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Detection of Diarrheagenic *Escherichia coli* Isolated Using Molecular Approaches

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Abstract: *Escherichia coli* strains are among the major bacterial causes of diarrheal illness. There are now seven classes of diarrheagenic *E. coli* (DEC), namely enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diarrhea-associated hemolytic *E. coli* (DHEC) and Cytolethal Distending Toxin (CDT)-producing *E. coli*. Due to the need for costly and labor-intensive diagnostic procedures, identification of DEC is difficult at standard laboratories. Therefore, Polymerase Chain Reaction (PCR) or dot blot has been used for genetic detection of DEC of 25 *E. coli* isolates from different sources. Amplification of *eae* (277 bp), *bfp* (266 bp), *stx1* (154 bp), *EAST* (94 bp), *stx2* (698 bp) and *elt* (450 bp) genes of a single product in separate reactions was produced. PCR showed ability to amplify and detected genes of the most common important categories of diarrheagenic *E. coli* isolates of different sources, it is possible implementation of this technique to diagnosis water, food-borne outbreaks related to *E. coli*. Dots blot and sequence analysis used to confirm the results of PCR.

Key words: *Escherichia coli*, diarrheal illness, diarrheagenic *E. coli*, enteroinvasive *E. coli*, cytolethal distending toxins

INTRODUCTION

Acquisition of virulence genes is believed to provide an evolutionary pathway to pathogenicity. Virulence mechanisms that characterize the pathogroups of *E. coli* are genetically determined by chromosomally located factors (pathogenicity islands, chromosomally inserted bacteriophages) or extra-chromosomal elements (plasmids) that encode diarrhea-associated characteristics like colonization ability, production of toxins and hemolysins (Aranda *et al*., 2004; Kaper *et al*., 2004). The main virulence factor of EHEC bacteria has been reported to be a production of Stx toxin(s) (Stx1 and/or Stx2) or their variants encoded by the *stx* genes (Kaper *et al*., 2004; Paton *et al*., 2004; Thorpe, 2004). ETEC were the most common *E. coli* group associated with tourist diarrhea with production of heat labile (LT) and/or stable (ST) toxin encoded by respective *lth* and *sta* genes (Olasz *et al*., 2005). Unlike other diarrheagenic *E. coli* strains, EIEC characteristically cause *Shigella*-like invasive infection, producing plasmid (pINV) mediated proteins encoded by *ipa*, *ial* genes, typically leading to bloody

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diarrhea (Nataro and Kaper, 1998; Aranda *et al*., 2004; Schmidt and Hensel, 2004). Strains of EAEC group are recognized by aggregative, localized adherence (LA: encoded by *bfpA*) of the bacterial cells on the microvilli and a production of EAEC toxin (EAST). DAEC, also known as cell-detaching *E. coli*, are characterized by diffuse adherence to epithelial cells, possession of adhesion fimbria encoded by a fragment of *daaC* gene, I-hemolysin production and cytotoxic necrotising factor 1 (Nataro and Kaper, 1998; Clarke, 2001). In addition, the Cytolethal Distending Toxin (CDT)-producing *E. coli* forms the latest group of enteric pathogens (Clarke, 2001). 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. The significant for public health a high percentage of *E. coli* isolates that demonstrated 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). The present study describe the method which a DNA hybridization procedure was used to detect *E. coli* isolate from human, water source, food and from animal samples. This technique can be used in surveys in combination with PCR, a Vero cell assay, or other screening tests and is particularly valuable when low frequencies of positive samples are expected. Samples are screened for *E. coli* by PCR and the positive isolates are identified on dot blot membrane.

MATERIALS AND METHODS

Sources of Isolates

Twenty five *E. coli* isolates as clinical, marine water, river water, 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 originally provided from Kula Lumpur Hospital (HKL), which marine water and river water 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 samples of animal source (deer, pig, goat and 2 chickens) were provided by Microbiology Department, Faculty of veterinary Medicine, University Putra Malaysia (UPM).

Maintenance of Bacteria

E. coli strains were stored at -80°C in Luria-Bertani (LB) broth containing 20% glycerol. Bacteria were recovered from frozen stocks and plated on LB agar and were never subcultured more than twice before DNA extraction.

DNA Extraction

All isolates were prepared by inoculating a single colony into 1 mL of LB broth and incubated at 37°C with shaking (~100 rpm) overnight. DNA was extracted using a QIGENE DNA purification kit.

