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Identification of *Aeromonas culicicola* by 16S rDNA RFLP

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Abstract

Studies were conducted on the improvement of *A. culicicola* identification. This species is phenotypically very similar to *A. veronii* biotype sobria, *A. sobria*, and *A. allosaccharophila.* The sequences of 16S rDNA of *A. culicicola* isolates show the highest similarity with *A. jandaei*, *A. veronii*, and *A. caviae*. Digestion of 16S rDNA PCR product with AluI and MboI restriction endonucleases allowed discriminating *A. culicicola* from all other *Aeromonas* species with the exception of *A. jandaei.* Additional digestion of 16S rDNA PCR product with BceAI showed a possibility of distinguishing *A. jandaei* from *A. culicicola.*

Key words: *Aeromonas culicicola*, 16S rDNA RFLP, identification

The genus *Aeromonas* comprises Gram-negative chemoorganoheterotrophic bacteria widely spread in the surface water, sewage (Schubert, 1991) and food (Hänninen and Sittonen, 1995; Palumbo, 1996). Some of the strains of the bacteria have been implicated as human pathogens causing gastroenteritis, soft-tissue and wound infections, pneumonia, and bacteraemia (Altwegg, 1999). Some members of *Aeromonas* sp. cause a broad range of infections in cold- and warm-blooded animals (Gosling, 1996).

The taxonomy of the genus *Aeromonas* has undergone continual change due to addition of newly described species and reclassification of existing taxa. In Bergey's Manual of Systematic Bacteriology, the genus has been divided into four species: *A. hydrophila*, *A. caviae*, *A. sobria*, and *A. salmonicida* (Popoff, 1984). DNA-DNA hybridizations have resulted in founding at least 19–20 hybridization groups (HGs) within *Aeromonas* sp. Some of them have names: *A. hydrophila* (HG 1), *A. bestiarum* (HG 2), *A. salmonicida* (HG 3), *A. caviae* (HG 4), *A. media* (HG 5), *A. eucrenophila* (HG 6), *A. sobria* (HG 7), *A. veronii* (HG 8/10), *A. jandaei* (HG 9), *A. schubertii* (HG 12), *A. trota* (HG 14), *A. allosaccharophila* (HG 15), *A. encheleia* (HG 16), and *A. popoffii* (HG 17). Two genomic groups, HG 11 and HG 13 are unnamed (Altwegg, 1999; Martinez-Murcia, 1999). Recently, three new species have been described: *A. culicicola* (Pidyar *et al.*, 2002), *A. simiae* (Harf-Monteil *et al.*, 2004) and *A. molluscorum* (Mińana-Galbis *et al.*, 2004).

Phenotypic similarity of strains belonging to different *Aeromonas* sp. genomic groups creates many problems with their identification and requires confirmation by using the molecular methods (Kaznowski, 1997). Soler *et al.* (2003) have found that phenotypically only 14.5% or 20.3% of strains were correctly identified by MicroScan Walk/Away and BBL Crystal Enteric/Nonfermenter systems, respectively. Recently, Figueras *et al.* (2005) using 16S rDNA-RFLP obtained results that were completely different from those obtained by using API 20NE. Several molecular methods have been proposed to help distinguishing of *Aeromonas* spp.: rybotyping (Hänninen and Sittonen, 1995), multilocus enzyme electrophoresis (Altwegg *et al.*, 1991), amplified fragment length polymorphism (AFLP) (Huys *et al*., 1996), restriction fragment length polymorphism of 16S-23S rDNA intergenic spacer (Łaganowska and Kaznowski, 2004), PCR-amplified length polymorphism in tRNA intergenic spacers (Łaganowska and Kaznowski, 2005) and restriction fragment length polymorphisms of 16S rDNA (16S rDNA RFLP) (Borell *et al.*, 1997; Figueras *et al.*, 2000).

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A. culicicola is a species proposed by Pidyar *et al.* (2002) upon the analysis of three strains. One of them was isolated from the midgut of mosquito *Culex quinquefasciatus*, whereas the other two from the midgut of *Aedes aegyptii*. Recently, Figueras *et al.* (2005) recovered 27 *A. culicicola* isolates from a drinking water supply in Spain. There are difficulties in the identification of this species because the isolates are phenotypically very similar to *A. veronii* biotype sobria, *A. sobria*, and *A. allosaccharophila* (Pidyar *et al.*, 2002; Figueras *et al.*, 2005). The presence of a cytolytic enterotoxin gene in *A. culicicola* strains, which is considered as a characteristic virulence factor in *Aeromonas* spp., indicate that this species may have significance for public health (Pidyar *et al.*, 2003; Figueras *et al.*, 2005).

