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A STUDY ON ENTEROHAEMORRHAGIC E. COLI O157: H7 ASSOCIATED WITH DIARRHEA AND HEMOLYTIC UREMIC SYNDROME IN CHILDREN

(With 6 Tables and one Figure)

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دراسة عن ميكروب الإيشريشيا القولونية المعوية النزفية والمصاحبة لحالات
الإسهال ومتلازمة انحلال الدم البوليوني في الأطفال

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يعتبر ميكروب *E. coli* O157:H7 من أهم الميكروبات التي تنتقل إلى الإنسان عن طريق الغذاء الملوث والتي تشكل خطورة على صحة الإنسان وخصوصاً في الأطفال حيث تعتبر متلازمة انحلال الدم البوليوني من المضاعفات الشائعة والخطيرة نتيجة للإصابة بهذا الميكروب. وقد أجريت هذه الدراسة لتحديد نسبة الإصابة بهذا الميكروب وكذلك نسبة حدوث متلازمة انحلال الدم البوليوني لدى مجموعة من الأطفال تعاني من حالات الإسهال المدمم والإسهال الغير المدمم. لذلك فقد تم جمع عدد ٢٠٠ عينة عشوائية من براز الأطفال بواقع ١٠٠ عينة من حالات الإسهال الغير المدمم ، ٥٣ عينة من الإسهال المدمم و ٤٧ عينة من حالات الإسهال المدمم المصاحب لمتلازمة انحلال الدم البوليوني من وحدة الجهاز الهضمي في مستشفى الأطفال بأسبوط في الفترة من أكتوبر ٢٠٠٣ إلى سبتمبر ٢٠٠٤. وقد تم عزل الميكروب بنسبة ١٦,٩٨% ، ١٧% و ٣% لكل من حالات الإسهال المدمم، حالات الإسهال المدمم والمصاحب لمتلازمة انحلال الدم البوليوني وحالات الإسهال الغير المدمم على التوالي. وقد ارتفعت نسبة الإصابة بين الأطفال في الريف (١١,٣%) عن المدينة (٥%) وتم عزل الميكروب من الأطفال التي تم تغذيتهم على منتجات ذات الأصل الحيواني بنسبة ١٧,٢٧% و ٢,٥% للألبان الصناعية. وقد اثبتت الدراسة أن غالبية المرضى من الأطفال كانت تتراوح أعمارهم بين (٧-٢٤ شهر) وكانت نسبة الإصابة بينهم (١٨,٣%) بينما كانت نسبة الإصابة (٥%) بين الأطفال التي تتراوح أعمارهم أقل من ٧شهور. كما أظهرت النتائج أن الإصابة كانت اعلى بين الذكور (١١,٤%) عن الإناث (٦,٦٧%). وقد تمت دراسة مدى حساسية العترات المعزولة، وأظهرت النتائج أن ٦٠% من العترات قاومت الكلورامفينيكول وان ٣٠% من العترات كانت مقاومة للأمبيسلين، ١٠% من العترات قاومت الأيرثرومييسين وان ٥% من العترات كانت مقاومة لكل من الجينتاميسين

والتيتراسيكلين، ودلت النتائج ان ٣٠% من العترات كانت تحمل البلازميد وتراوح وزنه الجزيئي بين ١,١ - ٤٥ ميغا دالتون. وقد تمت مناقشة الأهمية الصحية والوبائية لميكروب *E.coli* O157:H7 ومصادر التلوث المختلفة، هذا بالإضافة إلى مناقشة التوصيات لكيفية الحد من انتشار هذا الميكروب وكيفية الوقاية منه.

