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Phylogenetic Groups/B2 Subgroup Distributions, Serogrouping and Identification of Virulence Factors in Extended-Spectrum Cephalosporin-Resistant *Escherichia coli* Strains Isolated from the Stool of Healthy Children Under 10 Years Old

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Abstract

Background: Segregation of *Escherichia coli* (*E. coli*) into the phylogenetic groups was observed in the experiments so that group B2 contained the enteropathogenic *E. coli* (EPEC) strains and extraintestinal pathogenic *E. coli* (ExPEC).
Objectives: This study aimed to identify B2 phylogenetic groups in the extended-spectrum Cephalosporins resistant *E. coli* isolated from the stool of healthy children under 10 years old.
Methods: One hundred *E. coli* resistant to broad-spectrum Cephalosporins were collected from the feces of healthy children under 10. Subsequently, we grouped phylogenetic via PCR based on the genes *yjaA*, *chuA*, *arpA*, as well, as *TspE4.C2*. Then, according to Clermont et al.'s study, we used two individual multiplex PCRs for identifying B2 sub-groups (I-X subgroups). Serogroup typing with the 12 O-antigen was analyzed via PCR, and finally, 10 virulence genes (*cnf1*, *papG*, *ibeA*, *malX*, *usp*, *cdt*, *eae*, *bfp*, and *afa-Dr*) were identified with PCR.
Results: The age range of the healthy children was between 1 and 10 years. The B2 and unknown phylogroups were the most common strains in this study. The most common B2 subgroups were I (2%), IX (1%), V (8%), IV, V, VII (1%), IX, V (3%), IX, V, III, I (1%), IX, V, III, VII, I (%1), V, I (6%), V, III, I (3%), and V, III, VII (1%), with each subgroup carrying distinctive sets of ExPEC virulence markers. The results also showed that 29% of *E. coli* in the healthy children had *malX* and 23% had *papGII*. It was also found that 32% of the strains isolated from the healthy children had antigens O2 and 36% were unknown.
Conclusions: In this study, 27% of the strains belonged to B2 phylogroup and 6% to B1 phylogroup. Moreover, serogroups O2, O16, and O25 were predominant and belonged to B2 phylogroup. Moreover, *malX*, *papGII*, *usp*, *papGIII*, *aggR*, and *eae* virulence genes also had the highest to lowest supply among the tested strains, respectively. Moreover, B2 isolates were shown to have further virulence-related genes in comparison to the non B2 isolates.

Keywords: *EPEC*, B2 phylogroup, Cephalosporins Resistant, Healthy Children

1. Background

Escherichia coli is a commensal bacterium living in the intestinal tract of animals and humans (1). In particular, adherent-invasive *E. coli* (AIEC) pathotype was one of the key bacterial triggers of IBD (2). Certain strains are the member of the extraintestinal pathogenic *E. coli* (ExPEC). Besides, it is possible to classify *E. coli* strains into the phylogenetic groups, such as B1, A, C, B2, E, D, F, as well as clade I/II (3). Moreover, the ExPEC strains, in which diverse virulence factor genes are used to characterize, are the members of D and B2 phylogenetic groups fundamentally. Most *E. coli* types, including EPEC (carrying *bfp* and *eae* genes), may be commonly assigned to 1 of the 4 clusters of B1, A, D, as well as B2 (4). Among *E. coli* phylogenetic groups, B2 phylogroup is believed to be more important than others. These strains indicate more ability to persist in the gut microbiota compared to the ones in other *E. coli* phylogenetic groups, which may be caused by accumulating pathogenicity islands (PAIs) as well as ExPEC virulence genes (5). Furthermore, B2 strains have at least 10 phylo-

genetic sub-groups (I–X) or lineages, including I (STc131), II (STc73), III (STc127), IV (STc141), V (STc144), VI (STc12), VII (STc14), VIII (STc452), IX (STc95), and X (STc372) (6). Studies indicated the predominance of subgroups I (STc131), II (STc73), as well as IX (STc95) in a majority of ExPEC strains (7). The present study subtyped the phylogenetic group B2 strains previously isolated from the commensal feces of healthy children. Their serogroups, virulence markers, as well as abilities to persist in the microbiota are associated with certain B2 groups.

