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Research Article

Molecular Detection of Virulence Factors Encoding Genes In Escherichia coli Isolated From Diarrheal Children Under Five Years

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Article Info

Article history:

Received 29-4-2023

Received in revised form 8-12-2023

Accepted 8-12-2023

Available online13-12 -2023

Keywords:virulence factors, Escherichia coli, diarrhea, aEPEC, tEPEC

Abstract

The present study was carried out to investigate the bacteria that cause diarrhea in children, as well as the virulence factors possessed by Escherichia coli isolated from the stool of sample children under 5 years of age with diarrhea. The bacterial isolates were diagnosed using the Vitek 2 system, then 40 E. coli isolates were subjected to molecular detection by PCR. The results showed that the incidence of diarrhea in males 46 (57.5%) was higher than that of females 34 (45%), and the statistical analysis showed that there was a significant difference between infected males and females (P = 0.02) . Molecular detection results showed that 20 (50%) of bacterial isolates carry genes (STX1, STX2, eae, bfpA), which belong to the pathotypes of E. coli that cause diarrhea (DEC), and that 10 (50%) are bacterial isolates Enteropathogenic EPEC, and 10 (50%) isolates carrying two genes together (STX1 & STX2) belong to the entero hemorrhagic EHEC, and it was noted that there was no clear significant difference between the two strains (P > 0.05). Eight of bacterial isolates (80) carrying the gene (eae) belonging to atypical enteropathogenic aEPEC, and two bacterial isolates (20%) carrying two genes together (eae & bfpA) belong to the typical enteropathogenic tEPEC. Statistical analysis showed that there was a significant difference between the two strains of these bacteria (P = 0.05) .

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Peer review under responsibility of Iraqi Academic Scientific Journal and University of Kerbala.

Introduction

Diarrhea is a serious health problem and a major cause of child mortality, particularly in developing countries. The annual number of deaths due to diarrhea in developing countries is estimated at about 2.5 million [1, 2], most deaths from diarrhea occur due to severe dehydration and loss of fluids. Diarrhea is one of the most important causes of malnutrition in children under the age of five [3]. A local study in Babylon showed that improper disposal of sewage water, poor hand washing habits , and low family income are among the most common factors for acute diarrhea in children [4]. In addition, a local study acknowledged the need to enhance public health education for mothers of children, and to avoid the unnecessary use of modern antimicrobials [5]. The factors causing diarrhea can be summarized as bacterial, viral and parasitic factors, as the vast majority of bouts of diarrhea occur through viral causes that appear in the winter season [6]. The bacterial causative agents of diarrhea in children under the age of five are: *Shigella spp.*, Diarrheagenic *Escherichia coli* , *Salmonell spp* , *Vibrio cholera* , *Aeromonas spp.*, *Yersinia entrocolitica*, *Plesiomonas ssp* , *Compylobacter spp* and *Vibrio paraheamolyticus* [7]. Previous studies indicated that *E. coli* are the main cause of diarrhea in young children, followed by rotaviruses and parasites [8]. *E. coli* is a member of the enteric family which is deemed, as the common species among genus *Escherichia*. It lives in the intestinal tract of humans and animals as a normal flora, and is an opportunistic pathogen when the host's natural defenses are weak, especially in childhood or old age, causing diseases. Neonatal meningitis and septicemia are caused by uropathogenic *Escherichia coli* (UPEC), where pathogenic *E. coli* strains possess specialized virulence factors that increase the bacteria's ability to cause many diseases. Virulence factors of pathogens are grouped into two groups : adhesion structures which help bacteria to attach to the host tissues and to overcome the host's immune system, and endotoxins and exotoxins [9]. The current study was conducted to identify the causative agents of diarrhea in children, especially *E. coli*, by studying some genes such as (eae, bfpA, STX1, STX2) which encoded to the virulence factors for these bacteria.

Materials and methods

Stool sample collection

Stool samples (100) were collected from children under the age of 5 years, and 80 samples were collected from children suffering from symptoms of diarrhea and 20 stool samples were considered as control samples which collected from healthy children. The selection of samples was based on examining the stool sample under a light microscope, which contained pus cells or red blood cells . All these children were attended at the Children's Teaching Hospital in the Holy Karbala Governorate for the period from 2/2/2022 to 15/ 6/2022. Samples were transferred with a swab with (Cary-Blair) medium, then the samples were cultured on several different differential media [10].

