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Identification of Clinical *Aeromonas* Species by *rpoB* and *gyrB* Sequencing and Development of a Multiplex PCR Method for Detection of *Aeromonas hydrophila*, *A. caviae*, *A. veronii*, and *A. media*

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Conventional identification of *Aeromonas* species based on biochemical methods is challenged by the heterogeneous nature of the species. Here, we present a new multiplex PCR method directed toward the *gyrB* and *rpoB* genes that identifies four *Aeromonas* species, *A. hydrophila*, *A. media*, *A. veronii*, and *A. caviae*, and we describe the application of this method on a Danish strain collection.

Aeromonas spp. are highly adapted to aquatic environments and have been described as pathogenic to humans and animals. The genus *Aeromonas* consists of more than 20 valid species, of which *A. hydrophila*, *A. caviae* (synonymous with *A. punctata*), *A. media*, *A. veronii* bv. sobria, and *A. veronii* bv. veronii are of particular clinical significance, because they can cause gastroenteritis, wound and soft tissue infections, and septicemia (1). *Aeromonas* spp. may produce an array of virulence factors (e.g., cytolytic toxins with hemolytic activity and enterotoxins). Recent reviews suggested that only a subset of *Aeromonas* spp. are truly pathogenic and may be transmitted by hitherto unknown routes, and they proposed that further epidemiological and molecular studies are needed (2, 3). *Aeromonas* species identification has traditionally been performed by a combination of different biochemical tests. However, these are not always conclusive, since some *Aeromonas* species display heterogeneous biochemical properties; compared to molecular methods, the correct identification rate with biochemical tests has been shown to be very low (4, 5). Molecular species identification has been exploited by the 16S rRNA gene, either by restriction fragment length polymorphism (RFLP) (6–9) or direct sequencing (10, 11). However, due to insufficient interspecies sequence variation and heterogeneity among copies of ribosomal RNA operons in the same bacteria (10, 12, 13), this gene may not be an optimal target. A number of studies have shown that the sequences of several different housekeeping genes are able to differentiate this tight taxonomic group of organisms. These genes include an RNA polymerase B subunit (*rpoB*), an RNA polymerase D subunit (*rpoD*), and a DNA gyrase B subunit (*gyrB*) (10, 14–16). The objective of the present study was to identify species of *Aeromonas* by partial *gyrB* and *rpoB* sequencing in order to develop a multiplex PCR (mPCR) that targets the four most prevalent and clinically relevant species identified by sequence analysis on a Danish strain collection.

The strain collection used in this study was composed of 51 *Aeromonas* spp. collected from diarrheagenic patients during the period of 2005 to 2010. Each stool specimen was grown on enteric medium (Statens Serum Institut, Hillerød, Denmark) (17), and *Aeromonas* was identified by its distinct colony morphology, while further delineation of clinically relevant species was performed manually according to their biochemical characteristics, including Voges-Proskauer test results and lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, glucose (gas), esculin, and acid production from the fermentation of carbohydrates (4). Bacterial colonies were prepared for PCR and for *gyrB* and *rpoB* sequencing by 10 min of boiling in 10% Chelex 100 (Bio-Rad, Hercules, CA, USA) in 10 mM Tris-HCl and 1 mM EDTA (pH 8), followed by centrifugation and 10-fold dilution of the supernatant in PCR-grade water. PCR was performed in a total reaction volume of 25 μl containing the following reagents: 5 μl of template, 0.2 mM each dATP, dCTP, dGTP, and dTTP (GeneAmp; Applied Biosystems), 1× PCR buffer, 2.0 mM $MgCl_2$, 1.25 U Platinum *Taq* DNA polymerase (Invitrogen), and 0.5 μM each of primers up1 and up2r (18) or Pasrpob-L and Rpob-R (19) when *gyrB* or *rpoB*, respectively, was amplified. The amplicon size of the partial *gyrB* gene was 1,273 bp, and that of *rpoB* was 560 bp. In order to obtain reliable sequence quality in both directions, sequencing was performed by a combination of the PCR primers mentioned above and additional primers designed here for this purpose for *gyrB* (*gyrB*-F2[493], 5′-GAGGACTACAGCAAGAAGGCCA-3′; *gyrB*-R2[516], 5′-GACTTGGCCTTCTTGCTGTAGTC-3′) and for *rpoB* (*rpoB*-F2, 5′-CAACTTCGTCGGTGATCACA-3′; *rpoB*-R2, 5′-TGTGATCACCGACGAAGTGG-3′). The obtained partial *gyrB* and *rpoB* gene sequences were deposited in GenBank (see Table S1 in the supplemental material). Based on the sequences obtained in this study combined with GenBank sequences from Küpfer et al. (10), alignments of the *rpoB* and *gyrB* sequences were