Characterization of the gene encoding 16S rRNA is now well established as a method for identification of species and genera of bacteria (Martinez-Murcia *et al.*, 1992; Martinez-Murcia, 1999). Complete sequences of 16S rDNA of the members of *Aeromonas* sp. have been published (Martinez-Murcia, 1999; Pidyar *et al.*, 2002) and are available in GenBank. Analysis of 16S rDNA sequences of all *Aeromonas* species showed differences from 1 to 33 substitutes. Pidyar *et al.* (2002) have found that sequences of their *A. culicicola* isolates show the highest similarity with *A. jandaei* (only one substitution), *A. veronii*, and *A. caviae* (5 substitutions). Recently, Figueras *et al.* (2005) revealed in 16S rDNA of six strains of *A. culicicola* five of the variation (positions 457 to 476) and other two at positions 1011 and 1018.

The objective of our study was to improve *A. culicicola* identification by restriction fragment length polymorphism of 16S rDNA.

Three strains of *A. culicicola* previously described by Pidyar *et al.* (2002) and 18 type or reference strains representing other 16 hybridization groups were used in this study (Table I). Bacterial DNA was extracted by using Nucleo-spin C + T kit (Macherey-Nagel, Germany). The primers 5'-AGA GTT TGA ATC ATG GCT CAG-3' and 5'-GGT TAC CTT GTT ACG ACT T-3' (Borrell *et al.* 1997) were synthesized by Genset Oligos (Paris, France). PCR amplifications were carried out in a final volume of 50 µl with 100 ng of template DNA, 5 µl of 10×PCR buffer with $NH_4(S0_4)_2$, 50 pmol of each primer, 200 µM of dNTP mix, 2.5 mM of $MgCl_2$, and 2 U of Taq polymerase (Fermentas). The amplification involved initial denaturation step (93°C, 3 min), followed by 35 cycles of denaturation (94°C, 1 min), annealing (56°C, 1 min), and extension (72°C, 1 min). After the final cycle, extension at 72°C was allowed for 10 min. PCR products were precipitated by 96% cold (–20°C) ethanol, dried, and resuspended in 25 µl of sterile water (Borrell *et al*. 1997).

Table I
Bacterial strains used in the study

Hybridization group	Strain	Hybridization group	Strain
1	*A. hydrophila* ATCC 7966^T	9	*A. jandaei*: ATCC 49568^T, LMG 13065
2	*A. bestiarum* ATCC 51108^T:	11	*Aeromonas* sp ATCC 35941
3	*A. salmonicida* subspecies *salmonicida* LMG 3780^T	12	*A. schubertii* ATCC 43700^T
4	*A. caviae* ATCC 15468^T	13	*Aeromonas* sp. LMG 17321
5	*A. media* ATCC 33907^T	14	*A. trota* ATCC 49657^T
6	*A. eucrenophila* ATCC 23309^T	15	*A. allosaccharophila* CECT 4199^T
7	*A. sobria* CIP 7433^T	16	*A. encheleia* CECT 4342^T
8/10	*A. veronii* biotype sobria CDC 0437–84	17	*A. popoffii* LMG 17541^T
10/8	*A. veronii* biotype veronii ATCC 35624^T	18	*A. culicicola*: MTCC 3249, SH 2/5, SLH 21/5

Abbreviations: ATCC – American Type Culture Collection, Manassas,VA, USA; CDC – Centers for Disease Control, Atlanta, USA; CECT – Collection Espanola de Cultivos Tipo, Universitad de Valencia, Spain; CIP – Collection bacterienne de l'Institut Pasteur, Paris, France; LMG – Culture Collection, Laboratorium voor Microbiologie Universiteit Gent, Belgium; MTCC, SH, SLH – strains recived from dr Y. Shouche, Molecular Biology Laboratory, Pune University, Ganeshkhind, India.

Enzymatic digestions were performed by incubating 5 µl of the amplification product with 5 U of AluI and MboI (Fermentas) or BceAI (New England Labs) overnight at 37°C. Aliquots of 10 µl of each reaction mixture were mixed with 2 µl of loading buffer containing 0.09% bromophenol blue, 0.9% xylene cyanol FF, 60% glycerol and 60 mM EDTA, and the mixture was electrophoresed on 2.5% Micropore Nu agarose gel (Prona, Spain) in Tris-borate-EDTA buffer. Gels were stained with ethidium bromide (1 µg/ml) and documented with Bio-Print V.99 system (Vilbert Lourmat, France). Sizes of DNA fragments were cal-

culated using GelCompar II software (Applied Maths, Belgium) with MassRuler DNA Ladder Mix (Fermentas) as a molecular size reference.

