EPIDEMIOLOGY

Isolation and characterisation of Shiga toxigenic *Escherichia coli* strains from northern Palestine

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Shiga toxigenic *Escherichia coli* (STEC) isolates from symptomatic and asymptomatic patients in northern Palestine in 1999 were screened for serotype O157 and characterised for virulence genes by multiplex PCR assay. Of the 176 STEC isolates, 124 (70.5%) were of serotype O157. All these isolates carried the gene for Shiga toxin type 1 (stx_1) and 112 (90.3%) carried stx_2. The intimin encoding gene locus *eae* was detected in 16 isolates (12.9%) and the enterohaemolysin encoding gene, *hlyA*, in 18 (14.5%). Statistical analysis showed a significant association between the presence of *eaeA* and *hlyA*, either alone or combined with stx_1 and stx_2 genes in O157 isolates from symptomatic infection. ERIC-PCR analysis of DNA from 80 serotype O157 isolates revealed three major clonal populations.

Introduction

Shiga toxigenic *Escherichia coli* (STEC) are an important cause of gastrointestinal disease in man. Infection with these organisms may result in life-threatening complications such as haemolytic-uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura [1, 2]. Within the STEC family, certain strains, such as those of serotype O157 or those that have particular combinations of putative virulence factors, appear to be more virulent for man [1–3]. Numerous outbreaks of STEC-related disease have been attributed to serotype O157, although the latter accounts for only *c*. 60% of isolates in outbreaks [4]. Indeed, several strains of other serotypes have been implicated in both sporadic infections and outbreaks caused by STEC. In some studies, non-O157 strains have represented 20% [5] and 30% [6] of STEC isolated.

Several virulence factors have been described in STEC, including Shiga toxin type 1 (Stx1, encoded by stx_1), Shiga toxin type 2 (Stx2, stx_2), intimin (*eaeA*) and the plasmid-borne enterohaemolysin encoded by enterohaemorrhagic *E. coli* (EHEC) *hlyA* [2, 7–9]. The present study was initiated to assess the importance of STEC as an aetiological agent of acute diarrhoea among Palestinians. The presence of stx_1, stx_2, *eaeA*, *hlyA* and

O157 *rfbE* genes was tested for simultaneously by a multiplex PCR [2] and the clonal structure of the population of isolates was examined by genomic DNA fingerprinting by enterobacterial repetitive intergenic concensus-PCR (ERIC-PCR) [10].

Materials and methods

STEC isolates

A total of 250 stool samples was collected during an outbreak of diarrhoea between February and June 1999 in the north of Palestine. Samples were obtained from symptomatic patients, i.e., those with diarrhoea with or without blood or with clinical signs of HUS, and asymptomatic patients. Samples were plated on Mac-Conkey agar, incubated overnight at 37°C, and a loopful of growth from the first inoculation streak was suspended in 0.5 ml of distilled water and boiled for 10 min. After centrifugation of the lysate, the supernate was used in PCR.

Multiplex PCR

The stx_1, stx_2, *eaeA*, *hlyA* and O157 *rfbE* gene sequences were detected with the primer pairs described previously [2]. Details of the nucleotide sequence, the specific gene region amplified and the size of the PCR product for each primer pair are given in Table 1. Samples (5 μl) of each extract were amplified in 50-μl reaction mixtures with 35 PCR cycles each consisting of 1 min of denaturation at

Table 1. Primers used in multiplex PCR

Primer	Sequence (5′–3′)	Specificity	Amplicon size (bp)
stx1F	ATAAATCGCCATTCGTTGACTAC	nt 454–633 of A subunit coding region of stx_1	180
stx1R	AGAACGCCCACTGAGATCATC		
stx2F	GGCACTGTCTGAAACTGCTCC	nt 603–857 of A subunit coding region of stx_2 (including stx_2 variants)	255
stx2R	TCGCCAGTTATCTGACATTCTG		
eaeAF	GACCCGGCACAAGCATAAGC	nt 27–410 of *eaeA* (this region is conserved between EPEC and STEC)	384
eaeAR	CCACCTGCAGCAACAAGAGG		
hlyAF	GCATCATCAAGCGTACGTTCC	nt 70–603 of EHEC *hlyA*	534
hlyAR	AATGAGCCAAGCTGGTTAAGCT		
O157F	CGGACATCCATGTGATATGG	nt 393–651 of $rfbE_{O157:H7}$	259
O157R	TTGCCTATGTACAGCTAATCC		

nt, nucleotide; EPEC, enteropathogenic *E. coli*.