SUMMARY

Shiga toxin-producing *Escherichia coli* (STEC) is considered one of the most important emergent zoonotic food borne pathogens. A total of two hundred random samples were collected from children with acute diarrhea. 53 of them had blood in stools, 47 had blood in stools and associated with hemolytic uremic syndrome (HUS). The other hundred had no blood in their stools. Samples were collected from the gastroenterology unit of Assiut Children University Hospital during the period from October 2003 to September 2004. The present study was designed to estimate the incidence of STEC O157:H7 infection among diarrheal children with and without blood in stools, moreover percentage frequency of HUS among patients with positive STEC infection was evaluated and demographic and clinical characteristics of the STEC patients were investigated. Furthermore antibiotic resistance patterns of the recovered strains were studied and plasmid profile of the obtained isolates were performed to elucidate the relation between the obtained strains. *E.coli* O157:H7 could be detected in 16.98%, 17% and 3% of bloody diarrhea, bloody diarrhea associated with HUS and non bloody diarrhea, respectively. Ecological distribution of the examined children revealed that the rate of infection was higher (11.3%) in rural areas than in urban areas (5%). *E.coli* O157:H7 was recovered in children fed on bottle milk (2.5%) and animal products (17.27%) while it was not isolated from children with breast feeding. The majority of cases in the present study were in the age group of 7-24 months with a rate of (18.3%), followed by those in age group of <7months with a rate of 5%. It has been estimated that 60% of the isolated strains were resistant to ampicillin and 10% of them were resistant to erythromycin as well as 5% of the strains were resistant to gentamycin and tetracycline. It was revealed that out of the isolated 20 isolates of *E.coli* O157:H7, 6 (30%) harboured some copies of plasmids ranging in size from (1.1 to 45 MDa). The obtained strains harbouring plasmids were grouped into 6 plasmid profiles with different molecular weights and this results reveal the existence of a variety of clones which may indicate several sources of contamination.

Key words: *E.coli O157:H7, Diarrhea, Children, Hemolytic uremic syndrome, Antimicrobial susceptibility, plasmid profile.*

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC), especially of serotype O157:H7 is considered one of the most important emergent zoonotic food borne pathogens constituting a worldwide public health problem either in the form of individual cases of infections or outbreaks (Leclercq *et al.*, 2001). Epidemiological investigators revealed that different species of animals may act as a reservoir of *E. coli* O157:H7 (Nelson *et al.*, 1998). STEC infection in man have been associated with consumption of food of animal origin especially undercooked beef or raw milk including cow's, goat's and ewe's milk (Little and De Louvois, 1999; Rubin *et al.*, 1999; Stephan and Kuhn, 1999 & Sayed and Husein 2003). Moreover infection may also be waterborne or acquired via person to person transmission in such communities as nursing homes, day care centers, schools and hospitals (Nelson *et al.*, 1998 & Hashimoto *et al.*, 1999).

Shiga toxin- producing *E. coli* is associated with wide spectrum of clinical manifestation including non-specific diarrhea, hemorrhagic colitis and life threatening hemolytic uremic syndrome (HUS) which leads to acute renal failure in children and thrombocytopenic purpura, all of which are related to adherence of the pathogen to intestinal tract lining followed by production of one or more vero toxins which is implicated in vascular endothelial damage observed in hemorrhagic colitis and HUS patients (Gray, 1995; Nelson *et al.*, 1998 & Karpman *et al.*, 2001).

E.coli O157:H7 is the prototypic enterohaemorrhagic *E.coli* (EHEC) which produces potent cytotoxins known as shiga like toxins (SLT) or shiga toxins (STx). Shiga toxins are translocated from the bowel to the circulatory system and transported by leukocytes to capillary endothelial cells in renal glomeruli and other organs (Karmali, 2004). STx have been specifically implicated as a causal factor for HUS because cases with HUS were found to be associated with STx-producing strains; the toxin has been identified in the kidney of patients with the syndrome; and the toxin was found to be cytotoxic for renal endothelial and epithelial cells (Karpman *et al.*, 2001). The exact mechanism responsible for the syndrome, however, remains speculative. Endothelial injury is considered the primary pathogenic event in diarrhea associated HUS. Moreover, an acute inflammatory response in STEC

infection including leukocytes and inflammatory mediators was also suggested to play an important role in the pathogenesis of STEC induced hemolytic uremic syndrome (HUS) by enhancing the effect of shiga toxins produced by the organisms (Litalien *et al.*, 1999).

The present study was designed to estimate the incidence of STEC O157:H7 infection among diarrheal children with and without blood in stools, moreover percentage frequency of hemolytic uremic syndrome (HUS) among patients with positive STEC infection was evaluated and demographic and clinical characteristics of the STEC patients were investigated. Furthermore antibiotic resistance patterns of the recovered strains were studied and plasmid profile of the obtained isolates were performed to elucidate the relation between the obtained strains.

