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Research Article

Detection of Virulence Factors of *Escherichia coli* Strains Isolated from Children with Diarrhea

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Abstract

Background: *Escherichia coli* strains are one of the most important foodborne bacteria caused acute gastroenteritis and also considered an important bacterial agent of infantile infectious diarrhea.

This study was conducted during the period from March 2013 to March 2016 to investigate the presence of some virulence genes in 115 *Escherichia coli* isolates from children less than 10 years of age in Khartoum, Sudan.

Methods: One hundred and fifteen samples were randomly collected from children less than 10 years of age with diarrheal infection. All samples were cultured and *E. coli* strains were isolated. *Pal, fimH* and *eaeA* genes were identified in all samples using PCR. O157 (*rfb*) and H7 (*fliC*) strains were identified based on the presence of putative virulence factors and subtypes. Furthermore, sequence analysis was performed for *fliC* strains. Phylogenetic tree was constructed using NJ and UPGMA based on sequence of *fliC* strains and similar sequences available from NCBI database.

Results: *Pal* gene has been detected in all samples. 39.1% and 62.6% of the samples respectively have shown the *eaeA* and *fimH* genes. Prevalence of *fliC* was 42.6% and *rfb* was (4.4%), and 4.4% for both genes which is confirmation of *Escherichia coli O157:H7*. Furthermore, *fliC* gene showed suggestive association significant with diarrhea type ($p = 0.031$, $p = 0.05$ and $P = 0.012$, respectively). Results indicate that the samples sequenced belong to different strains of *E. coli O157: H7* and *E. coli O55:H*. These strains showed high similarities to the following strains: EC4115, EDL933, Sakai, SS17, SS52, TW14359, WS4202, 9234 and O55:H7 str. CB9615.

Conclusion: The prevailing strains of *E. coli* caused diarrheal disease among children in Sudan are *E. coli O157: H7* and *E. coli O55:H.*

Keywords: *Escherichia coli; Diarrhea; Acute Gastroenteritis*

Introduction

Acute gastroenteritis and diarrhea are common and costly problems that cause significant morbidity and mortality in children worldwide. An estimated case of diarrhea of about 1.7 billion occur annually worldwide among children under five [1,2]. Worldwide, the most common pathogens that cause this disease are: *Escherichia coli O157:H7, Salmonella spp., Shigella spp., Campylobacter spp., Listeria monocytogenes, Vibrio cholera, Yersinia enterocolitica, Rotavirus, Cryptosporidium spp., Entamoeba histolytica* and *Giardia intestinalis* (*lamblia*). These pathogens can cause potentially serious diseases which may be fatal, especially in children.

Escherichia coli is one of the most important bacterial agent of infantile infectious diarrhea [3]. In Sudan infant mortality due to diarrhea is 102 per 1000 live births and neonatal mortality is 51 per 1000 live births [4,5]. According to Saeed., *et al.* (2015), *E. coli* is the most common causative agent of diarrhea in Sudan. Saeed., *et al.* (2015) report about 48% of children under age 5 years were infected with pathogenic *E. coli* in Khartoum Sudan [5]. In Sudan, although diarrhea is one of the most common reasons for Children to visit healthcare clinics, but knowledge of the causative agents of these diarrhea cases is limited.

The diseases caused by a particular strain of *E. coli* that is rendered pathogenic by their ability to possess specific virulence factors, such as enterotoxin or adherent fimbriae, which are genetically encoded by plasmid DNA, chromosomal DNA, and bacteriophage DNA [6]. The E. coli group contains non-pathogenic commensal *E. coli* and diarrheagenic *E. coli* (DEC) types, such as Enteropathogenic *E. coli*(EPEC), Enterotoxigenic (ETEC), Enteroaggregative *E. coli* (EAEAC), Enterohaemorrhagic *E. coli* (EHEC) and Enteroinvasive *E. coli* (EIEC) [7]. These pathotypes are classified according to their specific virulence determinants. The *O157:H7* serogroup of *E. coli* (EHEC), produced a family of toxins known as Shiga toxin or verotoxin and is an important cause of bloody diarrhea (hemorrhagic colitis) and acute diarrhea among infants in developing countries [8]. The pathogenic *E. coli* have several virulence factors implicated in pathogenesis, such as a pathogenicity island called locus of enterocyte effacement LEE that encodes proteins, such as Intimin, an outer membrane protein encoded by *eaeA* [9], involved in attaching effacement [10] beside a serotyping based on the somatic antigen O encoded by *rfb* [9], the flagellar antigen H encoded by *fliC* [11], type 1 fimbria *fimH* [12] and peptidoglycan-associated lipoprotein *pal* [13].