Detection of *E. coli* Specific Target Genes by PCR

Fifty microliter containing 1X BST buffer (Biosynthec Inc. Malaysia), 1.8 mM $MgCl_2$ (Biosynthec Inc. Malaysia), 200 μm dNTPs (Fermentas Life Sciences), 5 IU taq polymerase (Biosynthec Inc. Malaysia), 10 pmoles of each primer and 200 ng μL^{-1} DNA template. The PCR programmer steps performed were initial denaturation at 94°C for 2 min, followed by 30 cycles of amplification steps consisting of denaturation at 94°C for 1 min, annealing at 64°C for 1 min and elongation at 72°C for 2 min. The amplification was ended with final extension at 72°C for 7 min. After

amplification, an aliquot of 10 μL reaction mixture was loaded into the wells of 1.4% agarose gel and electrophoresed, then stained with ethidium bromide and image was captured under UV illumination (Alpha Imager™ 2200, Alpha Innotech Corporation).

Dot Blots Hybridization

Dot blot assay was also used as an alternative method to verify the specificity of the above PCR assay. The purified PCR product of each primer was labeled with Hors Reddish Peroxidase (HRP) using the Direct Nucleic Acid Labeling Kit ECL (Amersham Pharmacia Biotech, UK) and used as probe hybridization to genomic DNA of *E. coli* isolates. Each isolates, 100 ng of purified PCR denatured by heating at 96°C for 10 min and spotted onto Hybond-N nylon membranes (Amersham Pharmacia Biotech, UK). DNA fixed onto filter membrane by UV cross-linking by incubating in a UV cross-linking chamber (UV-Cross linker UVC-500, USA) for 3 min at the room temperature. The prehybridization and hybridization temperature were both 42°C. All filters were pre-hybridized for 1 h in 5 × SSC (1.5 M sodium chloride, 0.15 M sodium citrate). Hybridization was carried out overnight with heat-denatured probe. Detection was performed using the phototope-star detection kit according to the manufacturer's instructions (Amersham Pharmacia Biotech, UK). Gram negative and Gram positive bacteria used as control.

RESULTS

Virulence Gene Profile

In *E. coli* isolates, stx_1, stx_2, *elt*, *ESAT*, *bfp* and *eae* genes are chromosomally encoded. PCR-based method for detection of above virulence genes were used. Firstly, to evaluate PCR carried out for 25 isolates from various sources were tested. If the result was negative, sample was considered as negative for diarrheagenic *E. coli*. On the other hand, if the 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 as described in Fig. 1-6. This study demonstrates that the virulence factor of *E. coli* of four different groups *stx1*, *stx2*, *elt*, *ESAT*, *bfp* and *eae* genes have been successfully amplified from several *E. coil* isolates (Table 1).

Sequence Analysis

Five of PCR products selected from 5 different sources with expected size 277 bp for the genes *bfp* gene a major structural subunit of bundle-forming pilus were confirmed by sequencing. The sequences obtained were compared to the sequence of *bfp* gene in the database by using NCBI Blast software and Biology Workbench 3.2 under CLUSTER W program (http://workbench.sdsc.edu/CGI/bw.cgi). The *bfp* gene sequences were aligned to the *bfpA* gene sequence under the accession numbers Z68186 as in Fig. 7. The alignments showed that the *bfp* gene sequences obtained were highly

Table 1: Six primers used in PCR for detection of diarrheagenic groups of *E. coli*

Sequence (5' to 3')	Target gene	Size (bp)	Reference
F-ATACTCCGATTCCTCTGG R-TTCTGCGTACTGCGTTC	*eae*	277	Present study
F-GCTT CAGG CAGA TACA GA R-GTTA CCCA CATA CCAC GA	*stx2*	698	Present study
F-GCTT GCTG CCAC CGTT A R-TGTT ATTG TTTG CTGG ACCT AC	*bpf*	266	Present study
F-TGTCGCATAGCGGAACCTCAC R-GCAGTCATTACATAAGAACGCCCAC	*stx1*	154	Present study
F-CACAGTATATCCGAAGGC R-CGAGTGACGGCTTTGTAG	*EAST*	94	Chakraborty *et al.* (2001)
F-GGCGACAGATTATACCGTGC R-CGGTCTCTATATTCCCTGTT	*Elt*	450	Stacy-Phipps *et al.* (1995)

