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Original Article

Serogroups and genetic diversity of diarrheagenic strains of *Escherichia coli*: a retrospective study

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Abstract

Introduction: Diverse serogroups of *Escherichia coli* cause sporadic cases and outbreaks of diarrhea among children. Our study aimed to evaluate the serogroups of diarrheagenic strains of *E. coli* that cause diarrheal disease in children under two years old, and clarify if the cases were sporadic or outbreaks.

Methodology: The retrospective study included 130 strains of pathogenic *E. coli*, isolated from children who were less than two years of age, and had diarrheal disease, between May 2016 and July 2019. The study was conducted in the Bacteriology Laboratory (County Clinical Hospital, Mureș, Romania). The 130 strains were sero-grouped using polyvalent and monovalent O antisera. Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) was performed to evaluate the similarity between different *E. coli* strains, and a simplex polymerase chain reaction (PCR) was performed to detect the presence of the *hlyA* gene that is specific to the enterohemorrhagic strains.

Results: After agglutination with polyvalent O antisera, slightly more than half of the strains (50.77%) were sero-grouped as Shiga toxin-producing *E. coli* (STEC), and the rest of the strains belonged to the Enteropathogenic *Escherichia coli* (EPEC) serogroups. Serogroup O157 was the most frequently identified (16.51% of the total number of typeable strains), and one strain was positive for *hlyA*. ERIC-PCR revealed a high diversity of strains, with an overall 50% similarity.

Conclusions: STEC serogroups were the most common strains causing diarrheal disease, and O-157 was the dominant serogroup identified. The strains included in our study presented high genetic diversity, suggesting that most of the cases were sporadic.

Key words: Diarrhea, *Escherichia coli*, EPEC, serogroup, PCR.

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Introduction

Acute diarrheal disease is a major cause of death worldwide. Although health education has improved and access to medical services is increasing, the acute diarrheal disease continues to be one of the most commonly encountered pathologies, especially in pediatric departments [1,2].

Enteropathogenic *Escherichia coli* (EPEC) causes watery diarrhea in children, and the pathogenicity of these strains is determined by the presence of an adhesin (intimin, encoded by the *eae* gene), or by the presence of a plasmid-encoded protein (EAF-EPEC adherence factor), both facilitating bacterial adhesion to the intestinal wall. In addition to EPEC, Shiga toxin-producing *E. coli* (STEC) strains may be involved in the etiology of diarrheal disease in children. The in vivo behavior of these bacterial strains differs depending on the type and complexity of the virulence factors, which also influences the clinical evolution of the disease [3–5].

Although *E. coli* is one of the most characterized bacteria (at phenotypic and molecular levels), it continues to be a major public health issue, causing serious infections in both adults and children. Previous studies have used different approaches for the classification and diagnostic protocols of the diarrheagenic strains of *E. coli*. O and H serotyping is still frequently used for identification due to its accessibility, and is especially useful for detecting pathogenic serotypes and outbreaks. However, this method is considered laborious due to the high diversity of antigens (53 H antigens and more than 187 O

serogroups), and misidentification of serogroups and false-negative results have been reported [6–10].

Diarrheagenic pathotypes include EPEC, STEC, Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), Diffusely Adherent *E. coli* (DAEC) or Adherent-Invasive *E. coli* (AIEC). 'Hybrid' pathotypes are also described, including Enteroaggregative Hemorrhagic *E. coli* (EAHEC) that exhibits virulence genes associated with STEC and EAEC (EAHEC serotype O104:H4, EAEC). Although all EHEC strains produce Shiga toxin (STEC), not all Shiga toxin-producing strains are classified as EHEC. The STEC strains rarely cause severe syndromes, such as hemolytic uremic syndrome, because virulence factors (hemolysin) are absent. Serotypes O157:H7 and O103:H21, although sometimes classified as STEC, are in fact EHEC, with specific virulence factors.

