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Detection the Phylogenetic groups of E. coli that isolated from diarrheal in children under five years and study their relationship of common serotypes in Baghdad hospitals

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ABSTRACT

The study included 50isolates of E.coli isolatesthat were obtained from (120) stool samples collected from children with diarrhea of both sexes who visited hospitals in Baghdad. E.coli serotypes were diagnosed according to serotyping method for different age groups of children under five years. The results were represented by (7) (14%) isolates of E.coli belonging to Anticoli 1 group, (10) (20%) isolates belonging to Anticoli 11 group, (5) (23%) isolates belonging to Anticoli 111 group and (22) (44%) isolates which included (2/22) (9%)belongs to serotypes O111, O78 by (2/22) (9%), and for each of the serotypes O25, O142, and O119 are (3/22) and by 14%, O55 (5/22)) at 23% and O44 (4/22) by 18%. As for the rest of the isolates, no results were given, which is represented by (28) (56%) unknown isolates which were not classified for any of the serotypes. Moreover, the results showed that the highest percentage of diarrhea was in males at (56%), while in females it was(44%); in addition the highest percentage of infection with E. coli in males and females was within the age group (1-12) months by (48%), followed by the age group of (12-24) months by (28%), and then the age group (36-48) by 14%, while the lowest rate of E.coli infection in males and females found in the age group (24-36) months by (6%) followed by the age group (48-60) monthsby(4%).The results of the phylogenetic groups detected several groups includinggroup A (2/50, 4%), B1 (0/50, 0%), B2 (1/50, 2%), C (12/50, 24%), D (6/50,12%), F (3/50,6%), Clade1 (0%) and E (26/50, 52%) which is the predominant group.

Keywords: *diarrhea, E. coli, serological test, phyllo-groups*

INTRODUCTION

Diarrheal diseases are a severe public health problem and causingthe morbidity and mortality in infants and young children in developing countries;the intestinal infections and diarrheal diseases are a major cause of hospitalization and a serious health problem. Diarrhea is a clinical syndrome of acute gastroenteritis resulting from disorders that cause a defect in the absorption of water and salts, which results in an increase in the movement of the intestinal muscles, causing an increase in the number of defecation that may exceed more than three times a day, as it is described as watery stool containing blood or mucus. Additionally, it has an unpleasant odor with different colors.

The color of liquid stool may be yellow, green or brown; the severe diarrhea usually causes damage to the mucous membranes of the intestinein infected patient, and it is a cause of death among children, especially in developing countries, as it is a fatal disease for children around the world, especially the regions of South Asia and Africa (Park et al., 2022; Abdullah., 2016). Recently, seven major groups have been detected based on the virulence genes and pathogenicity mechanisms; these include:Enterotoxigenic E.coli (ETEC), Dffuselyadhering E. coli (DAEC), Enteroaggregative E. coli (EAEC), Enterohemorrhagic E. coli (EHEC), Bacterial Enteroanvasive E. coli (EIEC), Enteropathogenic E. coli (EPEC). Furthermore, hybrid genotype (EHEC/EAEC or EA-HEC) with genetic recombination was reported during an outbreak of diarrhea in Germany (Rezouqi et al., 2016; Omolajaiye et al., 2020).

E.coli has many virulence factors that enable the bacteria to cause infection and disease in human; the most important of these are intestinal diseases represented by watery and bloody diarrhea which called diarrheagenic E. coli (DEC), urinary tract infection (UTIs) which called uropathogenic E. coli (UPEC), sepsis, meningitis and conjunctivitis(Khalil., 2016; Al-Kalifawi, 2013) Interestingly, some strains of E.coli have the ability to overcome the immune system and cause disease due to their possession of a set of encoded genes located at specific sites known as pathogen city islands (PAIs) (Al- Ibrahim& Hadi,2015; Hassan and Mahmood,2019).PAIs are more present in pathogenic strains of E.coli than in non-pathogenic strains, which code for a number of virulence factors such as adhesions, toxins, siderophores, capsules, lipopolysaccharides (LPS),Biofilm (Ghafil,2018), enzymes and type III secretory systems, TypeIII secretion systems, haemolysin, alkaline protease, and antibiotic resistance (Ramírez & Eckhard, 2022; Abdul-Ghaffar and Abu-Risha,2017).

