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NOTE

Extended method for discrimination of *Aeromonas* spp. by 16S rDNA RFLP analysis

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A previously described molecular method, based on 16S rDNA RFLP analysis, for the identification of *Aeromonas* spp. was unable to separate the species *Aeromonas salmonicida*, *Aeromonas bestiarum* and the recently described *Aeromonas popoffii*. In this study, the method has been extended with endonucleases *Alw*NI and *Pst*I for the identification of these species. A molecular frame for the identification of all known *Aeromonas* spp. is presented.

Keywords: *Aeromonas*, 16S rDNA RFLP, molecular taxonomy

The genus *Aeromonas* currently comprises 14 species (*Aeromonas hydrophila*, *Aeromonas bestiarum*, *Aeromonas salmonicida*, *Aeromonas caviae*, *Aeromonas media*, *Aeromonas eucrenophila*, *Aeromonas sobria*, *Aeromonas jandaei*, *Aeromonas veronii*, *Aeromonas schubertii*, *Aeromonas trota*, *Aeromonas allosaccharophila*, *Aeromonas encheleia* and *Aeromonas popoffii*), although the taxonomy of the group is not yet resolved. For example, even though *Aeromonas ichthiosmia* and *Aeromonas enteropelogenes* were previously synonymized with *A. veronii* and *A. trota*, respectively (Collins *et al.*, 1993), recently Bruckner *et al.* (1999) still considered *A. enteropelogenes* to be a valid species (Figueras *et al.*, 2000a). Other conflicting species are *Aeromonas punctata* and *A. encheleia*. In relation to the former, there is still discussion on the priority between this species and *A. caviae* (Carnahan & Altwegg, 1996). Huys *et al.* (1996, 1997b) suggest including DNA hybridization group 11 (HG11) within the species *A. encheleia*, and Graf (1999) also includes *Aeromonas* Group 501 (Hickman-Brenner *et al.*, 1988) within that species. However, a recent phylogenetic analysis of the genus *Aeromonas* considered the three to be separate taxa (Martínez-Murcia, 1999). Identification of *Aeromonas* spp. has long been controversial due to their phenotypic heterogeneity (Janda *et al.*, 1996; Abbott *et al.*, 1998). A number of approaches that have been applied to characterize the aeromonads have attempted a definitive species identification frame. Despite all these efforts, identification of some species is still a serious problem because the conventional biochemical tests are not always reliable and discrepancies remain between phenotypic and genetic groups (Janda *et al.*, 1996; Borrell *et al.*, 1997, 1998).

The 16S (or small subunit) ribosomal gene has proved to be a valuable tool in providing signature sequences for delineation and identification of most *Aeromonas* species (Martínez-Murcia *et al.*, 1992). Consequently, a number of species-specific DNA probes have been reported (Ash *et al.*, 1993a, b; Dorsch *et al.*, 1994; Oakey *et al.*, 1999; Khan *et al.*, 1999; Demarta *et al.*, 1999). A protocol was recently described based on the RFLP patterns of the complete PCR-amplified 16S rDNA gene that enabled identification of most (10 species) *Aeromonas* spp. by using two endonucleases (*Alu*I and *Mbo*I) simultaneously (Borrell *et al.*, 1997). Two additional enzymes, *Nar*I and *Hae*III, were necessary to distinguish the species *A. salmonicida*, *A. encheleia* from *Aeromonas* HG11. The discrimination of *A. salmonicida* from the recently described species *A. bestiarum* (Ali *et al.*, 1996) was not included in that study. The method described by Borrell *et al.* (1997) does not allow the identification of the new species *A. popoffii* (Huys *et al.*, 1997a). The objective of this study, therefore, was to extend our previously proposed identification pathway to provide a protocol for all known species of *Aeromonas*, including the two newly mentioned species.