Identification of Bacterial culture

For initial diagnosis of the bacterial isolates, several media including (MaConkey agar medium, EMB medium, X.L.D medium, TCBS medium, Sorbitol MacConkey medium and Chromium medium) were used to identify bacterial isolates according the phenotypic characteristics of the bacterial colonies such as color, shape, size, edges, and heights [10].

Microscopic identification

The shape and color of bacterial cells were tested where bacterial smears were prepared from overnight bacterial grew on the MacConkey agar [11] and slides were stained by gram stain, and examined using a light microscope.

Diagnosis of bacteria using the Vitek2 system :

The VITEK2 device, equipped by Bio Merieux, was used to conduct biochemical tests for these bacterial isolates. The VITEK2 device includes 48 types of biochemical tests that are used for the diagnosis of bacteria.

Molecular identification

Genomic DNA extraction

Genomic DNA was extracted from pure isolates of *E. coli* using a Kit according to the manufacturer's instructions(Addbio,). The concentration of DNA determined spectrophotometrically at 260 nm (Thermo Scientific NanoDrop 1000).

Amplification of the genes encoded to the virulence factors:

Primer sequences used to amplify fragments of (eae, bfpA, STX1 and STX2) genes are illustrated in Table 1.

Table 1: primers sequences and genes

Gene	Primers sequences		(bp)	Reference
eae	F	CTGAACGGCGATTACGCGAA	917	12
	R	CCAGACGATACGATCCAG		
bfpA	F	AATGGTGCTTGCGCTTGCTGC	326	12
	R	GCCGCTTTATCCAACCTGGTA		
STx1	F	ATAAATCGCCATTCGTTGACTAC	180	12
	R	AGAACGCCCACTGAGATCATC		
STx2	F	GGCACTGTCTGAAACTGCTCC	255	12
	R	TCGCCAGTTATCTGACATTCTG		

The following reagents were included in each PCR tube: 1 µl of primer F and 1 µl of R (10 pmol), 3 µl DNA template, 12.5 µl of ReadyMix Taq PCR Reaction and the final volume was completed to 25 µl by deionized water. The tubes were put in a thermocycler (Labnet USA) and PCR cycling was accomplished follow the conditions listed in (Table 2) [12]. The DNA amplicons were loaded on a 1.5 % agarose gel for 45 min at 90 mv alongside with a 1 kb ladder (Bio-Rad Laboratories).

Table 2: condition of thermal cycles of PCR

Gene	Initial denaturation	30 cycles			Extension
		Denaturation	Annealing	Extension	
eae	95 ◦C for 5 min	95 ◦C for 1 min	58 ◦C for 30 sec	72 ◦C for 1 min	72 ◦C for 10 min
bfpA	95 ◦C for 5 min	95 ◦C for 5 min	58 ◦C for 30 sec	72 ◦C for 1 min	72 ◦C for 10 min
STX1	95 ◦C for 5 min	95 ◦C for 5 min	50 ◦C for 30 sec	72 ◦C for 1 min	72 ◦C for 10 min
STX2	95 ◦C for 5 min	95 ◦C for 5 min	50 ◦C for 30 sec	72 ◦C for 1 min	72 ◦C for 10 min

Statistical analysis

All data are presented as mean ± SD of three replicates . An independent two-samples t-test and one-way ANOVA were used to determine the significant differences between two groups and three groups, respectively using Mini Tab statistical software version 16 (Pennsylvania). Duncan's multiple range test was used as a post hoc test to compare between means at $P \leq 0.05$.

