ETIOLOGY OF BLOODY DIARRHEA IN CHILDREN AND PREVALENCE OF *E. COLI* O157:H7

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ABSTRACT

Background

Infectious diarrhea continues to be a health burden worldwide especially in children living in developing countries. The main etiologies of bloody diarrhea in developing regions are *Entamoeba histolytica* and bacteria such as *Shigella* and other bacteria.

Objectives

To determine the causative agents of bloody diarrhea in children and the prevalence of Enterohemorrhagic *Escherichia coli* in children with bloody diarrhea.

Materials and Methods

A cross-sectional study conducted at Dr.Jamal Ahmad Rashid Teaching Pediatric Hospital in Sulaimani city from October 2018 to August 2019. We examined stool specimens from inpatient children with diarrhea using fresh mount. We further investigated the bloody stools by cultivation on several bacteriological media. Growth was identified and the causative agents were determind based on culture, Gram stain, biochemical tests, Serological test and VITEK® 2 system. Finally, we used multiplex PCR to identify EHEC O157:H7 and Shiga toxin genes.

Results

From 2589 fresh mount stool examination, 117 (4.5%) were identified as bloody diarrhea based on finding RBCs. Of the 117 cultivated stool specimens, bacteria were identified as the cause of bloody diarrhea in 73(62.4%), *E. histolytica*/ *E. dispar* in 36 (30.8 %), bacteria and *E.histolytica*/ *E. dispar* in 6 (5.1%), while in 2 (1.7%) specimen, the cause remain unidentified. The isolated bacteria were *Shigella* spp. (56, 69%), *Slamonella* spp. (11, 14%), Enteroinvasive *Escherichia coli* (6, 7%), *Campylobacter* spp. (3, 4%) and Enterohemorrhagic *Escherichia coli* (3, 4 %). Two EHEC showed shiga toxin type two gene.

Conclusion

Shigella spp. and *E. histolytica* were the most prevalent agents of bloody diarrhea in children aged 7 months to 12 years. Enterohemorrhagic *Escherichia coli* harboring shiga toxin type 2 gene was identified in bloody diarrhea but in fewer cases compared to other bacteria.

Keywords: *Children bloody diarrhea; E. histolytica; Shigella; Enterohemorrhagic Escherichia col; EHEC; O157:H7* .

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INTRODUCTION

Infectious diarrhea is a leading cause of morbidity and mortality in children in the developing countries [1]. Over 1.7 billion global cases of diarrheal disease are reported annually and these are associated with an estimated 2.2 million deaths [2]. Diarrheal diseases mostly affecting the developing countries, where unsafe water supplies, poor sanitation, and nutritional deficiencies play a role in spreading the disease [3].

Bloody diarrhea represents approximately 20-30% of all cases of diarrhea [4]. Bloody diarrhea (dysentery) means either the blood is visible in the stool, or blood and mucous are detected by microscopy [3]. Dysentery is a major cause of childhood morbidity and mortality, especially in developing countries in Africa, Asia, and Central and Latin America [4]. About 10% of the total diarrheal episodes globally have visible blood in the stools while, acute bloody diarrhea accounts for 5–15% of all diarrheal deaths in children aged under five years in low and middle-income countries [5].

Bloody diarrhea is transmitted by fecal-oral route through contaminated food and water, close personal contact or sexual contact with fecal exposure [6],or after ingestion of contaminated food from animal origin [7]. Several bacterial strains such as *Shigella*, Enteroinvasive *Escherichia coli* (EIEC), Enterohemorrhagic *Escherichia coli* (EHEC, or *E. coli* O15:H7), *Salmonella*, *Yersinia* and *Campylobacter* may cause dysentery in all regions of the world. In the developing tropical and semitropical regions, *Entamoeba histolytica* is an important cause of dysentery especially in older children and adults living in rural areas [8]. *Shigella* alone causes 10 to 15% of acute diarrhea and more than 50% of all the dysentery cases in children less than five years of age. In the developing countries, *Shigella flexneri* continues to be the most common bacteria, in contrast to the developed world where *Shigella sonnei* is more common [9].

