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TOTAL PROTEIN ELECTROPHORESIS AND RAPD FINGERPRINTING ANALYSIS FOR THE IDENTIFICATION OF AEROMONAS AT THE SPECIES LEVEL.

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SHORT COMMUNICATION

ABSTRACT

Fifteen well-defined strains of *Aeromonas* of thirteen species were analyzed by SDS protein electrophoretic analysis (SDS-PAGE) and random amplified polymorphic DNA analysis (RAPD). The comparison between the patterns obtained by both methods allowed differentiating all the strains. Clusters formed by the unweighted pair group method with arithmetic averages applied to protein data correlates with the genetic and biochemical information about the species. The results show that protein fingerprinting has the potential to differentiate *Aeromonas* species, but the low qualitative variation indicates that this technique is not efficient for the characterization of strains within a species. Conversely, RAPD fingerprinting allows the identification of strains but the high variability limits its potential as an aiding method for species identification.

Key words: *Aeromonas*, molecular markers, species identification

The genus *Aeromonas* comprises several species of oxidase negative and catalase positive, glucose-fermenting, facultative anaerobic, gram-negative, rod-shaped, motile and non-motile bacteria. They are widely distributed in nature, especially in aquatic environments, and have been isolated from a variety of raw foods. Several species of the genera have been associated with several diseases in both warm and cold blood animals (fishes, reptiles, etc.) (6). In humans, they are opportunistic pathogens causing gastroenteritis, and less commonly, cellulitis, wound infections, meningitis, otitis, peritonitis, endocarditis and septicemia (10).

The taxonomy of the genus *Aeromonas* is confuse and controversial (4). The need of a system for the identification and classification of *Aeromonas* isolates is justified by their ecological and clinical importance. Different methods as biotyping (22), isozyme electrophoretic analysis (21), DNA hybridization (23), lipopolysaccharide analysis (27), serotyping (7), ribosomal DNA typing (1), SDS-PAGE analysis of cell proteins (14,15), RAPD markers (16,17), AFLP fingerprinting (8), and PCR (18,19) have been used to type isolates. However, these methods are not generally accepted as standard systems for the evaluation of *Aeromonas* isolates, as a standard method should be simple, rapid, inexpensive, reliable, and applicable in any kind of routine laboratory. SDS-PAGE analysis of cell proteins and RAPD analysis, two methods that have most of these characteristics, have been tested for isolates identification (14, 15, 16, 17). However, few attempts have been made to evaluate their usefulness for the characterization of *Aeromonas* at the species level. Therefore, the purpose of this study was to compare the efficiency of SDS-PAGE and RAPD analysis for the differentiation of *Aeromonas* species.

The strains used in this work were: *A. hydrophila* (ATCC 7966 A, obtained from Dr. Naharro, ATCC 7966 B, obtained from the CCT; and CECT 398), *A. allosaccharophila* (ATCC 51208),

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A. caviae (ATCC 15468), *A. ichtiosmia* (ATCC 49904), *A. euchrenophila* (ATCC 23309), *A. enteropelogenes* (ATCC 49803), *A. trota* (ATCC 49657), *A. salmonicida* var. salmonicida (ATCC 33658), *A. media* (ATCC 33907), *A. veronii* (ATCC 35624), *A. encheleia* (CECT 4341), *A. sobria* (ATCC 43979), *A. hydrophyla* var. punctata (ATCC 14486), and clinical isolates of *Escherichia coli, Pseudomonas aeruginosa, Enterobacter cloacae, Salmonella typhimurium, Staphylococcus epidermidis,* and *Citrobacter* spp.

For SDS-PAGE analysis, cultures were grown over-night on LB broth (1.0 ml) and centrifuged on microtubes. The pellets were washed with water, and suspended in sample buffer. The proteins were dissociated by immersion for 5 min in boiling water. The samples were centrifuged to eliminate cell debris and used directly for electrophoretic separation (11).