Seventy-two strains from diverse origins were analysed, including the type strains of the species which could not be distinguished by previously described protocols and six additional strains (Table 1) identified as *A. veronii* in a recent study (Graf, 1999). Genomic DNA extraction and PCR amplification of the 16S rDNA was performed as previously described (Martínez-Murcia *et al.*, 1992; Borrell *et al.*, 1997). Computer analysis [using DIGEST and RESTRY programs

Table 1. Strains of *Aeromonas* spp. used in this study

Species	Strain*	Source
A. bestiarum	ATCC 51108^T, CECT 5200, CECT 5201, CECT 5202, CECT 5203, CECT 5204, CECT 895, CECT 896, CECT 5179, CECT 4239	Fish
	LMG 13662	Faeces
	CECT 5219	Cake
	CECT 5222, CECT 5223	Shellfish
	CECT 5224, CECT 5226, CECT 5228, CECT 5236	Drinking water
	CECT 5248, CECT 5211	Seawater
	CECT 5213, CECT 5212	River
	CECT 5214, CECT 5215, CECT 5239, CECT 5242, CECT 5205, CECT 5217, CECT 5247, CECT 5206	Reservoirs
A. salmonicida	ATCC 33658^T, CECT 4237, CECT 4236	Fish
	LMG 13448	Faeces
	LMG 18998	Wound exudate
	LMG 19037, CECT 5221, CECT 5218	Cake
	CECT 5225, CECT 5227	Shellfish
	CECT 5229, CECT 5232, CECT 5238, CECT 5230, LMG 19036	Drinking water
	CECT 5209, CECT 5220, CECT 5234	Seawater
	CECT 5231	Reservoir
	CECT 5249	River
A. popoffii	LMG 17541^T, LMG 17542, LMG 17543, LMG 17544, LMG 17545, LMG 17546, LMG 17547	Drinking water
	CECT 5235, CECT 5246,CECT 5245, CECT 5250	Reservoirs
	CECT 5251, CECT 5240, CECT 5243, CECT 5244	River
	CECT 5210	Seawater
A. encheleia	CECT 4342^T, CECT 4340, CECT 4341, CECT 4343	Fish
Aeromonas Group 501	ATCC 43946	Leg wound
Aeromonas HG11	ATCC 35941	Ankle fracture
A. veronii biogroup *sobria*	LMG 13068, LMG 13071, LMG 13073, LMG 13074, LMG 13695	Faeces
	LMG 13694	Unknown

*Abbreviations: ATCC, American Type Culture Collection; LMG, Belgian Coordinated Collection of Micro-organisms; CECT, Colección Española de Cultivos Tipo.

of PC/GENE (IntelliGenetics) and OMIGA restriction sites (Oxford Molecular)] of the complete 16S rDNA sequences of the type strains of all *Aeromonas* spp. was performed to select the most suitable restriction endonucleases for species discrimination. Products of digestions with enzymes *Alu*I and *Mbo*I or *Hae*III were electrophoresed on 4% Metaphor agarose (FMC BioProducts). Digestions performed with enzymes *Nar*I, *Pst*I and *Alw*NI were separated on 1·2% Seakem LE agarose (FMC BioProducts). The same protocol was applied in the case of the six mentioned strains from the study of Graf (1999) to confirm their identity. A further computer simulation of restriction enzyme *Alu*I was performed to recognize restriction fragments for all type strains within the part of the sequence (5′-end) amplified by Graf (1999), i.e. the first ca. 600 bp of the gene.

Fig. 1 shows a flowchart of the protocol for the identification of the 16 species of *Aeromonas*, including species HG11 and *Aeromonas* Group 501. Endonucleases *Alu*I and *Mbo*I provided different RFLP patterns for 10 species of *Aeromonas* which have already been published (Borrell *et al*., 1997) and for *Aeromonas* Group 501. However, *A. salmonicida*, *A. encheleia*, *Aeromonas* HG11, *A. popoffii* and *A. bestiarum* exhibited the same RFLP pattern. A third enzyme, *Nar*I, was needed to discriminate *A. bestiarum* and *A. salmonicida* from *A. encheleia*, *Aeromonas* HG11 and also from *A. popoffii*. The use of *Hae*III allowed the distinction of *Aeromonas* HG11 from *A. encheleia* and *A. popoffii*, separation of which was then accomplished using *Alw*NI. It was possible to differentiate *A. bestiarum* from *A. salmonicida* using either endonuclease *Pst*I or *Sfa*NI, but *Pst*I is recommended because of its price.

