Cryptosporidiosis in Kuwaiti children: association of clinical characteristics with Cryptosporidium species and subtypes

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To determine the association of clinical characteristics with Cryptosporidium types and subtypes, faecal specimens from 2548 children with diarrhoea were screened by microscopy for Cryptosporidium spp., and positive specimens were genotyped and subtyped by PCR-RFLP. A total of 87 of the 2548 children (3.4%) had cryptosporidial diarrhoea by microscopy and the majority (41.4%) of the infected children were in the 4–8-year-old age group. Molecular characterization of the 83 children studied further (4 had mixed infections and were not subtyped) showed that Cryptosporidium parvum was the most commonly identified species (73.5%) and consisted of three subtypes: Ila and Ild were the most common (80.3%), followed by Ile. Twenty-two (26.5%) of the children had Cryptosporidium hominis and showed three subtypes: Id was the most common (54.5%), followed by Ia (36.4%) and Ie. Associated clinical manifestations varied between C. parvum and C. hominis. Diarrhoea associated with subtype Id, the most commonly identified C. hominis subtype, was more severe than that associated with other subtypes. In conclusion, this study confirmed a very different Cryptosporidium genotype and subtype distribution compared with other tropical countries among Kuwaiti children with diarrhoea, with a predominance of C. parvum Ila and Ild. In addition, subtype Id of C. hominis was associated with more diverse and severe clinical manifestations in infected children, suggesting that parasite genetics may play an important role in the clinical manifestations of human cryptosporidiosis.

INTRODUCTION

Cryptosporidiosis is a major cause of diarrhoea in developing countries and generally causes self-limited watery diarrhoea in immunocompetent patients or chronic severe diarrhoea in immunocompromised individuals (Iqbal et al., 1999, 2001). Previous studies in various tropical countries have shown that children of <2 years of age are the most susceptible to Cryptosporidium infection, with the reported incidence ranging from 1.1 to 18.9% (Ajampur et al., 2007, 2010; Xiao et al., 2001).

A wide diversity of Cryptosporidium spp. and subtypes infect humans, and each may have a range of transmission routes of public-health significance (Ajampur et al., 2007, 2010; Geurden et al., 2009; Sulaiman et al., 2005). Recently, genetic characterization of Cryptosporidium spp. into allelic subtypes has helped molecular epidemiological studies enabling the better understanding of the transmission of cryptosporidiosis in humans, and the associations between different species and clinical manifestations and infection risk factors (Albert et al., 1995; Cama et al., 2007; Gatei et al., 2003; Geurden et al., 2009; Peng et al., 2003; Sulaiman et al., 2005; Xiao, 2010; Xiao et al., 2001).

Although cryptosporidiosis is prevalent in tropical regions, information on the molecular characterization of Cryptosporidium spp. is very limited. The majority of human infections in tropical countries are caused by Cryptosporidium hominis, indicating anthropogenic transmission as a major transmission route (Ajampur et al., 2010; Gatei et al., 2003; Leav et al., 2002; Peng et al., 2003; Xiao et al., 2001). The State of Kuwait is in a desert geographical region characterized by long, dry, hot summers and short, relatively warm winters. The main source of drinking water in Kuwait is piped, filtered, desalinated brackish water and/or bottled water. However, the majority of Kuwaiti families spend an extended period of time in winter camp sites traditionally set up individually in the desert during the months of December–March each year. These camp sites are supplied by overhead water storage tanks. The water from a local desalination plant is carried to these camp sites by water tankers.

Information on the prevalence rates of cryptosporidial infections in Kuwait is limited. An earlier study reported a unique endemicity of cryptosporidiosis in children with a
prevalence of Cryptosporidium parvum, suggesting that the transmission of cryptosporidiosis in Kuwaiti children differed significantly from other tropical countries (Sulaiman et al., 2005). However, little or no socio-demographic and clinical characteristics of these cases were documented.

In the present study, we determined the molecular characterization of Cryptosporidium spp. isolated from Kuwaiti children with diarrhoea, and analysed the association between clinical characteristics and socio-demographic data with specific Cryptosporidium spp. and subtypes. Water reservoirs were also screened for Cryptosporidium spp. to determine the source of transmission. Stool samples were collected from 2548 children with diarrhoea in Kuwait City between September 2005 and March 2008. The isolates were genotyped and subtyped using small subunit (SSU) rRNA-based PCR-RFLP (Ajjampur et al., 2007; Sulaiman et al., 2005).

