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Clinical relevance of virulence genes in *Campylobacter jejuni* isolates in Bahrain

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There are no data describing the genetic make-up of *Campylobacter* strains (an important aetiological agent of diarrhoea) circulating in the Arabian Gulf region. Here, the molecular characterization of two virulence genes in *Campylobacter jejuni* from Bahrain and the relationship with clinical infection are reported. Molecular screening for cytolethal distending toxin (*cdtB*) and invasion-associated marker (*iam*) genes was carried out on *C. jejuni* stool isolates collected from January 2002 to January 2004 in Bahrain. The molecular characterization was correlated with the patients' socio-demographic and clinical parameters. Of the 96 *C. jejuni* strains tested, 50 (52 %) were $cdtB^+/iam^+$, 30 (31 %) were $cdtB^+/iam^-$ and 16 (17 %) were $cdtB^-/iam^-$. Sixty-nine per cent (66/96) of patients were less than 3 years old, with significantly higher detection of $cdtB^+/iam^+$ and $cdtB^+/iam^-$ strains ($P < 0{\cdot}001$ and $P < 0{\cdot}01$, respectively) in this age group. Seventy patients (73 %) were symptomatic. In the group that were less than 3 years old, 62 and 85 % of those with $cdtB^+/iam^+$ and $cdtB^+/iam^-$ strains, respectively, were symptomatic compared with 100 % for those over 3 years of age. However, the presence of $cdtB^-/iam^-$ strains still resulted in clinical infection in the children under 3 years but not in the older patients. This is the first report describing the molecular characterization of virulence genes in *Campylobacter* isolates from this region. The findings indicate that strains of different virulence genetic make-up are circulating in the population, with children under the age of 3 years being most vulnerable. Further work on the molecular characterization, gene expression and determination of the invasive phenotypes of *C. jejuni* strains circulating in different regions is needed.

INTRODUCTION

Campylobacter jejuni is now recognized as a leading bacterial cause of food-borne disease in both developed and developing countries (Butzler, 2004). The spectrum of disease may range from mild, self-limiting, non-inflammatory diarrhoea to severe, inflammatory, bloody diarrhoea with pyrexia, abdominal cramps, bacteraemia and faecal leukocytes (Sack *et al.*, 2001). Extra-intestinal disease may also occur, particularly in those with underlying immunosuppression (Tee & Mijch, 1998). A notable complication of *C. jejuni* infection is the development of Guillain–Barré syndrome, which has a significant association with serological evidence of recent previous infection with *Campylobacter* (Mishu *et al.*, 1993). Several factors have been proposed to explain the varied clinical presentation associated with *Campylobacter* infection, and the phenotypic traits associated with different *C. jejuni* strains may be related to their genetic diversity. However, the precise mechanism of pathogenicity is yet to be elucidated fully.

Toxin production and the invasive capability of infecting strains may modulate the clinical presentation of *Campylobacter* enteritis. Cytolethal distending toxin (Cdt) production by *C. jejuni* was first described by Johnson & Lior (1988). CdtB, which is encoded by the *cdtB* gene, is the active subunit of the holotoxin, which causes cell distention and irreversible cell-cycle arrest (Eyigor *et al.*, 1999). In addition to its toxigenic effect, it has also been suggested that Cdt may play a role in invasion (Konkel *et al.*, 2001). The occurrence of inflammation, infiltration of the lamina propria by neutrophils and bacteraemia indicate that invasion is an important pathogenic mechanism in *Campylobacter* infection (Ketley, 1997). Another virulence gene linked with *Campylobacter* invasiveness is the invasion-associated marker (*iam*) gene. *In vitro* studies have shown that this chromosomal genetic marker of *Campylobacter* strains is associated preferentially with both adherence and invasion (Carvalho *et al.*, 2001).

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Abbreviations: BDFH, Bahrain Defence Force Hospital; Cdt, cytolethal distending toxin; GCC, Gulf Co-operation Council; iam, invasion-associated marker; SMC, Salmaniya Medical Complex.

The occurrence of *Campylobacter* as a cause of diarrhoeal illness in the Gulf Co-operation Council (GCC) countries has been shown to range from 1·6 to 28 % (Sethi *et al.*, 1989; al-Freihi *et al.*, 1993; Akhter *et al.*, 1994; Ismaeel *et al.*, 2002). However, there are no data describing the genetic make-up of *Campylobacter* strains circulating in this population. Here, we report the molecular characterization of two virulence genes in *C. jejuni* strains isolated in the Kingdom of Bahrain and the relationship with clinical infection.

