Quadruplex PCR for Simultaneous Detection of Serotype, Biotyp e, Toxigenic Potential, and Central Regulating Factor of *Vibrio cholerae* V

Hemant Kumar Khuntia, Bibhuti Bhusan Pal, and Guru Prasada Chhotray*

Regional Medical Research Centre, Chandrasekharapur, Bhubaneswar, Pin-751023, Orissa, India

Received 7 January 2008/Returned for modification 11 February 2008/Accepted 30 April 2008

A quadruplex PCR was developed for the simultaneous detection of genes specific for *Vibrio cholerae* O1 and/or O139 serogroup (*wbe* and/or *wbf*), cholera toxin A subunit (*ctxA*), toxin-coregulated pilus (*tcpA*), and central regulating protein ToxR (*toxR*) in a single tube reaction. This is a simple, rapid, and accurate approach for the detection of toxigenic *V. cholerae* O1 and/or O139 and can prevent the rapid spread of the disease by early detection.

Although there are many bacterial causes of diarrhea, only *Vibrio cholerae*, the etiological agent of cholera, has caused repeated pandemics (15). Of the more than 280 serogroups of *V. cholerae* identified so far (17), only two serogroups, O1 and O139, are known to cause epidemic and pandemic cholera (6). However, nonepidemic serogroups (non-O1 and non-O139), although not involved in cholera epidemics, can be pathogenic and are occasionally associated with small outbreaks of diarrheal diseases (9). Because untreated cholera leads to the onset of serious outbreaks and potentially great devastation, quick diagnosis and identification of the causative serogroup is necessary from a public health point of view. However, conventional methods of diagnosis are inconvenient for the early detection and characterization of cholera-causing vibrios through a battery of biochemical tests, toxin assay, and slide agglutination with specific antisera (11). In light of the need for quick diagnosis, monoplex PCR and multiplex PCR methods have recently been developed to characterize the serogroup (O1 and/or O139), biotype, toxigenic potential, and association with the regulatory system of *V. cholerae* strains (1, 5, 7, 4, 13, 15). These approaches are limited in that they do not provide simultaneous detection of the serogroup (O1 or O139), biotype (El Tor or Classical), toxigenic potential, and regulating factor. To address these limitations, we report here on the development of a quadruplex PCR assay that simultaneously targets the *wbe* (O1) and/or *wbf* (O139), *ctxA*, *tcpA* (El Tor or Classical), and *toxR* genes.

The targets selected were the *wbe* (O1) and/or *wbf* (O139), *ctxA*, *tcpA* (El Tor or Classical), and *toxR* genes. Specific primer pairs were used to identify serotype (O1 or O139) and biotype (O1, Classical, or El Tor). Primer pairs for *ctxA* (7), *tcpA* El Tor (7), *tcpA* Classical (10), *wbe* (5), *wbf* (5), and *toxR* (8) genes were selected from the literature. The quadruplex PCR assay utilizes six primer pairs and detects the presence of *wbe* (O1) and/or *wbf* (O139), *ctxA*, *tcpA* El Tor or Classical, and *toxR* genes generating amplification products of 192 and/or 449 bp, 302 bp, 472 or 620 bp, and 901 bp, respectively. Detection of *wbe* (O1) and/or *wbf* (O139) confirms the O1 and/or O139 serogroup (5), *tcpA* confirms whether the sample belongs to either the El Tor (7) or the Classical biotype (10), and *toxR* demonstrates its involvement in regulating the toxic genes and differentiates it from other vibrios.

