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SHORT COMMUNICATION

Prevalence and genotyping of *Cryptosporidium* isolated from HIV/AIDS patients in urban areas of Thailand

An important parasitic infection among HIV/AIDS patients is the cryptosporidiosis. *Cryptosporidium* is an intestinal protozoan that causes severe diarrhea and may lead to death in immunocompromised hosts. Cryptosporidiosis may occur sporadically or as outbreaks following zoonotic transmission from farm animals, person-to-person spread or the contamination of water supplied (Karanis *et al.*, 2007). New species and genotypes of the *Cryptosporidium* genus are being identified in recent years and there is evidence that more than one *Cryptosporidium* species are involved in human infections and disease (Hunter & Thompson, 2005). Currently, 16 *Cryptosporidium* species have been considered to be valid, and *C. hominis* and *C. parvum* appear to be most widely distributed (Plutzer & Karanis, 2009).

Cryptosporidiosis had been recognized as opportunistic infection in patients with AIDS. Studies on the prevalence of cryptosporidiosis in HIV/AIDS patients have mostly been restricted to those with diarrhea, or have been based on surveillance data. The occurrence of cryptosporidiosis increased worldwide due to the HIV/AIDS epidemic with the average prevalence rate in developing countries of 24% (range: 8.7–48%). Cryptosporidiosis is a significant infectious disease among the HIV/AIDS patients in Thailand, and the prevalence rate has been previously reported between 2.5% and 25% (Thamlikitkul *et al.*, 1987; Jongwutiwes *et al.*, 1990; Moolasat *et al.*, 1995; Uga *et al.*, 1998; Saksirisampant *et al.*, 2002; Jirapiyo *et al.*, 2002; Gatei *et al.*, 2002; Tiangtip & Jongwutiwes, 2002; Wiwanitkit & Srisuphanunt, 2006; Srisuphanunt *et al.*, 2008). Herein we describe the prevalence and *Cryptosporidium* species among of HIV/AIDS-infected patients with diarrhea from different hospitals in Bangkok, Thailand.

SUBJECTS AND METHODS

Collection of Fecal Material, Cryptosporidium Oocysts Purification and Microscopic Examination

Fecal samples were collected from 152 HIV/AIDS patients attending the outpatient department or admitting in Siriraj Hospital, Rajavithi Hospital and Chulalongkorn Hospital, in Bangkok. Informed consent was obtained from all the patients and the study protocol was approved by the Ethics Committee of Siriraj Institutional Review Board, Mahidol University, Chulalongkorn University and Rajavithi Hospital, Ministry of Public Health, Thailand. After the fecal material collection, *Cryptosporidium* oocysts from each sample were concentrated by Sheather's sucrose flotation technique and discontinuous sucrose gradient concentration. The pellets were stained by DMSO-modified acid fast stain (AFS) and followed by immunofluorescence antibody (IFA) method with a specific monoclonal antibody against an epitope of *Cryptosporidium* oocyst wall, and fluorescein isothiocyanate labelling as described by the manufacturer Monofluo® Kit *Cryptosporidium* (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). Microscopic examination was performed for all samples. The fecal samples were preserved in 2.5% potassium dichromate and kept at 4°C.

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An aliquot of 200 μl of the sample suspension in 2.5% potassium dichromate was taken, and processed for genotypic analysis. The positive samples of *Cryptosporidium* determined by microscopy (AFS and/or IFA) and detection were further subjected to count the oocysts by haemacytometer (Boeco, Hamburg, Germany). Duplicate haemacytometer counts were used for each sample.

Cryptosporidium Genomic DNA Extraction and Genetic Analysis

DNA was extracted from fecal samples using the Qiamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer descriptions, with the addition of three 10-min freeze–thaw cycles after resuspension in lysis solution in order to rupture the *Cryptosporidium* oocysts. Liquid nitrogen was used for freezing, and thawing was carried out at 70°C in Dry Thermo unit (DTU-2B; Taitec, Saitama-ken, Japan). DNA was eluted in 100 μl buffer (Qiagen) and stored at −20°C until use. All samples were subjected to the nested PCR analysis of the 18S SSUrRNA gene and sub-genotyping by direct DNA sequencing and/or by RFLP analysis.

