

SHORT REPORT

DETECTION OF *CRYPTOSPORIDIUM PARVUM* IN HIV-INFECTED PATIENTS IN MALAYSIA USING A MOLECULAR APPROACH

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Abstract. This cross-sectional study determined the prevalence of cryptosporidiosis in HIV-infected patients using polymerase chain reaction (PCR). Stool specimens were collected from HIV infected patients who were admitted to Hospital Raja Perempuan Zainab II, Kota Bharu, Malaysia, for various indications from December 2004 to December 2005. A modified acid-fast stain was performed on the direct stool smears, then the stool specimens were further tested using nested PCR targeting the 18S rRNA gene of *Cryptosporidium parvum*, with a built-in internal control (IC). Out of 59 samples, 11 were positives. Nested PCR identified a total of nine samples (16%) compared to microscopy, which identified only three samples. All PCR negative results showed IC amplicons, suggesting that these samples were true negatives and were not due to inhibition of PCR. This study highlights the importance of molecular diagnosis in determining the true prevalence and epidemiology of *C. parvum*.

INTRODUCTION

Cryptosporidium is a ubiquitous parasite that infects a wide range of vertebrates, including humans (Leav *et al*, 2003; Xiao *et al*, 2004). Cryptosporidiosis in immunocompetent people is usually a self-limited infection, but in immunocompromised individuals, such as HIV/AIDS patients, it often causes chronic or cholera-like diarrhea and can be fatal if not properly managed (Amadi *et al*, 2002). The prevalence of cryptosporidiosis in HIV-positive

patients with diarrhea differs quite markedly from one study to another, ranging from 0 to 100% with a median of 32% (Hunter and Nichols, 2002). These differences may be explained by differences in study design, geographical location, population group, sensitivity of laboratory methods or stage of the disease.

In Malaysia, *Cryptosporidium* is rarely detected in clinical specimens, probably due to the use of conventional methods which have low sensitivity (Ludin *et al*, 1991; Lim *et al*, 2005). Furthermore, there is no specific drug therapy for the disease apart from highly active antiretroviral therapy (HAART) and adequate hydration and water replacement therapy. Although the Federal Drug Authority (FDA), USA, has approved nitazoxanide (NTZ)

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for the treatment of cryptosporidiosis, the drug is still not available in Malaysia (Fox and Saravolatz, 2005).

For the past two decades, many studies have been carried out to evaluate new molecular diagnostic tools in order to improve the detection rate of *Cryptosporidium* in clinical samples. Among the molecular techniques, polymerase chain reaction (PCR) has received much attention. Hence, the present study was carried out to determine the true prevalence of *Cryptosporidium* among HIV-infected patients in Kelantan, Malaysia, using a PCR assay.

MATERIALS AND METHODS

Specimen collection

Sample size was calculated based on a single proportion method, and a total of 59 stool specimens were analyzed. All samples were obtained from HIV-infected patients who were admitted to Hospital Raja Perempuan Zainab II, a general hospital in Kelantan, Malaysia. Stool specimens were collected from adult patients using a universal container. Direct smear was performed (two slides) and the slides were fixed with methanol for microscopic examination. Another portion of each stool specimen was preserved in 2.5% potassium dichromate and kept at 4°C to be used for the PCR assay.

Microscopy examination

Microscopic diagnosis of *Cryptosporidium* was performed by modified Ziehl-Neelsen stain and observed with light microscopy. Specimens were tested blindly, meaning that a different individual was assigned to do the microscopic examination and PCR assay.

PCR amplification

i) **DNA extraction and nested PCR.** DNA was extracted by boiling the samples with polyvinylpyrrolidone (PVPP) as previously described (Morgan *et al*, 1998) with some

modifications. The primers used in this study were specific for the 18S rRNA of *C. parvum* (Sturbaum *et al*, 2001; Bialek *et al*, 2002).

ii) **Internal control.** An internal control (IC) was designed to co-amplify a 416 bp cloned fragment of latent membrane protein 1 (LMP1) of Epstein Barr virus (EBV) (Yap *et al*, 2007). The same amounts of IC template and primers were added to all PCR reactions. All PCR was conducted in a Mastercycler 5330 (Eppendorf, Hamburg, Germany). The PCR amplicons were analyzed by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide (10 mg/ml), visualized and captured with an image analyzer (Chemilmager, Alpha Innotech, California, USA). The PCR was considered positive when the 416 bp IC band and the 285 bp *Cryptosporidium* specific band were seen. When only the 416 bp IC band was observed, the PCR was considered negative. When both the 416 bp and 285 bp bands were absent, the PCR was considered inhibited and was subsequently repeated after 1/10 or 1/50 dilution of the sample DNA.

iii) **Detection and sequencing of semi-nested PCR amplicons.** The 285 bp nested PCR amplicons were purified using a gel extraction kit (Wizard SV Gel, Promega, USA) and sent for sequencing at Tech Dragon (Hong Kong). The DNA sequencing results were assembled and analyzed using the ContigExpress alignment program in VectorNTI version 9.0 software. All of the sequences were then subjected to a BLAST (blastn) search to determine their homologies using the GenBank database (National Center for Biotechnology Information). Sequences were aligned with currently available 18S rRNA gene sequences of *Cryptosporidium* using the CLUSTALW program.