Recent studies recommend that O:H serotyping needs to be confirmed with tests that can identify the specific virulence factors for each strain (escV for EPEC and STEC; bfpB for EPEC; stx1, stx2 for STEC; elt, estIa, estIb for ETEC; invE for EIEC; astA, aggR, pic for EAEC) [11–13].

Our study aimed to evaluate the serogroups of diarrheagenic strains of *E. coli* that cause diarrheal disease in children under two years old, and clarify if the cases were sporadic or outbreaks.

Methodology

The study included 130 pathogenic strains of *Escherichia coli*. The strains were collected between May 2016–July 2019 from diarrheic stools (originating from 0–2 years old children), in the Bacteriology Laboratory, County Clinical Hospital, Mureș, Romania (Ethics committee approval number: 16868/23.10.2020).

Serotyping

Stool samples collected from the children were inoculated on specific culture media used for routine stool culture: McConkey agar, Salmonella-Shigellaagar, and McConkey with sorbitol agar. The screening for EPEC was performed by randomly picking at least 6 lactose-positive colonies (with different morphotypes) from McConkey agar. The chosen colonies were isolated on nonselective media and then serotyped. Agglutination was performed to assess the serogroups. First, polyvalent O antisera (OK O pool antisera, SSI Diagnostica, Hillerød, Denmark) against pathogenic *E. coli* EPEC/STEC: Pool1 (O26, O103, O111, O145, O157) – STEC, Pool2 (O55, O119, O125ac, O127, O128ab) – EPEC, Pool3 (O86, O114, O121, O126, O142) – EPEC were used. The strains were stored in glycerol stocks at -70 °C. Subsequently, the strains were typed with monovalent sera (OK O single antisera, SSI Diagnostica, Hillerød, Denmark) corresponding to each group, as determined by the three polyvalent pools.

Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR)

The *E. coli* DNA was extracted using the boiling method. A bacterial colony from the overnight culture was mixed by vortexing for 15 seconds with 500 µL DNase free water in a sterile 2 mL microcentrifuge tube. The bacterial suspension was heated to 99 °C for 20 minutes, in a thermomixer. Immediately after, the suspension was frozen for 10 minutes at -20 °C, followed by centrifugation at 16000 x g for 5 minutes at 4 °C. Finally, 300 µL of the supernatant containing DNA was transferred to a new microcentrifuge tube and used as a DNA template.

The primers used for ERIC-PCR were ERIC1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) [14,15]. The PCR reaction was performed in a final volume of 25 µL containing the following: Master Mix 12.5 µL (DreamTaq Green PCR 2X, containing DreamTaq DNA polymerase, 2X DreamTaq Green buffer, dATP, dCTP, dGTP and dTTP, 0.4 mM each, and 4 mM $MgCl_2$), 9.5 µL DNase free water, 0.4 µM of each forward and reverse primers and 1 µL DNA template. The amplification was performed using the MiniAmpTM Thermal Cycler (Applied Biosystems, Waltham, MA, US): initial denaturation (5 seconds, 95 °C), 30 amplification cycles of denaturation (94 °C, 30 seconds), annealing (52 °C, 60 seconds), extension (72 °C, 2 minutes) followed by and a final extension (72 °C, 8 minutes). The amplicons were visualized by electrophoresis in 2% agarose gel containing 1 µL GelRed® nucleic acid gel stain (Biotium, Inc., Hayward, USA), in 1X TAE buffer, at 65 V for 2 hours. A DNA marker was used for each electrophoresis run (GeneRuler 100 bp DNA ladder, Thermo Fisher Scientific, Waltham, MA, USA)). The dendrogram was generated using GelJ v.2.0 software, based on the Dice similarity coefficient and the unweighted pair group method with arithmetic mean (UPGMA).

PCR

For the 18 strains described as O157, genomic DNA was extracted by the boiling method. For simplex PCR, the following primers GTAGGGAAGCGAACAGAG

Table 1. The distribution of the diarrheagenic *E. coli* strains that agglutinated with monovalent O sera.