METHODS

Study population, specimen collection and screening

Stool specimens. Stool samples were collected from 2548 children with diarrhoea aged between 6 months and 16 years during the period September 2005–March 2008. Children passing three to four watery stools in a 24 h period for at least 2 days were characterized as having diarrhoea. These children presented at outpatient clinics of the three local general hospitals. These hospitals cover a large population living in urban and semi-urban areas. Information on the consistency and number of stools passed each day by the patient was obtained from parents or guardians. Demographic information, area of residence, socio-economic status of the parents, contact with animals, and sources of drinking water and food, as well as the health status of the child, were also documented. The study was approved by the Research Ethical Committee of the Faculty of Medicine, Kuwait University.

Stool specimens were screened for Cryptosporidium by microscopy of acid-fast stained faecal smears (Iqbal et al., 2001). Faecal specimens were also cultured for enteric bacterial pathogens as described by Albert et al. (1995). Briefly, samples were plated on MacConkey agar, and Salmonella–Shigella agar, which were used for the isolation of Escherichia coli, Salmonella spp. and Shigella spp., whilst Campy-BAP was used for the isolation of Campylobacter jejuni. Rotavirus was detected by ELISA. Direct faecal smears were prepared with a drop of Escherichia coli Campylobacter jejuni Salmonella–Shigella and Albert et al. were also cultured for enteric bacterial pathogens as described by Iqbal et al. (1995). Briefly, samples were plated on MacConkey agar, which were used for the isolation of Escherichia coli, Salmonella spp. and Shigella spp., whilst Campy-BAP was used for the isolation of Campylobacter jejuni. Rotavirus was detected by ELISA. Direct faecal smears were prepared with a drop of physiologically saline and Lugol’s iodine-trichrome, and microscopy was carried out to detect Giardia and other protozoan parasites. Aliquots of Cryptosporidium-positive specimens were preserved at 4 °C in 2.5% potassium dichromate for molecular characterization.

Water specimens. Water from five different overhead water tanks (tanks A, C, F, G and H) was collected during 2007 and tested for coliform bacteria and Cryptosporidium oocysts. The water samples from the tanks were processed by the United States Environmental Protection Agency method 1623, and Cryptosporidium oocysts pellet were detected by immunofluorescent microscopy (US EPA, 2001). Briefly, water samples were filtered through an Envirochek HV filter using procedures described in method 1623. Cryptosporidium oocysts in the pellet for each sample were detected after staining with fluorescently labelled mAbs against Cryptosporidium and Giardia (Merifluor; Meridian Bioscience). DNA extraction from the oocysts was carried out using a QIAamp DNA mini kit (Qiagen) following the manufacturer’s protocol.

DNA isolation, identification of species, genotyping and subtyping. DNA was extracted from all Cryptosporidium-positive faecal specimens. The DNA lyase was extracted once with phenol/ chloroform/isoamyl alcohol solution, and purified using a QIAamp stool DNA mini kit (Qiagen) following the manufacturer’s protocols (Sulaiman et al., 2005).

The purified DNA of each specimen was subjected to PCR-RFLP at the SSU rRNA locus, which amplified an 834 bp fragment of the SSU rRNA gene by nested PCR and PCR-RFLP (Xiao et al., 2001). Briefly the SSU rRNA gene in 1.0 µl DNA was amplified by nested PCR. The PCR was followed by digestion of 10 µl of the secondary PCR product with SspI (New England BioLabs) and VspI (Gibco). The Cryptosporidium spp. or genotypes were determined by banding patterns following 2% agarose gel electrophoresis (Xiao et al., 2001). Cryptosporidium spp. and genotypes were labelled by comparing banding patterns with those published previously. Samples that were negative for SSU rRNA by PCR were analysed by PCR-RFLP at the COWP and TRAP-C loci (Sulaiman et al., 2005).

Each specimen was analysed at least twice in two independent PCR-RFLP analyses. DNA sequencing was carried out only on a few selected isolates for confirmation.

C. parvum and C. hominis subtyping. Subtyping of C. parvum and C. hominis was carried out by a GP60-based tool, which amplified an ~850 bp fragment of the GP60 gene, and amino acid sequence analysis (Alves et al., 2003). For specimens that failed to amplify, a smaller fragment (~400 bp) of the gene was amplified using primers as described previously (Sulaiman et al., 2005).