Different biochemical tests are routinely used for *Aeromonas* identification. These tests, although useful, are laborious, time-consuming and can give erroneous identification. Some of these conventional methods require the use of as many as 18 tests for species identification and six additional tests are necessary to differentiate the species included within the '*A. hydrophila*' complex, i.e. *A. hydrophila*, *A. bestiarum* and *A. salmonicida* (Janda *et al*., 1996). When biochemical tests were applied, only 10 *A. bestiarum* and six *A. salmonicida* strains were unequivocally identified from

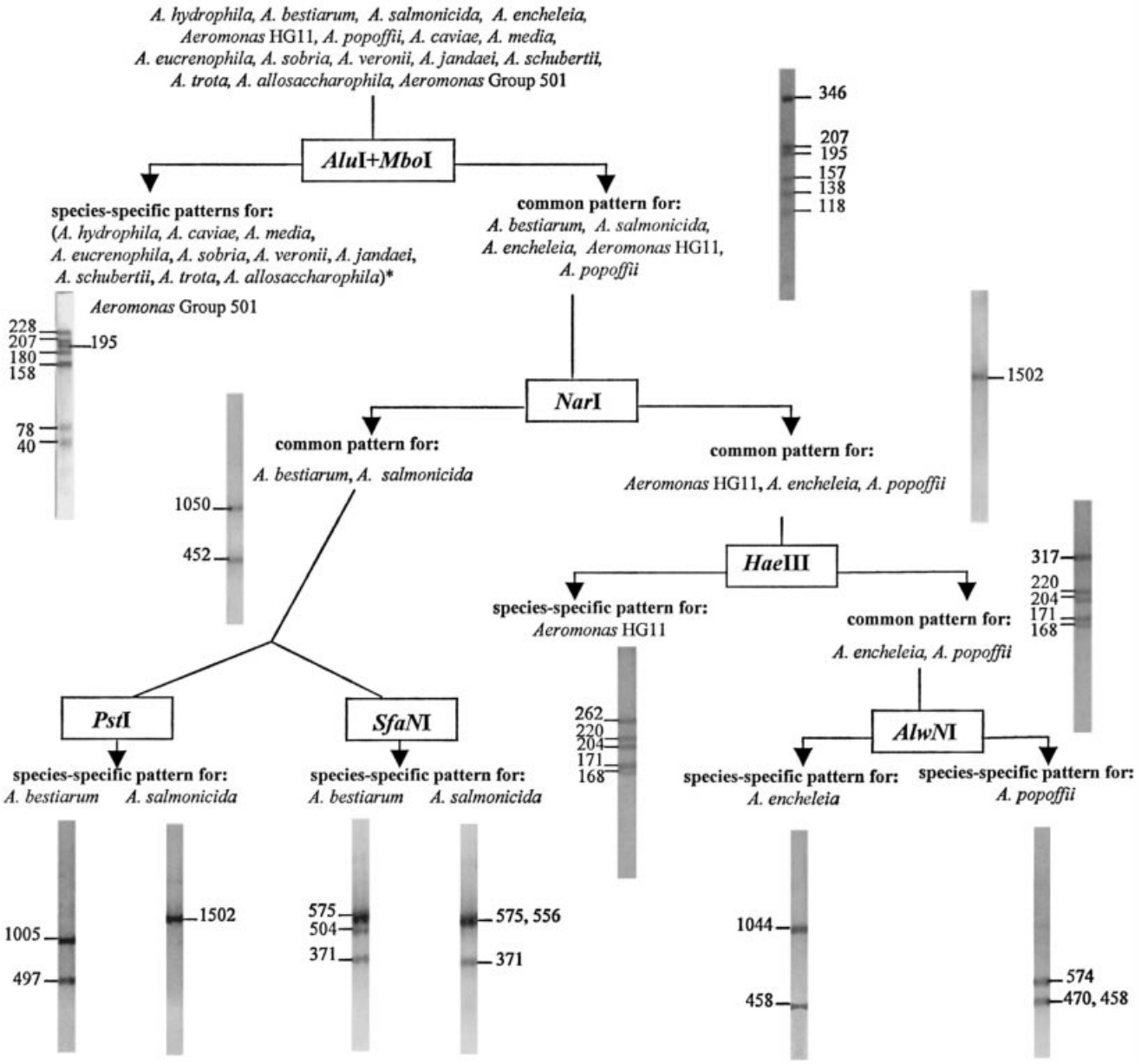

Fig. 1. Different steps for the identification of *Aeromonas* species by 16S rDNA RFLP analysis. Sizes are shown in bp. The species-specific patterns indicated by an asterisk, with the exception of *Aeromonas* Group 501, were illustrated by Borrell *et al.* (1997).

a total of 54 and 32 strains, respectively (Borrell *et al.*, 1998). This misidentification problem has now been overcome by the newly proposed molecular approach.

