介して首都圏へ移動することも否定できない．以上の理由から，今後も成田国際空港における疾病媒介蚊と蚊媒介性病原体の監視を継続することが重要であると思われる．

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# マラリア対策の進捗による感染状況の変化とフィールドでの迅速診断キットの限界

国立感染症研究所　寄生動物部
大前比呂思
帝京平成大学　ヒューマンケア学部
亀井喜世子
群馬大学大学院　医学系研究科・社会環境医学
中澤　港
Solomon Islands Medical Training & Research Institute
Bernard Bakote'e

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群馬大学大学院　医学系研究科・社会環境医学
中澤　港
Solomon Islands Medical Training & Research Institute
Bernard Bakote'e

**Key Words**：熱帯熱マラリア，三日熱マラリア，迅速診断キット

## はじめに

マラリア迅速診断試験法：Malaria Rapid Diagnostic Tests（RDTs）は，辺境のマラリア浸淫地で顕微鏡による検査が困難な場合，急性の発熱疾患の鑑別診断に有用である[1]。また，マラリア非浸淫地においても，トラベル・クリニックや検疫所における診断補助など，様々な用途で用いられる[1]。現在では，RDTsのための様々なキットが，一部の先進国にとどまらず，世界中の多くの国々で生産されるようになり，そのQuality Controlが必要な状況となったが，検出限界の目標としては，原虫密度100/μlが一つの指標となる[2]。

RDTsは，主にマラリア原虫の可溶性蛋白Histidine rich protein-2（HRP-2）を検出するタイプと，原虫性乳酸脱水素酵素Parasite lactate dehydrogenase（pLDH）を検出するタイプに分けられる。わが国では，前者としてNow ICT Malaria P.f/P.v，後者としてOpti MALが入手できるが，従来から，RDTsのSensitivity，Specificityは，熱帯熱マラリアに比し三

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**Epidemiologic Change of Malaria after the Progress of Control Program and Limitation of Malaria Rapid Diagnostic Tests in the Tropics**

Hiroshi Ohmae*　Kiseko Kamei**　Minato Nakazawa***　Bernard Bakote'e****

*Department of Parasitology, National Institute of Infectious Diseases*
***Faculty of Human Care, Teikyo Heisei University*
****Department of Social Ecological Medicine, Gunma University*
*****Solomon Islands Medical Training & Research Institute*

論文請求先：大前比呂思　〒162-8640　東京都新宿区戸山1-23-1　国立感染症研究所　寄生動物部

表1 各種 RDTs のマラリア原虫種別の Sensitivity と Specificity

| RDTs の種類 | Sensitivity | Specificity |
|---|---|---|
| ICT Malaria P. f./P. v. | *92 ～ 100% (*P.f*) | 84 ～ 100% (*P.f*) |
| | 75 ～ 89% (*P.v*) | 86 ～ 98% (*P.v*) |
| OptiMAL | 89 ～ 94% (*P.f*) | 88 ～ 98% |
| | 91% (*P.v*) | |
| Pan-R Malaria | 91 ～ 96% | 99% |

＊主に各製造会社の添付書類による。

表2 ソロモン諸島の調査におけるマラリア迅速診断試験法，OptiMAL と Pan-R Malaria の Sensitivity，Specificity の比較

| RDTs の種類 | | Sensitivity 実数 | Sensitivity % | Specificity 実数 | Specificity % |
|---|---|---|---|---|---|
| OptiMAL | *P. falciparum* | 4／7 | 57.1 | 164／165 | 99.4 |
| | *P. vivax* | 4／13 | 30.8 | 156／159 | 98.0 |
| Pan-R Malaria | *P. falciparum* | 4／7 | 57.1 | 164／165 | 99.4 |
| | *P. vivax* | 7／13 | 54.0 | 156／159 | 98.0 |

日熱マラリアの方が低くなる傾向が指摘されていた（表1）。また，原虫密度の低い感染者に関しては，マラリアの種別に関わらず Sensitivity が低くなるが，特に原虫密度 100/$\mu$l 未満の三日熱マラリアの場合，従来の Now ICT Malaria P.f/P.v では，殆ど検出できないことも指摘されている[3]。