Antimicrobial resistance such as third-generation cephalosporin-resistant (3GCR) in the commensal bacteria and acquiring virulence factors by these strains is worrisome in terms of its ability to spread to pathogens. These challenges result in increased healthcare costs and mortality. However, there is no sufficient data on the antimicrobial resistance profile, the virulence traits of these commensal strains, especially in a developing country like Iran. Therefore, the present study involves the identification profiles of these isolates from the stool of healthy children under 10 years old (8, 9).

2. Objectives

This study aimed to identify B2 phylogenetic groups in the extended-spectrum Cephalosporins resistant *E. coli* isolated from the stool of healthy children under 10 years old.

3. Methods

3.1. Ethical Statements and Bacterial Isolate

We performed this cross-sectional study in Tarbiat Modares University, Tehran, Iran, from January 2020 - February 2021. According to the research design, we examined 100 strains of *E. coli* resistant to broad-spectrum Cephalosporins (CTX, CAZ = 100% and ESBL genes (TEM = 26%, *CTX-M1* = 98%, SHV = 51%) isolated from the feces of healthy children under 10 years (57 males and 43 females). Subsequently, all the strains were transferred in Tryptic Soy Broth and incubated at 37°C overnight. In the next step, we streaked these strains on the MacConkey agar and performed incubation at a temperature of 37°C for 24 hours, and finally, pink colonies were subcultured on EMB (Eosin Methylene Blue), so that the colonies over them exhibited a green-metallic sheen color. Ultimately, we used a set of biochemical experiments for further confirmation. Then, in order to store them for a longer period, we used a tryptic soy broth consisting of 20% glycerol (provided by Merck Company: Germany) for storing the treated isolates at a temperature of -20°C.

3.2. Virulence Typing

Through the use of primers and PCR, particular genes were specified: cytolethal distending toxin (*cdt*), cytotoxic necrotizing factor 1 (*cnf1*), invasion of brain endothelium (*ibeA*), secretion autoinducer toxin (*sat*), uropathogenic-specific protein (*usp*), pathogenicity island marker (*malX*), Dr-antigen binding adhesins (*afa/Dr*), bundle-forming pili (*bfp*), shiga-like toxins (*stx*) intimin (*eae*), and transcriptional activator *aggR*. Table 1 presents the primers and temperature conditions.

3.3. Phylotyping Method and Detection of Phylogroups in Isolates

In this step, we applied PCR based method as described by Clermont et al. for determining nine phylogroups, e.g., B1, A, C, B2, E, D, F as well as clade I/II. Moreover, PCR was employed to amplify the key targeted genes such as *yjaA*, *chuA*, *arpA*, and *TspE4.C2*. To characterize the phylogroups E and C, we utilized the allele-specific PCR primers (3). Table 2 depicts the primers and temperature conditions.

3.4. Subtyping of B2 Subgroups

Utilizing PCR method, B2 subgroups, including I (STc131), II (STc73), III (STc127), IV (STc141), V (STc144), VI (STc12), VII (STc14), IX (STc95), and X (STc372)) were performed for 27 strains that were determined in Phylogrouping stage. Table 3 represents the primers and the product size.

3.5. Amplification of O-Serogroup

According to the analysis, the commonest serogroups of the extraintestinal *E. coli* entail O2, O1, O6, O4, O12, O7, O16, O15, O25, O18, O157, as well as O75 antigens that were examined with PCR. Table 4 reports the primers and the product size.

3.6. Statistical Analyses

SPSS21 and *t*-test were employed for analyzing the obtained data. Moreover, the confidence interval was considered to be 95% (CI = 95%), and P-value < 0.05 was considered to be significant.

3.7. Ethical Statement

As mentioned earlier, the present research was verified by the Ethical Committee of the Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran (IR.MODARES.REC.1399.085).