Materials and Methods

Sampling and *Escherichia coli* identification

This study was carried out to investigate 115 children less than 10 years old who were admitted with the Child Welfare Clinic to Ahmed Gasim Pediatric Hospital and Mohamed Alamin Hamid Pediatric Hospital during the period from December 2013 to March 2014. The ethical approval for this study was provided by the Ministry of Health, Research Administration.

Data of the patients were obtained using a questionnaire with the consent of the parents. Items of the questionnaire included: patient personal information, mothers information, water and food sources, Hygienity and clinical data (Diarrhea type and severity and dehydration severity adoptive WHO criteria (2017). The obtained data were analysed using the SPSS version 20.

The stool samples were collected from children who were admitted with diarrhea to the hospitals. Each stool sample was collected in a special sterile container, labelled and kept at 4°C, then cultured within 3 hours from collection.

The stool samples were culture onto Sorbitol MacConkey (SMAC) [14]. *E. coli O157* generally produced colorless colonies when cultured on this media.

About 2 ml of faeces was streaked on the surface of the Sorbitol MacConkey medium plate by using heated wire loops. The inoculated petri dishes were incubated for 24 hours at 370C. Sorbitol fermented by the non-pathogenic strains of *E. coli* formed red colonies and Pathogenic *E. coli* gave colorless colonies. On the following day, identification of the non-fermenting colonies and fermenting colonies was carried out by biochemical tests and then sub cultured in Nutrient Agar and kept in 200C [14,15]. It was then incubated in different biochemical media such as Urease Test [16], Indole [17] and Kligler Iorn Test (KIA) [18]. PCR confirmation of *E. coli* isolates and Detection of virulence factors.

The bacteria were grown in 10ml peptone water at 37^0C for 24 h and the DNA was extracted from bacteria using chloroform extraction and ethanol precipitation method [19,20]. DNA were measured using Nano-drop Spectrophotometer (ND.1000 v3.5.2).

The *pal* gene, *fimH* gene and *eaeA* gene were amplified using specific PCR primers [9,13,21] respectively. The *rfb* and *fliC* genes were amplified using a- Multiplex PCR specific primer [9,11] (Table 1).

Target Gene	Primer sequence (name)	Amplification conditions	Size (bp)
pal	ECPAL-L:5′- GGCAATTGCGGCATGTTCTTCC-3′ ECPAL-R: 5′- CCGCGTGACCTTCTACGGTGAC-3′	initial denaturation at 94^OC for 3 minutes, followed by 35 cycles of denaturation at 94^OC for 30s, annealing at 60^OC for 30s and elongation at 72^OC for 1.5 minutes.	280
fimH	Fim1: 5′- GAGAAGAGGTTTGATTTAACTTATTG-3′ Fim2: 5′- AGAGCCGCTGTAGAACTGAGG-3′	Initial denaturation at 95^OC for 4 minutes, followed by 40 cycles of denaturation at 94^OC for 60s, annealing at 60^OC for 60s and elongation at 72^OC for 70 and final extension at 72^OC for 5 minutes.	559
eaeA	F: 5′- GACCCGGCACAAGCATAAGC -3′ R: 5′- CCACCTGCAGCAACAAGAGG-3′	Initial denaturation at 95^OC for 1.5 minutes, followed by 35 cycles of denaturation at 95^OC for 1 minute, annealing at 61.9^OC for 1 minute and elongation at 72^OC for 1.5 minutes, and final extension at 72^OC for 1.5 minutes.	384
rfb O157	F: 5′- CGGACATCCATGTGATATGG -3′ R: 5′- TTGCCTATGTACAGCTAATCC -3′	Initial 41 denaturation at 94^OC for 5 minutes, followed by 35 cycles of denaturation at 94^OC for 60 second, annealing at 52^OC for 30 second, and elongation at 72^OC for 60 second, and final extension at 72^OC for 10 minutes.	259
fliC H7	F: 5′- GCGCTGTCGAGTTCTATCGAG-3′ R: 5′- CAACGGTGACTTTATCGCCATTCC-3′		625

Table 1: Primers and PCR conditions used for characterization of virulence genes, from diarrheal samples.