This study aimed to determine the serotypes and phylogenetic groups of E.coli causing diarrhea in children under five years.

MATERIALS AND METHODS

1- Patients Specimen Collection

A 120 stool samples were collected from children with diarrhea aged under five years from both sexes who attended hospitals, and under specialized medical supervision in Child Protection Teaching Hospital/Medical City, Child Central Teaching Hospital and Al Alawia Teaching Hospital in Baghdad provenance for the period from January 2022 to the end of April 2022.

2- Isolation and diagnosis

Thesamples were diagnosed using the following culture media: MacConkey agar and Eosin Methylene Blue Agar, in addition to biochemical assays which included Catalase, Oxidase and IMViC tests, while the final diagnosis of the isolates was performed using Vitek2 syste

3- The Serological diagnosis of bacterial phenotypes

The serotypes of E. coli isolated from diarrheal cases were diagnosed according to the serotyping method according to the manufacture’s protocol (sifin) among different age groups of children aged five years and under in both males and females at the Ministry of Health/Public Health Department/Central Public Health Laboratory.

4- Extraction of Genomic DNA

The bacterial DNA was extraction using HiPurA® Bacterial Genomic DNA Purification Kit® from collected isolates, according to the manufacturer's instructions.

5- Detection of Phylogeneticgroups genes of E.coliusing multiplex PCR device as shown in Table (1), which shows the sequence of specific primers.

TABLE 1: the sequence of the specific primers

Gene name		primer sequence(5-3)	Product size (base pair)	Reference
ChuA	F	ATGGTACCGGACGAACCAAC	288	Clermont and Colleagues(2013)
	R	TGCCGCCAGTACCAAAGACA		Clermont and Colleagues(2000)
YjaA	F	CAAACGTGAAGTGTCAGGAG	211	Clermont and Colleagues(2013)
	R	AATGCGTTCCTCAACCTGTG		
TspE4C2	F	CACTATTCGTAAGGTCATCC	152	Clermont and Colleagues(2013)
	R	AGTTTATCGCTGCGGGTCGC		
AceK	F	AACGCTATTCGCCAGCTTGC	400	Clermont and Colleagues (2004)
	R	TCTCCCCATACCGTACGCTA		
ArpAgpE	F	GATTCCATCTTGTCAAAATATGCC	301	Lescat and Colleagues (2012)
	R	GAAAAGAAAAAGAATTCCCAAGAG		
trpAgpC	F	AGTTTTATGCCCAGTGCGAG	219	Lescat and Colleagues (2012)
	R	TCTGCGCCCGGTCACGCCC		
trpBA	F	CGGCGATAAAGACATCTTCAC	489	Clermont and Colleagues (2008)
	R	GCAACGCGGCCTGGCGGAAG		

6- Detection the Phylogenetic groups genes inE.coli

The PCR mixture is consist of 12.5 µL of Master Mix prepared by Promega (USA); it includes the following: 1 µL of each F-primer and R-primer, 3 µL of DNA template and 2.5 µL of sterile deionized water(Promega, USA), and the total volume was 25 µL.Then, the PCR mixture tubes were mixed well using the vortex mixer and placed in the PCR machine. The optimal conditions for the PCR product amplification using Multiplex PCR are only included: one cycle for 4 minutes at 94 °C, 30 cycle that included 5 seconds at 94°C, 20 seconds at 57°C and 1 minute at 72°C. Then, 5 µL of PCR product was placed on agarose gel (2%)and the electrophoresis was performed at a potential difference of (100) volts for 60 minutes.

RESULTS AND DISCUSSION

After conducting the necessary tests to diagnose bacterial isolates, (50) isolates belonging to E.coli were obtained from diarrhea cases of children under five years out of (120) samples. The results of the current study also showed that the highest incidence of diarrhea was higher in males (56%) than found in females (44%), as shown in Figure (1).

