IDENTIFICATION OF *CRYPTOSPORIDIUM PARVUM* GENOTYPE FROM HIV AND NON-HIV FECAL SAMPLES BY PCR

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Abstract. In this study, specific primers of genotypes 1 and 2 were used to identify *Cryptosporidium parvum* genotypes in fecal samples. A total of 30 fecal HIV and non-HIV samples of *C. parvum* were examined by microscopic method, comprised of 7 samples from non-HIV children aged 8-12 years, 11 fecal samples from HIV-positive adults and 12 purified oocysts of *C. parvum* from HIV patients. Within this group of infected children, 5 were infected with genotype 1, while 2 samples were unclassified. In the HIV-positive adult patients, 7 samples were genotype 1, while 4 samples were unclassified. Of the 12 purified oocyst samples, 11 samples were positive for genotype 1, while only 1 purified oocyst sample was unclassified. The unclassified samples observed in our study may belong to other genotypes and no *C. parvum* genotype 2 was detected in our study population.

INTRODUCTION

Cryptosporidium is a coccidian parasite that is ubiquitous within its geographic distribution and the range of its vertebrate hosts. Recent molecular studies indicate that *C. parvum* is not a single uniform species but is composed of at least eight distinct genotypes that are morphologically identical (human, monkey, cattle, mouse, dog, pig, marsupial, and ferret) (Morgan *et al*, 2000; Xiao *et al*, 2000a). Molecular epidemiological studies show that the human genotype has been found exclusively in humans, while the cattle genotype has been found in humans and domestic livestock, such as cattle, sheep, and goats (McLauchlin *et al*, 2000; Morgan *et al*, 2000; Guyot *et al*, 2001).

Recently, molecular studies have provided significant evidence that the parasites causing zoonotic diseases and those causing the anthroponotic infections are genetically distinct and occur in two different transmission cycles (Awad-El-Kariem *et al*, 1995). Therefore, it can be postulated that in cases where human isolates carrying 'animal'-type markers are identified, infections were presumed to be the result of zoonotic spread, either directly or through contaminated food and water (Awad-El-Kariem, 1999).

In humans and many other mammals, *Cryptosporidium parvum* is recognized as a significant pathogen, primarily as a cause of acute, severe diarrheal illness. Infections with this parasite can lead to a chronic, life-threatening condition in immunocompromized individuals and to acute gastroenteritis and diarrhea in healthy people. Currently, there are no reliable and consistently effective anticryptosporidial therapeutic agents, and treatment relies mainly on maintaining the water and electrolyte balance and on nutrition (Sharpstone and Gazzard, 1996).

In HIV-seropositive cases and young children, identifying the isolate as a 'human' or 'animal' genotype is very important, not least for better understanding of the possible sources of infection, the relevance of anthroponotic versus zoonotic spread, and for the design of possible intervention strategies. The need, by epidemiologists, for a genotype-specific tool that can provide clues to the origin and possible modes of spread of *C. parvum* strains has been recognized recently (Arrowood, 1997).

Here, we report the genotyping of *Cryptosporidium parvum* isolates with human genotype specific primer and animal genotype specific primer by polymerase chain reaction (PCR) from HIV and non-HIV fecal samples. The primers used in this study have been shown to be *C. parvum*-specific (Sulaiman *et al*, 1999) and have been successfully used to directly differentiate between animal and human isolates based on the size of the PCR product (Morgan *et al*, 1996). However, it shares with other molecular typing protocols the disadvantage of being technically demanding.

MATERIALS AND METHODS

Samples

A total of 30 fecal HIV and non-HIV samples of *C. parvum* were examined by microscopic method,

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comprised of 7 samples from non-HIV children aged 8-12 years, 11 fecal samples from HIV-positive adults and 12 purified oocysts of *C. parvum* from HIV patients (Table 1). Purification of oocysts from fecal samples was performed by the method reported by Abe *et al* (2002a,b). DNA extraction was performed directly on the stool samples and purified oocyst samples by two menthods of extraction, the buffer lysis method described by Carnevale *et al* (2000) followed by phenol chloroform and ethanol precipitation, and also by the QIA Stool DNA Extraction Kit (Qiagen, Hilden, Germany).

