Characterisation of strains of *Aeromonas* spp. by phenotype and whole-cell protein fingerprint

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Summary. Sixty-eight isolates of *Aeromonas* spp. were examined biochemically and their cell proteins were analysed by silver-stained SDS-PAGE. Protein fingerprints did not correlate with phenotype. However, consideration of both phenotype and fingerprint showed clustering of epidemiologically related isolates. There was also evidence that similar strains could be found in infected people and water or other environmental samples.

Introduction

Aeromonas spp. are members of the Vibrionaceae. They are common in fresh surface water¹ and have long been recognised as occasional human pathogens, especially in soft tissue infections and septicaemia. In the last 10 years there has been increasing interest in their role as a cause of acute gastro-enteritis² although Koch's postulates have not been fulfilled (there is no animal model) and no outbreak attributable to aeromonads has ever been described. The relationship between water and human isolates, and between isolates from different patients is, therefore, of considerable interest. However, investigation of their epidemiology has been hampered by taxonomic confusion, and the difficulty of using conventional techniques such as serology to discriminate between different strains.

In studies during the 1960s and 1970s, the motile strains were commonly grouped together as "*A. hydrophila*", as distinct from "*A. salmonicida*", a non-motile, pigment-producing group which grew poorly or not at all at 37°C. On the basis of a numerical study of biochemical tests, Popoff and Véron³ described two species among the motile strains: *A. hydrophila*, with subspecies *anaerogenes* and *hydrophila*, and *A. sobria*. These groups came to be called *A. caviae*, *A. hydrophila* and *A. sobria*.⁴ This division of isolates appears to be of some biological significance. *A. caviae* is rarely haemolytic but *A. hydrophila* and *A. sobria* usually produce a haemolysin which is also probably a cytotoxin *in vitro* and an enterotoxin *in vivo*.^{5,6} Virulence in animal models² and human infections appears to be associated with *A. hydrophila* and *A. sobria* rather than *A. caviae*.^{7,8}

It has been shown that each of the three major

phenons include several DNA hybridisation groups (HGs) or genomic species.⁹ Twelve genomic species have so far been described.² Some authors have found a good correlation between phenotype ("phenospecies") and HG, with *A. hydrophila* belonging to HGs 1, 2 and 3, *A. caviae* to groups 4, 5 and 6 and *A. sobria* to groups 7, 8/10, 9 and 13.^{10,11} However, this may not always be the case, for Kuijper *et al.*¹² found that many strains identified as *A. hydrophila* belonged to HG 8, which is part of the *A. sobria* group. The issue has been further complicated by the description^{13–15} of more phenotypes, some of which correspond to HGs within the original three phenons and some of which are completely new species as determined by DNA hybridisation.¹⁶ Furthermore, radically different biotypes belong to the same HG. Mesophilic strains are now included in HG 3 with the former members of *A. salmonicida*, and HG 8/10 includes most clinical strains of *A. sobria* as *A. veronii* biotype *sobria* and some biochemically different and independently described isolates known as *A. veronii* biotype *veronii*.¹⁷ The aeromonads are clearly a highly heterogeneous group and it is likely that many more phenotypes and hybridisation groups remain to be described.

For epidemiological surveys it is impractical to perform DNA hybridisation on every isolate. A more sensible approach might be to identify isolates as *A. hydrophila*, *A. sobria* or *A. caviae*,⁴ use an electrophoretic fingerprinting technique to further characterise them, and reserve hybridisation for selected isolates.

Whole-cell protein fingerprinting has been used for both taxonomy and differentiation of strains within a species. Bacterial cell preparation is relatively simple and it is particularly suitable for screening large numbers of isolates. The following study was designed to maximise discrimination between strains by using a sensitive silver stain combined with high resolution digital analysis. The results were examined for their

Table. Scheme used to discriminate recently described phenotypes of *Aeromonas* spp.