Strains	Total	Pool 1	Pool 2	Pool 3
Number of strains that agglutinated with polyvalent O antisera	130	66 (50.77%)	29 (22.31%)	35 (26.92%)
Positive agglutination with monovalent O antisera (Number, % of total)	109 (83%)	58 (44.62%)	23 (17.69%)	28 (21.54%)
Positive agglutination with monovalent O antisera (% of pooled number)		87.88%	79.31%	80%
Negative agglutination with monovalent O antisera (Number, % of total)	21 (16.15%)	8 (6.15%)	6 (4.62%)	7 (5.38%)
Negative agglutination with monovalent O antisera (% of pooled number)		12.12%	20.69%	20%

(*hlyA* F) and AAGCTCCGTGTGCCTGAA (*hlyA* R) were used. The reaction was carried out in a 25 µL mixture of DreamTaq Green PCR Master Mix 2X (Thermo Fisher Scientific, Waltham, MA, USA) (12.5 µL containing DreamTaq DNA polymerase, 2X DreamTaq Green buffer, dATP, dCTP, dGTP and dTTP, 0.4 mM each, and 4 mM $MgCl_2$), DNase free water (9.5 µL), primers (0.4 µM of each forward and reverse primers) and genomic DNA (1 µL): denaturation (4 minutes, 94 °C), 30 cycles of denaturation (94 °C, 45 seconds), annealing (55 °C, 60 seconds), extension (72 °C, 1 minute) followed by a 7-minute extension (72 °C). The PCR products were separated on 1.5% agarose gel electrophoresis for 1 hr at 100 V and visualized using GelRed.

Statistical analysis

GraphPad QuickCalcs (on-line software, https://www.graphpad.com/quickcalcs/) was used for the statistical analysis (Fisher's test).

Results

Serotyping

Slightly more than half of the 130 tested strains were identified as pool 1 (66, 50.77%) after agglutination with polyvalent O antisera. The rest of the strains were distributed almost evenly in the serogroups of pool 2 (29, 22.31%) and pool 3 (35, 26.92%).

When the strains were retyped with monovalent O sera, the reaction was positive only for 83% of the tested strains. The number of strains that did not present positive agglutination for monovalent sera was similar in pool 1, pool 2, and pool 3 strains (8, 6, and 7 strains respectively) (Table 1).

Most of the strains were included in the pool 1 serogroups (STEC) (Table 2). O157 (pool 1) included 18 strains (16.51% of the total number of strains), followed by O145 (pool 1) and O26 (pool 1), both with 14 strains (12.84%, of the total number of strains). The smallest number of strains belonged to serogroup O125ac (pool 2, 2 strains) and O114 (pool 3, 1 strain).

Out of all the strains included in pool 1, the number of strains identified as O157 was significantly higher than the number of strains included in O103 (p = 0.0227) and O111 (p = 0.0045). Among the strains included in pool 2, the number of strains included in serogroup O127 was significantly higher than the other serogroups (p values: 0.0074 to 0.023). Among the strains included in pool 3, the number of strains identified as O86 was significantly higher than the

Table 2. The distribution of the diarrheagenic *E. coli* strains after serotyping with monovalent O sera.

Pool	Serogroup	Strains (number)	Strains (% from the total number of typeable strains)	Strains (% from the number of typeable strains included in each pool)
1	O26	14	12.84%	24.14%
	O103	7	6.42%	12.07%
	O111	5	4.59%	8.62%
	O145	14	12.84%	24.14%
	O157	18	16.51%	31.03%
2	O55	2	1.83%	8.7%
	O119	3	2.75%	13.04%
	O125ac	2	1.83%	8.7%
	O127	11	10.09%	47.83%
	O128ab	5	4.59%	21.74%
3	O86	11	10.09%	39.29%
	O114	1	0.92%	3.57%
	O121	9	8.26%	32.14%
	O126	3	2.75%	10.71%
	O142	4	3.67%	14.29%

number of strains identified as O114 (p = 0.0023) and O126 (p = 0.0286). There were no statistically significant differences between the number of strains identified as O157, O127, and O86, the serogroups that included the largest number of strains in each pool (p values: 0.2 to 0.58).

