ANTIBIOTIC RESISTANCE, PLASMID PROFILE AND RAPD-PCR ANALYSIS OF ENTEROPATHOGENIC *ESCHERICHIA COLI* (EPEC) CLINICAL ISOLATES

Wan Keng Fei[1], Son Radu[1], Cheah Yoke Kqueen[1], Patrick Guda Benjamin[2], Clemente Michael Wong Vui Ling[1], Hon Shui Fong[2], Abidah Harun[2], Lo Sze Khiong[2] and Liew Phen Kiew[2]

[1]Department of Biotechnology, Faculty of Food Science and Biotechnology, University Putra Malaysia, UPM Serdang, Selangor, Malaysia; [2]Hospital Miri, Miri, Sarawak, Malaysia

Abstract. Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of diarrhea among infants in developing countries. A total of 38 EPEC isolates, obtained from diarrhea patients of Hospital Miri, Sarawak, were investigated through plasmid profile, antibiotic resistance and randomly amplified polymorphic DNA (RAPD) analysis. From the 8 types of antibiotics used, all isolates were 100% resistant to furoxime, cephalothin and sulphamethoxazole and showed high multiple antibiotic resistant (MAR) indexes, ranging from 0.5 to 1.0. In plasmid profiling, 22 isolates (58%) showed the presence of one or more plasmids in the range 1.0 to 30.9 mDa. The dendrogram obtained from the results of the RAPD-PCR discriminated the isolates into 30 single isolates and 3 clusters at the level of 40% similarity. The EPEC isolates were highly diverse, as shown by their differing plasmid profiles, antibiotic resistance patterns and RAPD profiles.

INTRODUCTION

Diarrheagenic *E. coli* can be categorized into 6 groups: enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC) and diffuse adhering *E. coli* (DAEC) (José and Finlay, 2001). Though EPEC is a leading cause of infantile diarrhea among children aged between less than six months to 23 months, especially in the developing countries (Antai and Anozie, 1987; Renu *et al*, 1990; Albert *et al*, 1995; Donnenberg, 1995), it does happen when adults are exposed under the same environments (Ulshen and Rollo, 1980). The patients will vomit, experience stomach cramps and low fever, which is often accompanied by fluid loss (Levine and Edelman, 1984; Nataro and Kaper, 1998). EPEC is transmitted primarily by person-to-person spread, but outbreaks of EPEC disease have occasionally been linked to contaminated food and water (Bower *et al*, 1989; Viljanen *et al*, 1990; Wu and Peng, 1992).

A variety of antibiotics have been used to treat EPEC and have proved useful in many cases, but multiple antibiotic resistance is common among EPEC (Donnenberg, 1995). Many strains of *E. coli* carry resistance factors (R factors) that determine resistance to antibiotics and can be transferred among themselves or to other bacterial species (Linton *et al*, 1981) to establish multiple antibiotic resistance. The widespread use of antibiotics has been identified as a major factor accounting for the increased incidence of antibiotic resistance (Towner, 1982). Detection and monitoring of multi-antibiotic resistant EPEC is important to substantiate the choice of antibiotics for the treatment of infections cause by this organism.

EPEC pathogenicity is regulated by the product of a gene encoded on the 50 to 70 mDa EPEC adherence factor (EAF) plasmid (Nataro *et al*, 1985). EAF plasmid is mainly encoded bundle-forming pilus (BFP) in the process of localized adherence (Donnenberg, 1999). However, Knutton *et al* (1987) noted that the gene encoded for the A/E lesion was also found on the bacterial chromosomal within the locus of the enterocyte effacement (LEE) which encoded the type III secretory apparatus, such as intimin (Nataro and

Correspondence: Dr Son Radu, Department of Biotechnology, Faculty of Food Science and Biotechnology, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
Tel: +(603)-89468361; Fax: +(603)-89423552
E-mail: son@fsb.upm.edu.my

Kaper, 1998). It is also believed that the R-factor is transferred in the plasmid when EPEC infects the host and builds up resistance towards antibiotics. Random amplified polymorphism DNA (RAPD) has been used as a sensitive and efficient method for providing information on distinction and relatedness among the organisms (Welsh and McClelland, 1990; Williams *et al*, 1990). The polymorphisms produced can enable the detection of slight genetic differences between DNA samples tested.