Table 1. Primers and Amplicon Size (bp) for Detection of Virulence Genes

Target Gene	Primers, Genes, and Sequence (5'- 3')	Amplicon Size (bp)	Ref.
eae	F: GTAAGTCTCAAACGCAAGCAACCAC	229	(10)
	R: AACCTTGTTGTCAATTTTCAGTTCATCA		
bfp	F: AATGGTGCTTGCGCTTGCTGC	268	(10)
	R: GCCGCTTTATCCAACCTGGTA		
papGI	F: TCGTGCTCAGGTCCGGAATTT	461	(11)
	R: TGGCATCCCCCAACATTATCG		
papGII	F: GGGATGAGCGGGCCTTTGAT	190	
	R: CGGGCCCCCAAGTAACTCG		
papGIII	F: GGCCTGCAATGGATTTACCTGG	258	
	R: CCACCAAATGACCATGCCAGAC		
Stx1	F: TAAATCGCCATTCGTTGACTAC	180	(12)
	R: AGAACGCCCACTGAGATCATC		
Stx2	F: GGCACTGTCTGAAACTGCTCC	255	
	R: TCGCCAGTTATCTGACATTCTG		
cdt	F: TAAATGGAATATACATGTCCG	588	(13)
	R: TTTCCAGCTACTGCATAATC		
Cnf-1	F: GAACTTATTAAGGATAGT	543	(13)
	R: CATTATTTATAACGCTG		
ibeA	F; AGGCAGGTGTGCGCCGCGTAC	170	(14)
	R: TGGTGCTCCGGCAAACCATGC		
usp	F: CGGCTCTTACATCGGTGCGTTG	615	(15)
	R: GACATATCCAGCCAGCGAGTTC		
malX	F: GGACATCCTGTTACAGCGCGCA	930	(15)
	R: TCGCCACCAATCACAGCCGAAC		
aggR	F: GTATACACAAAAGAAGGAAGC	194	(16)
	R: ACAGAATCGTCAGCATCAGC		
afa-Dr	F: CGAAAACGGCACTGACAAG	230	(17)
	R: AGGCTTTCCGTGAATACAACC		

Table 2. Primers and Amplicon Size (bp) for Detection of Phylogroups

Target Gene	Primers, Genes, and Sequence (5'- 3')	Amplicon Size (bp)	Ref.
chuA	F: GAC GAA CCA ACG GTC AGG AT	279	(3)
	R: TGC CGC CAG TAC CAA AGA CA		
yjaA	F: TGA AGT GTC AGG AGA CGC TG	211	
	R: ATG GAG AAT GCG TTC CTC AAC		
TspE4.C2	F: GAG TAA TGT CGG GGC ATT CA	152	
	R: CGC GCC AAC AAA GTA TTA CG		
arpA	F: AACGCTATTCGCCAGCTTGC	400	
	R: TCTCCCCATACCGTACGCTA		

