A PROBLEM-SOLVING INTEGRATIVE APPROACH OF *ESCHERICHIA COLI* O157:H7 SEROTYPE DIAGNOSIS

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ABSTRACT

The diagnosis of *Escherichia coli* O157:H7 infection needs to be considered for all the patients who present with diarrhea and hemolytic uremic syndrome. In Romania, the routine method for detecting the *E. coli* O157:H7 members in stool cultures is still based on the use of slide agglutination tests to screen sorbitol non- fermenting colonies recovered from sorbitol-MacConkey medium.

This report focused on a sorbitol-negative *E. coli* strain presumptively assigned to the O157 serogroup during the routine clinical laboratory diagnosis that was referred to the reference laboratory for confirmatory testing and further genetic characterization. The PCR-based serotyping and virulence gene detection complemented by DNA sequencing showed that the strain did not belong to the O157 serogroup but qualified as enteropathogenic *E. coli*.

The screening of *E. coli* O157:H7 strains can result in a false-positive diagnosis and lead to inappropriate public health measures. Therefore, although the schemes of microbial detection and characterization may use various combinations of testing methods, depending on the laboratory level, the aim of investigations is to specifically identify the true pathogenic strains.

Keywords: *Escherichia coli* pathotype, VTEC/STEC, molecular identification

REZUMAT

Infecţia cu *Escherichia coli* O157:H7 trebuie suspicionată în cazul tuturor pacienţilor cu diaree şi sindrom hemolitico-uremic. În Romania, metoda utilizată în mod curent pentru detectarea în probele de materii fecale a membrilor serotipului O157:H7 se bazează pe teste de aglutinare pe lamă, aplicate coloniilor sorbitol-negative, izolate pe mediul MacConkey cu sorbitol.

Acesta este un raport despre o tulpină de *E. coli* sorbitol negativă, identificată prin algoritmul curent de diagnostic al unui laborator clinic ca fiind din serogrupul O157, care a fost trimisă laboratorului de referinţă pentru confirmare şi caracterizare suplimentară. Serotipizarea bazată pe PCR şi analiza genelor de virulenţă, completate de secvenţierea AND, au arătat că tulpina nu aparţine serogrupului O157, dar este enteropatogenă.

Modul de identificare a tulpinilor de *E. coli* O157:H7 poate genera rezultate false, care conduc la luarea unor măsuri de sănătate publică nepotrivite. Ca urmare, chiar dacă schemele de detecţie şi caracterizare microbiană pot include o combinaţie variată de teste, în functie de nivelul de expertiză al laboratorului, scopul final al investigaţiilor trebuie să fie identificarea adevăratelor tulpini patogene.

Cuvinte-cheie: prototip de *Escherichia coli*, VTEC/STEC, identificare moleculară

MATERIALS AND METHODS

E. coli strain and DNA preparation

A sorbitol-negative *E. coli* isolate recovered from the culture of a stool specimen collected from a child hospitalized with diarrhea was referred to the reference laboratory for further investigation. The strain was tested in the clinical laboratory where it had been isolated by slide agglutination with O157 antiserum and gave a positive result.

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O157 and H7 serology

Serotyping of both O (lipopolysaccharide) and H (flagellar) antigens was performed with commercially available antisera (SSI Diagnostica, Hillerød, Denmark). The O antigen was determined using antisera for slide agglutination produced against the EPEC and VTEC serogroups (Statens Serum Institute). The H7 was the only flagellar antigen sought. All the antisera were used according to the manufacturer's instructions. The strain was initially checked for autoagglutination with saline.