For the quadruplex PCR, all of the strains examined by a monoplex PCR, including controls, were grown in Luria-Bertani broth (Difco) at 37°C. As described previously (3), DNA was extracted by boiling the sample for 10 min, followed by storage at −20°C until use. A bacterial cell lysate was used as the source of DNA. To ensure that each individual primer pair was adequate for amplification, a single-target PCR assay was conducted prior to quadruplex PCR optimization with our control strains (Fig. 1). After confirmation of the specificity of each primer by monoplex PCR, we combined primer sets in different ratios and tested the control strains in several PCR cycling protocols. The optimized protocol was carried out with a 35-μl reaction mixture that contained 10× PCR amplification buffer (100 mM Tris [pH 9.0], 500 mM KCl, 0.1% gelatin (Bangalore Genei, India), 2.5 μl of magnesium chloride (25 mM); 2.5 μl each of 2.5 mM dATP, dCTP, dGTP, and dTTP (Bangalore Genei); 90 pmol each primer for *tcpA* (El Tor) and *tcpA* (Classical); 68 pmol each primer for *ctxA*, *wbe* O1, and *wbf* O139; 60 pmol primer for *toxR*; 1.2 μl of Taq DNA polymerase (Bangalore Genei); Milli-Q water to a final volume of 29.5 μl; and 5.5 μl of cell lysate (template DNA). Finally, the reaction mixture was overlaid with a drop of sterile mineral oil (Bangalore Genei). Amplification was carried out for 4 min at 94°C for the initial denaturation, followed by 30 cycles of 1.5 min at 94°C, 1.5 min at 55°C, and 1.5 min at 72°C, with a final round of 7 min at 72°C in a thermal cycler (Techne, England). The PCR product (12 μl) was visualized by using a UV transilluminator after electrophoresis in 2% agarose gels in Tris-borate-EDTA buffer at 100 V for 45 min and ethidium bromide staining (0.5 μg/ml).

To address the specificity of the quadruplex PCR, *V. cholerae* and strains belonging to the *Enterobacteriaceae* were subjected for quadruplex PCR (Table 1). Strains of *V. cholerae* O1,
O139, and non-O1/non-O139 were subcultured from the laboratory stocks that had been isolated and confirmed earlier at NICED, Kolkata, India, by a multiplex culture method, with the multiplex and quadruplex PCR assays confirmed earlier at NICED, Kolkata, India, by a multiplex culture method, with the multiplex and quadruplex PCR assays for all of the tested strains. All of the O139 strains, whereas non-O1 and non-O139 strains carried only the toxR gene. The analysis revealed that there was complete agreement between the results of the monoplex and quadruplex PCR assays for all of the tested strains. All of the non-O1 and non-O139 strains were subjected to repeated PCR assays, and we found that all were positive for the toxR gene.

Rapid identification and discrimination of O1 and O139 by the culture method, with the multiplex and quadruplex PCR assays indicating 100% specificity for our PCR.

To reveal the practical usefulness of the quadruplex PCR assay, 70 and 22 strains of the V. cholerae O1 and O139 strains, respectively, isolated from hospitalized diarrhea patients were compared to those obtained by the monoplex PCR assay. The monoplex and quadruplex PCR assays detected ctxA, tcpA, wbe and/or wbf, and toxR genes among all of the V. cholerae O1 and O139 strains, whereas non-O1 and non-O139 strains carried only the toxR gene.

To evaluate the practical usefulness of the quadruplex PCR assay, the quadruplex PCR was validated with 30 V. cholerae O1 strains and 10 V. cholerae O139 strains; these strains were confirmed earlier at NICED, Kolkata, India, by a multiplex PCR assay (3). The strains were subjected to quadruplex PCR, and the results were compared to those obtained by monoplex PCR. The quadruplex PCR assay showed 100% specificity in identifying the reference strains; most importantly, nonspecific bands were not visualized. The same results were observed when the DNA of the control V. cholerae strains was included in the quadruplex PCR assay.

For the analysis of the quadruplex PCR assay, 40 strains of V. parahaemolyticus were positive for cholera toxin. CT, cholera toxin.

CT, cholera toxin.

