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CHARACTERIZATION OF SHIGA-TOXIGENIC ESCHERICHIA COLI O157:NM ISOLATED FROM A CHILD WITH DIARRHEA

KARAKTERIZACIJA ŠIGA TOKSIN PRODUKUJUĆE ESCHERICHIA COLI O157:NM IZOLOVANE IZ DETETA SA DIJAREJOM

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Key words

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Ključne reči

Šiga toksin produkujuća *Escherichia coli*; serogrupa O157; lančana reakcija polimeraze; geni virulencije

Abstract

Shiga toxin-producing *Escherichia coli* O157 are the most important recently emerged group of food-borne pathogens. This serogroup is generally more virulent than non-O157 Shiga toxin-producing *E. coli* and cause severe human diseases: hemorrhagic colitis and hemolytic-uremic syndrome. We have isolated non-sorbitol fermenting *E. coli* from faeces of a child with mild diarrhea. The isolate was of O157:NM (NM, nonmotile) serotype. Molecular characterization was done by PCR detection of virulence genes. Genes for both types of toxins, Stx1 and Stx2, were detected, and *eaeA* and *hlyA* genes encoding intimin and enterohaemolysin, respectively. Beside the presence of all the most important virulence genes, bacteria cause only mild form of diarrhea.

INTRODUCTION

Shiga toxigenic *Escherichia coli* (STEC) strains are an important cause of gastrointestinal disease in humans. The severity of diseases vary from mild diarrhea with abdominal pain and vomiting, to hemorrhagic colitis (HC), and life-threatening hemolytic-uremic syndrome (HUS) [1]. STEC is a food-borne pathogen and undercooked ground beef and other contaminated beef products a main sources. Over 200 of different O:H serotypes of STEC have been isolated from cases of human disease [2]. Since its first identification as a pathogen in 1982 in the USA following an outbreak of HC, STEC O157:H7 strains have been implicated in sporadic cases and outbreaks of serious gastrointestinal disease and complications such as HUS worldwide [3, 4, 5]. HUS complicates about 10% of cases of *E. coli* O157:H7 infections, and may be fatal in 2-10% of cases [6]. Non-O157 STEC strains have been isolated from HUS patients, too. In general, the isolation rates of non-O157 STEC from serious diseases and outbreaks are lower than those of O157:H7.

STEC produce Shiga-like toxins (Stx) that are responsible for the principal manifestations of HC and HUS [7]. Based on serological methods and DNA sequencing, these toxins have been divided into two major subclasses, Stx1 and Stx2. Most strains of O157:H7 serotype produce Stx2 only, strains that produce both Stx1 and Stx2 toxins are occasionally found, and strains producing Stx1only are rare isolated [6]. Regardless of serotype, STEC strains that produce Stx2 are more commonly associated with severe disease compared to isolates producing Stx1 only or both toxins [8].

In addition to toxins, several other virulence factors contribute to the pathogenicity of O157:H7 STEC strains. One of them is plasmid-encoded enterohemolysin, the product of *hlyA* gene. Another is intimin, outer membrane protein required for intimate attachment of bacterial cells to enterocytes. Intimin is the product of *eaeA* gene, that is present on a large pathogenicity island termed the locus for enterocyte effacement (LEE). The LEE of strain EDL 933, which is a reference strain of serotype O157:H7, is about 43.4 kb long and contains 41 open reading frames. Beside *eaeA* gene, LEE contains genes that encode secreted proteins required for signal transduction and the development of the characteristic attaching-and-effacing lesions on enterocytes, genes that encode translocated intimin receptor (Tir), type III secretion apparatus, and others [9].

In the present study, an isolate of E. coli proved to belong to O157 serogroup, was analized by PCR for the presence of virulence genes.

MATERIAL AND METHODS

Case. Feces samples were collected from 1-year-boy who had mild diarrhea, in the last 20 days with diagnosis Enterocolitis prolongata. Child had mushy diarrhea with no blood, 2-3 number of stools per day without abdominal pain, fever, or vomiting and no sign of systemic complications. The child did not receive antibiotic treatment. In an interview with mother, she conveyed that the child consumed common food and family members had no history of diarrhea or recent travel. Unfortunately, we were unable to establish the source of infection in our patient.

One month after onset of illness, the three control stool samples were obtained from patient and were negative regarding to isolation *E. coli* O157. In a telephone call the patient's pediatrician conveyed that child had recovered from the illness without complication.

Bacteria isolation. Feces samples were processed for all common enteric pathogen, such as *Salmonella, Campylobacter, Shigella, Yersinia enterocolitica* and *Escherichia coli* O157 using MacConkey agar (Liofilchem, Italy), XLD agar (bioMérieux, France), cefixime tellurite-sorbitol MacConkey agar (Himedia, India), *Campylobacter selective agar* (Himedia, India), *Yersinia* selective agar (Himedia, India) and Selenite cystine broth (Liofilchem, Italy) as previously described [10]. After incubation for 18 hours at 37°C and *Yersinia* selective agar for 24 hours at 25°C, the plates were examined for mentioned enteric pathogens.

