Molecular analysis of Cryptosporidium species isolated from HIV-infected patients in Thailand

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Summary

Cryptosporidium isolates from diarrhoeal stools of human immunodeficiency virus (HIV)-infected patients in Thailand were genetically analysed by sequencing the variable region in the 18S rRNA gene. Twenty-nine isolates from four children and 25 adults attending King Chulalongkorn Memorial Hospital in Bangkok during 1996 and 2000 were analysed. All patients suffered from chronic watery diarrhoea and had low CD4+ lymphocytes (mean ± SD = 105.5 ± 133.2 cells/µl). Four Cryptosporidium species were identified, i.e. C. parvum (genotype 1), C. meleagridis, C. muris and C. felis occurring in 24, 3, 1 and 1 isolates, respectively. Oocysts of C. muris were significantly larger than oocysts of other species; C. felis was the smallest in these populations (P < 0.01). Sequences of the ITS1, 5.8S rRNA and ITS2 regions of C. muris and C. meleagridis identified in this study displayed unique sequences from those of other known species. Based on a limited number of isolates analysed, only C. meleagridis and C. muris were found in HIV-infected children, whereas the genotype 1 of C. parvum predominated in HIV-infected adults.

Keywords

Cryptosporidium, Thai, HIV, sequence, rRNA

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Introduction

One of the major etiologic agents causing prolonged diarrhoeal disease among patients infected with human immunodeficiency virus (HIV) is a coccidian parasite of the genus Cryptosporidium. Although cryptosporidiosis occurs in both immunocompetent hosts and immunocompromised patients, infections are more prevalent and clinically more severe in the latter (Tzipori & Griffiths 1998).

Precise species identification of Cryptosporidium has been a matter of debate as different isolates within the same species may possess overlapping features of host range, oocyst morphology and predilection site of infection (O’Donoghue 1995). Recent genetic analysis of the 18S rRNA sequences has revealed the multispecies nature of the genus Cryptosporidium. At least 10 species of Cryptosporidium (C. parvum, C. muris, C. uraia, C. felis, C. meleagridis, C. baileyi, C. serpentis, C. andersoni, C. saurophilum and C. nasorum) have been considered to be valid based on morphological, biological and molecular evidence (Fayer et al. 2000).

Despite the fact that C. parvum has been predominantly identified in human infections, other species of Cryptosporidium have been detected in diarrhoeal stools of patients (Pieniazek et al. 1999; McLauchlin et al. 2000; Morgan et al. 2000; Pedraza-Diaz et al. 2000). In this regard, different species/genotypes of Cryptosporidium can exhibit unique modes of transmission to humans (Peng et al. 1997). Importantly, outbreaks of cryptosporidiosis from contaminated food and water are not uncommon, resulting in significant morbidity and mortality among infected individuals (Fayer et al. 2000). Thus, the issue of species identification of Cryptosporidium is of public health importance for proper control measures as no effective anticryptosporidial drug has been available to date (Griffiths 1998; Xiao et al. 2000).

A proportion of HIV-infected patients suffer from protracted diarrhoea because of Cryptosporidium infections, resulting in a significant decrease in quality of life or death. Cryptosporidiosis has consistently been an important opportunistic infection among HIV-infected patients in...
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Thailand (Wanke et al. 1999). Although genotypic analysis of Cryptosporidium has been performed using clinical isolates from HIV-infected individuals derived from north America (Sulaiman et al. 1998; Widmer et al. 1998; Peñazek et al. 1999; Morgan et al. 2000), Europe (Bonnin et al. 1996; Caccio et al. 2000; Morgan et al. 2000; Pedraza-Diaz et al. 2001a,b), Central America (Sulaiman et al. 1998) and Africa (Morgan et al. 2000), no data are available on this issue among isolates from south-east Asia where HIV infection is highly endemic. Therefore, we characterized Cryptosporidium isolates from HIV-infected Thai patients by determination of oocyst dimension and sequencing of a variable region in the 18S rRNA gene. Results revealed that all four species of Cryptosporidium recognized to infect humans occurred in HIV-infected Thai patients.