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TABLE 1 Primers used in the mPCR method for *Aeromonas* species identification

Primer	Target organism (gene)	Sequence	Product (bp)	Primer concn (μM)
A-cav F(4)	*A. caviae* (*gyrB*)	5′-TGCTGCTGACCATCCGC-3′		0.5
A-cav R(74)		5′-GGTGCCTGCGGCTCG-3′	70	0.5
A-med F(176)	*A. media* (*gyrB*)	5′-GGCCAAGCGTCTGCGT-3′		0.2
A-med R(275)		5′-CGCCCTCGTAGCAGAAGTGA-3′	99	0.2
A-hyd F(533)	*A. hydrophila* (*gyrB*)	5′-AGTCTGCCGCCAGTGGC-3′		0.45
A-hyd R(677)		5′-CRCCCATCGCCTGTTCG-3′	144	0.45
A-Ver F(b1)	*A. veronii* (*rpoB*)	5′-CGTGCCGGCTTTGAAGTC-3′		0.15
A-Ver R(b1)		5′-GATCACGTACTTGCCTTCTTCAATA-3′	224	0.15
A-16S F(270)	*Aeromonas*	5′-CGACGATCCCTAGCTGGTCT-3′		0.05
A-16S R(731)	Universal (16S rRNA gene)	5′-GCCTTCGCCACCGGTAT-3′	461	0.05

constructed, and species-specific primers were designed for mPCR toward four *Aeromonas* species, *A. hydrophila*, *A. media*, *A. veronii*, and *A. caviae*. All sequence analyses were done by the CLC DNA Workbench software, version 6.5 (CLC bio, Århus, Denmark). The resulting mPCR procedure was performed in a 25-μl reaction mixture containing the following reagents: 1.5 μl of template, 0.2 mM each dATP, dCTP, dGTP, and dTTP (GeneAmp, Applied Biosystems), 1.2× PCR buffer, 2.0 mM $MgCl_2$, and 1.25

