Journal of Applied Sciences

ISSN 1812-5654

science alert

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Journal of Applied Sciences 7 (17): 2531-2534, 2007
ISSN 1812-5654

Simultaneous Detection of Enteropathogenic *E. coli* and Shiga Toxin-Producing *E. coli* by Polymerase Chain Reaction

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Abstract: A PCR for detection of two categories of diarrheagenic *Escherichia coli* was developed. This method proved to be specific and rapid in detecting virulence genes from enteropathogenic *E. coli* (EPEC) (*eae* and *bfp*) and Shiga toxin-producing *E. coli* (STEC) (*stx1*, *stx2* and *eae*) from seventy isolates of various sources. Present results confirm that it is possible and feasible to perform a simultaneous amplification of the virulence genes from two categories of diarrheagenic *E. coli* (STEC, EPEC) and that this technique becoming a novel diagnostic tool for future water food-borne outbreaks studies.

Key words: Polymerase chain reaction, enteropathogenic, genes, shiga toxin producing *E. coli*

INTRODUCTION

Recently, there was a common perception that pathogenicity traits in *Escherichia coli* are more the exception than the rule and *E. coli* was generally regarded as part of the normal lower intestinal flora. Three main types of clinical syndrome can result from infection with one of these pathotypes: enteric and diarrheal diseases, urinary tract infections and sepsis/meningitis. Three additional *E. coli* pathotypes, collectively called ExPEC (Russo and Johnson, 2003) are responsible for extraintestinal infections. ExPEC is composed of uropathogenic *E. coli* (UPEC) isolates that cause urinary tract infections, neonatal meningitis-associated *E. coli* (MNEC) and *E. coli* strains that cause septicemia (Bekal *et al*., 2003; Kaper *et al*., 2004). Pathotypes are defined based the presence of combinations of virulence and virulence-related genes; conversely, the pathotype of an uncharacterized strain can be inferred from its virulence gene profile (Kaper *et al*., 2004). EPEC and the majority of clinical isolates STEC harbor the Locus of Enterocyte Effacement (LEE), a pathogenicity island that is responsible for the phenotype of attaching-and-effacing (A/E) lesions (Dobrindt *et al*., 2004; Kaper *et al*., 2004). EPEC are a major cause of human infantile diarrhea predominantly in less-developed countries but are also identified with increasing frequency in industrialized areas (Afset *et al*., 2004; Kaper *et al*., 2004; Nguyen *et al*., 2006). These pathogens colonize the small intestine, induce the degeneration of epithelial microvilli and intimately adhere to the host cell. The central mechanism of EPEC pathogenesis is a lesion called 'attaching and effacing (A/E), which is characterized by intimate adherence of the bacteria to the intestinal epithelium (Nougayrède *et al*., 2003). The *eae* gene located in the pathogenicity island LEE and the *bfpA* gene located on a plasmid, called the EPEC Adherence Factor (EAF), have been used to classify this group of bacteria into typical and atypical strains (Kaper, 1996). The EAF plasmid harbors the bundle-forming pilus (*bfp*) operon, encoding the type IV pili responsible for localized adherence and the formation of microcolonies on host cells. Like EPEC the STEC responsible for sporadic infections as well as serious outbreaks worldwide, mostly harbor the LEE pathogenicity island. These pathogens cause an acute inflammation of the colon, resulting in hemorrhagic colitis with rare but serious sequelae including neurological disorders and the Hemolytic-Uremic Syndrome (HUS), the leading cause of acute renal failure in children (Karmali *et al*., 2003; Kaper *et al*., 2004). *E. coli* strains of the A/E genotype (*eae*) harbouring the EAF plasmid (*bfpA*), are classified as typical EPEC. Most such strains belong to certain O: H serotypes (Trabulsi *et al*., 2002). Strains of the A/E genotype, which do not posses the EAF plasmid (*bfpA*$^{-}$), are classified as atypical EPEC. *eae*-positive *E. coli* strains harbouring Shiga toxin genes

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(*stx1* and/or *stx2*) are classified as enterohaemorrhagic *E. coli*. EPEC and STEC strains is currently usually based on conventional serotyping with specific antisera in a time-consuming process demanding some technical expertise and the cross reaction with other gram negative pathogens. To enhance clear diagnostic and help in therapeutic measures, we developed a molecular approach based on multiplex PCR (MPCR) for the simultaneous detection of diarrheagenic *E. coli* belonging to STEC or EPEC.

