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Isolation of enterotoxigenic *Escherichia coli* from British troops in Saudi Arabia

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SUMMARY

Specimens from 181 patients with diarrhoea were examined by a Military General Hospital in a 3-month period during deployment of troops to Saudi Arabia in 1990/1. DNA probes for heat labile (LT) and heat stable (ST) enterotoxin genes identified enterotoxigenic *Escherichia coli* (ETEC) in 47 of the specimens (26%) and 49 ETEC strains were isolated. The majority (55%) belonged to a novel ETEC serotype having the O-antigen 159 and a flagellar antigen designated as a provisional new type. They produced ST and the coli surface associated antigen (CS)6. Strains of serotype O6:H16 represented 22% of the ETEC examined. They produced ST, LT and CS3 together with either CS1 or CS2. The remaining ETEC belonged to seven O:H serotypes. Overall, ST was the only enterotoxin gene identified in 73% of the ETEC and 67% of the strains expressed CS6 in the absence of other colonization antigens. Resistance to three or more antibiotics was observed in 53% of the ETEC, including most of the O159 strains.

INTRODUCTION

Travellers' diarrhoea is usually a mild self-limiting disease that is frequently suffered by visitors to developing or tropical countries and to regions with poor hygiene. The illness is usually of brief duration with loose stools accompanied by symptoms which include nausea, vomiting and abdominal cramps. Travellers' diarrhoea has important economic consequences for the tourist industry and for aid workers and military personnel who may be deployed from areas of good hygiene to those of lower standards. Strains of enterotoxigenic *Escherichia coli* (ETEC) are the most common cause of travellers' diarrhoea [1]. Studies that have examined the association of ETEC and diarrhoea in troops, students and Peace Corps volunteers in tropical areas have found ETEC in between 29 and 75% of diarrhoea cases [2–4]. Strains of ETEC are also of major importance in diarrhoeal disease affecting young children in developing countries and contribute significantly to the mortality of this group [5].

Enterotoxigenic *E. coli* belong to a range of serotypes that differ from those characteristically associated with the other major groups of pathogenic *E. coli* [6]. The strains produce one or more enterotoxins which may be heat labile (LT) or heat stable (ST). Production of enterotoxins results in a stimulation of net fluid secretion from the small intestine without the invasion of epithelial tissue. An initial stage in the pathogenesis of ETEC strains is their colonization of the small intestine. In many strains colonization factor antigens (CFA) or coli surface associated (CS) antigens have been identified that may mediate this process. Many of these antigens, including CFA/I, CS1, CS2, CS3, CS4, CS5 and several others are fimbrial or fibrillar structures [7], although apparently non-fimbrial antigens such as CS6 are also thought to be adhesive factors [8].

Hostilities in the Middle East during the period from late 1990 to the early months of 1991 resulted in the deployment of approximately 50000 British military personnel to the region and the establishment of medical support. Here we report the results of a study that used DNA probes for the ST and LT genes to examine the prevalence of ETEC in diarrhoea cases examined by a Military General Hospital in Saudi Arabia during this time.

MATERIALS AND METHODS

Patients and specimens

During the period of study from 6 November 1990 to early January 1991 stool specimens or rectal swabs were obtained from military personnel reporting with diarrhoea to the hospital, aid stations or field ambulances. From early in December 1990, patients were requested to complete a questionnaire to provide information on the nature, frequency and duration of the diarrhoea, and the presence of other symptoms including fever and abdominal cramp.

Initial screening of specimens

Stool specimens or rectal swabs were plated on MacConkey agar and examined for pathogens by the Pathology Unit of the hospital. Lactose non-fermenting colonies were tested with polyvalent salmonella and shigella antisera and identified biochemically using API (10 or 20E). Where a pathogen was identified, the organism was isolated, transferred to an agar slope and sent to the Laboratory of Enteric Pathogens (LEP) in London for confirmation of identity and further typing. For the remaining majority of the specimens from which no pathogen was isolated on initial plating, a sweep of coliform organisms from a MacConkey agar plate was subcultured to a slope and sent to LEP to be screened for the presence of ETEC.

On the basis of early laboratory results on the O-serogroups of strains of ETEC identified by DNA probing (see below), pooled polyvalent antisera directed against these and other common ETEC O-groups were subsequently sent to the hospital laboratory. These were used to screen lactose fermenting colonies by agglutination tests before specimens were sent to the LEP. Antiserum I contained antibodies to *E. coli* O groups 6, 78, 153, 159 and 167; antiserum II contained antibodies to O groups 27, 63, 115, 128 and 148 whereas antiserum III was directed against O groups 8, 15, 20 and 25.

*Confirmation of non-*E. coli *pathogens*

Organisms that had been provisionally identified as strains of salmonella were further investigated in the LEP by serological and biochemical methods [9]. Phage typing of *Salmonella typhimurium* was carried out as described previously [10]. The identify of *Shigella sonnei* and *Citrobacter freundii* was confirmed by standard techniques [11].

