IMMUNE RESPONSE IN DIARRHEAL PATIENTS AND ASYMPTOMATIC CARRIER WITH CS6-PRODUCING ENTEROTOXIGENIC *ESCHERICHIA COLI* INFECTION

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Abstract. Enterotoxigenic *Escherichia coli* (ETEC) is one of the major causes of diarrhea in children and travelers in developing countries. ETEC colonization factors (CFs) are virulence determinants considered as protective antigens and major targets for vaccine development against ETEC infections. One of the most prevalent CFs, coli surface antigen 6 (CS6), a non-fimbrial polymeric protein consisting of two major subunits, CssA and CssB, is produced by approximately 25-35% of ETEC worldwide. We could isolate only CS6-producing ETEC strains from two diarrheal patients and one asymptomatic carrier, but we could not detect CssA- or CssB-specific antibodies in the feces and blood of two patients convalescing from natural ETEC infection and of an asymptomatic carrier using western blotting. Therefore, in order to protect against infection with CS6-producing ETEC, protective levels of CS6 immunity should be incorporated in any future vaccines against ETEC.

Keywords: ETEC, colonization factor, CS6, immune response

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INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is a leading cause of diarrhea in travelers to endemic areas and in infants living in developing countries, accounting for approximately 200 million episodes of diarrhea and approximately 380,000 pediatric deaths annually (WHO, 2012). ETEC is transmitted via contaminated food and beverages. ETEC adheres to and colonizes the intestinal epithelium with colonization factors (CFs) and expresses heat-labile enterotoxin (LT) and/or heat-stable

enterotoxin (ST) (Nataro and Kaper, 1998; Kaper *et al*, 2004). These enterotoxins then attach to specific intestinal gut receptors, resulting in aberrations in fluid homeostasis mechanisms of epithelial cells (Nataro and Kaper, 1998; Kaper *et al*, 2004).

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), such as 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 (Gaastra and Svennerholm, 1996; Torres *et al*, 2005). Among 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, 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.

Diarrhea due to CS6-expressing ETEC strains is now recognized as one of the most common ETEC infections, and recent data show that approximately 25-35% of cases may be due to ETEC strains possessing this phenotype (Jiang *et al*, 2002; Shaheen *et al*, 2004; Al-Gallas *et al*, 2007; Puiprom *et al*, 2010). CS6 is a non-pilus polymeric protein and consists of two major subunits, CssA and CssB (Nicklasson *et al*, 2008; Tobias *et al*, 2008). Based on this information, CS6 has been selected as a target antigen for vaccine development, although little is known about the capacity of CS6 to induce immune responses in humans (Helander *et al*, 1998; Qadri *et al*, 2007). It is not clear if natural infec-

tion with CS6 may protect against further infection with CS6-producing ETEC, as detailed studies of the immune response to CS6 have not been carried out in diarrheal patients.

We therefore determined the immune responses to CS6, in particular CssA and CssB, in diarrheal patients and in an asymptomatic carrier with CS6-producing ETEC.

MATERIALS AND METHODS

Bacterial strains

Four ETEC strains were originally isolated from stool samples obtained from two Japanese diarrheal patients (three strains) and one Japanese asymptomatic carrier (one strain) in Thailand in 2008. Diarrheal patient #1 had been infected sequentially with ETEC RCC1 and ETEC RCC3 within half a year (Table 1). All strains were serogrouped with commercially available antisera (Denka Seiken, Tokyo, Japan) for specific somatic (O) antigens and stored in Luria-Bertani (LB) broth (Sambrook and Russell, 2001) containing 25% glycerol at -80ºC. ETEC H10407 was used as CFA/I-positive control strain and ETEC H10407P as CFA/I-negative strain.

Bacterial culture conditions

ETEC strains were routinely grown on heart infusion agar plates (Difco Laboratories, Detroit, MI) or in LB broth at 37ºC for 20 hours (Sambrook and Russell, 2001). For optimal expression of CF, ETEC strains were grown on CFA agar plates at 37ºC for 20 hours (Evans *et al*, 1977).

Detection of enterotoxin and CF genes

Enterotoxin (LT, STh and STp) and CF genes were detected using polymerase chain reaction (PCR) as previously described (Puiprom *et al*, 2010).

Salting-out (hydrophobicity) test

ETEC strains on CFA agar plates were subjected to a salting-out test as previously described (Honda *et al*, 1983). In brief, bacterial hydrophobicity was determined by observing cell clumping in a range of ammonium sulphate concentrations (0.25, 0.5, 1.0, 2.0 and 4.0 M). The lower the concentration of ammonium sulphate needed to induce cell clumping, the higher is the cell-surface hydrophobicity.

