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; Martínez-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* (Miñana-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 (Laganowska and Kaznowski, 2004), PCR-amplified length polymorphism in tRNA intergenic spacers (Laganowska 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 B S T R A C T**

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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 NH₄(SO₄)₂, 50 pmol of each primer, 200 µM of dNTP mix, 2.5 mM of MgCl₂, 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).

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### Table I

**Bacterial strains used in the study**

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

Abbreviations: ATCC – American Type Culture Collection, Manassas, VA, USA; CDC – Centers for Disease Control, Atlanta, USA; CECT – Collection Espanola de Cultivos Tipo, Universidad 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 received 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% Microapore 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 digestion 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)_{123}; 3’TGCCG (N)_{145}'. 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 to 17. A. schubertii (HG 12) and strain of unnamed HG 13 gave DNA fragments similar to A. jandaei.

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**