We did not find a commercial restriction endonuclease that would allow one-step distinguishing of all *Aeromonas* species. *A. culicicola* can be identified in two steps. In the first step, the 16S rDNA amplicon is digested with AluI and MboI restriction endonucleases according to Borrell *et al.* (1997) and Figueras *et al.* (2000), who have elaborated a scheme for distinguishing *Aeromonas* species upon restriction analysis of 16S rDNA digested with several endonucleases. DNA fragments of 16S rDNA digested with AluI and MboI allowed distinguishing *A. culicicola* from all other *Aeromonas* species with the exception of *A. jandaei*. These two species gave identical DNA fragments of 207, 195, 188, 158 138, 78, and 69 bp.

Sequences of 16S rDNA of six strains of *A. culicicola* have been described (Figueras *et al.* 2005). All the sequences contain thymine at position 254, whereas in *A. jandaei* there is cytosine at this position (Gen Bank). On-line analysis (http://www.restrictionmapper.org) showed a possibility of distinguishing *A. jandaei* from *A. culicicola* by BceAI digestion (New England), which recognizes sequence 5'ACGGC $(N)_{12}$3'; 3'TGCCG $(N)_{14}$5'. Electrophoresis of DNA fragments obtained after digestion with BceAI empirically proved the capability of this method. The sizes of the DNA fragments were 800, 322, 285, and 110 bp for *A. culicicola*, and 520, 322, 285, and 110 bp for *A. jandaei* (Fig. 1). Identification of *A. jandaei* and *A. culicicola* only upon digestion with BceAI, without preliminary differentiation from other *Aeromonas* species by AluI and MboI digestion, is not possible. PCR products of *A. culicicola* 16S rDNA digested with BceAI gave fragments similar in size to those for HGs 1 to 11, and 14–17. *A. schubertii* (HG 12) and strain of unnamed HG 13 gave DNA fragments similar to *A. jandaei*.

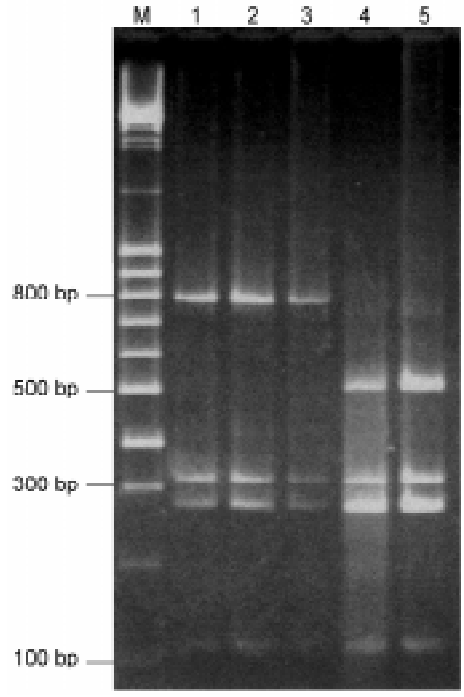

Fig. 1. 16S rDNA RFLP patterns of *A. culicicola* and *A. jandaei* strains, obtained by using BceAI endonuclease

Lanes: 1. A. *culicicola* MTCC 3249, 2 *A. culicicola* SLH21/5, 3. A. *culicicola* SH2/5, 4. *A. jandaei* ATCC 49568, 5. *A. jandaei* LMG 13063, M. Molecular size reference.

In conclusion, we propose BceAI treatment of PCR-amplified 16S rRNA gene for distinguishing *A. culicicola* and *A. jandaei*. This rapid method complements the identification scheme proposed by Borrell *et al.* (1997) and expanded by Figueras *et al.* (2000), which enables identification of *Aeromonas* sp. strains belonging to 17 hybridization groups.

Literature

Altwegg M., M.W. Reeves, R. Altwegg-Bissig and D. Brenner. 1991. Multilocus enzyme analysis of genus *Aeromonas* and its use for species identification. *Zbl. Bakt.* **275**: 28–45.

Altwegg M. 1999. *Aeromonas* and *Plesiomonas*, p. 507–516. In: P.R. Murray, E.J. Baron, M.A. Pfaller, F.C. Tenower, R.H. Yolken. (eds), Manual of Clinical Microbiology. ASM Press, Washington.

Borrell N., S.G. Acinas, M.J. Figueras and A.J. Martinez-Murcia. 1997. Identification of *Aeromonas* clinical isolates by restriction fragment length polymorphism of PCR-amplified 16S rRNA genes. *J. Clin. Microbiol*. **35**: 1671–1674.