Caco-2 adhesion test

ETEC strains on CFA agar plates were subjected to a Caco-2 adhesion test as previously described (Taniguchi *et al*, 2001). The adhesion indices are reported as percent Caco-2 cells with at least one adhering bacterium (index 1) and the average number of bacteria per cell (index 2) by counting 10 randomly chosen fields in three separate experiments.

Serum collection and fecal extraction

Within 10 days after recovery from diarrheal illness, venous blood samples (10 ml) were collected from the three subjects, and then sera were prepared and stored at -20°C until use. Informed consent was obtained from the subjects, in accordance with Osaka University guidelines for human experimentation. Fecal samples were extracted as previously described (Gaspari *et al*, 1988). In brief, 20 ml of watery stools were filtered through gauze and added 0.2 ml of Halt^{TM} protease inhibiter cocktail (Thermo Fisher Scientific, Rockford, IL). In the case of solid stools, 20 g of stools were added into 20 ml of phosphate-buffered saline (PBS) containing 0.2 ml of Halt^{TM} protease inhibiter cocktail, vortexed intensely with glass beads. All samples were then centrifuged at 8,000g for 10 minutes at 4°C, and the supernatants were stored in aliquots at -20°C until use.

Bacterial agglutination test

ETEC strains grown on CFA agar plates were suspended in PBS and 10 l aliquots of the bacterial suspension (10^8 bacteria/ml) were mixed with 10 l of serum or fecal extract on a glass slide. The mixtures were gently rotated for 2 minutes, and bacterial agglutination was observed visually (Honda *et al*, 1989).

Preparation of heat extracts

ETEC strains grown on CFA agar plates were suspended in PBS, incubated at 60°C for 20 minutes and then centrifuged at 8,000g for 20 minutes at 4°C. Supernatants were used as heat extracts (Wolf *et al*, 1989).

SDS-PAGE and western blotting

Whole-cell lysates and heat extracts were denatured by boiling for 5 minutes in a running buffer containing 2% sodium dodecyl sulphate (SDS), 1% 2-mercaptoethanol and 50 mM Tris-HCl (pH 7.5). Proteins were separated by 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to Immobilon^{TM}-P membranes (Millipore, Billerica, MA) using a semidry blotting apparatus (Oriental Instruments, Tokyo, Japan). Membranes were incubated for 1 hour in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and 5% skim milk. Membranes then were incubated for 1 hour with a 1:100 dilution of serum or a 1:4 dilution of fecal extracts in TBS-T, washed with TBS-T, and incubated for a further 1 hour with a 1:1,000 dilution of peroxidase-conjugated goat anti-human immunoglobulin (Ig) G or IgA antibodies (Cappel Laboratories, Cochranville, PA) in TBS-T containing 5% skim milk. Following another wash with TBS-T, protein bands were visualized using 4-chloro-1-naphthol (Sambrook and Russell, 2001).

Table 1
Characteristics of ETEC strains isolated from three diarrheal patients and an asymptomatic carrier.

Strain no.	Source	O group	PCR-based detection		Hydrophobicity	Caco-2 cell adhesion	
			Toxin	CF		Index 1 (%)	Index 2
RCC1	Diarrheal patient #1 [a]	O169	STp	CS6	n.a.[b]	85.2	43
RCC3	Diarrheal patient #1 [a]	O128	STh	CFA/I	0.25	75.6	15
RCC8	Diarrheal patient #2	O169	STp	CS6	n.a.[b]	88.4	50
V14	Asymptomatic carrier	O25	LT	CS6	n.a.[b]	83.8	45

[a] This patient was infected sequentially with RCC1 and RCC3 within half a year.
[b] Not agglutinated in 4.0 M ammonium sulfate solution.
Index 1, percent Caco-2 cells with at least one adhering bacterium.
Index 2, average number of bacteria per cell.

N-terminal amino-acid sequencing

After SDS-PAGE, proteins in the gel were transferred to an Immobilon™-P membrane and stained with Amide black. Stained protein bands were eluted from the membrane and amino acid sequence of the N-terminus was determined using Edman degradation method in an automated protein sequencer (492 Procise®; Applied Biosystems, Foster City, CA).

RESULTS

Characterization of ETEC strains

ETEC RCC1 was STp- and CS6-gene positive strain. ETEC RCC3 was STh- and CFA/I-gene positive. ETEC RCC8 was STp- and CS6-gene positive strain. ETEC V14 was LT- and CS6-gene positive (Table 1). Diarrheal patient #1 was infected with ETEC RCC1 and ETEC RCC3 within a half year.