The Multiplex-PCR was performed in a 25 µl amplification mixture using 2 µl of the DNA extracts from the bacterial culture. One µl of each primer in a Maxime PCR premix kit (i-tag) - (iNtRON Biotechnology, INC. www.Intronbio.com in a total volume of 25 µl. The premix kit containing 1x reaction buffer (10 xs), 2.5mM each deoxyribonucleotide Triphosphate (dNTPs), 2.5U i-taqTMDNA polymerase (5U/µl) and 1x gel loading buffer.

Eight µl of the PCR amplification product were electrophoresed in agarose (2%) containing 0.5 ug/ml ethidium bromide. The amplified PCR products were separated by electrophoresis at 83 V for 25 minutes in 1X TBE running buffer, visualized under UV at documentation system. 100bp DNA ladder was used as a molecular size marker.

Nucleotide Sequence and phylogenetic analysis

Amplified DNA from individual samples that showed unique bp (1000) than the target 625bp for *fliC* gene were sent for purification and sequencing by Bioneer Company in South Korea. The sequence were analysed and compared to the database from National Center of Biotechnology Information (NCBI) for Basic Local Alignment Search Tool (BLAST) [22]. The sequences were edited and aligned using Clustal W, [23], BioEdit software 7.2.5 [24] and CLC main workbench version 6 [25]. Species identifications were confirmed through Blast analysis (NCBI)) for some aligned samples, that showed high similarity with 9 strains of *E. coli*. The sequence of *fliC* gene of the size 625 bp and 1000 bp were aligned for phylogenetic analyses using CLC Genomic Workbench 8.5. [26] using Neighbor-Joining method [27] and UPGMA method [28] to give the relatedness and divergence between the different strains. About 1000 replications of bootstrap analyses supported the phylogenetic groupings.

Statistical Analysis

Data obtained from all tests were transferred to the Microsoft Excel spreadsheet (Microsoft 2013) for analysis. Then, the statistical analysis was performed using SPSS/20.0 software (SPSS Inc., Chicago, IL). P-values were calculated using chi-square test with SPSS version 20 [29] and multivariate techniques with R Studio 3.2.2 [30]. Statistical analysis was used to find any significant relationship for prevalence of E. coli and virulence strains between different samples. The P-value less than 0.05 was considered statistically significant.

Results and Discussion

Analysis of Age group and Association with Clinical Symptoms

Analysis of the age group showed that, age of the children vary between 0 - 10 years old, in which, diarrhea incidence and its subsequent dehydration severity was highest among children less than 3 years old (74.78%), and significantly different from the other age groups (Figure 1 and Table 2). These results agreed with findings reported by previous publications [31,32]. This might be due to immature immune system of children less than two years. These findings are in contrast to another study conducted in Khartoum [34], in which the higher infections were among children more than 2 years. Some researchers reported that, E. coli infection prevailed among children less than 5 years old [35].

Age Group	Dehydration Severity			
	Mild	Moderate	Severe	P ≤ 0.05
0 - ≤ 3	63.3%	73.9%	92.3%	
3 < 4	16.7%	6.5%	0.0%	
4 < 6	3.3%	10.9%	0.0%	
6 < 8	10.0%	4.3%	2.6%	
8 ≤ 10	6.7%	4.3%	5.1%	
	100.0%	100.0%	100.0%	0.017

Table 2: *Association of dehydration severity with age group.*

There is no association between the type of diarrhea (watery, mucoidy or bloody) and the age group. Type of diarrhea is affected directly by the infectious agent such as *E. coli, Shigella* sp, etc [36] (Table 3).

The incidence of the diarrhea cases was higher in male children (56%), than in female children (44%) (Figure 2). Although these outcomes agreed with many previous studies [37,38]. However, the ratio of male to female children affected by diarrheal disease is not statistically different, and hence, suggest that, male and female children were equally affected [38,39].

