Amplification by the polymerase chain reaction of a specific target sequence in the gene coding for Escherichia coli verotoxin (VTe variant)

W.M. Johnson 1, S.D. Tyler 2, G. Wang 1 and H. Lior 1

1 National Laboratory for Enteric Pathogens, and 2 National Laboratory for Special Pathogens, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada

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1. SUMMARY

Synthetic oligonucleotide primers were used in a polymerase chain reaction (PCR) protocol to target a specific sequence in the gene coding for the A subunit of Escherichia coli verotoxin (VTe-variant, VTev). This PCR protocol permits the VTe-variant target sequence to be distinguished from closely related sequences in the same coding regions for type 1, type 2, and type 2 variant E. coli verotoxins. This procedure will be a valuable adjunct to other DNA amplification techniques currently being used for molecular epidemiological studies of verotoxigenic E. coli.

2. INTRODUCTION

Strains of Escherichia coli producing a family of related cytotoxins are termed verotoxigenic E. coli or VTEC. VTEC have been closely associated with sporadic diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura [1] in human disease and also with porcine edema disease [2,3]. Six distinct E. coli verotoxins (VTs or Shiga-like toxins) are now recognized and include VT1 (SLT-I) [1], VT2 (SLT-II) [1], VTe (SLT-IIv) [1,3,4], VT2 variants type a and b (VT2vha and VT2vhb) [5], and VTe variant (SLT-IIva) [6,7]. Genes coding for these verotoxins have been cloned and the nucleotide sequences published for slt1 (VT1) [8], slt II (VT2) [9], slt IIv (VTe) [4,10], vtx2ha and vtx2hb (VT2vha and VT2vhb [11], and sltIIva (VTe) [7]. Nucleotide sequence data for the gene coding for VTe in E. coli H.I.8 (putative O128 serogroup) confirmed that this
gene was most closely related to that of VTe, sharing 98% homology in the B subunit gene and 70.6% homology in the A subunit gene [7]. Both VT1 and VT2 toxins have been clearly associated with human disease and are encountered in both bovine and porcine reservoirs. Strains producing VT2-variant (VT2v) have been isolated from cases of HUS and HC in addition to non-human sources (unpublished data), and the E. coli H.I.8 strain shown to produce VTev toxin was originally isolated from a case of infant diarrhea.

We recently described polymerase chain reaction (PCR) protocols which can detect and differentiate the family of VT genes [12-14], including a PCR application to differentiate the highly homologous genes coding for Shiga toxin and E. coli VT1 [15]. In the present report we describe a PCR protocol which will rapidly and specifically distinguish the target sequence in the A subunit gene of VTev gene from closely related genes in other VTEC.

3. MATERIALS AND METHODS

3.1. Bacterial strains and verotoxin determinations

With the exception of E. coli H.I.8, all of the E. coli strains and their sources were previously described in detail including serotypes, growth conditions, VT and enterotoxin determinations [12,13]. Cytotoxic activities were determined as TCD₅₀/μg protein in Vero and HeLa cells after 48 h of incubation. Protein was estimated by the Bio-Rad Protein Assay (Bio-Rad Laboratories (Canada Ltd., Ontario). Quantitative toxin neutralization assays were performed as previously described [12] using toxin-specific antisera to E. coli VT1, VT2, and Vte.

3.2. PCR

Procedures for nucleic acid (NA) isolation and amplification in the PCR were as previously reported [12,13]. The targeted NA sequence, comprising a 490-bp region spanning nucleotides 290–779 of the open reading frame of the slt IIva A subunit gene, was amplified with the synthetic oligonucleotide primers (Oligonucleotide Synthesis Laboratory, Queen's University, Kingston, Ontario, Canada) 5'-TGTGTGGGCTTGCTCT-TCAGC-3' and 5'-ATGCGCGGGTCATGGAACGT-3'. Thirty cycles of amplification were performed in a DNA Thermal Cycler (Perkin Elmer Cetus) using 10 ng NA under the following conditions: denaturation for 2 min at 94°C, annealing of primers for 2 min at 55°C and primer extension for 1 min at 72°C with autoextension. Reaction mixture (10 μl) was analysed by standard submarine gel electrophoresis (2% agarose; 5 V/cm) and the reaction products were visualized by staining with ethidium bromide (0.5 μg/ml in the running buffer).