Identification of ETEC by DNA probing

Growth from the agar slopes received in the LEP was streaked on MacConkey agar. Samples that failed to give coliform growth were not pursued further. Bacterial growth, comprising confluent areas and single colonies, was transferred by replica plating on to an 82 mm diameter nylon filter (Hybond-N, Amersham International) supported on a nutrient agar plate. After 4–6 h incubation at 37 °C the filters were prepared for DNA hybridization [12]. The presence of ETEC was detected with alkaline phosphatase-conjugated oligonucleotide probes directed against the ST and LT enterotoxin genes (SNAP system, DuPont) [13]. The ST probe consisted of oligonucleotides specific for the STA1 and STA2 genes [14]. Initially, filters were hybridized according to the manufacturer's instructions at 50 °C with a mixture of the ST and LT probes. They were washed at 45 °C, a temperature that allowed unambiguous detection of both ST-positive and LT-positive control strains. Up to five of the probe-positive colonies on the master plates were picked and serotyped with antisera to O-antigens 1–170 and H-antigens 1–56 [15]. Colonies were subsequently tested with separate ST and LT SNAP probes [13].

Characterization of ETEC strains

Strains were examined initially for colonization factors by their ability to cause mannose-resistant haemagglutination of bovine erythrocytes [16]. Production of CFA/I, CFA/III, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS17 and putative colonization factors (PCF) PCFO159, PCFO9 and PCFO166 was tested for by ELISA [17]. Strains were tested for resistance to antibiotics by the methods of Anderson and Threlfall [18]. The plasmid content of the strains was determined by agarose gel electrophoresis of plasmid DNA prepared by the method of Birnboim and Doly [19]. Molecular sizes of the plasmid present in the strains were measured relative to standard plasmids run on the same gel.

RESULTS

During the period of study, 168 samples of coliform growth and 13 cultures were received by the LEP from 181 separate patients. Two cultures were confirmed as *Citrobacter freundii*, an organism not usually considered an enteropathogen. There were four salmonella isolates: two of *S. emek* and a single strain of each of *S. grumpensis* and *S. typhimurium* phage type 141. Six of the remaining isolates were identified as *Shigella sonnei* and one contained *Klebsiella* species and *E. coli*.

Table 1. *Isolation of enterotoxigenic* E. coli *from 181 cases of diarrhoea among British military personnel in Saudi Arabia*

Type of enterotoxin produced by strain	No. of patients (%)
ST	34 (19)
LT	1 < 1
ST + LT	11 (6)
ST and mixed LT infection	1 < 1
	47 (26)

Identification of specimens containing ETEC

Preliminary tests with a mixed probe for ST and LT genes on coliform growth indicated that ETEC were carried by 47 patients (26% of total). Hybridization of individual bacterial colonies with the separate probes gave the results shown in Table 1. One individual appeared to have a mixed infection with both ST and LT strains and another patient provided samples at different times in which different ETEC were identified. A total of 49 strains was characterized further. The majority of specimens from which ETEC were isolated contained a very high proportion of probe positive organisms on the hybridized filters, but for some specimens, fewer than 20 colonies out of several hundred were detectable. Of the 47 patients from whom ETEC were isolated, 17 had completed a questionnaire describing their illness. The majority of patients (15/17) suffered soft or watery motions for between 1 and 5 days (14/17) and other symptoms included abdominal cramps (10/17) and fever (2/17).

Ninety-seven specimens had been screened with pooled polyvalent antisera directed against ETEC O-antigens; 24 were reported to contain *E. coli* that agglutinated with one or more of the sets. Thirteen of these specimens were confirmed as containing ETEC by DNA probing, but no colonies hybridizing with ST or LT genes were isolated from the remaining 11. DNA probes indicated that a further 13 of the 97 specimens contained ETEC, although these had not been detected using polyvalent antisera.

Properties of ETEC strains isolated

A summary of the properties of the ETEC strains isolated is shown in Table 2. The majority (27/49 or 55%) belonged to serogroup O159 and possessed an H antigen that was unidentifiable with currently available antisera, and was provisionally classified as a new flagellar type. Strains of serogroup O159 were isolated from specimens collected throughout the period of study. Strains of serotype O6:H16 represented 22% (11/49) of the ETEC identified. These organisms were isolated from samples obtained during 2 weeks in the early to mid-December period of the study. The remaining ETEC belonged to seven different serotypes and included four strains of serotype O169:H- obtained from specimens taken within an approximately 1 week period in mid to late December. Multiple drug resistance (resistance to three or more antibiotics tested) was found in 53% of the ETEC strains isolated and was mainly associated with the O159 strains. The predominant pattern of drug resistance was to ampicillin, streptomycin,

