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The Presence of Free Shiga-Like Toxins in Stool Specimens of Patients with Diarrhea During one Year Study

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Abstract

During one year study, 394 stool samples obtained from a random cases of diarrhea were examined for the presence of free shiga-like toxins Stx1 and Stx2 in Vero cells assay. Of 394 stool specimen supernatants tested, 2 (0.5%) gave positive results. The two stool supernatants positive on Vero cell line were wholly capable of being neutralized with the monoclonal antibody to Stx2. Broth cultures of strains isolated from the two positive stool samples were negative for Stxs-production in Vero cytotoxicity assay and by PCR.

Key words: *E. coli*, shiga-like toxins, diarrhea.

Shiga-like toxins-producing *Escherichia coli* (STEC) strains have been recognized as a significant human pathogens. Infections with STEC continues to be health problem in many countries since they may result in life-threatening complications such as hemolytic uremic syndrome (HUS) (Boyce *et al*., 1995; Karch *et al*., 1995). Serotype of STEC, the most commonly isolated from HUS patients, is *E. coli* O157:H7 which is characteristically unable to ferment sorbitol within 24 h and can be easily recovered from stool samples on sorbitol-MacConkey agar (Boyce *et al*., 1995; Krishnan *et al*., 1987). However, many other sorbitol-fermenting strains have been shown to produce shiga-like toxins. The detection of these strains among a multitude of nonpathogenic *E. coli* and other intestinal bacilli is a difficult task, and it very often fails in the presence of a low number of STEC organisms in stool sample. The probability of isolating STEC from stool samples of patients with STEC infection depends on the time between the onset of symptoms and the examination of stool specimen. STEC strains can be detected in stool of the patients for only a short time and also culturing stool sample for the STEC requires the testing of 10 to 20 colonies of *E. coli* (Boyce *et al*., 1995). The studies of Karmali *et al*. (1985) and other investigators (Krishnan *et al*., 1987; Maniar *et al*., 1990; Ritchie *et al*., 1992) strongly suggest that the detection of free shiga-like toxins in stool filtrates is the most specific and sensitive assay, and allows to detect all Stxs-producing organisms.

In the study, we used Vero cytotoxicity assay for the direct detection of Stxs in stool specimens obtained from patients with diarrhea.

During the period from 1 October 2002 through 31 October 2003, 394 stool samples obtained from unselected and untreated patients with diarrhea were collected. Patients come from different hospitals of Wrocław city, Poland. The mean age of patients was 12 years (range, 2 months to 42 years). A single fresh stool sample obtained from each patient 3 to 7 days after the onset of gastrointestinal symptoms was emulsifying in a 1:5 dilution with tryptic-soy broth supplemented with 100 mg streptomycin per mL to remove

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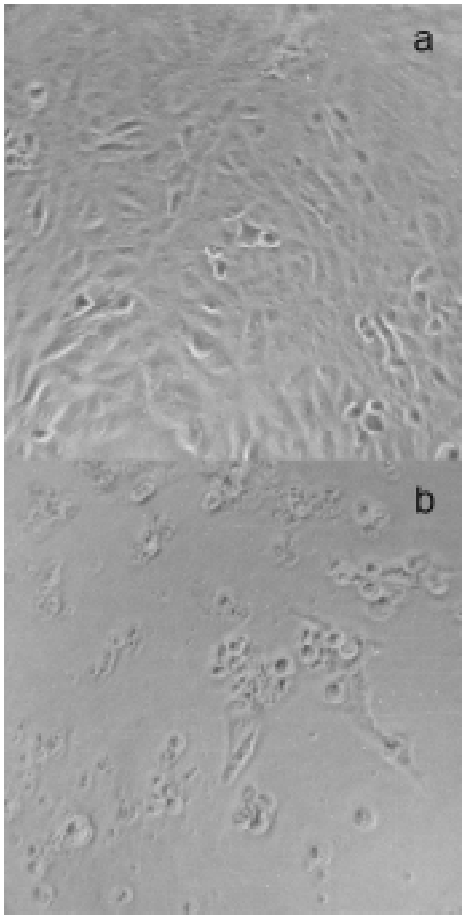

Fig. 1. Cytotoxic effect of shiga-like toxin Stx2 on Vero cells: a) cells not infected, b) cells infected with Stx2 – cytotoxic effect observed after 24 h

viable bacteria. The mixture was incubated at room temperature for 1 h and centrifuged at 5000 xg for 10 min. The supernatant was recentrifuged at 16000 xg for 30 min at 4°C, and assayed on Vero cells for the presence of Stxs. Vero cells were grown to a confluent monolayer in a 96-microdilute plates (NUNC A/S) in Minimal Essential Medium (MEM) supplemented with 5% fetal bovine serum (FBS) and antibiotic-antimycotic solution (100 U of penicillin, 100 μg of streptomycin and 0,25 μg of amphotericin B/mL, Gibco BRL) at 37°C in 5% CO_2 atmosphere. Before cytotoxicity test performance the cells were refeeded with fresh MEM supplemented with 2% FBS and antibiotic-antimycotic solution. The supernatant obtained from *E. coli* O157:H7 EDL933 strain was used as a positive control. Toxin neutralization test was performed by adding an equal volume of monoclonal antibodies to Stx1 and Stx2 (1:100 dilutions) separately or together to the fecal supernatants followed by incubation at 37°C for 1 h (the antibodies were kindly provided by Prof. L. Siegfied of PJ Safaric University, Institue of Medical Microbiology, Kosice, Slovakia). For cultivation of stool samples, sorbitol-MacConkey agar was used.

