JOURNAL OF CLINICAL MICROBIOLOGY, July 1997, p. 1671–1674 Vol. 35, No. 7
0095-1137/97/$04.00+0

Identification of *Aeromonas* Clinical Isolates by Restriction Fragment Length Polymorphism of PCR-Amplified 16S rRNA Genes

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Received 27 November 1996/Returned for modification 26 February 1997/Accepted 26 March 1997

Identification of *Aeromonas* species, emergent pathogens for humans, has long been controversial due to their phenotypic and genomic heterogeneities. Computer analysis of the published 16S rRNA gene sequences revealed that restriction fragment length polymorphism of the PCR-amplified 16S rRNA gene is a good and rapid way of assessing the identities of all known species of *Aeromonas*. The method was evaluated with the reference strains of all species (or DNA homology groups) and 76 clinical isolates of diverse origin. Most results from the two approaches were in agreement, but some discrepancies were discerned. Advantages over previous phenotypic and genetic methods are discussed.

Aeromonads are common microorganisms in freshwater and estuarine environments and have long been recognized as occasional pathogens of reptiles, fish, and different mammals (8, 14, 15, 21). They have been implicated in the etiology of a variety of systemic and localized human diseases (18), especially soft-tissue infections and septicemia. There is an increasing interest in their role as a cause of acute gastroenteritis, although Koch's postulates have not been fulfilled (there is no animal model) and no outbreak attributable to aeromonads has ever been described (25). *Aeromonas* species produce a variety of virulence factors (9, 17), and they are among the few microorganisms that can produce carbapenemases (28).

In the last decade, the classification of the genus *Aeromonas* has undergone major changes. In *Bergey's Manual of Systematic Bacteriology* the genus was divided into three mesophilic and motile species (*A. hydrophila*, *A. caviae*, and *A. sobria*) and the psychrophilic nonmotile species *A. salmonicida* (27). Extensive DNA-DNA hybridization studies have resulted in the recognition of 13 well-defined genomic species (8, 14, 15, 21). However, some discrepancies remain between observed DNA homology groups (DHG) and those obtained by phenotypic analysis. Three new genomic species, *A. allosaccharophila*, *A. bestiarum*, and *A. encheleia*, have recently been described (2, 12, 24). The proposed species *A. ichthiosmia* (30) and *A. enteropelogenes* (29) appeared to be identical to *A. veronii* and *A. trota*, respectively, on the basis of phylogenetic evidence (10). A comprehensive phylogenetic analysis of the genus *Aeromonas* by 16S rRNA gene sequencing has been reported (23, 24). Overall percent sequence similarity between the *Aeromonas* species was found to be very high, but all known named species possessed characteristic 16S rRNA sequences.

Although certain biochemical tests allowed for some improvements (1, 7), phenotypic identification of the genomic species mentioned above appeared to be difficult. A clear-cut differentiation of all species was not possible even by using chemotaxonomic markers (4, 17, 20) and ribotyping of restricted genomic DNA (22). Identification by PCR with 16S rRNA gene-targeted oligonucleotides has been evaluated for *A. sobria*, *A. schubertii*, and *A. jandaei* (5, 6) and for *A. hydrophila* and *A. veronii* (11). However, some species had identical sequences in this selected target region and therefore could not be distinguished.

We report a rapid method for identification of all known phylogenetically established *Aeromonas* species, based on the restriction patterns of the PCR-amplified 16S rRNA genes. The new method has been evaluated with the reference strains and applied to 76 biochemically characterized clinical isolates from different samples and sources.

MATERIALS AND METHODS

Bacterial strains and cultivation. The type strains of the *Aeromonas* species used in this study are listed in Table 1. A total of 76 *Aeromonas* strains were isolated from different clinical samples: stool, blood, soft tissue infection (cellulitis, wound exudates, and skin abscesses), urine, and pleural, joint, and ascitic fluids. They were collected from different geographical locations in Spain: 5 from Hospital Son Dureta, Palma de Mallorca; 11 from Hospital Sant Joan, Reus; 19 from Hospital Vall d'Hebrón, Barcelona; 14 from Hospital Sant Pau, Barcelona; 14 from Hospital Clinic, Barcelona; 8 from Fundación Jimenez Díaz, Madrid; and 5 from Hospital de la Princesa, Madrid. All strains were grown on Trypticase soy agar, incubated at 28 to 30°C for 24 h, and stored in 15% glycerol–Trypticase soy broth at −70°C.

