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Multiplex PCR-DNA probe assay for the detection of pathogenic *Escherichia coli*

Leigh Watterworth[a], Edward Topp[b], Heidi Schraft[a], Kam Tin Leung[a,*]

[a]*Department of Biology, Lakehead University, 955 Oliver Road, Thunder Bay, Ontario, Canada P7B 5E1*
[b]*Agriculture and Agri-Food Canada, London, Ontario, Canada N5V 4T3*

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Abstract

A multiplex PCR-DNA probing assay was developed to detect four major *Escherichia coli* virotypes. Six highly specific polymerase chain reaction (PCR) primer sets and DIG-labeled chemiluminescent probes were designed to target the Shiga-like toxin I and II genes (*stx*I and *stx*II) of verotoxigenic *E. coli* (VTEC), heat-stable and heat-labile toxin genes of enterotoxigenic *E. coli* (ETEC), adherence factor (EAF) of enteropathogenic *E. coli* (EPEC) and a fragment of the invasiveness plasmid (IAL) of enteroinvasive *E. coli* (EIEC). The primer pairs generate products of 350, 262, 170, 322, 293 and 390 bp in length, respectively. The multiplex primers and probes were tested for specificity against 31 pathogenic *E. coli* strains, nine nonpathogenic *E. coli* and non-*E.coli* enteric and environmental bacterial strains. The results showed a high degree of specificity of the primers and probes for strains from corresponding virotypes and no reaction with the nontarget bacterial strains. The proposed multiplex PCR-DNA probing assay provides rapid and specific detection of four major virotypes of *E. coli*.

Keywords: Multiplex-PCR; DNA probes; Pathogenic *E. coli*; VTEC/EHEC; ETEC; EPEC; EIEC

1. Introduction

Pathogenic *Escherichia coli* have become a significant health concern, especially *E. coli* O157:H7, having caused major outbreaks in water and food in the recent past which has increased the demand for a rapid detection method. Pathogenic *E. coli* can be divided in to six virotypes: enterohemorrhagic (EHEC), entertoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EaggEC) and diffusely adherent (DAEC). The division is based on the virulence factors they possess which initiate the disease symptoms (Smith, 1992). Verotoxigenic *E. coli* (VTEC) includes *E. coli* strains that contain Shiga-like toxin I and/or Shiga-like toxin II gene(s). EHEC is a subset of VTEC that also

* Corresponding author. Tel.: +1 807 343 8265; fax: +1 807 346 7796.
E-mail address: kam.leung@lakeheadu.ca (K.T. Leung).

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contains the *eae*A gene involved in the attachment of bacteria to enterocytes and the *hly*A gene which encodes a haemolysin. The four major *E. coli* virotypes used in this study are VTEC/EHEC, ETEC, EPEC and EIEC. VTEC/EHEC express numerous virulence factors, two of the most significant being the Shiga-like toxins (STX) I and II (Nakao and Takeda, 2000). ETEC carry the plasmid-mediated heat-stable (ST) and/or heat-labile (LT) toxins (Rappelli et al., 2001). EPEC rely on plasmid encoded enteroadherence factor (EAF) for the development of localized adherence necessary for infection (Tobe et al., 1999). EIEC infection is mediated by the plasmid encoded invasion associated loci (IAL; Rappelli et al., 2001). These virulence factors are the targets for the molecular detection of pathogenic *E. coli* in this study.

In general, diarrheagenic *E. coli* cannot be identified based on biochemical criteria alone as in most cases they are indistinguishable from nonpathogenic *E. coli*. The differentiation between *E. coli* virotypes requires the use of cell culture assays that are not typically part of a diagnostic routine (Rappelli et al., 2001). The current protocol for the identification of pathogenic *E. coli* O157:H7 is a time-consuming process (Okamoto et al., 1999) which involves the use of selective and differential growth media which take into consideration phenotypic traits characteristic of *E. coli* O157:H7, such as loss of sorbitol fermentation and β-glucuronidase inactivity (Cebula et al., 1995). The use of these characteristics alone is limited as the emergence of *E. coli* O157:H7 strains demonstrating sorbitol fermentation (Ware et al., 2000) and β-glucuronidase activity (Gunzer et al., 1992) have been found. Serotyping is often included in the battery of biochemical tests used to identify *E. coli* O157:H7. This method alone, however, cannot identify pathogenic *E. coli* since there is not always a correlation between pathogenicity and the O and H antigens expressed (Rappelli et al., 2001). This test also provides no information on the types of toxins produced and, in the case of anti-O157 sera, lack specificity as the anti-O157 sera cross-reacts with a variety of other bacterial species (Cebula et al., 1995; Hu et al., 1999). An additional downfall of the protocol is the reliance on culturing. The isolation and detection of pathogens from environmental samples may be difficult if cells have become stressed or injured (Lisle et al., 1998; Wang and Doyle, 1998). For these reasons, methods targeting DNA detection are being pursued.

The polymerase chain reaction (PCR) is a selective and sensitive method that rapidly amplifies specific regions of a gene. Although PCR is rapid and specific, the process could become cumbersome when being applied to numerous samples with various potential targets. To minimize time and materials, primers can be combined in a single reaction tube to form a multiplex-PCR (MP-PCR) which can simultaneously detect numerous target genes in a single sample.

The MP-PCR method has been used to identify and differentiate pathogenic *E. coli* strains in a number of studies. A method has been developed which can detect the presence of coliforms and *E. coli* in a sample (Bej et al., 1991). This method uses PCR to target the *lacZ* and *uidA* genes common in coliforms and *E. coli*, respectively. Numerous MP-PCR assays have been developed to detect pathogenic *E. coli*. Many of these methods target a single *E. coli* virotype, such as EHEC (Osek, 2002; Pollard et al., 1990; Paton and Paton, 1998, 1999; Feng and Monday, 2000) while others target a single serotype, for example, the infamous *E. coli* O157:H7 (Cebula et al., 1995; Fratamico et al., 1995, 2000, Venkateswaran et al., 1997; Hu et al., 1999; Nagano et al., 1998; Meng et al., 1997; Campbell et al., 2001; Wang et al., 2002). The MP-PCR assay has also been used to differentiate certain pathogenic *E. coli* from other pathogens that cause similar maladies, for example, ETEC or EIEC and *Shigella* (Frankel et al., 1989; Houng et al., 1997). Assays have been developed which attempt to differentiate *E. coli* virotypes by targeting virulence genes and other genes necessary for infection (Tsen and Jian, 1998; Tornieporth et al., 1995; Lang et al., 1994; Reid et al., 1999). Two studies were found which target the four major *E. coli* virotypes: EHEC, ETEC, EPEC and EIEC. These assays applied three (Rappelli et al., 2001) or four (Pass et al., 2000) different MP-PCR reactions targeting 8 or 11 different genes for the identification of the four different virotypes. The majority of these assays identify their target amplicons by agarose gel electrophoresis alone. It has been acknowledged that MP-PCR can produce nonspecific amplification as a product of mixing PCR primers (Pass et al., 2000; Rappelli et al., 2001).