

Genotypic Characterization of *Cryptosporidium* in HIV-Infected Patients in Thailand

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Abstract—*Cryptosporidium* is an intestinal protozoan parasite that causes cryptosporidiosis. It also has been recognized as an important opportunistic pathogen affecting HIV-infected patients. The disease can cause chronic diarrhea, decreased quality of life, and shortened survival in HIV-infected patients. The prevalence of cryptosporidiosis in HIV-infected patients is 5–50%. However, in Thailand information about the prevalence of each genotype and species of *Cryptosporidium* in HIV-infected patients is less available. In this study, genotypic characterization of *Cryptosporidium* isolated from HIV-infected patients in Thailand was investigated using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of 18 SSU rRNA gene. The fecal samples were collected during the period from 1999 to 2004. Among 110 *Cryptosporidium* isolates, five genotypes were identified which are *C. hominis* (36.4%), *C. parvum* (10%), *C. meleagridis* (19.1%), *C. felis* (16.4%) and *C. canis* (16.4%). These data indicate that extensive genotypic diversity among *Cryptosporidium* was observed among *Cryptosporidium* isolates and *C. hominis* is the predominant genotype in HIV-infected patients in Thailand.

Keywords—*Cryptosporidium*; Genotype; HIV

I. INTRODUCTION

Cryptosporidium, a protozoan parasite, is a causative agent of diarrheal disease called cryptosporidiosis reported from both developing and developed countries. Having a world distribution, cryptosporidiosis appears in both immunocompetent and immunocompromised individuals [1]. The infection is self-limiting in immunocompetent hosts, but can be severe, persistent or chronic life-threatening diarrhoea in immunocompromised patients, particularly in those suffering from the acquired immunodeficiency syndrome, AIDS [2–3]. The fecal-oral transmission can occur through ingestion of contaminated food or water and contacting infected humans or animals [4–5]. A high prevalence of cryptosporidiosis has been found in HIV-infected patients [5–6] though it varies depending on location, age of the study population, stage of disease and laboratory methods used [7].

There is extensive genetic variation within the genus *Cryptosporidium*. Using genotyping tools, five species of *Cryptosporidium* including *C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis* and *C. canis* have been shown to be responsible for most human infections both in immunosuppressed and immunocompetent patients [8–10]. Of these five species, *C. hominis* (previously known as *C. parvum* human genotype or genotype I) and *C. parvum* (previously known as *C. parvum* bovine genotype or genotype II) are the most commonly detected parasites in human cryptosporidiosis [11]. *C. hominis* is primarily associated with humans, whereas *C. parvum* is associated with a wide variety of host species including humans, cows and sheep. Moreover, these two species show geographic differences in their distribution [12]. In Thailand, information about the prevalence of species and genotype of *Cryptosporidium* in HIV-infected patients is less available. In the present study, we studied the prevalence of genotypes of *C. parvum* and other species in HIV-infected patients using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of 18 SSU rRNA genes. Results of the study showed that various *Cryptosporidium* species were found in HIV-infected patients.

II. MATERIALS AND METHODS

Isolates. One hundred and ten human fecal samples from HIV-positive patient previously shown to be positive for *Cryptosporidium* by light microscopy were used to determine the *Cryptosporidium* genotypes and species. The samples were collected from patients from 1999 to 2004 and were stored at -80°C prior to analysis.

DNA extraction. Extraction of genomic DNA was performed by using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Genomic DNA of each sample was kept at -20°C until used.

PCR-RFLP analysis of 18 SSU rRNA gene. Genotypic characterization of *Cryptosporidium* from humans was determined by PCR-RFLP analysis of 18 SSU rRNA gene.