Biochemical tests. Clinical isolates were considered presumptive *Aeromonas* spp. if they were gram negative and cytochrome oxidase positive, fermented D-glucose (triple sugar iron [TSI]), grew in nutrient broth–6% NaCl, and did not produce acid from inositol. Species identification was done by the scheme of Abbott et al. (1), based on the biochemical tests listed in Table 2, but bile esculin hydrolysis was done by another previously described method (8). Sugars were tested at 35°C. Salicin, D-sorbitol, and citrate were also tested at 30°C for the *A. hydrophila* complex (DHG 1, DHG 2, and DHG 3). Positive and negative controls were included.

Computer analysis of the 16S rRNA gene sequences. For restriction endonuclease selection, published 16S rRNA gene sequences (23, 24) (data for *A. encheleia* are from Martínez-Murcia [22a]) were analyzed by using the DIGEST and RESTRI programs of the PC/Gene package in a personal computer. Lists of the different digestion DNA fragments were obtained, and the analysis of theoretical banding patterns for *Alu*I and *Mbo*I revealed a good species discrimination. These enzymes do not differentiate the species *A. salmonicida*, *A. encheleia*, or *Aeromonas* spp. (DHG 11). For these species the enzymes *Nar*I and *Hae*III were selected.

DNA extraction and purification. DNA was extracted by using the InstaGene DNA Purification Matrix (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. The resulting DNA was diluted to a spectrophotometrically estimated concentration of 0.2 to 0.6 μg/μl and was kept at −20°C when stored.

Synthesis of primers. Primers 5′-AGAGTTTGATCATGGCTCAG-3′ (forward) and 5′-GGTTACCTTGTTACGACTT-3′ (reverse), at positions 8 to 27

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TABLE 1. DNA fragments obtained from the 16S rRNA gene sequence analysis of all known *Aeromonas* spp. (or DHGs) with endonucleases *Alu*I and *Mbo*I

Species	Reference strain	Presence of 16S rRNA gene RFLP fragments of the following size (bp):																						
		346	242	228	211	207	204	195	188	180	174	172	165	158	157	138	118	78	69	66	54	47	42	40	33
A. hydrophila (DHG 1)	CECT839^T	x				x		x					x		x	x			x	x	x		x		x
A. salmonicida (DHG 3)[a]	CECT894^T	x				x		x							x	x	x		x	x	x	x	x		x
A. caviae (DHG 4)	CECT838^T					x		x	x	x			x	x	x				x	x	x				x
A. media (DHG 5)	CECT4232^T					x		x			x	x	x		x	x			x	x	x		x		x
A. eucrenophila (DHG 6)	CECT4224^T				x	x		x			x	x				x	x		x	x		x	x		x
A. sobria (DHG 7)	CECT837^T					x		x			x			x	x	x	x		x	x	x	x	x		x
A. veronii biogroup *sobria* (DHG 8)[b]	CECT4247^T					x		x			x			x	x	x		x	x	x	x	x	x	x	x
A. jandaei (DHG 9)	CECT4228^T					x		x	x					x	x	x		x	x	x	x	x	x	x	x
A. schubertii (DHG 12)	CECT4241^T			x		x		x		x		x			x			x	x	x		x		x	x
A. trota (DHG 13)	CECT4255^T		x			x		x		x			x	x	x				x	x					x
A. allosaccharophila (DHG 14)	CECT4199^T					x	x	x			x	x			x		x		x		x	x	x		x

[a] Identical profiles for *A. bestiarum* CECT4247^T (DHG 2), *Aeromonas* sp. CECT4253^T (DHG 11), and *A. encheleia* CECT4342^T.
[b] Identical profile for *A. veronii* biogroup *veronii* CECT4257^T (DHG 10).

and 1509 to 1491, respectively, of the *Escherichia coli* numbering system, were synthesized in an Applied Biosystems 396 DNA/RNA synthesizer. The product was kept at 55°C overnight, vacuum dried, and resuspended in sterile distilled water to a final concentration of 50 pmol/μl.

PCRs. PCR experiments were carried out on an OmniGene thermal cycler (Hybaid). A final volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM (each) deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP; Pharmacia LKB Biotechnology), 2 U of *Taq* I DNA polymerase (Promega Corp.), a 2-μl aliquot of DNA sample, and 1 μl of each primer was added for every reaction. Reaction mixtures were overlaid with mineral oil (Light White Oil; Sigma). PCRs were performed under the following conditions: denaturation at 93°C for 3 min, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min. After the final cycle, extension at 72°C was allowed for 10 min. Negative controls to which no template DNA was added were also included in every set of reactions. PCR products were precipitated, dried, and resuspended in 25 μl of sterile water.