ERIC-PCR

ERIC-PCR provided fingerprints with a variable number of band patterns (1-13 bands) with molecular weights between 130 and 1300 bp. Most of the bands were observed between 200-500 bp, with the most prevalent band being at 320 bp. Most isolates presented 5 bands (29 strains), 6 bands (26 strains), or 4 bands (22 strains) (Figure 2). The dendrogram indicated a high diversity of strains, with an overall 50% similarity (Figure 3).

If the threshold was set at 60% similarity, the dendrogram yielded only 15 clusters (defined as A–O in Figure 3). In this case, there was only 1 cluster with 1 strain (H) and 3 clusters that included 2 strains (F, K, N). Cluster A included 57 strains (43.84%), most of them from pool 1 (29), followed by pool 2 (17) and pool 3 (11) strains. Serogroup O 26 represented 17.54% (10 strains) cluster A, while serogroup O 86 represented 8.77% of this cluster.

Taking into account a 70% similarity threshold, the dendrogram yielded a large number of clusters (52, defined as 1–52 in Figure 3). Thus, 15 (28.8%) clusters included only 1 strain and 14 clusters included 2 strains, representing one-third (33%) of the tested strains. Only

Figure 2. Representative image for ERIC-PCR patterns on agarose gel electrophoresis.

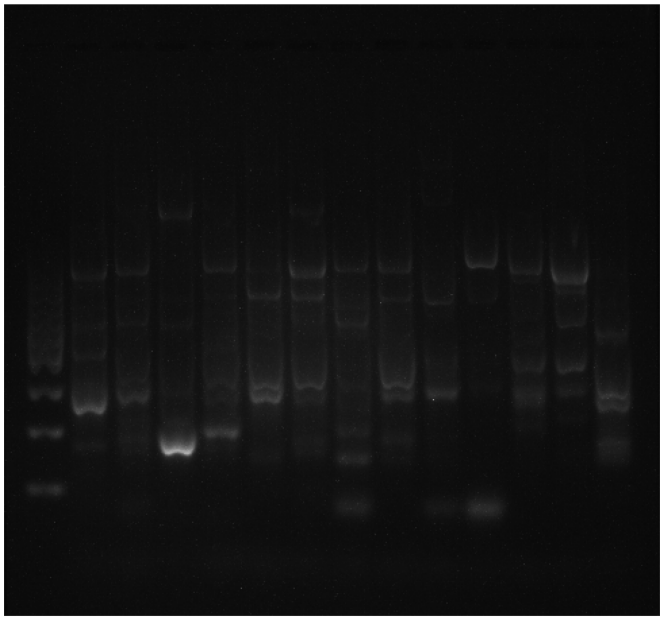
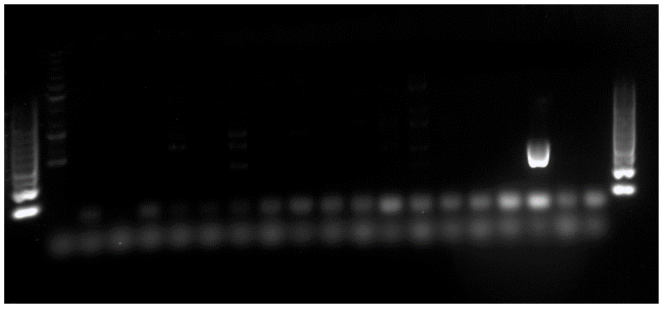


Figure 1. Simplex PCR electrophoresis gel image; strain 113 – positive band for the hlyA gene.



2 of the clusters with 2 strains included similar serogroups (cluster 19 – O157 and cluster 50 – O126). The largest clusters were the fifth (12 strains) and 6th (9 strains), but both included heterogeneous serogroups.

Identical strains (100% similarity) were observed in only two cases: strains 109 and 111 (both belonging to serogroup O121, pool 3) and strains 134 and 140 (both belonging to serogroup O86, pool 3).

