Plasmid content and localisation of the STaI (STaP) gene in enterotoxigenic *Escherichia coli* with a non-radioactive polynucleotide gene probe

A. BERTIN

Laboratoire de Pathologie Infectieuse et Immunologie, Institut National de la Recherche Agronomique,
Centre de Recherches de Tours, 37380 Nouzilly, France

Summary. Enterotoxigenic *Escherichia coli* (ETEC) strain P2200 of porcine origin possessed eight possibly plasmid-determined characters (K88⁺ Raf⁺ Hly⁺ Col⁺ Sm^r Tc^r Su^r STa⁺) and six plasmid DNA bands of 4.2–93 kb. Analysis of the spontaneous loss of characters and the results of matings with other *E. coli* strains revealed that the K88, Raf, Hly, Sm^r, Tc^r and Su^r characters could be transferred, and that the presence of the K88 and Raf characters was associated with an 83-kb plasmid. The presence and location of the STaI gene was investigated in several ETEC strains of bovine or porcine origin. Hybridisation with a non-radioactive polynucleotide probe associated the STaI gene with a plasmid in each strain; these plasmids were of 32–142 kb. In contrast, plasmids from a P2200 STa⁻ variant and plasmids from two STa⁻ variants of the bovine ETEC strain B41* (strain B41 obtained from a different source) did not hybridise with the probe. One of the B41*STa⁻ variants had lost the STa plasmid, whereas the second variant retained a plasmid of the same size which did not hybridise. In contrast, a third B41*STa⁻ variant retained a plasmid of the same size that still hybridised with the STaI probe. Plasmid DNA restriction fragment analysis, followed by hybridisation with the STaI probe, showed that the STaI gene was associated with 8.3-, 6.8- and 3.5-kb plasmid fragments in strain B41, and with 4.9-, 6.8- and 3.5-kb plasmid fragments in strain B41*, following digestion with *Eco*RI, *Bam*HI, or *Eco*RI + *Bam*HI, respectively. The STaI probe hybridised also with 12.2-kb *Eco*RI and 4.6-kb *Bam*HI fragments of the plasmid from the third B41*STa⁻ variant, that unexpectedly had given an initial positive hybridisation result. Different plasmid restriction fragment profiles were seen for strains B41 and B41*, the B41*STa⁻ variants and the transconjugant strains, thereby providing further evidence that molecular rearrangements of these plasmids can occur spontaneously.

Introduction

Several virulence factors have been identified in strains of enterotoxigenic *Escherichia coli* (ETEC). These include plasmid-encoded toxins that cause intestinal cells to lose water and electrolytes, and plasmid- or chromosome-encoded adhesins (CFA) that confer the ability to adhere to intestinal cells. Adhesins are often pili or pili-like structures that are antigenically identifiable. Different plasmid- or chromosome-encoded adhesins have been identified in ETEC strains of human origin. Similarly, adhesins K99 (in ETEC strains from calves, pigs and lambs) and K88 (in ETEC strains from pigs) are plasmid-encoded, whereas F41 (in ETEC strains from calves, pigs and lambs) is chromosome-encoded.^{1–3} More recently, another adhesin named 987P (in ETEC strains from pigs), originally thought to be encoded by

the chromosome, has also been found to be encoded by a plasmid.⁴ The identified toxins include heat-labile toxin (LT), which possesses similarities with cholera toxin, and several heat-stable toxins that are active either in the suckling mouse assay (STa) or only in the pig intestine (STb).⁵ Some structural and genetic heterogeneity has been found in STa toxins produced by bacteria of human and porcine origins.⁵

STa and LT toxins have been shown to be encoded by several different plasmids. Plasmids coding for STa + LT in human and pig strains of bacteria are usually considered to be highly related; plasmids coding for STa only, and probably those coding for LT only, are more heterogeneous. ETEC strains usually contain several distinct plasmid types, and adhesins have sometimes, but not always, been reported to be encoded by the same plasmid as toxin or antibiotic resistances. Recombination or transposition events have also been described.^{6–8}

In only a limited number of cases has toxin production been associated with a particular plasmid

and with other characters, largely because it is difficult to separate the various plasmids in a particular strain without a readily selectable genetic marker. DNA probes for toxin genes are now available, and these enable information to be obtained readily about the location, stability, recombination events, dissemination and occurrence of such genes in strains that do not express toxin.