Salting-out (hydrophobicity) test

The CFA/I-gene positive strain (ETEC RCC3) agglutinated at a relatively low concentration (0.25 M) of ammonium sulphate, indicating a high degree of cell-surface hydrophobicity (Table 1), which morphologically corresponds to a strain with rigid rod-shaped pili (Lüdi *et al*, 2006). None of the CS6-gene positive strains (ETEC RCC1, ETEC RCC8 and ETEC V14) agglutinated in 4.0 M ammonium sulphate, indicating a lower degree of cell-surface hydrophobicity which is consistent with strains having a non-pilus structure.

Caco-2 adhesion test

ETEC strains were tested for their ability to adhere to the Caco-2 cells, an established cell culture model for ETEC colonization (Rousset, 1986). These four ETEC strains (RCC1, 4, 8 and V14) adhered to Caco-2 cells with indices (index 1) of 85.2, 75.6, 88.4 and 83.8%, respectively and with an average number of bacteria per cell (index 2) of 43, 15, 50 and 45, respectively (Table 1 and Fig 1). The adherence indices of the CS6-gene positive strains were thus higher than those of the CFA/I-gene positive strain, consistent with a previous report of CS6-gene positive ETEC strains being more
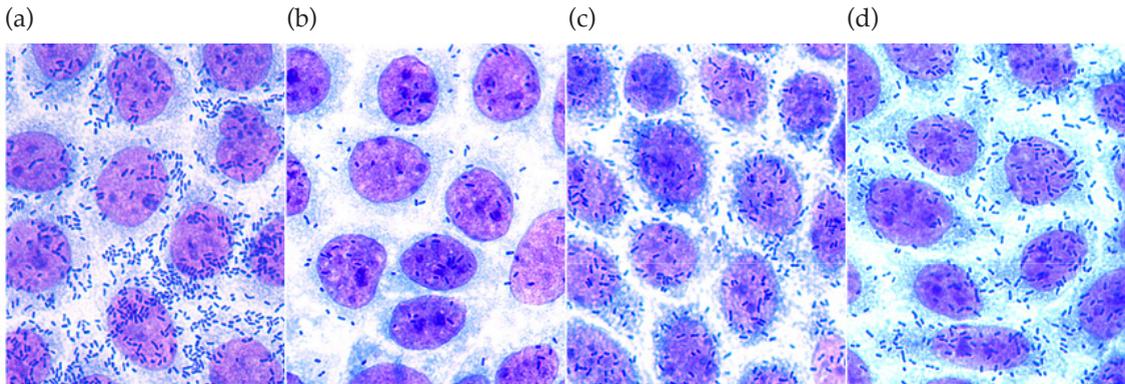
(a) (b) (c) (d)

Fig 1–Micrographs showing adhesion of ETEC strains to Caco-2 cells. (a) ETEC RCC1 (CS6-gene positive strain); (b) ETEC RCC3 (CFA/I-gene positive strain); (c) ETEC RCC8 (CS6-gene positive strain); (d) ETEC V14 (CS6-gene positive strain).

able to adhere to Caco-2 cells than other CF-gene positive ETEC strains (Puiprom *et al*, 2010).

Bacterial agglutination test

Serum 2 prepared from diarrheal patient #1 after infection with ETEC RCC3 agglutinated both ETEC RCC3 and ETEC H10407 (CFA/I-positive control strain), but not ETEC H10407P (CFA/I-negative strain derived from ETEC H10407) (Fig 2). Moreover, ETEC RCC3 and ETEC H10407 grown at 20ºC were not agglutinated with serum 2 (data not shown), indicating that this agglutination was indeed mediated by CFA/I, which is not expressed on the bacteria surface at low temperature (Halvorsen *et al*, 1997). On the other hand, CS6-gene positive ETEC strains were not agglutinated with any of the sera and as expected, none of the ETEC strains were agglutinated with serum 5 obtained from a healthy control.