Biochemical test has been used as first line detection of Bacteria, but, the Indole test is used widely for detection of E. coli specifically [39]. The majority of samples was positive Indole (87.8%) and KIA (88.7%) but negative urease (87.8%) [40] (Table 4). Indole is important in the identification of enterobacteria such as *E. coli* [17,41]. As far back as 1889, the indole test was used for detection of *E. coli* [5] and confirmation of *E. coli* with the indole test was undertaken in the UK [42].

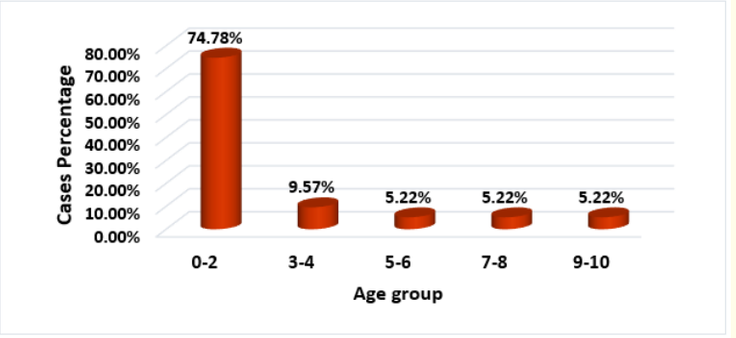

Figure 1: *A graph of age group distribution among participants less than ten years old.*

Age Group	Type of Diarrhea				
	Mucoidy	Mucoid/Bloody	Watery	Watery/Bloody	P ≤ 0.05
0 < 3	74.5%	66.7%	79.0%	100.0%	
3 < 4	8.5%	33.3%	4.8%	0%	
4 < 6	6.4%	0%	4.8%	0%	
6 < 8	6.4%	0%	4.8%	0%	
8 ≤ 10	4.3%	0%	6.5%	0%	
P ≤ 0.05	100.0%	100.0%	100.0%	100.0%	0.936

Table 3: *Association of diarrhea type with the age group.*

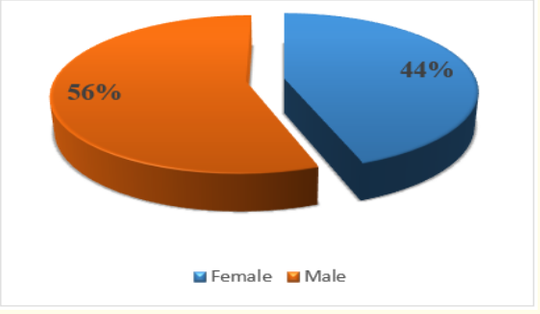

Figure 2: *Gender ratio of males to females of the participants.*

Number of Samples	Test					
	Indole		Urease		KIA	
	+	-	+	-	+	-
115 samples	101 (87.8%)	14 (12.2%)	14 (12.2%)	101 (87.8%)	102 (88.7%)	13 (11.3%)

Table 4: *The results of the biochemical tests for the sample collected.*

With regard to molecular characterization, five genes have been amplified in the samples tested. These genes are *pal, eaeA, fimH, fliC* and *rfb*. The first one is an indicator for enteric bacteria including *E. coli, Salmonella* and *Shigella* [13], and it was detected in all tested samples. The other genes, which are confirmative for pathogenic *E. coli* such as *eaeA* gene [7], *fimH* gene [43,44], and *fliC* gene [11,45], were detected in (85.2%) of the tested samples. Virulence genes such as *eaeA* and *fimH* were detected in (39.1%) and (62.6%) of the examined samples respectively. Discrimination of the incriminated serogroup of *E. coli* which was suggested to be prevailing in Sudan was checked by detection of *fliC* and *rfb* genes which are specific for *E. coli O157:H7* [46,47]. Both genes had been detected in about (42.6%) and (4.4%) of the samples respectively (Table 5) (Figure 3-6).

