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Identification of colonization factors of enterotoxigenic *Escherichia coli* with PCR-based technique

O. PUIPROM[1], S. CHANTAROJ[2], W. GANGNONNGIW[1], K. OKADA[1], T. HONDA[3], T. TANIGUCHI[1]* AND P. SAWANPANYALERT[2]

[1] *Section of Bacterial Infections, Thailand–Japan Research Collaboration Center on Emerging and Re-emerging Infections* (*RCC-ERI*), *National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand*
[2] *National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand*
[3] *Department of Bacterial Infections, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*

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SUMMARY

Colonization factors (CFs) mediate attachment of enterotoxigenic *Escherichia coli* (ETEC) to the intestinal mucosa and induce protective immunity against ETEC diarrhoea. We designed CF-specific polymerase chain reaction (PCR) primers, and developed a simple PCR-based genotypic CF identification method. ETEC strains ($n=17$) isolated from patients with diarrhoea in Thailand were examined for genotypical identification of CFs of ETEC strains. Coli surface antigen 6 (CS6) was the most common CF (29%), followed by CS13 (12%), colonization factor antigen I (CFA/I), CS2 and CS3, and CS17/CS19 (6% each), while 41% of the strains were negative. This simple PCR method for the detection of CF genes is useful for surveillance of ETEC infections in diagnostic laboratories.

Key words: Bacterial infections, diarrhoea, enteric bacteria, *Escherichia coli* (*E. coli*), molecular epidemiology.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is one of the major causes of diarrhoea in children and travellers in developing countries [1, 2]. The ability of ETEC to adhere to and colonize the intestinal epithelium is an essential step for pathogenicity in addition to its ability to produce heat-labile enterotoxin (LT) and/or heat-stable enterotoxin (ST). The colonizing ability of human ETEC depends on the presence of colonization factors (CFs) on the surface of the cells, which usually form pili, also known as fimbriae [3, 4]. Several types of colonization factor antigens (CFAs) and putative colonization factors (PCFs) have been identified on the basis of antigenic specificity and/or N-terminal amino-acid sequence of the major subunit (pilin), e.g. CFA/I, CFA/II, CS8 (originally CFA/III), CFA/IV, CS12 (PCFO159), CS13 (PCFO9), CS14 (PCFO166), CS15 (antigen 8786), CS17, CS18 (PCFO20), CS19 and CS20 [5, 6]. Of these, CFA/II and CFA/IV are heterogeneous and consist of a complex of different antigens named coli surface (CS) antigens. CFA/II is composed of CS1, CS2 and CS3, which are present in different permutations. Similarly,

* Author for correspondence: Dr T. Taniguchi, Section of Bacterial Infections, Thailand–Japan Research Collaboration Center on Emerging and Re-emerging Infections (RCC-ERI), National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi 11000, Thailand.
(Email: taniguti@biken.osaka-u.ac.jp)

CFA/IV is composed of CS4, CS5 and CS6. CFA/II-producing ETEC strains express CS3 alone or in combination with CS1 or CS2, while CFA/IV-producing ETEC strains express CS6 alone or together with CS4 or CS5. Although at least over 20 different CFs are known in human ETEC, there is still a substantial proportion of strains on which CFs have not been identified, and additional CFs may thus exist.

CFAs have been proposed as candidates for inclusion in a pilus vaccine against ETEC diarrhoea [7, 8]. In order to formulate such a vaccine, it is important to know the distribution of CFs on ETEC strains in different areas of the world. The identification and typing of CF is determined by serological test with specific monoclonal and/or polyclonal antibodies against each CF. However, such identification and typing method can be used in only a limited number of reference laboratories [9–11]. We therefore designed polymerase chain reaction (PCR) primers specific for each CF for the development of a simple PCR-based genotypic CF identification method. This method was applied to 17 randomly selected clinical isolates of ETEC strains in Thailand.