Serotyping, identification of bacteria. Three non-sorbitol fermenting colonies were selected and portion of these colonies serotyped by agglutination test using *E. coli* O157 antisera (IPHS, Serbia). Because all three colonies agglutinated with the O157antisera, these colonies were subcultured to blood agar and biochemically identified as *Escherichia coli* by a standard biochemical tests. Serotype O157 was tested by commercially available O157 and H7 antisera (SSI, Denmark). After three passages through motility test medium, the isolates were identified as NM. *E. coli* ATCC 35150 was used as a reference strain.

Strain presumptively identified as *E. coli* O157:NM was sent to Medical Military Academy for aditional molecular characterization.

Antibacterial susceptibility. Susceptibility testing for the antimicrobial drugs was done by a disk diffusion method on Mueller-Hinton agar (bioMérieux, France) according to CLSI [11]. Strain was tested for resistance to following antimicrobials (Bio Rad, France): ampicillin 10µg, amoxycillin/clavulanic acid 20/10 µg, cefotaxime 30 µg, ceftazidime 30 µg, cefpodoxime 10 µg, chloramphenicol 30 µg, ciprofloxacin 5 µg, gentamicin 10 µg, nalidixic acid 30 µg, tetracycline 30 µg, trimethoprim 5 µg, streptomycin 10 µg, sulphonamides 300 µg, and trimethoprim/sulfamethoxazole 1,25/23,75 µg. *E. coli* ATCC 25922 was used as a reference strain.

Detection of virulence genes by PCR. The extraction of DNA from *E. coli* was performed by the use of a QIAamp DNA Mini Kit for isolation of bacterial DNA (QIAGEN, Hilden, Germany). Virulence genes were detected by PCR in separate reactions as described below. The composition of the reaction mixture was as follows: 15 mM Tris-HCl (pH 8,0), 50 mM KCl, 2,5 mM MgCl2, 0,2 mM each of deoxynucleoside triphosphates, 0,2 mM each of primers, 5 µl of extracted DNA template, 1 U of *Taq* polymerase in a total volume of 50 µl. PCR conditions for stx1 and stx2 genes were 5 min for initial denaturation at 94ºC, followed by 35 cycles of 1 min at 94ºC, 1 min at 55ºC, and 1 min at 72ºC; and finally, 5 min at 72ºC. The conditions for *eaeA* and *hlyA* genes were 5 min for initial denaturation at 94ºC, followed by 35 cycles of 1 min at 94ºC, 1 min at 62ºC, and 1 min at 72ºC; and finally, 5 min at 72ºC. The amplified DNA was then analysed by electrophoresis on 1,5% agarose gel and visualised by ethidium bromide staining. The primers used for PCR amplification of stx1 gene were VT1c (5'-ACC CTG TAA CGA AGT TTG CG-3') and VT1d (5'-ATC TCA TGC GAC TAC TTG AC-3'), which amplified a 140 bp amplicon [12], and for amplification of stx2 gene were VT2c (5'-AAG AAG ATG TTT ATG GCG GT-3') and VT2d (5'-CAC GAA TCA GGT TAT GCC TC-3'), which amplified a 285 bp amplicon [13]. The primers used for PCR amplification of eaeA gene were EAE-AF (5'-GAC CCG GCA CAA GCA TAA GC-3') and EAE-AR (5'-CCA CCT GCA GCA ACA AGA GG-3'), which amplified a 384 bp amplicon [14], and for amplification of *hlyA* gene were HlyAF (5'-GCA TCA TCA AGC GTA CGT TCC-3') and HlyAR (5'- AAT GAG CCA AGC TGG TTA AGC T-3') which amplified a 534 bp amplicon [14].

RESULTS

Strain isolated from a feces of patient was identified as *E. coli* O157:NM, and was susceptible to all tested antimicrobial drugs.

In PCR amplification of a genomic DNA isolated from strain *E. coli* O157:NM with primers specific for main virulence genes, elicited amplicons were of the same sizes as those obtained from the positive control refernce strain *E. coli* ATCC 35150. In examined strain, genes encoding four main virulence factors of STEC and EHEC strains were detected: both Shiga-toxins Stx_1 and Stx_2, intimin, and enterohemolysin (Fig 1).

DISCUSSION

STEC serotypes differ in their pathogenic potencial. Serotypes that belong to enterohemorrhagic *E. coli* (EHEC), a subgroup of STEC, are highly pathogenic and cause severe human diseases. *E. coli* O157:H7 is the most frequent EHEC implicated as a cause of HUS. In a large study in Germany, among 524 EHEC isolated from HUS patients, 67.7% belonged to serotypes O157:H7 or O157:NM, and 32.3% belonged to 34 non-O157 serotypes [15]. Many clinical laboratories in Serbia do not routinely culture stool specimens for STEC. The recommendation of CDC for clinical laboratories is that all stools submitted for routine testing, be simultaneously cultured for *E. coli* O157:H7 and tested with an assay that detects Shiga toxins to detect non-O157 STEC [16]. Prompt laboratory identification of STEC strains is important because appropriate treatment early in the course of infection might decrease the risk for serious complications.