The objective of this study was to determine the antibiotic resistance, plasmid profiles and genotypic characterization of the EPEC strains isolated from apparently unrelated hospital patients by RAPD-PCR.

MATERIALS AND METHODS

Bacterial strains

Thirty-eight isolates identified as enteropathogenic *Escherichia coli* (EPEC) were isolated from patients by the forwarding laboratory at Hospital Miri, Sarawak, Malaysia. Table 1 shows details of the EPEC strains used in this study. Cultures of EPEC used in antibiotic resistance tests, plasmid and genomic DNA isolation were grown overnight in Luria Bertani broth at 37ºC at 300 x g.

Antibiotic resistance tests

The strains were tested for antibiotic resistance by the disk diffusion method, performed on Mueller Hinton agar plates. The antibiotic disks used in this study were purchased from Sigma Chemical Co, USA. The following antibiotic-containing discs were used: cephalothin (Kf, 30 μg), cefuroxime (Cxm, 30 μg), kanamycin (K, 30 μg), neomycin (N, 10 μg), nitrofurantoin (F, 300 μg), streptomycin (S, 10 μg), sulfamethoxazole (RL, 25 μg) and tetracycline (Te, 30 μg). The plates were incubated for 17-24 hours at 37ºC. Inhibitory zones were interpreted as recommended by the supplier. The multiple antibiotic resistance index of isolates is defined as a/b where 'a' represents the number of antibiotics to which the particular isolate was resistant and 'b' the number of antibiotics to which the isolate was exposed (Krumperman, 1983).

DNA isolation

Plasmid DNA was extracted by the alkaline lysis method, followed by electrophoresis, essentially as described by Sambrook *et al* (1989). The approximate molecular mass of each plasmid was determined by comparison with plasmids of known molecular mass of *Escherichia coli* V517 (Macrina *et al*, 1978). Prior to amplification by RAPD-PCR method, the chromosomal DNA of the EPEC strains was extracted by the mini-preparation method devised by Wilson (1989).

RAPD-PCR amplification

Ten randomly designed 10-mer primers with 50% G+C content (Genosys Biotechnologies Inc, USA), designated as Gen1-50-01 to Gen1-50-10 were screened and two primers, the GEN15002 (5′-CAATGCGTCT-3′) and GEN15009 (5′-AGAAGCGATG-3′) were selected for fingerprinting the EPEC strains, as they provided reproducible and discriminatory patterns. The RAPD-PCR reaction mixtures (25 μl) consisted of 2.5 μl of 10x PCR buffer, 0.5 μl of 10 mM dNTPs, 1.5 μl of 25 mM $MgCl_2$, 1 unit of *Taq* polymerase (Promega Co, USA), 5 pmol of primer and 1.0 μl (20-30 ng) of DNA template. The PCR amplifications were carried out with a Techne Thermal Cycler (Model HL-1). The cycling parameters were 3 minutes at 94ºC for predenaturation, 45 cycles each of 1 minute at 94ºC for denaturation, 1 minute at 36ºC for annealing, 2 minutes at 72ºC for extension and a final extension at 72ºC for 5 minutes. The PCR amplification products were visualized by running 15 μl of the amplification products on 1.2% agarose gel, which was stained with ethidium bromide and photographed under UV illumination.

Fingerprint pattern analysis

RAPD electrophoresis photos were scanned and the RAPD results were further analyzed with the computer software, RAPDistance Software Version 4.0. The genetic distances of the isolates were calculated with the Nei and Li (1979) formula. The dendrogram was drawn by using the unweighted pair group procedure (UPGMA) clustering and tree-building NJTREE program in the same computer software.