METHODS

Bacterial strains and growth conditions. From January 2002 to January 2004, 96 *C. jejuni* strains were isolated from the stools of in- and out-patients seen at the Bahrain Defence Force Hospital (BDFH) and Salmaniya Medical Complex (SMC), as well as out-patients attending primary healthcare facilities in the Kingdom of Bahrain. SMC and BDFH are national referral centres for specialist care, laboratory diagnosis and admissions. Symptomatic patients were defined as those who presented with diarrhoea (an increased number of loose bowel motions) with or without vomiting, fever and abdominal pain (Friedman & Isselbacher, 1998). For each patient, the attending physician completed a questionnaire designed to collect socio-demographic data as well as information on clinical parameters such as type and duration of symptoms, including the presence of nausea, vomiting, abdominal pain and fever. Abdominal pain was classified as mild, diffuse and/or crampy according to clinical judgement. Diarrhoea was defined as watery, malabsorptive or bloody depending on the appearance of the stools. Previous administration of antibiotics or chemotherapy (within the preceding month) was recorded.

Stool samples were processed at the microbiology laboratories at BDFH and SMC according to guidelines set out in the Bahrain National Quality Control Manual. All samples were cultured for *Salmonella*, *Shigella*, *Campylobacter*, *Aeromonas*, *Plesiomonas* and *Yersinia* as described previously (Jamsheer *et al.*, 2003). *Campylobacter* cultures were carried out using blood-free selective agar containing modified charcoal cefoperazone deoxycholate selective supplement (Oxoid). Plates were incubated at 42 °C for 48 h under microaerobic conditions (CampyGen; Oxoid). Suspected colonies were subcultured on chocolate agar plates and incubated microaerobically at 37 °C for 24 h (CampyGen; Oxoid). Negative cultures were reincubated for an additional 24 h. *C. jejuni* ATCC 33291 was used as a quality-control strain. *Campylobacter* isolates were identified to species level using routine biochemical tests (production of catalase, hippurate and indoxyl acetate), sensitivity to nalidixic acid and cephalotin and temperature preferences for growth at 37 and 42 °C, as described previously (Ismaeel *et al.*, 2002).

Microscopic assessment for parasites, cysts and ova was carried out by the formalin/ether concentration method and direct wet preparation (Ismaeel *et al.*, 2002). All samples from children under the age of 3 years were tested for the presence of enteropathogenic *Escherichia coli* as well as rotavirus and adenovirus using the Diarlex Rota-Adeno card agglutination test kit (Orion Diagnostics). The viral investigations were carried out at the Public Health Laboratory, Bahrain. All bloody stools were examined for enterohaemorrhagic *E. coli* irrespective of the patient's age.

Detection of *iam* and *cdtB* genes by PCR. Template DNA for PCR was extracted using the Chelex 100 boiling method, as described previously (Ismaeel *et al.*, 2005). Amplification of the *iam* locus was carried out in a mastermix volume of 40 µl containing 1 × DNA *Taq* polymerase buffer, 0·2 mM each deoxynucleotide triphosphate, 2 U *Taq* DNA polymerase (Boehringer) and 30 pmol each of forward primer 1·6F (5′-GCGCAAAATATTATCACCC-3′, nt 316–334 of the *iam* locus) and reverse primer 1·6R (5′-TTCACGACTACTATGCGG-3′; Interactiva GmbH). The amplification program consisted of 30 cycles of 1 min at 94 °C, 2 min at 42 °C and 3 min at 72 °C. The PCR product was detected by electrophoresis on a 2 % agarose gel and visualized under UV light after staining with ethidium bromide. The *cdtB* gene was detected as described previously (Ismaeel *et al.*, 2005) using degenerate primers VAT2 and WMI1 [5′-GT(A/C/G/T)GC(A/C/G/T)AC(G/C/T)TGGAA(C/T)CT(A/G/C/T)CA(A/G)GG-3′ and 5′-(G/A)TT(G/A)AA(G/A)TC(A/G/C/T)-CC(T/C)AA(T/G/A)ATCATCC-3′, respectively; Interactiva].