ところで，かつてマラリアの流行に悩まされたアジア，南太平洋諸国の多くでは，対策の成果により，最近マラリアによる死亡者数・罹病者数が大きく減少している[4]。そしてこれらの国に共通した傾向として，重症な熱帯熱マラリアが減少し，相対的に症状に乏しい三日熱マラリアの占める比率が増加していることもあげることができる[4]。

## 目 的

1990 年代には，サブ・サハラのアフリカ諸国と並ぶ熱帯熱マラリア浸淫地と言われてきた，パプア・ニューギニアやソロモン諸島においても，上記のアジア，太平洋諸国に共通した傾向は認められる。そこで，2007 年2月，ソロモン諸島のガダルカナル島北東岸のマラリア浸淫地において，近年，相対的に増加している低原虫密度の三日熱マラリア感染者の検出に，どの程度 RDTs が利用できるか検討した。三日熱マラリアに対する Sensitivity が比較的高いとされる Opti MAL と，昨年を発売されまだフィールドでの使用経験が少ない Pan-R Malaria の2種類の RDTs の結果を顕微鏡的検査による診断結果と比較検討した。

## 結 果

172 人の住民を対象として一斉検査を行ったところ，ギムザ染色した厚層塗沫標本による顕微鏡的形態診断により，7例の熱帯熱マラリアと 13 例の三日熱マラリアがみつかった。熱帯熱マラリア7例のうち迅速診断キットで検出されたのは4例にとどまったが，2種類のキットの結果は一致した（表2）。一方，三日熱マラリアでは2種類のキットの結果は一致せず，Pan-R Malaria では4例，Opti MAL では7例が診断されたにとどまった。

また，検出限界と原虫密度については，小数例での観察にとどまったものの，Opti MAL，Pan R-Malaria とも，熱帯熱マラリアにおいては，原虫密度 1,000/$\mu$l を超えた1例は検出できたが，100/$\mu$l に満たない6例のうち，検出できたのは3例にとどまった（表3）。一方，三日熱マラリアに関しては，Opti MAL を用いた場合，原虫密度 100/$\mu$l 以上の

表3　ソロモン諸島の調査においてみられたマラリア原虫密度と
OptiMAL と Pan-R Malaria の Sensitivity の関係

| 原虫密度 (No. of parasite／μl) | *P. falciparum* | | *P. vivax* | |
|---|---|---|---|---|
| | RDTs／顕微鏡 | Sensitivity (95% CI) | RDTs／顕微鏡 | Sensitivity (95% CI) |
| OptiMAL | | | | |
| ＜100 | 3／6 | 50 (20 − 80) | 4／10 | 40 (25 − 55) |
| 100 − 1,000 | − | − | 3／3 | 100 (34 − 100) |
| 1,000＜ | 1／1 | 100 | − | − |
| Total | 4／7 | 57 (40 − 74) | 7／13 | 54 (33 − 75) |
| Pan R Malaria | | | | |
| ＜100 | 3／6 | 50 (20 − 80) | 3／10 | 30 (15 − 45) |
| 100 − 1,000 | − | − | 1／3 | 33 (10 − 56) |
| 1,000＜ | 1／1 | 100 | − | − |
| Total | 4／7 | 57 (37 − 77) | 4／13 | 31 (63 − 100) |

3例について全例検出することができたが，Pan R-Malariaの場合は，原虫密度と検出率の間には明確な関連はみられず，全体で検出できたのも30%程度にとどまった。

## 考　察

原虫密度100/μl以下の例を多く含む熱帯熱マラリア感染者のRDTsのSensitivityに関する先行研究は，ICT Malaria P.f/P.vで66.1～86.2%，Opti MALで42.6～81.3%と幅がある[5)6)7)]。今回は熱帯熱マラリアの検出率は，Opti MAL，Pan R-Malariaとも，4/7の57.1%にとどまったが，原虫密度100/μl以下の例が多かったことを考えると，先行研究による結果と比して，極端に低かったわけではない。