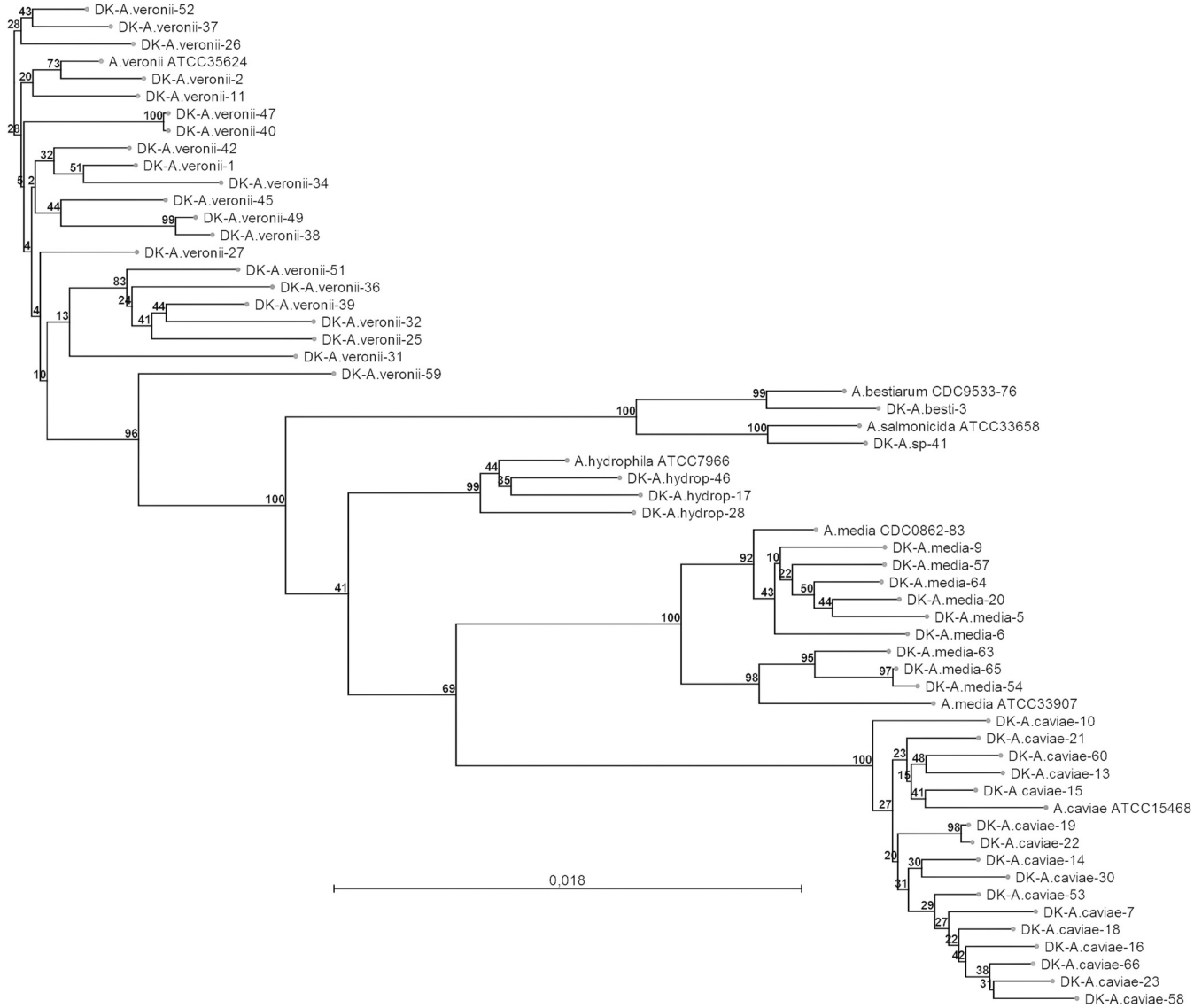

FIG 1 Neighbor-joining tree constructed from concatenated partial *gyrB* (1,113-bp) and *rpoB* (390-bp) sequences obtained from clinical strains in this study (accession numbers listed in Table S1 in the supplemental material) compared to reference sequences obtained from GenBank. Numbers at the nodes indicate bootstrap values as the percentage of 1,000 replicates. The scale bar indicates 1.8% sequence divergence.

U Platinum *Taq* DNA polymerase (Invitrogen); the primer concentrations are listed in Table 1, and the thermocycler conditions were as follows: 2-min hot start at 95°C, 6 cycles of 94°C for 40 s, 67°C for 50 s, and 72°C for 40 s, and 30 cycles of 94°C for 40 s, 65°C for 50 s, and 72°C for 40 s.

In the present study, we used *rpoB* and *gyrB* sequencing to determine the species identity of clinical *Aeromonas* isolates derived from patients suffering from gastroenteritis. In order to do so, we took advantage of previous findings where internal variations of the genes *rpoB* and *gyrB* were shown to be linked to phylogeny and, hence, correlated to species identity (10, 11, 14–16). However, due to variations in sequence quality and length, we decided to accept partial *rpoB* and *gyrB* sequences of 390 bp and 1,113 bp, respectively. With respect to the *rpoB* sequence, 390 bp is shorter than what was reported in previous studies, where a 560-bp sequence was used (10, 19), but phylogenetic analyses on reference strain sequences showed the same topologies with trees built from short (390-bp) and long (560-bp) sequences (data not shown).

Species identities of the clinical isolates were investigated by a combination of individual GenBank BLAST searches and alignment and phylogenetic tree construction for the individual sequences (data not shown) and for the concatenated sequences of *gyrB* plus *rpoB* (Fig. 1). With one exception among the 51 clinical isolates, the individual *gyrB* and *rpoB* gene sequences and the concatenated sequences resulted in the same species identifications. The one exception was a *gyrB* sequence (GenBank accession no. KJ747141) that was identical to *A. salmonicida*, while the *rpoB* sequence (KJ747178) may have originated from either *A. salmonicida* or *A. encheleia*. The remaining isolates were identified as *A. veronii* (n = 21, 41.2%), *A. caviae* (n = 16, 31.4%), *A. media* (n = 9, 17.6%), *A. hydrophila* (n = 3, 5.9%), and *A. bestiarum* (n = 1, 2%). The maximum interspecies divergences of *gyrB* and *rpoB* were found to be 10% (111 out of 1,113 nucleotides) and 9% (35 out of 390 nucleotides), respectively. The maximum intraspecies divergences of the *gyrB* gene were 5% (*A. veronii*, 53 nucleotides), 2.3% (*A. hydrophila*, 26 nucleotides), 2.5% (*A. caviae*, 28 nucleotides), and 3.1% (*A. media*, 35 nucleotides), and those of *rpoB* were 2.3% (*A. veronii*, 9 nucleotides), 1.5% (*A. hydrophila*, 6 nucleotides), 1.3% (*A. caviae*, 5 nucleotides), and 2.8% (*A. media*, 11 nucleotides).