This work reports the results of a study in which the phenotypic characters of an ETEC strain of porcine origin were correlated with its plasmid content following spontaneous loss of characters, and transconjugants obtained in matings with other *E. coli* strains were analysed. In addition, a non-radioactive STaI polynucleotide gene probe (termed STaP in strains of porcine origin) was investigated for its suitability to determine the occurrence and location of the STaI gene on plasmids from the porcine strain studied, together with other ETEC strains or STa⁻ variants of different origins.

Materials and methods

Bacterial strains

Strain P2200 was a K88⁺ ETEC strain of porcine origin.^{9,10} Strain B41 was a bovine K99⁺STa⁺ reference ETEC strain,¹¹ and strain B41* was supposedly identical, but was obtained from a different source;¹² these two strains had a different plasmid content.¹² Strain B41*C was derived from strain B41*, but had lost a 90-MDa (142-kb) plasmid, together with the ability to produce STa toxin and K99 adhesin.¹² Recipient strains used in plasmid transfers were: C600PK1046, (resistant to rifampicin, C600 rif^r); a nalidixic resistant mutant of another *E. coli* K12 (*E. coli* K12 nal^r); C13, a non-enterotoxigenic strain of *E. coli*;⁹ and a nalidixic acid-resistant mutant of strain B41*C.¹² The origins and characteristics of the porcine ETEC strains 431, 987 and 1676, and the bovine ETEC strains B44, B80 and B117 have been described previously.^{10,13-15} Each ETEC strain produced STa. Strain G157, provided by S. L. Moseley (University of Washington, School of Medicine, Seattle, Washington, USA), was used as a source of the STaI probe. Strain G157 carries plasmid pDAS101, comprising a 157-bp *Hinf*I fragment from pRIT1036 which had been blunt-ended by an end-fill reaction and cloned into the *Sma*I site of pUC8.

Characterisation of bacterial strains

Antisera specific for K99 and K88 were produced as described previously.^{9,12} Tests for the ability to ferment carbohydrates were made either with the API50 system (API System S.A., La Balme les Grottes, Montalieu-Vercieu, France) or in peptone water with phenol red. Production of haemolysin was detected on Sheep-Blood Agar (Difco, Detroit, MI, USA). Pro-

duction of colicins was detected by the agar-overlay method of Fredericq.¹⁶ Resistance to antibiotics was detected by placing antibiotic-containing disks (bioMérieux, Charbonnière Les Bains, France) on cultures spread on Mueller Hinton Agar (bioMérieux) plates. STa toxin production was determined by the suckling mouse assay.¹⁷ A strain giving a positive result in this test was designated STa⁺, and a strain giving a negative result was designated STa⁻.

Plasmid transfers

Donor and recipient strains were grown, without shaking, for 24 h at 37°C in 10 ml of Trypticase Soy Broth (bioMérieux). Equal portions (200 µl) of each culture were mixed and incubated for a further 18 h at 37°C. Transconjugants were selected on Trypticase Soy Agar (bioMérieux) supplemented with appropriate antibiotics: streptomycin 2.5 mg/L; sulphadiazine 100 mg/L; rifampicin 32 mg/L; nalidixic acid 40 mg/L.