SDS-PAGE and western blotting

The protein of 15.0 kDa (by SDS-PAGE) from ETEC RCC3 was identified as being CfaB (a major subunit of CFA/I) by Edman N-terminal amino acid sequencing

(data not shown) (Fig 3). ETEC RCC1 and ETEC V14 expressed 14.8- and 14.5-kDa protein, respectively, which was by N-terminal amino acid sequencing identified as being CssA and CssB (major subunits of CS6), respectively (data not shown). Western blotting corroborated the results of the bacterial agglutination test and this was utilized to verify the production of specific antibodies against major subunits, namely CssA and CssB, in the sera from the diarrheal patients and the asymptomatic carrier. Western blotting of whole cell lysates showed that serum 2 from diarrheal patient #1 following infection with CFA/I-gene positive ETEC RCC3 reacted with the 15.0-kDa CfaB and a number of other proteins (Fig 3, lane 2), and these bands were more numerous and intensely stained than those from CS6-gene positive ETEC RCC1 and ETEC V14 (Fig 3, lanes 1 and 3, respectively). A number of proteins in whole-cell lysates of CS6-gene positive ETEC RCC1 were recognized with serum 1 and serum 3 from diarrheal patients, but not with serum 4 from the asymptomatic carrier. Western blotting, failed to detect in sera specific antibodies against CssA and
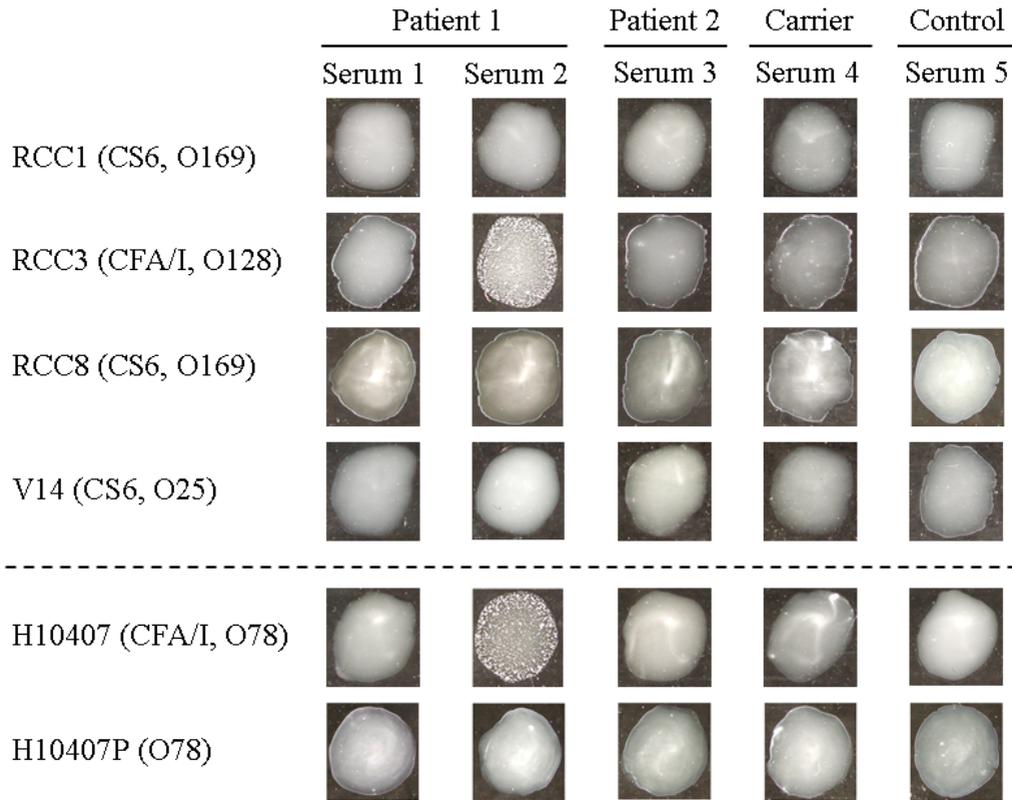
Fig 2–Bacterial agglutination test of ETEC strains with sera from two diarrheal patients and an asymptomatic carrier. Serum 1 and serum 2 were obtained from diarrheal patient #1 following infection with ETEC RCC1 and with ETEC RCC3, respectively; serum 3 was obtained from diarrheal patient #2 following infection with ETEC RCC8; serum 4 and serum 5 obtained from an asymptomatic carrier with ETEC V14 and a healthy volunteer without any pathogenic bacteria, respectively.

CssB. Unfortunately, we could not detect any bands by western blotting using fecal extracts (data not shown).