	The Gene				
	ECO Pal (280 pb)	*fimH* (559 bp)	*eaeA* (384 pb)	*fliC* (625pb)	*rfb* (259 pb)
Total	115 (100%)	72 (62.6%)	45 (39.1%)	49 (42.6%)	5 (4.4 %)

Table 5: *The percentage of the samples in which studied genes have been amplified.*

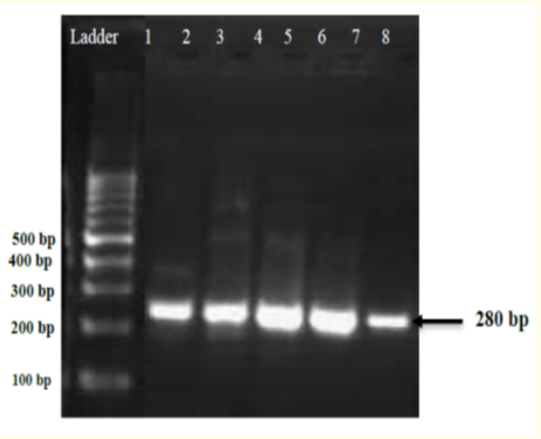

Figure 3: *Result of PCR assay, amplifying 280bp segment of pal gene Lanes 1-8; Lane Ladder: 100-bp DNA marker.*

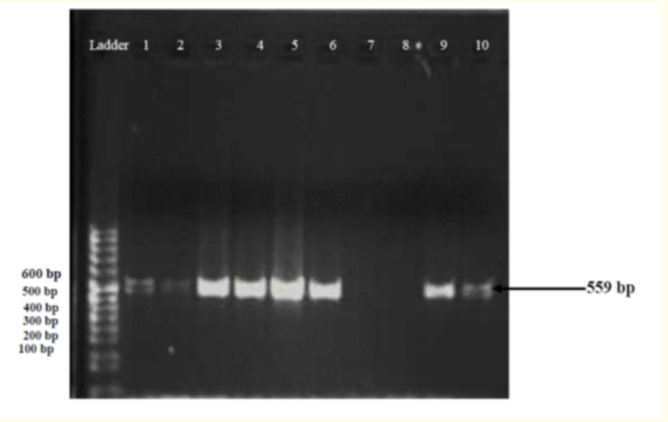

Figure 4: *Result of PCR assay, amplifying 559bp segment of fimH gene. Positive samples lanes: 1-6 and 9-10; Lane Ladder: 100-bp DNA marker.*

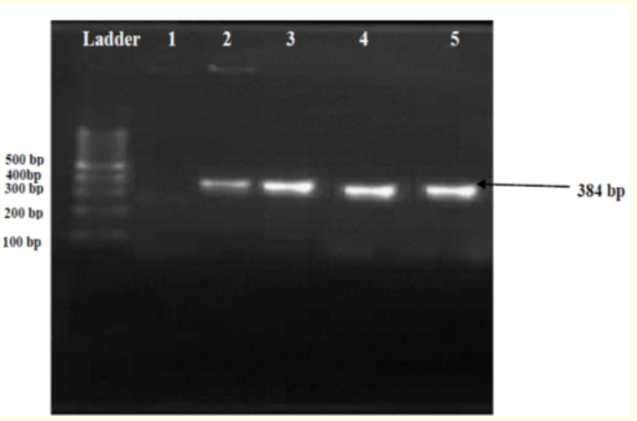

Figure 5: *Result of the PCR assay, amplifying 384bp segment of eaeA gene. Positive samples lanes: 2- ; Lane Ladder: 100-bp DNA marker.*

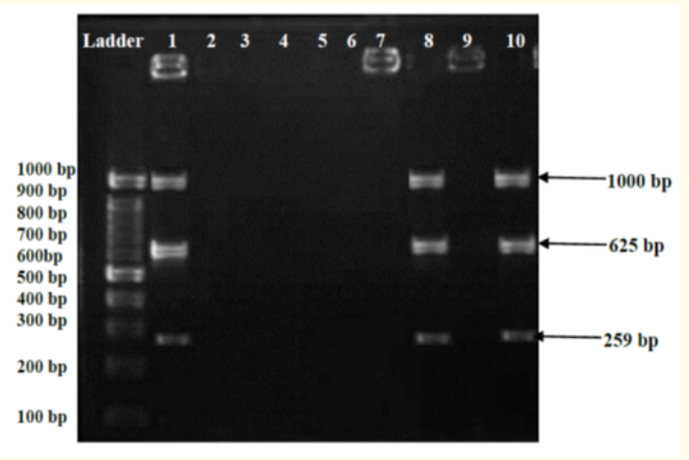

Figure 6: *Result of PCR assay, amplifying 625bp and 1000bp segment of fliC gene and 259bp segment of rfb gene. Positive samples lanes:1, 8 and 10; Lane Ladder: 100-bp DNA marker.*