Plasmid analysis

After alkaline extraction,¹⁸ the plasmid content of each strain was analysed by electrophoresis on vertical agarose 0.8% gels in a Protean gel apparatus (BioRad Laboratories, Paris, France). The sample slots contained 40 µl of a plasmid extract. Electrophoresis was normally at 100 V for 150 min in TAE buffer.¹⁹ Gels were stained for 30 min with ethidium bromide 0.5 mg/L and photographed under UV light on to Polaroid type 665 film. The molecular sizes of plasmids were estimated from a linear regression curve constructed from the mobility of standard plasmids of known molecular size.

Restriction fragment analysis

Plasmid DNA was extracted by the method of Birnboim and Doly,²⁰ and then digested with either *Eco*RI, *Bam*HI (Appligène, Illkirch, France), or both, in H buffer¹⁹ for 150 min at 37°C, followed by ethanol precipitation and resuspension in 15 µl of TAE. Electrophoresis was performed in TAE on agarose 1% gels in a horizontal gel electrophoresis system (Model H4; Bethesda Research Laboratories, Gaithersburg, MD, USA). Samples and fragments of known size (λ -*Bst*EII; Appligène) were electrophoresed for 18 h at 30 V, stained with ethidium bromide and photographed as described above.

Probe extraction and purification

Plasmid pDAS101 was extracted by the method of Holmes and Quigley,²¹ and was then digested with *Eco*RI and *Bam*HI as described above. The 157-bp fragment was isolated from a polyacrylamide 8% gel as described previously,²² but with the modification of Bialkowska-Hobrzanska.²³ Two cycles of purification

Table I. Plasmid content and phenotypic characters associated with porcine ETEC strain P2200 and its spontaneous variants

Bacterial strains	Plasmid content (kb)						Phenotype							
	93	83	55	6.3	5.2	4.2	K88	Raf	Hly	Col	Sm	Tc	Su	STa
Original strain P2200	+	+	+	+	+	+	+	+	+	+	R	R	R	+
Variants														
P2200 Raf ⁻	+	-	+	+	+	+	-	-	+	+	R	R	R	+
P2200 STa ⁻	+	+	+	+	+	+	+	+	+	-	R	R	R	-

Table II. Plasmid content and phenotypic characters associated with transconjugants obtained in matings with ETEC strain P2200

Recipient strains and transconjugants*	Plasmid content (kb)						Phenotype							
	93	83	55	6.3	5.2	4.2	K88	Raf	Hly	Col	Sm	Tc	Su	STa
C600 rif ^r	-	-	-	-	-	-	-	-	-	-	S	S	S	-
C600Raf ⁺ (A,D,F,I,J)	+	+	-	+	+	+	+	+	-	-	R	S	R	-
C600Raf ⁺ (B,C)	-	+	-	+	-	-	+	+	-	-	R	S	R	-
C600Raf ⁺ (E,G,H)	-	+	-	+	+	+	+	+	-	-	R	S	R	-
B41* <i>C</i> nal ^r	-	-	-	-	-	-	-	-	-	-	S	S	S	-
B41* <i>C</i> Raf ⁺ (1,3,4,5)	+	+	-	-	-	-	+	+	-	-	S	S	S	-
B41* <i>C</i> Raf ⁺ (2,6)	-	+	-	-	-	-	+	+	-	-	S	S	S	-
<i>E. coli</i> K12 nal ^r				NT			-	-	-	-	S	S	S	-
<i>E. coli</i> K12 Raf ⁺ (A)				NT			+	+	-	-	R	S	R	-
<i>E. coli</i> K12 Raf ⁺ (B)				NT			+	+	+	-	R	S	S	-
<i>E. coli</i> K12 Raf ⁺ (C)				NT			+	+	-	-	R	R	R	-

NT, not tested.

*Transconjugant designations are shown in parentheses.

were carried out and the homogeneity of the purified DNA fragment was verified on a polyacrylamide 5% gel.

