An-Najah National University
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Typing of Shiga Toxin-Producing *Escherichia coli* Isolates in Palestine by Multiplex and Arbitrarily Primed PCR.

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DEDICATION

TO

MY PARENTS, MY SISTERS

AND MY BROTHERS

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LIST OF DEFINITIONS

- EPEC: Enteropathogenic Esherichia coli.
- EHEC: Enterohemorrhagic Esherichia coli.
- Eagg. EC: Enteroaggregative Esherichia coli.
- ETEC: Enterotoxigenic Esherichia coli.
- EIEC: Enteroinvasive Esherichia coli.
- DAEC: Diffusely adhering Esherichia coli.
- HC: Hemorrhagic colitis.
- HUS: Hemolytic colitis.
- VTEC: Verotoxin producing Esherichia coli.
- STEC: Shiga toxin producing Esherichia coli.
- SLTEC: Shiga like toxin producing Esherichia coli.
- STX: Shiga toxin.
- EaeA: Attaching and effacing.
- Ehly: Enterohemolysin.
- KDa: Kilo Dalton.
- Gb_3: Globotriaosylceramide.
- SMAC: Sorbitol MacConkey agar.
- RBC: Red blood cell.
- PCR: Polymerase chain reaction.

ABSTRACT

Shiga toxinigenic *Escherichia coli* (STEC) comprises a diverse group of organisms capable of causing severe gastrointestinal and systemic diseases in humans. Within the STEC family, certain strains appear to be of greater virulence for humans, for example, those belonging to serogroup O157 and those with particular combinations of other putative virulence factors. One hundred seventy six Shiga toxigenic *Escherichia coli* (STEC) isolated from patients with either severe diarrhea or non-severe diarrhea in the northern Palestine in 1999 were characterized for virulence genes by multiplex PCR assay. Of the 176 STEC isolates, 124 (70.5%) belonged to the O157: H7 serotype. All of the STEC isolates were *stx1*$^+$, 140 (79.5%) were *stx2*$^+$. The *eae* locus was detected in 16 (9.1%) and the h*lyA*-encoding gene was detected in 18 (10.2%) of these isolates. Sixty-six of the 79 (83.5%) isolates obtained from individual with severe diarrhea, carried both the *stx1* and *stx2* toxin genes and 14 (17.7%) were *eae*$^+$, while 74 (76.3 %) of 97 isolates from individuals with non-severe diarrhea were *stx1*$^+$ *stx2*$^+$ and 2 (2.1%) carried *eaeA* encoding genes. The locus encoding the production of hemolysin was detected in 16 of 79 and 2 of 97 from severe diarrhea cases and non-severe diarrhea, respectively. Our results show a strong association between *stx1*, *stx2*, *eaeA* and *hylA* genes combined together and disease severity.

The combination of ERIC-PCR and multiplex-PCR analysis of 80 STEC isolates allowed us to define 9 clones among the isolates. Three major clones were found among 81.2% of all STEC isolates. The remaining clones were considered as sporadic since they were found only in a small number of isolates. An observation of primary concern is the widespread of the major clones throughout northern Palestine. Thus STEC infection was most probably due to the consequence of clonal transmission.

CHAPTER 1

INTRODUCION

Escherichia coli is a predominant nonpathogenic facultative anaerobic of the human colonic flora. Some *E. coli* strains, however, have developed the ability to cause disease of the gastrointestinal, urinary or central nervous system in even the most robust human hosts (Natro and Kaper, 1998).

Diarrhoeagenic strains of *E. coli* are classified in six categories; enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroaggregative (EAggEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), and diffusely adhering (DAEC) *E. coli* strains. This classification scheme is based on putative virulence factors, clinical syndromes, or other characteristic markers, such as the adherence phenotype (Dalla-costa *et al.*, 1998; Natro and Kaper, 1998).

Enterohemorrhagic *E. coli* (EHEC), also referred to as verotoxin producing *E. coli* and Shiga toxin-producing *E. coli*, is a pathogenic bacterium that causes diarrhea and hemorrhagic colitis (HC) and that lead to life-threatening systemic effects including hemolytic-uremic syndrome (HUS) (Philpott *et al.*, 1998). The triad of acute hemolytic anemia, thrombocytopenia and acute renal failure characterizes HUS (Natro and Kaper, 1998). The term "verotoxinogenic *E. coli*" or verocytotoxin-producing *E. coli* (VTEC) was derived from the observation by KonowalChunk *et al.*, 1997, that these strains produced a toxin that was

cytotoxic to Vero cells. An alternative nomenclature is "Shiga toxin-producing *E. coli*" (STEC [formerly SLTEC]). Which reflects the fact that one of the cytotoxins produced by these organisms is essentially identical at the genetic and protein levels to the Stx produced by *Shigella dysenteriae* type1 (O`Brien *et al.*, 1983). STEC and VTEC are equivalent terms, and both refer to *E.coli* strains that produce one or more toxins of the Stx family.

1.1 Virulent Factors of STEC

Several virulent factors contribute to the capacity of STEC strains to cause serious gastrointestinal disease in humans and complications such as HUS. These include Shiga toxins (Stx), an outer membrane protein (intimin), the product of chromosomal gene *eaeA* and a plasmid-encoded enterohemolysin (Ehly) (Bonnet *et al.*, 1998; Paton and Paton, 1998).

1.1.1 Shiga toxins (Stx)

The Stx family contains two major types, those that are immunologically similar to the Stx produced by *Shigella dysenteriae* type1 (Stx1) and those that are immunologically distinct from Stx produced by *Shigella dysenteriae type1* (Stx2) (Franck *et al.*, 1998; Scotland *et al.*, 1985; Strockbine *et al.*, 1986). A single STEC strain may produce Stx1 only, Stx2 only, or both toxins and even multiple forms of Stx2. While

Stx1 is highly conserved, sequence variations exist within Stx2. The different variants are known Stx2c, Stx2v, etc.(Natro and Kaper, 1998).

Members of the Stx family are compound toxins (the holotoxin is approximately 70-kDa), comprising a single catalytic 32-kDa which is proteolytically nicked to yield 28-kDa peptide (A_1) and a 4-kDa peptide (A_2). The A_1 peptide contains the enzymatic activity, and the A_2 peptide serves to bind the A-subunit to a pentamer of five identical 7.7 kDa B-subunits. The B pentamer binds the toxin to a specific glycolipid receptor, globotriaosylceramide or Gb_3, which is present on the surface of eukaryotic cells (Natro and Kaper, 1998).

Once bound to a target cell membrane, toxin molecules are internalized by a process of receptor-mediated endocytosis (Natro and Kaper, 1998; Sandvig *et al.*, 1994). The A_1 peptide has RNA N-glycosidase activity that removes a single adenine residue from the 28S rRNA (elongation factor 1) of eukaryotic ribosomes, thus, inhibiting protein synthesis and leading to the death of intestinal epithelial cells (Natro and Kaper, 1998).