Table 3. Primers and Amplicon Size (bp) for Detection of B2 Subgroups

Primer Designation	Primer Sequences	Target	Sub-group	Product Size	Ref.
pabBgpII.F	5′-GAGTCACTGCCAGAAATTGCA-3′	*pabB*	II	415	(6)
pabBgpII.R	5′-GGCGAAAGGCTTAAAATTGCACT-3′				
trpAgpIII.F	5′-GACGCGCTGGAATTAGGCTC-3′	*trpA*	III	255	
trpAgpIII.R	5′-ATCGGCAACCAGCACCGAAT-3′				
dinBgpVI.F	5′-CAGCGGTGGAGATGCGCGAT-3′	*dinB*	VI	652	
dinBgpVI.R	5′-CAGCGGTGGAGATGCGCGAT-3′				
icdgpVII.f	5′-GCGGTATTCGCTCTCTGAAT-3′	*icd*	VII	810	
icdgpVII.r	5′-CAATTAAATCAGCCGCTTCG-3′				
aesgpIX.f	5′-CCTGGCCTGCAACGGGAG-3′	*aes*	IX	160	
aesgpIX.r	5′-TCTGGCTGCGGATAAAAGAG-3′				
putPgpI.f	5′-GGTATCGCTTACTTTAACGG-3′	*putP*	I	373	
putPgpI.r	5′-ACCACCGGACCAAACGCC-3′				
trpAgpIV.f	5′-TGCCAGTGGAAGAGTCCGCT-3′	*trpA*	IV	261	
trpAgpIV.r	5′-CCGGGGCGGAAATACCAAAG-3′				
polBgpV.f	5′-GCCGTTTCGCCGAAGATAAA-3′	*polB*	V	530	
polBgpV.r	5′-TAATGATCTTCAGCGCCTGT-3′				
aesgpX.f	5′-GACCGTTGTGAATACTCTTCA-3′	*aes*	X	713	
aesgpX.r	5′-TATAACAGGGCGGCACATTT-3′				
chuAgene.1	5′-CGATACGGTCGATGCAAAAG-3′	*chuA*	Internal control	1013	
chuAgene.2	5′-TTGGACAACATCAGGTCATC-3′				

4. Results

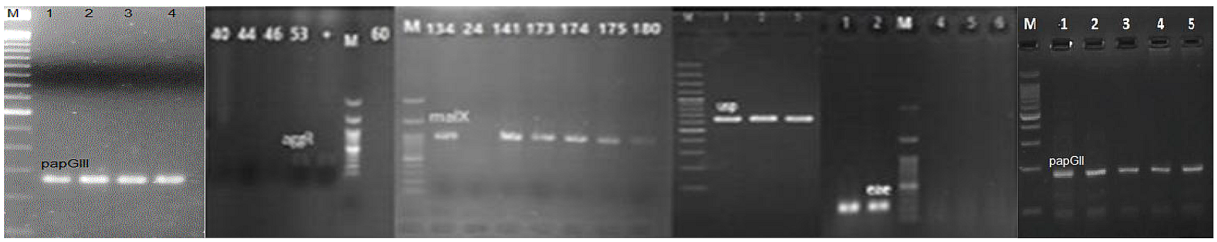
Of 100 isolates tested for virulence encoding genes, *papGII* 23% (n = 23), *papGIII* 8% (n = 8), *usp* 14% (n = 14), *aggR*, *eae* 1% (n = 1), *malX* 29% (n = 29), *bfp*, *stx1*, *stx2*, *cnf*, *afa-Dr*, *cdt*, *ibeA*, and *papGI* = 0 were recognized (Figure 1).

4.1. Detection of Phylogroups

As reported in Clermont et al.'s study, the researchers showed the generation of a specific profile by one of the quadruplex genotypes relative to the absence or presence of the 4 genes; for example, (+, -, +, -) demonstrates *arpA* (+), *chuA* (-), *yjaA* (+), and *TspE4.C2* (-). Considering this method, the percentage of phylogroup B1 was 6% (6/100), B2 was 27% (27/100), clade I/II was 1% (1/100), D/E was 16% (16/100), and E/clade I was 1% (1/100); also, 49% (49/100) of the strains were unknown. None of the isolates were recognized as phylogroups A, C, D, E, and F.

Table 4. Primers and Amplicon Size (bp) for Detection of O-Serogroup Amplification (O1, O2, O4, O6, O7, O12, O15, O16, O18, O25, O75, and O157)