RESULTS

Enteropathogenic *Escherichia coli* were isolated from various types of samples obtained from patients at the Miri Hospital in Sarawak, Malaysia (Table 1). The HVS samples, followed by wound swab and swab revealed a positivity rate

that was higher than other types of samples. The age distribution patterns are reported in Table 1. Antibiotic resistance tests showed that the EPEC isolates were resistant to most of the antibiotics tested (Table 1). The multiple antibiotics resistant (MAR) index ranged from 0.5 to 1.0, with EPEC isolates resistant to at least 4 of the antibiotics used for the test. All EPEC isolates showed 100% resistance to cefuroxime, cephalothin and sulfamethozaxole. This was followed by resistance to streptomycin (92.1%), neomycin (84.2%), tetracycline (76.3%), kanamycin

Table 1
Enteropathogenic *Escherichia coli* strains used in this study.

Strain no.	Sample type	Gender/age of patient (year)	Antibiotic resistant patterns	Plasmid profiles (megadalton)
EC01	Wound swab	F/37	CxmKfKNRlTe (0.75)	2.0
EC02	Swab	F/27	CxmKfFKNRlSTe (1.0)	ND
EC03	Higher vagina swab	F/31	CxmKfNRlSTe (0.75)	2.0
EC04	Swab	M/50	CxmKfKNRlS (0.75)	ND
EC05	Higher vagina swab	F/27	CxmKfNRlSTe (0.75)	22.7
EC06	Hand swab	M/30	CxmFKfKNRlSTe (1.0)	ND
EC07	PD fluid	M/-[a]	CxmKfNRlS (0.63)	2.6
EC08	Higher vagina swab	F/29	CxmKfKNRlSTe (0.88)	22.7
EC09	Higher vagina swab	F/29	CxmKfKNRlSTe (0.88)	7.0, 3.8
EC10	Eye swab	M/1 week	CxmKfNRlS (0.63)	6.2, 1.4
EC11	Wound swab	M/45	CxmKfKNRlSTe (0.88)	ND
EC12	Pus swab	M/1 month	CxmKfKNRlSTe (0.88)	22.7
EC13	Higher vagina swab	F/20	CxmKfRlSTe (0.63)	30.9, 6.2, 3.8
EC14	Swab	F/11	CxmKfNRlS (0.63)	ND
EC15	Urine	F/20	CxmKfNRlSTe (0.75)	27.9, 16.9, 9.3, 2.0
EC16	Higher vagina swab	F/37	CxmKfNRlSTe (0.75)	ND
EC17	Wound swab	M/30	CxmFKfKNRlSTe (1.0)	ND
EC18	Higher vagina swab	F/30	CxmKfNRlSTe (0.75)	ND
EC19	Higher vagina swab	F/18	CxmKfNRlSTe (0.75)	27.9, 9.3, 3.8, 2.0
EC20	Higher vagina swab	F/37	CxmKfNRl (0.50)	ND
EC21	Higher vagina swab	F/21	CxmKfKNRlSTe (0.88)	2.0
EC22	Tip	M/64	CxmKfNRlTe (0.63)	6.2
EC23	Higher vagina swab	F/-	CxmKfNRlSTe (0.75)	ND
EC24	Wound swab	M/49	CxmKfRlSTe (0.75)	6.2
EC25	Higher vagina swab	F/34	CxmKfNRlSTe (0.75)	ND
EC26	Wound swab	M/75	CxmKfNRlSTe (0.75)	ND
EC27	Wound swab	M/41	CxmKfNRlS (0.63)	17.0, 7.6, 2.6
EC28	Higher vagina swab	F/-	CxmKfRlS (0.50)	11.4, 3.8
EC29	Wound swab	M/23	CxmKfRlS (0.50)	5.6, 2.3
EC30	Swab	M/34	CxmFKfKNRlSTe (1.0)	2.6
EC31	Higher vagina swab	F/25	CxmFKfNRlSTe (0.88)	9.3, 4.6
EC32	Leg swab	F/13	CxmKfRlSTe (0.63)	9.0, 2.6
EC33	Higher vagina swab	F/54	CxmKfRlSTe (0.63)	7.6, 2.6
EC34	Swab	M/30	CxmKfKNRlSTe (0.88)	ND
EC35	Higher vagina swab	F/40	CxmFKfKNRlSTe (1.0)	ND
EC36	Wound swab	M/30	CxmKfNRlSTe (0.75)	ND
EC37	Higher vagina swab	F/24	CxmKfKNRlSTe (0.88)	9.3
EC38	Higher vagina swab	F/29	CxmKfNRlS (0.63)	ND

