Phenotypic methods for speciating clinical Aeromonas isolates

M H Wilcox, A M Cook, K J Thickett, A Eley, R C Spencer

Abstract
Aims: To establish the suitability of currently available phenotypic methods for speciation of clinical Aeromonas isolates in diagnostic microbiology laboratories.

Methods: Using 62 Aeromonas spp, three schemes based on biochemical reactions were compared: a series of conventional tests; a system based on the suicide phenomenon, comprising two tubes in total; and a commercially available test, API 20 NE, augmented with a plate assay for β haemolysin production. The whole cell and outer membrane protein (OMP) profiles of strains were examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE), according to the results of the above schemes, to determine the intra-species homogeneity.

Results: Ninety per cent of strains were identified satisfactorily according to conventional criteria. For these strains, agreement was obtained using the suicide phenomenon and API schemes in 93% and 88% of cases, respectively. The three schemes concurred for 82% of strains. Whole cell protein profiles were unsuitable for comparing strains within a species. However, OMP patterns were similar for 89% of A caviae and 63% of A hydrophila.

Conclusion: Phenospeciation of clinical Aeromonas isolates by the scheme based on the suicide phenomenon is simple to perform and accurate, and suitable for use in the diagnostic laboratory. OMP profiles are potentially useful for confirming the identity of A caviae and most A hydrophila, but not A sobria.

Methods
Sixty two Aeromonas spp were studied, comprising 27 and 29 faecal isolates from adults and children, respectively, who had been investigated for gastroenteritis in Sheffield. Six reference strains, including three kindly supplied by T Donovan (PHLS Laboratory, Ashford, Kent), and American Type Culture Collection strains ATCC 15468, 7966, and 43979 (A caviae, A hydrophila, and A sobria, respectively) were used. All strains were oxidase positive, fermentative Gram negative bacilli, resistant to both low (10 μg disc) and high (150 μg disc) concentrations of the vibrio-static agent 0129.

Phenospeciation (3 Clin Pathol 1992;45:1079-1083)
CONVENTIONAL BIOCHEMICAL METHODS
Specification was achieved according to the criteria of Popoff and Veron, modified by Janda et al. Briefly, the tests comprised: aesculin hydrolysis determined by inoculation of agar slants supplemented with aesculin (1 g l\(^{-1}\)) and ferric citrate (0.5 g l\(^{-1}\)) (30°C); Voges Proskauer reaction; acid production in arabinose and salicin peptone waters; gas production in glucose peptone water; glucose-nitrate oxidation; growth in the presence of potassium cyanide; and β haemolysin production determined by inoculation of Columbia agar (Oxoid) plates containing 5% sheep blood. All tests were incubated in air at 37°C (unless otherwise stated) for 18–24 hours.

SUICIDE PHENOMENON
The fermentation of glucose, with or without gas production and pelleting of bacteria (suicide phenomenon) was determined by inoculation of vessels containing nutrient broth (Oxoid) with 0.5% w/v glucose, 0.0015% w/v bromresol purple, and inverted tubes. Tests were read after incubation at 30°C for 18–24 hours. Additionally, for this method of specification aesculin hydrolysis, as determined above, was also tested. *A. hydrophila* is non-suicidal, aerogenic and aesculin positive; *A. sobria* is suicide variable, aerogenic and aesculin negative; *A. caviae* is suicidal, anaerogenic, and aesculin positive.

API 20NE AND HAEMOLYSIN PRODUCTION
Identification strips were used according to the manufacturer’s instructions and read after incubation at 30°C for 48 hours. As this method cannot be used to differentiate between *A. hydrophila* and *A. caviae* strains, haemolysin production (positive for *A. hydrophila* and negative for *A. caviae*) was also tested, as described above.7 13

SDS PAGE
Whole cell and OMP profiles were studied by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). Strains were cultured on a shaker in brain heart infusion broth (Oxoid) at 30°C for 18 hours, and were then centrifuged at 5000 x g for 20 minutes. Whole cell protein samples were prepared by resuspending the bacteria in distilled water to an optical density of 0.5 at 590 nm, and then centrifuging 10 ml of this suspension at 5000 x g for 20 minutes. Double strength sample buffer (150 μl) (4 g SDS, 20 ml glycerol, 10 ml 2-mercaptoethanol, 12.5 ml 1M TRIS-buffer (pH 6.8), and 20 mg bromphenol blue per 100 ml distilled water) was added to the deposit. After boiling for five minutes 150 μl of distilled water were added and boiling continued for a further five minutes. The supernatant fluid collected after centrifugation at 10 000 x g for 10 minutes represented the whole cell proteins.

