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Comparison of three molecular methods for typing *Aeromonas popoffii* isolates

Lara Soler[1], Maria Jose Figueras[1,*], Matilde R. Chacón[1], Josep Guarro[1] and Antonio J. Martinez-Murcia[2]

[1]Unitat de Microbiologia. Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Sant Llorenç 21, 43201 Reus, Spain; [2]Servicio de Diagnóstico Molecular. Escuela Politécnica Superior, Universidad Miguel Hernandez, Crta. Beniel Km.3,2, 03312 Orihuela (Alicante), Spain; [*]Author for correspondence (e-mail: mjfs@fmcs.urv.es; phone: 34-977759321; fax: 34-977759322)

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Abstract

Three typing methods, restriction fragment length polymorphism (RFLP) of the 16S-23S intergenic spacer region (ISR), PCR amplification of the enterobacterial repetitive intergenic consensus (ERIC) and of the repetitive extragenic palindromic units (REP), were evaluated for typing 26 isolates of *Aeromonas popoffii* from different geographical origins. When the methods were independently studied, ERIC showed the highest discriminatory power. When the methods were combined, the best combination of two methods was ERIC with REP since strains showed a tendency to cluster according to their geographical origin. However, this tendency was reinforced with the addition of ISR-RFLP.

Introduction

Members of the genus *Aeromonas* are gram-negative bacteria, and ubiquitous in aquatic environments, although they are also involved in a variety of human infections (Janda and Abbott 1998). *Aeromonas popoffii* is the most recently described species (Huys et al. 1997) and is commonly found in freshwater and seawater (Soler et al. 2002). Although to our knowledge there is no report of this species in clinical samples, the presence of common virulence factors such as aerolysin/hemolysin, serine protease, DNases and lipases in *A. popoffii* has been demonstrated (Soler et al. 2002).

Studies involving clinical and environmental *Aeromonas* strains using ribotyping have indicated that apparently unrelated strains can show identical patterns (Demarta et al. 2000) which, together with its laborious nature may be a shortcoming of the technique. Pulsed-field gel electrophoresis (PFGE) has been considered to be one of the best typing methods (Maslow et al. 1993). However, when compared with RAPDs for typing *A. hydrophila* isolates, the latter was simpler, cheaper, and quicker to perform, and consequently more suitable for epidemiological studies (Talon et al. 1998). Recently, restriction fragment length polymorphism (RFLP) of the 16S-23S intergenic spacer region (ISR) has been used successfully for typing *Aeromonas veronii* strains (Martínez-Murcia et al. 2000). However, this technique is laborious when compared with other typing methods based on the PCR amplification of short and repetitive sequences present on the genome of bacteria, such as enterobacterial repetitive intergenic consensus (ERIC) and repetitive extragenic palindromic units (REP) (Versalovic et al. 1991). These motifs are genetically stable and differ only in their copy number and chromosomal locations, making them a good target for strain differentiation (Hulton et al. 1991). RAPD and ERIC were used for typing *Aeromonas* isolates and both methods proved to be effective for demonstrating a nosocomial transmission of *A. hy-*

drophila (Davin-Regli et al. 1998). Despite that, there are only few comparative studies on the reproducibility and discriminatory power of molecular typing methods in *Aeromonas*.

We have evaluated three molecular methods (16S-23S ISR-RFLP, ERIC and REP) alone and in combination for typing isolates of *A. popoffii* of different origins. We have collected all the available strains of this species in order to evaluate their genetic diversity.

Materials and methods

Strains

We tested 26 *A. popoffii* strains, 7 from Belgium and Scotland (Huys et al. 1997), 8 from Switzerland (Demarta et al. 1999) and 11 from Spain (Soler et al. 2002) (Table 1). The latter were identified by 16S rDNA-RFLP. Strains received from other authors were also re-identified using the same methodology (Borrell et al. 1997; Figueras et al. 2000). The type strains of *A. hydrophila*, *A. bestiarum* and *A. salmonicida* were chosen as outgroups.