Statistical analyses. Statistical comparisons were made using Pearson’s χ² and Fisher’s exact tests using SPSS v.17.0.

RESULTS

Cryptosporidiosis in children

During the study period, stool specimens from 2548 children with diarrhoea were screened for Cryptosporidium oocysts. Cryptosporidium oocysts were detected in 87 children (3.4%) with diarrhoea, of which 63 (72.4%) had Cryptosporidium infection alone, whilst in others Cryptosporidium infection was associated with one or more enteric pathogens. Seven children were co-infected with rotavirus, three with Shigella flexneri, two with Campylobacter jejuni and Giardia duodenalis, and three with G. duodenalis. The children with Cryptosporidium-positive infection were between 6 months and 16 years of age, with a median age of 5.7 years (2.5±1.8). Forty-nine of the children were girls and thirty-eight were boys. Age-specific distribution of cryptosporidiosis showed the highest prevalence in the 4–8-year-old age group compared with the other age groups (36/87, 41.4%; P<0.05) (Table 1).

The majority of the population in Kuwait lives in urban areas with a clean piped water supply and has easy access to health facilities free of cost. There was no significant association between Cryptosporidium-positive cases and contact with animals.
Table 1. Distribution of Cryptosporidium genotypes and subtypes in Kuwaiti children by age

<table>
<thead>
<tr>
<th>Age group</th>
<th>No. of cases</th>
<th>C. parvum</th>
<th>C. hominis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of patients infected with subtype allele*</td>
<td>(61, 72.6 %)</td>
<td>(22, 26.5 %)</td>
</tr>
<tr>
<td></td>
<td>IIa</td>
<td>IIc</td>
<td>IId</td>
</tr>
<tr>
<td>&lt;2 years</td>
<td>13</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>2–3 years</td>
<td>14</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>4–8 years</td>
<td>36</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>9–11 years</td>
<td>9</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>12–16 years</td>
<td>11</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>83</td>
<td>29</td>
<td>12</td>
</tr>
</tbody>
</table>

*Four children who had mixed infections of C. parvum and C. hominis were not subtyped.

Seasonal variation of Cryptosporidium infection in children

The maximum number of cryptosporidiosis cases (52.9 %) was detected between the months of November and March each year, indicating a significant seasonal variation of cases (Fig. 1). However, it was not associated with any particular C. parvum or C. hominis subtype.

Cryptosporidium spp., genotypes and subtypes

DNA preparations of 83 samples positive by microscopy showed amplification in the secondary PCR and yielded products of the expected size (834 bp) in the nested PCR analysis of the SSU rRNA gene. DNA sequencing was used to confirm Cryptosporidium species/genotypes. RFLP analyses of the secondary PCR product using SpI and VspI identified C. parvum in 61 of the children (73.5 %) followed by C. hominis in 22 children (26.5 %) (Table 1). Four children had both C. parvum and C. hominis. Two samples that were negative by SSU rRNA PCR were identified by COWP PCR.

Subtyping of the 61 C. parvum and 22 C. hominis isolates showed that they belonged to six subtypes, three C. parvum and three C. hominis subtype families. The majority of the C. parvum isolates belonged to subtypes IIa (29, 47.5 %) and IId (20, 32.8 %), with the rest in IIc (12, 19.7 %) (Table 1). Subtyping of the 22 C. hominis isolates showed that Id was the most common (12, 54.5 %), followed by Ia (8, 36.4 %) and Ie (2, 9.1 %) (Table 1).

Association of clinical characteristics with C. parvum and C. hominis subtypes

There were no significant differences in demographics (gender, educational or socio-economic status of the parents) between C. parvum-infected children and those infected with C. hominis (Table 2). The majority of children had watery diarrhoea (>83 %) and dehydration was observed in <20 % of the cases. Fever was present in 21 % of cases, and 16 % complained of nausea and vomiting. Associated clinical manifestations varied between the different Cryptosporidium spp. Generally C. hominis infections were associated with more diverse and severe clinical manifestations of fever ($\chi^2$ value, 5.989; likelihood ratio, 6.513; likelihood ratio, 8.989; P=0.016) and diarrhoea (Table 2). In contrast, the majority of cases with C. parvum infections were associated with diarrhoea only. Nausea or vomiting was observed in <15 % of the infected children. The C. hominis-infected children presented with a greater duration and severity of diarrhoea than children infected with C. parvum: 68 versus 20 %, respectively (duration: $\chi^2$ value, 17.562; likelihood ratio, 17.073; P<0.001) (Table 2).