当初RDTsは，マラリア治療を前提とした発熱した患者で，熱性疾患の鑑別診断に使用されることが多かったが，2005年には世界中で2500万キットが使用されたと推測されるほど急速に普及が進む中，様々な用途に拡大されて利用される傾向がある[1)]。地域におけるマラリア浸淫状況を迅速・的確に把握するRapid assessmentや疫学的状況のモニタリング，治療後の効果判定などに積極的に利用されることも多い[7)]。しかし，今回の調査で，マラリア対策の進捗によって，低原虫密度の三日熱マラリア感染者数が相対的に増加している地域では，RDTsによる診断の限界も大きいことが確認された。

また，最近入手可能となったキット：Pan R-Malariaによってもその限界は克服されていないことがわかった。今後，検疫所などでRDTsを利用する場合にも，これらの問題点には十分留意する必要があろう。特に，三日熱マラリアの場合，熱帯熱マラリアに比して，市販されているRDTsの種類によって検出率や検出限界は大きく異なるので，今後はマラリア浸淫地のフィールドのみならず，マラリア非浸淫地の検疫所やトラベル・クリニックにおいても，目的に応じたキットの選択と利用が重要になると思われる。

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# Morphologic and Molecular Characterization of *Isospora belli* Oocysts from Patients in Thailand

Somchai Jongwutiwes,* Chaturong Putaporntip, Malee Charoenkorn, Takuya Iwasaki, and Takuro Endo

*Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; Department of Laboratory Investigation, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan; Division of Laboratory Medicine, National Iwate Hospital, Iwate, Japan; Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan*

*Abstract.* To investigate the extent of genetic heterogeneity in the genus *Isospora* infecting patients in Thailand, a total of 38 fecal samples containing *Isospora* oocysts from human immunodeficiency virus/acquired immunodeficiency syndrome patients (n = 30), corticosteroid-treated patients (n = 3) and immunocompetent individuals (n = 5) were recruited for analysis. Remarkable variation in the maximum width and length of oocysts both within and between isolates was observed. However, the average length-width ratio of oocysts was within the range for *I. belli* (> 1.2). *Ex vivo* sporogonic development of freshly passed oocysts in feces from three of these isolates was observed longitudinally, showing that 27% of these oocysts underwent complete sporulation. Interestingly, 95% of sporulated oocysts contained two sporocysts in an oocyst with four sporozoites in each sporocyst, and *Caryospora*-like oocysts, characterized by eight sporozoites enclosed by a single sporocyst, were also detected (5%). The small subunit ribosomal RNA (rRNA), 5.8S rRNA, internal transcribed spacer 1 (ITS-1), and ITS-2 were highly conserved, indicating that there were no cryptic species or extensive strain variation.

## INTRODUCTION

*Isospora belli* is a coccidian protozoa in phylum Apicomplexa that parasitizes epithelium of upper small intestine of humans and causes diarrheal disease. The entire life cycle of *Isospora* consists of asexual development and sexual reproduction that take place in the same host. Transmission of *I. belli* oocysts seems to be confined to the anthroponotic cycle because humans are the only known natural host.[1] The oocysts of *I. belli* usually require less than one day to a few days after passage from human intestine to complete sporogonic development and become infective.[2,3] Although both immunocompetent individuals and immunosuppressed patients are susceptible to infections, the prevalence of isosporiasis seems to occur more frequently in the latter. After the pandemic of human immunodeficiency virus-1 (HIV-1) infection, human isosporiasis has been more commonly identified as an opportunistic infection of the gastrointestinal tract of those who have low CD4+ lymphocyte counts (usually < 200 cells/μl).[4–6]

Human isosporiasis seems to be cosmopolitan in distribution, especially in tropical and subtropical regions such as Haiti, Mexico, Brazil, El Salvador, Venezuela, and southeast Asia.[1] However, the prevalence of this infection is occasionally underestimated because oocysts are usually excreted in small numbers or may not be found in spite of actual infection.[7] Moreover, the transparent appearance of *I. belli* oocysts could be overlooked in direct fecal smears.

