

## SHORT COMMUNICATION

## Detection of Cytotoxic Necrotizing Factor Types 1 and 2 among Fecal *Escherichia coli* Isolates from Brazilian Children with and without Diarrhea

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*The enteropathogenic role of cytotoxic necrotizing factor (CNF)-producing Escherichia coli was investigated by searching cnf genes among 2074 isolates from 200 children with and 200 without acute diarrhea in Brazil. Fourteen (7%) cases versus 10 (5%) control children carried at least one cnf positive isolate (P = 0.50) and most isolates expressed CNF type 1. DNA sequences of virulence factors of extraintestinal pathogenic E. coli (ExPEC) were detected in 78.6% of CNF1-producing isolates. Besides not being associated with human acute diarrhea, the CNF1-producing isolates here identified may represent potential ExPEC transitorily composing the normal intestinal flora.*

Key words: *Escherichia coli* - cytotoxins - cytotoxic necrotizing factor - adhesins

Cytotoxic necrotizing factor (CNF), a toxin firstly detected in extracts of *Escherichia coli* isolates from children with acute diarrhea in Italy, induces the progressive formation of multinucleated cells in certain cultured lines (CHO, Vero, HeLa, HEp-2), necrosis in the rabbit skin and in the mouse footpad, and death of mice inoculated by the intraperitoneal route. Two types of CNF have been identified (CNF1 and CNF2) and the cell activities of these toxins are due to their ability to constitutively modify the Rho proteins, a family of small GTPases that regulate the physiology of cell cytoskeleton (De Rycke et al. 1999).

The single structural gene encoding prototype CNF1 (*cnf1*) is located on a chromosomal pathogenicity island, which has also the codifying genes for  $\alpha$ -hemolysin (*hly*) and P-fimbriae or pyelonephritis-associated pilus (*pap*), whereas the *cnf2* gene is located on a high-molecular weight plasmid (De Rycke et al. 1999). CNF1 is frequently produced by extraintestinal pathogenic *E. coli* (ExPEC) isolated from humans with urinary tract infection (De Rycke et al. 1999, Russo & Johnson 2000, Boquet 2001) and occasionally detected in isolates from feces of children with diarrhea (Elliot et al. 1998, Okeke et al. 2000, Paciorek 2002). Production of CNF2 has been mainly found among isolates from calves with diarrhea or septicemia (Orden et al. 1999, De Rycke et al. 1999, Van Bost et al. 2001a).

The role of CNF-producing *E. coli* strains in diarrheal diseases has not been defined. Recently, Van Bost et al. (2001b) reported that CNF2-producing strains were able to colonize the intestines, cause long-lasting diarrhea, and invade the blood stream of newborn colostrum-restricted calves after experimental inoculation through the oral route. A previous report on the experimental infection of germfree colostrum-deprived newborn piglets showed no evidence for a pathogenic role of CNF1 in the development of diarrhea (Fournout et al. 2000). To date, there is no report on a comprehensively case-control epidemiological study of the association of CNF-producing *E. coli* strains with human diarrheal diseases. Therefore, such association was investigated by searching sequences homologous to the *cnf* genes among isolates from feces of children with and without acute diarrhea in the largest city of Brazil (São Paulo City). The characterization of all *cnf*-positive isolates identified with regard to the presence of established or putative virulence factors commonly detected in ExPEC is also reported.

A total of 2074 *E. coli* isolates from 200 children (1-4 years old) with bloody or non-bloody acute diarrhea (1030 isolates) and 200 age-matched non-diarrheic children (1044 isolates) was studied. *E. coli* strain J96 was included as the control for *cnf1*, *hly*, and *pap* genes (Blum et al. 1995). Strains S5 (Pérès et al. 1997), KS52 (Labigne-Roussel et al. 1985), and K-12 HB101 (pANN801-13) (Ott et al. 1986) were the controls for *cnf2*, afimbrial adhesin (*afa*), and S fimbrial adhesin (*sfa*) genes, respectively. *E. coli* laboratory strains HB101 and C600 were used as negative controls. All isolates and strains were stored in 15% glycerol at minus 70°C.