1.1.2. Intimin (Intestinal adherence factors).

The only potential STEC adherence factor that has been demonstrated to play a role in intestinal colonization in vivo is the 94- to 97-kDa OMP intimin encoded by the *eae* gene. Studies with *eaeA*-negative O157:H7 STEC mutants have shown that intimin is essential for the tight

binding of bacteria to host cells and actin reorganization in vitro (Donnenberg *et al.*, 1993; Louie *et al.*, 1993). There is no doubt that there is a strong association between carriage of *eaeA* and the capacity of STEC strains to cause severe human disease such as HC and HUS. (Beutin *et al.*, 1995; Willshaw *et al.*, 1994).

1.1.3. Enterohemolysin.

Beutin *et al.*, 1989 observed that a high proportion of STEC strains (89%) had a novel hemolytic phenotype that was distinct from that associated with the *E. coli* alpha-hemolysin (Hly). Strains producing this enterohemolysin (subsequently designated EHEC-Hly) produce small, turbid hemolytic zones on washed sheep RBC agar (supplemented with Ca^{2+}) after overnight incubation. Unlike alpha-hemolysin which is chromosomally encoded, EHEC-Hly was found to be encoded by the 60-MDa "virulence plasmid" (pO157) (Schmidt *et al.*, 1995). Enterohemolysin is found in nearly all O157:H7 strains and is widely distributed among non-O157 Stx-producing *E. coli* strains. Patients with HUS develop antibodies to enterohemolysin (Schmidt *et al.*, 1995), but there is no data indicating that it is involved in pathogenesis of disease. The role of enterohemolysin is still subject to speculation. Lysis of erythrocytes in vivo would release heme and hemoglobin, which enhance the growth of *E. coli* O157:H7 and could serve as a source of iron.

1.2. Source and Transmission of STEC.

Cattle have long been regarded as the principal reservoir of STEC strains. STEC strains are also prevalent in the intestinal tracts of other farm animals, including sheep, pigs, goats, dogs, and cats (Karmali, 1989; Kudva *et al.*, 1996). While many domestic animals carrying STEC are asymptomatic, certain STEC strains are capable of causing diarrhea in cattle, particularly calves (Smith and Scotland, 1988). High rates of colonization of *stx*-positive *E. coli* have been found in bovine herds in many countries (Burnens *et al.*, 1995; Griffin and Tauxe, 1991).

Contamination of meat with feces or intestinal contents during slaughter could be responsible for the initial contamination events. Grinding of the meat for hamburger would further mix the bacteria into the meat and thus make them less likely to be killed by surface searing of the meat during cooking (Karmali, 1989). Other proven food sources of STEC infection include unpasteurized dairy products, fruit and vegetable products, and municipal water supplies which presumably had come into contact with domestic animal manure at some stage during cultivation or handling (Nyholm *et al.*, 1996; Paton *et al.*, 1996). Person-to-person transmission of STEC is well documented during outbreaks and may also account for a significant proportion of sporadic cases (Grffin and Tauxe, 1991; Reida *et al.*, 1994). Another proved mode is secondary transmission, which may involve direct hand-to-hand contact (e.g., among children in day

care centers) or could be indirect, e.g., via contaminated water used for swimming (Ackman *et al.*, 1997).

1.3. Pathogenesis.

The incubation period of STEC diarrhea is usually 3 to 4 days, although incubation times as long as 5 to 8 days or as short as 1 to 2 days have been described in some outbreaks. Non-bloody diarrhea and crampy abdominal pain with low-grade fever or no fever characterize the classic illness caused by STEC at all. The diarrheic stool contains no leukocytes, which differentiate it from *Shigella* dysentery or EIEC strain infection. Within 1 or 2 days, the diarrhea becomes bloody and the patient experiences increased abdominal pain (Riley *et al.*, 1983). In most patients, the bloody diarrhea will resolve, but in about 10% of patients less than the age of 10 years and elderly patients, the illness will progress to hemolytic uremic syndrome (HUS). HUS is characterized by low platelet count (thrombocytopenia), hemolytic anemia, and kidney failure. Most patients will recover with appropriate supportive therapy, but 3 to 5% of affected children will die and about 12% to 30% will have severe complications including renal impairment, hypertension, or central nervous system manifestations (Griffin, 1995; Pickering *et al.*, 1994). Progression to HUS is more likely to occur in patients infected with *E. coli* O157:H7

who experience bloody diarrhea, fever, and elevated leukocyte count and who are very young or old (Martin *et al.*, 1990; Pavia *et al.*, 1990).

1.4. Diagnosis and Detection

As with other diarrhoeagenic *E. coli* strains, STEC strains may be identified by one of the four methods. These include (i) stool culture on highly differential medium, with subsequent serotyping, (ii) detection of the Shiga toxin in stool filtrate, (iii) detection of elevated antibody levels to Shiga toxin or other STEC antigens in serum and (iv) genotypic methods. In this study, we will present an overview of stool culture and PCR diagnostic methods

1.4.1. Culture techniques.

The agar medium most commonly used for the isolation of *E. coli* O157:H7 is SMAC agar (Farmer *et al.*, 1985; Jerse and Kaper, 1991). This medium contains 1 % sorbitol in place of lactose in the standard MacConkey medium. *E. coli* O157:H7 appears colorless on this medium. SMAC agar is not generally useful for Stx-producing *E. coli* strains of serotypes other than O157:H7 because there is no known genetic linkage between Stx production and sorbitol fermentation. However, one study found that all 19 Stx-producing *E. coli* strains isolated from HUS patients in Chile, including some O26, O111, and O55 strains, were sorbitol negative (Ojeda *et al*, 1995). Failure to ferment sorbitol is also not tightly

linked to other H types of the O157 serogroup, and several sorbitol-fermenting, Stx-producing O157:H- strains have been isolated from patients with HUS in Germany (Gunzer *et al.*, 1992). The commercially available Rainbow Agar O157 contains chromogenic substrates for D-glucuronidase and β-galactosidase may be used to screen for STEC. Glucuronidase-negative, galactosidase-positive, O157 strains appear as black colonies on this medium, whereas commensal *E. coli* strains are pink. It has also been claimed that some non-O157 STEC strains overproduce -galactosidase relative to -D-glucuronidase on this medium, giving the colonies a distinctive intermediate color (Ingram *et al.*, 1997). Again, it should be emphasized that isolation of a putative O157 strain is not a definitive diagnosis in itself, since isolates must be tested to confirm STX production.