Sodium-dodecyl sulphate polyacrilamide-gel electrophoresis (SDS-PAGE) was performed according to Laemmli (11) with a stacking gel containing 4.5% acrilamide and a resolving gel containing 12% acrilamide. Samples with 120 to 150 µg of proteins, as determined by mini Bradford method (2), were loaded in each track. Electrophoresis was performed at constant voltage of 80V for stacking gels and 150V for resolving gels. The gels were fixed for 15 min in an aqueous solution containing 7% glacial acetic acid and 30% methanol and stained over-night in 0.1% (w/v) Coomassie Brilliant Blue R-250 solution (3). All samples were prepared and examined in triplicate on different gels. After several destaining steps, the gels were photographed on a high intensity light box. The protein profiles were compared by eye. The proteins (bands) were listed as discrete character states per strain (presence/absence). Bands were considered identical only when their width, intensity and position were the same.

For randomly amplified polymorphic DNA analysis (RAPD), bacterial cultures were grown in 1.0 ml of LB broth at 18ºC for 24h, centrifuged at 15000xg for 5 min to pellet the cells. Total DNA was isolated by the method described by Pan *et al.* (20). DNA content of all samples was measured using spectrophotometer at 260 nm. DNA purity was evaluated by the 260/280 ratio and gel electrophoresis. All extracts were diluted to working solution of 10ng μl^{-1}.

The polymerase chain reaction DNA amplification protocol was a variation of that reported by Williams *et al.* (29). Reactions were performed in 25 µl volume containing 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 3 mM $MgCl_2$; 0.25% Triton-X-100; 1.25 mM of dNTP (Pharmacia LKB Biotechn.); 30 ng of single decamer primer (40 primers of kits A and B of Operon Techn.); 60 to 80 ng of genomic DNA; and 1.5 units of Taq DNA polymerase (Pharmacia LKB Biotechn.). DNA amplification was performed using a thermal cycler (model PTC100, MJ Research, Watertown, Mass.). The thermal cycle used was 94ºC for 1 min; then 45 cycles of 94ºC (1 min), 35ºC (1 min) and 72ºC (2 min), and finally 72º for 3 min. A negative control including all components except genomic DNA was included in all thermal cycle runs.

Following amplification, the RAPD products (20 µl) were loaded in 1.5% agarose gels in TBE buffer (89 mM Trisma-base, 89 mM boric acid and 8 mM EDTA) and resolved by electrophoresis. After electophoresis the amplification products were stained with ethidium bromide (0.5 µg ml^{-1}) and photographed under UV light. The size of amplification products were determined by comparison with Lambda DNA digested with *Eco*RI and *Hind*III restriction enzymes.

Bands were scored as present or absent. Bands that were not well defined were not included in the data set as these were assumed to be unreliable markers.

Total protein and RAPD data were analyzed using NTSYS-pc package, version 1.5 (26). Similarities were computed using the Jaccard´s coefficient, and strains were clustered by the unweighted pair-group method using arithmetic averages (UPGMA) in order to present the results in the form of dendrograms.

The fifteen *Aeromonas* strains evaluated yielded similar protein electrophoretic patterns with a high number of bands (>50 bands). However, based on the reproducibility and the criteria adopted for the analysis of the gels, the presence or absence of a total of 24 protein bands with molecular weights between 14000 and 65000 daltons were scored. The patterns obtained in three independent gels were very similar confirming the high reproducibility of protein fingerprinting analysis (14).

Protein profiles were very similar among the strains, and several strains exhibited characteristic proteins that may be useful markers for the identification at the species level: *A. allosaccharophila* (35.5 kDa), *A. sobria* (39.0 kDa), and *A. trota* (40.0, 27.0, and 24.5 kDa).

Using the unweighted pair group method with arithmetic averages for clustering, we identified a total of four clusters at the 70% hierarchical level (Fig. 1). *A. salmonicida* and *A.sobria* formed independent clusters, groupS 1 and 2, respectively. *A. salmonicida*, a non motile species included in the hybridization group 3 (24), and *A. sobria*, hybridization group 7, have been separated by different methods as immunoblotted SDS-PAGE gels (15,28), 16S rDNA sequencing (13), RAPD (17), and AFLP (9) *A. enteropelogenes* and *A. trota* cluster together within group 3. These data confirm previous results that considered these species as identical or similar by comparing their 16S rRNA sequence (5), and their AFLP profiles (8).