MATERIALS AND METHODS

Bacterial strains

A total of 17 ETEC strains were originally isolated from stool samples obtained from different patients with diarrhoea in Thailand between May and September 2006. All strains were serogrouped with commercially available antisera (Denka Seiken Co. Ltd, Japan) for specific somatic (O) antigens by an established method, and stored in Luria–Bertani (LB) broth [12] containing 25% glycerol at −80 °C at the Department of Medical Sciences, Ministry of Public Health, Thailand. Immediately before CF analyses, all strains were rechecked for enterotoxin (LT, STh and STp) genes by PCR, since it is well known that enterotoxin genes are usually co-localized with CF genes on virulence plasmids. Strains that did not possess any enterotoxin genes at the time of these analyses were therefore assumed to have lost their CF genes and omitted from this study.

Bacterial culture conditions

ETEC strains were routinely grown on heart infusion agar plates (Difco, USA) or in LB broth at 37 °C for 20 h [12]. For optimal expression of CF, ETEC strains were grown on CFA agar plates at 37 °C for 20 h [13].

Primer design

Nucleotide sequences of different major pilins were aligned to identify unique regions and utilized to design the CF-specific PCR primers (Table 1), except for the CS17/19-F and CS17/19-R primers that might recognize both CS17 and CS19 genes, since these nucleotide sequences are very similar. The specificity of the primers was tested by BLAST search.

DNA extraction and PCR

An overnight broth culture (10 μl) was added to 90 μl of distilled water, boiled for 5 min and centrifuged at 12 000 g for 10 min at 4 °C. The supernatant was used as the DNA template. PCR was performed with a 50-μl reaction mixture containing 1× PCR buffer (Takara Bio Inc., Japan), dNTP mixture (2·5 mM each of dATP, dTTP, dCTP and dGTP; Takara Bio Inc.), 0·5 μM of each primer, 10 μl of DNA template and 1·25 U of TaKaRa Ex Taq™ (Takara Bio Inc.), and the mixture was then subjected to PCR amplification using a PCR Thermal Cycler (Gene Amp PCR system 9700; Applied Biosystems, USA) under the following conditions: 95 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min for 35 cycles, with a final extension at 72 °C for 5 min. PCR products were subsequently subjected to 2% agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light.

Salting-out test (hydrophobicity test)

The salting-out test was performed as described by Honda *et al.* [14]. Bacterial hydrophobicity was determined by observing cell clumping in ammonium sulphate solutions at a range of concentrations (0·25, 0·5, 1·0, 2·0 and 4·0 M). The lower the concentration of ammonium sulphate, the higher the cell-surface hydrophobicity is.

Caco-2 adhesion test

ETEC strains were subjected to a Caco-2 adhesion test with the method described by Taniguchi *et al.*

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Table 1. *PCR primers used in this study*