Virulence genes of pathogenic bacteria are often located within genomic islands, bacteriophages, or large plasmids that enable gene transfer between bacteria strains. The clusters of genes may be flanked by insertion sequences, facili-

tating their disemination. Different combinations of virulence genes are often serotype-specific or subgroup-specific. Gene *eae* is present both in EHEC and in enteropahogenic *E. coli*, but in combination with stx genes only in EHEC. Population genetic analysis, using multilocus sequence typing (MLST) have shown that *E. coli* O157:H7 strains represent a single phylogenetic branch [9]. It has been postulated that pathogenic O157 lineage has evolved from an enteropathogenic *E. coli* O55:H7 group over the last 50 years by acquisition of shiga-toxin-converting phages and an O157 *rfb* gene cluster that encode O-antigen subunit.

Although O157 group is geneticaly compact, some strains of EHEC O157:NM are non-sorbitol-fermenting (non-SF) as EHEC O157:H7, and some strains are sorbitol-fermenting (SF). A few isolates of O157:NM serotype that are SF and *stx* gene negative, were found to belonge to enteropathogenic, not to enterohemorrhagic pathotype [17]. Some evidence suggests that SF-O157 is more frequently associated with HUS than non-SF strains [18, 19, 20, 21]. The *sfp* gene cluster, restricted to the large plasmid of SF EHEC O157, encodes Sfp fimbriae that mediate adherence of these bacteria to human intestinal epithelial cells [22]. Increased adherence of SF-O157 may in turn cause a more potent inflammatory host response, resulting in a higher risk for HUS. However, recently published work suggest that infection with SF-O157 results in less severe colitis than does the more common non-SF-O157 infection, and in a lower risk for HUS [23]. But patients infected with SF-O157 had anuria for longer periods and consequently had longer sessions of peritoneal and hemodialysis.

Our isolate was O157:NM serotype, and non-SF. Beside this it contained stx_1, stx_2, *eaeA*, and *hlyA* genes. It is known that EHEC strains with stx_2 toxin gene only are more virulent than strains that contain both *stx* genes or stx_1 gene only. Our strain is one of the rare EHEC O157 isolates that cause only mild diarrhea without hemorrhagia. We are not able to explain why genotipic potencial of this strain was not completely expressed. Maybe these results indicate that other virulence factors may contribute to increased likelihood of severe disease and HUS. Beside them are host factors such as pre-existing immunity, genetic factors, and others still unknown factors that could modulate host response to infection.

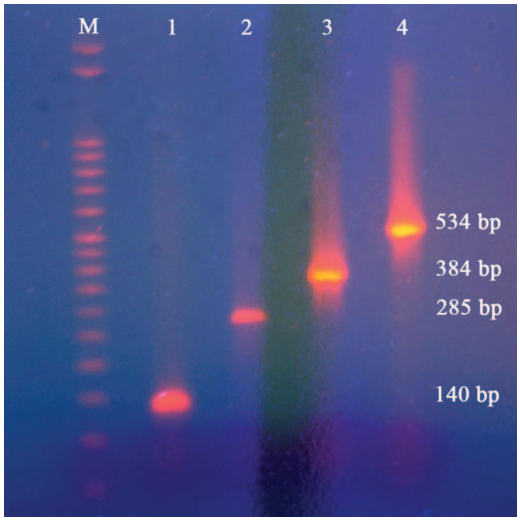

Fig. 1. PCR amplification of E. coli O157:NM strain with primers specific for virulence genes. Lane 1, stx1; lane 2, stx2; lane 3, eaeA; lane 4, hlyA; M, 50-bp DNA ladder.

Apstrakt

Escherichia coli O157 koja produkuje Shiga toksine, je najznačajniji patogen koji se prenosi hranom koji se nedavno pojavio. Ova serogrupa je generalno virulentnija nego ne-O157 *E. coli* koje produkuju Shiga toksine i izaziva teška oboljenja kod čoveka, kao što su hemoragični kolitis i hemolitično uremični sindrom. Iz stolice deteta sa umerenom dijarejom izolovali smo *E. coli* koja ne fermentuje sorbitol. Serotip izolata bio je O157:NM (NM, nepokretna). Molekularna karakterizacija rađena je detekcijom gena za virulenciju PCR metodom. Detektovani su geni za oba tipa toksina, Stx_1 i Stx_2, kao i *eaeA* i *hlyA* geni koji kodiraju intimin i enterohemolizin. I pored prisustva svih najznačajnijih gena za virulenciju, bakterija je izazivala samo blagu formu dijareje.

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