Table 2. *Properties of ETEC strains from Saudi Arabia*

Serotype	No. of strains isolated	Enterotoxin gene(s) carried	CFA or CS antigens produced	Drug resistance*	Molecular size of plasmids carried (kb)†	No. with plasmid profile
O159:H?	23	ST	CS6	ASSuTTm	136, 85	23
O159:H?	4	ST	CS6	—	137, 65	4
O6:H16	8	ST, LT	CS1, CS3	—	> 154, 54, 42, 5.	5
					112, 54, 40, 5,	2
					99, 51, 39, 5	1
O6:H16	2	ST, LT	CS2, CS3	ACSSuTTm	108, 88, 69, 6, 5, 4, 3	2
O6:H16	1	ST, LT	CS2, CS3	A	108, 71, 59, 42, 5, 4, 3	1
O169:H-	4	ST	CS6	—	122	4
O148:H28	2	ST	CS6	—	62, 45, 13, 9, 7, 5, 3, 2,	1
					88, 69, 46,	1
					9, 6, 5, 4	
O25:H42	1	ST	CS4, CS6	S	95	1
O128:H12	1	ST	CFA/I	ACSSuTTm	136, 84, 74	1
O64:H-	1	LT	Not known	S	95	1
O64:H5	1	LT	Not known	ST	> 154, 122, 2	1
O?:H10	1	ST	Not known	—	106, 80	1

* Symbols for drug resistance: A, ampicillin; C, chloramphenicol; S, streptomycin. SU, sulphonamides; T, tetracycline; Tm, trimethoprim.

† Measured by agarose gel electrophoresis. For sizes < 15 kb, the bands may represent more than one molecular form of the same plasmid.

sulphonamides, tetracycline and trimethoprim (ASSuTTm) but some strains of O6:H16 and one of serotype O128:H12 had additional chloramphenicol resistance (ACSSuTTm).

All of the serogroup O159 strains hybridized with the ST probe only and produced CS6. They appeared to fall into classes on the basis of drug resistance, but plasmid analysis showed that the two types were related. The drug-sensitive organism appeared to be derived from the resistant type by deletion of DNA from the 85 kb plasmid to leave a plasmid of 65 kb (Table 2). This was supported by the finding of occasional laboratory variants that lacked some of the drug resistance markers of the fully resistant strain and also carried smaller derivatives of the 85-kb plasmid.

All the strains of serotype O6:H16 hybridized with both the ST and LT probes and were differentiated into two major groups on the basis of drug resistance and CS antigen production. Strains belonging to both groups were isolated from the same batches of specimens. Most of the strains (8/11) were drug sensitive and produced CS1 and CS3. Five strains were indistinguishable by plasmid content and the remaining three appeared related to them by deletion of DNA from the largest plasmid species (Table 2). Three strains of serotype O6:H16 produced CS2 and CS3 and were drug resistant. Two of these organisms appeared identical, while the other was different in resistant pattern and plasmid content.

Four specimens yielded cultures of *E. coli* serotype O169:H- that appeared to be indistinguishable in their properties (Table 2). The isolates of serotype O148:H28 carried ST sequences and produced CS6 but differed in their plasmid

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content. Only one of the ETEC strains isolated in the study, that of serotype O128:H12, produced CFA/I. Two strains of serotype O64 were isolated, one of which had the H5 flagellar antigen whereas the other was non-motile. Both strains hybridized with the LT probe only, but they differed in drug resistance and plasmid profile. One of these organisms was isolated from the same specimen as a strain of serogroup O159. An antigen associated with bacterial colonization was not identified in the *E. coli* O64 strains or in another strain with an unidentifiable O antigen that hybridized with the ST probe.

Comparison of DNA probing and use of polyvalent antisera

For the 97 specimens that had been screened for ETEC with polyvalent antisera, it was possible to relate those results to the isolation of ETEC strains by DNA probing followed by serotyping. Only specimens found to contain ETEC of serogroups O6 and O159 were successfully identified by a polyvalent antiserum. Probe tests identified strains of O6 or O159 in 19 of the 97 samples and 13 of these had been scored as positive with polyvalent antiserum I. A further three specimens were positive with this antiserum but failed to give probe positive colonies when tested later. The other two antisera used indicated the presence of ETEC in eight samples but none of these yielded ETEC by DNA probing. These antisera failed to detect the presence of ETEC belonging to O serogroups 25, 128 and 148. ETEC strains belonging to the other seven serogroups identified were obtained from samples in which intestinal pathogens were not reported initially.