As discussed above, there is no definitive biochemical characteristic, which distinguishes STEC strains belonging to serogroups other than O157 from commensal fecal *E. coli* strains, a fact which significantly complicates the isolation of such organisms. However, nearly all O157 STEC strains and a significant proportion of non-O157 STEC strains produce EHEC-Hly. Strains producing EHEC-Hly are not hemolytic on standard blood agar but produce small, turbid hemolytic zones on washed sheep RBC agar. Alpha-hemolytic strains form large, clear zones of hemolysis on standard blood agar or washed sheep RBC agar plates. Production of EHEC-Hly has a high

positive predictive value, since Beutin et al., 1989 found no enterohemolytic strains among 267 fecal *E. coli* isolates which did not produce Stx. However, the predictive value of a negative EHEC-Hly result is low. Schmidt and Karch, 1996 investigated the enterohemolytic genotypic and phenotypic profiles of 36 O111:H STEC strains isolated from patients with HUS and diarrhea. Twenty strains were positive for EHEC-Hly; two additional strains were positive for EHEC-*hlyA* by DNA hybridization and PCR but were not hemolytic. The remaining 14 O111:H STEC strains were negative by both DNA hybridization and plating. The fact that a significant proportion of disease-causing STEC strains (44% in this particular study) are EHEC-Hly negative diminishes the usefulness of washed sheep RBC agar as a "generic" screen for primary isolation of STEC.

1.4.1. Identification of STEC by PCR

The PCR technique has been extensively used to detect *Stx* genes either in *Stx*-only techniques or in multiplex PCR techniques incorporating primers for *eae*, *ehx*, *uidA*, or *fliC* (Gannon *et al.*, 1997; Schmidt *et al.*, 1995). Some early studies used a single primer pair to detect both *stx1* and *stx2* (Paton *et al.*, 1993), but most methods now include two primer pairs that yield different-sized products for *stx1* and *stx2*. The various PCR techniques are highly sensitive and specific when used with bacterial colonies or cultures, but the use of PCR for direct analysis of stool samples

suffers from the same problems with background and inhibitory factors that are seen with other applications of PCR to stool samples.

PCR has also been used for the detection of genes encoding accessory virulence factors, such as *eaeA* and EHEC-*hlyA*, in STEC isolates (Schmidt *et al.*, 1995; Gannon *et al.*, 1993). This information may be of significance, because as discussed previously, there is a link between the presence of these genes and the capacity of an STEC isolate to cause serious human disease (Schmidt *et al.*, 1995). A two step multiplex PCR assay has been used for the detection and characterization of STEC O157. The first uses four primer pairs and detects the presence of stx_1, stx_2 (including variants of stx_2), *eaeA*, and EHEC-*hlyA*. The second assay has been used *rfb*-specific primers directed at a type-specific portion of the O157 *rfb* locus (the *rfbE* gene) (Paton and Paton, 1998).

1.5. Strain subtyping

STEC strains causing human disease belong to a very broad range of O:H serotypes (Karmali, 1989) (approximately 60 distinct O:H types), and the list has grown considerably since then. *E. coli* O157:H7 is the pathogen most frequently isolated from stool specimens with visible blood (Slutsker *et al.*, 1997). Some studies suggest that O157:H7 may cause 50 to 80% of all EHEC infections. However, the relative ease of isolation of this serotype on the basis of its inability to ferment sorbitol may be contributing to an

overestimation of its prevalence with respect to other STEC serotypes. Non-O157 STEC serotypes such, as serogroups O26, O91, O103, and O111 have been the predominant cause of human disease (Paton and Paton, 1998).

As with other bacterial pathogens, the use of molecular epidemiological techniques can be crucial in investigations whether cases Of STEC infections are linked to a common source or whether they represent sporadic and unrelated cases. Plasmid profiles have been used in several studies to distinguish strains of O157:H7, and although they have provided useful information, Paros *et al,* usually conclude that other methods provide better discrimination (Paros *et al.*, 1993). Sequence variation within *stx* genes has been used to distinguish different strains by using specific *stx* PCR techniques (Caprioli *et al.*, 1995; Franke *et al.*, 1995; Thomas *et al.*, 1993). Phage typing can separate O157:H7 strains into 66 different phage types (Frost *et al.*, 1993), but this technique is available only in reference centers that possess the typing phage. Arbitrarily primed PCR AP-PCR has been successfully used to discriminate O157:H7 strains (Madico *et al.*, 1995). This technique uses low-stringency PCR amplification with arbitrarily chosen oligonucleotide primers and allows any laboratory with a PCR machine to distinguish strains. Since STEC strains contain one or more large (60- to 70-kb) -like bacteriophages that contain the *stx* genes, variations in phage content and chromosomal

insertion sites can lead to strain differences that can be detected by Southern hybridization or PFGE techniques. Hybridization of genomic digests of *E. coli* O157:H7 strains (separated by conventional gel electrophoresis) with labeled DNA provides useful and discriminatory RFLP patterns (Paros *et al.*, 1993; Samadpour *et al.*, 1993), but this technique yields very complex patterns that can complicate the analysis of large numbers of strains (Samadpour 1995). PFGE has been used by several groups to investigate the molecular epidemiology of O157:H7 infections (Krause *et al.*, 1996; Meng *et al.*, 1995). PFGE is a more sensitive but more labor- and equipment-intensive technique than phage typing (Krause *et al*, 1996).

1.5.1. Polymerase Chain Reaction (PCR) Fingerprinting Techniques

PCR offers several advantages over other DNA-based typing methods: the need for only a few cells of the microorganism and a protocol involving fewer steps. Several PCR-based sub-typing methods have been reported in the past including arbitrarily primed PCR (AP-PCR) and PCR restriction fragment length polymorphism (PCR-RFLP).

As an alternative to the arbitrary approach, enterobacterial repetitive intergenic consensus sequences is also used as a basis for new approaches in PCR (ERIC-PCR). This method yields DNA molecules of various sizes, the differing lengths apparently reflecting differences in the distance between repetitive sequences in a given chromosome. When separated by

gel electrophoresis these molecules give a characteristic fingerprint so that different strains can be compared (Dalla-costa *et al.*, 1998; Tyler *et al.*, 1997).

1.6 Treatment and Prevention of STEC

Treatment of STEC disease is limited largely to supportive care (Nataro and Kaper, 1998); the availability of rapid and sensitive methods for diagnosis of STEC infection early in the course of disease has created a window of opportunity for additional specific therapeutic intervention. The objectives of therapeutic strategies would be threefold: (i) to limit the severity and/or duration of gastrointestinal symptoms, (ii) to prevent life-threatening systemic complications such as HUS, and (iii) to prevent the spread of infection to close contact (Paton and Paton, 1998). Antibiotic therapy might be expected to satisfy all three of the above goals (Paton and Paton, 1998). Vaccines provide an other alternative therapeutic strategy, however, non is currently available (Natraro and Kaper, 1998).