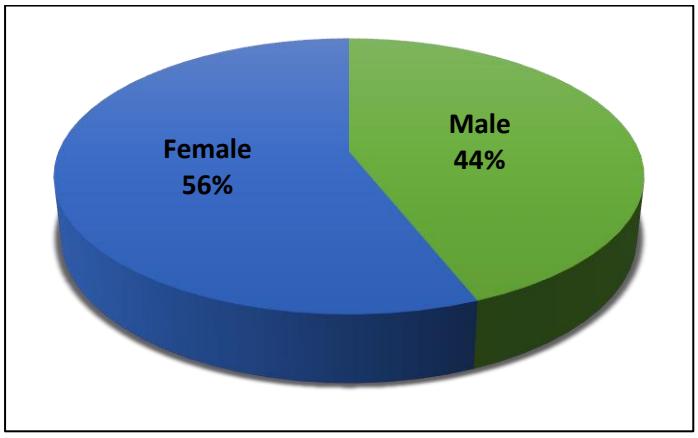

FIGURE 1: The Percentage of diarrhea infection in children of both male and female

The results of this study indicated that the highest rate of infection with E.coli was found in the age group (1-12) months by (48%), followed by the age group of (12-24) months by (28%), and the age group (36-48) by (14%). While the lowest rate of infection with E.coli was within the age group (24-36) months by(6%), and the age group (48-60) months by (4%), as shown in Table (2).

TABLE 2: The percentage of diarrhea infection of E. coli in males and Females in different age groups

Age	Total	(%)	Male No.	(%)	Female No.	(%)
1-12	24	48	12	24	12	24
12-24	14	28	9	18	5	10
24-36	3	6	1	2	2	4
36-48	7	14	5	10	2	4
48-60	2	4	1	2	1	2
Total	50		28		22	

The results of the current study were in agreement with the previous studies that observed a higher incidence of diarrhea among males compared to females, where the resultsof Al-Zubaidi (2021) study, which have been studied in Al-Najaf, showed a high incidence of diarrhea in males compared to females, which was 59% and 41% respectively. In addition, the results of this study were consistentwith Khairy et al. (2022) study that was conducted in the south Egypt, where it was found that 66 (20.6%) isolates were identified as DEC among children with diarrhea, represented by 16/66 (24.2%) in the age group less than one year; 22/66 (33.4%) in the age group 1 and 2 years and 28/66 (42.4%) are between 2 and 5 years. Moreover, Javadi et al. (2020) study reported that the rate of E.coli infection in children in the age group less than 3 years was for both males and females were 54% and 56% respectively, while the rate of infection for the age group 3-6 years in males and females were 27% and 26%. In contrast, the results of Emami et al. (2021) studydeclared that the infection rate in the age group 1-24 months was 69% of the 74% of those whose ages ranged from 1-59 months.

Many previous studies confirmed that this may be due to the incompleteness of the child's immune system and its dependence on the antibodies during breastfeeding, or through the acquisition of immunity, as Jafari et al.(2020) indicated that the antibodies decline in children after 5 months with the start of the weaning process and the using of plastic milk bottles for artificial feeding which may be another cause of infection due to contamination of these bottles or not sterilizing them periodically, which generates an environment suitable for the growth of bacteria and fungi, that leads to infection. Furthermore, it can be related to personal hygiene as well as water-borne diseases, as contamination of drinking water with feces is a major health problem that causes many cases of diarrhea, mainly in infants, and also the nature of the seasonal climate where viral and bacterial infections spread in the winter season, which is the time when samples were collected and the impact of climatic changes on them (Park et al., 2022).

The results of the sero-typing method, showed that the Entero pathogeing groups of E.coli are represented by (7) (14%) isolates of E.coli belong to Anticoli 1 group , (10) (20%) isolates belong to Anticoli 11 group , (5) (10%) isolates belong to Anticoli 111 group, and (22) (44%) isolates included (2/22) (9%) belong to serotypes O111, (3/22) (14%) belong to O142, and for each of serotypes O25, O142 and O119 was (3/22) (14%), O55 (5/22) (23%), O44 (4/22) (18%). While 28 (56%) isolates were unknown and not classified for any of the serotypesas shown in the Figure (2).