MATERIALS and METHODS

Collection of Samples:

Two hundred random stool samples were collected from children with acute diarrhea (53 of them had blood in stools, 47 had blood in stools and associated with HUS while the other hundred had no blood in stools). Samples were collected from the gastroenterology unit of Assiut Children University Hospital during the period from October 2003 to September 2004.

Complete case history was reported for each patient including feeding pattern, duration, frequency, consistency and the presence of visible blood in stools, presence of fever, convulsions or other neurological insults, and history of antibiotic administration before or during hospital stay. Diagnosis of the HUS was made according to Chandler *et al.*, (2002), using the following criteria:

Platelet count < 150,000/mm³, micro-angiopathic hemolytic anemia (hematocrit below 30% with evidence of reticulocytosis and fragmented erythrocytes on blood peripheral smear) and renal insufficiency as indicated by oliguria or anuria and azotemia. In all children with proven STEC O157:H7 infection, daily complete blood counts were obtained and renal function tests were performed for clinical purposes until the hemolytic uremic syndrome developed and resolved or until it became apparent that the infection was resolving without the occurrence of this complication.

Exclusion criteria:

Cases with diarrhea for more than 2 weeks, those with malnutrition, rickets, or suspected immune deficiency syndromes and

those with proven protozoal infection, hemorrhagic disorders or known chronic renal diseases were also excluded from the study.

Isolation and identification of *E.coli* O157:H7:

Enrichment Technique:

Stool samples were enriched in modified Tryptic Soya Broth (mTSB) supplemented with novobiocin (20 mg/liter). The inoculated broth was incubated at 37°C for 24 hours. (De Boor and Heuvelink, 2000).

Isolation on Sorbitol MacConkey agar:

Loopful from the incubated broth was streaked onto Sorbitol MacConkey agar plates and incubated at 37°C for 24 hours (De Boor and Heuvelink, 2000).

Identification of *E.coli* O157:H7:

Non sorbitol fermenter colonies were identified morphologically by Gram's stain and biochemically as *E.coli* according to Varnam and Evans, (1991) by the conventional IMViC (indole, methyl red, Voges Proskauer and Citrate utilization) and Triple sugar iron agar. A latex agglutination test (*E.coli* O157, Oxoid diagnostic reagents 620 M) was used for identification of *E.coli* serogroup O157 isolates. The Oxoid *E. coli* O157 latex was demonstrated by slide agglutination of *E.coli* strains possessing the O157 serogroup antigen according to Vernozy-Rozand, (1997). Bacto *E.coli* H7 antisera (Difco) was used to identify H7 strains according to manufacture's procedure.

Antibiotic susceptibility test:

The antibiotic sensitivity patterns were determined for the recovered strains by using the disc diffusion method (Schroeder *et al.*, 2002). The following antibiotic discs were used: ampicillin (10µg), chloramphenicol (30µg), erythromycin (15µg), gentamycin (10µg) and tetracycline (30µg).

Extraction of plasmid DNA:

E.coli O157:H7 strains were purified on Sorbitol MacConkey agar plates and incubated at 37 °C for 24 hours (De Boor and Heuvelink, 2000). Single colony was picked and inoculated in 10ml of Luria Bertani broth (LB) and grown in microaerophilic condition at 37°C for 10h. Plasmid extraction were done by using the alkaline lysis procedure as described by Woodford *et al.*, (1994).

Agarose gel electrophoresis:

10µl of the extracted plasmid was mixed with 10µl of loading buffer and the aliquots were loaded onto 0.7% agarose gel stained with ethidium bromide (0.5µg/ml). Electrophoresis was carried out at 90 v for

2-3h and visualized under UV transillumination (Biometra) at 320 nm and photographed (Woodford *et al.*, 1994). *E.coli* (V517) containing plasmids of molecular weight ranged from 1.4-35.8 MDa was used as molecular weight standard marker. The molecular weight of plasmids were calculated by blotting electrophoretic mobility of plasmid and the standard molecular weight marker.