PCR

The *hlyA* gene was amplified from only one out of the 18 strains that were identified as O157 (Figure 1), thus confirming that this strain was enterohemorrhagic.

Discussion

Theodor Escherich used the name *Bacterium coli commune* in his 1885 publication (reprinted in 1998), to describe a short rod, isolated from stools of infants; it was later named *Escherichia coli*, in 1954 [16–19]. The first serologic classification was proposed by Kauffman in 1944; these rules are still the basis for the modern modified protocols recommended for serotyping *E. coli* by somatic (O), flagellar (H), and capsular (K) surface antigens. A serotype of an *E. coli* strain is defined by a combination of O and H antigens. Serotyping is a widely used conventional method (once considered the gold standard method), that detects the O and H antigens for *E. coli* types. Recent studies draw attention to the multiple disadvantages of this method (laborious, expensive, non-typeable strains, cross-reactions), yet it is still a choice for many laboratories, mostly because of its availability. Agglutination (using antisera from rabbits) is an easy screening technique for detecting of *E. coli* pathotypes, while the more accurate and specific molecular methods are not available for routine diagnostics [2,10,19–22].

To reduce costs, some laboratories choose to screen for diarrheagenic *E. coli* groups using commercial polyvalent ‘pooled’ antisera. The reaction gives a

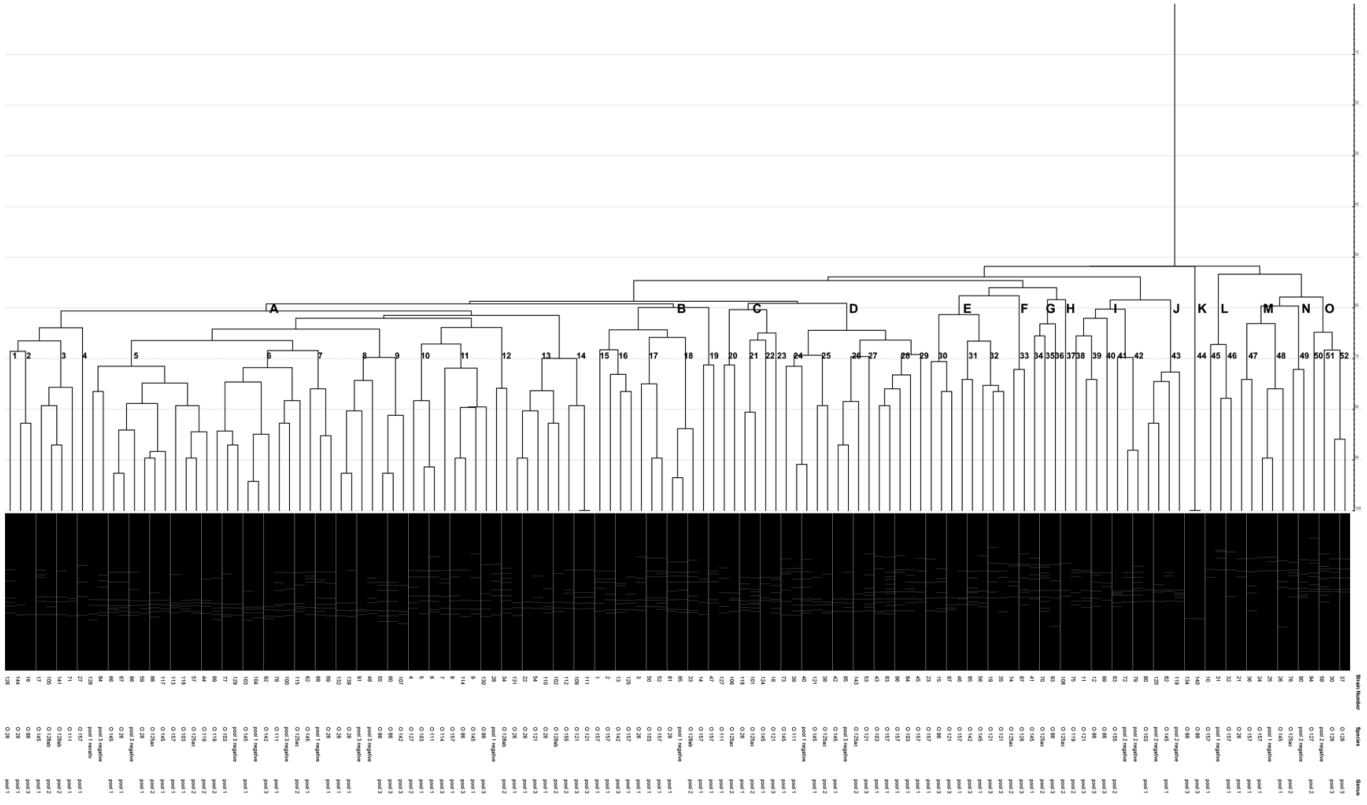
positive result if one of the antigens corresponding to the pooled antibodies is detected. Thus, in the first stage of this study, polyvalent antisera against pathogenic *E. coli* EPEC/STEC, pool 1 (O26, O103, O111, O145, O157) – STEC, pool 2 (O55, O119, O125ac, O127, O128ab) – EPEC, pool 3 (O86, O114, O121, O126, O142) – EPEC were used.

