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# Use of Restriction Fragment Length Polymorphism of the PCR-Amplified 16S rRNA Gene for the Identification of *Aeromonas* spp.

In the October 1999 issue of the *Journal of Clinical Microbiology*, Graf (4) presented a paper the results of which demonstrated variations in the restriction fragment length polymorphism (RFLP) patterns of the PCR-amplified 16S rRNA gene of *Aeromonas veronii* biovar sobria and possible false identifications resulting from this technique. The author pointed out that although 16S rDNA-RFLP had been proposed as a rapid method of identifying *Aeromonas* species (1), its precision needed to be evaluated with more reference strains, because only one reference strain had been tested for each species. In his work, Graf (4) used 62 strains from several collections to verify our protocol, but an RFLP method that was different from the one previously proposed by Borrell et al. (1) was used. Essential differences between the methods were as follows: (i) only a part of the 16S rRNA gene was amplified by Graf (600 bp at the 5′ end) instead of the complete gene as we proposed and (ii) the enzymes employed (*Alu*I, *Cfo*I, and *Mnl*I) to obtain the species-specific patterns were not the ones originally proposed. These important differences are not mentioned at all in Graf's paper (4). The simultaneous application of *Alu*I and *Mob*I allowed the separation of 10 species of *Aeromonas*, as stressed by Borrell et al. (1). The endonucleases in our study were the ones that, after computer analysis, targeted the species-specific regions within the entire 16S rRNA gene (8). The method was evaluated by using the type strain of each species, along with 76 previously biochemically identified strains (1), and it has also been used to characterize 55 strains of *A. veronii*, always producing unequivocal patterns (M. J. Figueras, A. J. Martinez-Murcia, N. Borrell, and J. Guarro, Abstr. 99th Gen. Meet. Am. Soc. Microbiol., 1999, abstr. C-401, p. 187, 1999). In Graf's paper (4), the criteria for selecting the enzymes are not mentioned but do not appear to be based on any previously computerized analysis of the 16S rRNA gene sequences of the type strains. This is evident from the nondiscriminatory patterns obtained for some species. For instance, strains ATCC 35941 and LMG 13076, which belong to *Aeromonas* sp. HG11, showed RFLP patterns identical to that of *Aeromonas encheleia* (1). However, there are eight nucleotide differences in the 16S ribosomal DNA (rDNA) sequence (9), and although *Aeromonas* sp. HG11 and *A. encheleia* are considered the same species by some authors (6), the proposal has not been formally validated since data contradicts the original description that is based on phenotype and DNA-DNA pairing (3). Theoretical computer analysis of the type strains also prevents misinterpretation, such as that caused by undigested fragments at the laboratory, as shown in the 325-bp band in lanes B and G of Fig. 1 in Graf's work (4), which must not appear.

As already noted in our paper (1), 16S-rDNA RFLP patterns different from those previously described may be expected if the digested sequence belongs to a new *Aeromonas* species or if the restriction sites in known species are affected by intraspecific nucleotide diversity. To date, the RFLP method we proposed has been successfully tested with more than 200 strains, including numerous reference strains, and when a different pattern has been encountered (unpublished results), it has corresponded to the newly described species *Aeromonas popoffii* (7). A recent study found variations in five nucleotide positions after the 16S rRNA gene of 12 *A. popoffii* strains was sequenced, but despite these variations, the existence of unique primary structures in the gene was recognized as useful for its identification (2).

The intraspecific heterogeneity reported by Graf may be due to the use of some reference strains that in previous papers showed contradictory results. An example of this is ATCC 43946, the strain that was wrongly included as *A. encheleia* in Graf's list. While DNA-DNA hybridizations of this strain show that it is closely related to *Aeromonas schubertii*, it belongs in fact to the *Aeromonas* group 501 (5), which has 30 nucleotides that are different in the 16S rRNA gene sequence from that of *A. encheleia* (9). Graf interprets the different patterns as a case of intraspecific diversity (4).

It is also worth mentioning that Graf misinterprets the variations in the biochemical behavior encountered in some *A. veronii* biovar sobria strains as being due to differences in the 16S rRNA gene (4), because phenotypic responses are never under the control of this gene.

In conclusion, Graf's paper (4) should be considered a modification of the original protocol (1) and in no way demonstrates its precision. We strongly believe that for a method to be validated and its precision to be demonstrated with more reference strains, as was Graf's aim (4), it must be followed in detail before any conclusions can be made. Finally, it would be a pity if confusing results obtained by Graf discouraged other researchers from applying the protocol as originally described (1).