Enterohemorrhagic *Escherichia coli* strains are the main foodborne, zoonotic, bacterial pathogens that may cause bloody diarrhea, and hemorrhagic colitis. In some circumstances, EHEC infection can lead to hemolytic uremic syndrome (HUS) and severe clinical complications, including kidney failure and thrombotic thrombocytopenic purpura [10]. Shiga-toxin producing *E. coli* (STEC) causes around 2.801.000 acute illnesses and lead to 3890 cases of HUS and 230 deaths [11]. *E. coli* O157:H7 are normal flora of the ruminants especially cattle[10].Transmission of EHEC is through ingestion of contaminated food or water due to improperly washed hands or following ingestion of contaminated foods from animal origin harboring the organism specially meat and meat products, as well as milk and dairy products, person to person contact and direct contact with animal [7].

The highest rates of sporadic *E. coli* O157:H7 enteric infections was reported in Canada with a rate of 13 per 100,000 [12]. In Europe, Scotland infection rates of approximately 4 cases per 100,000, while in Northern Europe infection rates are very low (e.g. 0.04 per 100,000 in Norway and Finland). In Asia, Japan infection rate of 2.74 per 100,000 was averaged between 1999 and 2004 [13].

The virulence factors contributing to intestinal colonization and establishment of disease in humans by EHEC include type III secreted proteins, factors mediating intimate adhesion (Tir/Intimin), and shiga toxins. These Factors are implicated in the formation of attaching and effacing lesions, which leads to the loss of microvilli from the intestinal brush border [14].*E. coli* O157 infection and HUS are largely pediatric illnesses, although they can occur at any age where 8% of STEC infection develops to HUS. Two to fifteen percent of HUS reported in children below five years and the case fatality rate was 3% to 5% [15].

In this study, we investigated the etiology of bloody diarrhea among hospitalized children with diarrhea and to determine the prevalence of EHEC O157:H7 in children bloody diarrhea.

MATERIALS AND METHODS

This cross sectional study was conducted on children complaining from bloody diarrhea who were admitted to Dr. Jamal Ahmad Rashid Pediatric Teaching Hospital in Sulaimani city during a period from October 2018 to August 2019. The study was approved by the Ethics Committee at College of Medicine, University of Sulaimani. Information regarding patient's age, gender, weight, residence, family status, source of water and consumption food, family history of diarrhea, etc. was acquired. Stool samples were collected in the sterile cup containers; the stool samples were preceded in less than one hour.

Stool specimens from all patients with diarrhea were examined macroscopically for color, consistency, presence of blood, mucus. Wet mount preparation with 0.9% saline and 0.5% Iodine were examined using higher power magnification of a light microscope for

white blood cells, red blood cells, cysts and trophozoites of protozoa, and bacteria [9, 16]. When specimen identified as bloody diarrhea, the stool was further examined using a smears with 1% carbolfuchsin for 30 seconds, and a second smear was stained with Gram´s stain using 0.3% carbolfuchsin as counter stain for five minutes. The stained smears were examined using 100 x magnifications for morphological appearance of *Campylobacter* spp. [17].

The stool specimens were cultivated for isolation of bacterial agents using different media. For isolation and identification of *Shigella* and *Salmonella* species, specimens were placed on MacConkey agar, Helton enteric agar, xylose lysine deoxycholate agar, and Salmonella Shigella agar, (all provided by Neogen, UK). The cultures were incubated at 37°C for 18-24 hours. *Salmonella* or *Shigella* species were detected based on colonial characteristic of lactose non-fermenter colorless colonies on MacConkey agar, green small colonies of *Shigella* on Helton enteric agar (*Salmonella* green and black center), *Shigella* red colonies(*Salmonella* red with a black center) on XLD, while on Salmonella Shigella agar, *Shigella* formed colorless colonies and *Salmonella* produced colorless with black center. The suspected colonies were confirmed by biochemical tests to identify *Shigella* and *Salmonella* species and confirmed by VITEK® 2system (Biomurex, France) [18-20].