DISCUSSION

A relationship between ETEC strains and travellers' diarrhoea was demonstrated by Rowe and colleagues [20] in a study of British troops in Aden. Serotyping showed that about 50% of the diarrhoea cases in newly arrived personnel was due to *E. coli* serotype O148:H28 that was subsequently shown to produce ST. In the present study, an ETEC strain was isolated by DNA probing in 26% of the diarrhoea cases examined, a value that falls at the lower end of the range of the estimated occurrence of ETEC in other reports [1–4]. The period of study coincided with the colder winter months in Saudi Arabia, conditions in which ETEC infection might be expected to be less prevalent. Specimens were initially examined under severely restricted conditions in the field and filters for DNA probing were prepared in the LEP, in some cases several weeks after the collection of stool samples. This might have resulted in failure to recover some ETEC strains.

Studies of diarrhoeal disease in American troops deployed in Saudi Arabia in late 1990 [21] identified ETEC in 21% of patients, a similar finding to that presented here. However, the incidence of shigella infections, principally due to *Shigella sonnei* was higher in US personnel (19% of patients [21]) than in British troops (3%). Enterotoxigenic *E. coli* have been reported as a cause of infantile diarrhoea in the Gulf region, although the isolation rate of approximately 9% [22] may be less than that in many developing countries.

In the study of Hyams and colleagues [21], the majority of ETEC strains isolated from US troops produced both ST and LT, with ST producers the next

most prevalent group. Serological identification of their 132 ETEC isolates [23] showed that they belonged to 32 O:H serotypes but only three of these (O6:H16, O128:H12 and O148:H28) were the same as those reported here. The ETEC strains isolated from American personnel in Saudi Arabia in 1990 showed similarities in O:H serotypes, enterotoxin types and colonization antigens to ETEC isolated in Egypt in 1989 [23]. Rations for British soldiers were provided almost exclusively from the usual UK suppliers whereas food for US personnel was supplemented with fresh produce from South West Asian countries and dairy products from Saudi Arabia [21].

The majority of ETEC strains isolated from British troops belonged to a novel ETEC serotype with the O-antigen 159 and a flagellar antigen that has been designated as a provisional new type. Strains of *E. coli* O159 have been found in studies of travellers' diarrhoea [6], in surveys of the occurrence of human ETEC in underdeveloped areas [24] and from outbreaks of *E. coli*-associated diarrhoea [25, 26]. These strains had the flagellar antigens 20 or 37, or were non-motile. The O159 strains in this report produced CS6 and differed from the *E. coli* serotype O159:H4 strains that produce the putative colonization factor PCFO159 [27]. Strains of serotype O6:H16 formed the second most prevalent group in the present study and have been commonly reported in travellers' diarrhoea [6, 16]. Although the specimens were collected over a short period of time, it appears unlikely that the infections were caused by a single strain. Plasmid analysis, drug resistance and CS antigen type indicated that at least two distinct clones of this serotype were associated with disease. Strains of serotype O6:H16 were the most frequent ETEC isolates from US troops in Saudi Arabia [23] but in contrast with our study, most strains produced CS2 and CS3.

The properties of the ETEC strains isolated in Saudi Arabia emphasize the importance of ST and CS6 sequences in virulence. Overall, ST was the only enterotoxin gene identified in 73% of the strains and CS6 was the single antigen identified that was associated with intestinal colonization in 67% of the ETEC. Almost one-third of the ETEC from American troops expressed CS6 alone, although there was no particular correlation with any one serotype [23]. The type of colonization factor found in human ETEC from different geographical areas may vary considerably but CS6 appears ubiquitous, either alone or with other antigens [22, 27]. Only three strains in this study (6%) had no identifiable colonization antigen, and two of them produced only LT. It has been suggested that strains of this type may produce less severe diarrhoea or may be non-pathogenic [24]. In the study of Wolf and colleagues [23] a much larger proportion of the ETEC strains (25%) did not express a colonization antigen detectable by the assays used.

DNA probing permits the simultaneous screening of several hundred colonies so that probe positive organisms can be detected at very low levels. Since probes for ETEC are directed against enterotoxin genes, the results are independent of the serogroups of the strains present. In contrast, the success of serological screening with pooled polyvalent antisera relies on the presence of the appropriate component antibodies. This test was useful for the detection of strains of serogroups O6 and O159 in 13 of 19 isolates that yielded these strains by DNA probing. However serological screening was not predictive for other ETEC and

some specimens apparently gave positive results not confirmed by probe tests. It is possible that some of these were false positive results or that some ETEC did not survive the interval between initial serological screening and DNA probing. The techniques were performed on only a proportion of specimens in separate locations under vastly different conditions. For these reasons we have not attempted quantitative estimates of the sensitivity and specificity of these tests.

The deployment of troops to Saudi Arabia was accompanied by infectious diarrhoea and our results confirm the importance of ETEC strains in a situation of this type. However, other classes of *E. coli* are associated with diarrhoeal disease and the importance of these is being investigated in those specimens from which an intestinal pathogen was not isolated.

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