Results

Morphological identification

The results showed that most isolates of *E. coli* are on the medium of Sorbitol MacConkey agar appeared pink, while other isolates of *E. coli* appeared pale in color because they do not ferment sorbitol sugar or ferment it late. On the medium of Hicrome Ec 0157: H, some isolates of *E. coli* appeared on the medium of the chromium agar in a purple color, or as for other strains of *E. coli*, their colonies appeared in a bluish-green color, and the isolates were identified according to [10]. Other bacterial species appeared, such as *Salmonella entric*, *Serratia plymuthica* and *Vibrio cholera*, MacConkey medium was also used to differentiate between Gram-negative bacteria that fermented lactose and non-fermented lactose. For example, *Salmonella entric* colonies appeared pale in color, and the colonies of *Vibrio cholerae* were also pale in color, while the colonies of *Serratia plymuthica* were red in color. Colonies of *E. coli* were colored yellow on XLD medium, as XLD medium is considered as a differential medium used to differentiate between organisms that produce hydrogen sulfide H_2S such as *Salmonella entrica* whose colonies were red with a pale pink halo around them, and this indicates the removal of carboxylate from lysine, which is a unique feature to distinguish it from the rest of the bacteria like *Proteus spp.*, that produces hydrogen sulfide H2S, and the colonies of *Vibrio cholerae* on XLD medium were pale in color, and the *Serratia plymuthica*, their colonies appeared yellow in color with black color, TCBS medium is a selective medium for all types of bacteria *Vibrio spp.* except for *Vibrio holisae*, as the colonies of *Vibrio cholera* were golden yellow, these results are consistent with what was mentioned in [10].

Microscopic identification

After staining with a Gram stain, bacterial smear were examined using a light microscope, all the cells of *E. coli* are short rods negative for Gram stain [9].

Diagnosis of bacteria using the Vitek2 system

For the purpose of diagnosis, 45 bacterial isolates out of 80 were tested using the Vitek 2 system, and the results indicated that 40 bacterial isolates were belonged to *E. coli*, one bacterial isolate to *Salmonella entrica*, one bacterial isolate to *Serratia plymuthica* , and 3 bacterial isolates belong to *Vibrio cholera.*

Molecular identification

The results showed that 20 (100%) *E.coli* isolates were belonged to the pathotypes of *E. coli* that cause diarrhea (DEC) , 10 (50%) bacterial isolates were EPEC, and 10 (50%) bacterial isolates which carried two genes together (*STx1* & *STx2*) belong to the intestinal hemorrhagic EHEC, and no significant differences ($P \geq 0.05$) were observed between the two strains , as shown in Figure 1.

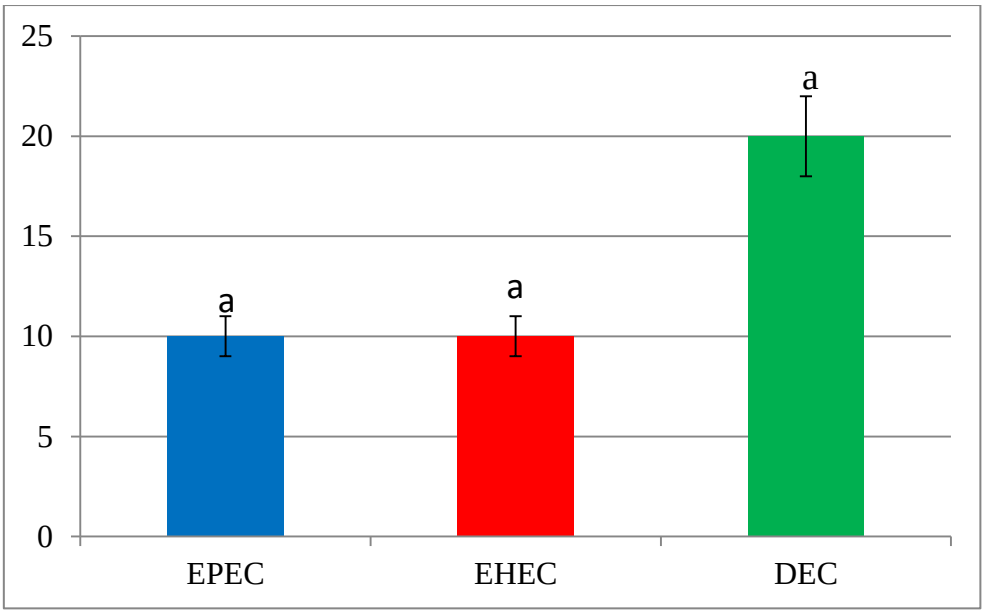

Figure 1: The number of DEC bacteria isolates and their strains (EHEC, EPEC) data are presented as mean ± SD. columns bearing similar letters do not give significant differences ($P \geq 0.05$) .