3.3. Restriction fragment length polymorphism (RFLP) analysis to establish amplicon integrity

As predicted from the sequence of the target NA [7], digestion of the amplicon generated using the primers described above should theoretically generate a RFLP pattern characterized by 197 bp and 293 bp fragments after digestion with the restriction endonuclease (RE), HaeIII. Aliquots (10 μl) of the amplicons recovered after the PCR were subjected to RE digestion using HaeIII (Gibco-BRL) as recommended by the manufacturer and the digested samples were analysed by gel electrophoresis as above.

3.4. Specificity of the PCR

The specificity of the VTev PCR protocol was evaluated using a battery of NA templates from reference E. coli strains, well-characterized VTEC, and Shigella dysenteriae which were as previously described [12–15].

4. RESULTS AND DISCUSSION

Primer-directed amplification was only observed in this PCR protocol using NA template from E. coli H.I.8. NA from all other VTEC, reference non-toxigenic, toxigenic, invasive, adherent and hemolytic strains of E. coli were consistently negative in this PCR targeting the A subunit of the VTev variant gene. Results of gels from the PCR using VTev primers are illustrated in Fig. 1 and include: E. coli H.I.8, VTev (lane b); E. coli 412, VTe (lane c); E. coli pEB1, VT2
Fig. 1. Occurrence and characterization of PCR products after primer-directed amplification of sequences targeted in the A subunit gene coding for VTev variant toxin. Lanes: a and h, 123-bp ladder (Bethesda Research Laboratories, Gaithersburg, MD); b, E. coli H.I.8 (VTev); c, E. coli 412 (VTe); d, E. coli pEB1 (VT2); e, E. coli O91:H21 (VT2vha & VT2vhb); f, E. coli H19 (VT1); g, E. coli H.I.8 amplicon digested with HaeIII (VTev).

The activity of extracellular verotoxin from E. coli H.I.8 was found to be 0.73 TCD₅₀/μg protein in HeLa cells and 2.9 TCD₅₀/μg protein in Vero cells. Complete neutralization of toxin from E. coli H.I.8 was observed using either anti-VTev or the anti-VT2 prepared using E. coli E32511, a reference strain for VT2-producing E. coli. Partial neutralization was observed in tests using the anti-VT2 prepared using E. coli pEB1, a genetic derivative of E. coli 933W. Anti-VT1 prepared using reference strain E. coli H19 was ineffective against VTev toxin. The relatively low level of cytotoxicity in HeLa cells and the complete neutralization by anti-VTev supports evidence that VTev is most closely related biologically with VTev toxin [6,7]. Complete neutralization with anti-VT2 prepared using E. coli E32511 may be attributable to the fact that both VT2- and VT2vha-coding B subunit genes are present in this strain [15,16]. Partial neutralization by anti-VT2 prepared using pEB1 confirms the published results of Gannon et al. [6,7]. These neutralization result suggest that, even immunologically, the VTev toxin shares more homology with VTev and VT2 toxins than with VT2.

Gannon et al. [7] selected three target regions of the sltIIva A subunit gene which failed to hybridize strongly with DNA from other VTEC, suggesting that the VTev gene differs from the other VT genes and confirming our more definitive results using the PCR. In view of the genetic and biological relatedness of VTev and VT2 toxins and remoteness from VT2 toxin, the VT nomenclature is more discriminatory than the Shiga-like toxin II terminology which refers to VT2 as SLT-II, VT2v as SLT-IIvh, VTev as SLT-IIv, and VTev as SLT-IIva. It is interesting that both VT2v- and VTev-producing E. coli have been associated with human disease and the ease, specificity and sensitivity of this VTev-specific PCR protocol will help establish the significance of these VTEC, particularly in enteropathogenic E. coli serogroups.

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REFERENCES