Genotyping of *C. parvum*

Genotyping of *Cryptosporidium* was performed using the genotype-specific primers that specifically amplify the undefined genomic DNA sequences of *C. parvum*. The set of primers used were the human-specific reverse primer, CP-HR (5´ CCT CTT TCC AAT TAA AGT TGA TG 3´) which amplified a 411-bp product from human isolates of *C. parvum* only, the animal-specific reverse primer CP-CR (5´ TCC AAA TTA TTG TAA CCT GGA AG 3´), which amplified a 312-bp product from animal isolates only and the forward primer, 021F (5´ GGT ACT GGA TAG ATA GTG GA 3´) which annealed to both human and animal isolates (Morgan *et al*, 1996).

PCR amplification was performed with modification from the original protocol. The PCR reactions were performed in a volume of 50 µl containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 2 pmol of CP-HR primer, 3 pmol of CP-CR primer and 6 pmol of 021F primer, 0.2 units of Taq DNA polymerase (Amersham), and 20 µl of the DNA sample. We used the PCR buffer supplied with the Taq DNA polymerase. Reactions were performed on a GeneAmp PCR System 9700 thermocycler (PerkinElmer, Foster City, CA).

Samples were denatured at 94°C for 2 minutes, and then subjected to 45 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. The DNA of *C. parvum* belongs to human and cattle isolate originating from a patient, was used as a positive control. Amplification products were subjected to electro-phoretic separation using 1.5% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator.

RESULTS

The results of the PCR studies are shown in Tables 1 and 2. PCR amplification by specific primers of

Table 1
Genotyping of 30 *Cryptosporidium parvum* isolates by using human genotype specific primer and animal genotype specific primer in polymerase chain reaction.

Sample number	Host	Source	Genotyping	Sample number	Host	Source	Genotyping
1	Non-HIV Child	Stool	G 1	16	HIV (+) Adult	Oocyst[a]	G 1
2	Non-HIV Child	Stool	G 1	17	HIV (+) Adult	Oocyst[a]	UN
3	Non-HIV Child	Stool	G 1	18	HIV (+) Adult	Oocyst[a]	G 1
4	Non-HIV Child	Stool	G 1	19	HIV (+) Adult	Oocyst[a]	G 1
5	Non-HIV Child	Stool	UN	20	HIV (+) Adult	Oocyst[a]	G 1
6	Non-HIV Child	Stool	G 1	21	HIV (+) Adult	Oocyst[a]	G 1
7	Non-HIV Child	Stool	UN	22	HIV (+) Adult	Oocyst[a]	G 1
8	HIV (+) Adult	Stool	G 1	23	HIV (+) Adult	Oocyst[a]	G 1
9	HIV (+) Adult	Stool	UN	24	HIV (+) Adult	Oocyst[a]	G 1
10	HIV (+) Adult	Stool	G 1	25	HIV (+) Adult	Oocyst[a]	G 1
11	HIV (+) Adult	Stool	G 1	26	HIV (+) Adult	Oocyst[a]	G 1
12	HIV (+) Adult	Stool	G 1	27	HIV (+) Adult	Stool	G 1
13	HIV (+) Adult	Stool	UN	28	HIV (+) Adult	Stool	UN
14	HIV (+) Adult	Stool	UN	29	HIV (+) Adult	Stool	G 1
15	HIV (+) Adult	Stool	G 1	30	HIV (+) Adult	Oocyst[a]	G 1

Notes: G 1 = Human genotype; UN= Unclassified; Oocyst[a] = purified oocysts

Table 2
Summary of genotyping of *C. parvum* by PCR.