RESULTS

Table 1: Incidence of *Escherichia coli* O157:H7 among the examined children

Type of Diarrhea	Examined samples	No.of infected children	%
Bloody	53	9	16.98
Bloody+HUS*	47	8	17
Non bloody	100	3	3
Total	200	20	10

*HUS: Haemolytic uremic syndrome.

Table 2: Ecological distribution of STEC infection among the examined children

Residence	Examined samples	No. of infected children	%
Rural	160	18	11.3
Urban	40	2	5

STEC: Shiga toxin producing *Escherichia coli*

Table 3: Feeding pattern of the examined children

Type of Feeding	No. of samples	No. of infected children	%
Breast	50	-	-
Bottle	40	1	2.5
Animal products*	110	19	17.27

* Children fed on breast milk or bottle milk together with animal products.

Table 4: Age-wise incidence of STEC infection among the examined children

Age-wise months	Type of Diarrhea										
	Bloody			Bloody + HUS			Non-bloody			Total	
	No. of samples	No. of infected children	%	No. of samples	No. of infected children	%	No. of samples	No. of infected children	%	No.	%
<7	12	2	16.7	17	1	5.9	31	-	-	3	5
7- 24	26	7	26.9	19	7	36.9	48	3	6.3	17	18.3
>24	15	-	-	11	-	-	21	-	-	-	-

STEC: Shiga toxin producing *Escherichia coli*

Table 5: Occurrence of STEC infection in male and female patients

Sex	Examined samples	No. of infected children	%
Male	140	16	11.4
Female	60	4	6.67

STEC: Shiga toxin producing *Escherichia coli*

Table 6: Antibiotic sensitivity pattern of the isolated STEC strains

Antimicrobial agents	Sensitive		Resistant	
	No./20	%	No./20	%
Ampicillin	14	70%	6	30%
Chloramphenicol	8	40%	12	60%
Erythromycin	18	90%	2	10%
Gentamycin	19	95%	1	5%
Tetracycline	19	95%	1	5%

STEC: Shiga toxin producing *Escherichia coli*

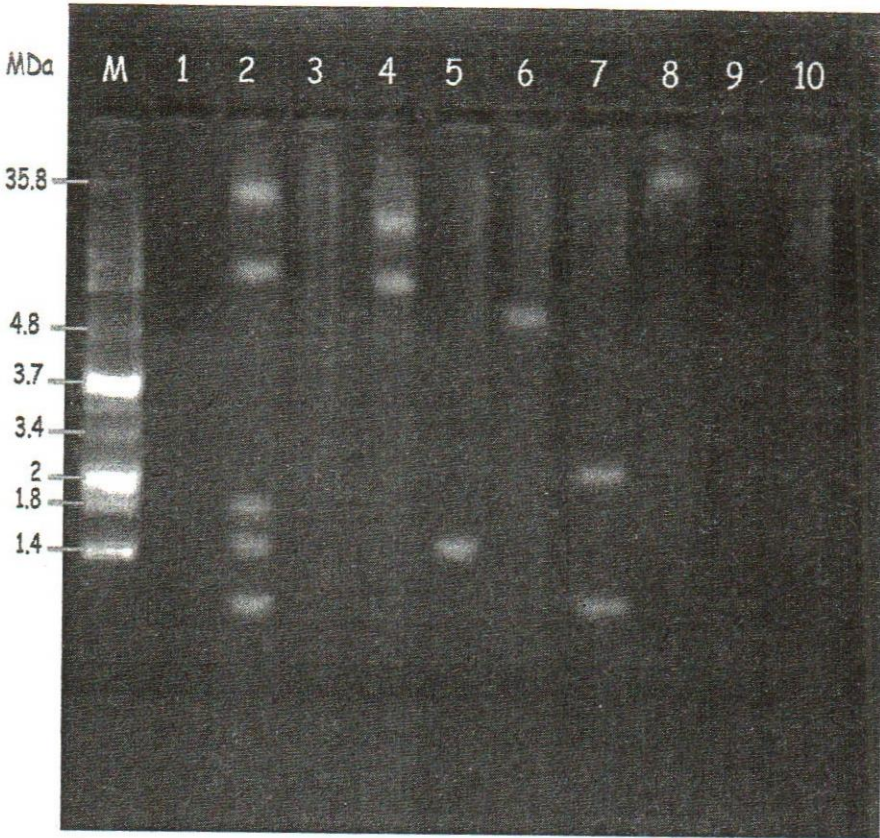


Figure 1: Plasmid profile of *E.coli* O157:H7 isolates.