For isolation and identification of *Campylobacter* species, the samples were inoculated on modified charcoal selective media agar (Liofilchem, Italy) and incubated at 42°C for 48-72 hours. *Campylobacter* species were identified by small gray colonies at microaerophilic condition, positive oxidase and catalase test, Gram-staining reaction and morphology [21, 22]. For isolation and identification of EIEC and EHEC, the specimens were inoculated on MacConkey agar (Neogen, UK) and Sorbitol MacConkey agar (SMAC) (Oxoid, UK) and incubated at 37°C for 24 hours. EIEC was identified by lactose non-ferment colorless colonies on MacConkey agar, and confirmed by agglutination test using poly-antisera (Baharafshan, Iran) [23]. EHEC was identified by growth of sorbitol non-ferment colorless colonies. The presumptive growth were confirmed by VITEK® 2 system where Gram Negative (GN) card was used according to the manufacturer's instructions [24].

For detection of shiga toxin genes in *E. coli* O157:H7, the suspected isolates were grown on trypticase soya broth (Neogen, UK) overnight at 37°C. DNA extraction was performed using DNA extraction kit (Genet Bio,Korea) according to manufacturer's instructions. Multiplex PCR was performed using primer set targeting *E. coli* O157:H7 *rfb* O157 (F: 5`CGGACATCCATGTGATATGG3`, R: 5`TTGCCTATGTACAGCTAATCC3`), *flic* H7 (F: 5`GCGCTGTCGAGTTCTATCGAGC3`, R: 5`CAACGGTGACTTTATCGCCATTCC3`) genes [25, 26]. Moreover, presences of virulence genes were detected by using specific primers for amplification of Shiga toxin one(*stx1*) gene (F: 5`ACA CTG GAT GATCTC AGT GG3`, R: 5`CTG AAT CCC CCT CCA TTA TG3`), *stx2*(F: 5`CCA TGA CAA CGG ACA GCA GTT3`, R:5`CCT GTC AAC TGA GCA CTT TG3`) [27].

Multiplex PCR carried in 20µL reaction containing 10 µL reaction master mix (HS Prime Taq DNA Polymerase 1 unit/10 µL, 2X reaction buffer) provided by Genet Bio, Korea, 2µL of each primer, 3 µLof water, and 3 µLof DNA template. Amplification for *rfb*O157 and *flic* H7 genes was done on a Techene thermo cycler (UK) using initial denaturation at 94°C for 3 min,30 cycles of denaturation at 94°C for 60 s, annealing at 62°C for 30 s, extension at 72°C for 60 s, and final extension at 72°C for 5 min. For *stx1*, *stx2* an initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 60 s, annealing at 53°C for 30 s, extension at 72°C for 60 s, and final extension at 72°C for 5 min. The amplified products were analyzed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining (0.5 g/mL). The results were recorded using the gel documentation system (Taiwan, Major Science).

RESULTS

From total tested 2589 stool specimens, 117 (4.5%) stool samples showed RBCs and identified as bloody diarrhea. Tables 1 show the characteristics of study samples (117 children).They included 69 males (58.9%) and 48 females (41 %), the age ranged from7 months to 12 years.

From the 117 bloody diarrhea specimens, bacteria were isolated from 73(62.4%), parasite trophzoites and cysts of *E.histolytica/ E.dispar* were found in 36 (30.8 %), while six (5.1%) specimens show both *E.histolytica/ E.dispar* and bacteria but in two specimens (1.7%) the causative agent remained unidentified. Apart from the amoebic cause of bloody diarrhea (42 *E.histolytica/*

E.dispar from 117 specimens), the pathogenic bacteria isolated were mostly *Shigella* spp. (56, 69%), followed by Non-typhoidal *Salmonella* spp. (11, 14%), EIEC (6, 7%), *Campylobacter* spp.(3, 4%), and EHEC O157:H7 (3, 4%), Table 2.