Endonuclease digestions, electrophoresis, and pattern analysis. Enzymatic digestions were performed by incubating 5 μl of the amplification products with 5 U of each enzyme (*Alu*I and *Mbo*I or *Nar*I and *Hae*III; Boehringer) and 2 μl of the corresponding 10× buffer (buffer A for *Alu*I and *Mbo*I and buffer M for *Hae*III and *Nar*I) in a total volume of 20 μl. The reaction mixture was incubated overnight at 37°C. Aliquots of 10 μl of each restriction reaction mixture were mixed with 2 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol), and the mixture was electrophoresed on a 4% Metaphor agarose gel (FMC BioProducts) in 0.5× TBE (Tris-borate-EDTA) buffer. Gels were stained with ethidium bromide (0.5 μg/ml) and photographed on a UV transilluminator. The molecular sizes of the fragments obtained (in base pairs) were estimated by using the GelBase Windows Software for the UVP's System 5000 (Ultra Violet Products), with Marker V (Boehringer) used as a molecular size reference.

RESULTS

Species of the genus *Aeromonas* exhibit very high levels of overall 16S rRNA gene sequence similarity; in nucleotides, this corresponds to 1 to 32 base differences (23, 24). Some species show very few differences, for example, *A. hydrophila* and *A. media* (three nucleotides) or *A. caviae* and *A. trota* (one nucleotide). Due to these circumstances, the search of endonucleases for differentiating 16S rRNA genes by restriction fragment length polymorphism (RFLP) analysis was not easy, and finally, a combination of two enzymes was needed. Endonucleases *Alu*I (AGCT) and *Mbo*I (GATC) were selected after a computer analysis of many restriction enzymes that are known to cleave the published 16S rRNA gene sequences of *Aeromonas* spp. The resulting DNA fingerprints (ranging from 33 to 346 bp) constituted specific patterns that could be used to identify strains to the level of phylogenetic species (Table 1). The RFLP patterns of the PCR-amplified 16S rRNA genes of some *Aeromonas* species or DNA homology groups are presented in Fig. 1. Only RFLP fragments ranging from 69 to 346 bp were taken into account. Duplicates of the type strain of *A. media* showed the same RFLP pattern (Fig. 1, lanes 4 and 6). The pattern of "*A. enteropelogenes*" was identical to that of *A. trota*, according to previously reported data (10). The three species *A. salmonicida*, *A. encheleia*, and *Aeromonas* sp. (DHG 11) could not be distinguished with these two enzymes, but they were differentiated by using enzymes *Nar*I (GGCGCC) and *Hae*III (GGCC). The cleavage site of *Nar*I was found only once, at position 452 of the 16S rRNA gene of *A. salmonicida*, but not in *A. encheleia* or *Aeromonas* sp. (DHG 11). Endonuclease *Hae*III cut 10 times in the 16S rRNA gene of *A. salmonicida* and *A. encheleia*, but one more site (position 465) was found in the sequence of *Aeromonas* sp. (DHG 11), resulting in the digestion of a DNA fragment of 317 bp and generating two fragments of 262 and 55 bp (data not shown).

Isolates from clinical samples that belonged to the genus

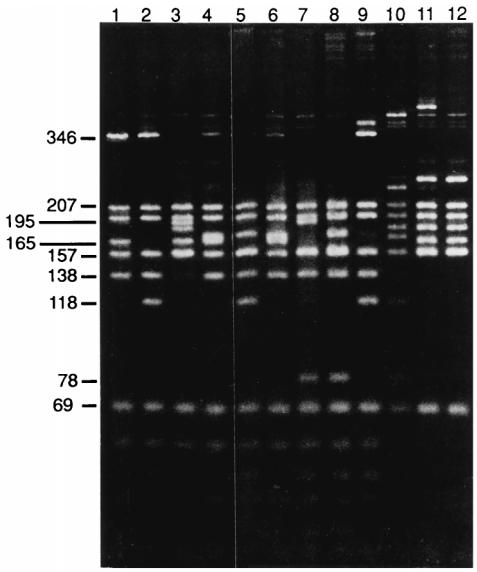

FIG. 1. Agarose gel showing the RFLP patterns, obtained by using endonucleases *Alu*I and *Mbo*I, of 16S rRNA genes amplified by PCR. Lanes: 1, *A. hydrophila* CECT839^T; 2, *A. salmonicida* CECT894^T; 3, *A. caviae* CECT838^T; 4, *A. media* CECT4232^T; 5, *A. sobria* CECT837^T; 6, *A. media* ATCC 33907^T; 7, *A. jandaei* CECT4228^T; 8, *A. veronii* biogroup *veronii* CECT4257; 9, *Aeromonas* sp. strain CECT4253^T (DHG 11); 10, *A. schubertii* CECT4241^T; 11, *A. trota* CECT4255^T; and 12, "*A. enteropelogenes*" DSM6394. Numbers on the left are molecular sizes (in base pairs).