Detection of virulence genes related to *E. coli* in most samples, indicate that pathogenic *E. coli* are the main cause of diarrhea. These findings agreed with many studies worldwide and in Sudan [3,5,33]. These results are in contrast to published papers [48].

In about (14.8%) of the samples, *fliC* and *fimH* genes were amplified together. Bekal., *et al*. (2003) reported the presence of both genes in *E. col*i isolates, and considered these genes as the virulence factor of EPEC strains [49]. Both *eaeA* and *fliC* genes were amplified together in (5.2%) of the samples. As *fliC* gene is specific for H antigen, which is found in certain strains of *E. coli* associated with EPEC and EHEC [11,50], and so the *eaeA* gene [7,11,51]. These finding suggest that, the causal agents of diarrhea in (5.2%) of the samples could be EPEC or EHEC strains.

On the other hand, *eaeA* and *fimH* genes were amplified in 11.3% of the samples and according to Osawa., *et al*. (2013), *fimH* gene found in all pathogenic *E. coli.* Hence, due to presence of *eaeA* gene in the same samples, these findings suggest that diarrheal infection might be caused by either EPEC or EHEC strains.

In 14.6% of the samples, the virulence genes *eaeA, fimH, rfb* and *fliC* were amplified together. Amplification of all these characteristics genes in the same samples, is an indicators for pathogenicity of *E. coli* prevailing in the tested samples (Table 6).

Association of the genotype with dehydration and diarrhea

There is no significant association between dehydration severity and the amplified genes. Dehydration is subsequent of diarrhea [52] (Table 7). On the other hand, diarrheal severity is not affected by the amplified genes under study (Table 8). Diarrheal severity -acute or persistent- is not distinct disease, but represent two end of continuum [53].

Gene	Dehydration Severity			
	Mild	Moderate	Severe	p-value
Pal	26.1%	40.0%	33.9%	-
fimH	20.8%	43.1%	36.1%	0.258
eaeA	26.7%	35.6%	37.8%	0.923
fliC	27.1%	33.3%	39.6%	0.979
rfb	0.0%	60.0%	40.0%	0.171

Table 7: *The association of dehydration severity with the amplified genes.*

Regarding the type of diarrhea (Watery, Mucoidy or Bloody), only *fliC* 625 have suggestive association with diarrhea type (Table 9). The *fliC* 625 gene is associated with EHEC Serotype *O157: H7 [11,50]*, and the type of diarrhea could be associated with *E. coli* strains.

Gene	Diarrhea Severity		
	Acute	Persistent	p-value
Pal	95.7%	4.3%	-
fimH	95.8%	4.2%	0.621
eaeA	93.3%	6.7%	0.299
fliC	95.8%	4.2%	0.655
rfb	100%	0%	0.797

Table 8: *The association of diarrhea severity with the amplified genes.*

The gene	Type of Diarrhea			
	Watery	Bloody	Mucoidy	P ≤ 0.05
Pal	53.9%	2.61%^{w}; 2.61%^{m}	40.9%	-
fimH	51.4%	2.8%^{w}; 2.8%^{m}	43.1%	0.920
eaeA	45.5%	4.5%^{w}; 2.3%^{m}	47.7%	0.338
fliC	47.1%	0%	52.9	0.012*
rfb	80%	0%	20%	0.617

Table 9: *The association between types of diarrhea with the amplified genes.*

^{m}: Refer to mucoidy bloody; ^{w}: Refer to watery bloody.

In this study, watery and mucoidy diarrhea were the prevailing type with very few cases suffering from combination of bloody watery or bloody mucoidy. Watery diarrhea is attributed to EPEC or EHEC strains [54], and these strains are characterized by *eaeA* [7,11,51], and *fliC* [11,50] genes, which have been amplified in about 45.5% and 47.1% of the samples with watery diarrhea (Table 9).