The recently described species *A. popoffii* has a very similar biochemical response to that of *A. bestiarum* and these species can only be separated by D-sucrose fermentation, lysine decarboxylase production and the use of DL-lactate as a sole energy and carbon source (Huys *et al.*, 1997a). A specific probe based on the 16S rDNA gene sequence has recently been designed for the identification of *A. popoffii* (Demarta *et al.*, 1999). The use of DNA probes to identify all *Aeromonas* spp. is costly and time-consuming, because of the need for a number of probes, and reliability is critical when sequence targets differ in so few nucleotides, e.g. *A. salmonicida* and *A. bestiarum* only show two nucleotide differences (Martínez-Murcia *et al.*, 1992). Our proposed scheme, also based on 16S rDNA sequencing, provides reliable and fast species identification of a large collection of isolates and can be rapidly achieved by simply digesting the complete PCR-amplified gene.

Demarta *et al.* (1999) reported variations in two or three nucleotide positions after sequencing the 16S rDNA of 12 *A. popoffii* strains. Despite these varia-

tions, unique primary structures exist in the gene which allow the identification of members of this species. In fact, a common pattern was obtained for all the *A. popoffii* strains tested, including those used in the original species description (Huys *et al.*, 1997a). As already noted in a previous publication (Borrell *et al.*, 1997), 16S rDNA RFLP patterns different to 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 intra-species nucleotide diversity, i.e. differences between strains of the same species. A common pattern, which differs from those previously reported (Borrell *et al.*, 1997), was obtained for the nine new isolates of *A. popoffii* that were included in this study.

Recently, Graf (1999) described a different 16S rDNA RFLP method using only the first ca. 600 bp of the gene and endonucleases *Alu*I, *Cfo*I and *Mnl*I to evaluate, according to the author, the precision of our original method (Borrell *et al.*, 1997) with 62 *Aeromonas* reference strains. This author reported diverse RFLP patterns within *A. veronii* and possible misidentifications of *Aeromonas* species suggesting that this was due to differences in the 16S rDNA gene sequences (Graf, 1999). These contradictory results have been investigated in our laboratory and broadly discussed elsewhere (Figueras *et al.*, 2000b). The intra-species heterogeneity reported by Graf (1999) appears to be due to a misidentification of the strains used. For example, in this study, six strains (Table 1) of the 11 considered by Graf to be *A. veronii* using our RFLP protocol were analysed; only two of them (LMG 13068 and LMG 13694) showed the pattern of *A. veronii* whereas the rest had that of *A. sobria*. In the same study, *Aeromonas* Group 501 (ATCC 43946) was considered to be *A. encheleia* (Graf, 1999), whereas these strains are distinct species with 30 nucleotide differences in the 16S rDNA gene (Martínez-Murcia, 1999). Graf also indicated that the use of a single enzyme, *Alu*I, can separate the species *A. veronii*, *A. caviae* and *A. hydrophila*. Further computer simulation on the 16S rDNA sequences of the type strains were carried out to confirm this statement; the endonuclease *Alu*I produced species-specific patterns only for *A. sobria*, *A. jandaei*, *A. schubertii* and *A. veronii* (although the latter had a pattern identical to that of *Aeromonas* Group 501). *A. caviae* and *A. hydrophila*, however, had identical patterns to other species. In summary, it is concluded that the main problem of Graf's method was that the enzymes were selected arbitrarily and not on the basis of a previous computerized analysis of the 16S rDNA gene sequences of the type strains of all species as described in our studies.

The method provided in this work, apart from being a reliable identifier of all known *Aeromonas* spp., can be highly useful in future studies for determining the real incidence of the recently described species *A. popoffii* and *A. bestiarum* obtained from different habitats. Species determination may be carried out rapidly and at reasonable cost. The use of the proposed protocol for research studies that need species identification is also encouraged.

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