Target Gene	Primers, Genes, and Sequence (5'- 3')	Ampliconsize (bp)	Ref
gndbis.f	5-ATACCGACGACGCCGATCTG-3	-	
rfb01.r	5-CCAGAAATACACTTGGAGAC-3	189	
rfb02a.r	5-GTGACTATTTCGTTACAAGC-3	274	
rfb018.r	5-GAAGATGGCTATAATGGTTG-3	360	
rfb016.r	5-GGATCATTTATGCTGGTACG-3	450	
rfb06a.r	5-AAATGAGCGCCCACCATTAC-3	584	
rfb07.r	5-CGAAGATCATCCACGATCCG-3	722	(18)
rfb04.r	5-AGGGGCCATTTGACCCACTC-3	193	
rfb012.r	5-GTGTCAAATGCCTGTCACCG-3	239	
rfb025a.r	5-GAGATCCAAAAACAGTTTGTG-3	313	
rfb075.r	5-GTAATAATGCTTGCGAAACC-3	419	
rfb015.r	5-TGATAATGACCAACTCGACG-3	536	
rfb0157.r	5-TACGACAGAGAGTGTCTGAG-3	672	



papGIII *aggR* *malX* *usp* *eae* *papGII*

Figure 1. PCR reactions for virulence encoding genes of *Escherichia coli* in all strains (*papGII*, *papGIII*, *usp*, *aggR*, *eae*, *malX* were positive), (*bfp*, *stx1*, *stx2*, *cnf*, *afa-Dr*, *cdt*, *ibeA*, *papGII* were negative); M: Ladder 1000 bp; *papGIII*, 258 bp; *aggR*, 254 bp; *malX*, 930 bp; *usp*, 615 bp; *eae*, 167 bp; *papGII*, 190 bp.

4.2. Analysis of B2 Subgroups

According to the analysis, the commonest B2 subgroups were determined among the *E. coli* strains in the healthy infants: I (2%), IX (1%), V (8%), IV, V, VII (1%), IX, V (3%), IX, V, III, I (1%), IX, V, III, VII, I (%1), V, I (6%), V, III, I (3%), and V, III, VII (1%).

4.3. Serogroup Typing in Escherichia coli Isolates

Table 5 presents the serogroup distribution in phylogroups of the *E. coli* strains isolated from the stool of healthy children below the age of 10. O2, O1, and O4 serogroups were the most abundant known serogroups among all the phylogroups, respectively. However, O157 was not in either of the strains (Figure 2).

4.4. Phylogenetic Groups and Pathogenicity Genes

Analysis of the relationship between virulence genes with the phylogenetic groups showed that the spread of each virulence gene enhanced largely in the phylogenetic group B2 than that in the remaining phylogenetic groups.

Table 6 demonstrates the relationship between phylogenetic groups and pathogenicity genes.

5. Discussion

The emergence and spread of third-generation cephalosporin-resistant *E. coli* in developing countries are a great concern. Healthy children colonized with ESBL-producing strains may transmit the resistant strains to other individuals, including the hospitalized patients (19). Based on four genes, namely *arpA*, *chuA*, *yjaA*, and *TspE4.C2*, *E. coli* strains could be categorized into the phylogenetic groups presented as follows: A, B1, B2, C, D, E, F, and clade I/II (3). Among these phylogroups, the phylogroup B2 mainly includes the strains which cause extraintestinal infections in humans as opportunistic

Table 5. Serogroup Distribution in Phylogroups of *Escherichia coli* Strains; B2 Phylogroups Had the Highest Serogroup and in Clade I/II, E/Clade I Phylogroups Had the Lowest Serogroups.

Phylogroups	Serogroups												
	O1	O2	O4	O6	O7	O12	O15	O16	O18	O25	O75	O157	Unknown
A	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	0	3	0	0	0	0	0	0	0	0	0	0	3
B2	1	5	1	0	0	0	0	5	1	2	1	0	11
C	0	0	0	0	0	0	0	0	0	0	0	0	0
D	0	0	0	0	0	0	0	0	0	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0	0
D/E	1	4	1	1	1	1	0	0	0	0	0	0	7
Clade I/II	0	1	0	0	0	0	0	0	0	0	0	0	0
E/clade I	0	0	0	0	0	0	0	0	0	0	0	0	1
Unknown	6	19	4	0	2	0	1	0	1	1	1	0	14