It is of note that infection with *I. belli* usually produces more aggressive and prolonged period of symptoms in patients with acquired immunodeficiency syndrome (AIDS) than in immunocompetent individuals.[1,6] Among immunocompetent patients with isosporiasis, infants and young children seem to have more severe manifestations than in adults. Meanwhile, chronic diarrheal symptoms persisting for more than a decade caused by *I. belli* infections have been reported in adults without concurrent immunosuppression.[1,8] Although the gastrointestinal symptoms of patients with isosporiasis usually subside within a few days after initiation of treatment with combination of trimethoprim and sulfamethoxazole, relapses have been observed in approximately half of symptomatic cases.[4,5] Thus, the spectrum of isosporiasis seems to be variable, partly depending on the host immune status whereas variation in virulence of parasite strains remains unknown.

A number of distinct species have been characterized for *Cryptosporidium*, another enteric coccidian protozoa that causes diarrheal illness in both humans and animals. Recent molecular epidemiologic studies have shown that cryptosporidia infecting humans belong to heterogenous species.[9–11] Most of these species are responsible for zoonotic transmission, and anthroponotic cryptosporidiosis is caused by *C. hominis*. The knowledge of speciation in cryptosporidia is of considerable importance for disease control and prevention, especially for those with an immunosuppressive status, because no documented effective anti-cryptosporidial drug is available.[11]

Despite the increased significance of enteric coccidiosis, comparatively little is known about species/strain variation in the genus *Isospora* infecting humans. To address this issue, we extracted the DNA from oocysts found in fecal samples of isosporiasis patients in Thailand and determined the sequences of the genes for small subunit ribosomal RNA (SSU rRNA), internal transcribed spacer (ITS), and 5.8S ribosomal RNA. We also analyzed morphologic features of oocysts of *I. belli* from patients with normal immune status and those with compromised immunity.

## MATERIALS AND METHODS

**Stool samples and background data.** We obtained stool samples positive for *I. belli* oocysts from individuals at King Chulalongkorn Memorial Hospital in Bangkok, Thailand, as a cross-sectional study between January 2002 and December 2004. Isosporiasis was diagnosed by the presence of characteristic oocysts in one or more stool samples by direct wet smear method, formalin-ethylacetate sedimentation, or modi-

* Address correspondence to Somchai Jongwutiwes, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. E-mail: fmedsjw@md2.md.chula.ac.th

fied kinyoun acid fast stain. Stool samples and clinical data of *I. belli*-infected cases were obtained after informed consent was obtained. These data included age, sex, presenting symptoms, history of illness, and relevant laboratory test results such as complete blood count and CD4+ lymphocyte count. Eosinophilia was determined based on a relative eosinophil count of more than 4 cells per 100 leukocytes. Diarrhea was defined as three or more unformed stools per day.

Aliquots of each *I. belli*-positive stool sample were preserved as described by adding approximately four volumes of absolute ethanol and storing at ambient temperature.[12] Whenever possible, fresh stool samples were collected for observing sporulation of oocysts. The ethical aspects of this study were reviewed and approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University.

**Morphometry of oocysts.** The dimension of each oocyst was determined by measuring maximum length and width with an Olympus (Center Valley, PA) BX51 light microscope at a magnification of 400. At least 20 oocysts were measured for each sample.

**Sporulation of oocysts.** Fresh stool samples were directly diluted with an equal volume of sterile water. The stool suspension of each sample was applied onto more than 50 clean glass slides and covered with 22 × 22 mm cover slips. The edges of the cover slips were tightly sealed, incubated at 25–30°C, and kept in humidifier boxes to minimize evaporation of fluid from the samples. A total of 100 oocysts for each isolate were examined every 6 hours for 20 days by light microscopy using a magnification of × 400.

**DNA extraction.** DNA of *I. belli* oocysts was extracted from either fresh stool samples or ethanol-preserved specimens by the method described for the extraction of *Cryptosporidium* oocysts,[11] except that the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used. Prior to DNA extraction of preserved samples, ethanol was removed by washing the samples with sterile water (by centrifugation 3 times at 1,600 × *g* for 15 minutes without stopping) and the pellet was resuspended in 5 mL of water. The DNA purification procedure was essentially as described in the manufacturer's instruction manual. The purified DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and stored at −20°C until used.