Interestingly, after this screening, 50.77% of the strains were identified as pool 1 (which contains STEC strains), despite the fact that all strains were isolated from children under 2 years of age. We expected to find strains that belong especially to EPEC-related O serogroups, as these are considered to be among the most important agents that cause persistent diarrhea (community, as well as nosocomial) in children worldwide [19,20,22].

The frequency of identifying EPEC as a cause of diarrhea is influenced by various factors, including geographic region, socioeconomic status, and diagnostic methods. EPEC is still considered the leading cause of diarrhea among little children in developing countries [23–25]. In developed countries, the prevalence of EPEC is decreasing, probably because molecular diagnostic techniques are used more often [3,26]. In a recent study, published in 2016 by Canizalez-Roman *et al*., diarrheagenic *E. coli* was the most common cause of diarrhea in children under 2 years of age, with EAEC strains being involved in almost half of the cases [27]. Another recent study by Zhou *et al*. in 2018, identified EPEC as the most frequent diarrheagenic pathotype, atypical EPEC being considered a dominant etiological agent for diarrhea in children from Central China [28]. In contrast, a study published by Chen *et al*. in 2014 identified EAEC as the most prevalent pathotype in southeastern China [29].

Serotyping for *E. coli* O and H antigens is a technique used to identify pathogenic strains, but due to the large variety of antigens, many strains remain uncharacterized [11,30]. When the strains included in our study were typed with monovalent O antisera, only 83% were assigned to O serogroups, while 21 remained unidentified, even when reaction with polyvalent antisera was positive. Two studies, published in 2013 and 2014 by Feng and Reddy, found that over 50% of the STEC and ETEC strains could be partially characterized or not characterized at all [31,32]. In another study, published in 2017 by Fierz *et al*., only 82.1% of the tested STEC strains were identified as O serotypes, using a molecular diagnostic protocol [33]. Even if our results are supported by data from the literature, it is worth mentioning that polyvalent antisera agglutination was performed as a part of

Figure 3. Dendrogram presenting the similarity between ERIC-PCR band patterns.



routine laboratory diagnostic, and all positive strains were stored in -70 °C, during the three years of study. Agglutination with monovalent antisera was performed at the end of the collection period, but even if it has been made from fresh overnight cultures, there is a possibility that the bacterial structure was affected by the prolonged storage time.

