Typing of *Aeromonas* species by polyacrylamide-gel electrophoresis of radiolabelled cell proteins

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Summary. A method for typing species of *Aeromonas* by ^{35}S -labelling and sodium-dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) of total soluble proteins is described. Radiolabelled-protein profiles from different strains were compared by use of the Dice coefficient. Typability was 100% and discrimination of pairs of differing strains was satisfactory with acceptable reproducibility. The results also yielded information on subgroups of phenotypic species.

Introduction

The development of a comprehensive typing method for *Aeromonas* is important both for the investigation of outbreaks in hospitals (Cookson *et al.*, 1984; Picard and Goulet, 1984) and for studying the epidemiology and taxonomy of the different species. Biotyping (Cookson *et al.*, 1984), esterase electrophoresis (Picard and Goulet, 1984) and haemagglutination patterns (Adams *et al.*, 1983) have been investigated as typing methods. None of them, however, has been fully evaluated with regard to the three main requirements of any method—typability, reproducibility and discrimination—and none has yet been generally accepted. Serological typing is possible but there is such heterogeneity amongst strains that it is difficult to raise a useful bank of sera. As many as 72% of strains have been untypable in some studies (von Graevenitz, 1984). We describe a new system for typing aeromonads. It is based on sodium-dodecyl sulphate polyacrylamide-gel electrophoresis of ^{35}S -methionine-labelled bacteria (radioPAGE) and was first described by us for typing *Clostridium difficile* (Tabaqchali *et al.*, 1984).

Materials and methods

Bacterial strains

In total, 91 isolates of species of *Aeromonas* from London and North Wales were identified and speciated

by previously described methods (Millership *et al.*, 1983; Barer *et al.*, 1986); 51 isolates were from water or other environmental specimens, 39 from human faeces and one from a perineal swab.

Sixty-three isolates were grouped into 12 different clusters, each of which consisted of isolates considered likely to be of the same strain because they had been isolated from patients and from the water supply in the same hospital, or from members of the same family, or from ponds in the same geographical area. The isolates in each cluster gave identical results or differed from each other in only one biochemical reaction. A further 28 isolates were thought likely to be unrelated to each other or to isolates of the above 12 clusters.

RadioPAGE method

Isolates were stored on nutrient-agar slopes and subcultured every 4–6 months for up to 3 years. They were subcultured from storage on to Horse Blood Agar (Gibco) and incubated overnight at 37°C in air. A portion of one colony of each isolate was inoculated into 100 μl of Methionine Assay Medium (Difco) containing 185 kBq (5 μCi) of ^{35}S -methionine (Amersham International) until the medium was slightly turbid. The samples were incubated at 37°C in air for 2 h and then subcultured to check for purity. After incubation, a 100- μl amount of double-strength sample buffer (Laemmli, 1970) was added to each sample and the soluble proteins dissociated by immersion for 3 min in boiling water.

Sodium-dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970) with a stacking gel containing acrylamide 30 g/L and a resolving gel, 0.75-mm thick, containing acrylamide 100 g/L. Each gel was loaded with 15- μl volumes of extracts from 12–16 samples and one ^{14}C -methylated-protein mixture (Amersham International). Electrophoresis was performed at constant currents of 10 mA for stacking gels and 15 mA for resolving gels. The gels were fixed overnight in an aqueous solution

Received 28 Jul. 1986; revised version accepted 3 Oct. 1986.

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containing glacial acetic acid 20% v/v and propan-2-ol 20% v/v and they were then vacuum-dried on to filter paper. Autoradiographs were developed after exposure for 24 h.

Analysis of autoradiographs

Protein profiles were compared by eye using the Dice coefficient (Dice, 1945) with the following criteria. Lines were considered identical only when their width and intensity, as well as their position, were the same. For adjacent bands on a track to be considered separate, a line of decreased density had to be visible across the whole track. Profiles were compared within gels and between up to four gels run in the same experiment, but it was not considered possible to compare gels from different experiments by eye because of variations in track lengths and overall densities.