Test	<i>A. veronii</i> * biotype <i>sobria</i>	<i>A. trota</i>	<i>A. hydrophila</i> group†	<i>A. veronii</i> biotype <i>veronii</i>	<i>A. caviae</i> group‡	<i>A. schubertii</i>
Aesculin hydrolysis	—	—	+	+	+	—
Arabinose fermentation	—	—	+	—	+	—
Salicin fermentation	—	—	+	+	+	—
Gas from glucose	+	+	+	+	—	—
H ₂ S production in GCTF	+	+	+	?	—	?
VP test	+	—	+	+	—	—
Lysine decarboxylation	+	+	±	+	—	+
Gluconate	+	—	±	?	—	—
Elastase production	—	—	+	—	—	—
Indole production	+	+	+	+	+	—
Sucrose fermentation	+	—	+	+	+	—
Ampicillin sensitivity	R	S	R	R	R	R
Mannitol fermentation	ND	ND	+	+	+	—
Cellobiose fermentation	ND	ND	—	±	?	—
Arginine dihydrolase	ND	ND	+	—	—	+
Ornithine decarboxylase	ND	ND	—	+	—	—

ND, not done.

* Includes *A. sobria* and *A. jandaei*.† Includes *A. salmonicida* and *A. hydrophila*.‡ Includes *A. media*, *A. eucrenophila* and *A. caviae*.

ability to distinguish strains both within and between some currently described phenotypes.

Materials and methods

Media

Culture media were supplied by Oxoid except those for fermentation, decarboxylase and dihydrolase tests which were supplied by Southern Group Laboratories. Incubations were overnight in air at 37°C except where stated otherwise.

Bacterial strains

Sixty isolates of *Aeromonas* spp. from water, food and people in the London area, a colonial variant of one isolate and a replicate of another, and six reference strains—*A. caviae* ATCC 13136, *A. hydrophila* ATCC 7965, *A. sobria* ATCC 9071, *A. veronii* ATCC 35622, *A. schubertii* ATCC 43700 and *A. media* ATCC 33907—were examined. Isolates were stored in nutrient broth with glycerol 20% v/v in liquid nitrogen and subcultured on horse-blood agar.

Isolates were identified as aeromonads if they were gram-negative rods, oxidase positive, fermentative, completely resistant to a 150-µg disk of the vibriostatic agent O/129 (Oxoid), and failed to grow in salt 6% broth yet gave an appropriate biochemical profile for "*A. hydrophila*" in API 20E trays (API system, La Balme Les Grottes, 38390 Montalieu-Vercieu, France). Isolates were then examined as described previously⁵ to differentiate the phenotypes *A. caviae*, *A. hydrophila* and *A. sobria*.⁴ Briefly, examinations were made for: lactose fermentation on MacConkey agar; fermentation of L-arabinose and salicin; pro-

duction of gas from glucose; elastase production; hydrogen sulphide production;¹⁸ decarboxylation of lysine (Moeller's method); hydrolysis of aesculin in aesculin agar; β-haemolysis on horse-blood agar; growth at 42°C on horse-blood agar; the Voges-Proskauer test by the Barritt method; and oxidation of glucuronic acid. Further differentiation of phenotypes was attempted by inclusion of indole and sucrose tests from the API 20E trays and determinations of sensitivity to ampicillin¹⁹ defined as a minimal inhibitory concentration (MIC) of < 2 mg/L. Isolates differing from the nearest ideal phenotype by two or more tests were examined further for D-mannitol and cellobiose fermentation in peptone water sugars and formation of ornithine decarboxylase and arginine dihydrolase (Moeller's method).