Patient groups	Genotype 1	Genotype 2	Unclassified	Total
Non-HIV children	5	0	2	7
HIV adults				
1. Stool samples	7	0	4	11
2. Purified oocysts	11	0	1	12
Total	23	0	7	30

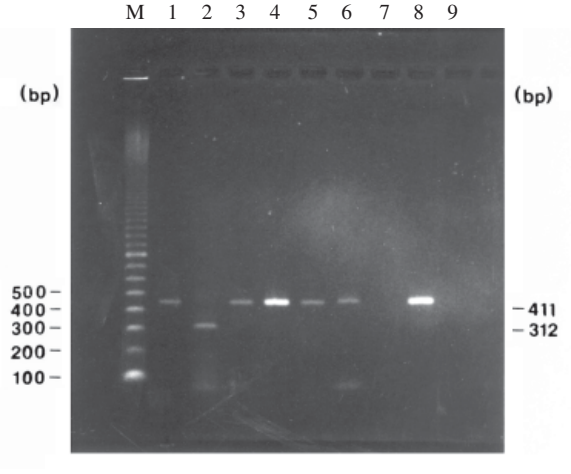

Fig 1- DNA extracted from fecal samples from non-HIV children, aged 8-12 years amplified using the 021F, CP-CR, and CP-HR diagnostic primers. Lane M = molecular marker (100 bp ladders), lane 1= positive control (human isolate), lane 2 = positive control (animal isolate), lanes 3 to 6 and lane 8 = amplified products (411 bp) which indicate the human isolates, lanes 7 and 9 = unclassifed samples.

genotype 1 and 2 showed that within the group of infected children, 5 were found to be infected with genotype 1, while 2 samples were unclassified, as shown in Fig 1. In the HIV positive adult patients, 7 samples were genotype 1, while 4 were unclassified (Fig 2). Of the 12 purified oocysts samples, 11 samples were positive for genotype 1, while only 1 purified oocyst sample was unclassified, as shown in Fig 3. The unclassified samples observed in our study may possibly belong to the other genotypes or be due to the low specificity of microscopic method. No *C. parvum* genotype 2 was detected in our study population.

DISCUSSION

Results of recent molecular characterization studies have shown that there are extensive genetic differences among different *Cryptosporidium* species, as well as within *C. parvum*. The inter- and intraspecies differences observed between the *Crytosporidium* species have given rise to the opportunity to develop molecular diagnostic tools to identify *Cryptosporidium* spp and also for genotyping purposes. To date, at least two species-specific and seven genotyping-specific protocols have been described (Sulaiman *et al*, 1999).

Using genotype-specific primers by polymerase chain reaction which directly differentiate between animal and human isolates based on the size of the PCR product (Morgan *et al*, 1996), we detected a total of 23 samples belonging to the human genotype (genotype 1); 7 samples were unclassified, no sample was found of the animal genotype (genotype 2). These finding shows that the cause of cryptosporidiosis in most of the patients in our study group was *Cryptospordium parvum* belonging to the human genotype, which is also similar to studies conducted elsewhere (Pieniazek *et al*, 1999; McLauchlin *et al*, 2000; Tiangtip and Jongwutiwes, 2002), which agree that the human genotype is the predominant genotype, causing most cases of cryptosporidiosis in humans worldwide.

Since the primer used in our study could only differentiate between animal and human genotypes, the unclassified samples observed may belong to the other genotypes, as many studies have shown that other genotypes of *Cryptosporidium* can also infect humans, especially HIV-infected persons (Pieniazek *et al*, 1999; McLauchlin *et al*, 2000) and children (Xiao *et al*, 2001). The other genotypes of *Cryptosporidium* that have been reported in humans are the *C. parvum* dog genotypes, genotypes identical to *C. felis, C.*