OMPs were prepared by resuspending bacterial cells, cultured as above, in 5 ml 50 mM TRIS-HCl buffer (pH 7.4) to an optical density of 1.5 at 590 nm. Suspended bacteria were lysed by sonication over ice for two one minute periods at 12 μm peak-to-peak amplitude (MSE Scientific Instruments, Crawley, England). After centrifugation at 5000 x g for 10 minutes to remove cell debris OMPs were separated from the supernatant fluid by adding 0.5 ml 20% w/v Sarkosyl (Sigma, Poole, England) and incubating at room temperature (20°C) for 30 minutes. OMPs were collected by centrifugation at 50 000 x g for one hour at 4°C, and then resuspended in 50 μl 50 mM TRIS-HCl buffer. Single strength sample buffer (150 μl) was added, and then boiled as detailed for whole cell preparations.

Whole cell and OMPs were separated on 12% SDS PAGE gels (Mini Protean II Biorad, Hemel Hempstead, England), loading 10 μg protein per lane. Molecular size standards (Sigma) were run concurrently. Following electrophoresis, gels were fixed and stained with Coomassie brilliant blue R250.

Results
The results of the three biochemical schemes for speciating *Aeromonas* spp are compared in fig 1. Of 62 strains studied, six (10%) gave inconclusive results according to the conventional scheme and are not included in the

![Figure 1 Phenotypic characterisation of aeromonads using three different methods of speciation. The figures inside circles represent the number of strains identified as a particular phenotype. The figures outside circles show the numbers of strains changing identity (dotted lines) from the convetional scheme results. Strains identified as either *A. hydrophila* or *A. caviae* by API 2ONE were divided according to haemolysin production.](image-url)
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removing analysis. Janda et al Defined eight primary and nine supplementary phenotypic properties for identifying clinical isolates of Aeromonas spp. The six strains that could not be satisfactorily identified gave inappropriate results for at least two primary reactions. However, the six strains were morphologically similar to the remainder of Aeromonas isolates and satisfied the broad criteria detailed in the methods section. Fifty two out of 56 strains (93%) gave the same species identification in the conventional and suicide phenomenon schemes; the corresponding figure for the API 20NE plus haemolysin assays was 49 (88%) strains. The three schemes gave concordant results for 82% of strains tested. Four and two strains not identifiable by the conventional scheme were speciated as A sobria by the API and suicide schemes, respectively. All 27 strains identified by the conventional scheme as A caviae were haemolysin negative, while 15 out of 16 A hydrophila were haemolysin positive; seven (77%) A sobria were also haemolysin positive.

Gas production from the fermentation of glucose was frequently not detected using glucose peptone water. For 12 strains gas production was reproducibly negative in the latter method, but positive in the suicide tubes which contained nutrient broth and glucose, and these were incubated at 30°C instead of 37°C. A separate experiment showed that the most critical factor was the temperature of incubation, gas production being more reliable at 30°C as opposed to 37°C.

The whole cell protein profiles contained in excess of 40 bands which made comparison between species and strains difficult, and species specific patterns could not be identified with confidence (data not shown). The OMP profiles of 62 Aeromonas spp were grouped according to the identity of strains using the conventional biochemical scheme of speciation. For two of the three phenospecies, namely A caviae (fig 2) and A hydrophila (fig 3), striking similarities were noted between most isolates. A characteristic four band OMP profile was seen in 24 out of 27 (89%) A caviae, consisting of proteins with mean molecular weights (range) of 54 (51–60), 44 (40–48), 31 (27–35) and 29 (26–33) kilodaltons. A different, but again characteristic, four band protein profile was observed in 10 out of 16 (63%) A hydrophila, consisting of 52 (50–55), 44 (42–64), 33 (31–35) and 28 (27–30) kilodalton proteins. As seen in A caviae, the two high molecular weight OMPs were expressed more strongly than the remainder. Two isolates identified by conventional biochemical criteria as A caviae had OMP profiles typical of A hydrophila (tracks 3 and 4 from the right, fig 2). Both strains were haemolysin negative and identified as A caviae by the API 20NE scheme, and in the suicide phenomenon one was characterised as A caviae and the other “unknown”. A sobria isolates had strongly heterogenous OMP profiles (fig 4), with one or two strongly expressed proteins within the high molecular weight ranges noted for the other two phenospecies. Of the six isolates not identified by conventional criteria, two had OMP profiles similar to the A hydrophila pattern (tracks 1 and 2 from the left, fig 5), while the remainder were unrecognisable. Both of the former isolates were identified as A hydrophila by the suicide phenomenon and API 20NE schemes.

Discussion

The large increase over the past decade in the number of publications on the genus Aeromonas has heralded the acceptance of several new genospecies and phenospecies. Similarly, studies now point to pathogenic roles for Aeromonas spp that were previously unrecognised. For example, we and others have provided evidence that A caviae is implicated as a cause of gastroenteritis in children. A hydrophila and A sobria can cause skin and soft

Figure 2 Outer membrane protein profiles of 27 strains identified as A caviae by conventional criteria. A caviae type strain ATCC 15468 is in track 1 (from the left). Molecular weights are indicated at the far right side.
tissue infection and septicemia in both immunocompromised and immunocompetent individuals. In vivo and in vitro studies indicate that the latter phenospecies are inherently more virulent that *A caviae.*15-17 Unless diagnostic microbiology laboratories are encouraged to isolate and speciate these potential pathogens, further clarification of the behaviour of *Aeromonas* spp will be impeded.