Electrophoresis and staining of proteins

Four or five colonies of each isolate were emulsified in 1 ml of distilled water, sodium dodecyl sulphate (SDS) was added to a final concentration of 2% w/v, and the samples were boiled for 3 min. The protein concentration was then adjusted with sample buffer²⁰ until a 1 in 10 dilution of the sample in water had an A₂₈₀ of 0.1 SD 0.01. Samples were then diluted with an equal volume of sample buffer and stored at -20°C for up to 6 weeks before electrophoresis in polyacrylamide gels. This was performed by the method of Laemmli²⁰ at a constant current of 10 mA when the sample was in the stacking gel (acrylamide 3%) and 15 mA when in the resolving gel (acrylamide 10%). The temperature of electrophoresis was controlled at 18 ± 1°C by a thermal recirculation unit to prevent "smile" effects. Gels had 16 or 20 tracks with mol. wt protein markers placed in the exterior lanes and in every third lane. Electrophoresis was continued until

Strain no.	Phenotype (4) (Serotype)	Source	Atypical characters
0142	caviae	pondwater	
1653	caviae	pondwater	
0150	caviae	human faeces	
0159	caviae	human faeces	
0141	hydrophila	pondwater	
0124	hydrophila	tap water	Elastase -ve, Arabinose -ve
*0133	caviae (03)	intensive care unit, kitchen drain, hospital X	
0117	hydrophila	tap water, hospital X	
*0123	caviae (03)	throat, patient, ICU, hospital X	
1231	caviae	colonial variant of 0123	
*0121	caviae (AX23)	tap water, hospital X	Gas +ve
*1281	caviae	tap water, hospital X	
*0135	hydrophila (03)	enteral feeds, ICU, hospital X	
0161	caviae	human faeces	
1651	unknown	pondwater	
0143	caviae	surface floodwater	
*0144	hydrophila	tap water, hospital X	Elastase -ve, Arabinose -ve
*0113	caviae	unknown	
0047	caviae	human faeces	
0066	caviae	human faeces	
0071	caviae	surface water	
0039	caviae	human faeces	Gas +ve
*0057	sobria	human faeces	
1321	caviae	kitchen drain, ICU, hospital X	
*1324	caviae	kitchen drain, ICU, hospital X	
0118	sobria	tap water, hospital X	
0027	sobria	human faeces	
0051	sobria	human faeces	sucrose -ve
0045	sobria	human faeces	
0139	sobria	human faeces	
*0032	caviae	human faeces	
*0134	caviae	enteral feed, ICU, hospital X	
*0029	caviae	human faeces	
*0074	sobria	human faeces	
ATCC 35622	veronii HG 8/10		
0022	sobria	human faeces	
ATCC 7965	hydrophila		
0138	hydrophila	nasogastric feed equipment, ICU, hospital X	
0154	caviae	human faeces	
0126	caviae	tap water, hospital H	
0016	caviae	human faeces	
0120	caviae	tap water, hospital H	
*ATCC 43700	schubertii HG 12		
*0007	hydrophila	human faeces	
*0040	caviae (AX20)	human faeces	
ATCC 33907	media HG 5a		
0100	hydrophila (026)	tap water, hospital X	Elastase -ve, Arabinose -ve
0116	hydrophila	London river water	
*0115	hydrophila	replicate of 0116	
0092	hydrophila	tap water, hospital X	
ATCC 13136	caviae		
0058	caviae	human faeces	
*0048	caviae	human faeces	
*0018	sobria	human faeces	
*1382	hydrophila	feeding equipment, ICU, hospital X	
*1381	hydrophila	feeding equipment, ICU, hospital X	
0106	caviae (03/AX21)	tap water, hospital F	
0090	caviae	tap water, hospital H	Gas -ve
0088	caviae	water, public baths	
*0096	caviae	tap water, hospital H	
*0101	caviae (AX27)	tap water, hospital H	
0097	caviae (AX24)	tap water, hospital X	
0095	caviae	tap water, hospital X	
*ATCC 9071	sobria HG8/10		
*0023	hydrophila	human faeces	
*0026	hydrophila	human faeces	Gas +ve
*0079	hydrophila	water, public baths	
0031	caviae	human faeces	

* = Isolates sent to reference laboratory for confirmation of identity and serotyping

Fig. 1. The protein fingerprints, characteristics and source of the 68 *Aeromonas* isolates studied. Fingerprints were normalised with respect to the mol.-wt markers, high frequency artefact was removed by application of a low pass Fourier filter, and contrast was enhanced before photography from the computer screen.

the bromophenol blue tracking dye was 1 cm from the bottom. Proteins were then stained with silver,²¹ with some minor modifications to the original technique.