MATERIALS AND METHODS

Sources of isolates: Twenty five *E. coli* isolates as clinical, marine, river, food and animal were studied from five different sources in Malaysia 2003-2004. The clinical (pus, 2 urine and 2 stool) samples were studied from the specific culture of Microbiology Laboratory there is takes were originally provided from Kula Lumpur Hospital, which were marine and river isolates were collected from Costrica beach, Sunggi linggi river Nergeri Sembilan State. The food (raw milk, chess, yogurt and 2 raw meats) sample was selected randomly from different restaurant in Seri Serdange area, Selangor state. The last sample of animal source (deer, pig, goat and 2 checken) was provided by Microbiology Department, Faculty of Veterinary, UPM.

Development PCR for Detection of *E. coli* specific target genes and primer design: For the development of a multiple PCR, we designed primer pairs (Table 1). In order to specific detection of targets of *E. coli* isolates, we chose four marker genes (*eae*, *bfp*, *stx1* and *stx2*) exhibiting the highest degree of homology among the corresponding sequences found in the databases. The MPCR was performed in a 50 μL reaction mixture consisting of 2.5 μL of 10 X Ampli Buffer A, 0.45 μL of 50 mM $MgCl_2$(Vivantis, Malaysia), 0.50 μL, dNTPs, 0.5 μL of each forward and reverse primers (Research Biolabs, Singapore), 2.0 μL genomic DNA sample and finally 0.2 μL Taq DNA polymerase. Thermocycling conditions (Biometra-TRIO Thermoblock. Germany) were as follows: 95°C for 2 min, followed by 30 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 7 min. After amplification, an aliquot of 10 μL reaction mixture was loaded into the wells of a 1.4% agarose gel and electrophoresed; then stained with ethidium bromide and images was captured under UV illumination (Alpha Imager™ 2200, Alpha Innotech Corporation).

Table 1: Four designed primers were used in the multiplex PCR for detection of diarrheagenic groups of *E. coli*

Sequence (5' 3')	Target gene	Size (bp)	Reference
F-ATACTCCGATTCCTCTGG			
R-TTCTGCGTACTGCGTTC	*eae*	277	This study
F-GCTT CAGG CAGA TACA GA			
R- GTTA CCCA CATA CCAC GA	*stx2*	698	This study
F- GCTT GCTG CCAC CGTT A			
R- TGTT ATTG TTTG CTGG ACCT AC	*bpf*	266	This study
F-TGTCGCATAGCGGAACCTCAC			
R-GCAGTCATTACATAAGAACGCCCAC	*stx1*	154	This study

RESULTS

Firstly, to evaluate multiplex PCR carried out for 25 isolates from various sources were tested. If the result was negative, the sample was considered as negative for diarrheagenic *E. coli*. On the other hand, if the multiplex PCR was positive, the sizes of bands on the gel were compared with the marker, in order to identify the suspected *E. coli* in the isolates. Minimum criteria for determination of *E. coli* were defined as shown in the (Fig. 1) the presence of *stx1* and/or *stx2* for STEC the