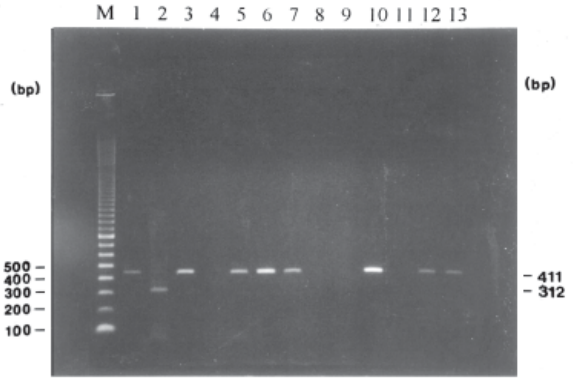

Fig 2- DNA extracted from fecal samples from HIV-positive adults amplified using the 021F, CP-CR, and CP-HR diagnostic primers. Lane M = molecular marker (100 bp ladders), lane 1= positive control (human isolate), lane 2 = positive control (animal isolate), lanes 3, 5, 6, 7, 10, 12 and 13 = amplified products (411 bp) which indicate the human isolates, lanes 4, 8, 9 and 11= unclassifed samples.

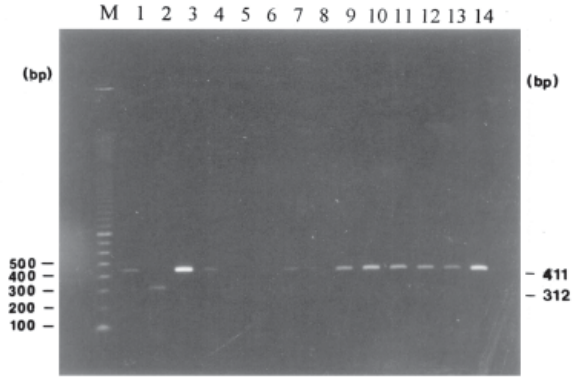

Fig 3- DNA purified oocysts of *C. parvum* from HIV patients amplified using the 021F, CP-CR, and CP-HR diagnostic primers. Lane M = molecular marker (100 bp ladders), lane 1= positive control (human isolate), lane 2 = positive control (animal isolate), lanes 3, 4, 6 to 14 = amplified products (411 bp) which indicate the human isolates, lane 5 = unclassifed sample.

meleagridis (Pieniazek *et al*, 1999; McLauchlin *et al*, 2000; Xiao *et al*, 2001) and the *C. muris* (Tiangtip and Jongwutiwes, 2002). The increasing reports of the discovery of these new *Cryprosporidium* genotypes in human cryptosporidiosis should cause the existing diagnostic tools to be reevaluated. However, the unclassified samples observed in this study may also be due to the low specificity of the microscopic method of *C. parvum* detection, and PCR-RFLP (Xiao *et al*, 2001) may be needed to confirm the result.

Genotyping results also showed that no *C. parvum* genotype 2 was detected in our study population, which is similar to reports elsewhere in Thailand (Tiangtip and Jongwutiwes, 2002). However, since the sample sizes used in each of the studies above were small, we were unable to conclude that *C. parvum* genotype 2 does not infect the Thai population, as many other studies have shown that *C. parvum* genotype 2 does exist and infects humans in many other parts of the world (McLauchlin *et al*, 2000; Morgan *et al*, 2000; Xiao *et al*, 2000; Guyot *et al*, 2001). Therefore, further study is needed to investigate the prevalence of genotype 2 in the Thai population, as well as the other genotypes.

In summary, our study revealed that the majority of the patients in our study population was infected with *C. parvum* genotype 1, while no infections with *C. parvum* genotype 2 were observed. Therefore, the results of our study may indicate that *C. parvum* human genotype (genotype 1) is the most predominant genotype in the Thai infected population, which epidemiologically, suggests that the most likely frequent mode of transmission of *Cryptosporidium parvum* infection in the general population is from human to human or anthroponotic modes of transmission. However, large scale genotyping of *Cryptosporidum* species in the Thai infected population should be performed to support this hypothesis.

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