16S-23S rDNA ISR-RFLP (ISR-RFLP) analysis

Genomic DNA extraction and PCR amplification of the 16S-23S ISR was performed as previously described (Martínez-Murcia et al. 2000), although in the present study we employed a different enzyme combination. The 16S-23S ISR amplified product was digested with the enzymes *Hinf*I-*Cfo*I, *Hinf*I-*Taq*I and *Alu*I. Digested products were electrophoresed on 4% Metaphor agarose (FMCS Bioproducts Europe, Denmark) for 6 h on Tris-acetate-EDTA buffer. Molecular

Table 1. Isolates used in the study and genotypes determined by 16S-23S ISR RFLP, REP and ERIC-PCR analyses.

16S rDNA RFLP	Isolate	Origin	Types				
			16S-23S ISR-RFLP			REP-PCR	ERIC-PCR
			*Hinf*I-*Cfo*I	*Hinf*I-*Taq*I	*Alu*I		
A. hydrophila	CECT 839T	Tin of milk with fishy odour	1	1	1	1	1
A. bestiarum	CECT 4227T	Fish	2	2	2	2	2
A. salmonicida	CECT 894T	Atlantic salmon	3	3	3	3	3
A. popoffii	LMG 17541T	Drinking water production plant (Oelegem); Belgium	8	7	6	4	4
A. popoffii	LMG 17542	Drinking water production plant (De Blankaart); Belgium	6	4	9	5	5
A. popoffii	LMG 17543	Drinking water production plant (Snellegem); Belgium	7	10	5	6	6
A. popoffii	LMG 17544	Drinking water production plant (Eeklo); Belgium	8	7	6	7	7
A. popoffii	LMG 17545	Drinking water production plant (Snellegem); Belgium	8	7	5	8	8
A. popoffii	LMG 17546	Drinking water service reservoir (Undy Station); Scotland	8	8	6	9	9
A. popoffii	LMG 17547	Drinking water production plant (Turriff); Scotland	9	9	6	11	11
A. popoffii	F533E	Tap water (Pregassona); Switzerland	10	10	10	10	10
A. popoffii	F548B	Tap water (Vinagello); Switzerland	10	10	10	10	10
A. popoffii	F548C	Tap water (Vinagello); Switzerland	10	10	10	10	10
A. popoffii	F600B	Tap water (Vinagello); Switzerland	10	10	10	10	10
A. popoffii	F600C	Tap water (Vinagello); Switzerland	10	10	10	10	10
A. popoffii	F539A	Tap water (Vinagello); Switzerland	10	10	10	10	10
A. popoffii	F479E	Fountain swab (Vezio); Switzerland	8	6	4	12	12
A. popoffii	F498B	Tap water (Cadro); Switzerland	8	10	8	13	13
A. popoffii	137	Baells Reservoir; Spain	8	9	6	14	14
A. popoffii	159	Segre River; Spain	5	13	4	15	15
A. popoffii	203	Seawater. El Masnou-Barcelona; Spain	8	9	6	16	16
A. popoffii	210	Seawater. Rivermouth of Llobregat; Spain	8	9	6	17	17
A. popoffii	254	Noguera Pallaresa River; Spain	6	11	11	18	18
A. popoffii	274	Noguera Pallaresa River; Spain	10	6	6	19	19
A. popoffii	366	Freshwater. Riera de Merles; Spain	4	13	6	20	20
A. popoffii	370	Baells Reservoir; Spain	8	5	7	21	21
A. popoffii	594	Freshwater. Riera de Merles; Spain	9	14	6	14	22
A. popoffii	32	Seawater. Rivermouth of Fluvia; Spain	11	15	12	14	23
A. popoffii	638	Fluvia River; Spain	8	12	6	14	24

Weight Marker V (Roche, Madrid, Spain) (5 μl) was electrophoresed twice in each gel.

REP and ERIC analysis

DNA extraction was performed with the Instagene Matrix (Bio-Rad Laboratories, California, USA). The DNA was quantified with the GeneQuant *pro* (Amersham Pharmacia Biotech, Cambridge, England) and an amount of 100 ng was used per PCR reaction in a GeneAmp PCR System 2400 (Perkin Elmer, Foster City, USA), using primers and conditions previously described (Vila et al. 1996). Samples (25 μl) of each PCR end product were electrophoresed on polyacrylamide 10% w/v gels at a voltage of 56 mA for 4 h. AmpliSize™ Molecular Ruler 50–2,000 bp Ladder (Bio-Rad) (8 μl) was electrophoresed twice in each gel.