Initial taxonomic studies based on 59 biochemical characteristics, of which seven were found to be of discriminatory value, described two mesophilic *Aeromonas* spp. Later modifications assigned clinical isolates to one of three phenospecies *A hydrophila, A caviae, and A sobria*; this terminology has been applied in this study. Several new phenospecies such as *A trota* and *A jandaei* have been proposed, but these are rarely recovered from clinical specimens.18-19 It is now appreciated that within each phenospecies more than one DNA hybridisation group exists. Nevertheless, most clinical isolates represent DNA groups 1, 4, and 8, which correspond to the phenospecies outlined above.1

Three biochemical phenospeciation schemes have been compared which are amenable for use in the diagnostic laboratory. The gold standard results were taken as those obtained from the conventional scheme, although such assumptions may be flawed. This scheme is time consuming and laborious to perform, and does not easily lend itself to the occasional speciation of small numbers, because of problems with the limited shelf-life of media. When handling large numbers of strains it becomes most unwieldy. Furthermore, the reproducibility and subjectivity of tests such as growth in the presence of potassium cyanide (noted also in the present study) is apparent.20 The variable behaviour of *Aeromonas* spp at 30°C compared with 37°C has been described elsewhere, and the choice of incubation temperature varies between studies.21, 22 Our observations of the variable results of gas production from the fermentation of glucose confirm these findings.

The API 20NE and haemolysin assays were, by contrast, easy to perform. However, the results of this approach to speciation concurred with the conventional scheme for 88% of isolates, compared with a figure of 93% for the suicide phenomenon scheme (p > 0.1). It was interesting to note that the percentage acceptability of the API profile number bore no relation to the correct speciation of *A caviae/A hydrophila* isolates. Hence a strain that had either a high or low probability of being *A hydrophila* could be speciated as *A caviae,* for example. The production of β haemolysin was, however, an accurate discriminator between such isolates. The speciation scheme based on the presence or absence of the suicide phenomenon (the susceptibility of *A caviae* and some *A*...
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sobria to acidic conditions) was accurate, simple, and inexpensive to perform. It requires only basic reagents and in total comprises only two tubes per strain tested. When describing this method of phenospeciation, Namdari and Bottone tested 210 clinical and environmental isolates and found that their results “paralleled” those achieved by conventional criteria.1,2 Whether strains that were only satisfactorily identified by the latter method were included in this analysis is unclear. A simplified key for speciating Aeromonas which can distinguish eight phenotypes using seven tests has recently been described.20 However, several of these species are rarely encountered clinically and the key incorporates antibiotic susceptibility testing using the Kirby-Bauer technique which is not favoured in Europe.

We were interested to compare the results of the above methods with the Aeromonas spp protein profile phenotypes to determine the intra-species homogeneity. Several studies have examined the protein profiles of Aeromonas spp, with widely differing patterns being obtained.8 21 23-25 For example, the number of whole cell proteins clearly identified by SDS PAGE has varied from about six to 50.21 23-25 Our results agree with the higher figure and, particularly when using mini-gel apparatus, this makes the interpretation of profiles difficult. We were unable to identify species specific whole cell protein profiles. An earlier report observed about 20 Aeromonas OMPs, which gave somewhat “busy” protein profiles.7 Our extraction methods identified about four to six strongly expressed OMPs, and distinct patterns were noted for 89% A caviae and 63% A hydrophila, but not for A sobria. Clearly, differing methods of protein extraction, including centrifugation speeds and cell lysis techniques, will influence the purity of OMP preparations, and hence, together with the sensitivity of detection methods, will determine the number of protein bands observed by SDS PAGE. Two unidentifiable isolates and two that were speciated as A caviae by conventional criteria had OMP patterns typical of A hydrophila, and the true identity of these Aeromonas spp remains uncertain.

We have not attempted to redefine or subdivide the phenospecies A caviae, A hydrophila, and A sobria as this was not the aim of the study, and phenotypic criteria alone are insufficiently discriminatory for this purpose. We are presently investigating the potential of genotypic methods such as ribotyping for grouping Aeromonas spp, which may be able to clarify such problems. It seems clear that within the three phenospecies heterogenous strains exist, although this fact does not detract from the broad virulence properties ascribed to date to each group.9 OMP profiles are, however, a useful means of confirming the phenospeciation of A caviae and some A hydrophila isolates, as determined by simple biochemical schemes such as that based on the suicide phenomenon.

The latter technique is particularly suitable for use in the diagnostic microbiology laboratory.


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