Extracted DNA of *Cryptosporidium* from stool specimens was amplified for 18 SSU rRNA gene using the nested PCR technique. Primary and secondary PCR were carried out using primers and conditions described by Xiao et al. [13]. The primary PCR, performed with primers 18SFor1 (5'-TTCTAGAGCTAATACATGCG-3') and 18SRev1 (5'-CCCATTTCCTTCGAAACAGGA-3') amplified a 1,300-bp fragment. The primary PCR mixtures contained 1 µl of template DNA, 1 x PCR buffer (Promega, USA), 3 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate (dNTP), 1 µM each primer, 2.5 U of *Taq* DNA polymerase, and 2 of nonacetylated bovine serum albumin (BSA; 10 mg/ml; Promega, USA) in a 50 µl reaction volume. Primary PCR cycling conditions consisted of an initial denaturation of 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 59°C for 45 s, and 72°C for 60 s, with a final extension of 72°C for 7 min. The secondary PCR, performed with primers 18SFor2 (5'-GGAAGGGTTGTATTTATTA GATAAAG-3') and 18SRev2 (5'-AAGGAGTAAGGAACA ACCTCCA-3'), amplified an 830-bp fragment within the primary PCR product. The reaction conditions were similar to those described above for the primary PCR, with the exception that 1 µl of the primary PCR product was used as the template and the MgCl₂ concentration was 1.5 mM. Cycling conditions for the secondary PCR consisted of 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 58°C for 90 s, and 72°C for 2 min. The secondary PCR product was visualized by gel electrophoresis using 2% agarose gel and documented on high density printing paper using Uvisave gel documentation system I (Uvitech, Cambridge, UK). Digestions of the PCR products were performed using two restriction enzymes, *VspI* and *SspI* (Promega, USA.) at 37°C overnight, and separated by 2% agarose gel electrophoresis.

III. RESULTS

The 18 SSU rDNA amplified from fecal samples showed amplicon of the expected size (Fig. 1). These PCR products were used for the genotypic determination by RFLP (Fig. 2). Genotypic characterization of *Cryptosporidium* isolated from HIV patients is shown in Table 1. The majority of isolates (40/110) were identified as *C. hominis* (36.4%), follow by *C. meleagridis* (19.1%), *C. felis* (16.4%) and *C. canis* (16.4%), and *C. parvum* (10.0%), respectively.

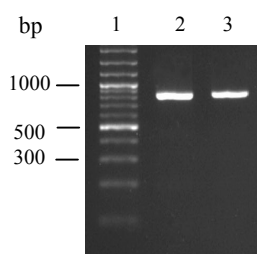


Fig. 1 Secondary PCR amplification products of 18 SSU rDNA of *Cryptosporidium* isolates. Lane 1 was molecular weight marker. Lanes 2-3 were 18 SSU rDNA amplicons.

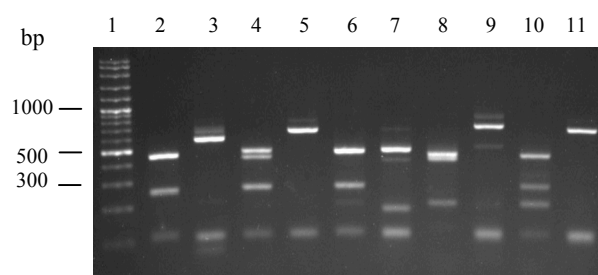


Fig. 2 Differentiation of *Cryptosporidium* by RFLP analysis of PCR product of the 18 SSU rDNA. Lane 1 was molecular weight marker. Lanes 2-3 was *C. hominis*. Lanes 4-5 was *C. parvum*. Lanes 6-7 was *C. meleagridis*. Lanes 8-9 was *C. felis*. Lanes 10-11 was *C. canis*. PCR product in lanes 2, 4, 6, 8 and 10 was digested with *SspI* and PCR product in lanes 3, 5, 7, 9 and 11 was digested with *VspI*.

TABEL 1. Distribution of 110 *Cryptosporidium* isolates from HIV patients using PCR-RFLP analysis of 18 SSU rRNA gene.

| Species or genotype | Number of samples (%) |
|-----------------------|-----------------------|
| <i>C. hominis</i> | 40 (36.4%) |
| <i>C. parvum</i> | 11 (10.0%) |
| <i>C. meleagridis</i> | 21 (19.1%) |
| <i>C. felis</i> | 18 (16.4%) |
| <i>C. canis</i> | 18 (16.4%) |

IV. DISCUSSION

Cryptosporidium is a common protozoan parasite found in feces of human and other animals worldwide. The prevalence of *Cryptosporidium* infection varies in different regions of the world and in different study populations. The prevalence of *cryptosporidium* infection in HIV-infected patients varied from 2.2-52.7% [5-6, 14-20]. In Thailand, there were few reports with prevalence ranging from 8.8-94.4% among HIV-infected individuals [21-24].