Image comparison, analyses and reproducibility

Gel images were saved as TIFF files, normalized with the above mentioned molecular size markers, and further analysed by BioNumerics software, version

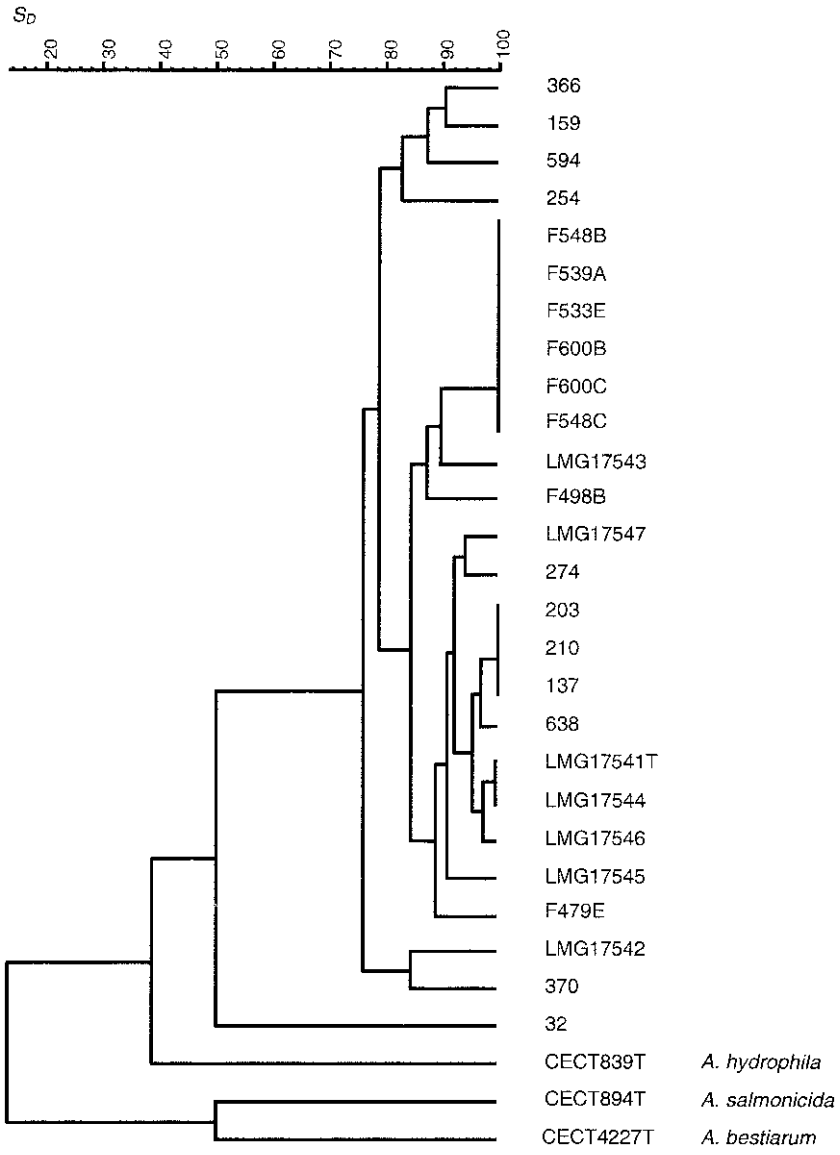

Figure 1. S_D/UPGMA cluster analysis based on the combined *Hinf*I-*Cfo*I, *Hinf*I-*Taq*I and *Alu*I ISR-RFLP produced patterns.