The results showed that among ten enteropathogenic (EPEC) isolates, 8 (80%) of which were bacterial isolates carrying the gene (eae) belong to atypical enteropathogenic (aEPEC) , and two isolates (20%) carried two genes together (eae & bfpA) belong to bacteria typical enteropathogenic (tEPEC). Statistical analysis revealed that there was a significant differences between the two types of these bacteria as shown in Figure 2 ($P = 0.05$) .

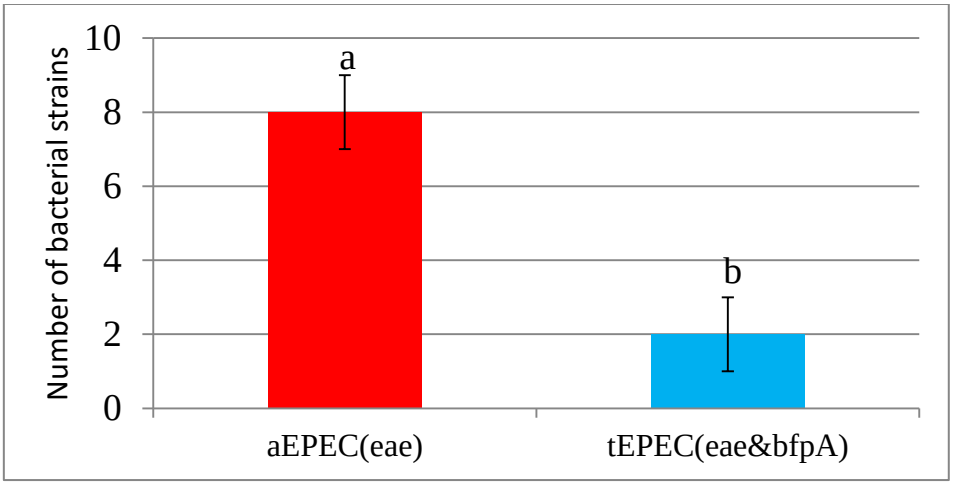

Figure 2: shows the number of isolates of EPEC types, which are typical tEPEC and atypical aEPEC .

PCR products were loaded in gel electrophoreses to detect the genes including *eae*, *bfpA* ,*STx1* and *STx2* genes, these genes were detect in EPEC with size of (917, 326,180, 255) bp, respectively shown in Figures 3, 4, 5 , 6

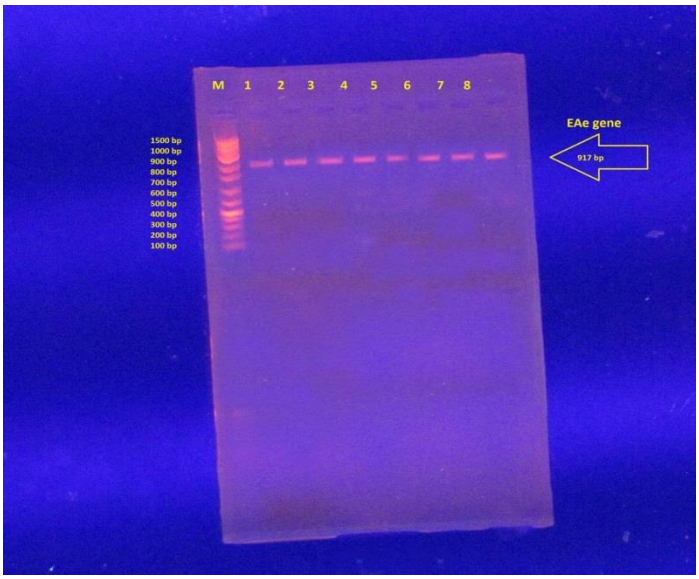

Figure 3: shows the electrophoresis of the PCR product of the *eae* gene (917 bp) of EPEC isolates in 1.5% agarose and 100V for 30 min. The numbers from 1 to 8 represent the number of bacterial isolates carrying the *eae* gene.