Mucoidy diarrhea is attributed to EPEC [54] or EIEC strain [54,55], but in this study, the amplified genes with mucoidy diarrhea are correlated only with EPEC strain. Mucoid diarrhea found in 47.7% and 52.9% with *eaeA* and *fliC* genes respectively (Table 9).

Bloody diarrhea is attributed to (EHEC) *O157:H7* [56], and this strain could by molecularly characterized by amplification of *eaeA* gene [51], which was amplified in about 6.8% of the samples with bloody diarrhea (Table 9).

Sequence analysis

34% of samples amplified for *fliC* gene showed the 1000bp fragment (A1000 strain) along with the 625bp fragment (A7, A11, O12 and O22 strains).

The results of sequence analyses of these fragments were subjected to NCBI BLAST analysis, shown similarity with common serogroups of *E. coli O157:H7* and *E. coli O55:H7.* Furthermore, strains of serogroup *O157:H7* are EC4115, EDL933, Sakai, SS17, SS52, TW14359, WS4202, 9234 and serogroup *O55:H7* str. RM12579. Those strains with exception of *O55:H7* str. RM12579 are reported to be associated with EHEC [57].

Phylogenetic Tree Analysis

Analysis of phylogenetic tree by NJ and UPGMA methods revealed a very strong association between the presence of the *fliC* 625bp and 1000bp fragments and *E. coli O157:H7* and *E. coli O55:H7* serogroups (Figure 7 and 8). The A1000 strain has been mentioned for the first time as a product of amplification of fliC gene. This might be an ancestor from cluster A or split from cluster B. Serogroup E. coli O157: H7 is known to be descendant from *E. coli O55:H7* serogroup [58,59]. There is a close association between four sequenced samples A7, A11, O12 and O22 with *O157:H7* str. WS4202 and O157:H7 str. 9234 (Cluster A) and they are all diverged from str. RM12579 *O55:H7* and A1000 (sequenced sample). These outcomes in agreement with previously mentioned ancestral relation between O55 and O157 [60,61]. On other hand, the sequenced sample A1000 was found to be closely related to Cluster A, but a little bit far from them. Other strains of O157:H7 in Cluster B (str. EC4115, str. EDL933, str. Saki, str. TW14359, str. SS17, and str. SS52) found to be closely related to each other [62] and far diverged from cluster A.

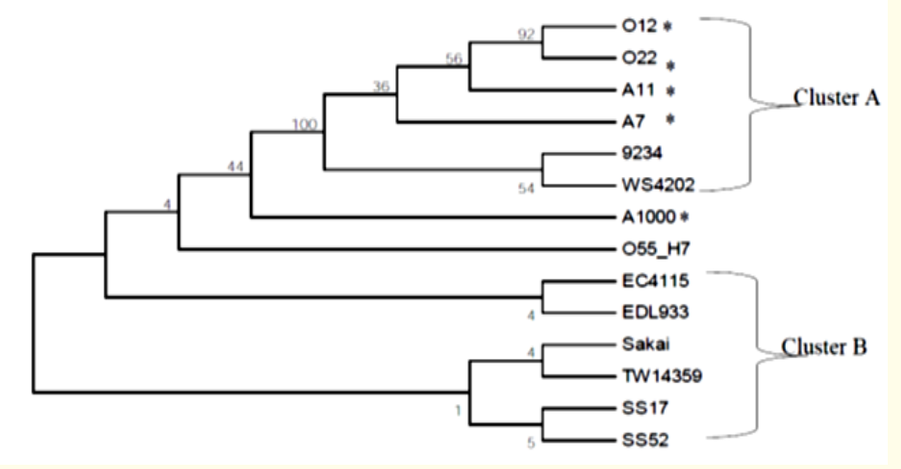

Figure 7: *Phylogenetic tree of 9 E. coli strains and 5 sequenced samples, based on genetic distance analysis of fliC gene 625 and 1000bp sequencing. The sequenced samples and the strains were grouped into two clusters A (O12, O22, A11, A7, str. 9234 and str. WS4202) and B (str. SS52, str.tW14359, str.SS17, str. Sakai, str.EDL93 and str. EC4115) depending on the evolutionary distance. The tree was constructed with Neighbor-Joining method. The numbers at the nodes are bootstrap confidence values based on 1000 replicates. (*) Indicates for the sequenced tested sample.*