Briefly, gels were fixed overnight in methanol 50% v/v, washed in water for 30 min and soaked in fresh silver diamine solution (0.36 g NaOH, NH₄OH 35%

Fig. 2. Cluster analysis of 68 isolates of *Aeromonas* spp. including replicates. Horizontal axis is the hierarchical level in percentage similarity. Species names are according to Popoff and Véron⁴ except for the named reference strains. The hybridisation group (HG) of the latter is stated where known.

w/v 1.4 ml, silver nitrate 20% w/v 4 ml, made up to 100 ml in water) for 15 min with constant shaking. Gels were then washed twice in water, placed in fresh reducing solution (citric acid 1% v/v 2.5 ml, formaldehyde 36% w/v 0.26 ml made up to 500 ml with water) and proteins were visualised within 10–20 min. The reaction was stopped with acetic acid 5% v/v and

the gel was de-stained in fresh Farmer's solution (sodium thiosulphate 0.6 g, sodium carbonate 0.3 g and potassium ferricyanide 0.1 g in 100 ml of water) until all bands had disappeared. Gels were washed until all the yellow Farmer's solution was eluted, fixed in three 1-h washes of methanol 50% v/v and stained as before; the final development of bands occurred in

6–8 min. Stain development was controlled by inclusion of a control strain of known band pattern. Care was taken to ensure that the final gel dimensions were within ± 1 cm of those before staining began. All isolates were examined in duplicate or triplicate on different gels.

Analysis of gels

The detailed method has been published elsewhere.²² Briefly, gels in a plastic bioassay dish (Nunc) were placed on a high intensity light box (Wardray) and recorded with a charge coupled device (CCD) camera (Model 90, Datacopy Corp., USA) with a 55 mm Micro-Nikkor lens and Hoya HA50 infra-red filter giving 1728×2240 picture elements on a 256 point grey scale. Recording was controlled by an IBM PC/AT compatible microcomputer running under MS-DOS with "WIPS" software supplied with the camera. All further image processing was with bespoke software. Gels were viewed in detail and the unwanted margins were deleted with programs compiled by the Microsoft C compiler v 5.1. They were then transferred from the MS-DOS to a Xenix partition of the hard disk. Tracks were located automatically and a one-dimensional density plot was produced by taking an average across the track at each point along its long axis. The plot lengths were normalised to 1024 points by a linear fit of $\log_e$ mol. wt on segments. Background density variation was corrected by a Fourier routine to apply a high pass step filter. Density plots were then compared by Fourier routines to calculate the correlation between pairs of plots and derive a similarity coefficient. Cluster analysis of the similarity matrix produced was by a Euclidean distance agglomerative algorithm.

Serotyping

Twenty-seven isolates were sent to the Division of Enteric Pathogens, Central Public Health Laboratory, Colindale, for confirmation of phenotype and serotyping.

Results

Thirty-six of the isolates were designated *A. caviae*, 16 as *A. hydrophila* and nine as *A. sobria*. One isolate could not be placed in any recognised group. Isolates were then re-examined as in the table. One sucrose-negative isolate resembling *A. sobria* was found; however, it was also lysine-negative by both API and conventional tests and, therefore, did not correspond to the original description of *A. jandaei*. No isolates of *A. veronii*,¹⁷ *A. schubertii*¹⁶ or *A. trota*¹⁴ were identified. Three gas-forming isolates, resembling *A. caviae* but which may be *A. eucrenophila*, were identified.²³

Only nine (33%) of the 27 isolates examined were typable by serology. Three isolates were of serogroup

O3, and one of O26 and five, including one reacting with two antisera, belonged to provisional new serogroups. Therefore, serotyping was abandoned.