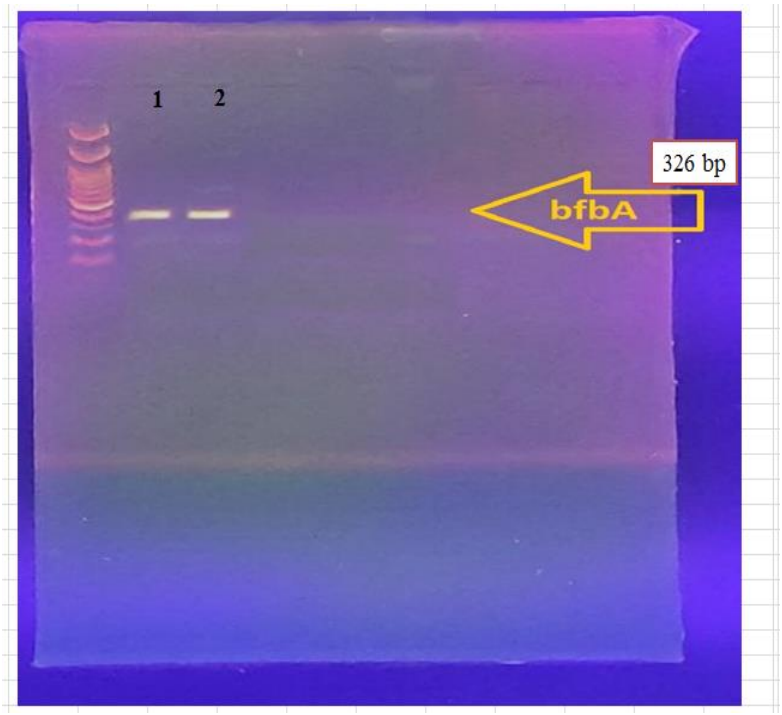

Figure 4: shows the electrophoresis of the PCR product of the bfpA gene (326 bp) of EPEC isolates in 1.5% agarose and 100V for 30 minutes. The numbers from 1 to 2 represent the number of bacterial isolates carrying the bfpA gene .

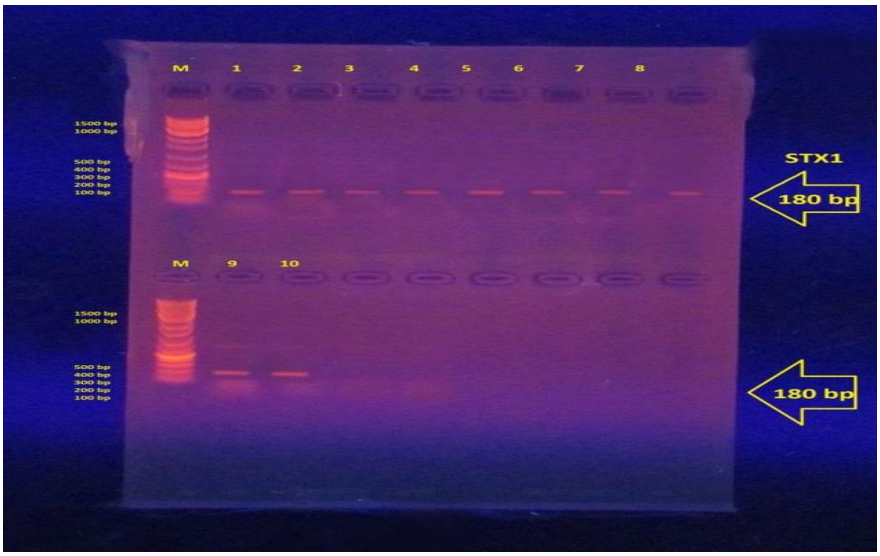

Figure 5: shows the electrophoresis of the PCR product of the STX1 gene (180 bp) of EHEC isolates in 1.5% agarose and 100V for 30 minutes. The numbers from 1 to 10 represent the number of bacterial isolates carrying the STX1 gene.