Materials and methods

Sources of Cryptosporidium samples

Twenty-nine isolates of Cryptosporidium from HIV-infected patients presenting with chronic diarrhoea who sought medical treatment at King Chulalongkorn Memorial Hospital in Bangkok during 1996 and 2000 were included, and background demographic, clinical and laboratory data were retrieved for analysis. A minimum sample of 1 g of stool was collected from each Cryptosporidium-infected individual. In addition to stool examinations for parasites by direct smear method, formaldehyde–ether sedimentation technique and modified kinyoun acid fast stain, freshly submitted stool samples from these patients were cultured for routine enteric pathogens as previously reported (Wanke et al. 1999). Each patient submitted two to three stool samples collected on consecutive days. This study was conducted with the approval of the Ethical Review Committee of Research, Faculty of Medicine, Chulalongkorn University. Informed consent was obtained from each participant at the time of sample collection.

Oocyst isolation and DNA extraction

Oocyst isolation was determined by measuring 30 oocysts from each isolate under 1000× magnification as described by Jongwutiwes et al. (2002). Stool samples were preserved in 75% ethanol and kept at ambient temperature until study. This storage condition has been previously evaluated in our laboratory to retain the morphology of Cryptosporidium oocysts and be suitable for molecular analysis (Jongwutiwes et al. 2002). Purification of oocysts was performed by the sugar flotation procedure (Kim et al. 1992), and washed thrice with sterile water. Cryptosporidium DNA was isolated from the sediment using a Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) with a slight modification by adding 300 μl of Cell Lysis Buffer supplied in the kit and was subsequently subjected to disruption by vortexing with glass beads, diameter 425–600 μm (Sigma, St. Louis, MO, USA), for 15 min. After ethanol precipitation, DNA was dissolved in 20 μl of TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) buffer and kept at –30 °C until use.

Polymerase chain reaction (PCR)

Amplification of the C. parvum 18S rRNA gene by PCR was performed in 20 μl reaction mixture containing Cryptosporidium DNA, 200 μM each dNTP, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 2 mM MgCl2, 1.25 unit Taq DNA polymerase (Promega) and 0.2 μM of each primer. The PCR primers were CR-P1: 5'-CAGGGAGGTAATGACAAAGAA-3' (nucleotide positions 437–456 after the HCNV4 sequence, GenBank accession number AF093489) and CR-P2: 5'-TCAGCTTGGACCCATACCT-3' (nucleotides 1070–1089). The thermal cycler profile contained 35 cycles of 94, 50 and 72 °C for 40, 40 and 60 s, respectively. One microlitre of primary PCR was used as template for nested PCR which was done with essentially the same amplification conditions except using primers CR-P3: 5'-ATTGGAGGGCAAGTCTGGTG-3' (nucleotides 525–544, GenBank accession number AF093489) and CPB-DIAGR (Johnson et al. 1995). The PCR-amplified 18S rRNA gene of Cryptosporidium was analysed by electrophoresis on 2% agarose gels, stained with ethidium bromide and visualized under UV transilluminator.

The internal transcribed spacer 1 (ITS1), 5.8S rRNA and ITS2 Type A regions of Cryptosporidium were amplified using the outer primers, CR18F: 5'-AGTCTTATGCCAATACAGGTCTGTGAT-3' (nucleotides 2857–2883 of the KSU-1 sequence, GenBank accession number AF040725) (Le Blancq et al. 1997), and CR28R: 5'-TCAGTTACTAAGGAAATCTGATTGATT-3' (nucleotides 4471–4497) for primary PCR. The inner primers for nested PCR were CR18F-I: 5'-CTAAGCAGGTGCAGCAG-3' (nucleotides 2913–2939) and CR28R-I: 5'-ATGCTTAAAGGTCTGGTGAT-3' (nucleotides 4422–4448). The PCR profile contained 35 cycles of 94, 55 and 72 °C for 1, 1, and 2 min, respectively. After purification with GFX™ PCR, DNA and Gel Band Purification Kit (Pharmacia, Piscataway, NJ, USA), DNA was used as template for sequencing. Positive and negative DNA templates for controls were as previously reported (Jongwutiwes et al. 2002).
DNA sequencing