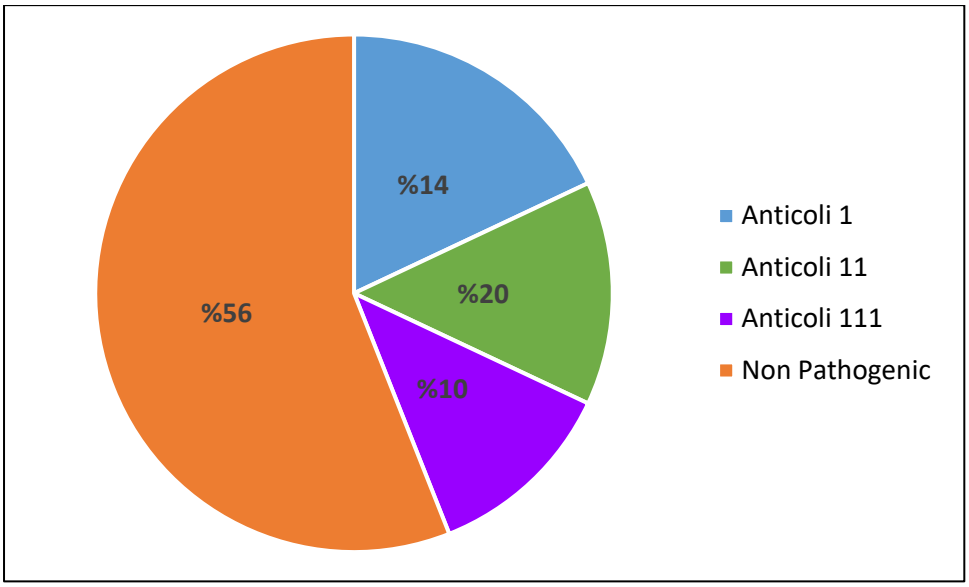

FIGURE 2: The percentage of E. coli isolates isolated from diarrhea cases using sero-typing method

The results of this study were consistent with Park et al., (2022) study that isolated 75 isolates of E.coli from diarrheal cases in South Korea, where it wasfound that 36 (48%) isolates did not classified for any of the serotypes, while 39 (52%) isolates belong to the serotypes O166 (6/75) by 8.0%, O18 (2/75) by 2.7%, and each of the serotypes O24, O20, and O8 (4/75) by 5.3%. The high percentage of variation and heterogeneity between E.coli isolates is due to the difference in the number of samples and geographical location; previous studies also indicated that 14 serotypes except O157 became general, which are represented by O26, O45, O55, O91, O103,O140, O111, O113, O118, O121, O128, O145, and O153 (Park et al.2022).

In this study, the results of the sero-typing method observed that the number of enteropathogenic E. coliisolates was (22) (44%) belong to the different groups represented by Anti coli 111, while (28) (56%) isolates were not classified for any serotypes.In general, the results obtained in the current study by using multiplex PCR test that is fast and reliable compared to serotyping tests, especially when examining a large number of isolates, and it is suitable technique in the case of an epidemic that requires rapid diagnosis. However, the using of serotyping test is necessary in observational studies when information on isolates is required (Alfinn et al., 2022).

To determine the phylogenetic groups of all E. coli isolates, a Clermont quadru-plex PCR method was used to determine seven coding genes: chuA, yjaA, TspE4.C2, arpA, arpAgpE, trpA and trpBAas well as the control elements for E. coli grouping into several groups (A, B1, B2, C, D, E, F and Clade I) . In addition, chuA genewas determined which encodes for the iron-regulated blood transfusion protein;yjaAgene of unknown function, and TspE4C2 gene sequence located within the gene encoding the lipase enzyme. The evolutionary group is represented by eight groups: A, B1, B2, C, D, E, F and clade I, which includes five strains or clades (I-V in E. coli strains (Ahumada-Santos et al., 2020).