Aims of the Study

STEC is now seen as a pathogenic species with remarkable versatility in its ability to cause disease in humans and animals. Outbreaks of disease due to STEC can affect thousands of individuals and can engender national and international headlines. Pathogen-specific virulence factors have been discovered that adversely affect a wide range of eukaryotic cell processes including protein synthesis, cell division, ion secretion, and transcription.

Epidemiological studies of STEC are necessary to identify possible clusters of cases, which is important in terms of infection control policy. Detection of a discriminative marker would allow a more selective implementation of infection control measures in order to prevent dissemination of STEC.

The present study was initiated to assess the importance STEC as a possible etiological agent of acute diarrhea among Palestinians. Simultaneously, the presence of stx1, stx2, eaeA, hlyA and O157 *rfb*E genes are to be tested by a previously described multiplex PCR procedure (Paton and Paton, 1998). Clonal clustering of the isolates are to be determined by the combination of genomic DNA fingerprinting performed by ERIC-PCR and the results of multiplex PCR (Dalla-costa *et al.*, 1998). In addition, the resistance rate of the STEC isolates against several antibiotics is to be evaluated.

CHAPTER II

MATERIALS AND METHODS

2.1 Subjects and study design

This study comprises 250 stool specimens of human origin often associated with diarrhea. These samples were collected between February and June 1999 from microbiological laboratories (6 private laboratories and 3 hospital laboratories) in three different cities (Tulkarm [n=73], Nablus [n=128], and Jenin [n=49]) in the northern part of the West Bank (Palestinian area)

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2.2 Sample collection and processing

For the STEC assay, fecal samples were streaked out on MacConkey agar plates. *E. coli*-like colonies were isolated and tested by two step multiplex PCR assay. The first assay uses four primer pairs and detects the presence of stx_1, stx_2, *eaeA*, and *hlyA*. The second assay is using a primer directed at a type-specific portion of the O157 *rfb* locus (*rfbE*). The isolate (s) was subsequently identified based upon biochemical properties. Their ability to ferment sorbitol was also tested on SMAC.

2.3 Examination of STEC isolates by multiplex PCR

Preparation of bacterial lysates.

At least 10 colonies were suspended in 100 µl of sterile distilled water. The suspension was then heated at 99°C for 10 min and cooled to

room temperature. Following centrifugation of the lysate, 5 µl of the supernatant was used in PCR.

Primers for PCR.

Details of the nucleotide sequence, the specific gene region amplified, and the size of the PCR product for each primer pair used in multiplex PCR are listed in Table 1.

Amplification procedures

PCR assay 1 (Detection of stx1, stx2, eaeA and hylA)

Samples (2-5 µl) of each bacterial extract were amplified in 50-µl reaction mixtures containing 200 µM of deoxynucleoside triphosphates (dNTPs), approximately 250 nM of each primer, and 1 U of *Taq* polymerase (Promega, USA), in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2mM $MgCl_2$, and 0.1% Triton X-100 were used. PCR mixture was overlaid with 50 µl of mineral oil. DNA amplification was performed in a Stuart Scientific thermocycler, with the following thermal cycling profile. 35 PCR cycles, each consisting of 1 min of denaturation at 95°C; 2 min of annealing at 65°C for the first 10 cycles, descremenating to 60°C by cycle 15; and 1.5 min of elongation at 72°C, incrementing to 2.5 min for cycle 25 to 35.

PCR assay 2 (Detection rfbE).

PCR amplification was comprised of the following three steps. An initial denaturation of 1 cycle at 95°Cfor 5 min, followed by 35 cycles,

each consisting of 30 s at 94°C, 30 s at 66 °C, 30 s at 72°C, and finished by extension cycle for 10 min at 72 °C.

PCR products:

25 µl were mixed with 2 µl of agarose gel loading dye and separated on a 2% agarose gels containing 0.25 µg ethidium bromide per ml, run at 100V for 1 hr. A 100-bp DNA ladder was used as a molecular size marker. Gels were photographed on a 392-nm-wavelength transilluminator and band patterns were compared visually. Lanes with a single band of the appropriate size (180 bp [stx_1], 255 bp [stx_2], 384 bp [*eaeA*], 534 bp EHEC *hlyA* and 259 bp $rfbE_{O157:H7}$) were considered positive (Paton and Paton, 1998).

2.4 ERIC-PCR

We used ERIC-2 arbitrary primer, consisting of 22 bp, (5′AAGTAAGTGACTGGGGTGAGCG 3′) which in pilot experiments had shown a good ability to discriminate STEC strains for typing purposes. PCR was performed in 25-µl vol containing 10 mM Tris HCl pH 9.0, 50 mM KCl, 1.75 mM MgCl2, 0.1 mM Triton X-100, 250 µM of each dNTP, 1µM ERIC-2, 1 unit of AmpliTaq DNA polymerase (Promega, USA) and 5 µl of the prepared DNA lysate. PCR mixture was overlaid by 50 µl of mineral oil. DNA amplification was performed in a Stuart Scientific thermocycler, with the following thermal cycling profile. Initial

denaturation at 94°C for 5 min was followed by 40 cycles of amplification (denaturation at 94°C for 60 sec, annealing at 25°C for 60 sec, and extension at 72°C for 90 sec) ending with a final extension at 72°C for 5 min. PCR products (25 µl) were mixed with 2 µl of agarose gel loading dye and separated on a 2% agarose gels containing 0.25 µg ethidium bromide per ml, run at 100V for 1 hr. A 100-bp DNA ladder was used as a molecular size marker. Gels were photographed on a 392-nm-wavelength transilluminator and band patterns were compared visually. Patterns that differed by one or more DNA bands were considered as different type.

2.5 Susceptibility testing

Antimicrobial susceptibilities of the strains were determined by disk diffusion mainly as described by Bauer *et al.* (Bauer *et al*,1966). The following antibiotics were used: Gentamycin ($10\mu_g$), norfloxacin ($10\mu_g$), imipenem ($10\mu_g$), ampicillin ($10\mu_g$), and tazopactum. ($100\ \mu_g$), amikacin ($30\mu_g$) and pipracillin ($100\mu_g$). All antibiotics were purchased from (Oxoid, England). Zones of inhibition to antibiotics were determined in accordance with the interpretive standards outlined by the National Committee for Clinical Laboratory Standards (NCCLS, 5th ed., Pa 1993).

Table 1. PCR primers

Primer	Sequence of primers(5 –3)	Specificity[a]	Amplicon size (bp)
Assay 1			
Stx1 F	ATAAATCGCCATTCGTTGACTAC	nt 454-633 of A subunit coding region of stx1	180
Stx1 R	AGAACGCCCACTGAGATCATC		
Stx2 F	GGCATGGTCTGAAACTGCTCC	nt 603-857 of A subunit coding region of stx2	255
Stx2 R	TCGCCAGTTATCTGACATTCTG		
EaeAF	GACCCGGCACAAGCATAAGC	nt 27-410 of eaeA	384
EaeAR	CCACCTGCAGCAACAAGAGG		
HlyAF	GCATCATCAAGCGTACGTTCC	nt 70-603 of hlyA	534
HlyAR	AATGAGCCAAGCTGGTTAAGCT		
Assay 2			
O157 F	CGGACATCCATGTGATATGG	nt 393-651 of rfb- $E_{O157:H7}$	259
O157 R	TTGCCTATGTACAGCTAATCC		

[a] nt, nucleotide; F, forward; R, reverse; bp, base pair.