Amplification of *Cryptosporidium* DNA by Nested PCR

The 18S SSUrRNA nested PCR was performed according to Nichols *et al.* (2003) and as it has been previously applied by Plutzer and Karanis (2007). Briefly, firstly the PCR product of 655–667 bp and secondly PCR product of 434 bp were amplified in standard mixtures of 25 μl containing 400 nmol (1 μl) of each SSU rRNA specific primer, 200 μM dNTP (2.5 μl), 1.5 mM MgCl2 (3 μl) and 2.5 U (0.25 μl) HotstarTaq DNA polymerase (Qiagen) and 5 μl of DNA template. The templates were subjected to 35 amplification cycles as it has been previously described in Plutzer & Karanis (2007). For *Cryptosporidium* species sequencing and genotyping, the PCR products were further processed for the identification of *Cryptosporidium* species by direct DNA sequencing and/or by RFLP analysis using two endonucleases (*Ssp*I and *Vsp*I; Promega, Madison, WI, USA). Each reaction mixture contained 10 μl of master mix and 10 μl of secondary PCR product. Restriction digestion was carried out at 37°C for 2 hours and the digestion fragments were analysed using 2% agarose gels stained with ethidium bromide. Thirty-three samples were found positive by nested PCR and all of these samples were successfully genotyped. 18S SSUrRNA PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and applied as templates for sequencing using the forward and reverse primers of the nested (secondary) PCR. Sequencing was conducted either commercially sequenced by the Bioservice Unit in Bangkok, Thailand, or using the Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. An ABI Prism 3100 Genetic Analyzer automated sequencer (Applied Biosystems) was used to analyse the sequencing reactions. Obtained sequences were compared with published sequences of *Cryptosporidium* species and genotypes on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences of the 25 samples used in this study have been deposited in the GenBank database under the accession number from GU944826 to GU944851 and the other seven contained *C. hominis* (with sequences matching GenBank accession no. AY 458612.1) and one contained *C. meleagridis* (with sequences matching GenBank accession no. AF112574.1).

Statistical Analysis

A statistical analysis based on a two-sided Fisher's exact test was performed with SPSS 17.0 for Windows software to investigate the possible association between the results obtained by microscopy (AFS and/or IFA) and PCR. The chi-square test was used to examine for the correlation among these three methods. The result were considered to be significant at alpha=0.05.

RESULTS

The Diagnosis of *Cryptosporidium* Infections in HIV/AIDS Patients Samples during the Present Study

Microscopy

Cryptosporidium oocysts in HIV/AIDS patient samples observed microscopically after the DMSO-modified AFS technique. The stained oocysts appeared bright red round to ovoid bodies against a pale green background, containing elongated naked sporozoites and followed by IFA method with brilliant green fluorescence, correct shape and size of the fluorescein isothiocyanate-labelled objects and clearly visible oocyst wall. Also empty oocysts have been observed. When these techniques were used to investigate all 152 samples from the investigated HIV/AIDS patients for *Cryptosporidium* spp. infections, the total number of positive samples by one of each account methods between microscopy (AFS and/or IFA) and/or PCR was 50 (32.9%). *Cryptosporidium* spp. was detected by AFS in 28 out of 152 HIV/AIDS patients and in 41 out of 152 HIV/AIDS patients examined by IFA. The prevalences were 18.4% and 27% by AFS and IFA, respectively.

