A DNA probe specific for *Aeromonas* colonies

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**Abstract**

Members of the genus *Aeromonas* are important enteropathogens. Commercial identification systems are often unable to correctly identify *Aeromonas* strains and misidentification as *Vibrio* spp. is common. A digoxigenin-DNA probe based on a 237 bp of the glycerophospholipid-cholesterol acyltransferase gene has been tested in a colony hybridization assay. The probe hybridized with all *Aeromonas* species tested (*n* = 16) but not with strains of other enteropathogenic bacteria (*n* = 20). The probe allowed the unequivocal identification of *Aeromonas* in primary isolation media within 36 h. © 2002 Elsevier Science Inc. All rights reserved.

1. Introduction

The genus *Aeromonas* comprises oxidase-positive, facultative anaerobic, Gram-negative bacilli. Some species have been described as causative agents of various gastrointestinal and, to a lesser extent, extraintestinal diseases (Janda & Abbott, 1998; Altgwegg, 1999). The commercial biochemical systems currently employed in clinical laboratories for the identification of Gram-negative enterobacteria (API 20E, Vitek, MicroScan etc.) are unable to correctly identify *Aeromonas* spp. and confusion with *Vibrio* spp. (*V. fluvialis, V. damsela* or *V. cholerae*) is common (Overman et al., 1985; Overman and Overley, 1986; Abbott et al., 1998; Vivas et al., 2000). The confusion with *V. cholerae* is of special relevance due to its public health significance (Abbott et al., 1998).

Given the increasing importance of *Aeromonas* in clinical infections (Janda & Abbot, 1998) and the limitations of the existing methods, there is a need to design accurate systems that can unequivocally identify these microorganisms.

Molecular methods tend to be more accurate, the vast majority of them having been focused on species identification by using either the 16S rRNA gene (Ash et al., 1993; Dorsch et al., 1994; Borrell et al., 1997; Demarta et al., 1999; Figueras et al., 2000) or virulence associated genes such as aerolysin (Husslein et al., 1992; Khan et al., 1999) and lipases (Cascon et al., 1996). These methods are useful and suitable in well-equipped research laboratories, but clinical laboratories need cheaper and faster methods for unequivocal detection of *Aeromonas*, especially at the genus level.

Glycerophospholipid-cholesterol acyltransferase (GCAT) is an unusual extracellular lipase released by members of the genus *Vibrio* and *Aeromonas* (MacIntyre et al., 1979). The enzyme is considered one of the potential virulence factors that can contribute to the pathogenesis of *Aeromonas* in fish (Lee and Ellis, 1990). The gene has been cloned and sequenced in strains of *A. salmonicida* and *A. hydrophila* (Thornt et al., 1988; Nerland, 1996). In a previous study, where the gene presence was investigated by PCR, we found it in practically all *Aeromonas* strains tested, including representatives of all species (Chacón et al., 2001, submitted for publication).

The aim of the present study was to evaluate if the 237 bp fragment of the GCAT gene could be used to distinguish *Aeromonas* sp. from other common enteropathogens in colony hybridization assays.

2. Materials and methods

2.1. Bacteria strains and media

A total of 36 bacteria strains belonging to 8 different genera were tested, although from 5 of them only one strain was assayed. The strains used in this study are listed in Table 1. The strains of the different species (up to 5 colonies) were inoculated in groups of 9 onto petri dishes (9 cm
The media used were: tryptic soy agar (TSA) (Difco, Barcelona, Spain), 5% human agar blood, MacConkey agar (Difco), Salmonella-Shigella agar (SS) (Difco), xylose lysine deoxycholate agar (XLD) (Difco) and Hektoen enteric agar (Difco). The plates were incubated at 37°C for 18–24 h.

2.2. Colony blot preparation

The colonies grown in the above mentioned media were blotted to a positively charged autoclaved nylon membrane (Boehringer Mannheim, Barcelona, Spain). The membrane was then transferred to a filter paper saturated with 10% SDS (Sodium dodecyl sulfate) for 3 min, followed by an incubation on a second filter paper soaked with 0.5 N NaOH-1.5 M NaCl for 5 min. The membrane was then neutralized by placing it on a filter paper soaked with 1 M Tris Base, pH 7 and 1.5 M NaCl for 5 min followed by another incubation of 5 min on a new filter saturated with 10× SSC (1.5 M NaCl-0.15 M sodium citrate pH 7.2). Finally the membrane was UV-crosslinked for 3 min on an UV-transiluminator, rinsed in sterile double-distilled H₂O and air dried until used.

