Biochemical characteristics, enterotoxigenicity and susceptibility to antimicrobial agents of clinical isolates of *Aeromonas* species encountered in the western region of Saudi Arabia

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Summary. The biochemical characteristics, enterotoxigenicity and susceptibility to antibiotics are reported for 22 strains of *Aeromonas* species isolated from clinical specimens in the Western Region of Saudi Arabia. *Aeromonas caviae* was the species most frequently observed; a high proportion of these strains fermented lactose, whereas lactose fermentation was not observed in strains of *A. hydrophila* and *A. sobria*. Enterotoxigenicity, as judged by cytotoxicity in tissue culture was observed in three of four *A. hydrophila* strains and six of seven *A. sobria* strains, but in only one of 11 *A. caviae* strains. Two schemes for the biochemical assessment of enterotoxigenicity were found to be in 91% and 86% agreement respectively with cytotoxicity studies and in 95% agreement with each other. No single biochemical test correlated fully with enterotoxigenicity, but 86% of strains that oxidised gluconate produced a cytotoxin. Most strains were inhibited by concentrations of gentamicin, amikacin, chloramphenicol and tetracycline achievable in plasma. Most strains were resistant to broad-spectrum penicillins and many were also resistant to cefuroxime and cefoxitin.

Introduction

Aeromonas spp. have long been recognised as fish pathogens. More recent awareness of their role in human disease, notably diarrhoea, has renewed interest in the taxonomy of the genus which has been in a state of confusion. The only generally accepted species is *Aeromonas salmonicida*, the aetiological agent of furunculosis in salmonid fish, which has never been implicated in human disease. Recently, numerical taxonomy and studies of polynucleotide relatedness have established the existence of at least three other species or biotypes: *A. hydrophila*, *A. sobria* and *A. caviae*, and it is probable that further subgroups within each species remain to be established (Popoff and Véron 1976; Popoff *et al.*, 1981).

Individual species of *Aeromonas* have been associated with particular clinical syndromes, the frequency of which varies geographically and seasonally (Champsaur *et al.*, 1982; Gracey *et al.*, 1982); however, reports on individual species remain sparse. Certain strains of *Aeromonas* produce an

enterotoxin (Sanyal *et al.*, 1975), and this factor is reported to be highly associated with certain biochemical characteristics (Burke *et al.*, 1982; Turnbull *et al.*, 1984).

The present study sought to establish the biochemical characteristics, enterotoxigenicity and antimicrobial susceptibilities of *Aeromonas* spp. encountered in clinical specimens in the western region of Saudi Arabia.

Materials and methods

Clinical isolates of *Aeromonas* spp. were identified by the API 20E system (Analytab Products, La Balme-les-Grottes, Mantalieu-Vercien, France). Most strains were isolated by the method recommended by Millership and Chattopadhyay (1984). Strains of *Aeromonas* were identified to species level according to the expanded system of Popoff and Véron (1976), as described by Janda *et al.* (1984).

Biochemical characteristics

In addition to those characteristics observed on primary identification, tests for aesculin hydrolysis, production of acid from lactose and salicin, and of gas from glucose, and oxidation of gluconate, were performed as

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described by Cowan (1974). Haemolysis of sheep and human erythrocytes was detected on Columbia Agar base (Oxoid CM331) containing the respective erythrocytes at a concentration of 5%. Haemolysis was assessed according to the criteria of Furniss *et al.* (1978). Production of deoxyribonuclease was determined on DNAase Test Agar (Oxoid). For the detection of lecithinase, plates of Nutrient Agar (Oxoid CM3) containing egg yolk emulsion (Oxoid SR47) 5% were spot inoculated and incubated at 37 °C for 5 days.

The ability of strains to utilise substrates provided as the sole carbon source was studied by use of the API C auxographic system (Analytab Products). Tests were incubated at 30 °C and examined daily for 3 days.