3.1. Patients and Clinical Features

The PCR product of *stx*-encoding genes (STEC isolates) was identified in 176 (70.4%) of the 250 stool samples analyzed in this study. No geographic clustering of *stx* gene-positive samples was found, but if distribution frequency of PCR-positive stool samples is grouped by source, the following account can be found (Figure 1). The frequency was 67 % for Tulkarm, 73 % Nablus and 69 % Jenin.

Table 2 presents the general and clinical features of patients and characteristics of the STEC isolates. The median age was 10.8 (range, 1 to 80 years), and 98 (55.5%) were male. Based on clinical information available from the donors, STEC isolates were classified into two categories. The first category (non-severe diarrhea) comprises 97 (55.1%) isolates from healthy persons and from patients with uncomplicated non-bloody diarrhea. The second category (severe diarrhea) comprises 79 (44.9%) isolates from patients with bloody diarrhea. Abdominal pain occurred in 59 (33.5%) and fever (body temperature of >38C°) was observed in 30 (17.1%) patients. Of the 176 STEC isolates, 124 (70.5%) belonged to the O157:H7 serotype, all of the STEC isolates were *stx1*$^{+}$, 140 (79.5%) were *stx2*$^{+}$. The *eae* locus was detected in 16 (9.1%) and the hlyA-encoding gene was detected in 18 (10.2%) of these isolates.

3.2. Age trends in carriage of STEC

To determine the impact of age on our results, we divided the study population into seven age groups as shown in Figure 2. Rate of carriage of STEC was high among children under 10 years of age (79 %). The carriage rate declined to 71% at age > 60 years.

3.3. Association between virulence factors of STEC isolates and disease severity

The overall distribution of virulence factors in 176 human STEC isolates obtained from individuals with severe diarrhea or with non-severe diarrhea is summarized in Table 3. Sixty-six of the 79 (83.5 %) isolates obtained from individual with severe diarrhea, carried both the *stx1* and *stx2* toxin genes and 14 (17.7 %) were eae^{+}, while 74 (76.3 %) of 97 isolates from individuals non-severe diarrhea were $stx1^{+}$ $stx2^{+}$ and 2 (2.1 %) carried *eaeA* encoding genes. The locus encoding the production of hemolysin was detected in 16 of 79 and 2 of 97 from severe diarrhea cases and non-severe diarrhea, respectively.

By using this typing method, we distinguished five distinct genotypes among all of the STEC isolates analyzed. The genetic profile (M4) $stx1^{+}$ $stx2^{+}$ was the most prevalent (117 of 176 isolates; 66.5%), followed by (M5) $stx1^{+}$ (36 of 176 isolates; 20.5%). The data presented in

Table 3 clearly showed an association among isolates with the genotype M1 (*stx1*$^{-}$ *stx2*$^{+}$*eaeA*$^{+}$ *hlyA*$^{-}$) and disease severity.

3.4. Cluster analysis of STEC isolates by AP-PCR pattern and Multiplex PCR

Represented 80 STEC isolates were ERIC-PCR analyzed to study the epidemiology of STEC. The STEC isolates fell into 5 distinct patterns on AP–PCR identified E1 through E5 (Table 4). AP-PCR pattern E1, E2 and E4 were represented by 42 (52.5%), 11 (13.8%) and 17 (21.3%), respectively. The remaining AP-PCR patterns (E3 and E5) were found in four and six isolates respectively. The five patterns are shown in Figure 3.

The combination of AP-PCR and Multiplex-PCR analysis allowed us to define 9 clusters among the isolates (Table 4). The high degree of heterogeneity should be considered together with the clear evidence of 3 major clusters among the STEC isolates. E1::M4 was the most common cluster included 38 isolates, E2::M5 cluster included 10 isolates and E4::M4 cluster included 17 isolates. E1::M4, E2::M5, and E4::M4 isolates widespread throughout northern Palestine. Together, E1::M4, E2::M5, and E4::M4 clones represented 81.2 % of all STEC isolates. The remaining clones were considered as sporadic since they were found only in a small number of isolates. 59 of the 80 STEC isolates (72.5 %) were from community source.

3.5. Resistance to antibiotics

One hundred eighteen isolates of STEC, identified in this study, were tested for resistance to tazopactum, ampicillin, pipracillin, norfloxacin, gentamycin, amikacin and imipenem (Table 5). The most common resistance was to ampicillin and tazopactum (98[83%]) each, followed by pipracillin (96[81%]), norfloxacin (70[59%]), gentamycin (65[55%]), amikacin (56[48%]) and imipenem (31[26%]). Resistance to at least 3 drugs was found in 105 isolates (89 %).

Table 5 also shows the antibiotic resistance patterns encountered among the isolates. Only 4 isolates (3.4%) were susceptible to all antibiotics tested. The remaining 114 (96.6%) were associated with eleven different patterns of resistance.

The percentages of resistance among *eaeA* positive STEC and *eaeA* negative STEC were respectively, 18.7% and 19.7% for 5 antibiotics; 25% and 17.7% for 6 antibiotics; and 56.3% and 3.9% for 7 antibiotics. Resistance to 6 or more antibiotics occurred more frequently in the group with *eaeA* positive than in *eaeA* negative group (Table 6).

Table 2. General and clinical data of patients during the acute phase and characteristics of the STEC strains isolated from patient' stool specimens

Characteristics	No. (%)
Gender	
Male	98 (55.5)
Female	78 (44.3)
Age (years) median (min., max.)	10.8 (1, 80)
Clinical Feature	
Severe diarrhea	79 (44.9)
Non-severe diarrhea	97 (55.1)
Abdominal pain	59 (33.5)
Fever [a]	30 (17.1)
Headache	13 (07.4)
STEC characteristics [b]	
O157: H serotype	124 (70.5)
Stx1	176 (100)
Stx2	140 (79.5)
EaeA	16 (9.1)
HylA	18 (10.2)

[a] Body temperature of > 38C°
[b] Detected by PCR

Table 3. Overall distribution of virulence factors in 176 STEC isolates isolated from individuals with severe and non-severe diarrhea by multiplex PCR

STEC genotype[a]					No. of isolates		
Stx1	*Stx2*	*eaeA*	*hlyA*	Genotype	Total (%) (n=176)	Severe diarrhea (n=79)	Non-severe diarrhea (n=97)
+	+	+	+	M1	11(06.3)	10	1
+	+	+	-	M2	5 (02.8)	4	1
+	+	-	+	M3	7 (04.0)	6	1
+	+	-	-	M4	117 (66.5)	46	71
+	-	-	-	M5	36 (20.5)	13	23

[a] +, gene present; -, gene absent

Table 4. Cluster analysis of 80 STEC isolates by AP-PCR pattern and Multiplex PCR.