95°C, 2 min of annealing at 65°C for the first 10 cycles, decreasing to 60°C by cycle 15 with elongation for 1.5 min at 72°C, rising to 2.5 min from cycles 25 to 35. PCR products were separated by electrophoresis in agarose 2% gels and stained with ethidium bromide. Strains ATCC 43890 and ATCC 43894 were used as positive control strains for stx_1 and stx_2 respectively. Distilled water served as a negative control.

At least 10 *E. coli*-like colonies from primary plate PCR-positive cultures were retested by the same PCR protocol. Isolates were confirmed as *E. coli* by the API 20E system (bioMérieux, Marcy L'Etoile, France) and tested for sorbitol fermentation on sorbitol MacConkey agar (SMAC). The O157 antigen of isolates was confirmed by agglutination with a specific latex reagent (Oxoid).

ERIC-PCR

PCR was performed with primer ERIC2, 5′AAG-TAAGTGAC TGGGGT GAGCG3′ [10] and crude heated isolates in 25-μl reaction mixtures with 5 μl of template DNA. Initial denaturation was at 94°C for 5 min followed by 40 cycles of amplification (denaturation at 94°C for 60 s, annealing at 25°C for 60 s and extension at 72°C for 90 s) ending with a final extension at 72°C for 5 min. Separated PCR products in agarose gels were visualised as above and patterns that differed by one or more DNA bands were considered to be different ERIC-types.

Antimicrobial susceptibility

The susceptibility of isolates to antimicrobial agents was determined by disk diffusion [11] in accordance with National Committee for Clinical Laboratory Standards [12]. The following antibiotics (μg) were used: gentamicin (10), norfloxacin (10), imipenem (10), ampicillin (10), tazobactam (100), amikacin (30) and piperacillin (100).

Statistical analysis

Significant differences were calculated by the χ^2 test and statistical significance was defined as $p < 0.05$.

Results

STEC was identified in 176 (70.4%) of 250 stool samples. Of the 176 STEC isolates, 120 were from symptomatic and 56 from asymptomatic patients. Abdominal pain was reported by 59 patients (33.5%) and a temperature of $>38°C$ in 30 (17.1%). One hundred and twenty-four (70.5%) of the STEC isolates were sorbitol negative and of serotype O157. All the O157 isolates were positive for stx_1 and 112 (90.3%) for stx_2; *eae* was detected in 16 (12.9%) and *hlyA* in 18 (14.5%). Among the 52 non-O157 isolates, 28 carried stx_1 or stx_2, or both, and 24 had stx_1 alone (Table 2). The distribution of genes in the O157 isolates from symptomatic and asymptomatic patients showed no evidence ($p > 0.05$) to support an association between stx_1 and stx_2, or stx_1 alone, and the pathogenic potential

Table 2. Distribution of genes encoding virulence factors in 176 STEC isolates and ERIC-PCR patterns of 80 STEC isolates from symptomatic and asymptomatic patients

STEC genotype						Number of isolates from		ERIC-PCR
stx_1	stx_2	*eaeA*	*hlyA*	Total	Serogroup	symptomatic	asymptomatic	pattern (number of isolates)
+	+	+	+	11	O157	10	1	E5 (5), E3 (4)
+	+	+	−	5	O157	4	1	…
+	+	−	+	7	O157	6	1	E5 (1)
+	+	−	−	89	O157	55	34	E1 (42), E2 (1), E4 (17)
+	−	−	−	12	O157	7	5	E2 (10)
+	+	−	−	28	Non-O157	19	9	…
+	−	−	−	24	Non-O157	19	5	…

+, gene present; −, gene absent.