PCR and Species Identification by PCR-RFLP

By nested PCR, *Cryptosporidium* species infections were diagnosed in 33 of 152 HIV/AIDS patients analysed with a prevalence rate of 21.7%. There was overlap between microscopy (AFS- and IFA-positive) and PCR-positive detection of *Cryptosporidium* in 20 samples (these samples contained between 3 and 200 oocysts from 10 μl out of the 1 ml concentrated sample). Out of the 11/20 samples which harboured more than 100 oocysts, all were found to be PCR-positive. Subsequently RFLP and direct DNA sequencing results identified *C. hominis* in eight samples, *C. parvum* in one sample and *C. meleagridis* in the others. The relationship between microscopy (AFS and/or IFA) and PCR from 152 HIV/AIDS patients' samples is shown in Table. There was a significant association between the results obtained by microscopy of either AFS or IFA and PCR procedures (chi-square test, $P<0.05$). Using RFLP analysis and direct DNA sequencing technique, 16 positive samples were *C. hominis*, 12 samples with *C. parvum* infection, 3 samples *C. meleagridis*, 1 sample with *C. felis* and 1 sample with an infection of *C. canis*.

DISCUSSION

The prevalence of cryptosporidiosis in hospitalized HIV/AIDS patients during the present study was 18.4% by AFS. AFS staining could be very useful for the screening of cryptosporidial infections to improve diagnosis and clinical epidemiology in HIV clinical cases as also has been reported from some countries, e.g. Zambia (Chintu *et al.*, 1995), south Italy (Brandonisio *et al.*, 1999), Korea (Guk *et al.*, 2005) and south India (Ramakrishan *et al.*, 2007). A prevalence of 27% by IFA was observed in our study and this represents the first report of the *Cryptosporidium* detection by IFA in clinical samples in Thailand. PCR and sequencing were applied to identify and genotype the species. IFA is the most sensitive technique. We could successfully identify *Cryptosporidium* species from HIV/AIDS-positive patients in 16 samples with *C. hominis*, 12 samples with *C. parvum* infection, 3 samples with *C. meleagridis*, 1 sample with *C. felis* and 1 sample with an infection of *C. canis*. In our study, *C. hominis* has been slightly more

TABLE. *Association between the detection of* Cryptosporidium *by microscopy and PCR in samples of HIV/AIDS patients*

Microscopy AFS and/or IFA	PCR		
	Positive	Negative	Total
Positive	29	12	41
Negative	4	107	111
Total	33	119	152

commonly identified than *C. parvum* among HIV/AIDS patients in central Thailand, with concordance to the prevalence of 30.0% with *C. hominis* infection in a report from Saksirisampant *et al.* (2009) and Llorente *et al.* (2007). Not only *C. hominis* infects humans, but it has been also found in animals such as dugongs, lambs and cattle (Smith *et al.*, 2005). A higher number of positive samples were identified by microscopy: 41 positive samples AFS and/or IFA in comparison to 33 positive samples by PCR showing a discrepancy of 10.5%. This difference may be explained by taking into consideration the assumption of empty oocysts in the samples as it has been observed during the microscopically examinations. Another factor that may affect the results obtained by PCR is the existence of inhibitors in the fecal material.

The present results indicated *C. hominis* and *C. parvum* as the prominent species in the investigated HIV/AIDS patients in Thailand. Most HIV/AIDS-positive patients attending hospitals in Bangkok are from urban or slum areas in the city and neighbouring provinces. However, epidemiological data on exposures (e.g. urban/peri- and urban/provincial dwelling) were not available during these investigations. Transmission ways of human infections of *C. parvum* directly via food and water contamination and livestock are not excluded. The HIV/AIDS-positive patients might obtain *Cryptosporidium* oocysts from the direct contact with domestic animals or via nosocomial infections. This is presumably due to direct transmission of *C. parvum* from animals to animals and/or from animals to humans. Moreover, *Cryptosporidium* infections in other mammals, pets, aves and insects might be transmitted to humans. Bangkok is a big city and has a lot of stray and/or domestic animals. In agreement with the reports of other authors, molecular epidemiology studies indicated that the proportion of *C. parvum* infections in humans is much higher in rural (43%) than in urban areas (19%) (Learmoth *et al.*, 2004). Furthermore, livestock or domestic animals have been incriminated in human infections with *C. parvum* by both direct and indirect transmission (Becher *et al.*, 2004). *Cryptosporidium* oocysts can be dispersed on pasture directly by animals or by spreading the manure on pasture (Sischo *et al.*, 2000), propagating infection over vast areas or in surface water. Our results support the hypothesis that humans may receive *Cryptosporidium* oocysts by contamination of food and drinking water (Srisuphanunt *et al.*, 2008, 2009, 2010) or domestic animals and these data represent a substantial report of HIV/AIDS-confirmed cases of cryptosporidiosis in Thailand. It is not excluded that the course of cryptosporidiosis in the investigated patients depends on different *Cryptosporidium* subtypes and this should be a subject of future studies to confirm individual differences or correlations of symptoms in the affected persons.

