

Development of duplex-PCR for identification of *Aeromonas* species

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ABSTRACT

Introduction: The number of reports of intestinal infections caused by *Aeromonas* spp. has increased significantly in recent years. In most clinical laboratories, identification of these bacteria is carried out by general phenotypic tests that sometimes do not accurately differentiate *Aeromonas* and *Vibrio*. **Methods:** A duplex-polymerase chain reaction (PCR) was developed directed to 2 targets identifying *Aeromonas* spp. pathogenic to humans. **Results:** The duplex-PCR results were reproducible and specific for *Aeromonas* spp. pathogenic to humans. **Conclusions:** This method will allow differentiation between *Vibrio* and *Aeromonas* spp. in patients with in cholera-like symptoms and can also be used in water quality monitoring.

Keywords: *Aeromonas*. Identification. Polymerase chain reaction.

Aeromonas spp. are gram-negative aquatic bacteria involved in infections such as pneumonia, sepsis, hemolytic uremic syndrome, septic arthritis, and more recently they have been reported causing intestinal infections¹.

Aeromonas diagnosis in most clinical laboratories, especially in developing countries, is based on phenotypic methods. The oxidase test is used for differential diagnosis from Enterobacteriaceae and a series of other biochemical tests are used for differential diagnosis from *Vibrio* and *Plesiomonas*². The results are imprecise and *Aeromonas* is often misclassified, being mainly misidentified as *Vibrio*, which similarly grows on thiosulfate citrate bile salts sucrose (TCBS) and is oxidase positive³. Commercial systems for bacterial identification such as API20E and Vitek have proven useless for *Aeromonas* identification^{3,4}. Hence, the role of *Aeromonas* as an etiologic agent of infection remains underestimated².

Several molecular methods for genotypic identification of *Aeromonas* spp.⁵ are now available⁶⁻⁹. However, most of them are species-specific and targeted to potential virulence genes. Hence, they are unable to recognize non-virulent *Aeromonas* species.

Here we describe a duplex polymerase chain reaction (PCR) that provided timely and accurate identification of medically important *Aeromonas* spp. by amplification of genes encoding

glycerolphospholipid: cholesterol acyltransferase (*gcat*) and small subunit (16S) recombinant DNA (rRNA).

Preliminary tests were performed using reference strains of *Aeromonas* spp. most commonly involved in human diseases (*A. caviae*, *A. hydrophila*, *A. jandaei*, *A. media*, *A. veronii*, and *A. trota*) as well as *Vibrio* species of major medical importance (*V. cholerae*, *V. alginolyticus*, *V. fluvialis*, *V. furnissi*, *V. mimicus*, *V. parahaemolyticus* and *V. vulnificus*). We also tested 40 strains of *Aeromonas* spp. that were isolated from feces of patients with diarrhea.

Bacterial cultures were provided by the Bacterial Culture Collection of Health Importance/IOC/FIOCRUZ. *Aeromonas* strains were identified genotypically by restriction fragment length polymorphism (RFLP)⁶ and *Vibrio* isolates were typed by biochemical and serological methods. Extraction of chromosomal DNA from the cultures was performed as previously described¹⁰.

Some authors^{11,12} have suggested that all *Aeromonas* spp. harbor *gcat*, but others report that some do not^{7,13,14}. Therefore, we included the second primer targeted to the 16S gene. Primer sequences *gcat*-f: 5'-ctctggaatccaagtatcag-3' and 5'-*gcat*-r ggcaggttgaacagcagtatct-3' were previously described¹⁵, and 16S-f 5'-accgagcggttgataagt-3' and 5'-16S-r ggcaacaaggacaggggt-3' were designed for the present study. For primer design, *Aeromonas* spp. 16S gene sequences were collected from the European Molecular Biology Laboratory (EMBL) database (accession nos. X60411, X60412, X60415, X60416, FJ998417, HM007582, AB034760, AJ224309, and FJ998415) and aligned by MegAlign (DNASTar), and the conserved regions within the gene were selected.

Duplex-PCR reactions were prepared in a total volume of 25µL containing 50mM KCl, 10mM Tris-HCl, 2.5M MgCl₂, 400mM of each dNTP, 40pmol GCAT primers, 20pmol 16S primers, 1U *Taq* DNA polymerase (Promega), and 20ng DNA.

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The amplifications were performed in a Biometra T-3000 Genetic Analyzer thermal cycler programmed for 35 cycles of 1 min at 94°C, 1 min at 54°C, 1 min at 72°C and a final 5 min extension at 72°C. Ten microliters of PCR products were electrophoresed in a 1% agarose gel containing SYBR Safe DNA gel stain (Invitrogen) at 100V for 1h, visualized on an ultraviolet (UV) transilluminator, and photographed using the Kodak 1D image analysis version 3.5 (Digital Kodak Science).