TABLE 2. Biochemical characteristics of the *Aeromonas* species identified by RFLP analysis of the 16S rRNA gene

Phylogenetic sp.[a]	Phenotypic sp.[b]	Result of the following biochemical test[c]:										
		L/O/A	BEA	GAS	VP	A/M/S	RAM	SOR	SALI	LAC	MAN	CIT
A. hydrophila (12)[d]	*A. hydrophila* (10)[d]	+/−/+	+	+	+	+/+/+	−	−	+	−	ND	ND
	Unidentified (1)	+/−/+	−	+	+	+/+/−	ND	ND	−	ND	ND	ND
	A. veronii biogroup *sobria* (1)	+/−/+	−	−	+	+/+/+	+	−	−	−	ND	ND
A. veronii (35)	*A. veronii* biogroup *sobria* (30)	+/−/+	−	±	+	−/+/+	ND	ND	−	ND	ND	ND
	A. hydrophila (3)	+/−/+	+	+	+	−/+/+	ND	ND	+	ND	ND	ND
	Unidentified (1)	−/−/+	−	−	+	−/+/+	ND	ND	ND	ND	ND	ND
	Aeromonas sp. DHG 2 (1)	+/−/+	+	+	+	+/+/+	−	−	−	−	ND	ND
A. caviae (22)	*A. caviae* (15)	+/−/+	+	−	−	ND	ND	ND	ND	ND	−	+
	A. caviae/*A. media* (4)	−/−/±	±	−	−	+/+/+	−	−	−	±	+	+
	A. hydrophila (1)	+/−/+	+	+	+	+/+/+	ND	ND	ND	ND	ND	ND
	A. veronii biogroup *sobria* (1)	+/−/+	ND	+	+	−/+/+	ND	ND	ND	ND	ND	ND
	Unidentified (1)	−/−/−	+	−	−	ND	ND	ND	ND	ND	ND	ND
A. sobria (1)	Unidentified (1)	+/−/−	−	−	−	+/+/+	ND	ND	ND	ND	ND	ND
A. media (2)	*A. caviae*/*A. media* (2)	−/−/+	−	+	−	+/+/+	−	−	−	±	+	+
A. salmonicida (4)[e]	*Aeromonas* sp. DHG 2 (3)	±/−/±	+	±	±	+/+/+	ND	ND	ND	±	−	−
	A. salmonicida (1)	−/−/−	+	+	+	+/+/+	−	+	−	−	−	+

[a] Determined by analysis of 16S rRNA genes.
[b] Following RFLP tests.
[c] L/O/A, lysine decarboxylase/ornithine decarboxylase/arginine dihydrolase; BEA, bile esculin hydrolysis; GAS, gas production from TSI; VP, Voges-Proskauer test; A/M/S, acid from arabinose/acid from mannitol/acid from sucrose; RAM, acid from D-rhamnose; SOR, acid from D-sorbitol; SALI, acid from salicin; LAC, acid from lactose; MAN, acid from mannose; CIT, utilization of citrate; ND, not done.
[d] The numbers of isolates tested are indicated in parentheses.
[e] Acid from sorbitol and utilization of citrate were determined at 30°C.

Aeromonas were subjected to biochemical characterization following the scheme proposed by Abbott et al. (1), and the results are listed in Table 2. The same strains were analyzed by RFLP of PCR-amplified 16S rRNA genes with the selected enzymes *Alu*I and *Mbo*I. Most results from the two different approaches were in agreement, but some discrepancies appeared (Table 2). Two presumed *A. veronii* isolates were classified as *A. hydrophila* and *A. caviae* by RFLP analysis. Three *A. veronii* strains and a strain of *A. caviae*, as shown by the 16S rRNA gene restriction patterns were biochemically characterized as *A. hydrophila*. Six strains that were named *A. caviae* or *A. media*, biochemically indeterminate (mannose and citrate positive), were classified by RFLP analysis as *A. caviae* (four strains) and *A. media* (two strains). The four biochemically unidentified strains showed the RFLP patterns of *A. hydrophila* (DHG 1), *A. veronii* (DHG 8/10), *A. caviae* (DHG 4), and *A. sobria* (DHG 7). Biochemical and RFLP PCR tests were repeated and the results were confirmed.