All isolates yielded a protein fingerprint with 30–40 bands. The patterns obtained are shown in fig. 1. They have been digitally processed and contrast enhanced with merging of some closely spaced bands to simplify visual examination.

Reproducibility

Similarity coefficients of different tracks of the same isolate had a β distribution of mean 88% (SD 8%) within gels and 80% (SD 8%) between gels. The greater variation between gels is well known and is usually corrected by careful selection of the limits within which bands in different tracks are considered identical. This is not possible with Fourier correlation; therefore, within-gel similarity coefficients were multiplied by 0.9 so that results of within- and between-analysis could be combined. All known replicates fell within the expected cluster identified at the 93% level in fig. 2.

Cluster analysis

Five isolates of *A. sobria* clustered together at the 97% level but otherwise, as is apparent from fig. 1, fingerprints were not related to phenospecies. This was also true for the newer phenospecies. Isolates of very different phenotype could have similar or identical protein fingerprints, whereas some of those of similar phenotype had different fingerprints.

Clusters of isolates from the same or related sources are grouped together (fig. 2). At the 93% hierarchical level, 19 phenons were defined, of which 10 included two or more phenotypically similar isolates from the same source (nos. 1, 3, 6, 7, 8, 12, 14, 15, 16 and 17). Phenon three was especially complex. It included four environmental isolates of *A. hydrophila*, two of which were from the same hospital, and three phenotypically similar isolates of *A. caviae*, two known to be of the same serotype, also from that hospital.

Discussion

We analysed 1024 data points along each track to resolve all the visible protein bands. This large number made analysis very sensitive to minor shifts in band positions. For this reason, Fourier correlation, rather than Pearson-product moment correlation, was used to calculate similarity coefficients because the former is less sensitive to some forms of positional shifting.²² Reproducibility was acceptable and made fingerprinting highly discriminatory. This method, when considered with the phenotypes of *A. caviae*, *A. hydrophila* and *A. sobria*, permitted the 60 isolates to be divided into 33 groups.

Whole-cell protein fingerprints usually correlate

well with phenotype.²⁴ Therefore, the isolates were examined with regard to the generally accepted phenotypes.⁴ Isolates were then re-examined according to the criteria in the table. Tests in commercial kits are not generally recommended but some were included because they were used in the original descriptions of *A. veronii*¹⁷ and *A. schubertii*.¹⁶ Although separation of all the recognised genotypes of *Aeromonas* spp. by phenotype has been claimed,¹¹ usually the genospecies within the *A. caviae* and *A. hydrophila* phenotypes are not distinguished.² *A. jandaei* can be distinguished from the others by only a few tests, including some in commercial kits; therefore, it was not added to the table.

Whichever phenotyping system was used, there was little relation between protein fingerprint and phenotype, probably because of the increased sensitivity of silver staining for detecting protein bands over that of Coomassie Blue. A previous study of this genus²⁵ and species within other genera,²⁶ with autoradiography to detect proteins, also showed considerable variation between strains of the same phenospecies. Usually gram-negative species showed variations in band positions between strains, whereas strains of gram-positive species showed more variation in relative

band densities but fewer differences in band position (unpublished data). This suggests that fingerprints may be a function of cell-wall or outer-membrane proteins, which are known to vary between strains of the same species.

It is also unlikely that fingerprints are related to the DNA hybridisation group because the two reference strains of HG 8/10, *A. sobria*¹¹ and *A. veronii*¹⁷ are widely separated and the latter is clustered with the reference strain of *A. hydrophila* at the 95% level.

There is evidence that human and environmental strains may be similar. Phenon 3 included two isolates of *A. caviae* serotype 3—one from a throat swab and one from the kitchen drain on the same ward. Phenons 7 and 8 included a series of isolates of the same phenotype, some from human sources, including faeces, and some from mains water. It has long been assumed that isolates associated with cases of gastroenteritis are acquired from water or food. All the patients were resident in the same community receiving a common mains water supply, which may be a source of aeromonads.¹

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