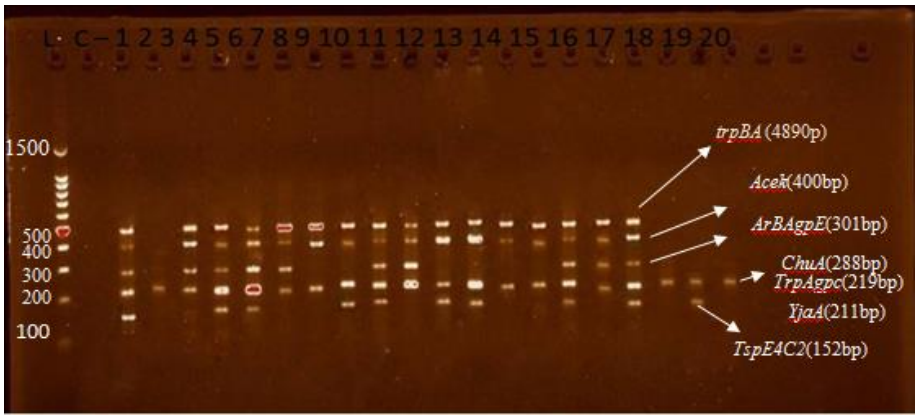

FIGURE (1-A): The Quadru-plex PCR electrophoresis of trpBA (489bp), Acek (bp 400), ArBAgpE (bp301), ChuA (bp 288), TrpAgpc (bp219), YjaA (bp211) and TSPE4C (bp152) of E.coli isolates on agarose gel (2%)at potential difference of 100 V for 60 minutes. The ladder (L) (100-1500 bp) for isolates under 1-20.

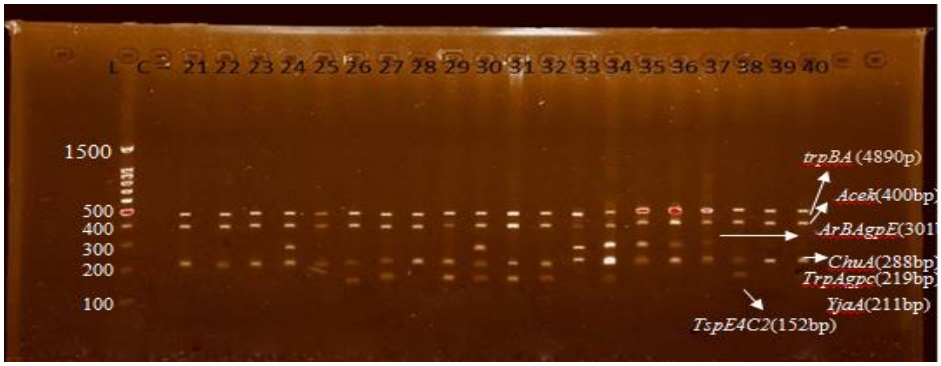

FIGURE (2-A): The Quadru-plex PCR electrophoresis of trpBA (489bp), Acek (bp 400), ArBAgpE (bp301), ChuA (bp 288), TrpAgpc (bp219), YjaA (bp211) and TSPE4C (bp152) of E.coli isolates on agarose gel (2%) at potential difference of 100 V for 60 minutes. The ladder (L) (100-1500 bp) for isolates under 21-40.

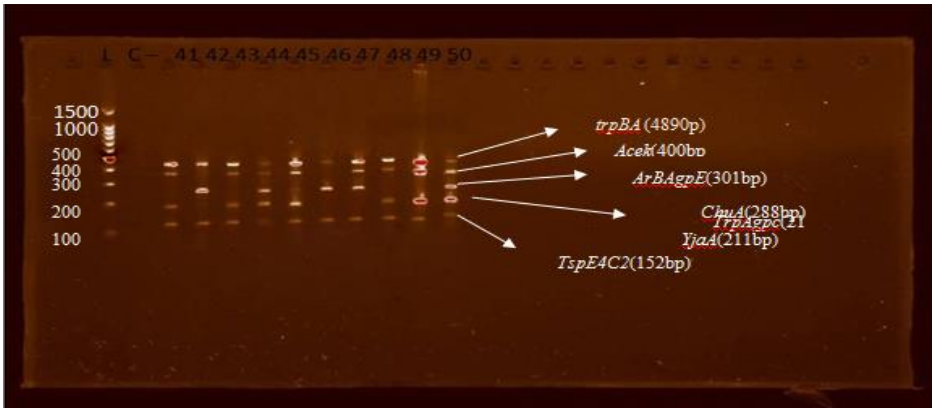

FIGURE (3-A): The Quadru-plex PCR electrophoresis of trpBA (489bp), Acek (bp 400), ArBAgpE (bp301), ChuA (bp 288), TrpAgpc (bp219), YjaA (bp211) and TSPE4C2 (bp152) of E.coli isolates on agarose gel (2%) at potential difference of 100 V for 60 minutes. The ladder (L) (100-1500 bp) for isolates under 41-50.