DISCUSSION

In the present study we have applied the biochemical tests proposed by Abbott et al. (1) to 76 clinical isolates also characterized by RFLP analysis of the PCR-amplified 16S rRNA gene. Most results from both approaches were in agreement, but some discrepancies were discerned. For example, a strain biochemically identified as *A. veronii* (bile esculin hydrolysis and acid from salicin negative; Table 2) showed the RFLP pattern of the genomic species *A. hydrophila*. Vice versa, three strains initially identified as *A. hydrophila* (bile esculin hydrolysis and acid from salicin positive) showed the RFLP patterns of *A. veronii*. This suggests that the hydrolysis of esculin and fermentation of salicin may lead to incorrect determinations, particularly for *A. hydrophila* and *A. veronii*, as reported previously (21). Two strains phylogenetically identified as *A. caviae* showed two differences from the usual biochemical pattern of this species (gas production from TSI and Voges-Proskauer test positive; Table 2) and, therefore were initially supposed to be *A. hydrophila* and *A. veronii*. Additional tests for separating species of the *A. hydrophila* complex (DHG 1, DHG 2, DHG 3) have recently been proposed, but problems with the *A. caviae* and *A. media* pair still remain (19).

Of the currently described *Aeromonas* spp. (or DHGs) all except *A. eucrenephila* (DHG 6) and *A. sobria* (DHG 7) have been recovered from clinical material. Similar to a previous survey (21), the species most frequently found in our clinical samples were *A. veronii* (46%), *A. caviae* (29%), and *A. hydrophila* (16%), covering ca. 90% of all strains isolated. The reasons why these species predominate in clinical material remain unknown, although it may be due to their relative pathogenicity for humans. Although it is unusual in clinical samples, we have isolated a strain that showed the 16S rRNA gene RFLP profile of *A. sobria* (DHG 7), the biochemical determination of which was uncertain, and therefore, its identity remains unresolved. Perhaps clinical strains of this genomic species have previously been misidentified because of their phenotypic intraspecific heterogeneity (17). Methods such as serotyping (19), sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins (31), and phage typing (3) are very useful for differentiating individual strains, but they are too sensitive for species delineation. Generally, phenotyping (18), multilocus enzyme electrophoresis (4), and cellular fatty acid composition (17, 20) correlated well with DNA-DNA hybridization, but a clear-cut differentiation of all species was not possible. Ribotyping of restricted genomic DNA (22) and the AFLP method (16) may be used for identification, but a number of intraspecies heterogeneities in the DNA patterns appeared and results should be interpreted carefully. Differenti-

ation of *Aeromonas* spp. by randomly amplified polymorphic DNA analysis has recently been reported (26). Although the randomly amplified polymorphic DNA method has largely been used for typing strains, patterns are often too diverse within a species (13) and, therefore, may lead to an incorrect species determination. The determination of 16S rRNA sequences represents an unequivocal means of determining bacterial taxa above the species level (32). The 16S rRNA genes of all known *Aeromonas* spp. (except *A. bestiarum*), although highly similar, showed unique primary structures (23, 24). In those previous studies, the DHG 8-DHG 10 pair revealed identical sequences, indicating that they are two biogroups of the same species, *A. veronii*. Identical sequences were also found in *Aeromonas* sp. strain CIP 7430 (DHG 2) and *A. salmonicida* (DHG 3) (23); this problem still remains to be solved. Identification by sequencing the small-subunit rRNA is time-consuming and too expensive for large-scale isolations. Oligonucleotide probes designed from highly variable regions may help in the rapid screening of isolates, but a number of different probes are required. Moreover, identical target regions make it difficult to differentiate the pairs *A. caviae-A. trota*, *A. eucrenophila-A. media*, and *A. hydrophila-A. encheleia* (5, 6, 11, 23).

The new method developed in the present study may serve to identify almost all known *Aeromonas* spp. This strategy provides information about many variable sequence sites, and RFLP patterns different from those in Table 1 may be expected if restriction sites are affected by intraspecific nucleotide diversity or the digested sequence belongs to an *Aeromonas* sp. not yet described. Species determination, on the basis of phylogenetic relationships, may be carried out in a short period of time at a reasonable cost for a large number of isolates. To our knowledge this is the first reported method that, at the sequence level, makes the identification of *Aeromonas* spp. possible, rapid, and reliable without the need for sequencing. We consider this method of invaluable potential for further investigations of *Aeromonas* epidemiology and its pathogenic implications in humans.

ACKNOWLEDGMENTS

This research has been supported by grant FIS 96/0579 from the Spanish Ministry of Health and a grant from CIRIT.

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