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REFERENCES

Becher, K. A., Robertson, I. D., Fraser, D. M., Palmer, D. G., & Thompson, R. C. A. (2004). Molecular epidemiology of *Giardia* and *Cryptosporidium* infections in dairy calves originating from three sources in Western Australia. *Veterinary Parasitology*, **123**, 1–9.

Brandonisio, O., Maggi, P., Panaro, M. A., Lisi, S., Andriola, S., & Acquafredda, A. (1999). Intestinal protozoa in HIV-infected patients in Apulia, South Italy. *Epidemiology and Infection*, **123**, 457–462.

Chintu, C., Luo, C., Baboo, S., Khumalo N. B., Mathewson, J., DuPont, H. L. & Zumla, A. (1995). Intestinal parasites in HIV-eropositive Zambian children with diarrhea. *Journal of Tropical Paediatrics*, **41**, 149–152.

Gatei, W., Suputtamongkol, Y., Waywa, D., Ashford, R. W., Bailey, J. W., Greensill, J., Beeching, N. J. & Hart, C. A. (2002). Zoonotic species of *Cryptosporidium* are as prevalent as the anthroponotic in HIV-infected patients in Thailand. *Annals of Tropical Medicine and Parasitology*, **96**, 797–802.

Guk, S. M., Seo, M., Park, Y. K., Oh, M. D., Choe, K. W. & Kim, J. J. (2005). Parasitic infections in HIV-infected patients who visited Seoul National University Hospital during the period 1995–2003. *Korean Journal for Parasitology*, **43**, 1–5.

Hunter, P. R. & Thompson, R. C. A. (2005). The zoonotic transmission of *Giardia* and *Cryptosporidium*. *International Journal of Parasitology*, **35**, 1181–1190.

Jirapinyo, P., Ruangsiri, K., Tesjaroen, S., Limsathavourat, S., Sripiangjan, S. & Junnoo, V. (2002). High prevalence of *Cryptosporidium* in young children with prolonged diarrhea. *The Southeast Asian Journal of Tropical Medicine and Public Health*, **24**, 730–733.

Jongwutiwes, S., Kraivichian, P., Kulkumthorn, M., Sitthichareonchai, P. & Jaroenkorn, M. (1990). Cryptosporidiosis among orphanage children in Thailand: a one year prospective study. *The Southeast Asian Journal of Tropical Medicine and Public Health*, **21**, 458–464.

Karanis, P., Kourenti, C. & Smith, H. (2007). Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. *Journal of Water and Health*, **5**, 1–38.

Learmonth, J. J., Ionas, G., Ebbett, K. A. & Kwan, E. S. (2004). Genetic characterization and transmission cycles of *Cryptosporidium* species isolated from humans in New Zealand. *Applied and Environmental Microbiology*, **70**, 3973–3978.

Llorente, M. T., Clavel, A., Goñi, M. P., Varea, M., Seral, C., Becerril, R., Suarez, L. & Gomez-Lus, R. (2007). Genetic characterization of *Cryptosporidium* species from humans in Spain. *Parasitology International*, **56**, 201–205.

Moolasart, P., Eampokalap, B., Ratanasrithong, M., Kanthasing, P., Tansupaswaskul, S. & Tanchanpong, C. (1995). Cryptosporidiosis in HIV infected patients in Thailand. *The Southeast Asian Journal of Tropical Medicine and Public Health*, **26**, 335–338.