Patterns of clinical manifestations also varied among C. hominis subtype families. Infections with subtype IId were associated with fever (41 %), dehydration (27 %) and more severe diarrhoea (55 %) (Table 2). Diarrhoea associated with subtype IId, the most commonly identified C. hominis subtype, was more severe than that associated with other subtypes. Ten of the fifteen patients (67 %) with diarrhoea of >6 days duration were infected with C. hominis subtype IId and nine of them (90 %) had severe diarrhoea with more than five watery stools a day (P<0.001) (Table 2). Interestingly, 9 of the 12 (75 %) C. hominis subtype IId cases had spent a considerable time at their desert camp sites. There was no increase in the duration and/or severity of diarrhoeal symptoms in children who were co-infected with rotavirus, S. flexneri, Campylobacter jejuni or G. duodenalis.

The association between cryptosporidial diarrhoea and the water source was studied only during 2007. The main source of drinking water in Kuwait is piped and/or bottled water. However, the majority of Kuwaiti families spend an...
Table 2. Association of socio-demographic and clinical features of infected Kuwait children, with Cryptosporidium genotypes and subtypes

<table>
<thead>
<tr>
<th>Patient data</th>
<th>C. parvum</th>
<th>C. hominis</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IIa</td>
<td>IIC</td>
<td>IIId</td>
</tr>
<tr>
<td>Total number</td>
<td>83</td>
<td>29</td>
<td>12</td>
</tr>
<tr>
<td>Socio-demographic features</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>37</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Female</td>
<td>46</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>Parents’ education status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>11</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Primary</td>
<td>29</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Secondary</td>
<td>43</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>Stay in winter camps</td>
<td>29</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Contact with animals</td>
<td>9</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Clinical features</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>18</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Nausea/vomiting</td>
<td>13</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Dehydration</td>
<td>16</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Duration of diarrhoea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2–5 days</td>
<td>56</td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>&gt;6 days</td>
<td>27</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Severity of diarrhoea†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–4</td>
<td>62</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>&gt;5</td>
<td>21</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

*P values were calculated to associate demographic and clinical features with Cryptosporidium spp. derived by Pearson χ² and Fisher’s exact tests using SPSS v.17.0.
†Maximum number of stools per day.

extended period of time in winter camp sites traditionally set up individually in the desert during the months of December–March each year. Traditionally, no animals or pets are kept in or around these camp sites except for a few camels for pleasure rides. These camp sites are supplied by overhead water storage tanks. The water from a local desalination plant is carried to these camp sites by water tankers. The water from five different overhead water tanks was collected and tested for coliform bacteria and Cryptosporidium oocysts. The water sample from one tank (tank G) was positive for Cryptosporium oocysts, and five members of the same family staying at this camp suffered from C. parvum subtype IIa infection.

DISCUSSION

This is, to the best of our knowledge, the first report from the Middle East to document the role of genetically characterized Cryptosporidium spp. and subtypes in clinical manifestations in children with diarrhoea in Kuwait. Cryptosporidium oocysts were detected in 3.4 % of children with diarrhoea compared with ~10 % in an earlier study (Iqbal et al., 2001). We could not explain the difference in prevalence of Cryptosporidium infection; however, clusters of cases from small outbreaks may have been included in the earlier study. Most epidemiological studies in tropical countries have shown the highest prevalence of infection in children of <2 years of age (Iqbal et al., 1999; Katsumata et al., 1998). In this study, the highest prevalence of cryptosporidiosis was seen in significantly older children, and similar findings have been reported previously from Kuwait, Saudi Arabia and the United Arab Emirates (Al Braiken et al., 2003; Iqbal et al., 2001). The delayed Cryptosporidium infection is attributed to better hygiene and socio-economic conditions in Kuwait and Saudi Arabia.

The majority of cryptosporidiosis cases (~53 %) in Kuwait occurred during the dry months of November–March. In tropical countries, the highest prevalence of Cryptosporidium infection is usually associated with the rainy season, and water-borne transmission is considered a major route in these areas (Ajjampur et al., 2010; Peng et al., 2003; Xiao et al., 2001). There is no regular rainy season in Kuwait and thus the possibility of water-borne transmission is minimal. However, the period of high prevalence of infection did correspond to the ‘winter desert camping’ season in Kuwait when a large number of families spend an extended period of time in desert camps supplied with hundreds of overhead water tanks. The water to these tanks is supplied from a local desalination plant. The water from one of the five water
tanks tested showed *C. parvum* oocysts of subtype IIa. Five members of the same family staying at this camp suffered from *C. parvum* subtype IIa infection. Earlier, a study showed water-tank-specific *C. hominis* subtype Ia infection in a rural community in India (Ajjampur et al., 2007). A significant number of water tanks must be checked for cryptosporidial oocysts to conclusively rule out any possible water-related transmission at these winter desert camps.

