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Detection of *eae, bfpA, espA* Genes on Diarrhoeagenic Strains of *Escherichia coli* Isolates

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Abstract

The *Enteropathogenic Escherichia coli* (EPEC) is one of pathogenic strain of diarrheagenic *E. coli* group in children and infant that occurs in developing countries. The significant virulence factors in pathogenic EPEC are *eaeA* (*E. coli attaching-effacing*), *bfpA* (*bundle-forming pilus A*) and *espA* (*encoding secreted protein A*) genes. The use of DNA probes to detect the virulence genes in *E. coli* in Indonesia is not common yet. In this experiment the gene fragments of *eae, bfpA,* and *espA* were used as probes to detect the EPEC among *E. coli* isolates from stool specimensin of diarrheic children attending Public Health Centers in Yogyakarta. The DNA samples were isolated from 49 diarrheagenic *E. coli* isolates. The DNA probes of *eae, bfpA* and *espA* were obtained by amplification of DNA fragment of EPEC O126 using PCR technique. Furthermore, those probes were used to identify the presence of those genes among *E. coli* isolates using hybridization technique. The results showed that 42 (85.7%) isolates were *espA*$^-$, 25 isolates (51%) were *eaeA*$^-$ (EPEC strains). Therefore among 25 isolates of EPEC, 20 isolates (80 %) among EPEC were *bfpA*$^-$ (typical EPEC strains).

Keywords : DNA probe, *eae, bfpA, espA*, EPEC.

Introduction

Enterophatogenic *Escherichia coli* (EPEC), an important paediatric diarrhea pathogen, employs multiple adhesins to colonize the small bowel and produces characteristic 'attaching and effacing' (A/E) lesions on small intestinal enterocytes. EPEC adhesions that have been associated with A/E adhesion and intestinal colonization include bundle-forming pili (BFP), EspA filaments and intimin (Cleary *et al.*, 2004). The genetic determinants for the production of A/E lesion are located on the locus enterocyte effacement (LEE), a pathogenicity islands that contains the genes encoding intimin, a type III secretion system, a number of secreted (Esp) proteins, and the translocated intimin receptor named Tir (Trabulsi *et al.*, 2002). The locus enterocyte effacement can be divided into three functional regions : first region encoding for a type III secretion system; the second region containing the genes *eae* and *tir,* and the third region containing the genes *espD, espB*, and *espA*. The *eae* genes encodes an outer membrane protein, intimin which is essential for intimate attachment of the bacterium to the host cell. The type III secretion system is involved in the secretion of protein EspA, EspB, EspD and Tir. EspA is encoded by the *espA* gene and forms a filamentous structure on the bacterial surface through which EspB, EspD and Tir are secreted (Shaw *et al.*, 2001). The EspB and EspD proteins are thought to be incorporated into the host cell cytoplasmic

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membrane, where they form a pore through which other bacterial effector molecules, such as Tir, enter the host cell. Tir is the receptor for intimin, which is translocated via the EspA filament and EspB / EspD pore into the host cell and incorporated into the membrane. As well as interacting with intimin, this protein is also involved in promoting cytoskeletal action rearrangement in the host cell (Kuhne *et al.*, 2004).

The *eae* gene, which is located in the 'locus of enterocyte effacement; (LEE) pathogenicity islands, and the *bfpA* gene, located on a plasmid called the EPEC adherence factor (EAF), have both been used for identification of EPEC and for subdivision of this group of bacteria into typical and atypical EPEC. *E. coli* strains with the A / E genotype (*eae*$^+$) that harbour the EAF plasmid (*bfpA*$^+$) are classified as 'typical EPEC'; most of these strains belong to certain O : H serotypes. Strains with the A / E genotype that do not posses the EAF plasmid (*bfpA*$^-$) are classified as 'atypical EPEC' (Nataro and Kaper, 1998). For many years, diagnosis of EPEC was based on O : H serotype identification (Levinde & Edelman, 1984). During the last two decades, the pathogenic mechanism of EPEC infection has been clarified, this has resulted in a change in diagnostic methods from serogrouping to phenotypic and genotypic methods (Afset *et al.*, 2003).

Identification of diarrheagenic *E. coli* strains requires that these organisms be differentiated from non pathogenic members of the normal flora. Serotypic markers correlate, sometimes very closely, with specific categories of diarrheagenic, however these markers are rarely sufficient in and on themselves to reliably identify a strain as diarrheagenic. In addition to its limited sensitivity and specificity, serotyping is tedious and expensive and is performed reliably only by small number of reference laboratories. Thus, detection of diarrheagenic *E. coli* has focused increasingly on the identification of characteristic which themselves determine the virulence of these organisms. This may include *in vitro* phenotypic assays which correlate with the presence virulence factor. Indeed, molecular method remain the most popular and most reliable techniques for differentiating diarrheagenic strains from non pathogenic members of the stool flora and distinguishing one category from another. Substantial progress has been made both in the development of nucleic acid-based probe technologies as well as PCR methods (Bekal *et al.*, 2003).