Direct DNA sequencing of each purified PCR product was performed using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase. Each sequencing reaction contained 8.0 μl of terminator ready reaction mix, 300–500 ng of template DNA, and 3.2 pmol of primer in a total reaction volume of 20 μl. Cycle sequencing was performed according to the manufacturer’s protocol using an ABI PRISM™ 310 DNA sequencer. The DNA sequence was determined from both directions using primers CR-P3 and CPB-DIAGR for the 18S rRNA gene fragments. Sequences of the ITS1, 5.8S rRNA and ITS2 were analysed using primers CR18-FI, CR28-RI, 5.8SF (5’-GTCTTGGTTCTCATAACGAT-3’) (positions 3769–3788 of the KSU1 sequence) and 5.8SR (5’-AGTT-CACATTGCTTATCGCA-3’) (positions 3805–3824). The sequence of each isolate was verified by direct sequencing of a newly amplified PCR product from the same DNA sample.

Data analysis

Differences in oocyst dimensions were determined by Student’s t-test. Significance was defined at the 1% level. Sequence alignment and comparison were performed using the GENETYX MAC™ version 8.0 with manual adjustment. A phylogenetic tree was constructed by the neighbour-joining method based on Kimura’s distance with bootstrap confidence.

Nucleotide sequence accession numbers

The nucleotide sequences of Cryptosporidium isolates reported in this paper are available in the EMBL, GenBank™ and DDBJ data bases under the accession numbers AF356786–AF356814 for the 18S rRNA gene, and AF381167–AF381170 for the ITS1, 5.8S rRNA and ITS2 sequences.

Results

Cryptosporidium isolates

Twenty-five Cryptosporidium isolates were collected from adults and four isolates from children. Of these, 22 were male. The mean ± SD age was 31.1 ± 13.9 years (range = 2–55). Thirteen isolates were from Bangkok residents and the rest were from other parts of Thailand as listed in Table 1. All but two patients had CD4+ lymphocytes < 200 cells/μl (mean ± SD = 105.6 ± 133.2) (Table 1). Isolates CR8, CR19, CR21 and CR29 were from children who acquired HIV infection through vertical transmission. The other isolates were from adult patients who infected with HIV through either sexual contact or injection drug use. Twenty-one patients suffered from other co-infections, mostly oesophageal candidiasis and pulmonary tuberculosis.

Analysis of variable region in the 18S rRNA gene

Comparison of the polymorphic region in the 18S rRNA gene spanning 470–491 bp has shown that isolates in this study belonged to four species of Cryptosporidium based on perfect sequence identity with those previously reported of known species. The genotype 1 of C. parvum was identified to be the most prevalent species occurring in 24 isolates and found only in adult patients. Three other isolates, i.e. CR8, CR21 and CR29, were identical with C. meleagridis while isolates CR1 and CR19 belonged to C. felis and C. muris, respectively. Remarkably, HIV-infected children harboured only C. meleagridis and C. muris while none of C. parvum was detected in this age group. Comparison of species distribution of Cryptosporidium among HIV-infected individuals from different geographical origins is shown in Table 2. Although C. parvum, either genotype 1 or 2, is predominantly encountered worldwide, C. felis and C. meleagridis comprise almost 10% of all species identified.

Sequences of the ITS1, 5.8S rRNA and ITS2 Type A regions of C. muris and C. meleagridis

Sequence analysis has shown that isolates CR8, CR21 and CR29, belonging to C. meleagridis, gave identical sequences in the ITS1, 5.8S rRNA and ITS2 regions while isolate CR8, identified as C. muris, displayed a different sequence. Comparison with previously published sequences of C. parvum genotype 1 (H1 strain) (GenBank accession number AF093012), genotype 2 (C13 strain) (GenBank accession number AF093008) and C. felis (Cat1 strain) (GenBank accession number AF093013) has shown that the ITS1 and ITS2 regions exhibited extensive sequence variation but the 5.8S rRNA gene showed high sequence homology (Figure 1). Cryptosporidium felis and C. muris were genetically related based on analysis of the ITS1, 5.8S rRNA and ITS2 regions using the neighbour-joining method while C. meleagridis was closer to C. parvum. A concordant result was obtained by analysis of the 18S rRNA gene (data not shown).
Oocyst dimensions

Isolate CR19 (C. muris) possessed the largest oocysts, whereas isolate CR1 (C. felis) had the smallest mean values of length and width. Other isolates exhibited similar oocyst dimensions (Table 3). Importantly, the mean length and width of C. felis and C. muris were significantly different from each other (P < 0.01). However, the oocysts of isolates genetically identified to be C. meleagridis and C. parvum were of similar dimensions.