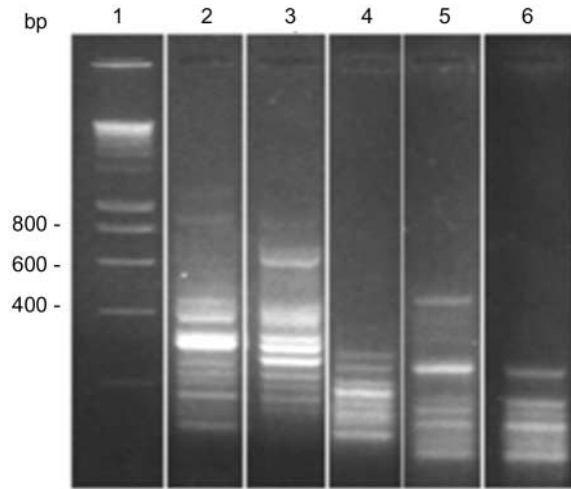**Fig. 1.** Representative ERIC-PCR profiles of DNA from STEC isolates. Lane **1**, molecular sizes marker (100-bp ladder DNA); **2**, pattern E1; **3**, E2; **4**, E3; **5**, E4; **6**, E5.

of the isolate (Table 2). Stx_1 alone and stx_1 plus stx_2 were present, respectively, in similar proportions of O157 isolates from symptomatic patients (100%, 91.5%) and asymptomatic patients (100%, 88.1%). However, *eaeA*- and *hlyA*-encoding genes were significantly more frequent in isolates from symptomatic cases (17.1% and 19.5%, respectively) ($p < 0.05$ for each) than from asymptomatic cases (4.8% each). A strong association was found between the combination of stx_1, stx_2, *eaeA* and *hylA* genes and the patient source of O157 isolates ($p < 0.05$).

ERIC-PCR analysis of 80 isolates of serotype O157 revealed five distinct DNA patterns, E1–E5 (Fig. 1). Patterns E1, E2 and E4 were represented by 42 (52.5%), 11 (13.8%) and 17 (21.3%) isolates, respectively; pattern E3 was restricted to 4 isolates and E5 to 6 isolates (Table 2). Repeat testing of five isolates by ERIC-PCR 2 months apart showed that the DNA patterns were stable and reproducible.

Of the seven antimicrobial agents tested, resistance to ampicillin and tazobactam was present in 83% of STEC isolates with resistance to piperacillin in 81%, norfloxacin 59%, gentamicin 55.1%, amikacin 48% and imipenem 26%. Resistance to five or more drugs was found in 49% of the isolates.

Discussion

STEC, especially those of serotype O157, are increasingly isolated from severe diarrhoeal disease and constitute a serious medical problem for many patients [1, 3]. This study is the first to address the prevalence of STEC in stool samples from the northern part of Palestine where poor hygienic conditions are common; 70.4% of stool samples were positive for STEC and 70.5% of these isolates were serotype O157.

These results are consistent with data from Finland [5] which showed O157 to be the major STEC serogroup. Although strains of O157 predominated, the proportion of non-O157 isolates (29.5%) among STEC in Palestine is similar to reported frequencies in Finland (20%) and Belgium (30%) [5, 6]. The stx_1 and stx_2 genes were very frequent among O157 isolates from Palestine but there was a low frequency of *hlyA* and e*aeA*. To our knowledge, O157 isolates with these characteristics are rare globally. In many studies, isolates with a genetic profile of *eaeA*, *hlyA* and stx_1, either alone or combined with stx_2 have been the most prevalent [5, 13, 14]. However, strains similar in genetic profile to those described here have been reported to occur sporadically [14]. The frequency of serotype O157 isolates with this complement of genes might be explained by the expansion of three major clones, defined by ERIC-PCR analysis, which together accounted for 87.5% of the isolates.

A relationship between the virulence factors carried or expressed by O157 isolates and their pathogenic potential for man has been proposed. Strains producing Stx2 appear to be more virulent for man than those producing only Stx1 [3, 5, 15, 16]. This view is not supported by the toxigenic profiles of O157 isolates from Palestinian patients. Here, O157 isolates typically carried stx_1, either alone or with stx_2. However, a significant association was observed between both *hlyA* and e*aeA*, either independently or with stx_1 and stx_2 genes and the patient source of O157. These results confirm the observations of a previous study [3] and support the suggestion of synergy between the adhesin intimin, enterohaemolysin and Shiga toxin.

The antimicrobial susceptibility patterns of the STEC isolates are a cause for concern as almost half were resistant to five or more agents. Similar rates of resistance have been reported [5]. The high rate in northern Palestine may be due, in part, to selective pressure resulting from uncontrolled use of broad-spectrum antibiotics compounded by a lack of antibiotic prescribing policy and the sale of antibiotics over-the-counter to the general public.

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