The use of DNA probes in hybridization technique to detect the gene target on diarrheagenic *E. coli* in Indonesia has not been used routinely. The aim of this study is to identify the presence of virulence factor genes: *eaeA* (*E. coli attaching-effacing*), *bfpA* (*bundle-forming pilus*) and *espA* (encoding *secreted proteinA*) on *E. coli* strains among *E. coli* isolates from stool specimens of diarrheic children attending Public Health centers in Yogyakarta using the hybridization technique and DNA probes of *eae, bfpA* and *espA* .

Materials and Methods

Samples

Samples (stool specimens) were collected from dirrheic children under 5 years of age attending Public Health Centers in Yogyakarta. EPEC O126 was used as the positif control and *E.coli* ATCC 10536 non EPEC strain was used as the negative control, both were derived from Biofarma Laboratory, Bandung, Indonesia. Three set of primers *espA*, *bfpA* and *eae*, LB media, M9 (minimal media), assay media, PCR Core kit (Roche), Agarose, Dig DNA Labeling and Detection Kit (Roche).

Culture and identification methods

Culture and biochemical identification of *E. coli* were done according to standard microbiological methods (Madigan *et al.*, 2002).Polymerase Chain Reaction (PCR) assay

The *eae, bfpA* and *espA* probes were prepared by PCR amplification of the genes on EPEC O126. The following specific primers were used for PCR :

a. Specific primer *eae* (Aranda *et al.*, 2004), was received from Alpha DNA
   eae 1 : 5' – CTGAACGGCGATTAC GCGAA – 3'
   eae 2 : 5' – CCAGACGATACGATC CAG – 3'
b. Specific primer *bfpA* (Aranda *et al.*, 2004), was received from Alpha DNA
   bfpA 1 : 5' – AATGGTGCTTGCGCTT GCTGC – 3'
   bfpA 2 : 5' – GCCGCTTTATCCAACCT GGTA – 3'
c. Specific primer *espA* (Knutton *et al.*, 1998) was received from Cybergere Ab
   Forward : 5' - GCG AGT ACT TCG ACA TC – 3'
   Reverse : 5' - TTA TTT ACC AAG GGA TAT – 3'

The amplification was carried out in volumes of 25 ml according PCR Core Kit manual with thermocycler. The amplification conditions were as follows: pre denaturation *eae* gene, 1 cycle of 95°C, 5 min, followed by 40 cycles of 95°C, 30 s, 50°C, 1 min, 72°C, 75 sec with a final extension step of 72°C, 10 min. The *bfpA* genes : pre denaturation 1 cycle of 95°C, 5 min, followed by 40 cycles of 95°C, 30 s, 57°C 1 min, 72°C, 1 min, then a final extension step of 72°C, 10 min. The *espA* genes : pre denaturation 1 cycle of 94°C, 5 min, followed by 40 cycle of 94°C, 30 s, 50°C, 1 min, 72°C, 1 min, then a final extension step of 72°C, 10 min. PCR products were then electrophoresed on 2.5% agarose gel, stained with ethidium bromide and visualized by UV light. The amplification of fragment DNA could be isolated and identified with marker DNA.

DNA hybridization

Diarrheagenic *E. coli* were tested by hybridization with the specific DNA probes *eaeA*, *espA* and *bfpA*. The DNA hybridization reaction was carried out according to Dig Labeling Kit recommendation. The EPEC O126 strain was used as positive control for *bfpA*, *eae*, and *espA* probes, whereas the non EPEC strain (*E. coli* ATCC 10536) was used for negative control.

Results and Discussion

The *eae, bfpA* and *espA* genes amplification

The results of *eae, bfpA* nd *espA* genes amplication from EPEC O126 strain are shown in Figure 1. The DNA marker was used to detect the size of the amplification products, as follows : the fragment of gene *espA* gene detected as a band of 579 bp, *eaeA* gene detected as a band of 917 bp and *bfpA* gene detected as a band of 326 bp. Those fragments were used as probes on dot blot hybridization

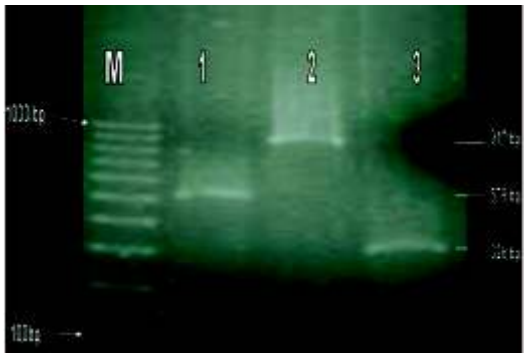

Figure 1. The result of genes amplification *espA*, *eaeA*, *bfpA* on EPEC O126. M = the DNA marker, 1 = *espA* gene, 2 = *eae* gene, 3 = *bfpA* gene

The *eaeA, bfpA* and *espA* genes detection

All 49 *E. coli* isolates were tested by hybridization technique using specific DNA probes of *eaeA*, *bfpA*, and *espA* as shown on Table 1. and Figure 2, 3, and 4. Among the 49 *E. coli* isolates, 42 isolates (85.7 %) were *espA*

(Figure 4.), 25 isolates (51%) were *eae*- (Figure 2.) and among those, 20 isolates (80%) were *bfpA*- (Figure 3.).