The different bands of E. coli help to classify or separate them into one of eight phylogenetic groups, as shown in Figures (1-A, 2-A, 3-A). Human fecal samples of children under five years were diagnosed using a new phylogenetic group mapping method which shows that about 76% of E. coli isolates belong to the newly described phylogenetic groups including C, E, F and clade I. The arpA gene is also found in all groups of E. coli isolates except the strains belonging to groups B2, F and clade I, while the trpA and arpArgE genes are specific to groups C and E (Lescat et al., 2012).Studies have been shown that E. coli strains associated with extra-intestinal infection usually belong to phylogenetic groups B2 or D, and commensal isolates of E. coli are generally related to A and B1 groups (Emami et al.,2021).

There was a high distribution of E. coli causing diarrhea associated with different strain groups among the children, which confirms the importance of future monitoring of the distribution of sequences and virulence factors of E. coli and the detection of pathological patterns of E. coli that cause diarrhea, as it is estimated that one billion cases of diarrheal infections occur worldwide every year (Alfinn et al., 2022) , Table (4) shows the percentages of phylogenetic groups of E.coli in the current study.

TABLE 4: The percentage of phylogenetic groups of E. coli

Phylogenetic groups	No.	Percentage (%)
A	2	4%
B1	-	0%
B2	1	2%
C	12	24%
D	6	12%
E	26	52%
F	3	6%
Clade1	-	

The results of the current study revealed that the phylogenetic group E was the predominant (52%, 26/50) for isolates that possess the genes arpA, TrpBA, ChuA, ArBAgpE andTspE4c2.STEC has been classified into two main groups, O157 which causes hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUC), and non-life-threatening O157, which are represented by a group of serotypes in the current study which are (O111:K58, O55:K59,O119:K69,O25:K11,O142:K86) according to the serotyping method as shown in Table (5) which shows the distribution of serotypes of the phylogenetic group of E. coli from diarrheal cases of children less than five years old. E. colistrains causes of food-borne diseases, food-producing animals such as cows and chickens which are the main reservoirs of many foodborne pathogens that produce Shiga toxin, which cause millions of cases of sporadic diseases and chronic complications. Moreover, the large and difficult outbreaks in many countries and between countries, the scale of this problem is illustrated by the large proportion of 1.5 billion annual cases of diarrhea in children under 5 years of age caused by microorganisms that cause intestinal diseases, which lead to more than 3 million deaths annually (Alfinn et al., 2022).

Phylogenetic group C,which containing arpA and yjaA genes, was represented by (12/50, 24%) of the isolates in the current study, which is the second most prevalent group. The phylogeneticgroup C has been newly described and is closely related to phylogenetic group B1; although there are few genetic differences between them. Possible sources of DEC in this group may be human or animal fecal matter (Lescat et al., 2012).According to the results of this study, this group includedserotypes (O78:K80 and O119:K69); then both groups were followed by phylogenetic group B2 (1/50,

2%) for isolates that possess chuA, yjaA and TspE4.C2 genes.Interestingly, the strains of this group are pathogenic and associated with lethal external-intestinal infection, which included the serotypes (O55:K59 and044:K74) according to the current study; the members of this group are usually isolated from carnivores and herbivores (Emami et al., 2021). In addition the phylogenetic group F was detected with a percentage of (3/50, 6%), which possessed chuA gene, which included serotypes (O142:K86 and O25:K11). On the other hand, the phylogenetic group A possessed arpA and yjaA genesby (4%, 2/50), which includes serotype O44:K74. The main source may be the likely source of DEC in the human fecal phylogenetic group (Berthe et al., 2013).