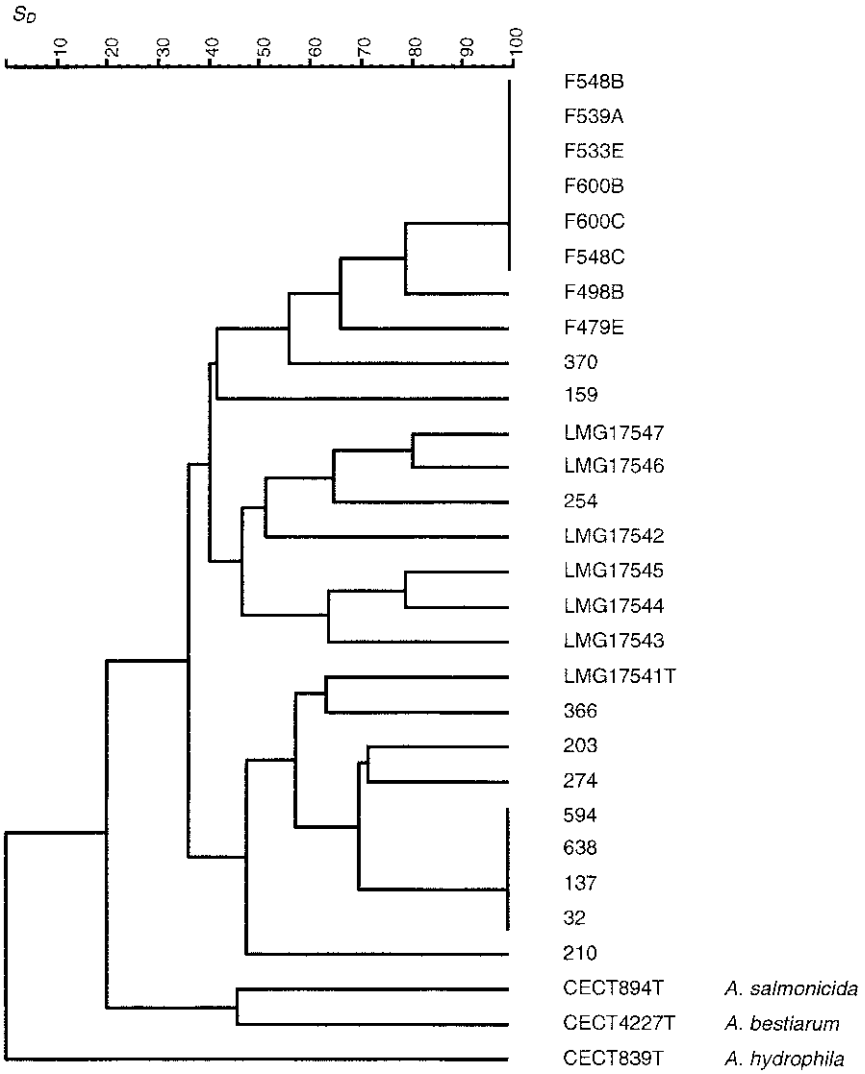

Figure 2. S_D/UPGMA cluster analysis based on the REP-PCR produced patterns.

1.5 (Applied Maths, Kortrijk, Belgium). To construct the dendrograms, levels of similarity between the profiles were calculated by using the band-matching Dice coefficient (S_D) and the cluster analysis of similarity matrices was calculated with the unweighted pairgroup method with arithmetic averages (UPGMA). Isolates were assigned to a different type when any band differences were observed.

Three different assays using duplicates of three different strains (LMG17542, F539A and F479E) were performed to analyze the reproducibility of the REP and ERIC methods. In the first, DNA extraction and PCR were performed in the same experiment and electrophoresis in the same gel for all strains. In the second, only electrophoresis was done in different gels for the duplicate strains. In the third, DNA extraction, PCR and electrophoresis were done in separate experiments for each duplicate. A band-matching tolerance of 75% was chosen, and similarity matrices of whole densitometric curves of the gel tracks were calculated using the pair-wise Pearson's product-moment correlation coefficient (r-value) (Vinuesa et al. 1998). In the present study, DNA extraction, PCR and electrophoresis were done in the

same experiment whenever possible, otherwise, strains already analysed were included as controls in subsequent experiments.