PCR-based assays for genes encoding determinants for serotype and virulence factors

A conventional touchdown PCR with previously designed primers for the *wzx* sequences unique to serogroup O157 was used [6]. The DNA template was made by suspending a loop full of culture in 200 µl of water and heated for 15 min in boiling water. The 50 µl PCR mix contained 3 µl of crude DNA template, 1x Taq Polymerase buffer (Promega) with 3 mM $MgCl_2$, 0.3 mM of each deoxynucleoside triphosphates, 15 pmols of each primer, and 2U of GoTaq G2 Flexi DNA Polymerase (Promega). The PCR conditions were 95°C for 15 min; 10 cycles of 95°C for 30 s, 68–59°C (decrease 1°C /cycle) for 20 s, 72°C for 52 s, followed by 35 cycles of 95°C for 30 s, 59°C for 20 s, 72°C for 52 s and 72°C for a final 1 min extension.

A multiplex PCR with previously published primers was additionally performed for defining the O157:H7 serotype. The main target genes were *rfbE*O157 and *fliC*H7 and the 16S rRNA encoding gene as internal positive control [7]. The PCR volume adjusted to 25 µl contained: 1x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1.5 U Taq DNA polymerase (Promega) plus primers (0.1 µM of the *E. coli* 16S rRNA (E16S) primers, 0.4 µM fliC primers, and 1 µM rfbE primers) and 3 µl of bacterial lysate. The PCR program consisted in an initial denaturation step at 95°C for 3 min followed by 30 cycles of amplification with denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, ending with a final extension at 72°C for 7 min.

Commercial primers (SSI Diagnostica, Hillerød, Denmark) designed to detect virulence encoding genes associated with the diarrheagenic *E. coli* strains were used. Specifically, the following genes were targeted in a multiplex PCR: *eae* (intimin), *vtx1* (verotoxin 1), *vtx2* (verotoxin 2), *elt* (heat-labile enterotoxin), *est* (heat-stable enterotoxin), and *ipaH* (invasive plasmid antigen). The PCR was performed in a total reaction volume of 20 µl containing 1 x Multiplex PCR Master Mix (Qiagen), 4 µl primer mix, and 4 µl of bacterial lysate. Amplification conditions comprised 95°C for 15 min, followed by 35 cycles of 94°C for 50 s, 57°C for 420 s, and 72°C for 50 s, and finally 72°C for 3 min.

PCR for the detection of the gene coding for enterohemolysin was performed using previously published primers [8].

PCR was performed by addition of 5 ml of this bacterial cell suspension to a reaction mixture containing 30 pmol of each primer per ml, 5 ml of 10-fold-concentrated polymerase synthesis buffer, 200 mM (each) deoxynucleoside triphosphate, and 2.0 U of Taq polymerase (Amersham Laboratories, Buckinghamshire, United Kingdom). The samples were filled up with water to a final volume of 50 ml. After an initial denaturation of 5 min at 94°C, the samples were subjected to 30 cycles of amplification, each of which consisted of 30 s at 94°C, 90 s at 57°C, and 90 s at 72°C. F

Amplicons were analyzed by electrophoresis on agarose 2% w/v gels using standard conditions, followed by staining with ethidium bromide.

DNA sequencing

The amplicon resulted from the PCR of the *wzx* gene, previously purified with Nucleospin Gel and PCR Clean-up (Mackerey Nagel), was sequenced using the PCR primers and BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Cycle sequencing reaction products were further purified using DyeEx 2.0 Spin kit (Qiagen) and analyzed on a SeqStudio Genetic Analyzer instrument (Applied Biosystems), according to the manufacturer's instructions. Raw sequences were visually inspected and edited using BioEdit free software and the

consensus sequence was identified using the Basic Local Alignment Search Tool (BLAST) program (https://blast.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

Serotyping based on the combination of the principal cell surface components, the O-antigens and flagellar H-antigens, is still used for diagnosis and surveillance purposes and for distinguishing strains during outbreaks. The method is a time-consuming and demanding task with several limitations among which cross reactivity of the antisera or non-typeability of many strains. In Romania, by virtue of the fact that O157:H7 clone has been considered to cause the vast majority of severe infections, O157 serotyping remains the most commonly employed method to screen for VTEC in the clinical laboratories. Mostly, owing to the cost of testing, the screening, performed only if a specific request is received and restricted to O157 antigen, is not confirmed by titration. Moreover, nucleic acid amplification methods that can rapidly determine the presence of genes encoding determinants for major virulence factors such as verocytotoxin genes are not routinely used.

