used as an aid in the discrimination of *Aeromonas* strains unlike, for example, the patterns of mannose-resistant and eluting haemagglutinating activity shown by strains of *Escherichia coli* for which it has been possible to define accurately conditions both for their optimal production and for their detection (Crichton and Old, 1980).

If only one blood-cell species, such as that of man, is selected for use in haemagglutination tests to detect bacterial haemagglutinins, two problems will be encountered: (i) haemagglutinins, though produced, may not react at all with human red cells; and (ii) even if human red cells are agglutinated, several distinct mechanisms may be involved in their haemagglutination. Thus, from the present study of strains grown under different conditions in parallel and tested for haemagglutination with a range of red-cell species in the separate presence of L-fucose, D-galactose or D-mannose, it was clear that the observed 'mannose only' inhibition of human red-cell agglutination resulted, in many cases, from the combined activities of the MS-HA (strongest with fowl or guinea-pig red cells) and the FMS-HA (with human red cells).

Although the classification of Schubert (1974) has been followed in this paper, it should be mentioned that other classification schemes are available. Thus, whereas Schubert (1974) considered that the motile aeromonads, i.e., those other than *A. salmonicida*, belonged to the two species *A. hydrophila* and *A. punctata* each with its own distinct subspecies, Popoff and Véron (1976) on the basis of computer-assisted analysis described two different species, *A. hydrophila* and *A. sobria*, and, furthermore, they included strains of *A. punctata* in the species *A. hydrophila*. Again, more recent work based on polynucleotide-sequence relationships has indicated the existence of three separate groups, each of which has been afforded species status: *A. hydrophila*, *A. sobria* and *A. caviae* (Popoff *et al.*, 1981). Of the 31 isolates of *A. hydrophila* examined in this study, 14 could have been classified as *A. sobria* according to the scheme of Popoff *et al.* (1981) (Ms J. Robinson, personal communication). Nevertheless, all of these 31 isolates, whether or not they produced enterotoxin and however named, had similar haemagglutinating activities. In contrast, nine of the 10 isolates of *A. punctata* ss. *caviae* (*A. caviae*) showed a relative absence of haemagglutinating activity, a finding which perhaps supports their delineation as a separate species. It is possible, of course, that the haemagglutinating activities observed among this small number of isolates from a limited geographical area reflect merely that some of them have a common origin and are members of a clone. That being so, it is obvious that future studies should include strains isolated in other parts of the world.

Although haemagglutinins are widely distributed in both clinical isolates and free-living saprophytes, interest has been directed increasingly in recent years towards the possible rôle of haemagglutinins in the establishment of infections in man or animals. In the study of strains which form different types of haemagglutinins with distinct but overlapping affinities, it is often difficult to identify both the separate presence and properties of each haemagglutinin. It is obvious from the present study that the establishment of any correlation between the different haemagglutinins in *Aeromonas* species and fimbrial or non-fimbrial material is for future study and is likely to prove difficult. However, before any pathogenic rôle is ascribed to any particular adhesin, discussed here or elsewhere, it would seem wise to culture strains under a wide variety of conditions and to test them with a range of fresh, and tanned, red cells in order to identify as fully as possible the haemagglutinins produced *in vitro*. In that way it should also be possible to avoid assigning undue importance to putatively different haemagglutinin types that in reality reflect no more than different amounts of the same haemagglutinin produced under inadequately defined cultural conditions.

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