Virulence factor	Primer	Sequence (5′ to 3′)	Product size (bp)	GenBank accession no.
LT	LT-F	ATGACGGATATGTTTCCACTTCTC	393	S60731
	LT-R	AACCTTGTGGTGCATGATGAATCC		
STh	STh-F	TTCACCTTTCGCTCAGGATGCTA	168	M29255
	STh-R2	CACCCGGTACAAGCAGGATT		
STp	STp-F	TCCCCTCTTTTAGTCAGTCAACTG	176	M58746
	STp-R	TTAATAACATCCAGCACAGGCAGG		
CFA/I	CFAI-F	TTAAACTTGCTGATACACCACAGC	150	M55661
	CFAI-R	CATTTACACCGGATGCAGAATATC		
CS1	CS1-F	TTTTGAAGCTCACACCATCAACAC	201	X62879
	CS1-R	CTCGAAGCAAAGTTCAGATCATTG		
CS2	CS2-F	TGCATATCTTCCAGGAGAGAAAAG	254	Z47800
	CS2-R	TAGTTGCAGAAACTGTCTCTACAC		
CS3	CS3-F	ACTGGCGTTTCTAATACTTTGGTG	289	X16944
	CS3-R	CTAAATGTTCGTTACCTTCAGTGG		
CS4	CS4-F	TAGAATTAACCTATTCACCTGCGG	326	AF296132
	CS4-R	AGAAACGACCCCACTATAATTTCC		
CS5	CS5-F	CTGAACAGTTGAATATCACCCTTG	360	AJ224079
	CS5-R	CTGCCTTGGCATTCATATCAATAG		
CS6	CS6-F	AGCGACTAAAAACTTCCCAGTATC	390	U04844
	CS6-R	TAGTAACCAACCATAACCTGATCG		
CS7	CS7-F	ATATACCGTTTACTCCTGGTACTG	431	AY009095
	CS7-R	TACCGGAGCTACAAAGTTAATAGC		
CS8 (CFA/III)	CS8-F	TTCTGGGGATTATCGGAACAATTG	497	D37957
	CS8-R	TGTAGTATTATCAGTAGCAGCCAG		
CS12 (PCFO159)	CS12-F	TATCTTGTAAACCAGTCAGGTGAC	154	AY009096
	CS12-R	ACGGAATAACCTGATCAGGTAAAG		
CS13 (PCFO9)	CS13-F	AGGTGGTGCTGTAAATAGTGTTTC	182	X71971
	CS13-R	TTTTCCCAGCACTAATACCTGAAG		
CS14 (PCFO166)	CS14-F	CAGTGTTCTAACCCAGATTTACAC	219	AY283611
	CS14-R	TCTTTACTATTCGAAACACCTGCC		
CS15 (8786)	CS15-F	GGTCTATGTTTCAGGAGATGATAC	250	X64623
	CS15-R	GAATGTTGCTGTATATTCTCCAGC		
CS17/CS19	CS17/19-F	TATCGCATTAACCTATTCTTCGGC	289	AY515609
	CS17/19-R	TTGTGTGTCTGCATGAATCGTAAG		AY288101
CS18 (PCFO20)	CS18-F	ATATTGCTTATGTGCTTGCGGATG	331	U31413
	CS18-R	GTTGCAGAGGCTTTAATAGTCAAC		
CS20	CS20-F	CCTTTGCCAGGTAAAAACAGATG	439	AF438155
	CS20-R	ACGAATGGTCAAAACACCAGTTG		
CS21 (Longus)	CS21-F	TCTTGGCATTATCGGTACGATTG	474	AF004308
	CS21-R	ACAGACATATCTACACCAGTTGC		
CS22	CS22-F	TAGTAGGTGATGTTGCCACTGTT	407	AF145205
	CS22-R	TGTTGCTGTATATGTTCCAGCAG		

[15]. The adhesion indices were presented as the percentage of Caco-2 cells with at least one adhering bacterium (index 1) and the average number of bacteria/cell (index 2) by counting 10 randomly chosen fields in three separate experiments.

RESULTS AND DISCUSSION

This study focused on the examination of 17 randomly selected clinical isolates of ETEC in a collection of *E. coli* strains kept at the Department of

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Table 2. *Characteristics of ETEC strains isolated from patients with diarrhoea in Thailand*

Strain no.	O group	PCR		Hydrophobicity (M)	Caco-2 cells	
		Toxin	CF		Index 1 (%)	Index 2
EC18/49	O1	LTh	—	n.a.*	89·8	86·8
EC19/49	Untype	LTh	—	n.a.*	72·6	16·7
EC60/49	Untype	LTh	CS13	0·5	31·3	0·8
EC428/49	O126	STh	CFA/I	0·25	72·8	18·6
EC711/49	Untype	LTh	CS17/CS19†	0·25	63·2	8·1
EC859/49	O159	STp	CS6	n.a.*	84·3	48·4
EC860/49	O159	STp	CS6	n.a.*	88·7	42·3
EC861/49	O159	STp	CS6	n.a.*	81·4	38·8
EC909/49	O8	LTh	—	n.a.*	64·2	2·6
EC910/49	O8	LTh	—	n.a.*	58·8	1·5
EMEC21-1/49	O6	STh	CS2, CS3	0·25	38·6	0·9
EMEC43/49	Untype	LTh	—	n.a.*	77·9	3·7
EMEC54/49	Untype	LTh	CS13	0·5	37·3	1·6
EMEC107-2/49	Untype	STh	—	0·25	58·4	1·2
EMEC115/49	Untype	STh	—	0·25	51·9	2·4
EMEC134/49	O148	STp	CS6	n.a.*	89·5	44·8
EMEC140-1/49	O159	STp	CS6	n.a.*	82·3	31·4

* Not agglutinated in 4·0 M ammonium sulphate solution.
† PCR cannot distinguish between CS17 and CS19.