The results showed that the two most common causes of bloody diarrhea were *E. histolytica/E. dispar* and *Shigella* spp. According to these two groups, we compared characteristics like sex, age group, residence, family history, recurrence, water source, and fever. We also compared the frequency of diarrhea, duration of diarrhea, and finding RBCs and pus cells in stool examination (Table 3). Statistically significant relation was found between *Shigella* and duration of diarrhea, RBCs, and pus cells in stool.

The results of multiplex PCR on EHEC O157:H7 is shown in Figure 1. The amplicons for *rfb* O157,*flic* H7,*stx1* and *stx2* yields different band size of 259bp, 625bp, 614bp, and 779bp respectively. The results for *rfb* O157, *flic* H7 gave band within band size in all three isolates, while only two strain were positive for *stx2* and all were negative for *stx1* which show in Figure 1.

Table 1. The characteristics of the study samples (*n*=117).

Characteristics	Frequency	Percentage
Sex		
Male, $\overline{X}$ age = 4.62 y	69	59
Female, $\overline{X}$ age = 5.15 y	48	41
Age (Year)		
A (≤1)	19	16.2
B (>1≤5)	53	45.3
C (>5)	45	38.5
Diarrhea duration (Days)		
≤ 2	38	32.5
> 2	79	67.5
Diarrhea frequency (Times)		
< 5	27	23.1
5-10	70	59.8
>10	20	17.1
Bloody diarrhea in family		
Yes	18	15.4
No	99	84.6
Recurrent diarrhea		
Yes	14	12
No	103	88
Residence		
Urban	69	67.5
Suburban	38	32.5
Living quality standard		
Moderate	73	62.4
Poor	44	37.6
Source of water		
Tap water	91	77.8
Filter water	9	7.7
Spring water	2	1.7
Well water	6	5.1

Table 2. The frequency and percentage of identified pathogenic bacteria from 81 stool specimens.

Bacteria	Number	Percentage
***Shigella* spp.**	56	69
***Salmonella* spp.**	11	14
EIEC	6	7
EHEC (O157:H7)	3	4
***Campylobacter* spp.**	3	4
Unidentified	2	2
Total	81	100

Table 3. The differences between *Shigella* spp. (*n*=54) and *E. histolytica*/*E. dispar* (*n*=36) in regard of frequency of diarrhea , duration, RBCs and pus cells observed in stool examination.

	Cause	Mean	Median	SD	*P*-value
Frequency of diarrhea					
	Shig. spp.	8.41	8	2.514	0.223
	Ent. his./dis.	7.69	8	2.96	
Duration of diarrhea					
	Shig. spp.	2.37	2	0.808	0.043
	Ent. his./dis.	2.78	2.5	1.07	
RBCs					
	Shig. spp.	18.56	20	9.536	< .001
	Ent. his./dis.	11.81	10	8.42	
Pus cells					
	Shig. spp.	18.43	20	6.717	0.019
	Ent. his./dis.	15	20	6.61	