The fourth group was subdivided at the 85% similarity level into three subgroups. Subgroup 4.1 was formed by *A. euchrenophila* and *A. encheleia*, two species that cluster together when analyzed by AFLP (8), but are separated in different hybridization groups, group 6 and 16 (4, 9). Subgroup 4.2 was formed by *A. hydrophila* CECT 398 and *A. veronii* ATCC 35625, with 98% similarity between them, and *A. caviae* ATCC 15468, *A. ichtiosmia* ATCC 49904 and *A. media* ATCC 33907, all from the *A. caviae* group (12, 24). Subgroup 4.3 was formed by the two samples of *A. hydrophila* ATCC 7966, one obtained

from the "Coleção de Culturas Tropicais", and the other one from the Animal Pathology Department, University of León, confirmed by their protein fingerprinting as identical, and *A. allosaccharophila* ATCC 51208, a new species proposed by Martinez-Murcia *et al.* (13).

For RAPD analysis an initial screening of primers was performed. DNA of *A. hydrophila* CECT 839 and *A. trota* ATCC 49657 were amplified using the 40 decamer primers of the kits A and B of Operon Technologies. The results obtained in these first experiments were used to select 14 primers that gave at least four intense amplification products for each species: OPA-02, OPA-04, OPA-09, OPA-11, OPA-19, OPB-04, OPB-06, OPB-07, OPB-10, OPB-12, OPB-13, OPB-15, OPB-16 and OPB-17.

The selected primers were used to analyze the 15 strains of *Aeromonas*. Using the 14 selected primers 290 RAPD bands were scored (4 to 18 bands per primer), ranging in size from 100 to 2700 base pairs. Of the total bands scored, 280 (95%) were polymorphic. This percentage of polymorphic bands (95%) is extremely high when compared with the variation reported at the species level for other organisms, but is comparable to that obtained Oakey *et al.* (17) when comparing the RAPD patterns for some species of *Aeromonas*. Despite the high variation observed, RAPD profiles exhibited ten bands that were common to all the *Aeromonas* species, and absent in clinical isolates of *Escherichia coli, Pseudomonas aeruginosa, Enterobacter cloacae, Salmonella typhimurium, Staphylococcus epidermidis*, and *Citrobacter* spp. These bands can be cloned and sequenced to design PCR primers that may be useful for the rapid detection of aeromonads. This approach has been previously used to design PCR primers for the identification of *A. salmonicida* (18) and *A. hydrophila* (19).

The ability to differentiate all tested strains by RAPD suggests that this technique may be practically applied for the identification of *Aeromonas* isolates, being necessary for this purpose, the amplification with one or two selected primers. Even showing very different profiles, ten bands were common to all the strains.

Comparing the RAPD patterns of the 15 *Aeromonas* strains evaluated, we construct a matrix that was used to calculate the Jaccard´s similarity between strains. As expected, the highest similarity (96.77%) was obtained between the two representatives of *A. hydrophila* ATCC 7966. The lowest similarity was observed between *A. enteropelogenes* and *A. hydrophila punctata* (20.17%).

The unweighted pair group method with arithmetic averages applied to the RAPD data, did not allow the clear separation of clusters among the tested strains. Similar results were obtained by Huy *et al*. (8) using AFLP analysis. These results support