Statistical analysis. Data were entered in Microsoft EXCEL and analysed using the SPSS version 12 statistical package. Statistical significance was calculated using a chi square test.

RESULTS

During the study period, 96 *C. jejuni* strains were isolated. Fifty (52 %) strains were $cdtB^+/iam^+$, 30 (31 %) were $cdtB^+/iam^-$ and 16 (17 %) were negative for both genes. Fig. 1 shows the characteristic 495 bp band for *cdtB* and the 518 bp band for *iam* following electrophoresis. Sixty-nine per cent (66/96) of the patients were under the age of 3 years; 56 % had faecal leukocytes and 12 % had blood in the stool. None of the patients was found to have parasites in the stool and neither rotavirus nor adenovirus was detected. Apart from one patient with $cdtB^+/iam^+$ *C. jejuni* who also had *Shigella*, and two patients with $cdtB^+/iam^-$ strains with concomitant *Salmonella* infection, no other bacterial infection was detected.

The detection of strains positive for one or a combination of two virulence genes was significantly higher in children under the age of 3 years ($P < 0{\cdot}01$ and $P < 0{\cdot}001$, respectively; Table 1). Strains negative for both genes were not detected in the 3- to 15-year-old group and their detection in the other two age groups was comparable. Although there was a preponderance of male patients (57 males versus 39 females) and over 60 % of strains with virulence genes were from males, the detection of strains negative for both genes was comparable in both sexes (Table 1). The majority of strains in all three virulence categories were isolated from patients of Bahraini nationality.

A total of 70 patients (73 %) was found to be symptomatic (Table 2). Symptomatic patients were defined as those presenting with diarrhoea (an increased number of loose bowel motions) with or without vomiting, fever and abdominal pain (Friedman & Isselbacher, 1998). Asymptomatic patients were those without diarrhoea who either had non-specific symptoms such as abdominal discomfort and flatulence or were being tested as part of a routine recruitment screening. No correlation was found with previous administration of antibiotics and none of the patients had received chemotherapy. No specific correlation was found between the type of diarrhoea (watery or bloody) and culture positivity for *Campylobacter*. Remarkably, all patients over the age of 3 years who were infected with strains positive for one or both virulence genes were symptomatic (Table 2). In

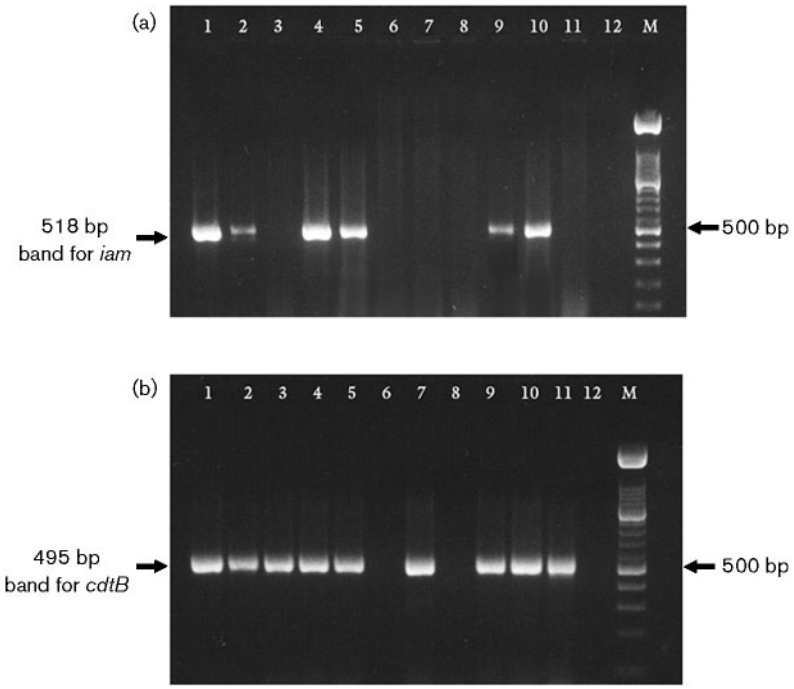

Fig. 1. (a) Electrophoresis of PCR products for the *iam* gene. Lanes 1 and 2 show the 518 bp product from a known iam^+ control (in-house BDF 833 *C. jejuni* strain). Lanes 4, 5, 9 and 10 show the results from strains positive for the *iam* gene. Lane M, 100 bp marker. (b) Electrophoresis of PCR products for the *cdtB* gene. Lanes 1 and 2 show the 495 bp product from a known $cdtB^+$ control (in-house BDF 14 *C. jejuni* strain). Lanes 3–5, 7 and 9–11 show the results from strains positive for the *cdtB* gene. Lane M, 100 bp marker.