Coefficients between pairs of isolates thought likely to be the same or likely to be different, and between pairs from different species, were plotted on frequency histograms. Reproducibility was assessed from the differences between coefficients of pairs of isolates in repeated experiments when the strains had been prepared from storage on each occasion and by comparing the categories to which these pairs were assigned in repeated experiments (see *Results*). Discrimination was analysed on the assumption that each different source should have at least one distinguishable strain of *Aeromonas*; thus, the expected and actual numbers of types could be compared. Discrimination was also assessed by the proportion of pairs of isolates expected to be different that were, in fact, distinguishable.

Relationships between isolates were further defined by computer-aided cluster analysis of the similarity coefficients by the method of variance (Delabre *et al.*, 1973). Standards were not used in the groups so analysed. Thus, the dendrograms constructed illustrate similarities only within a group and do not indicate identity of isolates.

Results

All isolates were typable and each yielded a labelled-protein profile of 40–60 visible bands with mol. wts (10^3) between 14 and 200. The frequency histograms for Dice coefficients between pairs of related and non-related isolates in each species are shown in fig. 1. Isolates of *A. caviae* showed a clear bimodal distribution with some coefficients clustering at 80–100% and others at 20–60%. The high proportion of supposedly non-identical pairs clustering near 100% occurred because water isolates from three separate hospitals proved indistinguishable by this method.

Isolates of *A. hydrophila* behaved like those of *A. caviae*; thus, identical pairs grouped at 75–100% and non-identical pairs at <60%. There was no clear pattern for *A. sobria*, but the similarity

coefficients of 21% of pairs were <70% and for 75% of pairs were <80%.

Reproducibility

The mean difference between coefficients of 71 repeatedly tested pairs of isolates was 9.4%, the modal value was 11% and the range was 20%. Thus, truly identical pairs could have a minimum similarity coefficient of 80%. That corresponded to a mean of 5, and a maximum of 10, differences between these pairs and accorded with the data from frequency histograms. It suggested that pairs of isolates of *A. caviae* and *A. hydrophila* could be categorised as follows: (1) those with a similarity coefficient of >80% were considered indistinguishable; (2) those with a similarity coefficient of 70–80% were of doubtful identity; and (3) those with a similarity coefficient <70% were considered to be definitely different. With these criteria, none of the 71 pairs differed in category on repeated testing.

Discrimination

It was possible to separate the species *A. caviae* and *A. hydrophila* into clusters and individual strains. If the relationship of a pair of isolates was in doubt, the relationships of the isolates of the pair to isolates of other pairs were examined. Thus, for example, if a pair of isolates A and C had a similarity coefficient of 70–80%, but isolate A was indistinguishable from isolate B and isolate B from isolate C, then isolate A was considered to be indistinguishable from isolate C. The proportion of pairs of isolates expected to be different, and which could be distinguished (i.e., a similarity coefficient <70%), was 76%. In one experiment 20 human isolates of *A. caviae* from 17 different sources were assigned to 19 different types. The relationships among the 19 types are illustrated in fig. 2a, with the duplicate strain omitted. Although four pairs of isolates appeared very similar, they were, nevertheless, distinguishable by the above criteria. In another experiment, eight human and 32 water isolates of *A. caviae* from four apparently different sources were distinguished as being isolates of 7 human and 7 water strains; on the other hand, 21 water isolates from three hospitals were indistinguishable. Relationships amongst some of the isolates examined in this experiment were further defined by cluster analysis, as shown in fig. 2b.

Seventeen isolates of *A. hydrophila* from five sources were shown to belong to 11 separate types. The proportion of pairs of isolates thought to be different, and which could be distinguished, was

Fig. 1. Frequency histograms of similarity coefficients between pairs of related and unrelated isolates of species of *Aeromonas*—□, related pairs; ■, unrelated pairs.

96%. Water isolates of *A. hydrophila* and *A. caviae* did not appear similar to isolates from human faeces.

Taxonomy

All isolates of *Aeromonas* had basically similar protein profiles; 12 of the more intense bands, or sets of bands, were reproducible between different

experiments and relatively constant for all isolates (fig. 3). However, further delineation into phenotypic species was not at first possible. For isolates of *A. caviae* in particular, similarity coefficients within the species were of the same order as those found for strains of different species.