The results of this studyrevealed the distribution of serotypes of the phylogenetic groups isolated from cases of diarrhea in children under five years old, as shown in Table (5); these findings were consistent with the results of Jafari et al.(2020) study, which was conducted in Tehran in Iran on 65 isolates of E. coli isolated from cases of diarrhea and their results was represented by E group (17/65, 26%) group which was the predominant, followed by B1 group (13/65, 20%), B2 (9/65, 13.8%), C (8/65, 12%), D, Agroup (2/65, 3%) and clade I (3%, 2/65). while the results of Alfine et al.(2022)study, that conducted on 79 isolates of E. coli isolated from cases of diarrhea in southern Africa, found that the result was represented by B2 group (24/79, 30%), B1 (18/ 79, 22%), C (10/79, 13%), E (5/79, 6%), group A, D (6%) and group F (1%), and B2 was the predominant group. Moreover, the study of Iranpour et al.(2015) in Iran observed lower prevalence rates of serotype groups which including 0.7% and 4.6% respectively for group E isolates in comparison to the results of this study.

Whereas the studies of Abdul-Razzaq and Abdul-Lateef (2011) in Iraq, and Katongole et al. (2019) study in Ugandawere reported that phylogenetic group A was the predominant group. Furthermore, the study of Snehaa et al.(2021) in East Delhi in India on 200 samples of children aged between 0.5-5 years suffering from acute diarrhea, found thatgroup A was (50%), B1 (20%), D (20%), then C, B2, F (10%) and E (0%). The result Emami et al. (2021) study which conducted Fars in Iran on 850 children of both sexes, observed that the percentages of groups were for group A (0%), B1 (13%), B2 (56%), and D (31%);the findingsof Khairy et al. (2020) study in the south of Egypt on 66 isolates of E. coli isolated from children aged less than 5 years old, and revealed that groups A (47%) was the predominant group followed bygroup B2 (44%), D (9%) and B1(0%).

These differences in the distribution of phylogenetic groups in the current study and comparison with other previous studies could be due to differences in geographical areas, host health status, nutritional factors, patterns of antibiotic using, and genetic factors, as well as differing epidemiological importance of E. coli pathogens in children according to geographical area, it can influence on the distribution of E. coli strains groups in humans and animals (Galal, 2021).

TABLE 5: The distribution of serotypes of phylogenetic groups of E. coli isolated from diarrhea cases of children under five years.

No.	Isolate No.	Serotypes	Phylogenetic group
1	E.1	Enter pathogenic E.coli type II (O111:K58)	E
2	E.2	Enter pathogenic E.coli type I(O142:K86)	F
3	E.5	Enter pathogenic E.coli type III (O25:K11)	E
4	E.9	Enter pathogenic E.coli type II (O111:K58)	E
5	E.10	Enter pathogenic E.coli type II (O55:K59)	E
6	E14.	Enter pathogenic E.coli type I(O44: K74)	A
7	E.16	Enter pathogenic E.coli type III (O78: K80)	C
8	E.17	Enter pathogenic E.coli type I(O44: K74)	C
9	E.18	Enter pathogenic E.coli type III (O25:K11)	F

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10	E.19	Enter pathogenic E.coli type II (O55:K59)	B2
11	E.24	Enter pathogenic E.coli type I(O119:K69)	E
12	E.26	Enter pathogenic E.coli type III (O78:K80)	D
13	E.28	Enter pathogenic E.coli type I(O119:K69)	E
14	E.31	Enter pathogenic E.coli type I(O119:K69)	C
15	E.34	Enter pathogenic E.coli type III (O25:K11)	E
16	E.36	Enter pathogenic E.coli type II (O55:K59)	E
17	E.38	Enter pathogenic E.coli type II (O55:K59)	E
18	E.41	Enter pathogenic E.coli type II (O55:K59)	C
19	E.42	Enter pathogenic E.coli type I(O44: K74)	E
20	E.46	Enter pathogenic E.coli type I(O44: K74)	B2
21	E.47	Enter pathogenic E.coli type III (O78: K80)	E
22	E.48	Enter pathogenic E.coli type I(O142:K86)	D

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