Southern transfers

Gels were first treated successively with: 0.25 M HCl for 15 min; 0.5 M NaOH, 1.5 M NaCl for two 30-min periods; and 0.5 M Tris-HCl, pH 7.0, 3.0 M NaCl for two 20-min periods. DNA was then transferred to Nylon Hybond-N membranes (Amersham France, Les Ulis, France) in 10 × SSC (SSC was 150 mM NaCl, 15 mM sodium citrate, pH 7.0), and was fixed by baking at 80°C for 2 h.

STaI gene detection

Purified STaI probe DNA was labelled by random priming with digoxigenin (Non-Radioactive DNA Labelling and Detection Kit; Boehringer-Mannheim, Meylan, France), and hybridised with plasmid DNA transferred previously to Hybond-N membranes as

described above. Pre-hybridisation (3 h) and hybridisation (overnight) were performed in the absence of formamide at 68°C. The probe concentration in the hybridisation medium was 50 or 100 ng/ml.

Results

Derivatives of strain P2200 were selected by spontaneous loss of phenotypic characters (STa, Raf). These were analysed for their plasmid content and were compared with transconjugants obtained in matings with other strains of *E. coli* (tables I and II). Six plasmid DNA bands were identified in strain P2200. Although the association of characters with the presence of a particular plasmid DNA band was not easy, the K88 and Raf characters could be associated with the presence of the 83-kb plasmid. The 55-kb plasmid was neither transferred nor eliminated, and the 93-, 5.2- and 4.2-kb plasmids did not appear to be associated with the characters studied. It was not possible to link the STa character to the presence of a

Fig. 1. Plasmid content (A) and Southern blot following hybridisation with the STaI probe (B) of selected strains and transconjugants. Lane 1, strain B41; 2, strain B41^{*}; 3, strain B41^{*}Tc^s1; 4, strain B41^{*}Tc^s2; 5, transconjugant C600Tc^s1; 6, transconjugant C600Tc^s2; 7, strain G157 (pDAS101).

Fig. 2. Plasmid content (A) and Southern blot following hybridisation with the STaI probe (B) of selected strains of *E. coli*. Lane 1, strain C13; 2, strain B44; 3, strain B80; 4, strain B117; 5, strain 431; 6, strain 987; 7, strain 1676; 8, STaI⁻ variant of strain P2200; 9, strain P2200; 10, strain B41^{*}; 11, strain G157 (pDAS101).

particular plasmid DNA band. A further observation was that C600 Raf⁺ transconjugants also acquired the ability to ferment melibiose.

The location of the STaI gene in several ETEC strains of bovine or porcine origin was investigated. Strain B41 possessed four plasmid DNA bands.¹² The 105-kb (67-MDa) plasmid was associated with the presence of K99 antigen and production of STa toxin, whereas the 87-kb (55-MDa) plasmid was associated with resistance to streptomycin, tetracycline and sulphonamides. In contrast, all these phenotypic characters were encoded by a 142-kb (90-MDa) plasmid in strain B41^{*}.¹² Of three STa⁻ derivatives of strain B41^{*} selected by loss of tetracycline resistance, one had lost the 142-kb plasmid and its associated characters (strain B41^{*}C); the other two derivatives, B41^{*}Tc^s1 and

B41^{*}Tc^s2, retained resistance to streptomycin and sulphonamides.¹² These resistances were transferred by conjugation to *E. coli* C600 to produce transconjugants designated C600Tc^s1 and C600Tc^s2, respectively. Strains B41^{*}Tc^s1 and B41^{*}Tc^s2 retained a plasmid DNA band migrating at about the same level as that of strain B41^{*} (fig. 1A). Results obtained with the STaI probe showed that the 142-kb plasmid from strain B41^{*}Tc^s1 retained the ability to hybridise with the probe, whereas the plasmid from strain B41^{*}Tc^s2 did not hybridise (fig. 1B). Corresponding results were obtained with transconjugants C600Tc^s1 and C600Tc^s2 (fig. 1B).

Further hybridisation experiments showed that each enterotoxigenic strain in the study carried a plasmid that hybridised with the STaI probe (figs. 2A and B).