This finding suggested that the group of strains defined biochemically as *A. caviae* contained one or more subdivisions. The two groups (I and II) of *A.*

Fig. 2. Dendrograms constructed from cluster analyses of two groups of *A. caviae*: a, unrelated strains from human faeces; b, strains from two different hospitals. Isolates from hospital X were: ■, from human faeces; ●, from taps and drains in a ward kitchen; and ○, from mains water elsewhere in hospital X. Isolates from hospital Y were: □, from water.

caviae that were apparent on cluster analysis (fig. 2a) were also distinguished by differences in the intense bands present at positions 7 and 11 (fig. 3).

The species *A. hydrophila*, on the other hand, appeared to be more homogeneous than *A. caviae*. However, one strain of *A. hydrophila* differed markedly from the other strains, showing differences in the intense bands at positions 3 and 8. All isolates of *A. hydrophila* differed from those of *A. caviae* and *A. sobria* because the former had a broader, more intense band at position 7. Although differences between isolates of *A. sobria* and those of group I of *A. caviae* were not marked, minor differences in the bands at positions 9 and 10 were consistently found with isolates of these groups.

Discussion

RadioPAGE has proved to be a valuable method for typing *C. difficile* (Tabaqchali *et al.*, 1984), methicillin-resistant strains of *Staphylococcus aureus* (Stephenson *et al.*, 1986) and coagulase-negative staphylococci (Stephenson and Tabaqchali, 1986). In the present study, radioPAGE has been used to type isolates of species of *Aeromonas* from human and environmental sources. Typability was 100%, discrimination was good and reproducibility was acceptable. Each strain appeared to

have a unique profile or "fingerprint". *A. sobria* appeared to be more homogeneous than *A. caviae* or *A. hydrophila*, but because all isolates of *A. sobria* had come from a similar geographical area, some apparently unrelated strains may have been isolates of the same strain. It seemed reasonable to assume that pairs of isolates with similarity coefficients < 70% were distinguishable, but there were insufficient pairs available for further analysis.

A Dice coefficient of 70% was taken as the point below which isolates could be considered to be of totally different strains. The doubtful area of 70–80% represented the overlap containing both different strains with similar profiles and identical isolates with a similarity coefficient of < 80%. The complex protein profile of *Aeromonas* was also difficult to interpret visually, and some subjective bias was inevitable. That might be eliminated, and information on variations in intensities analysed, if the data could be processed by computer. A computer-linked radioscaner is currently being developed.

The finding of a common type of *A. caviae* in three separate hospitals suggested that it was present in the water before distribution and the water authority confirmed that all hospitals were served by the same treatment works. Similar observations have been made elsewhere (Burke *et al.*, 1984). We have not, however, been able to find

Fig. 3. Protein profiles obtained by radioPAGE of isolates of *Aeromonas*—track 1, *A. caviae* group II; 2–5, *A. caviae* group I; 6–8, *A. sobria*; 9, atypical strain of *A. hydrophila*; 10 and 11, typical strains of *A. hydrophila*; 12 and 13, identical strains of *A. caviae* from two members of the same family. The 12 relatively constant and intense bands (▶) are designated 1–12 from the top of the gel; *, mol. wt markers.

similar human isolates, as might have been expected if *Aeromonas* was present in tap water. Accordingly, the assumption that human aeromonads were acquired from drinking water is of doubtful validity.

One advantage of radioPAGE is that relationships between isolates can be expressed in numeric form, allowing analysis by average and single-linkage methods. Thus, it is possible to avoid the kind of nonsensical conclusions that, although pairs of isolates A and B and B and C are related, isolates A and C are not themselves related. When many

isolates are examined, the large amount of data produced can be sorted quickly by computer and this is particularly important with coefficients in the “doubtful” area.

This kind of analysis may also help to distinguish taxonomic subgroups such as those within the species *A. caviae*, a division indicated by results from DNA-homology studies (Popoff *et al.*, 1981). Thus, radioPAGE is a powerful tool for the distinction of individual strains of bacteria and for their separation into larger groups.

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