[a]Age not available; ND=none detected.

(36.8%) and nitrofurantoin (15.8%). Overall, 7.9% were resistant to four types of antibiotics, 26.3% to five types of antibiotics, 31.6% to six types of antibiotics, 21.1% to seven types of antibiotics and 13.2% to eight types of antibiotics (Table 1). From the total 38 isolates, 22 showed the presence of one or more plasmids (Table 1). The isolates harbored plasmids ranging from 1.0 to 30.9 mDa. To better characterize the EPEC strains in the current investigation, all the EPEC isolates were subjected to DNA fingerprinting by RAPD-PCR, using primers GEN1-50-02 and GEN1-50-09. The RAPD-PCR accurately differentiates the strains by means of the number and position of the amplified DNA fragments, which are visible in the gels (data not shown). Accordingly, the greater the differences in the individual patterns, the more unrelated the strains are from one to another. When the results of the two primers were combined in a single dendrogram, all the 38 isolates were seen to represent individual isolates at 0% similarity, but forming two major clusters (Fig 1).

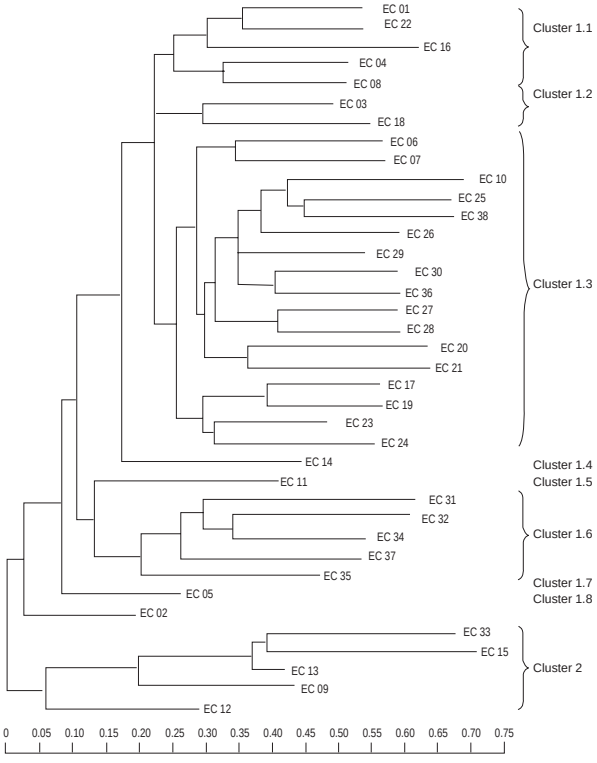

Fig 1–UPGMA-based dendrogram from RAPD profiles of the enteropathogenic *Escherichia coli* strains.

DISCUSSION

In this study, the most represented age patterns for positivity for the isolation of the EPEC strains were adults between 21-30 years old. This showed that adults also carried EPEC in their bodies but seldom expressed the symptoms of illness. It is believed that adults acquire immunity to EPEC. However, certain groups of adults, such as pregnant women, the elderly and persons having low antibody defenses have higher risk of being infected by EPEC. The higher rate of EPEC isolation was linked to the positivities of the HVS samples collected from the female patients, and suggests that this microorganism may represent one of the exacerbating factors for urinary tract infections in humans. Many previous studies showed that EPEC easily infects infants, but in this study, almost all isolates were isolated from adults or older patients. The majority age group ranged from 21 to 30 years old. This situation is not unexpected, as the previous study by Ulshen and Rollo (1980) showed that adults have the same risk of being infected by EPEC if exposed to the same environment.