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Hybridization with dig-GCAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. hydrophila ATCC°7966T</td>
<td>Milk tin</td>
<td>+</td>
</tr>
<tr>
<td>A. bestiarum ATCC51108T</td>
<td>Fish</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida ATCC33658T</td>
<td>Fish</td>
<td>+</td>
</tr>
<tr>
<td>A. caviae ATCC15468T</td>
<td>Guinea pig</td>
<td>+</td>
</tr>
<tr>
<td>A. media ATCC33907T</td>
<td>Fish</td>
<td>+</td>
</tr>
<tr>
<td>A. eucrenophila ATCC23309T</td>
<td>Fish</td>
<td>+</td>
</tr>
<tr>
<td>A. sobria ATCC43970T</td>
<td>Fish</td>
<td>+</td>
</tr>
<tr>
<td>A. veronii ATCC35624T</td>
<td>Sputum</td>
<td>+</td>
</tr>
<tr>
<td>A. fandaei ATCC49568T</td>
<td>Faeces</td>
<td>+</td>
</tr>
<tr>
<td>Aeromonas HG°11 ATCC35941</td>
<td>Ankle suture</td>
<td>+</td>
</tr>
<tr>
<td>A. schuberti ATCC43700T</td>
<td>Abscess</td>
<td>+</td>
</tr>
<tr>
<td>A. trota ATCC49657T</td>
<td>Faeces</td>
<td>+</td>
</tr>
<tr>
<td>A. encheleia ATCC51929T</td>
<td>Fish</td>
<td>+</td>
</tr>
<tr>
<td>A. allosaccharophila ATCC51208T</td>
<td>Fish</td>
<td>+</td>
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<tr>
<td>A. popoffi LMG°17541T</td>
<td>Drinking water production plant</td>
<td>+</td>
</tr>
<tr>
<td>Aeromonas Group 501 CECT°5178</td>
<td>N.S.</td>
<td>+</td>
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<tr>
<td>Vibrio fluvialis ATCC33809T</td>
<td>Faeces</td>
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</tr>
<tr>
<td>V. vulnificus ATCC27562T</td>
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<td>-</td>
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<td>V. vulnificus ATCC33147</td>
<td>Diseased fish</td>
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</tr>
<tr>
<td>V. vulnificus CECT 4602</td>
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<td>V. cholerae NO-01 CECT 557</td>
<td>River water</td>
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<td>V. cholerae NO-01 CECT 653</td>
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<td>V. parahaemolyticus ATCC17802T</td>
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<tr>
<td>V. parahaemolyticus CECT 612</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>Escherichia coli O:157:H7 CECT 4076</td>
<td>Human hemorrhagic colitis</td>
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<td>E. coli O:128: K86(B) CECT 742</td>
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<td>Pseudomonas aeruginosa ATCC25668</td>
<td>Human clinical isolate</td>
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<tr>
<td>Salmonella typhimurium ATCC13311</td>
<td>Food poisoning</td>
<td>-</td>
</tr>
<tr>
<td>Shigella flexneri CECT585</td>
<td>Dysentery</td>
<td>-</td>
</tr>
<tr>
<td>Plesiomonas shigelloides CECT 4262T</td>
<td>Faeces</td>
<td>-</td>
</tr>
<tr>
<td>Yersinia enterocolitica CECT 754</td>
<td>Faeces</td>
<td>-</td>
</tr>
</tbody>
</table>

a ATCC, American Type Culture Collection.
b HG, Hybridization Group.
c LMG, Belgian Coordinated Collections of Microorganisms, Belgium.
d CECT, Colección Española de Cultivos Tipo.
e N.S., Not specified.
the PCR amplifications and the amplified sequence are shown in Figure 1. PCR amplification conditions were those of Chacon et al. (2001, submitted for publication), although the annealing temperature was adjusted to 65°C so that the primers gave only a specific signal for Aeromonas DNA. Briefly, PCR amplification was performed in a final volume of 50 μl containing 1.2–2 μg of DNA, 0.3 mM deoxynucleotide triphosphate mix, 1× PCR buffer (Gibco-BRL, Barcelona, Spain), 1.8 mM MgCl₂, 0.1 μM of primers and, 2.5 units of Taq polymerase (Gibco-BRL). PCRs were carried out in a Perkin-Elmer GeneAmp 2400 PCR System by using the following program: 3 min denaturation cycle at 95°C, 35 melting cycles at 94°C for 1 min, annealing for 1 min at 65°C, followed by a final elongation at 72°C for 1 min. The PCR products (12 μl) were analyzed on 1.5% agarose gel (Sigma-Aldrich Ltd., Madrid, Spain) and electrophoresed on 1× Tris-Borate-EDTA for 2 h at 60 V. The PCR product was visualized under UV light.

