SHORT COMMUNICATION

Detection of the CS20 colonization factor antigen in diffuse-adhering *Escherichia coli* strains

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Abstract

We analyzed a randomly selected group of 30 diffusely adherent (DAEC), 30 enteropathogenic, 30 enteroaggregative, and five Shiga toxin-producing *Escherichia coli* strains isolated from children with diarrhea. Enterotoxigenic *E. coli* (ETEC) colonization factors (CFs) were evaluated by a dot-blot assay using 21 CF-specific monoclonal antibodies. Out of 95 non-ETEC strains, three DAEC were found to express coli surface antigen 20 (CS20). No other *E. coli* expressed CFs. We confirmed the three CS20-positive strains as ETEC-negative by repeat PCR and as toxin-negative by ganglioside-GM1-enzyme-linked immunosorbent assay. To our knowledge, this is the first study that has identified currently recognized CFs in non-ETEC diarrheagenic *E. coli* strains identified using molecular methods. CFs may be an unrecognized relevant adherence factor in other *E. coli*, which may then play a role in pathogenesis and the immune response of the host.

Enterotoxigenic *Escherichia coli* (ETEC) is one of the main causes of pediatric diarrhea in developing countries and in travelers' diarrhea. The main virulence factors of ETEC are the heat-stable (ST) and heat-labile (LT) enterotoxins, and the colonization factor antigens (CFs or CFAs) (Qadri *et al.*, 2005; Walker *et al.*, 2007; Svennerholm & Tobias, 2008). CFs are antigenic fimbriae that enable bacteria to adhere to and colonize host intestinal mucosa. The 25 CFs characterized in ETEC (Walker *et al.*, 2007) have been infrequently described in other diarrheagenic *E. coli*. The aim of this study was to determine the presence of CFs in non-ETEC diarrheagenic *E. coli* strains.

We analyzed a randomly selected group of 30 out of 36 diffusely adherent *E. coli* (DAEC), 30 out of 56 enteropathogenic *E. coli* (EPEC), 30 out of 111 enteroaggregative *E. coli* (EAEC), and five out of five Shiga toxin-producing *E. coli* (STEC) isolated previously during a passive surveillance diarrhea study of 1034 children under 12 months of age in Lima, Peru (Ochoa *et al.*, 2009). For sampling, we prepared a list of all positive strains by category, assigned them a correlative number, and performed a selection of random numbers using Microsoft Excel (simple random sampling). In the case of STEC, we included all five of the isolated strains, due to the low number of isolates. Five *E. coli* colonies per patient were analyzed by a multiplex real-time PCR method using previously validated specific primers for each pathotype: EAEC (*agg*R), ETEC (*lt*, *st*), EPEC (*eaeA*), STEC (*stx1*, *stx2*), DAEC (*daa*D), and enteroinvasive *E. coli* (*ipa*H) (Guion *et al.*, 2008).

Two colonies from each strain were tested for CFs by a dot-blot assay using 21 monoclonal antibodies: CFA/I, CS1–CS7, CS8 (CFA/III), CS12 (PCF0159), CS14 (PCF0166), CS17,

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14. ABSTRACT

We analyzed a randomly selected group of 30 diffusely adherent (DAEC), 30 enteropathogenic, 30 cnteroaggregativc, and five Shiga toxin-producing Eschericlria coli st rains isolated from children with diarrhea. Enterotoxigenic E. coli (ETEC) colonization factors (CFs) were evaluated by a dot-blot assay using 21 CFspecific monoclonal antibodies. Out of 95 non-ETEC strains, th ree DAEC were found to express coli surface antigen 20 (CS20). No other E. coli expressed CFs. We confirmed the three CS20-positive stra ins as ETEC-ncgat ive by repeat PCR and as toxin-negative by ganglioside-G M 1-enzyme-linked immunosorbent assay. To our knowledge, th is is the first study that has identified currently recognized CFs in non-ETEC diarrheagenic E. coli strains identified using molecular methods. CFs may be an unrecognized relevant adherence fac tor in other E. coli, which may then play a role in pathogenesis and the immune response of the host.

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CS18, CS20, CS19, PCF039 (P19A6), PCF071 (P8C1), Ag150 (P1F2), Fim4264 (P3H5), Fim4089 (P5H10), and Fim7162 (P7H5), as described previously (López-Vidal *et al.*, 1990; Viboud *et al.*, 1993; Sjöling *et al.*, 2007). The monoclonal antibodies were provided by Dr A.-M. Svennerholm (University of Gothenburg, Sweden) and Drs S. Farid and H. Shaheen (NAMRU-3, Cairo, Egypt). CF-positive strains were confirmed to lack ETEC-associated toxins by repeat PCR (*lt* and/or *st* genes) (Guion *et al.*, 2008) and by a ganglioside-GM1-enzyme-linked immunosorbent assay (ELISA) (LT) and inhibition GM1-ELISA (ST) (Svennerholm & Wiklund, 1983; Svennerholm *et al.*, 1986; Sjöling *et al.*, 2007). Serotyping was performed for somatic (O) and flagellar (H) antigens using an *E. coli* antisera kit (Denka Seiken, Tokyo, Japan).