DISCUSSION

This study involved an examination of four ETEC strains isolated from stool samples obtained from two Japanese diarrheal patients (three strains) and one Japanese asymptomatic carrier (one strain) in Thailand in 2008. Natural immunity appears to protect against ETEC infection. In developing countries, the incidence of ETEC diarrhea decreases with age, and for travelers the impact of diarrhea diminishes in proportion to the length of stay in an endemic area (DuPont *et al*, 1976; Black *et al*, 1981). Challenge with an ETEC strain was found to offer protection against rechallenge with the same strain (Levine *et al*, 1979; Evans *et al*, 1988). CS6-producing ETEC is now the most prevalent ETEC strain in various regions of the world (Jiang *et al*, 2002; Shaheen *et al*, 2004; Al-Gallas *et al*, 2007; Puiprom *et al*, 2010), but little is known about the natural immune responses
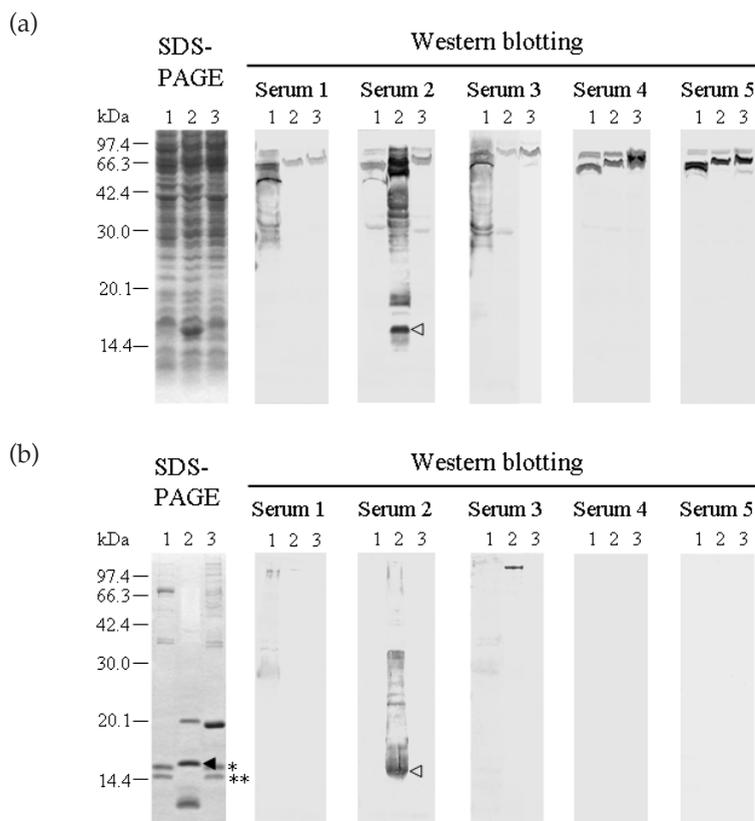
Fig 3–Western blotting of whole-cell lysates (a) and heat extracts (b) with sera from two diarrheal patients and an asymptomatic carrier. Lane 1, ETEC RCC1 (CS6-gene positive strain); lane 2, ETEC RCC3 (CFA/I-gene positive strain); lane 3, ETEC V14 (CS6-gene positive strain). The sources of sera used are described in the legend to Fig 2. Dark arrowhead, single asterisk and double asterisks indicate CfaB (major subunit of CFA/I), CssA and CssB (major subunits of CS6), respectively, which were identified by Edman N-terminal amino-acid sequencing. Empty arrowheads indicate the expected position of CfaB (15.0 kDa) as determined using anti-CFA/I antiserum. Molecular size markers are shown on the left.

against CS6-producing ETEC in humans (Helander *et al*, 1998; Qadri *et al*, 2007), and it is not clear whether anti-CS6 responses can protect against reinfection with CS6-producing ETEC, as no detailed studies of immune response to the major subunits of CS6 (CssA and CssB) have been carried out in ETEC-infected patients. Our study provided evidence that infection with a CS6-producing ETEC did not induce immune responses to

major subunits of CS6, so it seems likely that only vaccination with an adjuvant is needed in order to induce immunity to major subunits of CS6 in human. Most CS6-producing ETEC strains express ST (LT/ST or only ST), but ST is a non-immunogenic low-molecular-mass peptide (2 kDa). We therefore propose that major subunits of CS6 should be considered as vaccine compounds for any ETEC vaccine development.

In summary, we have shown that a patient infected with rigid rod-shaped pilus CFA/I-producing ETEC raised a significant antibody response to a CFA/I-major subunit, CfaB, and that the non-pilus polymeric protein CS6 had low antigenicity in humans. Although CS6 is now considered a candidate for a vaccine against ETEC infection, additional studies are needed to establish a viable strategy to induce an immune response to CS6, which is capable of preventing diarrhea and eliminate carrier status.

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