Only 19/51 (37.3%) *Aeromonas* isolates were correctly identified by the phenotypic method. Of the 18 *Aeromonas* isolates correctly identified by the phenotypic method, 2 (66.7%) were *A. hydrophila*, 2 (9.5%) were *A. veronii*, and 15 (93.8%) were *A. caviae* (for further details, see Table S1 in the supplemental material). In particular, high discrepancies were observed for *A. veronii* and *A. media*, which may be explained by the phenotypic resemblance between *A. veronii* bv. sobria and *A. hydrophila* (both species negative for ornithine decarboxylase) and *A. media* and *A. caviae* (almost identical biochemically), respectively (4, 5). In order to subclassify *A. veronii* strains, tests for ornithine decarboxylase, arginine dihydrolase, salicin, esculin, and tartrate, for example, can be applied. The 21 molecularly identified strains of *A. veronii* belonged to *A. veronii* bv. sobria when biochemically tested. *A. veronii* bv. sobria and *A. caviae* have been shown to be the most prevalent *Aeromonas* spp. associated with traveler's diarrhea, which includes symptoms of watery diarrhea, abdominal cramps, and fever (20).

The mPCR method was designed toward four species, *A. hydrophila*, *A. media*, *A. veronii*, and *A. caviae*, and all 49 isolates in the present study were correctly identified. Figure 2 shows an example of four strains analyzed by the mPCR method. *A. bestiarum* and *A. salmonicida* or *A. encheleia* were not included in the analysis because they had little or no clinical significance and few sequences available with relatively high sequence variation; hence, no optimal primer design was possible. Therefore, if isolates investigated by the present mPCR method do not generate an amplicon, they should be subjected to, e.g., *rpoB* and/or *gyrB* sequencing in order to obtain correct species identification. In conclusion, the present mPCR method offers easy and precise identification of *Aeromonas* species that may improve routine diagnostics and shed new light on the controversial clinical and epidemiological aspects of this genus. Especially significant is the identification of *A. media* and *A. veronii*, which, according to this study, are underdiagnosed when phenotypic methods for species determination are applied.

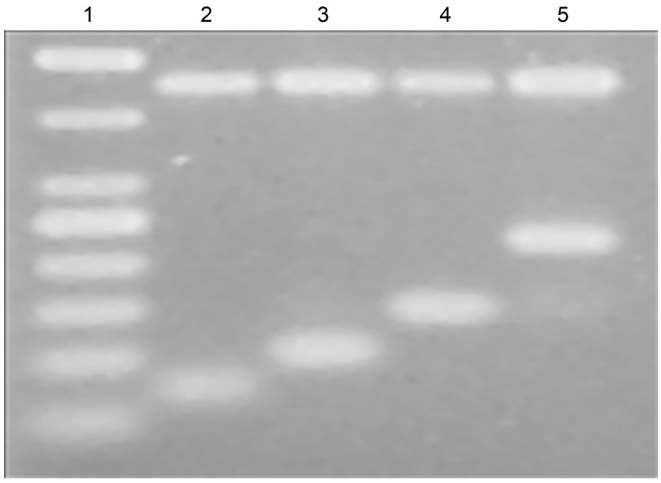

FIG 2 Four clinical isolates analyzed by mPCR for *Aeromonas* species identifications. Each primer set was designed to be specific for the particular species and to have a unique amplicon size when analyzed by agarose gel electrophoresis. Also included in the mPCR was a primer set targeting 16S rRNA genes, which served as an internal PCR control (461 bp). Lane 1, 50-bp DNA marker; lane 2, *A. caviae* (70 bp); lane 3, *A. media* (99 bp); lane 4, *A. hydrophila* (144 bp); and lane 5, *A. veronii* (244 bp).

Nucleotide sequence accession numbers. New sequences determined in this study were deposited in GenBank under accession numbers KJ747109 to KJ747187, KJ775031, and KJ775032 (for further details, see Table S1 in the supplemental material).

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