The distribution of *Cryptosporidium* genotypes in a population is an indication of the potential infection source (Learmonth et al., 2004; McLauchlin et al., 2000). The Kuwaiti children were infected almost exclusively with *C. parvum*, in contrast to the majority of studies conducted in other tropical countries that showed a dominance of *C. hominis* in children (Ajjampur et al., 2010; Gatei et al., 2003; Leav et al., 2002; Molloy et al., 2010). However, some studies in the Middle East have also reported a high prevalence of *C. parvum* infection (Hijjawi et al., 2010; Mahgoub et al., 2004; Sulaiman et al., 2005). This study also confirmed the presence of a unique endemcity of cryptosporidiosis in Kuwaiti children with a predominance of *C. parvum* (Sulaiman et al., 2005). In Iran, two studies reported a significant predominance of *C. parvum* (Meamar et al., 2007; Pirestani et al., 2008). Recently, in Jordon, of 44 isolates that were typed, 50% were *C. parvum* and ~45% were *C. hominis* (Hijjawi et al., 2010). Although the predominance of *C. parvum* infection in a population has been linked to zoonotic transmission (Sulaiman et al., 1998; Xiao et al., 2001), the majority of infected Kuwaiti children had no direct contact with animals. There is limited information on the prevalence of cryptosporidiosis among farm animals. In one study conducted in Basra, Iraq, cryptosporidiosis was diagnosed in 20% of cattle; however, no single positive case was detected among camels (Mahdi & Ali, 2002). Furthermore, recent subtyping studies have shown that not all *C. parvum* infections in humans are due to zoonotic transmission (Alves et al., 2003; Mallon et al., 2003).

Currently, the 60 kDa glycoprotein gene (*gp60*) is the most suitable and widely used genetic marker for detection of *Cryptosporidium* spp. infecting humans (Jex & Gasser, 2010). The subtyping of *Cryptosporidium* isolates from Kuwaiti children showed a very limited distribution of subtypes with predominance of only two *C. parvum* subtypes, IIa and IId (~80%), among the *C. parvum*-positive cases, and *C. hominis* subtypes Id (~55%) and Ia (36%) among the *C. hominis*-positive cases. This distribution of subtypes is very different from earlier studies in Europe, Portugal, Northern Ireland and South Africa, in which the subtypes were more or less evenly distributed among the common allele families (Alves et al., 2003; Glaberman et al., 2002; Leav et al., 2002). The subtype IIa has been detected in both humans and ruminants. In addition, ten children were infected with subtype IIC, which has been seen only in humans. The significance of this contradiction is not fully clear. More extended analysis of different environmental samples is needed to investigate the modes of transmission of infection in Kuwaiti children.

Our findings showed that *C. hominis*-infected children presented with more severe and diverse clinical manifestations of fever and diarrhoea than children infected with *C. parvum* spp. (*P*<0.001). Similar findings have been reported from India and Peru (Ajjampur et al., 2007; Cama et al., 2008). However, in the present study only *C. hominis* subtype Id was associated with more severe diarrhoea: >90% of cases infected with *C. hominis* subtype Id presented with severe diarrhoea lasting for >6 days. This finding is supported by an earlier study that showed that subtype Id was more virulent than other *C. hominis* subtype families in Peruvian human immunodeficiency virus-positive people (Cama et al., 2007). No such association of clinical severity was observed with *C. parvum*-infected children in this study.

In conclusion, our study confirms a very different *Cryptosporidium* genotype and subtype distribution compared with other tropical countries, with a predominance of *C. parvum* subtypes IIa and IId among Kuwaiti children with diarrhoea. However, the importance of this relative to animal sources cannot be assessed because of the lack of data on the incidence in animals and potential sources of exposure. In addition, subtype Id of *C. hominis* was associated with more diverse and severe clinical manifestations in infected children, suggesting that parasite genetics may play an important role in the clinical manifestations of human cryptosporidiosis.

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