The GCAT-PCR amplified fragment (237 bp) was cleaned using Concert™ Rapid PCR Purification System (Life Technologies, Barcelona, Spain), and 25 ng of the clean fragment was labeled using Digoxigenin labeling kit following the manufacturer’s instructions (Roche Diagnostics, Barcelona, Spain).

2.4. Colony hybridization with the DIG-labeled probe

The nylon membranes were hybridized with the denatured digoxigenin-labeled GCAT probe overnight at 68°C using DIG Easy Hyb solution (Roche Diagnostics); followed by washes at room temperature 2 × 5 min in 2× SSC (Standard Saline Citrate)-0.1% SDS; at 68°C 2 × 15 min in 0.5% SSC-0.1% SDS and, a final wash 2 × 15 min in 0.1% SSC-0.1% SDS at the same temperature. Membrane detection signal was performed using the Digoxigenin detection kit as recommended by the manufacturer (Roche Diagnostics).

3. Results and Discussion

In a previous study we demonstrated that the GCAT gene was present in 98% of the 234 strains of Aeromonas of environmental and clinical origin representing all species (Chacon et al., 2001, submitted for publication). This demonstrates that this gene is widely distributed. Therefore, it was considered a good candidate to be tested as a genus probe. The primers designed, for the GCAT gene, amplified a fragment of the expected size (237 bp) and no other amplicons were generated. Lower annealing temperatures (56°C, 58°C, 60°C and 62°C) weakly amplify a fragment of the same size in some Vibrio isolates (data not shown). This drawback was overcome when the PCR temperature was increased to 65°C.

Table 1 shows the results obtained by testing the DIG-labeled GCAT 237 bp fragment on colony blot assays in the type strains of all the species (n = 14), in 2 reference strains of Aeromonas and in 20 strains of other genera. The probe was proven to be specific for Aeromonas because it did not react with strains from other genera tested. Furthermore, the three Aeromonas strains (one A. bestiarum and two A. schubertii) that were negative in a previous study where the presence of the GCAT gene was investigated by PCR (Chacon et al., 2001, submitted for publication) gave a positive signal here. The failure to amplify the gene in those strains in the mentioned study could have been due to a possible mismatch of the primers.

To evaluate if the specificity of the probe could be influenced by the composition of the culture media routinely used in clinical laboratories to recover bacteria from stool samples, we tested four of the most common media i.e., MacConkey, SS, blood agar and Hecktoen. Equal results were found in all the media tested. Figure 2 illustrates an example of the hybridization results obtained when colo-
nies of *Aeromonas* and other enteropathogens were grown on blood agar (Figure 2 A). A positive signal was observed on the replica filter for *A. hydrophila* and *A. veronii*, but the probe did not hybridize with *V. cholerae*, *V. parahaemolyticus* or *A. veronii* (Figure 2 B).

The DNA probes reported to date for the detection of *Aeromonas* are species-specific (Husslein et al., 1992; Ash et al., 1993; Dorsh et al., 1994; Cascon et al., 1996; Demarta et al., 1999) and nothing is known about their possible cross-reactivity with other enteric bacteria. The use of these probes to detect *Aeromonas* strains could be costly and time consuming because of the need to test more than one probe, and no probes have yet been designed for all the species.

To our knowledge, there is only one oligonucleotide DNA probe for the genus *Aeromonas*, based on the 16S rRNA gene. It has been used for fluorescent "in situ" hybridization (FISH) of these microorganisms in water samples (Kämpfer et al., 1996). However, this probe has not yet been tested in all the recent *Aeromonas* spp. This probe was recently used to detect possible *Aeromonas* strains present in biofilms, although no signal was obtained by FISH in any sample tested (MacDonald and Brözel, 2000).

Although clinical identification of *Aeromonas* by conventional techniques can be completed in 72 h the results, as we have already mentioned, are frequently unreliable. Confusion with *Vibrio* spp. could be avoided by using two complementary tests, i.e., growth in nutrient broth in the presence of 6% NaCl and susceptibility to the vibriostatic agent O/129 (2,4-diamino-6, 7-disopropylpteridine), although their performance is still more time consuming. The main advantage of the probe here described is that it can give unequivocal results within 36 h and requires no specific skill by the end user for its application and interpretation.

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**REFERENCES**