contrast, a proportion of children under the age of 3 years infected with strains in these same virulence categories remained asymptomatic, although this was not statistically significant. Among the 16 $cdtB^-/iam^-$ strains identified, nine were isolated in children under the age of 3 years and the remaining seven strains were from those over the age of 15 years. In sharp contrast to the clinical picture observed for patients harbouring strains positive for one or two virulence genes, all those in the >15-year-old group who were positive for $cdtB^-/iam^-$ strains were asymptomatic. However, these $cdtB^-/iam^-$ strains resulted in significant clinically evident infections in the <3-year-old group (P <0·001; Table 2) and the proportion of symptomatic infections was at a level comparable to those seen with $cdtB^+/iam^+$ and $cdtB^+/iam^-$ strains.

DISCUSSION

These findings provide data correlating the presence of virulence gene combinations in *C. jejuni* with the demographic and clinical parameters of the patients from whom the strains were isolated. In addition, this is the first report describing the molecular characterization of *Campylobacter* strains from the Arabian Gulf (GCC) region. A total of 96 patients with *Campylobacter* enteritis was identified during the study period and this low number probably reflects the fact that routine *Campylobacter* screening was only introduced in 2002 after a pilot study showed detection of this micro-organism in 1·6 % (7/426) of children with diarrhoea (Ismaeel *et al.*, 2002). It is possible that during the early phase of implementation of procedures for optimal

Table 1. Detection of *C. jejuni* strains with virulence genes among patients of different age groups, gender and nationalities

Percentages in parentheses indicate the proportion of $cdtB^+/iam^+$, $cdtB^+/iam^-$ and $cdtB^-/iam^-$ strains found in each of the age group, gender and nationality categories. A significantly higher distribution of $cdtB^+/iam^+$ and $cdtB^+/iam^-$ *C. jejuni* strains was found among children under 3 years of age.

Virulence gene combination (number of samples)	Age (years)			Sex		Nationality	
	<3	3–15	>15	Male	Female	Bahraini	Non-Bahraini
$cdtB^+/iam^+$ (n=50)*	37 (74 %)	3 (6 %)	10 (20 %)	30 (60 %)	20 (40 %)	33 (66 %)	17 (34 %)
$cdtB^+/iam^-$ (n=30)†	20 (67 %)	10 (33 %)	0	20 (67 %)	10 (33 %)	23 (77 %)	7 (23 %)
$cdtB^-/iam^-$ (n=16)	9 (56 %)	0	7 (44 %)	7 (44 %)	9 (56 %)	13 (81 %)	3 (19 %)
Total (n=96)	66	13	17	57	39	69	27

*P <0·001; †P <0·01.

Table 2. Distribution of symptomatic presentation among patients of different age groups with strains of different virulence categories

Symptomatic patients were defined as those who presented with diarrhoea with or without vomiting, fever and abdominal cramps. Numbers in parentheses indicate the number of symptomatic patients as a proportion of the total number of patients in that age group with *C. jejuni* strains of the corresponding virulence category. No significant difference in the number of symptomatic infections was seen in the different age groups with strains positive for one or both virulence genes. Compared with the other age groups, those in the <3-year-old age group with $cdtB^-/iam^-$ strains had a significantly higher number of symptomatic infections.

Strains with virulence genes	Symptomatic patients in different age groups		
	<3 years	3–15 years	>15 years
$cdtB^+/iam^+$	62 % (23/37)	100 % (3/3)	100 % (10/10)
$cdtB^+/iam^-$	85 % (17/20)	100 % (10/10)	0/0
$cdtB^-/iam^-$	78 % (7/9)*	0/0	0 % (0/7)

*P <0·001.

specimen collection, transport and analysis some cases might have been missed.