PCR pattern			isolates	Source[a]			Type	
AP-PCR	Multiplex PCR	Cluster	no.	T	N	J	Nosocomial	Community
E1	M4	E1::M4	38	13	15	9	14	24
E1	M3	E1::M3	2	2	-	-	-	2
E1	M2	E1::M2	2	2	-	-	-	2
E2	M4	E2::M4	1	-	1	-	-	1
E2	M5	E2::M5	10	5	4	1	3	7
E3	M5	E3::M5	4	1	2	1	1	3
E4	M4	E4::M4	17	5	9	3	3	14
E5	M1	E5::M1	5	2	3	-	1	4
E5	M3	E5::M3	1	-	1	-	-	1

[a] T, Tulkarm; N, Nablus; J, Jenin

Table 5. Pattern of resistance to individual antibiotics and multiple antibiotic resistance among 118 STEC isolates.

	NOR.	IMP.	AMP.	TZP.	PRL.	CN.	AK.
No. (%) of Resistant isolates	70 59 %	31 26 %	98 83 %	98 83 %	96 81 %	65 55 %	56 48 %

	No. of antibiotics			
	2	3	4	≥5
No. (%) of isolates resistant to two or more of antibiotics	114 (96.6 %)	105 (89.0 %)	75 (63.6 %)	58 (49.2 %)

Resistance patterns	No. (%)	Resistotype
NOR IMP AMP PRL TZP CN AK	13 (11.0%)	R-1
NOR AMP PRL TZP CN AK	22 (18.6%)	R-2
AMP PRL TZP CN AK	05 (04.2%)	R-3
NOR IMP AMP PRL TZP	18 (15.3%)	R-4
NOR TZP CN AK	07 (05.9%)	R-5
NOR AMP PRL TZP	10 (08.5%)	R-6
TZP CN AK	09 (07.6%)	R-7
AMP PRL CN	07 (05.9%)	R-8
AMP PRL TZP	12 (10.2%)	R-9
AMP TZP CN	02 (01.7%)	R-10
AMP PRL	09 (07.6%)	R-11
Susceptible [a]	04 (03.4%)	R-12

[a] Susceptible to all antibiotics

CN, Gentamycin; NOR, Norfloxacin; IMP, Imipenem; AMP, Ampicillin; TZP, Tazopactum; AK, Amikacin; PRL, Pipracicllin.

Table 6. *eaeA* gene carrying isolates and its association with resistance to antibiotics in 118 STEC isolates.

Gene *eaeA* (No. isolates)	No. (%) of isolates resistant to antibiotics		
	5	6	7
-*eaeA* positive (16)	3 (18.7)	4 (25)	9 (56.3)
-*eaeA* negative (102)	20 (19.7)	18 (17.7)	4 (3.9)

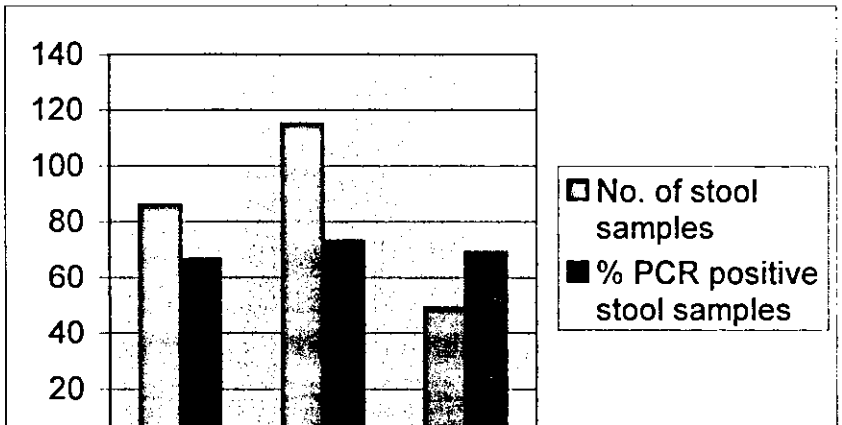

Figure 1. Distribution of 176 STEC strains isolated from stool samples of 250 according to the source and infection.

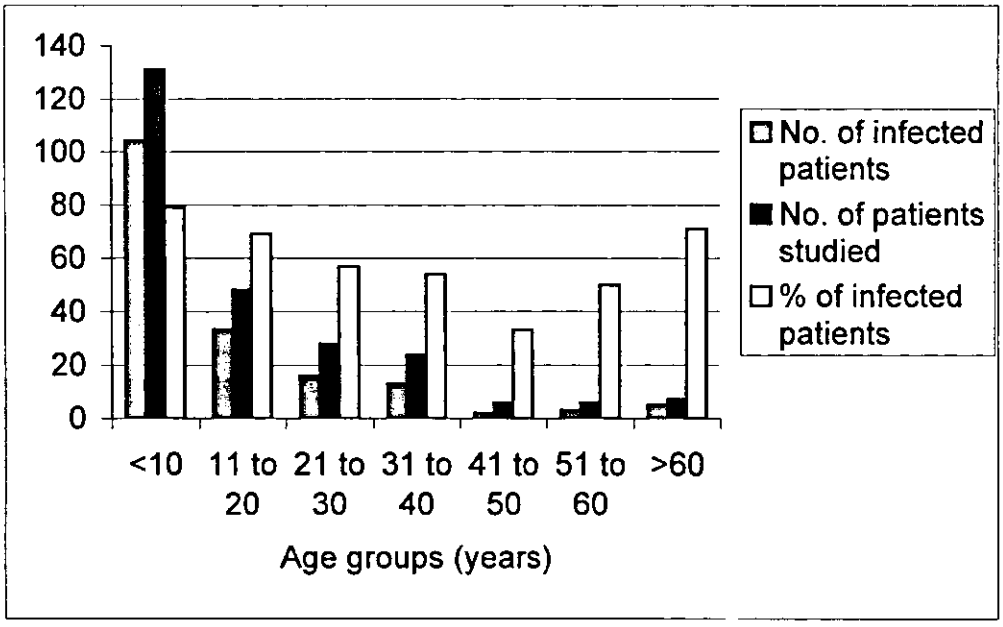

Figure 2. Distribution of 176 STEC isolates according to age group (years)