*** P-values analyzed by Student's independent T test.**

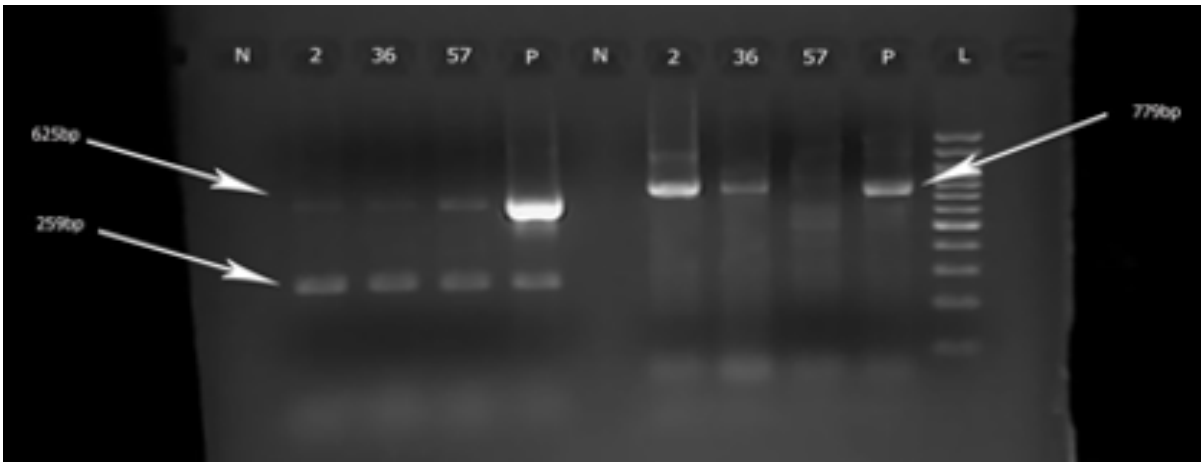

Figure 1 Agaroes gel electrophoresis 2% of m-PCR products. From the left to right shows negative control, samples number 2, 36, and 57for*rfb* O157, *flic* H7, positive control (E.coli O157:H7 ATCC 43894). Negative control, samples number 2, 36, and 57 that was positive (2 and 36) for *stx2*. S*tx1* was negative in all three, positive control for *stx2*, and DNA marker 100-1000bp.

DISCUSSION

Acute infectious diarrhea accounts for significant morbidity and mortality in all regions in the world and among all ages with over 2 million death occurring each year, particularly among children younger than five years [28]. Dysentery is considered as a main cause of childhood morbidity and mortality in developing countries [29]. The causes of acute dysenteric diarrhea vary by population and geography [30]. We carried out this study to identify the causes of bloody diarrhea among children. The results showed that amoebic dysentery is still prevalent in our region but not as dysentery caused by *Shigella* spp. From bloody stool, we isolated different bacteria including *Shigella*, *Salmonella*, EIEC, EHEC O157:H7 and *Campylobacter*, in children aged from 7 months to 12 years.

The prevalence of bloody diarrhea among children with diarrheas, who mostly settled in urban areas, was 4.5%. This rate was less than previously reported in Kurdistan Region(8.2 %) [31]. Although we found that the prevalence of bloody diarrhea is increasing with increased age of the child and was highest among children aged 1-5 years, in males compared to females, however no statistically significant difference between age groups and sex was found, this finding agree with other local studies [32, 33].

From our results, bacteria rather than *E.histolytica/E. dispar* were responsible for bloody diarrhea, this was similar to a pervious study from Bolivia [33]. *Shigella* and *E. histolytica/E. dispar* were the main causes of bloody diarrhea, comparing to a previous local study the prevalence of *Shigella* infection has increased while, *E. histolytica/E. dispar* gradualy decreased [34]. However, this result goes with another study conducted by Al- Jnabee [35].

Shigella is invasive bacteria that multiply within colonic epithelial cells causing inflammation, mucosal ulceration, and bleeding. the symptoms associated with this pathogen vary from mild watery diarrhea to severe dysentery accompanied with abdominal pain, cramps, fever and stools containing blood and mucus [36]. Our findings with *Shigella* infection showed statistically significant relation with fecal RBCs and pus cells, findings indicating colonic inflammation as previously reported [37, 38]. The high communicability, person to person contact, and low infection dose of *Shigella* may explain its high predominance among organism causing bloody diarrhea and make it difficult to control [36].

Entamoeba histolytica infection rate varies among in developing countries, affected by socioeconomic status, hygienic conditions, population, and transmission is often related to contaminated food and water [39]. In our study, the prevalence of *E. histolytica* changed according age, sex, residence, socioeconomic, and water source. *E. histolytica/E. dispar* was documented in 42.9% of children aged 1-5 or 5 -12 year, higher compared to children below one year. This was agreement to a local study [40], and was more in males as showed also by Victor et al. [39]. This may be attributed to lifestyles as boys are more active, and likely to interact more with contaminated environments [41]. Also, in our study infection was more from urban zone compared to suburban and this is similar to Harb et al. study [42].