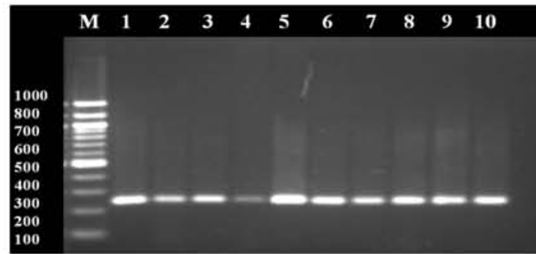

Fig. 1: PCR amplification of EPEC (*bfp* 266 bp) isolates. Lane 1 (stool), lane 2 (urine), lane 3 (chess), lane 4 (raw meat) lane 5 (river), lane 6 (pig), lane 7 (marine), lane 8 (chicken) lane 9 (goat) lan 10 ATCC (23519), lane 11 negative control. Lane M represents 100 bp DNA marker ladder (Fermentas Life Sciences)

Fig. 2: PCR amplification of EPEC (*eae* 277 bp) isolates. Lane 1 (stool), lane 2 (urine), lane 3 (chess), lane 4 (raw meat) lane 5 (river), lane 6 (pig), lane 7 (marine), lane 8 (chicken) lane 9 (goat) lan 10 ATCC (12810), lane 11 negative control. Lane M represents 100 bp DNA marker ladder (Fermentas Life Sciences)

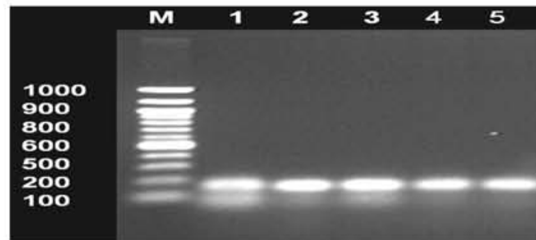

Fig. 3: PCR amplification of EPEC (*stx1* 154 bp) isolates. Lane 1 (stool), lane 2 (chess), lane 3 (marine), lane 4 (river), lane 5 (pig). Lane M represents 100 bp DNA marker ladder (Fermentas Life Sciences)

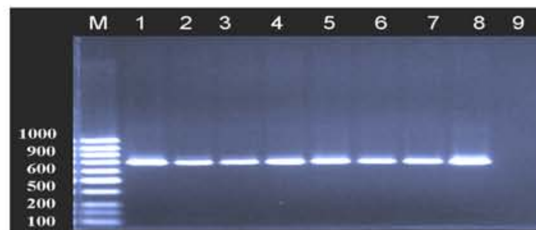

Fig. 4: PCR amplification of EPEC (*stx2* 698 bp) isolates. Lane 1 (stool), lane 2 (urine), lane 3 (chess), lane 4 (raw milk), lane 5 (river), lane 6 (pig), lane 7 (marine), lane 8 (deer), lane 9 negative control. Lane M represents 100 bp DNA marker ladder (Fermentas Life Sciences)

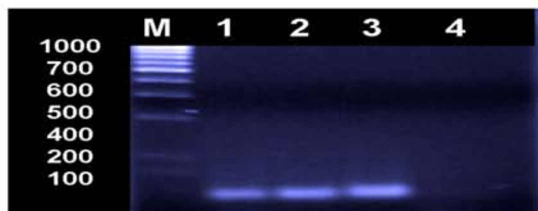

Fig. 5: PCR amplification of EPEC (*EAST*94 bp) isolates. Lane 1 and (stool), lane 3 (pig) lane lane 4 negative control. Lane M represents 100 bp DNA marker ladder (Fermentas Life Sciences)

Fig. 6: PCR amplification of EPEC (*elt* 450 bp) isolates. Lane 1 (urine), lane 2 (stool), lane 3 (chess), lane 4 (raw milk), lane 5 (river), lane 6 (marine), lane 7 (pig), lane 8 (chicken) lane 9 (deer), lane 10 (chicken) lane11negative control. Lane M represents 100 bp DNA marker ladder (Fermentas Life Sciences)