Nichols, R. A., Campbell, B. M. & Smith, H. V. (2003). Identification of *Cryptosporidium* spp. oocysts in United Kingdom non-carbonated natural mineral waters and drinking waters by using a modified nested PCR restriction fragment length polymorphism assay. *Applied and Environmental Microbiology*, **69**, 4183–4189.

Plutzer, J. & Karanis, P. (2007). Genotype and subtype analyses of *Cryptosporidium* isolates from cattle in Hungary. *Veterinary Parasitology*, **146**, 357–362.

Platter, J. & Karanis, P. (2009). Genetic polymorphism in *Cryptosporidium* species: an update. *Veterinary Parasitology*, **165**, 187–199.

Ramakrishna, K., Shenbagararathai R., Uma, A, Kavitha, K, Rajendran, R. & Thirumalaikolundusubramanian, P. (2007). Prevalence of intestinal parasitic infestation in HIV/AIDS patients with diarrhea in Madurai City, South India. *Japanese Journal of Infectious Diseases*, **60**, 209–210.

Saksirisampant, W., Eampokalap, W., Rattanasrithong, B., Likanonsakul, M., Wiwanitkit, L. & Nasingkarn V. (2002). A prevalence of *Cryptosporidium* infections among Thai HIV-infected patients. *Parasitology*, **85**, 424–428.

Saksirisampant, W., Prownebon, J., Saksirisampant, P., Mungthin, M., Siripatanapipong, S. & Leelayoova, S. (2009). Intestinal parasitic infections: prevalence's in HIV/AIDS patients in a Thai AIDS-care centre. *Annals of Tropical Medicine and Parasitology*, **103**, 573–581.

Sischo, W. M., Atwill, E. R., Lanyon, L. E. & George, J. (2000). Cryptosporidia on dairy farms and the role these farms may have in contaminating surface water supplies in the northeastern United States. *Preventive Veterinary Medicine*, **43**, 253–267.

Smith, H. V., Nichols, R. A., Mallon, M., Macleod, A., Tait, A., Reilly, W. J., Browning, L. M., Gray, S. W., Reid, S. W. & Wastling, J. M. (2005). Natural *Cryptosporidium hominis* infections in Scottish cattle. *Veterinary Record*, **156**, 710–711.

Srisuphanunt, M., Suvedyathavorn, V., Suputtamongkol, Y., Arnantapunpong, S., Wiwanitkit, V. & Satitvipawee, P. (2008). Potential risk factors for *Cryptosporidium* infection among HIV/AIDS patients in central areas of Thailand. *Journal of Public Health*, **16**, 173–182.

Srisuphanunt, M., Saksirisampant, W. & Karanis, P. (2009). Detection of *Cryptosporidium* oocysts in green

mussels (*Perna viridis*) from shell-fish markets of Thailand. *Parasite*, **16**, 235–239.

Srisuphanunt, M., Karanis, P., Charoenca, N. Boonkao, N. & Ongerth, J. E. (2010). *Cryptosporidium* and *Giardia* detection in environmental water of southwest costal areas of Thailand. *Parasitology Research*, **106**, 1299–1306.

Tiangtip, R. & Jongwutiwes, S. (2002). Molecular analysis of *Cryptosporidium* species isolated from HIV-infected patients in Thailand. *The Southeast Tropical Medicine and International Health*, **7**, 357–354.

Thamlikitkul, V., Tepmongkol, M., Lamon, C., Sripochang, S., Rungnapawate, W. & Suvajeejarum, T. (1987). Cryptosporidiosis in Siriraj Hospital, Bangkok, Thailand. *The Southeast Asian Journal Tropical Medicine and Public Health*, **18**, 229–232.

Uga, S., Kunaruk, N., Rai, S. K. & Watanabe, W. (1998). *Cryptosporidium* infection in HIV-seropositive and seronegative populations in southern Thailand. *The Southeast Asian Journal of Tropical Medicine and Public Health*, **29**, 100–104.

Wiwanitkit, V. & Srisuphanunt, M. (2006). Cryptosporidiosis occurrence in anti-HIV-seropositive patients attending a sexually transmitted diseases clinic, Thailand. *Tropical Doctor*, **36**, 64–64.