Tabel 1. The Result of hybridization method with DNA probes *espA*, *eaeA* and *bfpA*.

No.	Isolate	*espA*	*eaeA*	*bfpA*	No.	Isolate	*espA*	*eaeA*	*bfpA*
1.	884	-	-	-	26.	852	+	+	+
2.	883	+	-	-	27.	851	+	-	-
3.	881	-	-	-	28.	850	+	-	-
4.	880	+	-	-	29.	849	+	-	-
5.	879	-	-	-	30.	848	+	-	-
6.	878	+	-	-	31.	847	+	+	+
7.	877	+	+	-	32.	846	+	+	+
8.	876	+	-	-	33.	845	+	+	+
9.	875	+	+	-	34.	838	+	+	+
10.	874	+	-	-	35.	837	+	-	-
11.	872	+	-	-	36.	836	+	+	+
12.	871	+	+	-	37.	835	+	-	-
13.	870	-	-	-	38.	834	+	+	+
14.	869	-	-	-	39.	833	+	+	+
15.	868	+	+	+	40.	791	+	-	-
16.	867	+	-	-	41.	803	+	+	+
17.	866	+	-	-	42.	804	+	+	+
18.	867	+	+	+	43.	808	+	+	-
19.	866	+	-	-	44.	811	+	+	+
20.	863	+	-	-	45.	Isolat A	+	+	+
21.	859	+	-	-	46.	Isolat B	+	+	+
22.	858	+	+	+	47.	EPEC 114	-	-	-
23.	857	+	-	-	48.	K +	+	+	+
24.	855	+	+	+	49.	EPEC 142	+	+	+
25.	854	+	+	+	50.	EPEC 126	+	+	+
					51.	K -	-	-	-

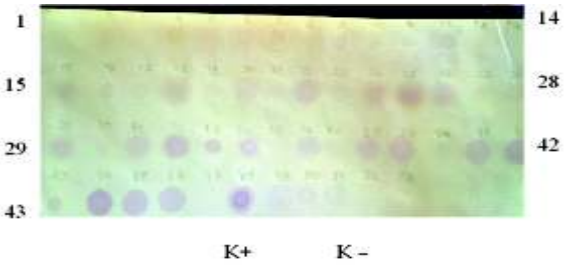

Figure 2. The result of hybridization with DNA Probe *eaeA*. K + = positive control (EPEC O126) , K - = negative control

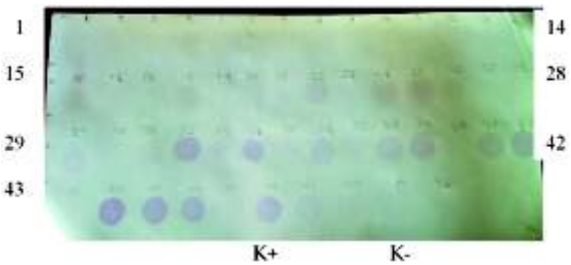

Figure 3. The result of hybridization with DNA Probe *bfpA*. K + = positive control (EPEC O126) , K - = negative control

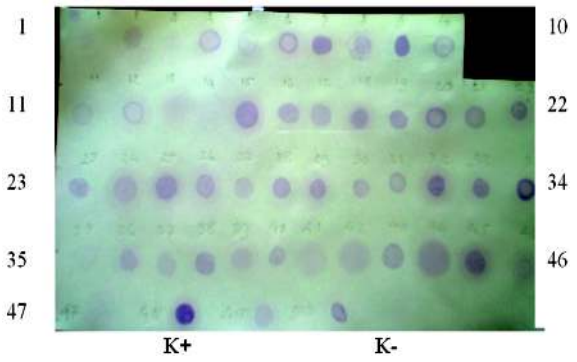

Figure 4. The result of hybridization with DNA Probe *espA*. K + = positive control (EPEC O126) , K - = negative control

The characteristic genetic profile of typical EPEC isolates is based on *eaeA*$^+$ and *bfpA*$^+$, while atypical EPEC were *eae*$^+$ and *bfp*A$^-$. This study showed that 51% of *E. coli* isolates from diarrheic children were identified as EPEC that consist of 20% Typical EPEC and 80% Atypical EPEC. This results was in agreement with the results of other studies which had shown that 30 to 40% of infant diarrhea can be attributed to EPEC in developing countries (Naro and Kaper, 1998).

Conclusion

DNA probes of *eae, bfpA* and *espA* could be used for detection of typical and atypical EPEC of diarrheagenic *E. coli* isolates, instead of using serotyping method.

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