Results and discussion

Aeromonas popoffii, the latest described species of the genus, was originally isolated from drinking water production plants and reservoirs in Belgium and Scotland (Huys et al. 1997). Recently more isolates were recovered from the same origin in Switzerland (Demarta et al. 1999) and Spain (Soler et al. 2002). In the present study we have investigated the genetic relatedness of all those isolates using three different molecular methods (ISR-RFLP, ERIC and REP). The genotypes obtained are summarized in Table 1 and Figure 1–5 show the dendrograms obtained. The type strains of *A. hydrophila*, *A. bestiarum* and *A. salmonicida* each presented a unique genotype and formed 3 clear outgroups with all methods tested. It is interesting to note that 6 of the 8 isolates from Switzerland (F548B, F548C, F600B, F600C, F539A and F533E) showed identical patterns with the three methods (Table 1, Figures 1–5) Figures 6 and 7 illustrate the identical patterns obtained with ERIC

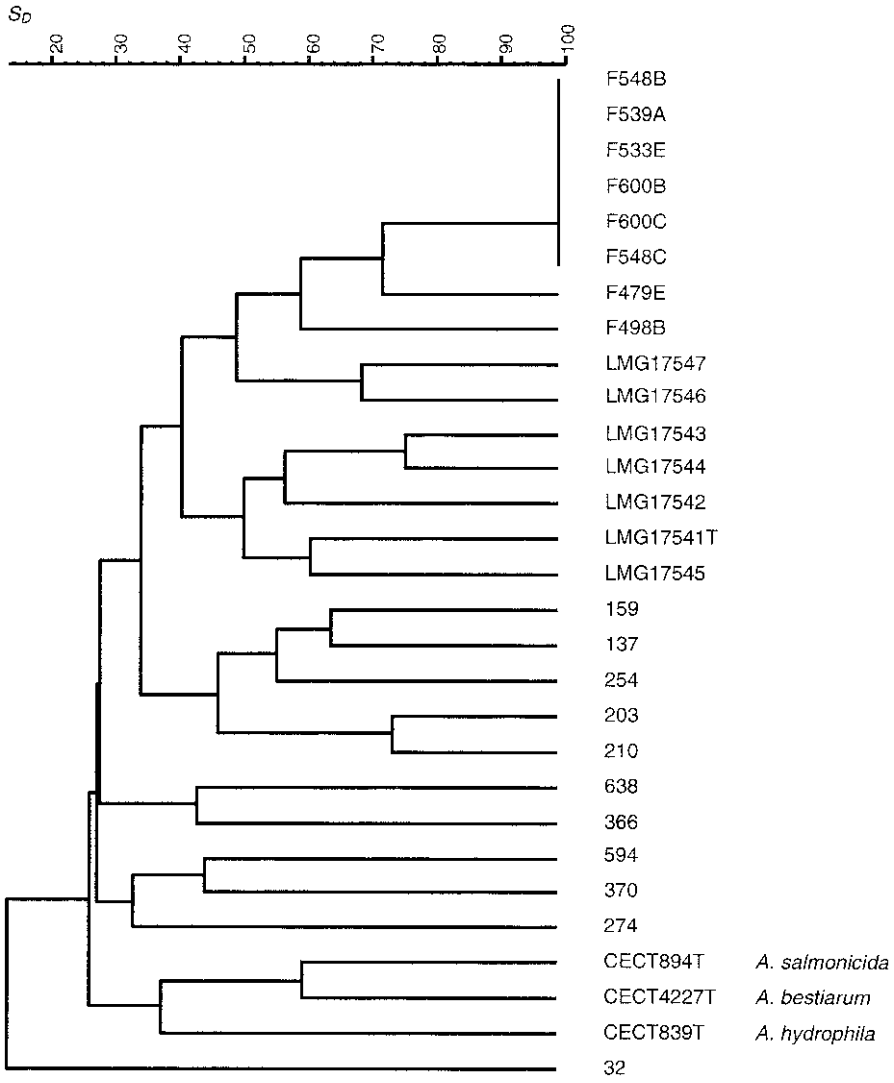

Figure 3. S_D/UPGMA cluster analysis based on ERIC-PCR produced patterns.

and REP for those isolates. These isolates also presented identical biochemical responses and antimicrobial susceptibility patterns which seems to indicate that they belong to the same clonal group. Therefore, we assume that, the 8 isolates from Switzerland (Demarta et al. 1999) represented only 3 strains.

