SHORT COMMUNICATION

Laboratory detection of verocytotoxin-producing *Escherichia coli* in the official food control in Slovakia

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Summary
The National Reference Centre (NRC) of Environmental Microbiology, Bratislava, Slovakia has been involved in the European Network of National Reference Laboratories for the official food control on *Escherichia coli* and Vero(Shiga) toxin-producing *Escherichia coli* (VTEC) under the authority of the European Community Reference Laboratory (EU-RL) in Rome since year 2006. In relation to the outbreak of hemorrhagic colitis in Germany in 2011, which was caused by *Escherichia coli* of serotype O104:H4, EU-RL regularly provided the laboratories in its network an up-to-date information concerning analytical procedures and organized the development of a protocol for the detection of VTEC. NRC analysed, during that period, approximately 45 samples of suspected bacterial strains of *Escherichia coli* isolated from food samples and biological materials. The strains were analysed for the presence of *vtx1* and *vtx2* genes encoding verocytotoxins, and *eae* gene encoding the protein intimin. Using conventional and qualitative real-time polymerase chain reaction, isolates from food samples were found to be serogroups O128 and O146, and isolates from biological materials were found to be serogroups O26 and O119. The presence of genes encoding verocytotoxins was not detected, only the presence of *eae* gene. None of *E. coli* isolates was O104:H4.

Keywords
Escherichia coli; verocytotoxin-producing *E. coli*; outbreak *E. coli* O104: H4; serogroup

Escherichia coli is classified as a member of the *Enterobacteriaceae* family of gamma-proteobacteria, which are gram-negative, facultatively anaerobic, sporeforming, motile or non-motile bacteria. *E. coli* bacteria are ordinarily commensal organisms, but six pathotypes of diarrheagenic *E. coli* have been recognized, each with distinct phenotypic and genetic traits [1]. Three general clinical syndromes result from infection with inherently pathogenic *E. coli* strains: urinary tract infection, sepsis/meningitis and enteric/ diarrheal disease [2]. The species *Escherichia coli* is divided into several serogroups according to the structure of surface antigens. Serogroups of *E. coli*, which cause most often food-borne disease, are O157, O26, O103, O91, O145, O111, O113, O104, O128 and O146. One group of the pathogenic *E. coli* are verocytotoxin-producing *E. coli* (VTEC), sometimes referred to as Shiga toxin-producing *E. coli* (STEC). This group comprises several *E. coli* serotypes capable of producing vero/shigacytotoxins (Vtx1/Stx1, Vtx2/Stx2). They cause distinctive clinical manifestations such as febrile bloody diarrhea with severe abdominal pain (hemorrhagic colitis, HC) and microangiopathic hemolytic anemia with renal failure (hemolytic uremic syndrome – HUS) [3].

In early May 2011, an outbreak of diarrhea with associated HUS began in northern Germany [4] and cases were subsequently reported in 15 other countries. As of July 22, a total of 3167 cases of non–hemolytic uremic syndrome (16 deaths) and 908 cases of HUS (34 deaths) caused by verocytotoxin- producing *E. coli* were

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reported, according to the German Protection against Infection Act. Several groups reported that the outbreak was caused by VTEC strain belonging to serotype O104:H4, with virulence features that are common to the enteroaggregative *E. coli* (EAggEC) pathotype [5]. Epidemiological investigations suggested that the outbreak was caused predominantly by contaminated sprouts produced by a farm in Lower Saxony [6]. The outbreak was associated with a number of atypical features such as older age of patients who were mainly women, a higher proportion of patients developing HUS, and a high incidence of neurological symptoms [7]. The ability of certain pathotypes of *E. coli* to colonize agriculturally important domestic animals and survive in meat products makes these organisms a particularly common cause of foodborne infections [8]. In addition, certain *E. coli* strains were shown to colonize plant tissues following contamination of soils or irrigation water from infected herds or wildlife, resulting in large outbreaks that were attributed to sprouts or contaminated vegetables [9, 10].