This report chose to describe the practices used by the reference laboratory to confirm the identity of a presumptive VTEC *E. coli* strain and the challenges posed to the identification achieved by both phenotypic and genotypic methods. The definitive characterization of the strain relied on traditional and molecular serotyping coupled with an assay for specific virulence genes enabling the determination of O- and H-group, pathotype, and the strain's pathogenic potential.

The PCR-based virulence genotyping targeted specific virulence factors of the major diarrheagenic *E. coli* groups [1], including the *eae*, *vtx1* and *vtx2* genes. The intimin encoding gene is part of a pathogenicity island designated locus of enterocyte effacement (LEE), integrated in the chromosome of different clonal lineages represented mostly by enteropathogenic *E. coli* (EPEC) but also by some VTEC strains. Intimin is required for intimate bacterial adhesion to epithelial cells inducing a characteristic histopathological lesion defined as "attaching and effacing" (A/E) [9]. The phage-encoded genes *vtx1* and *vtx2* are found specifically in VTEC conferring the ability to produce verocytotoxins (also known as Shiga toxins), the major virulence factors responsible for the damages of several organs as kidney, brain, liver, and pancreas [10]. The investigated strain displayed a genotype that qualified it as EPEC based on the presence of *eae* gene and lack of *vtx* genes. Moreover, the absence of the plasmid-encoded enterohemolysin, a putative virulence factor associated with VTEC, confirmed the identification [11]. The strain failed to agglutinate with all the commercial antisera used against the most common EPEC and VTEC serogroups, including O157 serogroup. The molecular serotyping, performed as indicated by two previously published protocols, confirmed that the strain did not belong to the O157:H7 serotype. Yet, the PCR-based protocol targeting only the O157 antigen generated an amplicon with a higher-than-expected molecular weight (Fig. 1) which further sequenced proved to be a 161-bp DNA fragment with 100% similarity with the *adhE* gene encoding a multifunctional protein of *E. coli*, amplified due to mis-priming [12]. Consequently, the studied strain did not belong to O157:H7 serotype and, moreover, lacked the *vtx* genes characteristic of VTEC. Nevertheless, it qualified as an EPEC that could have caused diarrhea especially since the infected patient was a three-year old child [1]. Regarding the strain's sorbitol negative phenotype, although usually considered as highly sensitive and predictive for O157:H7 clone, it was also observed in a small percentage of *E. coli* strains belonging to other serotypes and pathotypes such as EPEC [13]. This EPEC strain was such an example. Therefore, the *E. coli* O157 could be falsely identified by the screening procedure based on conventional serotyping and the inability to ferment sorbitol. The confirmatory tests rejected the suspicion and prevented inappropriate and unnecessary public health measures. At the same time, the inconclusive result obtained in one of the molecular serotyping assays underlined that none of

the aforementioned diagnosis approaches is without drawbacks.

CONCLUSIONS

Essentially, there is no absolute characteristic of pathogenic *E. coli*, and therefore testing algorithms need sufficient inclusivity to capture emerging strains. While the schemes of microbial detection and characterization may use various combinations of testing methods, depending on the laboratory level, the ultimate goals for public health investigations are aimed at specifically identifying the true pathogenic strains.

Fig. 1. Results of the PCR for wzx_{O157} gene (amplicon size 133 bp). Lane 1: PCR product generated by the presumptive O157 *E. coli* strain, lane 2: Positive control (O157:H7 strain), lane 3: negative control (H_2O), lane 4: 100-bp DNA ladder

Conflict of interests: No conflict of interest to declare.

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