Table III. Plasmid content and hybridisation properties of the *E. coli* strains studied

Bacterial strain	Approximate plasmid sizes (kb)	Plasmid hybridising with STaI probe (kb)
B41	105, 87, 4.9, 3.6	105
B41*	142, 4.9, 3.6	142
B41*Tc ^s 1	142, 4.9, 3.6	142
B41*Tc ^s 2	142, 4.9, 3.6	None
C600Tc ^s 1	142	142
C600Tc ^s 2	142	None
B44	107, 88, 10.1	107
B80	107, 7.9, 3.3	107
B117	95	95
431	110–105, 61, 6.5, 3.5	110–105
987	118, 104, 57, 32, 5.8, 4.7	32
1676	102, 71, 11.2, 3.5	102
P2200	93, 83, 55, 6.3, 5.2, 4.2	93
P2200 STa ⁻	93, 83, 55, 6.3, 5.2, 4.2	None
C13	107	None

The plasmids carried by the non-enterotoxigenic strain C13 and an STa⁻ derivative of strain P2200 did not hybridise with the STaI probe (figs. 2A and B). Table III summarises the results obtained from several agarose gels regarding the plasmid content and hybridisation properties of the strains studied.

To investigate the genetic modifications which might be occurring, plasmid restriction profiles were compared for strains B41*, B41*Tc^s1, B41*Tc^s2, C600Tc^s1 and C600Tc^s2. The small plasmids (3.6 and 4.9 kb) were not cut by the restriction enzymes used in these experiments. The complex data obtained following restriction endonuclease treatment of the other plasmids (results not shown) demonstrated differences between the original strains B41 and B41*, as well as

Table IV. Summary of hybridisation results obtained with the STaI probe and plasmid restriction fragments from selected strains

Strain	DNA fragments (kb) hybridising with the STaI probe		
	<i>Eco</i> RI	<i>Bam</i> HI	<i>Eco</i> RI + <i>Bam</i> HI
B41	8.3	6.8	3.5
B41*	4.9	6.8	3.5
B41*Tc ^s 1	12.2	4.6	4.6
C600Tc ^s 1	12.2	4.6	4.6

between the STa⁻ derivatives and transconjugants obtained following transfer to *E. coli* C600.

Hybridisation results obtained with the STaI probe confirmed that the plasmid from strain B41*Tc^s2 had lost the STaI gene, because no hybridisation occurred with any fragment (figs. 3A, B, C). In contrast, hybridisation occurred with a 12.2-kb *Eco*RI fragment and a 4.6-kb *Bam*HI fragment from strain B41*Tc^s1. The STaI gene was located to different *Eco*RI fragments, 8.3 and 4.9 kb respectively, from strains B41 and B41*, but was found on the same 6.8-kb *Bam*HI and 3.5-kb *Eco*RI + *Bam*HI fragments from these strains. The results are summarised in table IV.

Discussion

Each ETEC strain studied was found to contain between one and six plasmids, and in each strain it was possible to use the non-radioactive polynucleotide probe to localise the STaI gene to one of the plasmids,

Fig. 3. Southern blots of plasmid DNA from selected strain and transconjugants, digested with restriction endonucleases and hybridised with the STaI probe. Digest: (A) *Eco*RI; (B) *Bam*HI; (C) *Eco*RI + *Bam*HI. Lane 1, strain B41; 2, strain B41*; 3, strain B41*Tc^s1; 4, strain B41*Tc^s2; 5, strain B41*C; 6, transconjugant C600Tc^s1; 7, transconjugant C600Tc^s2; 8, strain G157 (pDAS101) linearised by *Eco*RI.

ranging in size from 32 to 142 kb. Strain P2200 contained six plasmids, of which a 93-kb plasmid hybridised with the STaI probe. Although one of the STa⁻ derivatives isolated from strain P2200 apparently retained this plasmid, no hybridisation was detected. One possibility is that strain P2200 might contain more than one plasmid that migrated at this level, and there was evidence for this possibility on some of the gels examined (results not shown). With some other strains, a few anomalies were also observed when compared with previous studies which examined the location of the STaI gene with radioactively-labelled probes.^{4,24} However, it has been reported²⁵ that the STa gene is located on a transposon, and this could account for any apparent changes in its precise location.