Discussion

Our analysis has revealed that Cryptosporidium isolates from HIV-infected patients in Thailand comprise heterogeneous species. Based on morphological data, only C. muris and C. felis displayed markedly different oocyst dimensions while C. parvum and C. meleagridis were indistinguishable. Therefore, upon microscopic examination, the oocyst dimension > 5.5 × 7.5 μm has the possibility to be C. muris. Although C. andersoni contains an overlapping size of oocysts, it has not yet been identified in human infection (Fayer et al. 2000). However, oocyst morphology per se does not provide adequate data for a precise identification of the species.

Sequence analysis of the variable region in the 18S rRNA gene has shown that most of the Thai isolates belonged to C. parvum (82.8%) which is consistent with previous reports from different geographical origins (Bonnin et al. 1996; Sulaiman et al. 1998; Widmer et al. 1998; Pieniazek et al. 1999; Caccio et al. 2000; Morgan et al. 2000; Pedraza-Diaz et al. 2001a,b). Despite a limited number of isolates analysed in this study, the species of Cryptosporidium other than C. parvum were not uncommonly encountered in HIV-infected patients in Thailand. This is in agreement with reports by others that Cryptosporidium populations infecting immunocompromised individuals exhibit more genetically diverse species than those found in immunocompetent hosts (McLauchlin et al. 1999; McLauchlin et al. 2000; Xiao et al. 2000). On the other
hand, the distribution of Cryptosporidium species among HIV-infected hosts derived from diverse geographical origins differs from place to place. For example, the genotype 1 of C. parvum outnumbers the genotype 2 in USA (Sulaiman et al. 1998; Widmer et al. 1998; Pieniazek et al. 2000; Caccio et al. 2000; Morgan et al. 2000) and Kenya (Morgan et al. 2000) whereas the two genotypes are almost equally detected in HIV-infected isolates from France (Bonnin et al. 1996). In the UK and Italy, only isolates possessing the genotype 2 are identified (Caccio et al. 2000) whereas none except one patient was co-infected with another enteric pathogen (Table 1). This study has identified C. meleagridis only in isolates from HIV-infected children although the number of isolates analysed in this age group is small. Furthermore, one child was infected with C. muris while none of C. parvum was identified in this age group. Recently, morphologically identified C. muris has been detected in stools of two Indonesian girls without any gastrointestinal symptom (Katsumata et al. 2000). The present report provides both morphological and molecular evidences for C. muris infection in human and suggests its role in diarrhoeal disease among HIV-infected patients. On the other hand, all C. parvum populations identified in adult patients belonged to human genotype. The reason underlying such peculiar distribution remains obscure. However, the modes of acquisition of Cryptosporidium in adults could be different from those in children. Exposure to as yet unknown animal reservoirs among children could be more common than in adult patients because only one zoonotic species of Cryptosporidium was identified in the latter. No apparent association between pets owned by these patients and the occurrence of zoonotic species of Cryptosporidium in these patients could be confirmed as only one case reported to have pets in his home. Nevertheless, stray dogs and cats are common in Thailand although no surveillance has been conducted concerning Cryptosporidium-infected rate in these animals. On the other hand, several lines of evidence have suggested the roles of interferon-γ and CD4+ lymphocytes in mediating the initial resistance and resolution of a C. parvum infection (Theodos 1998). Therefore, impaired immunity in the advanced stage of HIV-infection could lead to an establishment of infection with various species/genotypes of Cryptosporidium.