Toxigenicity assay

A modification of the tissue-culture method of detecting *Aeromonas* enterotoxin described by Cumberbatch *et al.* (1979) was used. Fresh cultures of *Aeromonas* strains were inoculated into 10 ml of Brain Heart Infusion Broth (BHI) and incubated for 18 h at 37 °C in a flask shaker (Griffin and George Ltd). Cultures were then centrifuged at 3000 rpm for 30 min and the supernate was passed through a 0.2-µm filter (Sartorius Instruments Ltd). Cell-free filtrates were stored at 4 °C and used within 48 h.

Toxin activity was demonstrated in HeLa cell monolayers maintained in Minimal Essential Medium containing fetal calf serum 2%, penicillin 100 units/ml, polymyxin 100 units/ml, nystatin 50 units/ml and streptomycin 0.1 µg/ml. Cell-free filtrates or BHI broth (negative control) were added to give a final dilution of 1 in 5. Tubes were incubated for 24 h at 37 °C and examined at 3, 5 and 24 h. All assays were performed in parallel. Samples producing shrinking or vacuolation of at least 50% of cells were considered to have elicited a cytotoxic response and to be enterotoxigenic.

Minimum inhibitory concentrations (MICs)

Aeromonas strains were grown overnight at 37 °C in Mueller Hinton Broth (Oxoid) and diluted in peptone water so that $c 10^4$ cfu were inoculated by a multipoint inoculator (Denley Instruments Ltd) on dried Mueller Hinton Agar (Oxoid) plates containing doubling dilutions of the following antibiotics: amoxycillin, amikacin, azlocillin, cefoxitin, cefuroxime, chloramphenicol, gentamicin, mezlocillin, piperacillin, tetracycline and ticarcillin. *Escherichia coli* NCTC 10418 was used as control organism.

Results

Characterisation of strains

A total of 22 strains of *Aeromonas*, identified according to the criteria of Popoff and Véron (1976) as *A. hydrophila* (4 strains), *A. sobria* (7 strains) and

Table I. The clinical sources of *Aeromonas* strains

Clinical source	Number of strains		
	<i>A. hydrophila</i>	<i>A. sobria</i>	<i>A. caviae</i>
Diarrhoeal faeces	4	6	10
Sputum	0	1	0
Midstream urine	0	0	1
Total	4	7	11

A. caviae (11 strains), was isolated from clinical specimens. All but two of the strains were isolated from faeces (table I).

Extended biochemical profiles of the *Aeromonas* strains are shown in tables II and III. All strains hydrolysed arginine and produced indole; all produced acid from glucose, mannitol and sucrose. None produced ornithine decarboxylase, urease or tryptophan deaminase, or acid from inositol, sorbitol, rhamnose or melibiose. Seven of the *A. caviae* strains fermented lactose and all of the *Aeromonas* strains, with the exception of one *A. caviae* strain, produced lecithinase.

The ability of the strains to utilise various carbohydrates as sole carbon source is shown in table III. All strains were able to utilise glucose, glycerol, 2-keto-D-gluconate, galactose and sucrose. No strain was able to utilise xylose, adonitol, xylitol, inositol, sorbitol, melezitose or raffinose.

Enterotoxin production by *Aeromonas* strains

Ten (45%) of the 22 *Aeromonas* strains were cytotoxic for HeLa cells: three of four *A. hydrophila* strains; six of seven *A. sobria* strains; and one of eleven *A. caviae* strains (table IV). The toxigenic *A. caviae* strain was a urinary isolate; the remaining toxigenic strains came from faeces.

The biochemical characteristics suggested by Burke *et al.* (1982) and Turnbull *et al.* (1984) as markers of enterotoxigenicity are indicated in table II. Both schemes failed to predict cytotoxin production in one strain of *A. caviae* and both falsely predicted toxigenicity in a strain of *A. sobria*. The scheme of Turnbull *et al.* (1984) additionally predicted toxigenicity in a non-cytotoxic strain of *A. hydrophila* (table IV). Amongst markers of toxigenicity suggested by both schemes, oxidation of gluconate was demonstrated in nine of ten strains that produced a cytotoxin, but also in two of 12 strains in which cytotoxin was not demonstrated; a positive

Table II. The biochemical characteristics of *Aeromonas* spp. from the western region of Saudi Arabia