Of 394 stool samples tested, 2 (0.5%,) were positive in Vero cytotoxicity assay. In the shiga-like toxins neutralization tests the two stool sample supernatants were completely capable of being neutralized with the antibodies to Stx2. The two Stxs-positive stool samples after thawing were plated on sorbitol-MacConkey agar and a twenty sorbitol-positive colonies were tested for the cytotoxicity on Vero cell line and for the presence of genes encoding Stxs by PCR. There were no sorbitol-non-fermenting isolates in the stool samples.

In the study, evidence of STEC infection could be demonstrated by direct detection of free shiga-like toxins in stool supernatants in 2 (0.5%) of the 394 patients with diarrhea. The cytotoxic effect of the two stool supernatants was considered to be specific for Stxs as it was completely neutralized by monoclonal antibodies to Stx2 in spite of lack of isolation of STEC strains from the stool samples. This may be because of the stool samples were frozen at –70°C and than thawed before culturing or the period of the time from the onset of the infection to the stool samples obtainment was too long. It has been shown that the amount of fecal STEC organisms decline rapidly and these strains can be detected in patients' stool for a short time (Ritchie *et al*., 1992; Karch *et al*., 1992). The infectious dose of STEC of some serotypes *e.g. E. coli* O157:H7 or O26:H11 is very low, and excretion of a low number of these bacteria makes their detection in stool specimens by cultivation on solid media impossible (Karch *et al*., 1995). According to study Karmali *et al*. (1985) the median durations of shedding of *E. coli* O157:H7 were 13 days (range, 2 to 62 days) in patients with diarrhea. In the study, the stool samples were obtained 3 to 7 days after the onset of diarrhea.

Previous study of STEC strains isolated in Wrocław city showed the presence of shiga-like toxin-producing *E. coli* among O26 serogroupe strains (Sobieszczańska *et al*., 2000). The most common method for screening of STEC infection is the cultivation of stool samples on sorbitol-MacConkey agar (Boyce *et al*., 1995). Since up to now we have not isolated from stool samples examined sorbitol-negative *E. coli* of serotype O157:H7 in our region. Taking into consideration that STEC strains belong to a very diverse range of serogroups and that other than *E. coli* intestinal bacilli strains are STxs-producing, it seems that detection of free shiga-like toxins is the best method for screening infections with both STEC O157:H7 and non-O157 strains as well as other STXs-producing organisms (Ritchie *et al*., 1992; Piérard *et al*., 1999).

In the study, in spite of lack of isolation of shiga-like toxins-producing strains from the two positive stool samples, we were able to confirm the two cases of STEC infection by neutralization assay with monoclonal antibodies to Stxs.

Literature

Boyce T.G., A.G. Pemberton, J.G. Wells and P.M Griffin. 1995. Screening for *Escherichia coli* O157:H7 – a nation-wide survey of clinical laboratories. *J. Clin. Microbiol*. **33**: 3275–3277.

Karch H., T. Meyer, H. Rüssmann and J. Heesemann. 1992. Frequent loss of shiga-like toxin genes in clinical isolates of *Escherichia coli* upon subcultivation. *Infect. Immun*. **60**: 3464–3467.

Karch H., H. Rüssmann, H. Schmidt, A. Scharzkopf and J. Heesemann. 1995. Long-term shedding and clonal turnover of enterohemorrhagic *Escherichia coli* O157 in diarrheal diseases. *J. Clin. Microbiol*. **33**: 1602–1605.

Karmali M.A., M. Petric, C. Lim, R. Cheung and G.S. Arbus. 1985. Sensitive method for detecting low numbers of verotoxin-producing *Escherichia coli* in mixed cultures by use of colony sweeps and polymyxin extraction of verotoxin. *J. Clin. Microbiol*. **22**: 614–619.

Krishnan C., V.A. Fitzgerald, S.J. Dakin and R.J. Behme. 1987. Laboratory investigation of outbreak colitis caused by *Escherichia coli* O157:H7. *J. Clin. Microbiol*. **25**: 1043–1047.

Maniar A.C., T. Williams, C.M. Anad and G.W. Hammond. 1990. Detection of verotoxin in stool specimens. *J. Clin. Microbiol*. **28**: 134–135.

Piérard D., G. Cornu and W. Proesmans. 1999. Hemolytic uremic syndrome in Belgium: incidence and association with verocytotoxin-producing *Escherichia coli* infections. *Clin. Microbiol. Infect*. **5**: 16–22.

Ritchie M., S. Partington, J. Jessop and M.T. Kelly. 1992. Comparison of a direct fecal shiga-like toxin assay and sorbitol-MacConkey agar culture for laboratory diagnosis of enterohemorrhagic *Escherichia coli* infection. *J. Clin. Microbiol*. **30**: 461–464.

Sobieszczańska B.M., R. Gryko and C.W. Małek. 2000. Isolation of verotoxigenic strains of *Escherichia coli* O26 in Poland. *Clin. Microbiol. Infect. Dis*. **6**: 226–231.