Duplex-PCR reproducibility was assessed by quadruplicate assays with 4 *Aeromonas* reference strains (*A. hydrophila* ATCC 7966^T, *A. veronii* ATCC 35624^T bio *veronii*, *A. caviae* ATCC 15468^T, and *A. hydrophila* IOC 11036), and specificity was assessed employing 6 reference *Aeromonas* and 7 *Vibrio* spp. isolates. As noted, 40 clinical strains of *Aeromonas* spp. were also included in the tests. Although there are more than 30 *Aeromonas* species described, only 6 are commonly found to be involved in human infections¹, and differential diagnosis is clinically challenging. These clinically important species were among those included in the present study.

The 2 target segments of *gcat* (237 bp) and 16S (~ 600 bp) were amplified in all *Aeromonas* reference strains tested (**Figure 1**) (**Figure 2**, lanes 1-8) as well as in the 40 clinical isolates mentioned (data not shown). The *gcat* gene was not amplified in any *Vibrio* species tested (**Figure 2**, lanes 9-16). However, faint bands corresponding to 16S were seen with *V. cholerae* non-O1/non-O139, *V. alginolyticus*, *V. mimicus*, and *V. parahaemolyticus* (**Figure 2**, lanes 10, 11, 14, and 15, respectively).

Vibrio spp. were included because of their biochemical and serological similarities to *Aeromonas*, which, as noted, have previously made differentiation difficult. Although the 16S gene was amplified in some of the *Vibrio* species, it did not hinder

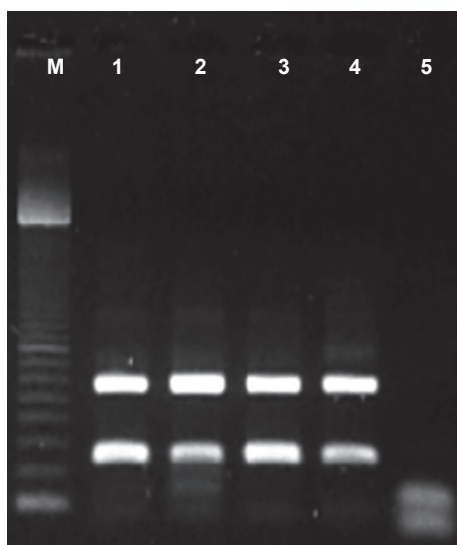


FIGURE 1 - Duplex-PCR reproducibility. Lanes: M: 100 bp molecular marker; 1: *Aeromonas caviae* ATCC 7966; 2: *Aeromonas veronii* ATCC 35624; 3: *Aeromonas caviae* ATCC 15468; 4: *Aeromonas hydrophila* IOC 11036; 5: negative control.

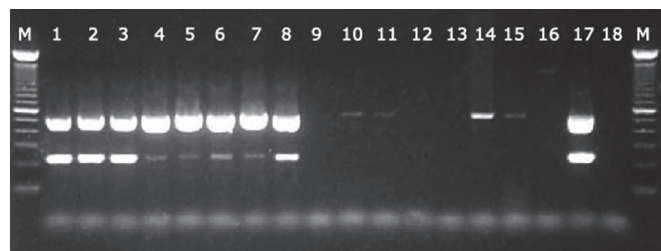


FIGURE 2 - Duplex-PCR specificity. Lanes: M: 100 bp molecular marker; 1: *Aeromonas caviae*; 2: atypical *Aeromonas caviae*; 3: *Aeromonas hydrophila*; 4: *Aeromonas jandaei*; 5: *Aeromonas media*; 6: *Aeromonas veronii*; 7: atypical *Aeromonas veronii*; 8: *Aeromonas trota*; 9: *Vibrio cholerae* O1; 10: *Vibrio cholerae* non-O1/non-O139; 11: *Vibrio alginolyticus*; 12: *Vibrio fluvialis*; 13: *Vibrio furnissi*; 14: *Vibrio mimicus*; 15: *Vibrio parahaemolyticus*; 16: *Vibrio vulnificus*; 17: positive control; 18: negative control.

the efficacy of the test, which recorded samples positive for *Aeromonas* only when both of the targeted genes were amplified.

The duplex-PCR method introduced here showed high reproducibility and specificity for *Aeromonas* spp. Therefore it should be useful as an alternative to phenotypic methods for identifying these bacteria and allowing a presumptive differentiation between the Aeromonadaceae and the Vibrionaceae that are commonly involved in human infections.

Rigorous validation of the technique should be sought by increasing testing with clinical *Aeromonas* isolates and other gram-negative oxidase positive bacteria strains. However, we consider publication of these preliminary results necessary because they indicate that laboratory identification of *Aeromonas* spp. can be improved with the duplex-PCR method we describe.

If validated, this duplex-PCR method can be employed to more effectively evaluate the incidence of *Aeromonas* in human enteric disease during routine diagnosis versus the traditional phenotypic procedures. It will allow a better understanding of the emerging role *Aeromonas* species in the pathogenesis of enteric infections and assist in guiding appropriate control measures.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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