Medical Sciences, Ministry of Public Health, Thailand. The characteristics of these 17 ETEC strains are summarized in Table 2.

PCR of toxin genes

The ETEC strains were rechecked for enterotoxin (LT, STh, STp) genes by PCR, which resulted in the detection of the genes encoding LT (eight strains, 47%) or STp (five strains, 29%) or STh (4 strains, 24%). No positive strain was detected which possessed genes encoding both LT and ST (STh or STp) (Table 2).

PCR of CF genes

Figure 1 shows the results of agarose gel electrophoresis of PCR-amplified products for detection of the CS6 gene. ETEC EC859/49, ETEC EC860/49, ETEC EC861/49, ETEC EMEC 134/49 and EMEC 140-1/49 yielded specific PCR-amplified products with the expected size (390 bp) for the CS6 gene (Fig. 1, lanes 6–8, 16, 17). Since some non-specific PCR-amplified products were observed in the lanes of CS6-negative strains, we attempted to optimize the PCR conditions for the examination of several important parameters such as annealing temperature, primer concentrations and magnesium ion concentration. Unfortunately, the findings did not improve significantly. Of the 17 ETEC strains, five were CS6-gene positive (29%), followed by CS13-gene positive (12%), CFA/I-, CS2 and CS3- and CS17/CS19-gene positive (6% each), while seven strains (41%) were

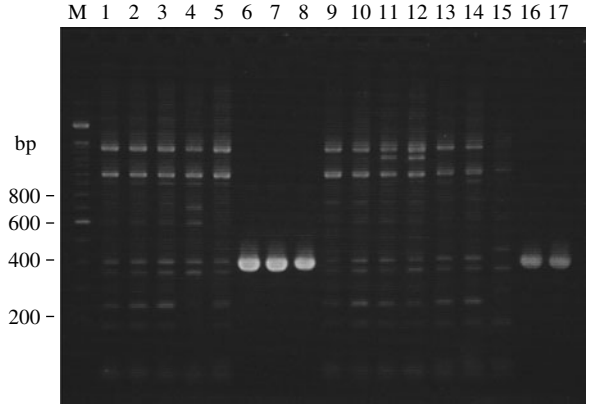

Fig. 1. Agarose gel electrophoresis showing PCR-amplified products for detection of CS6 gene in clinical isolates of ETEC strains. CS6-gene primers CS6-F and CS6-R were used. Lane M, 100-bp DNA ladder marker (Invitrogen, USA); lanes 1–17, clinical isolates of ETEC strains correspond to those listed in Table 2.

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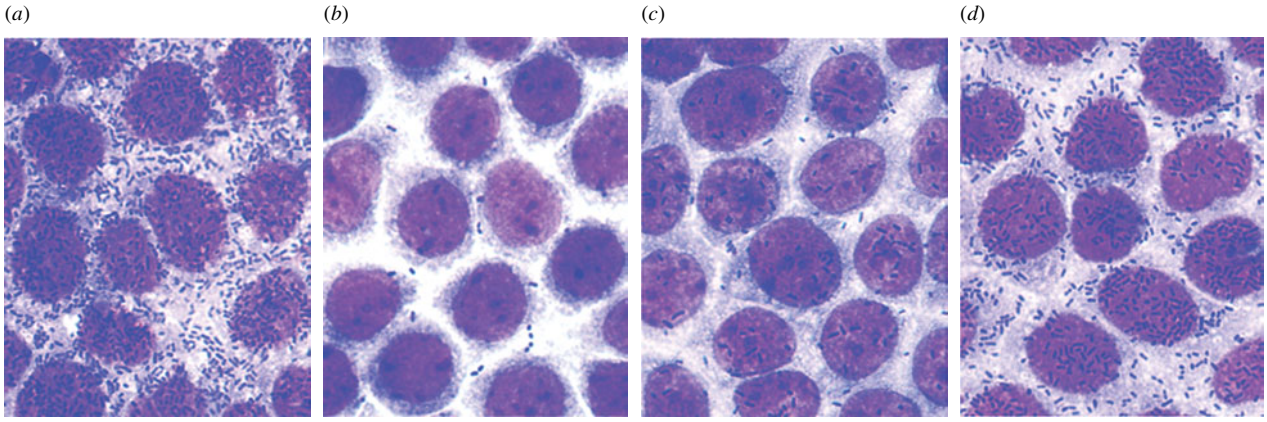