Figueras M.J., L. Soler, M.R. Chacon, J. Guerro and A.J. Martinez-Murcia. 2000. Extended method for discrimination of *Aeromonas* spp. by 16S rDNA RFLP analysis. *Int. J. Syst. Bacteriol.* **50**: 2069–2073

Figueras M.J., A. Suarez-Franquet, M.R. Chacon, L. Soler, M. Navarro, C. Alejandre, B. Grasa, A.J. Martinez-Murcia and J. Guarro. 2005. First record of the rare species *Aeromonas culicicola* from a drinking water supply. *Appl. Environ. Microbiol*. **71**: 538–541

Gosling P.J. 1996. *Aeromonas* species in disease of animals, p. 175–196. In: B. Austin, M. Altwegg, P.J. Gosling, S.W. Joseph (eds). The genus *Aeromonas*. J. Wiley and Sons, Chichester, New York, Brisbane, Toronto, Singapore.

Harf-Monteil C., A. Le Fleche, P. Riegel, G. Prevost, D. Bermond, P.A.D. Grimont and H. Monteil. 2004. *Aeromonas simiae* sp. nov., isolated from monkey faeces. *Int. J. Syst. Bacteriol.* **54**: 481–485.

Hänninen M.L. and A. Sittonen. 1995. Distribution of *Aeromonas* phenospecies and genospecies among strains isolated from water, foods or from humans clinical samples. *Epidem. Infect*. **115**: 39–50.

Huys G., R. Coopman, P. Janssen and K. Kersters. 1996. High-resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting. *Int. J. Syst. Bact*. **46**: 572–580.

K a z n o w s k i A. 1997. Numerical taxonomy and DNA-DNA hybridizations of *Aeromonas* strains isolated from human diarrheal stool, fish and environment. *System. Appl. Microbiol.* **20**: 458–467.

Ł a g a n o w s k a M. and A. K a z n o w s k i. 2004. Restriction fragment length polymorphisms of 16S-23S rDNA intergenic spacer of *Aeromonas* spp. *System. Appl. Microbiol*. **27**: 549–557

Ł a g a n o w s k a M. and A. K a z n o w s k i. 2005. Polymorphism of *Aeromonas* spp. tRNA intergenic spacer. *System. Appl. Microbiol*. **28**: 222–229

M a r t i n e z - M u r c i a A.J., S. B e n l l o c h and M.D. C o l l i n s. 1992. Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: lack of congruence with results of DNA-DNA hybridizations. *Int. J. Syst. Bact*. **42**: 412–421.

M a r t i n e z - M u r c i a A.J. 1999. Phylogenetic positions of *Aeromonas encheleia*, *Aeromonas popoffii*, *Aeromonas* DNA hybridization Group 11 and *Aeromonas* Group 501. *Int. J. Syst. Bact*. **49**: 1403–1408.

M i ñ a n a - G a l b i s D., M.M. F a r f á n, C. F u s t é and J. G a s p a r L o r é n. 2004. *Aeromonas molluscorum* sp. nov. isolated from bivalve molluscs. *Int. J. Syst. Bact*. **54**: 2073–2078

P a l u m b o S.A. 1996. The *Aeromonas hydrophila* group in food, p. 287–310. In: B. Austin, M. Altwegg, P.J. Gosling, S.W. Joseph (eds). The genus *Aeromonas*. J. Wiley and Sons, New York, Chichester, Brisbane, Toronto, Singapore.

P i d i y a r V., A. K a z n o w s k i, N. B a d r i N a r a y a n, M. P a t o l e and Y. S h o u c h e. 2002. *Aeromonas culicicola* sp. nov. from the midgut of *Culex quinquefasiatus*. *Int. J. Syst. Evol. Microbiol*. **52**: 1723–1728.

P i d i y a r V., K. J a n g i d, K.M. D a n y a n a n d a, A. K a z n o w s k i, J.M. G o n z a l e z, M.S. P a t o l e and Y.S. S h o u c h e. 2003. Phylogenetic affiliation of *Aeromonas culicicola* MTCC 3249^T based on *gyrB* gene sequence and PCR-amplicon sequence analysis of cytolytic enterotoixin gene. *System. Appl. Microbiol.* **26**: 197–202.

P o p o f f M. 1984. Genus III: *Aeromonas*, p. 545–547 In: N. R. Krieg, J. G. Holt (eds). Bergey's Manual of Systematic Bacteriology, Wiliams and Wilkins, Baltimore, London.

S c h u b e r t R.H.W. 1991. Aeromonads and their significance as potential pathogens in water. *J. Appl. Bacteriol. Symp. Suppl*. **70**: 131S–135S.

S o l e r L., F. M a r c o, J. V i l a, M.R. C h a c o n, J. G u e r r o and M.J. F i g u e r a s. 2003. Evaluation of two miniaturized systems. MicroScan W/A and BBL Crystal E/NF, for identification of clinical isolates of *Aeromonas* spp. *J. Clin. Microbiol.* **41**: 5732–5734