Strains with three combinations of virulence genetic make-up, $cdtB^+/iam^+$, $cdtB^+/iam^-$ and $cdtB^-/iam^-$, were identified, indicating that *C. jejuni* strains of diverse pathogenic potential are circulating in this population. The genes that we targeted are important for toxin production and invasiveness, both of which are pathogenicity mechanisms in *Campylobacter* infection (Ketley, 1997; Wassenaar, 1997). The *cdtB* gene is associated with toxin production, and the clinical manifestations of *Campylobacter* enteritis are in keeping with the toxigenic effect of cytotoxins. Of particular clinical relevance is the fact that this toxigenic effect may be enhanced by pre-exposure to the antibiotics of choice for treatment of *Campylobacter* infection (Ismaeel *et al.*, 2005). Furthermore, although studies have been carried out identifying these genes in individual *Campylobacter* strains, there is a paucity of data describing the molecular epidemiology of these virulence genes in clinical isolates obtained in different countries. Although *in vitro* work has shown that the *iam* gene is associated with adherence and invasion, there are only two reports describing the identification of this virulence gene in clinical isolates (Carvalho *et al.*, 2001; Rozynek *et al.*, 2005).

Diarrhoea is the hallmark of *Campylobacter* enteritis, but asymptomatic carriage of this pathogen has been described, particularly in developing countries where infection tends to be endemic (Blaser, 1997; Taylor *et al.*, 1988; Ketley, 1997). Therefore, in this study only patients presenting with diarrhoea were classified as symptomatic. This is particularly relevant in children, where the occurrence of symptoms such as fever, vomiting and abdominal pain may be due to various viral illnesses and not indicative of *Campylobacter* enteritis. Indeed, the majority of patients were under the age of 15 years, in keeping with the fact that *Campylobacter* enteritis in non-industrialized nations is seen mainly in children, with attenuation of symptoms with increasing age (Blaser, 1997; Ketley, 1997). However, our findings showed that children under the age of 3 years are the most vulnerable population for *Campylobacter* infection, as 69 % (66/96) of the patients were in this age group and the strains isolated from these children were mostly positive for the virulence genes. This is consistent with reports that this is a high-risk age group for diarrhoeal illness of both viral and bacterial aetiology (Kosek *et al.*, 2003). In addition, infection with strains negative for both virulence genes also resulted in symptomatic infection in this age group. This was in sharp contrast to the finding that older patients with $cdtB^-/iam^-$ strains were asymptomatic. Although the occurrence of symptoms with $cdtB^-/iam^-$ strains might reflect the presence and expression of other virulence genes, we cannot completely rule out possible infection with other pathogens or false-negative results in the virological investigations. However, the fact that a wide spectrum of micro-organisms was investigated and that tests for rotavirus and adenovirus were carried out in a referral laboratory suggests that these scenarios are unlikely.

A proportion of children under the age of 3 years was found to be asymptomatic, even though strains positive for one or two virulence genes were isolated from their stools. Again, this is markedly different from the picture seen with the older age groups, where all of those infected with $cdtB^+/iam^+$ and $cdtB^+/iam^-$ strains were symptomatic. Breast-feeding, which protects against childhood enteric infections, is encouraged culturally in GCC countries and it is not unusual to find children being breastfed up to the age of 2 years (Alnasir, 1992; Ogbeide *et al.*, 2004). In addition, a number of infant milk formulas on the market contain added prebiotics and probiotics, which are also protective against childhood diarrhoea (Senok *et al.*, 2005). These factors could explain this reduced level of symptomatic infection in this very young age group.

The observed male preponderance in the study population was not statistically significant and therefore not suggestive of a gender-related susceptibility to infection. Furthermore, although there is a large expatriate population in the country, the majority of the patients were of Bahraini nationality and this probably reflects access to care rather than any real epidemiological difference.

This study provides baseline data on the molecular epidemiology of two *C. jejuni* virulence genes and the relationship with clinical *Campylobacter* infection. Although we have demonstrated the presence of these genes, their expression was not characterized. Differences in gene expression, which may occur as a result of mutations or interplay between host and environmental factors, may also explain the variation in symptomatic presentation in the age groups. However,

although it has been suggested that *C. jejuni* may upregulate one set of genes whilst downregulating another during *in vitro* culture (Konkel *et al.*, 1993), the precise mechanisms involved have not been clarified fully and it is yet to be determined whether this occurs *in vivo*. As the effect of any combination of genes may be strain dependent (with host and environmental factors influencing their expression), thus giving rise to differences in phenotypic appearance of virulence characteristics, further work on molecular characterization and gene expression, as well as the invasive phenotypes of *C. jejuni* strains circulating in different populations, is needed.

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