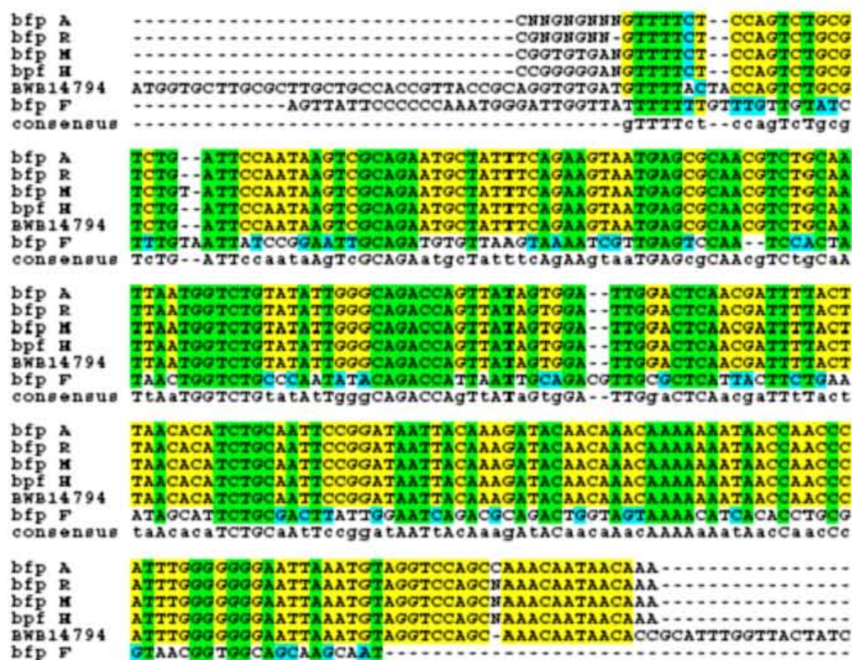

Fig. 7: Sequence analysis of *bfp* gene by CLUSTAL W program (C-clinical, M-marine, R-river, F-Food, A-animal. The nucleotide analysis by CLUSTAL W multiple alignments showed that the gene sequenced from five different isolates and published gene has a big altered the base sequence of the isolates deleted or inserted. BWB14794 *bfp* gene the published gene (Completely Conserved Residue: Green, Identical Residues: Yellow Similar Residues: Cyan, Different Residues: white)

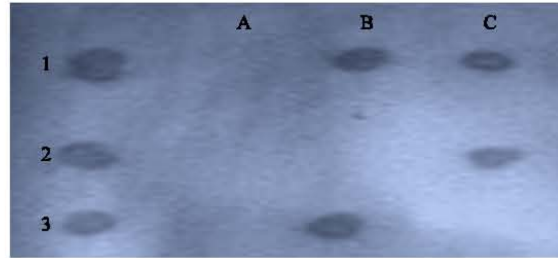

Fig. 8: Dot blot of 9 *E. coli* isolates. The horse radish peroxidase-labeled polymerase chain reaction product of *bfp* gene was used as probe. DNA chromosomal from *E. coli* isolates were used as targets. There were 3 positive control strains, (ATCC) (dot1-3). Dots A1 (*Vibrio cholera*), A2 (*Salmonella* sp.) and A3 (*Staphylococcus aureues*) are negative. Dots B1 (stool), B3 (stool) and C1 (meat), C2 (pig) are positive. B2 (marine) and C3 (river) did not show any dot blot signals

homologous (97-99%) to the sequence under accession number. Considerable variation in sequences among the isolates, which showed that although, the positive results produced band at the position 266 bp, the sequences showed nucleotide variations indicating strain differences.

Dot Blot Assay

DNA from PCR-positive strains showed a strong hybridization signal. No hybridization signal was detected by dot blot analysis of DNA from marine and river water isolates. The dot blot results of the *bfp* gene are shown in Fig. 8.

Specificity of *E. coli* Specific Probe

Specificity test of dot blot hybridization was used Horse Radish Peroxidase (HRP) specific DNA fragment from 3 isolates of different genera of Gram-positive and Gram-negative bacteria *Vibrio cholera*, *Salmonella* sp. and *Staphylococcus aureus* isolates were used as negative controls. Three positive controls (1) ATCC (23519), (2) ATCC (12799) and (3) ATCC (23520).