Test	Number of strains giving positive reactions			
	Total (n=22)	<i>A. hydrophila</i> (n=4)	<i>A. sobria</i> (n=7)	<i>A. caviae</i> (n=11)
Aesculin hydrolysis*	18	4	3	11
Arabinose (acid)*†	10	1	0	9
Salicin (acid)*	13	4	0	9
Lactose (acid)	7	0	0	7
Amygdaline (acid)	11	1	2	11
Sucrose (acid)	22	4	7	11
Glucose (gas)†‡	9	3	6	0
Voges-Proskauer*†‡	13	4	6	3
Lysine decarboxylase*†	8	3	5	0
β -galactosidase	21	4	6	11
Citrate utilisation	7	2	5	0
Gelatin hydrolysis	19	4	7	8
Lecithinase*	21	4	7	10
Haemolysis of sheep erythrocytes*	12	4	7	1
Haemolysis of human erythrocytes†	10	4	6	0
Deoxyribonuclease	20	4	6	10
Gluconate oxidation†‡	11	4	7	0

* Characters of value in identifying clinical isolates of *Aeromonas* spp. (Janda *et al.*, 1984).

† Characters of positive relationship to enterotoxicity (Turnbull *et al.*, 1984)

‡ Characters of positive relationship to enterotoxicity (Burke *et al.*, 1982)

Table III. Growth of *Aeromonas* strains in the presence of various carbohydrates as the sole carbon source

Substrate	Number of strains able to grow			
	Total (n=22)	<i>A. hydrophila</i> (n=4)	<i>A. sobria</i> (n=7)	<i>A. caviae</i> (n=11)
Glucose	22	4	7	11
Glycerol	22	4	7	11
2-Keto-D-gluconate	22	4	7	11
L-arabinose	10	1	0	9
Xylose	0	0	0	0
Adonitol	0	0	0	0
Xylitol	0	0	0	0
Galactose	22	4	7	11
Inositol	0	0	0	0
Sorbitol	0	0	0	0
Methyl-D-glucoside	7	3	4	0
N-Acetyl-D-glucosamine	11	3	5	3
Cellobiose	10	1	1	8
Lactose	3	0	0	3
Maltose	21	4	7	10
Sucrose	22	4	7	11
Trehalose	19	4	7	8
Melezitose	0	0	0	0
Raffinose	0	0	0	0

Table IV. The enterotoxigenicity of *Aeromonas* spp.

Species	Number of strains	Number of enterotoxigenic strains by stated criteria		
		Cytotoxicity	Burke <i>et al.</i> (1982)	Turnbull <i>et al.</i> (1984)
<i>A. hydrophila</i>	4	3	3	4
<i>A. sobria</i>	7	6	7	7
<i>A. caviae</i>	11	1	0	0
Total	22	10	10	11

Table V. MICs of 11 antibiotics for the *Aeromonas* strains

Antibiotic	Minimum Inhibition Concentration ($\mu\text{g/ml}$)											
	All strains (n = 22)			<i>A. hydrophila</i> (n = 4)			<i>A. sobria</i> (n = 7)			<i>A. caviae</i> (n = 11)		
	range	MIC50	MIC90	range	MIC50	MIC90	range	MIC50	MIC90	range	MIC50	MIC90
Amoxycillin	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128
Amikacin	1-8	4	8	4-8	4	8	2-8	4	8	1-8	8	8
Azlocillin	16-> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	16-> 128	> 128	> 128
Cefoxitin	< 0.25-> 256	8	> 256	0.5-> 256	32	> 256	< 0.25-> 256	2	> 256	< 0.25-32	8	32
Cefuroxime	< 0.25-> 256	4	32	< 0.25-> 256	4	> 256	< 0.25-32	1	32	0.5-8	4	8
Chloramphenicol	0.5-> 8	1	4	1	1	1	0.5-1	1	1	2-> 8	2	> 8
Gentamicin	0.5-4	0.5	1	0.5-1	0.5	1	0.5-1	0.5	1	0.5-4	1	1
Mezlocillin	8-> 128	> 128	> 128	> 128	> 128	> 128	8-> 128	> 128	> 128	8-> 128	32	> 128
Piperacillin	2-> 64	> 64	> 64	> 64	> 64	> 64	32-> 64	> 64	> 64	2-> 64	16	> 64
Tetracycline	1-> 16	2	4	1-2	1	2	1-2	1	2	1-> 16	2	> 16
Ticarcillin	128-> 256	> 256	> 256	128-> 256	> 256	> 256	128-> 256	> 256	> 256	256-> 256	256	> 256