An analysis of the antibiotic resistance patterns showed that none of the antibiotics tested was effective against all the strains used in this study. For example, in this study several of the EPEC strains were resistant to nitrofurantoin, a commonly prescribed drug for urinary tract infections. The presence of antibiotic-resistant EPEC in humans in the community is probably influenced by many factors, and they might act as a reservoir for infections by multi-resistant strains in hospitals. Our earlier studies indicate that resistant isolates of *E. coli* and/or genes encoding resistance can be transferred among animals and environmental isolates (Son *et al*, 1996; 1997). The resistance of EPEC isolates towards eight types of antibiotics tested was high and the multiple antibiotic resistance (MAR) index values ranged from 0.5 to 1.0. These relatively high MAR index values suggested that EPEC are more likely to have a predisposition to develop resistance under conditions of antibiotic selective pressure. These results confirmed data reported by other authors, indicating that EPEC are frequently and increasingly demonstrating multiple resistance to antibiotics tested (Antai and Anozie, 1987). The high incidence of antibiotic-resistant isolates of EPEC may be due to the widespread

use of antibiotics. The continuous use of even a single antibiotic over a period of weeks or months will select bacteria with resistance to different kinds of antibiotics in addition to the one in use. Conjugation that may occurr among species or between species, will build resistance diversity towards traditional antibiotics and resistance to antibiotics can be increased from the native version (Grabow *et al*, 1974; Tamanai-Shacoori *et al*, 1995). At the present time, we cannot exclude that the multiple antibiotic resistant strains colonizing different sites of the patients examined may represent a post-treatment effect that is strain selection due to the eradication of the more susceptible strains. In the absence of selective pressure, it would be expected that isolates with special resistance features would only be present in low number. This is an interesting point to be analyzed in follow-up studies.

Multiple antibiotic resistance can occur even in the absence of plasmid or transposon. The study published by Levy (1992) showed that research done with plasmid- and transposon-free *E. coli* was resistant up to seven types of antibiotics, including tetracycline. This higher level of resistance may be caused by initial mutation located in a single site on the *E. coli* chromosome. Since none of the EPEC isolates used in this study had been examined for their ability to transfer their antibiotic resistance phenotypes, it is not possible with certainty to correlate the presence of plasmid with antibiotic resistance. In addition, the antibiotic resistant and plasmid profile patterns do not show any direct correlations. The association of pathogenicity and the absence/presence of plasmid, especially the EAF plasmid in EPEC isolates, has been described by Kaper (1996). A study by Levine *et al* (1985) showed that the EAF plasmid was lost in a high proportion of colonies recovered from the stools of volunteers. Donnenberg *et al* (1998) estimated the plasmid is lost *in vitro* under direct selective pressure but still showed pathogenicity in patients with lower positive stool cultures. Recently, Teophilo *et al* (2002) also reported on the absence of plasmid in the isolates with multiple antibiotic resistance. Brown *et al* (1991) suggested, that under laboratory conditions, the absence of antibiotics in the culture media probably enhances plasmid instability.

For the purpose of epidemiological analysis, it is of great importance to use methods with high discriminatory power, excellent reproducibility and ease of interpretation and use. With the data analysis done with UPGMA clustering and tree building NJTREE program in the RAPDistance Software Version 4.0, a dendrogram was generated with genetic distance (Fig 1). Basically, all 38 EPEC isolates were divided into 2 main clusters and 8 sub-clusters in the main cluster. The main cluster consisted of about 87% of the total isolates. This showed that most of the isolates may have originated from a similar lineage. For example, 5 pairs of isolates that showed close relatedness were EC04-08, EC06-07, EC25-28, and EC27-28. According to their sources of isolation, isolate EC27 was isolated from the wound swab of a male patient, while EC 28 was isolated from the higher vagina swab of a female patient. This may suggest that RAPD-PCR can relate different strains of EPEC that may have infected different patients, and may be useful in the determination of possible nosocomial infection. The discriminatory power of RAPD-PCR can be shown by the ability to differentiate isolates at certain genetic distance/levels. At the level of 40% similarity, combination of the 2 primers was able to differentiate 38 isolates into 30 single isolates and 3 clusters (Fig 1). Besides EPEC in this study, RAPD-PCR has been used widely with other microbial isolates and plant cultivars to study the relatedness among the isolates, such as *Vibrio vulnificus* (Warner and Oliver, 1999), *Aeromonas* genomospecies (Oakey *et al*, 1996) and *Glycine max* (Chowdhury *et al*, 2000).