Non-typhoidal Salmonella (NTS) was also isolated in our study; NTS prevalence was 14% among bacterial causes of bloody diarrhea. This rate was approximately similar(10.3%) to Harb et al. study [43], but higher than what reported from India (3.3%) [9], while NTS were the most common bacterial isolates in childhood gastroenteritis (17.7%) in Mexico study [44].NTS may cause severe invasive disease, where infants and children less than five years of age are the most vulnerable. In most African countries, NTS are among the common bacteria, responsible for bloodstream infections in children. Death related to invasive NTS among hospitalized children ranged from 4.4% to 27% [45]. NTS usually causes self-limiting gastroenteritis [46]. However, in children, the elderly and immunocompromised patients, severe invasive disease with complicated extra-intestinal illness and bacteremia [47]. In this study, NTS infections were significantly related with fecal RBCs and pus cell, these findings were reported previously [48].

Campylobacter infection is one of the most widespread infectious diseases. In some developing countries campylobacteriosis is endemic especially in children [49]. In our study, the prevalence of *Campylobacter* infection was low (4%) compared in to other developing countries, where *Campylobacter* came secondly in children bloody diarrhea (23%) as in Kenya [50], while lower rate (3.7%) reported from Egypt [51].

The diarrhea accompanying *C. jejuni* infection is considered as inflammatory, with fever, and stools usually contain pus cells, gross or microscopic blood [52]. In our study, *Campylobacter* infection was associated with fever, vomiting, abdominal pain, pus cells and RBCs [22].

In our study, we isolated three sorbitol-negative *E. coli* (O157:H7), these were also confirmed by PCR. They constituted 4% among the isolated bacteria. EHEC infection is not common, this was also reported [53], where from 126 *E.coli*, only 11 isolates were EHEC at percentage of 4.78% in children gastroenteritis. However, another study from Iraq [54] showed a prevalence of 18.5%, more in children under one year, a result higher than ours where the three children with EHCE were under five years. We could not relate the source of infection in EHEC to food contaminated with cattle and patients showed variable clinical features beside their bloody diarrhea but they not developed HUS.

The EHEC isolates were grown on SMAC and confirmed by PCR detection of both *rfb* O157 and *flic* H7 genes. This is indicated usefulness of culture on SMAC. Also by PCR we detected *stx2* virulence gene in two EHEC. The *stx2* genotype strains apparently influences pathogenic ability and the variants of *vtx2* has been found to be significantly more common in strains isolated from patients who had developed HC and HUS than did the other *stx* gene variants [55]. A study detected more *stx2* (68.4%) than *stx1*(10.5%) from the 19 *E. coli* 0157:H7 serotypes isolated from bloody diarrhea [24].

Enteroinvasive *E.coli* (EIEC) was another isolate bacterium from bloody stool with rate of 7%. This figure is similar to other studies (7.4%) from Iran [56],and 5.6%from India [57], but is lower to Thailand (0.6%) and Chile (1.9%) studies [58, 59]. Some EIEC strains have remarkable phenotypic and genotypic similarity with *Shigella* species. They are usually non motile, lactose negative and lysine-decarboxylase negative [60]. In our study, all EIEC were lactose non-fermenter. This outcome was shown previously [61], and Vieira et al. showed that 36 EIEC isolates were all lactose-negative and only seven were lactose-positive [23].

In conclusion, bloody diarrhea constituted 4.5 % of admitted children with diarrhea. *Shigella* spp.and *E. histolytica* were the most prevalent agents of bloody diarrhea in children aged 7 months to 12 years. Other bacteria including Non-typhoidal *Salmonella* spp., EIEC, and *Campylobacter* spp. were reported. EHEC harboring shiga toxin gene type two genes was identified in bloody diarrhea but in fewer cases compared to other bacteria.

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