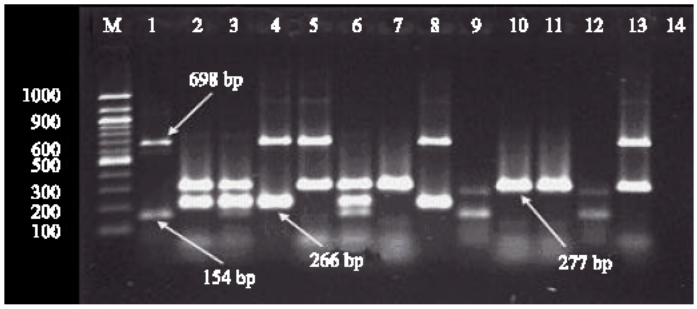

Fig. 1: Multiplex PCR amplification of *E. coli* isolates from different sources. Lane 1(stool) STEC *stx2* and *stx1*: Lane 2 (urine) EPEC (*eae* and *bfp*): Lane 3 (chess) STEC (*eae*, *bfp* and *stx1*): Lane 4 (raw meat) STEC (*stx2* and *bfp*): Lane 5 (river), STEC (*stx2* and *eae*): Lane 6 (pig), STEC (*eae*, *bfp* and *stx1*): Lane7 (marine) STEC (*eae*): Lane 8 (chicken) EPEC (*bfp*): Lane 9 (goat) STEC (*bfp* and *stx1*): Lane 10 and 11 (marine) EPEC (*eae*): Lane 12 ATCC(12810): Lane 13 ATCC (23519): Lane 14 negative control. Lane M represents 100 bp DNA marker ladder (Fermentas Life Sciences)

presence of *bfp* and *eae* for EPEC. This study demonstrates that the virulence factor of *E. coli* of four different strain *stx1*, *stx2*, bfp and eae, gene have been successfully amplified from several *E. col* isolates.

DISCUSSION

The application of multiplex PCR will provide the ease, speed and economic advantage over the single PCR reaction for detecting multiple virulent genes if present in any one isolate. However, known virulence factors alone cannot explain the pathogenicity of *E. coli* strains causing diarrhea (Olivier *et al*., 2004). Thus, this study provides the first opportunity to study the distribution of virulence genes within a defined *E. coli* isolates from various sources in the absence of selection bias regardless limited number of isolates, the ability to more broadly generalize these results needs to be assessed in future. Numerous MPCR methods have been developed for the identification of *E. coli* pathotypes (Aranda *et al*., 2004; Pass *et al*., 2000; Rappelli *et al*., 2001; Rich *et al*., 2001; Watterworth *et al*., 2005). However, most of the MPCR methods in the literature harbor limitations in terms of the number of targeted genes, specificity, the resolution of amplified fragments in agarose electrophoresis and nonspecific amplification. Recently, an MPCR has been introduced by Kimata *et al*. (2005) that targets 12 genes to differentiate between diarrheagenic *E. coli* pathotypes. However, real-time PCR and DNA array (Garrido *et al*., 2006) approaches have the advantage of higher sensitivity but are usually quite expensive. Therefore, in the present study, a novel single MPCR has been developed that allows for detection of EPEC and STEC isolates, in a straightforward and robust reaction. Several rounds of redesign and optimization, four highly specific primer pairs were developed which, combined in one reaction, gave rise to amplicons that are well resolved by agarose gel electrophoresis. The main challenge of present study to achieve a multiplex PCR is the possibility for primer dimers and artifacts product. So, it is necessary to design primers with close annealing temperatures, to begin the program with a hot start and to use reference strains to determine reaction specificity. The eight primers developed in the present study proved to be specific for the corresponding four genes, since we could not observe any cross-priming or the amplification of nonspecific DNA fragments. Further evaluation of the tetrameric MPCR we recommended to investigate with large number of environmental and clinical *E. coli* isolates to ensure highly specific and reliable. Detection of *E. coli* isolates present in water sources used for drinking or recreation could be an important tool in the development of strategies to better protect public health. In terms of public health, it is also significant that a high percentage of ExPEC isolates was found; these pathogens, which can also be part of the human and animal intestinal flora (Johnson and Russo, 2002), are responsible for an estimated 40,000 deaths and annual expenditures of at least $2.6 billion in the United States alone (Russo and Johnson, 2003). Therefore, the result clearly indicates that there is a need to better understand the public health implications of *E. coli* carrying virulence genes in recreational waters. Multiplex PCR presented here is an alternative assay in practical and rapid diagnostic tool for simultaneously detection of diarrheagenic *E. coli* in a single reaction tube. More studies are necessary to evaluate the contribution of diarrhoeagenic *E. coli* to the human disease burden as well as among the environment.

ACKNOWLEDGMENTS

We are grateful to Faculty of Veterinarian Department of Microbiology, University Putra Malaysia for their support in supplying us the *Escherichia coli* isolates of animal sources. We also thank the colleagues of Medical Microbiology Department, Faculty of Medicine and Health Sciences, UPM.

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