FIG. 1. Ethidium bromide-stained agarose gel electrophoresis of quadruplex PCR discriminates biotype El Tor from the Classical serogroup and discriminates O1 from O139 and simultaneously detects ctxA and toxR genes. Lane M, 100-bp DNA ladder (Bangalore Genei); quadruplex PCR product, lane 1, ctxA, tcpA, (Classical), toxR, and wbe (O1) gene-positive V. cholerae O1 biotype Classical 569B; lane 2, ctxA, tcpA, (El Tor), toxR, and wbe (O1) gene-positive V. cholerae O1 biotype El Tor strain 20 (VC20); lane 3, JP1; lane 4, Pu1372 (from water stool); lanes 5 through 7, ctxA, tcpA (El Tor), toxR, and wbf gene-positive V. cholerae O139 strains SG24, KH3, and Pu1416 (from water stool), respectively; lane 8, ctxA, tcpA, (El Tor), toxR, wbe (O1), and wbf (O139) gene-positive V. cholerae mix infection Pu1442; simplex PCR product, lanes 9 through 14, ctxA, tcpA (El Tor), toxR, wbf, toxR, and tcpA (Classical) genes, respectively; multiplex PCR product, lane 15, ctxA and tcpA (El Tor) gene-positive VC20; lane 16, ctxA and wbe gene-positive wbe V. cholerae O139 strain SG24; lane 18, ctxA, toxR, wbf, and wbf gene-positive V. cholerae mix infection Pu1442; lane M, 100-bp DNA ladder (Bangalore Genei).

that is, Salmonella enterica serovar Typhi, clinical (n = 4); Salmonella enterica serovar Paratyphi A, clinical (n = 6); Shigella dysenteriae type 1, clinical (n = 6); Shigella sonnei, clinical (n = 5); enteroinvasive Escherichia coli, clinical (n = 5); enterotoxigenic Escherichia coli, clinical (n = 12); enterohemorrhagic Escherichia coli, clinical (n = 5); Aeromonas hydrophila, clinical (n = 4); and Aeromonas hydrophila, environmental (n = 5).
strains is essential for prompt institution of effective antimicrobial chemotherapy and are critical steps in preventing the rapid spread of cholera. Earlier studies have demonstrated the utility of PCR for determining a single gene to detect serotype, biotype, or toxigenic potential (4). Later, one multiplex PCR reported the detection of \textit{wbe} (O1) and/or \textit{wbf} (O139) and \textit{ctxA} (5), while another group reported the presence of \textit{tcpA} and \textit{ctxA} (7) and did not show the three genes simultaneously in a single tube reaction. Hexaplex PCR assay (14) detected six genes (\textit{ctxA}, \textit{tcpA}, \textit{ace}, \textit{zot}, \textit{toxR}, and \textit{stx}) but failed to differentiate the strains belonging to serogroups O1 and O139, which is the prime need in diagnosis. All of these previous investigations failed to amplify four gene targets—\textit{ctxA}, \textit{tcpA}, \textit{wbe} (O1) and/or \textit{wbf} (O139), and \textit{toxR}—in a single tube reaction, which requires the performance of a separate PCR. To transcend this limitation, our quadruplex PCR is a successful tool for determining simultaneously the presence of the \textit{ctxA} gene, the \textit{tcpA} El Tor and/or Classical gene, the \textit{wbe} (O1) and/or \textit{wbf} (O139) gene, and the \textit{toxR} gene. This one-step quadruplex PCR is a very useful tool for detecting serotype, biotype, toxigenic potential, and regulatory factor in rapid, simple, specific, and accurate manner. Early diagnosis using this technique will hopefully reduce the threat of sudden widespread outbreaks of cholera.

This research was supported and funded by ICMR, New Delhi, India, to the Regional Medical Research Center (RMRC), Bhubaneswar, India.

We thank S. K. Kar, RMRC, for his kind support and encouragement and G. B. Nair, National Institute of Cholera and Enteric Diseases, Kolkata, India, for providing \textit{V. cholerae} standard strains.

REFERENCES