STEC was the most frequent group of diarrheagenic *E. coli* in our study. In fact, after agglutination with monovalent antisera, the most frequently identified (18 strains were in the O157 serogroup, 16.51% from the total number of typeable strains, 13.84% from the total number of strains), followed shortly by serogroups O145 and O126 (14 strains each, representing 12.84% from the total number of typeable strains and 10.76% from the total number of strains). One of the strains identified as O157 was *hlyA* positive, and therefore an enterohemorrhagic strain. Falup-Pecurariu *et al*., also identified STEC as the etiological agent for 6.4% of cases of diarrhea in children in Romania, in 2019 [5]. A systematic review published in 2018 by Valilis *et al*. found that the five serogroups that we identified as STEC, were involved, among other serogroups, in numerous (674) worldwide outbreaks (between 1995 and 2017) caused by non-157 STEC strains. During the same period, non-157 STEC strains caused more than 2700 cases in endemic settings around the world; in 8.7% of these cases hemolytic uremic syndrome appeared; serogroup O26 was the most frequent etiological agent involved in outbreaks and in the endemic cases [34].

O127 and O86 were the most prevalent among the EPEC serogroups identified in our study, and were represented, by 11 strains each (10.09% from the total number of typeable strains and 8.46% from the total number of strains). A recent study by Usein *et al*. identified a large set of diverse O serogroups among EPEC strains, including O26 and O157, using molecular methods [35]. These studies highlight the importance of implementing molecular methods for routine diagnostic and epidemiological purposes (community or nosocomial outbreak) since serotyping can provide frequent cross reactions and mis-identification.

Based on decades of epidemiological research, ERIC-PCR can be considered as a method of choice, because it is a simple and rapid technique that provides information about the similarity/clonality in different bacterial strains. The technique is based on amplification of genomic DNA sequences (127 bp imperfect palindromes) included between conserved repetitive regions. The distribution of these sequences varies between different species and between strains from the same species[14,36,37].

In our study, ERIC-PCR provided fingerprints with a variable number of PCR products ranging in size from 130 bp to 1300 bp. The most frequently encountered products were between 200–500 bp. Ramazanzadeh *et al*. in 2013 published similar findings while characterizing *E. coli* strains isolated from a hospital environment. Their study identified electrophoretic fingerprints with 6-15 bands, ranging from 100 to 1400 bp. The most frequently identified bands were between 200 and 800 bp [38]. ERIC-PCR is not considered a highly discriminatory technique, especially for strains typed under different conditions. To confirm similarities between different strains, PFGE (Pulsed Field Gel Electrophoresis) is the "gold standard" method, but this laborious and expensive method is not available, or practical, for most laboratories [39]. PCR methods, such as ERIC-PCR or REP-PCR (repetitive extragenic palindromic-PCR) are cheaper, faster, more easily available, therefore, these can be considered as good options for screening for the clonality of the isolates, based on their discriminatory power [40–42]. Even so, the diversity of the ERIC-PCR fingerprints is not generated only by the technique, it can be influenced, also, by the high diversity between *E. coli* strains, that are often isolated from different sources. As an example, in a study published in 2017, the authors typed by ERIC-PCR strains of *E. coli* isolated from animal feces; they observed fingerprints with 0 to 46 bands, ranging from 380 bp to 3280 bp, with the predominant fragments of 1200 and 2900 bp, very different from those obtained in our study and the study published by Ramazanzadeh *et al*. [43].

As expected, because the strains included in our study were collected over three years, they presented a high diversity with an overall similarity of only 50%, suggesting that most of these cases were sporadic, caused by nonrelated strains. There was no suspicion of clonal distribution or epidemic outbreak. When the similarity threshold was set at 70% (as most ERIC-PCR protocols recommend), the dendrogram revealed a large number of clusters (50), almost a third of them being represented by clusters with only one or two strains. Similar high diversity was observed in other studies, regardless of the chosen technique for typing *E. coli* strains [39,44].

Conclusions

STEC serogroups were the most frequent diarrheagenic *E. coli* strains that caused diarrheal disease in children under two years of age in our study.

Serogroup O157 was the dominant serogroup. The strains included in our study presented a high level of genetic diversity, indicating that most of the cases were sporadic.

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Ethics approval: The study protocol was approved by the Mureș County Clinical Hospital Local Ethics Committee (no. 16838/23.10.2020).

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