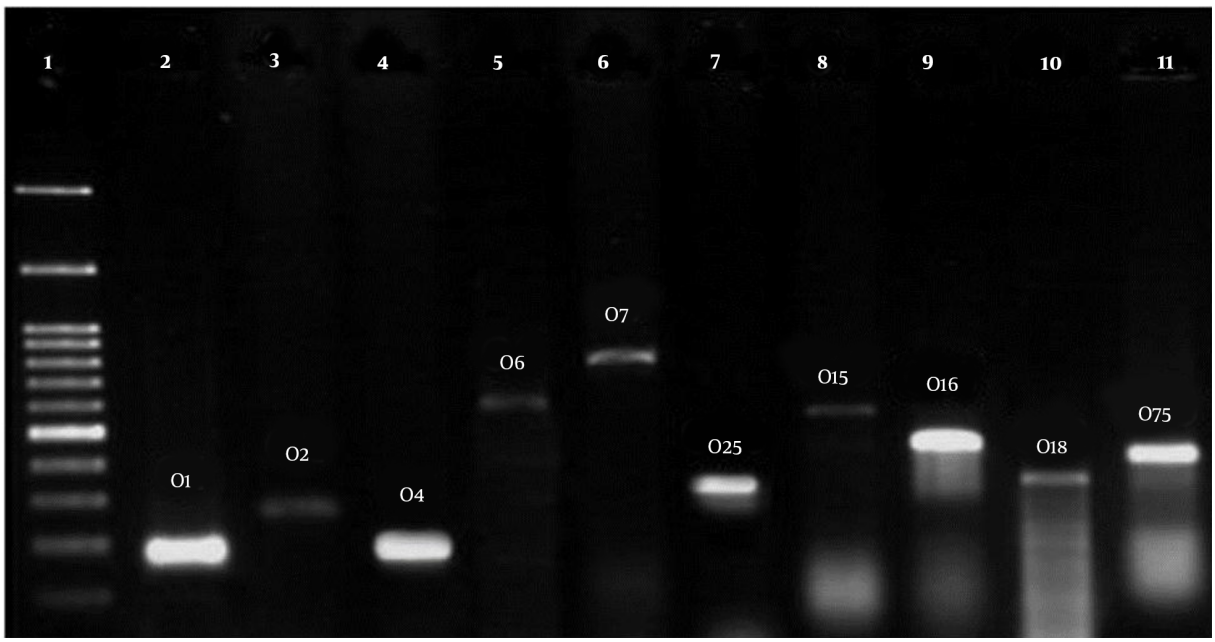


Figure 2. PCR reactions for Serogrouping of *Escherichia coli* in all strains (O1, O2, O4, O6, O7, O12, O15, O16, O18, O25, O75, and O157). Lane: 1, ladder 1000 bp; 2, 189 bp; 3, 274 bp; 4, 193 bp; 5, 584 bp; 6, 722 bp; 7, 313 bp; 8, 536 bp; 9, 450 bp; 10, 360 bp; 11, 419 bp.

pathogens (18). The present study was conducted to investigate the virulence factors, determine phylogroups, B2 subgroups, and serogroups of *E. coli* strains resistant to the broad-spectrum Cephalosporins isolated from the fecal samples of healthy children below 10 years of age. The study showed that 27% of the strains belonged to B2 phylogroup and 6% to B1 phylogroup. Among B2 phylogroup strains, the highest frequency belonged to subgroup V (*polB* genes). The *malX*, *papGII*, *usp*, *papGIII*, *aggR*, and *eae* virulence genes, respectively, had the highest to lowest supply among the tested strains. However, among B2 phylogroup strains, only *malX*, *papGII*, *usp*, and *papGIII* virulence genes were reported. We observed that 14 strains were positive for *usp* genes (is prevalent among strains causing urinary tract infections), one strain was positive for *eae* gene (a marker for EPEC pathovar), one strain was