Ribosomal RNA gene analyses have frequently been used to determine phylogenetic and taxonomic relationships among various parasites [25-26]. Specifically, RFLP evaluation of the SSU rDNA, a relatively simple, rapid method, has increasingly been used for genetic comparison of various isolates of protozoan parasite. This method is a useful tool to genetically characterize the organism. Moreover, previous study indicates that PCR-RFLP analysis for SSU rDNA was more sensitive than for COWP (*Cryptosporidium* oocyst wall protein) [10] because a *Cryptosporidium* contains five copies of the SSU-rRNA gene [27-28]. The ability to distinguish between species and genotype is an important epidemiological tool that can use to understand the transmission, possible pathogenicity and zoonotic potential of the organism.

Recent studies indicate that at least 23 valid *Cryptosporidium* species have been found in humans and variety of animals [29-30]. The two main species of *Cryptosporidium* that infect immunocompetent and immunocompromised individual are *C. hominis* and *C. parvum*. *C. hominis* is found almost exclusively in humans, whereas *C. parvum* is zoonotic species. The occurrence of both species in humans has provided evidence that both anthroponotic and zoonotic cycles can occur in human infections [31-32]. Several studies indicated that immunocompromised individuals are susceptible to a wide range of *Cryptosporidium* species and genotype [32-34]. At least seven species including *C. parvum*, *C. hominis*, *C. meleagridis*, *C. felis*, *C. muris*, *C. canis* and *C. suis* have been found in HIV-infected patients [9-10, 33-34]. In Thailand, the result of this study and previous studies showed that six species of *Cryptosporidium* isolates have been found in HIV-infected patients (i.e. *C. parvum*, *C. hominis*, *C. meleagridis*, *C. muris*, *C. felis*, and *C. canis*) [22, 35-36]. In this study, only five different species have been identified and *C. hominis* (36.4%) is the most frequently isolated species, which is in agreement with the previous studies in Thailand [22, 35]. However, one study in difference area of Thailand reported occurrence of *C. parvum* [36]. Other studies have also found that *C. hominis* is more responsible for human infection than *C. parvum* in many countries such as Iran (76%), Peru (79%) South Africa (82%), USA (67%) Spain (64%) and Japan (68%) [10, 18, 33, 37-39]. In contrast, *C. parvum* is predominant in other countries such as France (51%), U.K. (62%), Switzerland (54%) Malaysia (72%), Portugal (53%) and Kuwait (94%) [9, 34, 40-43]. This conflict could be explained by a different geographic distribution. *C. hominis* is the main cause of cryptosporidiosis in our geographical area, which suggests that human-to-human transmission is the main mode of spreading.

Other species that also infect human but less commonly include *C. meleagridis*, *C. felis*, *C. canis* and *C. suis*. In the present study, *C. meleagridis*, *C. felis*, and *C. canis* were identified in 21 (19.1%), 18 (16.4%), and 18 (16.4%) of HIV-infected individuals, respectively. Previous study in different study population of Thailand reported *C. meleagridis* in 10%, *C. felis* in 3% and *C. muris* in 3% [35]. The study in France found that *C. meleagridis*, *C. felis* and *C. muris* were detected in 6%, 14% and 3%, respectively, of HIV-infected individuals [34]. It has been reported that *C. meleagridis* and *C. felis* were less frequent (1%) in HIV-infected patients in Spain [10]. The study in Peru found *C. meleagridis* in 4%, *C. canis* in 9% and *C. felis* in 9% of HIV infected persons [15].

In conclusion, this study documented the distribution of *Cryptosporidium* species in HIV-infected patient in Thailand. The result showed that five *Cryptosporidium* species were identified and *C. hominis* was responsible for the majority of *Cryptosporidium* infection in the study population. In addition, a few zoonotic species were identified. Future studies are required to determine the diversity of subgenotype of *Cryptosporidium* isolates from HIV-infected patients in

Thailand and to understand the full public health significance of *Cryptosporidium* species and subgenotypes.

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