A previous study has shown that the ITS1, 5.8S rRNA and ITS2 regions exhibit considerable genetic divergence

<table>
<thead>
<tr>
<th>Country</th>
<th>C. parvum (genotype 1)</th>
<th>C. parvum (genotype 2)</th>
<th>C. parvum (‘dog’ genotype)</th>
<th>C. meleagridis</th>
<th>C. felis</th>
<th>C. muris</th>
<th>Total</th>
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<td>USA</td>
<td>34</td>
<td>6</td>
<td>1</td>
<td>3</td>
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<td></td>
<td></td>
<td></td>
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<td>9</td>
<td>Caccio et al. 2000; Pedraza-Diaz et al. 2001a; Pedraza-Diaz et al. 2001b</td>
</tr>
<tr>
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<td>6</td>
<td>7</td>
<td></td>
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<td></td>
<td></td>
<td>13</td>
<td>Bonnin et al. 1996</td>
</tr>
<tr>
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<td>2</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td></td>
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<td>13</td>
<td>Morgan et al. 2000</td>
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<td></td>
<td>8</td>
<td>Caccio et al. 2000</td>
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<tr>
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<td>2</td>
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<td></td>
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<tr>
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<td></td>
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<td></td>
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<td>4</td>
<td>Sulaiman et al. 1998</td>
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<td></td>
<td></td>
<td>3</td>
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<td>1</td>
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<tr>
<td>Total</td>
<td>75</td>
<td>36</td>
<td>1</td>
<td>7</td>
<td>8</td>
<td>1</td>
<td>128</td>
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</table>
among species of *Cryptosporidium* (Morgan *et al*. 1999). The unique sequences of the regions of *C. muris* and *C. meleagridis* in this analysis lend further support to the validity of these species and provide a fine resolution of genetic differences among species and strain of *Cryptosporidium*. Although extensive sequence divergence based on the ITS1 and ITS2 regions may not infer the phylogeny accurately, the resulting phylogenetic tree gave similar information on genetic relatedness among species of *Cryptosporidium* to those based on the 18S rRNA sequences (Xiao *et al*. 1999) (data not shown). Despite the occurrence of intraspecies variation within both genotypes 1 and 2 of *C. parvum* using microsatellite DNA analysis (Caccio *et al*. 2000), no evidence of genetic recombination between genotypes has been reported based on other genetic markers (Peng *et al*. 1997; Spano *et al*. 1998). The virtually identical sequence of the 18S rRNA gene in three clinical isolates of *C. meleagridis* in Thailand and that of an isolate from a turkey in Hungary (Sreter *et al*. 2000) has suggested a limited genetic variation within the species across disparate geographical areas and in different hosts.

*Cryptosporidium*-associated chronic diarrhoeal illness commonly occurs in late stage of HIV infection or advanced HIV disease when CD4+ counts are <200 cells/μl (Navin *et al*. 1999). The high prevalence of co-infections with other opportunistic pathogens among HIV-infected Thai patients was similar to those reported by Morgan *et al*., being 75.8 and 68.8%, respectively (Morgan *et al*. 2000) although the mean number of CD4+ lymphocytes is lower in the latter. Efforts to decrease morbidity because of these infections depend upon the effectiveness of specific antimicrobial agents, administration of highly active antiretroviral treatment and, more importantly, disease prevention. Therefore, further molecular analysis of *Cryptosporidium* isolates from both HIV-infected patients and immunocompetent hosts from diverse geographical origins together with surveillance of animal and environmental reservoirs will be required.

**Acknowledgements**

This study was supported by research funds from Postgraduate School of Chulalongkorn University and Ministry of University Affairs, Bangkok, Thailand, to R. Tiangtip for analysis of the 18S rRNA gene; and from the National Research Council of Thailand (grant 0745/001) to S. Jongwutiwes for analysis of the ITS1, 5.8S rRNA and ITS2 regions. We are grateful to all patients who participated in this study; to Udomsak Tangchaisuriya, Vit Suvannadhat, Urassaya Pattanawong and Tweesak Tia for encouragement. The excellent technical assistance from Sutin Yentakarm and Malee Charoenkorn is gratefully acknowledged.

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polymorphism analysis of a repetitive DNA sequence. *FEMS Microbiology Letters* 137, 207–211.


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