Table 6. The Relationship of Phylogenetic Groups and Pathogenicity Genes

Phylogenetic Group	Virulence Gene Pattern (No. Isolate)
B1	*papGII* (3), *aggR* (1)
B2	*malX* (17), *usp* (10), *papGIII* (4), *papGII* (9)
Clade I/II	-
D/E	*malX* (2), *papGII* (2)
E/clade I	*papGII* (1)
Unknown	*malX* (10), *usp* (4), *papGII* (1), *eae* (1)

[a] B2 phylogroup has the highest virulence factors, and no virulence factors were observed in clade I/II.
[b] The *aggR* gene was observed in group B1 and *eae* in an unknown phylogenetic group, while *papGII* was common to all phylogroups.

positive for *aggR* gene (a marker for *EAEC* pathovar), 29 strains were positive for *malX* gene (it is enriched in the strains causing extraintestinal infections). The results of serogroup determination based on O antigen indicated that the highest frequency was observed in the serogroups O1, O2, and O4 among all phylogroups, yet serogroups O2, O16, and O25 were predominant among phylogroup B2 strains. Nowrouzian et al. reported that among 140 commensal *E. coli* B2 strains, 47% of the Pakistani strains and 84% of the Swedish strains belonged to a major B2 subgroup (subgroups I-X) (7). Alizade et al. conducted a study on 216 strains of *E. coli* isolated from fecal samples of the patients with watery diarrhea. Although most strains belonged to B1 phylogroup, most strains contained the broad-spectrum beta-lactamase gene (*CTX-M1*) (20). Nojoomi and Ghasemian addressed *E. coli* strains isolated from the feces of the healthy children, about 98% of *E. coli* strains had *CTX-M1* gene, which indicates the difference between serogroup and antigenic profile of the strains and the virulence genes (21). Our results indicate the diversity of phylogroups among *E. coli* strains with the predominance of B2 group, and none of the isolates were recognized as phylogroup A, C, D, E, and F. This is, while in another similar study in Tunisia on community fecal carriage of broad-spectrum cephalosporin-resistant was B1, D, B2, and A phylogroups, respectively (22). It is the first study in Iran that showed a higher frequency of B2 phylogroup than other phylogroups among the *E. coli* isolates from fecal samples of the healthy children, while most similar studies were performed on urine, feces, and or blood samples of children or adults with urinary tract infection, diarrhea, and or sepsis. According to the obtained findings herein and similar articles, it seems that phylogroup B2 strains play a major role in the extraintestinal infections and carrying resistance genes that can result in the dissemination in hospital settings, and increase frequency in nosocomial infections with human health impact. Meanwhile, their frequency in healthy individuals is a function of lifestyle, customs, geographical area, nutritional status, and level of public health. The frequency of virulence genes in these bacteria varies depending on their invasive conditions or coexistence with the host, how they interact with the host immune system, and the infection site. The obtained findings suggested the evolution of features in the group B2 *E. coli* strains, which enable them to survive in the complicated context of the humans' intestines.

5.1. Conclusions

As shown in this research, we addressed the typing of the phylogenetic group B2 strains isolated from the commensal gut microbiota of healthy children under 10 years old. Virulence genes were more prevalent in group B2, carrying resistance genes, and the virulence factors like *usp* and *papG* by these strains can result in extraintestinal infections and increased frequency in nosocomial infections with the human health impact and leave few treatment options for the infections when caused by these strains in these children.

Footnotes

Authors' Contribution: S. N. P., B. B., M. M. developed the idea, designed the study, M. M. and Z. A. collected the samples, M. M. analyzed the data, and drafted the manuscript. A. M. M. and M. K. reviewed and revised the manuscript. All authors read and approved the final manuscript.

Conflict of Interests: The authors declare that they have no competing interests.

Data Reproducibility: The data presented in this study are openly available in one of the repositories or will be available on request from the corresponding author by this journal representative at any time during submission or after publication.

Ethical Approval: The present research was verified by the Ethical Committee of the Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran (Code: IR.MODARES.REC.1399.085, Link: ethics.research.ac.ir/EthicsProposalView.php?id=155115).

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Informed Consent: Written informed consent was obtained from each child's parent or legal guardian.

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