Our study, using ISR-RFLP, ERIC and REP demonstrated that the isolates LMG17546 (genotype 8/8/6/9/9) and LMG17547 (genotype 9/9/6/11/11); LMG17541^{T} (genotype 8/7/6/4/4) and F498B (genotype 8/10/8/13/13) are different, disagreeing with results obtained by ribotyping in the previous studies (Huys et al. 1997; Demarta et al. 1999). This confirms that ribotyping is not discriminatory enough as already indicated by other authors (Maslow et al. 1993; Demarta et al. 2000). Patterns for the rest of the isolates investigated were strain-specific or not, depending on the discriminatory power of the method. For instance, an identical ISR-RFLP type was obtained for isolates 203, 210 and 137 (Figure 1), while they showed specific patterns with the other two methods (Figures 2, 3). This may be considered a shortcoming of the ISR-RFLP method and additionally, as already commented, it is more complex to perform. The remaining fifteen isolates presented strain-specific ISR-RFLP patterns. The usefulness of the ISR-RFLP method was demonstrated for typing *A. veronii* in a previous study by our group. By using

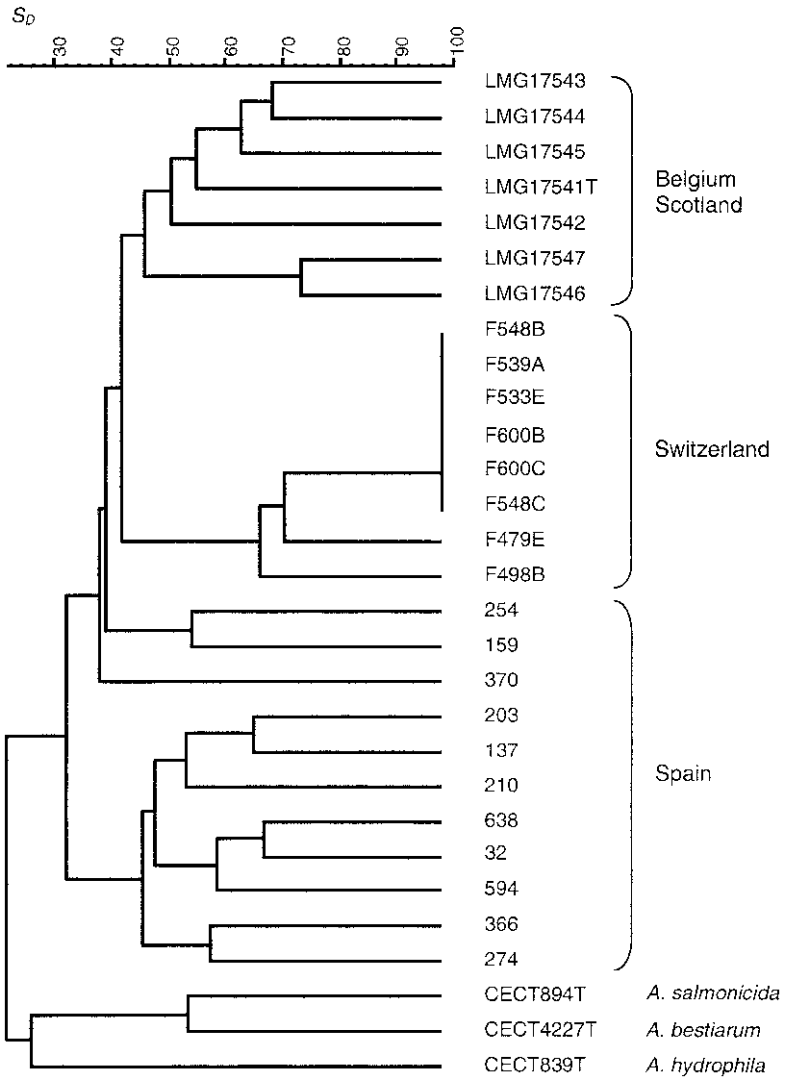

Figure 4. S_D/UPGMA cluster analysis based on the combined REP-PCR and ERIC-PCR produced patterns.

this method, we were able to recognize an identical clone from the same patient in sequential stool samples. In addition, we were able to trace related strains within a drinking water supply (Martínez-Murcia et al. 2000). However, in that study some unrelated strains also showed identical patterns.

When using the REP method, a total of 21 types were obtained (Table 1), type 14 being common to four strains (594, 638, 137 and 32) from different sources (Figure 2). However, they presented different types with the other two methods (Figures 1, 3).