Bacterial colony blots were prepared and hybridized under stringent conditions with labeled DNA probes as previously described (Maas 1983). The *cnf* and *hly* probes used were the 335 bp *Pst*I-*Cla*I fragment of pEOSW1

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(Oswald et al. 1994) and the ~6 Kb *AvaI*-A fragment of pSF4000 (Welch et al. 1983), respectively. Probes for *pap*, *sfa*, and *afa* consisted of amplified products (328, 410, and 750 bp, respectively) generated by PCR from prototype strains as previously described (Le Bouguenec et al. 1992). For detection and characterization of cytotoxic activity, all *cnf* probe positive isolates were submitted to cytotoxicity and neutralization assays with rabbit antiserum specific for CNF (types 1 and 2) in HeLa cells as previously described (Marques et al. 2003). Statistical analysis was performed with the Epi Info version 5.01b software (Dean et al. 1991) and a *P* value less than 0.05 was considered to show a significant difference.

Among the 2074 *E. coli* isolates, 73 (3.6%) harbored *cnf* sequences (43 isolates from children with diarrhea and 30 isolates from children without diarrhea). The frequency of detection of at least one *cnf* positive isolate among the 200 case children and the 200 controls were 14 (7%) and 10 (5%), respectively (*P* = 0.50). Culture supernatants from all *cnf* positive isolates induced multinucleation of HeLa cells. The CNF1 anti-serum neutralized the activity expressed by 70 isolates (65 complete and 5 partial neutralizations), whereas the anti-CNF2 neutralized the activity of 3 isolates (2 complete and 1 partial neutralization). Among the CNF1-producing isolates, 55 (75.3%) hybridized with the *pap* probe and all harbored *hly* and *sfa* sequences, but none had *afa* homologous sequences. As shown in the Table, 3 different genetic profiles were found among the *cnf* positive isolates and the most frequent profile was *cnf*, *hly*, *pap*, *sfa*. None of the CNF2-producing isolates harbored DNA markers other than *cnf*. Seventeen out of the 24 children with CNF-producing isolates had more than one isolate studied and all isolates showed the same genetic profile.

Ten CNF1-producing *E. coli* isolates had been previously characterized as carriers of DNA sequences and/or producers of one type of cytolethal distending toxin (Marques et al. 2003) and expression of this toxin accounted for the partial neutralization of toxic activity by anti-CNF1 obtained with the preparations from 5 isolates. Production of another type of cytolethal distending toxin

may also account for the partial neutralization obtained for one CNF2-producing isolate since Pérès et al. (1997) found DNA sequences of both CNF2 and cytolethal distending toxin in the same plasmid of some *E. coli* strains.

The lack of association between CNF-producing *E. coli* strains and acute diarrhea in the population studied was corroborated by the finding of established enteropathogenic agents in the feces of 11 (79%) children with diarrhea (data not shown). The characterization of the fecal CNF1-producing isolates indicated that all might be regarded as potential ExPEC composing, at least transitively, the normal intestinal flora.

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TABLE

Genetic profiles and type of cytotoxin produced by 73 fecal *Escherichia coli* isolates with DNA sequences of cytotoxic necrotizing factor

Genetic profiles <sup>a</sup>	Number of isolates (%)	Type of cytotoxin <sup>b</sup>
<i>cnf</i> , <i>hly</i> , <i>pap</i> , <i>sfa</i>	55 (75.3)	CNF1 <sup>c</sup>
<i>cnf</i> , <i>hly</i> , <i>sfa</i>	15 (20.6)	CNF1
<i>cnf</i>	3 (4.1)	CNF2

*a*: *cnf*, cytotoxic necrotizing factor gene; *hly*, alpha-hemolysin genes; *pap*, pyelonephritis-associated pilus genes; *sfa*, S fimbrial adhesin genes; *b*: determined by neutralization of cytotoxic activity by specific antisera to CNF1 or CNF2; *c*: ten isolates carried DNA sequences of cytolethal distending toxin and expression of this toxin was detected in five isolates by neutralization of cytotoxic activity with specific antiserum.

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