RESULTS

A total of 59 stool samples were analyzed by microscopy and nested PCR. Out of the

59 samples, eleven were positive; eight were identified using nested PCR alone; two, using microscopy only; and one using both methods (see Fig 1 for representative results of nested PCR). There were two samples that were positive by microscopy yet were repeatedly negative by nested PCR. The primers used in the nested PCR were targeted towards the hypervariable region of the 18S rRNA gene; hence, these negative results were probably the result of mutation or variation in the primer binding region of the 18S rRNA gene of the oocyst. A positive PCR test showed both genomic (285 bp) and IC (416 bp) amplicons. All negative samples, except sample number 14, showed the IC amplicon but not the genomic amplicon, suggesting that these samples were true negatives and were not inhibited. The amplification result in lane number 14 was not valid, since the IC band was absent. For sample number 12, the IC band was not as clear as the other bands, suggesting the presence of inhibitors. Both samples were repeated after dilution and were confirmed negative (data not shown). The amplicon products were confirmed as *C. parvum* by DNA sequencing, BLAST and CLUSTALW analysis.

DISCUSSION

Cryptosporidiosis is still considered an emerging infectious disease and is becoming more prevalent in immunocompromised populations (Hunter *et al*, 2003; Helmy *et al*, 2006). This is related to the use of more sensitive techniques for detection of the organism, especially in developed countries (Sunnotel *et al*, 2006; Amar *et al*, 2007; Hung *et al*, 2007). Determining the true prevalence of cryptosporidiosis in any region is important for epidemiological data; hence, a more sensitive and reliable technique is required to improve the detection rate. PCR offers one of the most sensitive methods for detecting *Cryptosporidium*, but its sensitivity in fecal material can be greatly reduced by the presence of poorly defined inhibitors of PCR.

Out of 59 samples analyzed, nine (16%) were positive by nested PCR technique. The prevalence of *Cryptosporidium* in HIV patients was higher compared to a recent study conducted in a similar population at Kajang Hospital, Kuala Lumpur, Malaysia, which had a prevalence of 3% (Lim *et al*, 2005). In Malaysia, few studies have been conducted to determine the prevalence of cryptosporidiosis in children and in immunocompromised patients,



Fig 1—Nested PCR amplification of stool samples for detection of *Cryptosporidium parvum*. Positive results were indicated by the presence of a 285-bp product and 416-bp internal control (IC) product. Lane M, 100 bp DNA ladder; lane 1, negative control (containing all PCR reagents but no target DNA added); lane 2, unloaded well; lane 3, positive control (genomic DNA from ATCC sample); lanes 4 through 25, clinical samples. Lanes 11, 20 and 24 all show positive results and lanes 12 and 14 PCR inhibition.

such as HIV-infected patients and cancer patients. Ludin *et al* (1991) studied stool samples from 836 patients with diarrhea and acute gastroenteritis in the pediatric ward, Penang General Hospital, Malaysia and found only 36 samples (4.3%) were positive for *Cryptosporidium*, and the prevalence was higher in children with diarrhea and vomiting than in children with acute gastroenteritis alone. Another study conducted by Menon *et al* (2001) showed a prevalence of only 0.9%.

The low prevalence rate in these previous studies could be explained by differences in the studied populations and the methods of detection used. Two of the studies highlighted above (Ludin *et al*, 1991; Lim *et al*, 2005) used conventional microscopy or immunofluorescence staining for diagnosis and this could be one of the reasons for the low detection rate. Furthermore, Ludin *et al* (1991) studied the prevalence of *Cryptosporidium* in different age groups (children) and non-HIV patients. Studies have shown that the prevalence of *Cryptosporidium* in children can range from 3.5% to 17% (Akyon *et al*, 1999; Banwat *et al*, 2003; Samie *et al*, 2006) with the highest prevalence rate obtained from using PCR as the diagnostic tool.

The prevalence of cryptosporidiosis in HIV patients also varies among studies, depending on where the study was conducted, the age of the populations studied, the stage of the disease and the laboratory methods used. The prevalence of cryptosporidiosis in HIV patients in the nearest country, Thailand, varied from 8.5% to 12.8% (Chokephaibulkit, 2001; Saksirisampant *et al*, 2002; Pinlaor *et al*, 2005). Prevalence of cryptosporidiosis in other regions such as African countries, ranges from 3.9% to 73.6% (Endeshaw *et al*, 2004; Tumwine *et al*, 2005; Sarfati *et al*, 2006).

In this study, PCR detected another eight positive samples that were considered negative by microscopy. This finding clearly identified the requirement for a more sensitive and

standardized method for detection of *Cryptosporidium*, as this will give us a true picture of its prevalence compared to conventional methods. The IC proved to be valuable in identifying the presence of PCR inhibitors in fecal samples and should also prove useful for identifying false-negatives in a clinical parasitology laboratory setting.

Although our study provided important information, there are important limitations that merit discussion. First, this was a laboratory-based study, and we did not correlate the findings with the patients' clinical data such as clinical presentation, stage of the disease, treatment and outcome. Second, direct microscopic examination was done without prior concentration since the fecal materials received were not adequate for this technique.

In summary, from the results of our study, we recommend the use of PCR in epidemiological and prevalence studies in order to improve the detection rate of cryptosporidiosis. It is also necessary to include an internal control in each PCR reaction in order to exclude false negative results.

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