With the ERIC-PCR method, a total of 24 types were obtained (Table 1) and all strains showed a distinct pattern, with the exception of the six mentioned isolates from Switzerland (Figure 3). These results confirm ERIC-PCR as the most discriminatory of the three methods investigated. The REP and ERIC methods have been broadly applied for typing purposes in several genera of bacteria (Vila et al. 1996; Sander et al. 1998; Marshall et al. 1999). Of those, only ERIC-PCR has been applied in *Aeromonas*, proving to be useful for tracing a nosocomial infection produced by *A. hydrophila* (Davin-Regli et al. 1998).

It has been argued that the reproducibility of the ERIC and REP methods depends upon standardization. Important parameters are the quantification of DNA and primers, the annealing temperature, the type of *Taq* DNA polymerase, $MgCl_2$ concentration and

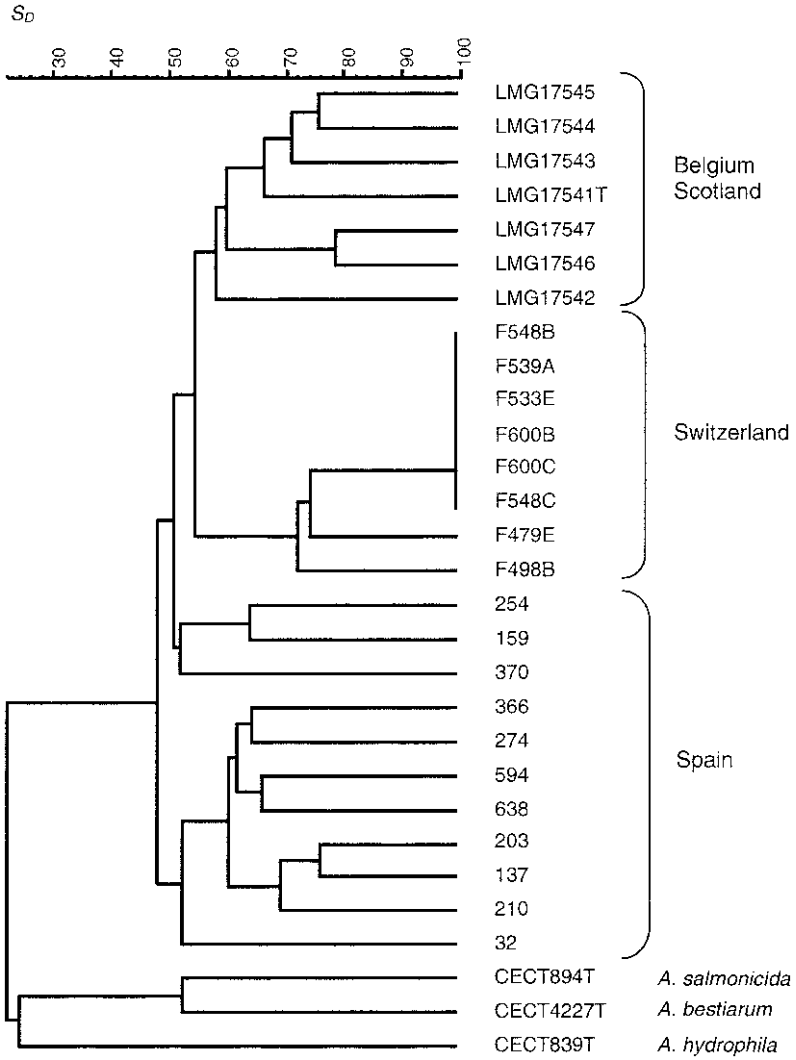

Figure 5. S_D / UPGMA cluster analysis based on the combined *Hinf*I-*Cfo*I, *Hinf*I-*Taq*I and *Alu*I ISR-RFLP, REP-PCR and ERIC-PCR produced patterns.