The location of the STaI gene was investigated in detail for strains B41, B41*, B41*Tc^s1 and B41*Tc^s2, as well as in the transconjugants C600Tc^s1 and C600Tc^s2, following restriction endonuclease digestion of plasmid DNA. Since it was first isolated, several reports have described different plasmid contents and associated characters for strain B41.^{26,27} In the present study, the STaI gene was localised to different plasmid *Eco*RI fragments in strains B41 and B41* (8.3 and 4.9 kb, respectively), but to the same *Bam*HI (6.8-kb) and *Eco*RI + *Bam*HI (3.5-kb) fragments. When considered with the data published previously, this provides further evidence for rearrangements of plasmid DNA, perhaps associated with the presence of a transposon. The STaI probe hybridised to a 4.6-kb *Bam*HI plasmid fragment from strain B41*Tc^s1 (an STa⁻ derivative of strain B41*) and the corresponding C600 transconjugant, thereby indicating the presence of at least a portion of the STaI gene, but did not hybridise with the other STa⁻ derivative, B41*Tc^s2, and its corresponding C600 transconjugant.

Previous studies have used DNA probes to identify the presence of the STaI gene in colony hybridisation experiments. Sommerfelt *et al.*²⁸ found that the digoxigenin system, as used in the present study, was suitable for colony hybridisation experiments with the addition of an extra blocking step with pre-hybridisation solution. This step was necessary to avoid a high background signal following hybridisation. In the present study, it was shown that this additional step could be omitted if hybridisation experiments were performed with Southern blots of crude plasmid DNA extracts. Higher probe concentrations could be used than those used by Sommerfelt *et al.*²⁸ (2.4 ng/ml), and the hybridisation mixture could be re-used several times without loss of sensitivity.

Overall, the study demonstrated that, although it was not always easy to associate particular phenotypic characters with the presence of a particular plasmid, a non-radioactively labelled polynucleotide probe was an effective means of characterising the location of the STaI gene on plasmid DNA isolated from ETEC strains and their derivatives.

Part of this work was presented at a meeting of the Société Française de Microbiologie held at the Institut Pasteur, Paris, France on 14 and 15 May 1990. I am grateful to Dr S. L. Moseley for providing strain G157 and to P. Menanteau for her participation in the characterisation of bacterial strains.