M: *E.coli* V517 marker. Plasmid bearing isolates:2,4,5,6,7,8

Lane 2: (31, 12 , 1.9 , 1.6, 1.1 MDa) ; lane 4: (25, 10.5 MDa);

lane 5: (1.5 MDa); lane 6: (7 MDa); lane 7: (2.7 , 1.2 MDa);

lane 8: (45 MDa).

DISCUSSION

Episodes of diarrhea continues to be a major cause of childhood mortality among children in developing countries (Kosek *et al.*, 2003). Most episodes of bloody diarrhea among children in developing countries result from intestinal infection and nearly all of these are caused by invasive bacteria (WHO, 1994). Compared to the most other foodborne illness, infections involving STEC O157:H7 or other EHEC strains are particularly serious with life threatening post-diarrheal

disorder. The infection caused by this organism have probably increased in incidence during the past several decades (Tarr, 1994).

The present study emphasizes the importance of STEC O157:H7 in children with diarrhea. It has been estimated that the incidence of STEC O157:H7 was 10% among the examined children (Table 1). It was isolated from 3%, 16.98% and 17% of the examined children with nonbloody diarrhea, bloody diarrhea and bloody diarrhea accompanied with HUS, respectively as illustrated in (Table 1). It has been estimated that the attack rate of STEC infection among children ranged from 0.1% to 71% (Griffen and Tauxe, 1991). In addition Pai *et al.*, (1988); Ramotor *et al.*, (1995) and Buteau *et al.*, (2000) & Loirat, (2001) isolated STEC from children with diarrhea with a rate of 3.2 %, 0.6 %, 12% and 85%, respectively. The differences in the reported prevalence rates could be attributed to difference in the contamination rates in the studied areas.

Haemolytic uremic syndrome is defined by triad of features: acute renal failure, thrombocytopenia and microangiopathic haemolytic anaemia. Although there are different varieties of HUS, the most common form, by far is classical ; or D+(diarrhea associated) which is a leading cause of acute renal failure in childhood (Nelson *et al.*, 1998). The presence of blood in diarrheal stools in children is presumed to be a sign of invasive enteric infection that carries a risk of serious morbidity and mortality (WHO, 1994). Stools from patients with STEC infection are sometimes described as "all blood and no stool" thus simulating gastrointestinal hemorrhage (Tayler and Monnens, 1998). Results of the present study revealed that among patients with STEC infection who had bloody diarrhea, (17%) developed the HUS within a week of the first symptoms, while none of the nine cases of bloody diarrhea developed the syndrome (Table 1). The incidence of HUS among STEC infected children was variably reported by several authors including (Boyce *et al.*, 1995) who reported HUS in 6% of infected cases usually within 2-12 days of the onset of diarrhea. However, both Wong *et al.*, (2000) and Chandler *et al.*, (2002) indicated that HUS developed soon after the onset of diarrhea in 15% of children infected with STEC.

Previous epidemiological investigations contributed that contact with farm animals especially with cattle as well as contamination of food and water were considered the most important risk factors for acquiring STEC infection (Michel *et al.*, 1999). Ecological distribution of the infected children in the present study (Table 2) revealed that the incidence of infection was higher in rural areas (11.3%) than in urban

areas (5%). These results reflect the high contamination rates with STEC in rural areas. Our results are in agreement with that reported by Poitrineau *et al.*, (1995). Furthermore feeding pattern of the examined children illustrated in (Table 3) indicates that infection was high (17.27%) among children fed on breast or bottle milk together with animal products than those who fed on bottle milk only (2.5%). In addition non of the children fed on breast milk were infected. The obtained results indicates the role of animal products as a source of infection.