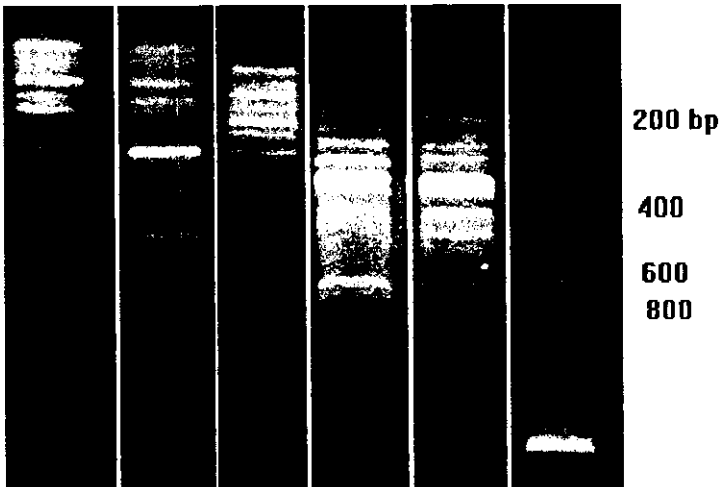

Figure 3. ERIC-PCR patterns of 80 STEC isolates. Lane 1, Molecular sizes marker (100-bp ladder DNA), some of the molecular sizes of the marker are indicated on the right side; Lane 2, pattern E1; Lane 3, pattern E2; Lane4, pattern E3; Lane 5, pattern E4; Lane 6, pattern E5.

CHAPTER IV

DISCUSSION

Shiga toxinigenic *Escherichia coli* (STEC) strains are a diverse group of organisms associated with severe gastrointestinal and systemic diseases in humans. Within the STEC family, STEC strains belonging to serogroup O157, have frequently been associated with cases of severe STEC disease, including hemolytic-uremic syndrome (HUS) (Nataro and Kaper, 1998; Paton and Paton, 1998). Illness caused by STEC occurs sporadically, in small clusters and outbreaks, and may be transmitted in a variety of ways, including through food and water and through person-to-person and animal-to-person (Keene *et al.*, 1994; Reida *et al.*, 1994).

The frequency with which STEC infection are being reported continues to increase, probably reflecting both a greater interest in this pathogen and a real increase in its incidence and geographic spread (Nataro and Kaper, 1998). Accordingly, the present study was initiated to assess the importance of STEC as a possible etiological agent of acute diarrhea among Palestinians and to establish clonal analysis among the collected isolates. Our results revealed that among northern Palestinian patients, the rate of STEC was 70.4% (176 / 250). This finding indicates that STEC infection is a serious problem in Palestine, as that reported in several countries (Nataro and Kaper, 1998; Boyce *et al.*, 1995). In our bacterial collection of 176 isolates of STEC, no geographic clustering of STEC was found throughout northern Palestine.

Age stratification showed that the highest isolation rate of the STEC (79%) was identified among child-hood age (<10 years). The rate decline to 71% at age > 60 years. This observation was in accord with the observations of an earlier study (Karmali, 1989; Nataro and Kaper, 1998). This could be partially due to the suppresser immune system and the formation of the antibodies against STEC strains as the age of patients increased; also most of the adult patients recover after appropriate supportive therapy.

The isolation rate of STEC from males (55.5%) was slightly higher than females (44.5%). The relative high incidence of STEC infection among males may be due to the frequent contact of males with animals, the reservoir of STEC (Nataro and Kaper, 1998). Severe diarrhea was observed in 44.9% of patients compared to non-severe diarrhea that was 55.1%. This observation is more likely due that STEC infection is self-limited. Thus, diarrhea leads to wash bacterial cells from the gastrointestinal tract allowing the immune system of the patient to overcome the disease. Abdominal pain and fever (body temperature of >38C°) occurred in 33.5% and 17.1% patients, respectively. These results were in accord with the observations of other studies in which abdominal colic and fever was more frequently among infected individuals (Dutta *et al.*, 1999).

It is now recognized that O157: H7 STEC is a dominant serotype in many parts of the world and historically has been the type most commonly associated with large outbreaks (Paton and Paton, 1998). Several studies found that O157: H7 cause 50 to 80 % of all STEC infections, and the most common of serious STEC disease in Europe and North America. Studies of large outbreaks caused by O157:H7 STEC indicate that roughly 5 to 10 % of individuals with diarrhea progress to HUS (Nataro and Kaper, 1998). In this study, we found that O157: H7 in 124 (70.5 %) of the STEC isolates, regardless of the severity of the clinical manifestations. Our results confirm and extend the finding of Keskimaki *et al.*, 1998, who reported that STEC serotype O157:H7 became the major STEC type. On the other hand, our findings suggest that the O157:H7 clone has become widely distributed throughout Palestine and that these strains can emerge a public health problem. This has important diagnostic implications, emphasizing the need for diagnosis procedures that allow detection of infection with O157:H7. The high frequency of O157:H7 infection raises the question of the therapeutic management of infected patients. This consideration is all the most important because antibiotic therapy may make the disease worse by inducing the temperate phage carrying the *stx* gene to enter the lytic cycle. This would produce multiple copies of the gene in each affected bacterium and could lead to an increase in translation of *stx*, the opposite of the desired effect. An additional alarming feature is that children and elderly

people infected with O157 positive STEC are at a higher risk for developing potentially fatal complication. Although strains of serotype O157:H7 was far more common than the others, non-O157 infections was notable (24.8 %), indicating the need for further studies to evaluate the significance of non-O157 in human disease.

In the present study, we used two multiplex PCR for the detection and characterization of STEC. Assay 1 detects the presence of *stx1*, *stx2*, *eaeA* and *hlyA*, generating PCR products of 180, 255, 384 and 534 bp, respectively. These primers amplify all *stx2* variants currently deposited with GenBank. Similarly, the *eaeA* primers are specific for a region at the 5' end of the gene that appears to conserve among all STEC strains examined. Moreover, the STEC *hlyA* primers were potential to eliminate the possibility of cross-reaction with the gene encoding *E.coli* alpha-hemolysin (Paton and Paton, 1998). Assay 2 uses portions of the *rfb* (O-antigen- encoding) region of *E.coli* serotype O157: H7 generating PCR product 259 bp. The *rfb* primers directly target O-antigen-encoding genes, and the assay is not dependent on clonal association between particular variants of unrelated genes (e.g., *eaeA*) and STEC strains of a given serotype. Thus, all O157: H7 strains tested positive, there was no reaction with clonally related STEC strains (Patricia *et al.*, 1998; Paton and Paton, 1998).