References

1. Gaastra W, De Graaf FK. Host-specific fimbrial adhesins of noninvasive enterotoxigenic *Escherichia coli* strains. *Microbiol Rev* 1982; **46**: 129–161.
2. Klemm P. Fimbrial adhesins of *Escherichia coli*. *Rev Infect Dis* 1985; **7**: 321–340.
3. De Graaf FK, Mooi FR. The fimbrial adhesins of *Escherichia coli*. *Adv Microb Physiol* 1986; **28**: 65–143.
4. Schifferli DM, Beachey EH, Taylor RK. The 987P fimbrial gene cluster of enterotoxigenic *Escherichia coli* is plasmid encoded. *Infect Immun* 1990; **58**: 149–156.
5. Thompson MR. *Escherichia coli* heat-stable enterotoxins and their receptors. *Pathol Immunopathol Res* 1987; **6**: 103–116.
6. Gyles CL, So M, Falkow S. The enterotoxin plasmids of *Escherichia coli*. *J Infect Dis* 1974; **130**: 40–49.
7. Elwell LP, Shipley PL. Plasmid-mediated factors associated with virulence of bacteria to animals. *Annu Rev Microbiol* 1980; **34**: 465–496.
8. Smith HR, Scotland SM, Rowe B. Genetics of *Escherichia coli* virulence. In: Sussman M (ed) *The virulence of Escherichia coli*. London, Academic Press. 1985: 228–269.
9. Bertin A. Virulence factors of enterotoxigenic *Escherichia coli* studied in the infant mouse model. *Ann Rech Vet* 1983; **14**: 169–182.
10. Duchet-Suchaux MF, Bertin AM, Menanteau PS. Susceptibility of Chinese Meishan and European Large White pigs to enterotoxigenic *Escherichia coli* strains bearing colonization factor K88, 987P, K99, or F41. *Am J Vet Res* 1991; **52**: 40–44.
11. Smith HW. The bacteriology of the alimentary tract of domestic animals suffering from *Escherichia coli* infection. *Ann NY Acad Sci* 1971; **176**: 110–125.
12. Bertin A. F41 antigen as a virulence factor in the infant mouse model of *Escherichia coli* diarrhoea. *J Gen Microbiol* 1985; **131**: 3037–3045.
13. Duchet-Suchaux M. Le souriceau, modèle d'étude de la diarrhée colibacillaire. *Ann Microbiol (Inst Pasteur)* 1980; **131B**: 239–250.
14. Duchet-Suchaux M, Le Maitre C, Bertin A. Differences in susceptibility of inbred and outbred infant mice to enterotoxigenic *Escherichia coli* of bovine, porcine and human origin. *J Med Microbiol* 1990; **31**: 185–190.
15. Duchet-Suchaux M. Protective antigens against enterotoxigenic *Escherichia coli* O101: K99, F41 in the infant mouse diarrhoea model. *Infect Immun* 1988; **56**: 1364–1370.
16. Fredericq P. Actions antibiotiques réciproques chez les enterobacteriaceae. *Rev Belge Pathol Med Exp* 1948; **19** Suppl IV: 1–107.
17. Dean AG, Ching YC, Williams RG, Harden LB. Test for *Escherichia coli* enterotoxin using infant mice: application in a study of diarrhea in children in Honolulu. *J Infect Dis* 1972; **125**: 407–411.
18. Kado CI, Liu S-T. Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol* 1981; **145**: 1365–1373.
19. Davis LG, Dibner MD, Battey JF. Basic methods in molecular biology. Amsterdam, Elsevier Science Publishing Co. 1986.
20. Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 1979; **7**: 1513–1523.
21. Holmes DS, Quigley M. A rapid boiling method for the preparation of bacterial plasmids. *Anal Biochem* 1981; **114**: 193–197.
22. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning. A laboratory manual. New York, Cold Spring Harbor Laboratory. 1982.
23. Bialoskova-Hobrzanska H. Detection of enterotoxigenic *Escherichia coli* by dot blot hybridization with biotinylated DNA probes. *J Clin Microbiol* 1987; **25**: 338–341.
24. Casey TA, Moon HW. Genetic characterization and virulence

- of enterotoxigenic *Escherichia coli* mutants which have lost virulence gene *in vivo*. *Infect Immun* 1990; **58**: 4156–4158.
25. So M, Heffron F, McCarty BJ. The *E. coli* gene encoding heat stable toxin is a bacterial transposon flanked by inverted repeats of IS1. *Nature* 1979; **277**: 453–456.
26. Meyers JA, Sanchez D, Elwell LP, Falkow S. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. *J Bacteriol* 1976; **127**: 1529–1537.
27. So M, Boyer HW, Betlach M, Falkow S. Molecular cloning of an *Escherichia coli* plasmid determinant that encodes for the production of heat-stable enterotoxin. *J Bacteriol* 1976; **128**: 463–472.
28. Sommerfelt H, Grewal HMS, Bhan MK. Simplified and accurate nonradioactive polynucleotide gene probe assay for identification of *Escherichia coli*. *J Clin Microbiol* 1990; **28**: 49–54.