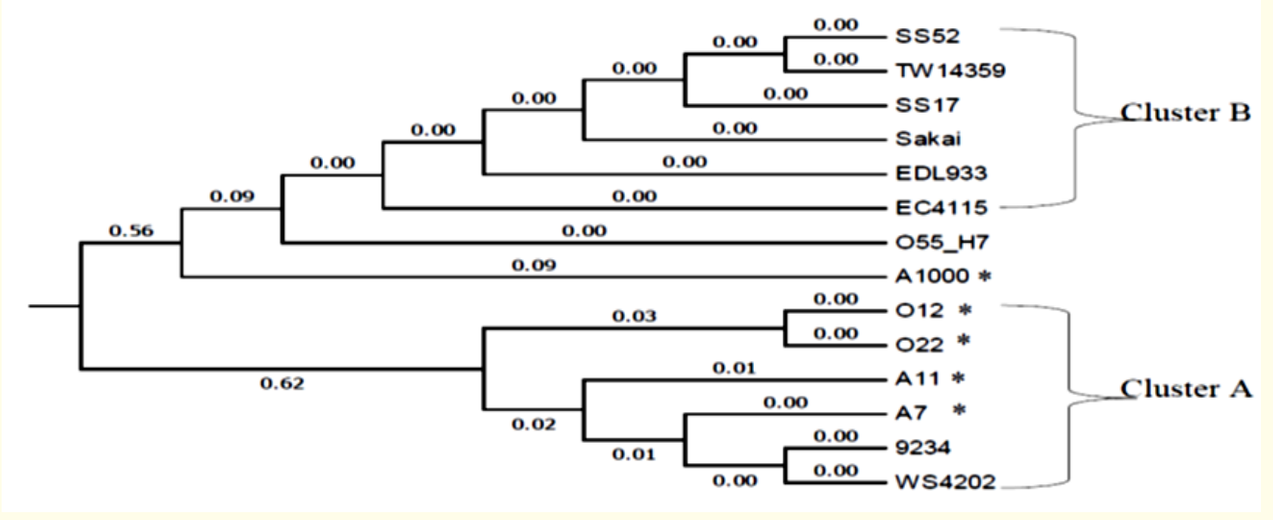

Figure 8: *Phylogenetic tree of 9 E. coli strains and 5 sequenced samples, based on genetic distance analysis of fliC gene 625 and 1000bp sequencing. The tree was constructed with UPGMA method. The sequenced samples and the strains were grouped into two clusters A (O12, O22, A11, A7, str. 9234 and str. WS4202) and B (str. SS52, str.tW14359, str.SS17, str. Sakai, str.EDL93 and str. EC4115) depending on the evolutionary distance. The numbers at the nodes are represent the branch length (Evolutionary distance). (*) Indicates for the sequenced tested sample.*

The sequenced samples (A7, A11, A12, A22 and A1000) was published on NCBI GeneBank in Feb 18, 2018 with the ACCESSION GenBank: MG574560, MG574561, MG574562, MG574563 and MG574564 respectively.

Conclusion

The main causal agent of diarrhea among children in Khartoum is either EPEC (*O55:H7*) or EHEC (*O157:H7*) serogroups. Results have also suggested that the serotype *E. coli O157:H7* might be one of the prevailing bacteria in Sudan. (34%) of diarhheal samples collected amplified new band for the fliC gene, the 1000bp fragment, along with the diagnostic band 625bp fragment. Results of the fliC gene

sequence indicate relationship between sequenced fliC fragments and strains of *E. coli O157:H7* (EC4115, EDL933, Sakai, SS17, SS52, TW14359, WS4202, 9234) and *E. coli O55:H7* (str. RM12579). All these findings enlighten some aspects about diarrheal diseases and their causal agents in Sudan and help in a better clinical diagnosis for the Study participants.

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Conflict of Interest

This is to declare that the authors have no conflict and interest.

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