The results of this study indicate that the EPEC strains are highly diverse, despite the fact that many of the isolates share the same antibiotic resistance patterns. Indirectly, this may suggest that the resistance patterns are not associated with major genetic alterations, and that the diversity observed can be attributed to other factors. Hence, RAPD fingerprinting technique has great potential for the characterization of clinical isolates that may experience genomic changes before or after resistance developed. In conclusion, this study shows a frequent occurrence of multi-resistant strains and the presence of a wide diversity of EPEC strains isolated from humans in a hospital.

ACKNOWLEDGEMENTS

This study was supported by the Malaysian

Government through the IRPA grant mechanism.

REFERENCES

Albert MJ, Faruque SM, Faruque ASG, *et al*. Controlled study of *Escherichia coli* diarrheal infections in Bangladeshi children. *J Clin Microbiol* 1995; 33: 973-7.

Antai SP, Anozie SO. Incidence of infantile diarrhea due to enteropathogenic *Escherichia coli* in Port Harcourt metropolis. *J Appl Bacteriol* 1987; 62: 227-9.

Bower JR, Congeni BL, Cleary TG, *et al*. *Escherichia coli* O114: Nonmotile as a pathogen in an outbreak of severe diarrhea associated with a day care center. *J Infect Dis* 1989; 160: 243-7.

Brown DJ, Threffall EJ, Rowe B. Instability of multiple drug resistance plasmid. *Epidemiol Infect* 1991; 106: 247-57.

Chowdhury AK, Srinives P, Tongpamnak P, Saksoong P. Identification of cultivars of vegetable soybeans [*Glycine max* (L.) Merr.] by RAPD Markers. *SABRAO J Breeding Gen* 2000; 32: 63-72.

Donnenberg MS. Enteropathogenic *Escherichia coli*. In: Blaser MJ, Smith PD, Ravdin JI, Greenberg HB, Guerrant RL, eds. Infections of the gastrointestinal tract. New York: Raven Press, 1995.

Donnenberg MS, Tacket CO, Losonsky G, *et al*. Effect of prior exerimental human enteroppathogenic *Escherichia coli* infection on illness following homologous and heterologous rechallenge. *Infect Immun* 1998; 66: 52-8.

Donnenberg MS. Interactions between enteropathogenic *Escherichia coli* and epithelial cells. *Clin Infect Rev* 1999; 28: 451-5.

Grabow WOK, Prozesky OW, Smith LA. Drug resistant coliforms: call for review of water quality standards. *Water Res* 1974; 8: 1-9.

José LP, Finlay BB. Pathogenic *Escherichia coli*. In: Groisman EA, ed. Principles of Bacterial Pathogenesis. California: Academic Press, 2001.

Kaper JB. Definition of EPEC. *Rev Microbiol* 1996; 27: 130-3.

Knutton S, Baldini MM, Kaper J, McNeish AS. Role of plasmid-encoded adherence factors in adhesion of enteropathogenic *Escherichia coli* to HEp-2 cells. *Infect Immun* 1987; 55: 78-85.

Krumperman PH. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Appl Environ Microbiol* 1983; 46: 165-70.

Levine MM, Edelman R. Enteropathogenic *Escherichia coli* of classic serotypes associated with infant diarrhea: epidermiology and pathogenesis. *Epidemiol Rev* 1984; 6: 31-51.