It has been estimated that the peak age-related frequency of STEC associated diarrhea and HUS was reported in young children (Nelson *et al.*, 1998). The highest incidence of STEC infection was recorded in children less than 5 years, and the HUS has been reported to be a more likely complication in younger children (Salmon *et al.*, 1999). The increased rate of STEC infection and HUS in children less than 5 years old suggests that immunity could play some role, moreover, younger age children may be highly susceptible to greater toxicity of STEC (Nelson *et al.*, 1998). However, protective immunity has not been demonstrated and even children without immunologic abnormalities have been infected more than once (Elliott *et al.*, 2001). The majority of cases in the present study (Table 4) were in the age group of 7-24 months (18.3%) with a rate of 26.9% , 36.9% and 6.3% for children with bloody diarrhea, bloody diarrhea associated with HUS and non bloody diarrhea, respectively. However the incidence of STEC O157:H7 in the age group of <7 months was (5%) as illustrated in (Table 4) with a rate of 16.7% and 5.9% for children with bloody diarrhea, bloody diarrhea associated with HUS, respectively. Our results are in parrallel with that reported by Poitrineau *et al.*, (1995) & Tozzi *et al.*, (2003). In addition results in (Table 5) declared that STEC O157:H7 was isolated from 11.4% and 6.67% of the examined males and females, respectively. Simillar findings were reported by Poitrineau *et al.*, (1995).

Antimicrobials are routinely used for disease prevention and growth promotion in animal production. This practice leads to the emergence and dissemination of antimicrobial resistance in STEC which poses a public health threat. (Witte, 1998 & Schroeder *et al.*, 2002). The obtained results in (Table 6) declared that 60% of the obtained strains were resistant to chloramphenicol followed by 30% of the strains were resistant to ampicillin and 10% of them were resistant to erythromycin as well as 5% of the strains were resistant to gentamycin and tetracycline. Simillar findings were reported by several authors

(Meng *et al.*, 1998; Aarestrup and Wegener, 1999; Galland *et al.*, 2001 & Zhao *et al.*, 2001). These results indicate the presence of multiple antibiotic resistant strains of *E. coli* O157:H7 which is considered a public health hazard.

Plasmid profile is considered as a useful strain molecular marker that distinguish the epidemic clone of a particular pathogen and help to identify specific vehicles of infection. (Wachsmuth *et al.*, 1991 & Taguchi *et al.*, 2000). It was investigated that out of the isolated 20 isolates of *E. coli* O157:H7, 6 (30%) harboured some copies of plasmids ranging in size from (1.1 to 45 MDa) as illustrated in (Figure 1). The obtained strains harbouring plasmids were grouped into 6 plasmid profiles with different molecular weights (lane 2: 31, 12, 1.9, 1.6, 1.1 MDa); lane 4: (25, 10.5 MDa); lane 5: (1.5 MDa); lane 6: (7 MDa); lane 7: (2.7, 1.2 MDa); lane 8: (45 MDa) and this results indicate the existence of a variety of clones which may point to the presence of several sources of contamination and the recovered isolates were epidemiologically different.

Conclusion and recommendation:

Shiga toxin producing *Escherichia coli* (STEC) infection was found to be relatively high among hospitalized children pointing to the role of food of animal origin especially milk and milk products which is considered as the most important source of transmission of STEC. The basic elements of food hygiene in the home and institutional setting need to be advocated aggressively in public education campaigns, especially through the popular media. The data presented by this study reinforce the importance of keeping STEC out of food chain at the farm level, preventing its dissemination in the food manufacturing process and cooking food of animal origin adequately to prevent infection at point of consumption. Moreover, infection with this organism may cause severe disease and complications including HUS with fatal outcome, so it is recommended that stool specimens from all patients with a history of acute bloody diarrhea should be cultured for STEC O157:H7. Early detection of cases with STEC infection can prevent additional cases particularly in hospitalized children and those attending day care centers. Separation of the infected children until two consecutive stool cultures are negative for STEC can prevent further transmission. It is highly recommended that during the week after the onset of acute bloody diarrhea, patients with documented STEC infection should be monitored for signs and symptoms of the HUS such as pallor, oliguria, peripheral blood smears, blood counts and urine analysis during this period.

However, the most important prevention measure is supervised by hand washing and health education of mothers and caregivers of infected children. The policy of using empirical antibiotics for treatment of diarrheal cases with blood in stools before results of stool culture and sensitivity testing should be revised. It is recommended not to give antibiotics to children who are infected with STEC unless it is approved by stool culture and sensitivity testing for the appropriate antibiotic treatment.

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