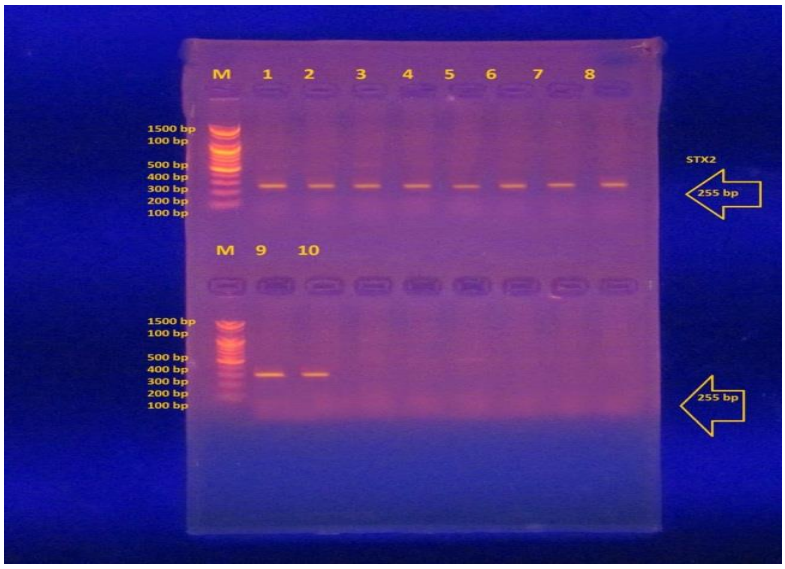

Figure 6: shows the electrophoresis of the PCR product of the STX2 gene (255 bp) of EHEC isolates in 1.5% agarose and 100V for 30 minutes. The numbers from 1 to 10 represent the number of bacterial isolates carrying the STX2 gene.

Distribution of pathotypes of *E. coli*

The results showed that the EPEC were present in a higher percentage in males 6 (60%) than their presence in females 4 (40%), but with non-significant differences (P >0.05), and the enterohemorrhagic (EHEC) were present in females 7 (70%) more than males 3 (30%), , as shown in Figures 7 and 8, respectively (P = 0.053).

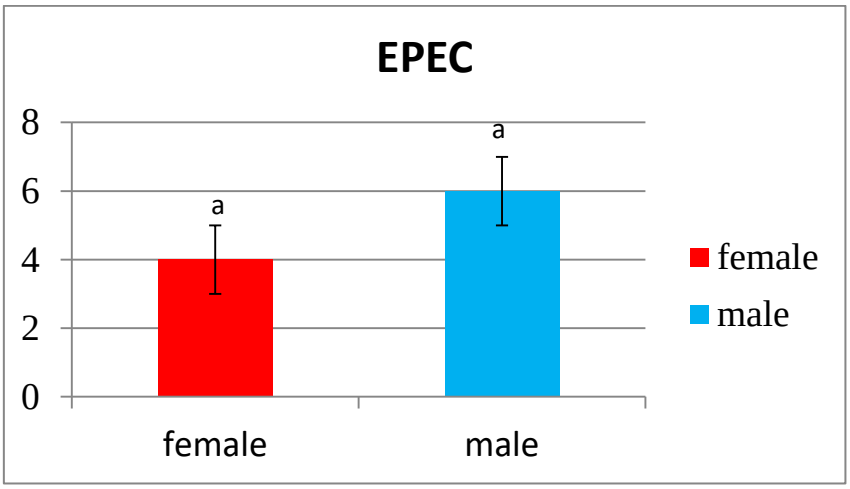

Figure 7: shows the distribution of EPEC isolates between males and females. Columns bearing the same letters do not give significant differences ($P \geq 0.05$) .