Levine MM, Nataro JP, Karch H, *et al*. The diarrheal response of humans to some classical serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. *J Infect Dis* 1985; 152: 550-9.

Levy SB. Antibiotic resistance: microbial adaptation and evolution. In: The antibiotic paradox: how miracle drugs are destroying the miracle. New York: Plenum Press, 1992.

Linton AH, Timoney JF, Hinton M. The ecology of chloramphenicol-resistance in *Salmonella typhimurium* and *Escherichia coli* in calves with salmonella infection. *J Appl Bacteriol* 1981; 50: 115-29.

Macrina FL, Kopecko DJ, Jones KR, Ayers DJ, McCowen SM. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. *Plasmid* 1978; 1: 417-20.

Nataro JP, Scaletsky ICA, Kaper JB, Levine MM, Trabulsi LR. Plasmid-mediated factors conferring diffuse and localized adherence of enteropathogenic *Escherichia coli*. *Infect Immun* 1985; 48: 378-83.

Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* 1998; 11: 142-201.

Nei M, Li WH. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA* 1979; 76: 5269-73.

Oakey HJ, Ellis JT, Gibson LF. Differentiation of *Aeromonas* genomospecies using random amplified polymorphism DNA polymerase chain reaction (RAPD-PCR). *J Appl Bacteriol* 1996; 80: 402-10.

Renu S, Dumrong C, Jitvimol S, Riichi S, Peter E. Nationwide surveillance program to identify diarrhea-causing *Escherichia coli* in children in Thailand. *J Clin Microbiol* 1990; 28: 469-72.

Sambrook J, Fritsch EF, Maniatis M. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989.

Son R, Rusul G, Karim MIA. Plasmid and antimicrobial resistance transfer from poultry strains to aquatic strains of *Escherichia coli*. *J Vet Malaysia* 1996; 8:7-15.

Son R, Rusul G, Karim MIA. Conjugal transfer of plasmids and antibiotic resistance among *Escherichia coli* isolated from animals in a rural area in Sarawak (Malaysia). *J Appl Microbiol* 1997; 82: 240-4.

Tamanai-Shacoori Z, Arturo M, Pommepuy M, Mamez C, Cormier M. Conjugal transfer of natural plasmids between *Escherichia coli* strains in sterile environmental water. *Curr Microbiol* 1995; 30: 155-60.

Teophilo GND, Virira RHSF, Rodrigues DP, Menezes FGR. *Escherichia coli* isolated from seafood: toxicity and plasmid profile. *Intern Microbiol* 2002; 5: 11-4.

Towner KJ. Resistance to trimethoprim among urinary tract isolates in United Kingdom. *Rev Infect Dis* 1982; 4: 456-60.

Ulshen MH, Rollo JL. Pathogenesis of *Echerichia coli* gastroenteritis in man–another mechanism. *N Engl J Med* 1980; 302: 99-101.

Viljanen MK, Peltola T, Junnila SYT, *et al.* Outbreak of diarrhea due to *Escherichia coli* O111:B4 in schoolchildren and adults association of Vi antigen-like reactivity. *Lancet* 1990; 336: 831-4.

Warner JM, Oliver JD. Randomly amplified polymorphism DNA analysis of clinical and environmental isolates of *Vibrio vulnificus* and other *Vibrio* species. *Appl Environ Microbiol* 1999; 65: 1141-4.

Welsh J, McClelland M. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Asids Res* 1990; 18: 7213-18.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 1990; 18: 6531-5.

Wilson K. Preparation of genomic DNA from bacteria. biology. In: Ausubel FM, Brent RE, Kingston RE, *et al*, eds. Current protocols in molecular biology. New York: John Wiley and Sons, 1989.

Wu S-X, Peng R-Q. Studies on an outbreak of neonatal diarrhea caused by EPEC O127: H6 with plasmid analysis restriction analysis and outer membrane protein determination. *Acta Paediatr Scand* 1992; 81: 217-21.