DISCUSSION

Molecular biology technique will provide the ease, speed and economic advantage over the conventional methods for detecting virulent genes of isolate if present. However, known virulence factors alone cannot explain the pathogenicity of *E. coli* strains causing diarrhea (Olivier *et al.*, 2004). *E. coli* is the most completely characterized prokaryotic model organism and one of the dominant indicator organisms of food and water quality indicator yet comparatively little are known about the structure of *E. coli* populations in their various hosts. Genetic diversity has been exploited by selection and adaptation so that pathogenic strains have tended to become host specific, with strains identified in scouring pigs being phenotypically and serotypically different from those that cause diarrhea in humans (Hart *et al.*, 1993). Within each host species, genetic differences can still be found in different pathogenic isolates. For example, *E. coli* strains associated with intestinal disease are genotypically different from those that cause extraintestinal disease. Unanswered question is how do virulence genes continue to be acquired or lost in each adapted individual's evolutionary pathway? Commensal *E. coli* isolates on the other hand have attracted very little attention because they are not overtly involved in causing disease. Nonpathogenic, could potentially also harbor virulence genes but are incapable of causing disease because they lack the appropriate virulence gene combinations. 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*., 2003; 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 (Nougayrede *et al*., 2003). To increase the sensitivity and specificity, 2 sets of PCR primers were used to detect EPEC in this study. One detects *bfp* gene and the other detects EAF plasmid. BFP is encoded on EAF plasmids, which share extensive homology among various EPEC strains. The importance of the EAF plasmid in human disease was shown by Levine (1987). The *eae* gene located in the pathogenicity island Locus of Enterocyte Effacement (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 4 pili responsible for localized adherence and the formation of microcolonies on host cells. Since we did not perform functional assay of the isolates, it is possible that strains which did not express the virulence genes or exhibit sequence variation in the primer binding sites were not detected. Molecular studies are needed to identify sequences specific for these strains particularly *elt*, *ESAT* gene in order to fill this information gap. 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. The presence of virulence genes among human and in the environments isolates of *E. coli* might not be interpreted as a process of active virulence gene acquisition. The present study suggest both human and environment for several years acquired and maintained these genes as part of a survival mechanism to engender greater diversity and hence increase their survival capability in the host. Genomic DNA blotted on a membrane and can be detected by dot blot of the hybridized signal, offers a rapid, simple, specific and accurate miniature system suitable in water-foodborne area. The continuing weakness of the methods used to isolate food-borne bacterial pathogens is their inability to isolate bacteria from samples that are positive as determined by very sensitive screening assays (Cerqueira-Campos *et al*., 1986; Peterkin *et al*., 1991; Todd *et al*., 1999). The membrane-based assay was performed optimized to be simple and performed in 1 to 2 h to test the *bfp* gene of amplified product. When tested with PCR product, the DNA probe provided to be specific and sensitive, revealed strong hybridization signals with most of *bfp*-positive *E. coli* strains. Stringent hybridization and washing conditions ensured high specificity and none of the *bfp*-negative bacterial strains revealed positive signals. While, Samadpour *et al*. (1990, 1994) were used a colony blot procedure with limit detection which involved radiolabeling to identify *E. coli* in several different kinds of foods (surimi-based salad, raw goat milk and blueberries) and found that 17% of 294 food samples contained *stx1* and/or *stx2* genes; these samples mainly came from beef, veal, pork and lamb, but they also came from chicken, turkey, fish and shellfish. The *E. coli* specific membrane assay developed in this study was very specific to *E. coli* as the probe sequence did not show positive signal for a variety of Gram positive and Gram negative bacterial tested which confirmed with sequencing analysis. The potentially serious water quality and food hygienic processing, rapid detection of the infectious agents based on the detection and identification the biondicator is essential for achieving favorable management outcomes. 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 *E. coli* isolates was found; these pathogens, which can also be part of the human and animal intestinal flora (Johnson and Russo, 2002). On the other hand, the important of *E. coli* as indicator of water quality which relies on the assumption that its presence in water is a direct evidence of fecal contamination and indicates the possible presence of pathogens such as *Salmonella* sp., *Shigella* sp., pathogenic

E. coli and enteroviruses, including hepatitis A (Donald and Harwood, 2007). Therefore, there is a need to better understand the public health implications of *E. coli* carrying virulence genes in recreational waters.

CONCLUSION

To better understand the public health implications of *E. coli* carrying virulence genes in recreational waters with the increasing demand for Hazard Analysis and Critical Control Point (HACCP) plans, risk assessments and health hazard evaluations, quantitative procedures for assessing *E. coli* contamination will be required. For this purpose, the PCR in combination with dot blot methods could be modified to obtain direct detect *E. coli*, although the limit of detection would be increased.

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