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**Author's Reply**

I appreciate the interest in my recent study (6). After carefully reading the comments, I do not believe that they affect or alter the conclusions that I made. I also need to clarify that I never stated that RFLP-PCR of the 16S rRNA gene should not be used for the identification of *Aeromonas* species; I stated "However, verification of species identification with biochemical tests is still appropriate for clinical diagnosis in light of the differences reported in our study" (6). Indeed, we have used this approach in combination with biochemical tests to identify environmental *Aeromonas* strains (11). Nor did I state or imply that the 16S rRNA gene was responsible for changing biochemical characteristics of the strains. The original contribution of Borrell et al. (2) was clearly cited as a powerful method, and the methodological differences are very clear and apparent to the reader, even when only the figures are examined. The conclusion I would hope that readers will take home from my paper is that analysis of both the 16S rRNA gene sequences and the biochemistry will produce a more rigorous identification than either alone.

The most important discovery in the study was that three *Aeromonas veronii* biovar sobria reference strains produced an unexpected RFLP pattern that was not predicted from computer analysis while four reference strains produced the expected pattern (6). Thus, one can reasonably assume that not all *A. veronii* biovar sobria strains will produce the expected pattern. Because I used the restriction endonuclease *Alu*I, as did Borrell et al. (2) in their study describing the use of RFLP-PCR of the 16S rRNA gene for *Aeromonas* identification, my results are applicable, relevant, and important to their study as well. As long as *Alu*I is included, even amplifying a larger fragment of the 16S rRNA gene and digesting the DNA with a mixture of the restriction endonucleases *Alu*I and *Mbo*I will not change the fact that some *A. veronii* biovar sobria reference strains identified by DNA-DNA hybridization will produce a different pattern than expected.

The results of my study (6) underline the importance of using a large enough set of well-characterized reference strains while establishing an identification method, instead of using a few reference strains and a collection of strains identified biochemically. In the field of *Aeromonas*, we are aided by valuable previous studies that have identified many strains by DNA-DNA hybridization and characterized them by using biochemical tests (3, 9), multilocus enzyme electrophoresis (1), and molecular methods (7). I examined those studies and searched for reference strains that covered the range of diversity, especially within the species of *A. veronii* biovar sobria.

There are four reasons why one might want to exercise caution when using the 16S rRNA gene sequence for identifying *Aeromonas* species. First, Martinez-Murcia et al. (10) reported in 1992 that the actual sequence difference between *Aeromonas* species can be very slight. For example, the *Aeromonas trota* strain ATCC 49659$^T$ differs from the *Aeromonas caviae* strain NCIMB 13016$^T$ by a single nucleotide and the *Aeromonas hydrophila* strain ATCC 7966$^T$ differed from the *Aeromonas media* strain ATCC 33907$^T$ by 3 nucleotides (10). Second, the phylogenetic trees that have been constructed using the 16S rRNA gene sequence suggest different degrees of relatedness than those inferred from DNA-DNA hybridization studies (10). Third, interestingly, it has been proposed that crossing-over of ribosomal sequences has occurred several times in *Aeromonas* (12). Fourth, it has been reported that for some bacteria, intraspecific variation in the 16S rRNA is not uncommon (4) and this was recently shown to occur in *Aeromonas popoffii* (5). Anyone interested in rigorously identifying *Aeromonas* strains must be aware of these valid, published concerns and their consequences when applying an approach that relies on 16S rRNA gene sequences.

In response to some of the more detailed issues, such as which strains to include under the species *Aeromonas encheleia*, we followed the recommendations from a study performed by investigators from three institutions that involved several approaches (8); thus, there was no need to include additional restriction enzymes to confuse the identification. In regards to the critique of faints bands supposedly resulting from the incomplete digestion of the samples, it should be noted that there is an alternative explanation and that is that the strain analyzed carries different 16S rRNA alleles that result in different RFLP patterns. Finally, when I interpreted the results in regard to the species identification, I specifically stated that I excluded single variants (6). This was to ensure that if a strain is reclassified or falsely classified it does not affect the final conclusions. In addition, the identity of 10 reference strains with the unusual patterns was verified by biochemical analysis to ensure that I had received and analyzed the correct strains (6). All of this was done in the interest of an accurate identification scheme.

As suggested in my report, my group has sequenced the 16S rRNA gene of several strains with unexpected patterns and these results are consistent and support the conclusions of our study (J. Graf and R. Troller, unpublished data). Hopefully, this will help to address some of these issues in a scientific manner. RFLP-PCR of the 16S rRNA gene is a valuable tool in the identification of *Aeromonas* strains; however, it remains my concern that by relying solely on an approach based on the 16S rRNA sequence, investigators may leave their studies open to criticisms that could be avoided if they would apply biochemical tests to independently verify their results.

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