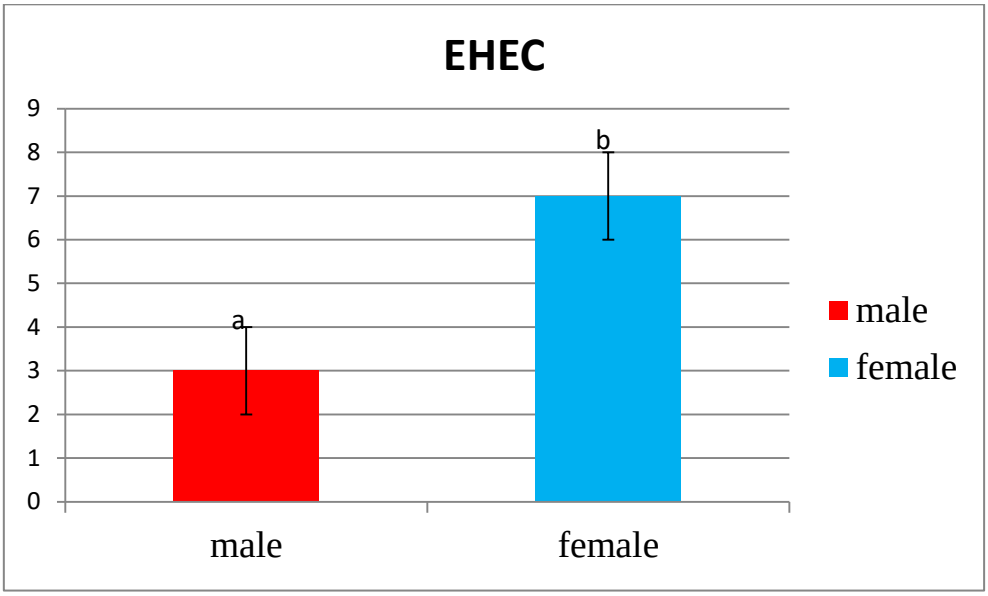

Figure 8: shows the distribution of EHEC isolates between males and females. Columns having different letters show significant differences ($P \leq 0.05$).

The results showed that EPEC were isolated in higher rate in children with age of (1-11) months with 6 bacterial isolates, whereas the lower isolation was in number 1 in the age group of (24-35) and (36-47) years. The results showed that EHEC were not present in the age group (1-11) months, whereas EPEC were missed in the age group of (48-59) months. The statistical analysis revealed that the isolation ratio of EHEC was significantly higher in the groups of (24-35) and (48-59) months compared to other age groups and EPEC ($P \leq 0.05$) as shown in Figure 9.

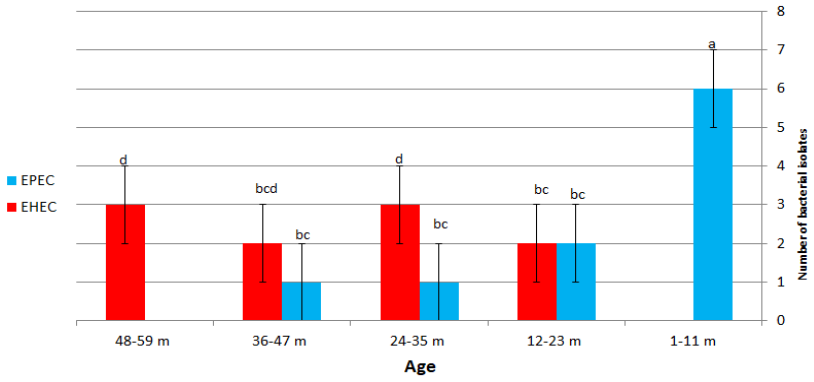

Figure 9: shows the distribution of pathological patterns between age groups. Columns having the different letters are significantly differences ($P \leq 0.05$)

Discussion

The data obtained from the current study regarding to DEC bacteria are consistent with the previous studies [13,14]. On the other hand, the results of the current study are disagreement with the results of [12] in Egypt. The results of the current study for the percentages of EPEC and EHEC are consistent with the results of the previous study in South Africa [15] and are disagreed with the results [16] in China. The reason for difference in the proportions of DEC bacteria types with other studies may be due to its association with several factors such as climate, individual hygiene level, and geographical location [17]. The results of tEPEC, aEPEC, EPEC) are consistent with results of [16, 18]. In contrast, the results regarding the distribution of the proportions of the EPEC strain of bacteria are disagreement with [12]. The results for EHEC are consistent with findings reported in [19], while the current results are disagreement with findings of [20]. The reason for the difference in prevalence ratios of EHEC with previous studies may be the climate was raised, as it was found that the increase in the ambient temperature corresponds to a higher incidence of *Escherichia coli* that cause diarrhea [21]. The results of the current study regarding the presence of EPEC and EHEC in males and females are consistent with the results reported in [22]. In addition, the data obtained from the current study regarding the presence of EPEC in the age groups are consistent with the findings of [23] but differed to the data reported in [24]. The current study is consistent with the presence of EHEC in the age groups obtained by a previous study [23] while was not in agreement with the study of [18].

Conclusions

It can be concluded that high ratio of aEPEC more than tEPEC could be indicate to their source of infection which may be not from human source. In addition, some strains of *E. coli* carried the *eae* gene only and some carried the eae gene with the *bfpA* gene .

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