MATERIAL AND METHODS

Bacterial isolates were obtained from the Regional Public Health Authorities in Slovak Republic, after being collected from the food chain in Slovakia and from biological materials by standard culture methods ISO 9308-1 and ISO 16649-2 on non-selective and selective media, followed by biochemical analysis [11, 12].

From bacterial isolates, DNA was extracted using two methods: the lysis by heating and using the bacterial DNA extraction Kit (Ecoli, Bratislava, Slovakia). The kit was used according to the manufacturer's instructions. Isolated DNA was examined by polymerase chain reaction (PCR) using specific primers to amplify PCR products for the presence of verotoxins *vtx1* (131 bp), *vtx2* (128 bp) and serogroups O157 (133 bp), O26 (268 bp), O128 (782 bp), O146 (378 bp), O103 (320 bp), O91 (105 bp), O145 (418 bp), O111 (829 bp), O104 (100 bp). PCR was performed using Mastercycler gradient PCR cycler (Eppendorf, Hamburg, Germany). The thermal programmes for the detection of individual serogroups were according to the instructions of EU-RL VTEC Method [13]. Isolates were also examined for the presence *eae* gene and O113 serogroup by "touchdown" PCR amplification using specific primers eaeF and eaeR to amplify a specific DNA fragment of 102 bp, and 113F and 113R to amplify a specific DNA fragment of 593 bp, respectively. PCR assays were performed using iCycler (BioRad, Hercules, California, USA) using a programme consisting of 10 cycles of 95 °C for 1 min, 65 °C for 2 min, 72 °C for 90 s, 11–15 cycles of 65–60 °C for 2 min, 15–25 cycles of 60 °C for 2 min and 25–35 cycles of 72 °C, followed by the final 2.5 min polymerization. PCR products were analysed by agarose gel electrophoresis in a gel of 2.5% agarose (Amresco, Solon, Ohio, USA). Amplification and detection of genes encoding verocytotoxins, *eae* gene and serogroups were also performed in a IQ5 Multicolor Real-time PCR Detection System (BioRad). Sequences of primers and TaqMan probes targeting the verocytotoxins, *eae* gene and serogroups were according to EU-RL VTEC Method [14], and fluorogenic dyes were used according to the equipment. The temperature programme consisted of denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s.

RESULTS AND DISCUSSION

A total of 5093 samples of foodstufs, 17197 water samples and 28228 environmental samples were analysed for the presence and quantity of *Escherichia coli* by laboratories of Regional Health Authorities in Slovakia in 2011. The samples were analysed in order to control water (drinking water, pools), in frames of the official food control and surveillance of food-borne diseases. The presence of *Escherichia coli* was determined in 5283 samples (10.5%). Forty-five suspect samples of bacterial strains of *E. coli* were analysed under this study and results are presented in Tab. 1. Samples were identified in National Reference Centre (NRC) of Environmental Microbiology, Bratislava, Slovakia in relation to the outbreaks or due to identification of isolates that represented a potential health risk to humans.

Isolates were confirmed as a *E.coli*-positive by standard culture methods. All samples created typical blue-green colonies on TBX (tryptone bile X–glucuronide) chromogenic agar, which detected the glucuronidase activity. Isolates were also indol-positive, non-capable of urea hydrolysis, and their ability to ferment lactose and other saccharides with production of gas was confirmed. Simultaneously, further single suspect colonies were analysed by additional biochemical tests. One strain of *E.coli* delivered to NRC was not vital probably because of bad transport conditions.

Serogrouping of O157, O145, O111, O103, O26, O121, O91, O113 and O104 *E. coli* strains

Tab. 1. Identification of serogroups within 45 suspect *Escherichia coli* isolates.