Fig. 2. Micrographs showing adhesion of ETEC strains to Caco-2 cells. (*a*) ETEC EC18/49 (CF-gene negative strain); (*b*) ETEC EC60/49 (CS13-gene positive strain); (*c*) ETEC EC428/49 (CFA/I-gene positive strain); (*d*) ETEC EC859/49 (CS6-gene positive strain).

negative for other CF genes (Table 2). Four of the five CS6-gene positive strains, each isolated from a different patient, were O159 and STp-gene positive. We intend to analyse these strains in our laboratory for any epidemiological clonal relationships. Two CS13 and LTh-gene positive strains will also be investigated.

Previous surveillance of ETEC strains in different areas of the world showed wide variations in CFs [16, 17]. In the present study, diarrhoea due to CS6-producing ETEC is now recognized as one of the most common and important ETEC infections (25–35%) in the world [9–11]. Our data for the strains in Thailand, obtained between May and September 2006, are consistent with these findings in that 29% of the cases may be due to this phenotype.

Salting-out test (hydrophobicity test)

Of the 17 ETEC strains, seven (41%) agglutinated at a relatively low concentration (<0·5 M) of ammonium sulphate, indicating a high degree of cell-surface hydrophobicity (Table 2). CFA/I-, CS2-, CS13- and CS17/CS19-gene positive strains corresponded to highly hydrophobic cells, which are morphologically pilus structures known as rigid rod-shaped pili or flexible pili, whereas the two hydrophobic strains (EMEC 107-2/49 and EMEC 155/49) could not amplify CF genes as determined by PCR. None of the CS6-gene positive strains agglutinated in 4·0 M ammonium sulphate, indicating a lower degree of cell-surface hydrophobicity, which is consistent with the fact that CS6 is known as a non-pilus structure [18].

Caco-2 adhesion test

ETEC strains were tested for the ability to adhere to the Caco-2 cells, an established cell culture model for ETEC colonization. For example, the ability of ETEC EC18/49 (CF-gene negative strain), ETEC EC60/49 (CS13-gene positive strain), ETEC EC428/49 (CFA/I-gene positive strain) and ETEC EC859/49 (CS6-gene positive strain) to adhere to the Caco-2 cells is demonstrated in Figure 2. The ETEC strains adhered to the Caco-2 cells with indices (index 1) of 89·8, 31·3, 72·8 and 84·3%, respectively, and with an average number of bacteria/cell (index 2) of 86·8, 0·8, 18·6 and 48·4, respectively. Adherence indices of CS6-gene positive strains were higher than those of other CF-gene positive strains (Table 2). Interestingly, the Caco-2 adhesion test also revealed that ETEC EC18/49 was negative for any CF genes and non-hydrophobic strains in the salting-out test, but adhered to the Caco-2 cells, suggesting that ETEC EC18/49 may posses an as yet unknown adherence factor. We intend to investigate this further.

Several phenotypic and genotypic identification methods have been developed for the identification of CFs [9–11, 16, 17]. The bacterial agglutination assay using anti-CF antibodies can be performed quickly, but it requires specific antibodies. Genetic identification methods such as DNA hybridization can also be used, but they require enzyme-labelling of the probes. Recently, and independently from our study, Rodas *et al*. described the development of multiplex PCR assays for identification of 19 ETEC CFs, and concluded that the PCR-based identification method was superior to the phenotypic colony dot-blot assay

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using anti-CF antibodies, since false-negative results were found in the latter. [19]. In view of their report and our present study, the PCR assay is a simple and effective method with several advantages compared to other methods for CF detection, we therefore recommend this system for elucidating the epidemiology of ETEC infection, and for aiding the development of an ideal CF vaccine against ETEC diarrhoea.

In summary, CS6-producing ETEC is the most prevalent strain of ETEC in Thailand, which is similar to the situation reported in various developing countries [9–11]. Most CS6-producing ETEC strains express ST (LT/ST or only ST), but immunity to ST is difficult to stimulate. CS6 should therefore be considered as a target for any ETEC vaccine development.

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DECLARATION OF INTEREST

None.

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