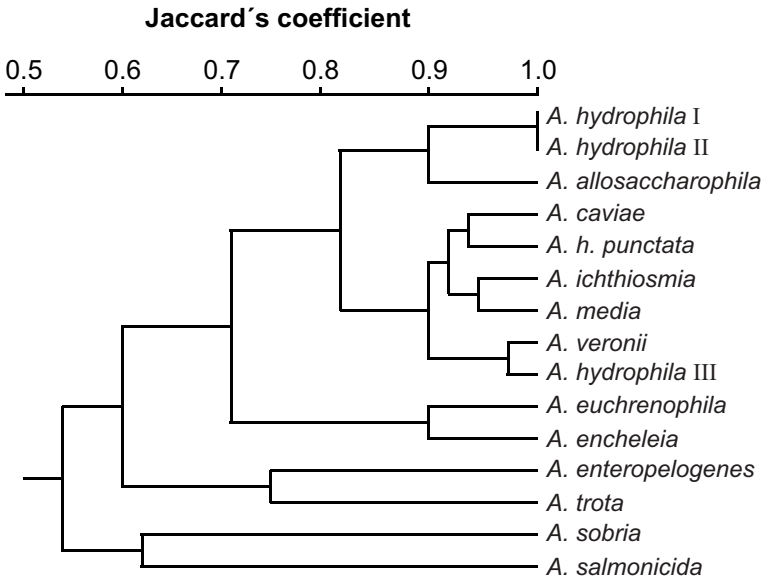

Figure 1. Dendrogram based on UPGMA analysis of the phenetic similarity between fifteen *Aeromonas* strains as determined by their protein profiles on SDS-PAGE. (*A. hydrophila* I and II – ATCC 7966 A and B, respectively; *A. hydrophila* III- CECT 398).

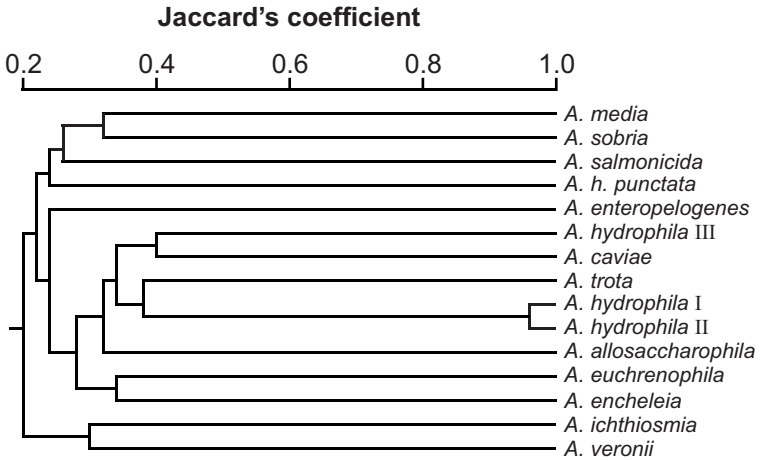

Figure 2. Dendrogram based on UPGMA analysis of the phenetic similarity between fifteen *Aeromonas* strains as determined by 290 RAPD bands. (*A. hydrophila* I and II – ATCC 7966 A and B, respectively; *A. hydrophila* III- CECT 398).

the existing classification of *Aeromonas* in several species, since each type strain gave different RAPD patterns.

The present results shows that RAPD analysis in *Aeromonas*, with the set of primers tested, even efficient for the discrimination among isolates (17) is not useful for the characterization of strains at the species level and the evaluation of relationships among *Aeromonas*. Conversely, the results obtained by the analysis of cell protein profiles correlates with the genetic and biochemical data previously reported by other authors, using different analytical methods, and can be used as a rapid, inexpensive and reliable system to help in identification and taxonomy of *Aeromonas* isolates.

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RESUMO

Análise eletroforética de proteínas totais e marcadores de RAPD na identificação de *Aeromonas* ao nível de espécie

Quinze linhagens de *Aeromonas* pertencentes a treze espécies foram avaliadas através de eletroforese de proteínas totais (SDS-PAGE) e segmentos de DNA amplificados ao acaso (RAPD). A comparação entre os padrões obtidos por ambos métodos permitiu diferenciar todas as linhagens. Agrupamentos formados com base nos dados protéicos mostraram relação com informações bioquímicas e genéticas a respeito das espécies. Os resultados mostraram que análises protéicas têm potencial para diferenciar espécies de *Aeromonas*, mas a baixa variação qualitativa indica que esta técnica não é eficiente para a caracterização entre linhagens dentro de espécies. Ao contrário, marcadores de RAPD permitem identificar linhagens, mas a alta variabilidade limita seu potencial como método auxiliar na identificação de espécies.

Palavras-chave: *Aeromonas*, marcadores moleculares, identificação de espécies.

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