Regional PHA	Number of suspected samples			Number of confirmed samples		Note
	Total	Food	Biological material	Non-pathogenic serogroup	Pathogenic serogroup	
Bratislava	20	20	—	16 + 1 (spontaneous agglutination)	2 / O128 (sterilized pickles and cucumber salad) 1 / O146 (herbal tea)	
Nitra	16	8	8	14	2 / O119 (biological material)	Enteropathogenic *E. coli*
Levice	7	—	7	4 + 1 (spontaneous agglutination)	1 / O26 (biological material)	1 non-vital bacterial strain
Trenčín	2	2	—	2	—	

PHA – Public Health Authority.

was done using conventional PCR methods. As a positive control for the identification of serogroup-associated genes, genomic DNA isolated from reference *E. coli* strains was used. The isolates provided by EU-RL VTEC in the framework of the proficiency testing programmes were used as reference strains belonging to other serogroups besides "top 5". The genes were absent in all isolates except for the serogroup O26, which was positive in one *E. coli* strain isolated from a biological material. The results were confirmed using qualitative real-time PCR (data not shown).

The positive results from molecular serogrouping were confirmed by slide agglutination method using commercially available antisera. Serogroups O146, O128 and O119 were only identified by slide agglutination in all unidentified samples. Two isolates from biological materials were identified as belonging to serogroup O119, two strains from cucumber and sterilized pickles were identified as belonging to serogroup O128, and one sample isolated from herbal tea as belonging to serogroup O146. Two samples agglutinated spontaneously in saline (Tab. 1).

Real-time PCR assay was also performed to identify VTEC strains. The method based on the detection genes encoding toxins Vtx 1,Vtx2 and the protein intimin, was used upon preliminary optimization using reference VTEC strains, regarding annealing temperature in temperature gradient, DNA concentrations and conditions giving a high endpoint fluorescence and low C_T (cycle threshold) values (data not shown). In our isolates, genes encoding verocytotoxins were not detected in any *E. coli* strain, only *eae* gene was detected in two isolates from biological materials. The *eae* gene was also confirmed using conventional PCR and the strains were defined as the enteropathogenic E. *coli* (EPEC).

PCR techniques are generally known as specific and sensitive methods, therefore the *E. coli* strains were tested for the presence of *vtx1, vtx2* genes encoding verocytotoxins, *eae* gene encoding intimin and serogroups by conventional or real-time PCR. The conventional PCR reaction for the detection of serogroup O104 was based on the detection of O-antigen-associated gene encoding the enzyme wzx flipase [8]. *Escherichia coli* O104:H4 is characterized as non-haemolytic, *vtx1*$^-$, *vtx2*$^+$ and *eae*$^-$, which does not fit to the definition of VTEC as these strains are always *eae*-positive. This new finding underlines the peculiarity of this serotype associated with a serious epidemic in Europe.

Detection of pathogenic *E. coli* is important for microbiological food safety within the official food control. Recently, several significant epidemics caused by this pathogenic microorganism took place in EU, and this epidemics has an increasing character. Confirmation of suspect isolates from human, food and environmental sources is necessary for epidemiological surveillance in EU. Therefore, it is useful to develop and implement the specific detection methods for this pathogen. NRC of Environmental Microbiology, Bratislava, Slovakia annually participates in interlaboratory studies within the European Network of National Reference Laboratories (NRL) organized by EU-RL for *E. coli*/VTEC in cooperation with Reference Centre of WHO for *E. coli*/VTEC (Statens Serum Institut, Copenhagen, Denmark) under European Centre for Disease Prevention and Control (ECDC), whose task is to prepare

and verify protocols for the detection of *E. coli* pathogroups, in order to ensure uniform standard diagnostic methods in all NRL of EU member states. Whereas the national clinical laboratory has not been established in Slovakia, NRC is able to provide identification of *E. coli* strains isolated from biological materials, associated with potentional diseases. In this study, *E. coli* O104:H4 associated with the outbreak in Germany was not detected in food samples or in biological materials in Slovakia.

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