MIC50, MIC90 = concentration inhibiting 50% and 90% of strains respectively

Voges-Proskauer reaction was observed in eight of the cytotoxin-producing strains and in four of the cytotoxin-negative strains. Haemolysis of human erythrocytes, suggested as a marker of toxigenicity by Turnbull *et al.* (1984), was found in eight cytotoxin-producing strains and in two non-producers.

Antibiotic susceptibility tests

The ranges of MICs of 11 antibiotics for the 22 *Aeromonas* strains are shown in table V. Nearly all the strains were resistant to concentrations of amoxycillin, azlocillin, mezlocillin, piperacillin and ticarcillin achievable in serum. However, the MICs of gentamicin, amikacin, tetracycline and chloramphenicol were generally within achievable serum levels. MICs of the β -lactamase-stable cephalosporins, cefuroxime and cefoxitin, varied widely between strains.

Discussion

With increased appreciation of the pathogenicity

of *Aeromonas* species it is probable that attempts to isolate the organism in the clinical laboratory will become more common. Although the strains grow readily on many lactose-containing media such as MacConkey's agar, reports of oxidase-variable reactions by some lactose-fermenting *Aeromonas* strains complicate the use of such media. (McGrath *et al.*, 1977; Overman *et al.*, 1979; Hunt *et al.*, 1981). This study has shown lactose fermentation to be a characteristic of most strains of *A. caviae*, and since this species is rarely enterotoxigenic, the use of MacConkey's agar may still play a role in screening for enterotoxigenic *Aeromonas* species.

In agreement with the criteria of Janda *et al.* (1984), biochemical characters found in the present study to be of use in species identification included: aesculin hydrolysis; the production of acid from arabinose and salicin; the production of gas from glucose; a positive Voges-Proskauer reaction and the production of lysine decarboxylase. Lecithinase production was commonly observed in all three species and plays no role in species identification. Failure to oxidise gluconate, to utilise citrate; to haemolyse sheep erythrocytes, and to utilise methyl-

L-glucoside as sole carbon source, together with the ability to produce acid from lactose and to utilise L-arabinose as sole carbon source, were characteristics useful in differentiating *A. caviae* from *A. hydrophila* and *A. sobria*.

Studies in which cytotoxicity was used as the criterion of toxigenicity showed *A. hydrophila* and *A. sobria* to be more virulent than *A. caviae*: nine of 11 (82%) strains of *A. hydrophila* and *A. sobria* produced cytotoxin, whereas only one of 11 strains of *A. caviae* appeared toxigenic. No single biochemical marker corresponded exactly with cytotoxicity, although most strains that oxidised gluconate produced a cytotoxin. Moreover, the schemes suggested by Turnbull *et al.* (1984) and Burke *et al.* (1982) provided both false positive and false negative predictions of enterotoxigenicity. Error rates of 14% and 9% respectively were observed for the two schemes and such predictive schemes should clearly be interpreted with caution.

Gastrointestinal infections with *Aeromonas* spp. are generally self-limiting, but antimicrobial treat-

ment may be necessary in serious infections, particularly systemic infection in immunocompromised patients. The antimicrobial susceptibility patterns observed in strains isolated in Saudi Arabia were similar to those reported from Great Britain, Canada and the United States of America (Smith, 1980; Fainstein *et al.*, 1982; Gray, 1984). Most strains were resistant to broad-spectrum penicillins and many were also resistant to cefuroxime and cefoxitin. However the MIC results indicate that the aminoglycosides, gentamicin and amikacin, as well as tetracyclines and chloramphenicol, should be suitable for those cases of *Aeromonas* infection in which antimicrobial therapy is necessary.

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