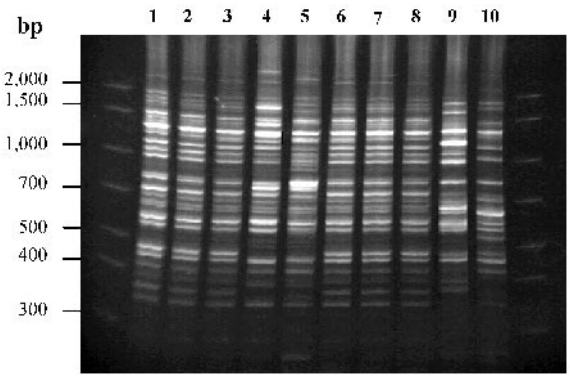

Figure 6. ERIC-PCR of *A. popoffii* strains. 1, F533E; 2, F600C; 3, F600B; 4, F498B; 5, F479E; 6, F548B; 7, F548C; 8, F539A; 9, 254; 10, 137

the thermocycler used (Tyler et al. 1997). We evaluated the reproducibility of these methods for three strains in duplicate, with the above mentioned parameters constant. When DNA extraction and PCR were performed in the same experiment and electrophoresis in the same gel for all strains, a correlation coefficient (*r*-value) of 0.92 to 0.96 was obtained for ERIC, and 0.90 to 0.95 for REP. DNA extraction and PCR done in the same experiments and duplicate strains electrophoresed in different gels lead to *r*-values of 0.78 to 0.86 for ERIC and 0.84 to 0.86 for REP. However, when DNA extraction, PCR and electrophoresis were performed in separate experiments, the *r*-values obtained were 0.50 to 0.67 for ERIC and 0.70 to 0.79 for REP. These results agree with those obtained by Vila et al. (1996) and Vinuesa et al. (1998) and indicate that the ERIC and REP methods are highly reproducible if DNA extraction, PCR and electrophoresis are done simultaneously for the strains being compared.

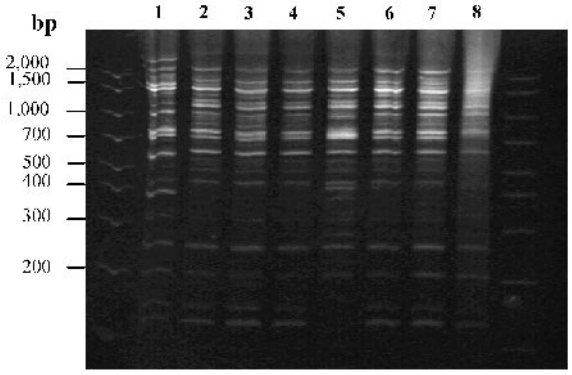

Figure 7. REP-PCR of *A. popoffii* strains. 1, F479E; 2, F600C; 3, F539A; 4, F533E; 5, F498B; 6, F548C; 7, F548B; 8, F600B.

Some studies have demonstrated that when different typing methods are combined, a higher resolution is obtained than when they are used individually (Vinuesa et al. 1998; Metha et al. 2001). Therefore, we evaluated the results obtained by pairing the different methods. Results obtained when combining ISR-RFLP and REP, ISR-RFLP and ERIC (data not shown) or ERIC and REP (Figure 4), produced a clear differentiation among all the strains. These results suggest that to avoid misinterpretations in epidemiological studies using either ISR-RFLP or REP, a combination of the methods is necessary. In addition, the ERIC and REP combination showed a tendency of the strains to cluster according to their geographical origin (Switzerland, Belgium, Scotland and Spain), although some freshwater isolates from Spain (254, 159 and 370) formed an intermediate group (Figure 4). When adding ISR-RFLP to the ERIC and REP combination each strain again had a unique profile (Figure 5). Furthermore, the overall topology of the dendrogram was maintained and reinforced both the tendency of strains to cluster according to their geographical origin and the genetic proximity of all *A. popoffii* strains. Isolates from Spain showed the highest intraspecific diversity. This could be attributed to the origins of the samples from Spain (sea water, rivers and reservoirs), as strains from the other geographical areas were all from freshwater (Huys et al. 1997; Demarta et al. 1999). Interestingly, two isolates (254 and 274) from the same river sample showed different ISR-RFLP, ERIC and REP types, indicating that different clones coexist in the same place.

According to our results, when using only one technique, ERIC-PCR was the most discriminatory, faster and easier to perform than ISR-RFLP and REP-PCR. The best combination of two methods found in our study was ERIC with REP because strains showed a tendency to group according to their geographical origin. However, this tendency was reinforced with the addition of ISR-RFLP.

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