Several studies have shown a large spectrum of variability in virulence factors make up in STEC populations, and many researchers have attempted to correlate the presence of specific recognized or putative virulence factors with disease or severity of disease (Boerlin *et al.*, 1998). Some investigators have suggested that strains, producing Shiga toxin type 2 appear to be of greater virulence for humans than those producing only Shiga toxin type 1 (Paton and Paton, 1998). This hypothesis is not supported by toxigenic profiles of STEC isolates from Palestinian patients with severe diarrhea or non-severe diarrhea. In these patients, STEC isolates have typically carried either *stx1 stx2* or *stx1* alone. However, a clear association of *hlyA* and e*aeA* virulence factors of STEC and their pathogenic potential for human was observed. The *hlyA* and *eaeA* were detected in 16 isolates (20.3 %) and 14 isolates (17.7 %) of the severe diarrhea group versus 2 isolates (2.1 %) for each gene of the non-severe diarrhea group. A strong association between *stx1*, *stx2*, *eaeA* and *hylA* genes combined together and disease severity was observed; the 4 genes were detected in 10 isolates from severe cases versus a single isolate from the non-severe group. These results confirm the observations of a previous study (Boerlin *et al*, 1998), thus supporting the hypothesis of the synergism between the adhesin intimin, enterohemolysin and Shiga toxin.

Recently, a large number of reports describing the use of ERIC-PCR for genetic typing of medically important microorganisms, including STEC

(Dalla-costa *et a.l*, 1998). The procedure is used with increasing frequency, facilitated by general applicability and high speed. However it was suggested that analysis of isolates from diverse geographic organism should be done for better evaluation of the method (VanBelkum, 1994). Accordingly, the ERIC-PCR method was evaluated by typing 80 isolates obtained from different areas of Palestine. The multiplex PCR method was used to confirm the results of ERIC-PCR on the same isolates.

In this study arbitrary primer ERIC-2 used in the field of *E.coli* typing (Dalla-costa *et al.*, 1998) was applied. When primer ERIC-2 was used, 5 patterns of 80 strains could be distinguished. The combination of the two PCR methods (Multiplex and ERIC-PCR) provided a high resolution typing and allowed us to define 9 different clones. Three major clones E1::M4 E2::M5 E4::M4, however, sharing common ERIC-PCR as well as a common multiplex PCR pattern, represented 81.2% of all STEC isolates. The remaining clones were considered as sporadic since they were found only in a small number of isolates. An observation of primary concern is the widespread of the major clones throughout northern Palestine. Thus STEC infection was most probably due to the consequence of clonal transmission. The majority of the isolates (59 of 80) were from community source. Thus indicating that STEC is almost likely due to community acquired infection.

The antimicrobial susceptibility patterns of STEC showed multiple antibiotic resistance. Almost 49% of the isolated strains were resistant to five or more drugs (Table 5). This high rate is likely due, in part, to selective pressure resulting from uncontrolled, unwise, and frequent administration of broad-spectrum antibiotics. This is promoted by the lack of antibiotic policy and a situation where antibiotics are sold over-the-counter. Extensive antimicrobial usage induces STEC strains of different genotypes to undergo multiple mutations and acquire resistance genes (Rice *et al.*, 1991).

Among the β-lactam antibiotics tested, imipenem behave as the most potent antibiotic. The highest activity of imipenem seems to be related to its stability against most β-lactamases and it is a rapid permeant (Rice *et al,* 1991).

The eaeA-positive strains were significantly resistance to 6 and more antibiotics, which means that the *eaeA* genes may be increasing the antibiotic resistance in these STEC strains (Table 6). A previous study suggested that resistant to antibiotics may be linked to virulence (Duta *et al.*, 1999).

Recommendations and guidelines for management of STEC infection

The emergence of STEC is a serious problem, and several strategies have been proposed to try to tackle it. Prevention should be the ultimate solution, as will vaccination programs. Measures are also required to maximize the microbiological safety of our food. Meat products can be made safe by thorough cooking; dairy products and fruit juices can be pasteurized. However, salad vegetables, which have been the source of several serious outbreaks, present a problem in the absence of food irradiation.

There are three key approaches to overcoming the problem posed by multiple drug resistance of STEC. The first is the on going community-based surveillance programs to define the extent of drug-resistant STEC infections. The second is the restrictive use of newer and broad-spectrum antibiotics that promote resistance. The third is the development of improved rapid diagnostic procedures so that specific treatment can be given where needed and withheld when unnecessary.

While most PCR-based tests are presently used to diagnose viruses or bacteria whose identification is tedious. The time is ripe for the use of these tests to identify STEC and its antibiotic resistance genes. If physicians

could have in hand the identity of the microorganism and its resistance profile at the same time that they have the clinical findings, antibiotic prescription rates could drop down dramatically, and when antibiotics are needed, more targeted and inexpensive antibiotics could be used.

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الخلاصة

الإشيريشيات القولونية المنتجة لسموم الشيقيلا تكوّن مجموعة مختلفة من الكائنات التي تسبب الأمراض الخطيرة للجهاز الهضمي و أمراض الأجهزة في الإنسان. ضمن عائلتها, سلالة تبدو أكثر خطورة للإنسان, مثل تلك السلالة التي تنتمي الى نوع O157:H7 و تلك التي ترتبط جزئيا بالعوامل الممرضة. مئة وستة وسبعون من ال STEC عزلت من مرضى بالإسهال الشديد أو الإسهال الغير شديد من شمال فلسطين في سنة 1999 والتي درست خصائصها للجينات الممرضة بواسطة طريقة تفاعل البلمرة المتعددة للبادئات (Muultiplex PCR). من هذه العزلات ال 176 STEC, 124 (70.4%) تنتمي إلى نوع O157:H7, كل عزلات STEC تحتوي على جين stx1, 140 (79.5%) تحتوي على stx2, موقع eaeA أكتشف في 16 (9.1%) و جين hlyA أكتشف في 18 (10.2%) من العزلات.

ستة وستون من العزلات ال 97 التي عزلت من مرضى بالإسهال الشديد, تحمل الجينات stx1 وstx2 معا و 14 منها حملت جين eaeA, بينما 74 من العزلات ال 97 التي عزلت من مرضى الإسهال الغير شديد تحتوي على stx1 و stx2 معا و2 من العزلات حملت جين eaeA. جين ال hly أكتشف في 16 من ال 79 و 2 من ال 97 من حالات الإسهال الشديد و الإسهال الغير شديد, بالتوالي.

نتائجنا تظهر قوة العلاقة بين ترابط الجينات(stx1, stx2, eae. Hly) معـا و شـدة خطورة المرض.

الربط بين نتائج التحـاليل multiplex-PCR و ERIC-PCR (باسـتخدام البادئ التلقائي) ل80 عزلة STEC تسمح لنا بتعريف 9 أنـواع فيـها. 3 أنـواع رئيسية موجودة بنسبة 81.2 % من العزلات. بقية الأنواع تعتـبر نـادرة لأنـها موجودة فقط بأعداد قليلة, من الملاحظ أن الأنواع الرئيسية واسـعة الإنتشـار فـي شمال فلسطين لذلك من المحتمل أن يكون سبب إنتقال مرض ال STEC هو انتقـال جيني (clonal transmission).