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 Plesiomonas2. 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 identification3,4. 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 authors11,12 have suggested that all Aeromonas spp. harbor gcat, but others report that some do not1,13,14. Therefore, we included the second primer targeted to the 16S gene. Primer sequences gcat-f: 5’-ctctggaatcccaagtatcag-3’ and 5’-gcat-r ggcaacaaaggacaggggt-3’ were designed for the present study. Therefore, 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 (DNAsstar), 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.
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 7966T, A. veronii ATCC 35624T bio veronii, A. caviae ATCC 15468T, 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 infections, 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 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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REFERENCES