Three CF-positive strains were identified in 95 non-ETEC strains (3%). All CFs were coli surface antigen 20 (CS20); the three strains were all DAEC (three of 30 DAEC, 10%). All other *E. coli* were negative for CFs. The presence of CS20 was significantly more frequent in DAEC than in other non-ETEC diarrheagenic *E. coli* (10% vs. 0%, $P < 0.05$). Repeat PCR for *lt* and *st*, the genes encoding for the ETEC-associated toxins LT and ST, was negative, as was a GM1-ELISA specific for LT and ST themselves. All of the CS20-positive DAEC strains showed a diffuse adherence pattern in the HEp-2 cell assay, similar to the other 28 DAEC clinical isolates analyzed in our laboratory. All three CS20-positive DAEC strains were nontypeable by O/H serotyping, which was performed twice using a commercial *E. coli* antisera kit (Denka Seiken). The CS20-positive DAEC strains were from acute diarrheal episodes (mean duration 7 days) in children with a mean age of 4 months. DAEC were the only pathogens isolated from each of these three episodes of illness.

In order to characterize the CS20-positive DAEC strains, we first attempted to demonstrate the transferability of a putative CS20-encoding plasmid to other *E. coli* strains. These conjugal transfer experiments were carried out using *E. coli* J53-2 (*pro*, *met*, Rif^R) as the recipient strain, as described previously (Soufi *et al.*, 2009). Briefly, overnight cultures of donor and recipient strains were grown in Mueller–Hinton broth at 37 °C. These strains were then mixed in a 1 : 10 proportion and incubated at 37 °C for an additional 4 h. The resulting mixture (0.1 mL) was spread onto Mueller–Hinton agar plates containing rifampin (150 mg L^{-1}) plus streptomycin (50 mg L^{-1}). Plates were again incubated at 37 °C for 48 h. Rifampin-resistant transconjugants growing on the selection medium were recovered and subjected to antibiotic susceptibility testing and a dot-blot assay using the CS20 monoclonal antibody to confirm the possible acquisition of plasmid-related genes. Transfer of the resistant phenotype was successful as demonstrated by the presence of novel drug resistance in the transconjugant strain by routine disk-diffusion methods, but we were unable to detect evidence of CS20-like fimbriae by a dot-blot assay in the recipient strains. The CS20-positive DAEC strains were further characterized in Dr Savarino's laboratory at the Enteric Diseases Department at the Naval Medical Research Center, Silver Spring, MD. PCR and sequencing of the whole-cell lysates and of the isolated plasmid preparations were performed using primers for the *csn*A gene, which encodes the major CS20 subunit (CS20-F: CCTTT GCCAGGTAAAAACAGATG and CS20-R: ACGAATGGT CAAAACACCAGTTG) (Puiprom *et al.*, 2010). The strain WS7179A (a CS20-positive sequenced strain) was used as the positive control. Testing of both the whole-cell lysates and isolated plasmid preparations was negative in the DAEC strains, however. The reason for this is unclear. We hypothesize that the CS20-like fimbria detected by the dot-blot assay in these three DAEC strains may not be encoded by a transferable plasmid, as is the case in wild-type ETEC, and may thus be chromosomal. Additionally, the gene encoding this CS20-like molecule may not appear to be directly homologous to *csnA* and thus would not be detected by primers specific for that gene.

Many pathogenic bacteria have the ability to adhere to specific host tissues to colonize. Bacterial adherence is mediated in part by polymeric adhesive fibers termed 'pili' or 'fimbriae,' which facilitate the initial attachment to epithelial cells and subsequent colonization of the host. The CFs are prominent examples of these structures and are most often designated by number as coli surface antigens, with the exception of CFA/I and certain others. These virulence determinants mediate bacterial adherence to intestinal mucosa. Studies of ETEC strains have shown that the most common CFs are CFA/I and combinations of CS1–CS6 (Qadri *et al.*, 2005). Protective immunity to CFs may occur following multiple natural infections, leading many investigators to believe that vaccines against ETEC infections should contain the immunogenic B subunit of the LT and a combination of the most common CFs (Qadri *et al.*, 2005; Walker *et al.*, 2007; Svennerholm & Tobias, 2008). The gene encoding the major subunit of CS20, *csn*A, is located on a plasmid and shows a high degree of affinity to the major fimbrial subunits of CS12 and CS18, as well as to F6 (also referred to as 987P), a CS from a porcine ETEC strain (Valvatne *et al.*, 1996, 2004).

In this study, we found evidence of three DAEC strains expressing CS20-like molecules. DAEC strains are identified on the basis of their diffuse adherence pattern on cultured epithelial cells. One of the adherence factors of DAEC is the surface fimbria (designated F1845) that is responsible for the diffuse adherence phenotype in a prototype strain. These fimbriae are homologous with members of the Afa/Dr family of adhesins (Le Bouguénec & Servin, 2006), which are identified by hybridization to a specific probe, *daaC*, common to operons encoding Afa/Dr adhesions.

To our knowledge, this is the first study that has searched for the most currently recognized CFs in non-ETEC diarrheagenic *E. coli* strains identified using molecular methods.

Earlier studies have looked at CFA/I and CFA/II in non-ETEC *E. coli* strains identified by serology, with variable results (Cravioto *et al.*, 1982; Iyer *et al.*, 1983; Caron *et al.*, 1990).

The presence of CFs in other diarrheagenic *E. coli* has multiple potential implications. CFs may be an unrecognized relevant adherence factor in other *E. coli*, which may then play a role in pathogenesis and the immune response of the host. Candidate vaccines for ETEC based on common CFs and the B subunit of LT could potentially protect against other enteric pathogens expressing CFs. Further studies are needed to evaluate the presence of CFs or CF-like molecules in a larger number of pathogenic *E. coli*.

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