**Polymerase chain reaction (PCR).** The DNA fragment spanning the SSU rRNA, ITS-1, 5.8S rRNA and ITS-2 regions of *I. belli* was amplified by a nested PCR using primers whose sequences were derived from the 5' portion of the SSU rRNA gene of *I. belli* isolate CI1 (GenBank accession no. U94787) and the 5' portion of the 28S rRNA gene of *I. felis* (GenBank accession no. U85705). Sequences of the outer pair of primers were Iso-18SF0, 5'-CTGGTTGATCCTGCCAGTA-3' and Iso-28SR0, 5'-AAGGCTCAATCAAGAACCTCCG-3'. Sequences of the inner pair of primers were Iso-18SF1, 5'-GATCCTGCCAGTAGTCAT-3' and Iso-28SR1, 5'-TGAAGCTAATCCCTCTTCC-3'. We used the same thermal cycling profiles for both primary and secondary PCR: denaturation at 94°C for 40 seconds, annealing at 64°C for 40 seconds, extension at 74°C for 5 minutes for 35 cycles of amplification. DNA amplification was performed by using a GeneAmp 9700 PCR thermal cycler (Applied Biosystems, Foster City, CA). To minimize the error introduced in the sequences during PCR amplification, we used ExTaq DNA polymerase (Takara, Shiga, Japan) that has efficient 5' → 3' exonuclease activity to increase fidelity and shows no strand displacement. The size of PCR product was examined by electrophoresis in a 1% agarose gel and visualized with an ultraviolet transilluminator (Mupid Scope WD, Japan). The PCR product was purified by using the QIAquick PCR purification kit (Qiagen).

**Subcloning.** The GeneJET™ PCR Cloning Kit was used for ligating the purified PCR product to the plasmid vector pJET1/blunt (Fermentas, Burlington, Ontario, Canada). After incubation at 15°C for 6 hours, the reaction mixture was precipitated, redissolved in 10 µL of double-distilled water, and transformed into *Escherichia coli* strain JM107 by electroporation using an *E. coli* pulser apparatus (Bio-Rad Laboratories, Hercules, CA). Recombinant DNA from positive clones was prepared by using the QIAGEN plasmid mini kit (Qiagen).

**DNA sequencing.** DNA sequences were determined directly from both PCR-purified templates and plasmid subclones. Sequencing analysis was performed in both directions for each template using the Big Dye Terminator version 3.1 Cycle Sequencing Kit on an ABI310 Genetic Analyzer (Applied Biosystems). Overlapping sequences were obtained by using sequencing primers (available upon request). Whenever one substitution occurred, the sequence was re-determined using PCR products from two independent amplifications from the same DNA template.

**Data analysis.** Sequences were aligned according to their primary and secondary structures by the CLUSTAL X and the MARNA programs, respectively.[13,14] Phylogenetic construction was performed by the neighbor-joining method using the Kimura-2 parameter and 1,000 bootstrap iterations as implemented in the MEGA version 3.1 program.[15] To analyze the evolutionary relationship, homologous sequences of other coccidian parasites available in the GenBank database were incorporated for comparison: *I. belli* (AF106935), *I. ohioensis* (AF029303), *I. suis* (U97523), *I. orlovi* (AY365026), *I. felis* (L76471), *Toxoplasma gondii* (M97703 and L49390), *Cryptosporidium parvum* (AF093490 and AF015773), *Cyclospora cayetanensis* (U40261 and AF301391), *Neospora caninum* (U16159 and L49389), *Hammondia heydorni* (AF317282), *H. hammondi* (AF096498), *Eimeria tenella* (U40264), and *E. papillata* (AY779501). New nucleotide sequence data obtained in this study have been deposited in the GenBank database under accession nos. DQ060658–Q060683.

## RESULTS

**Patient profiles.** Between January 2002 and December 2004, stool samples from 39,510 individuals were submitted to parasitology laboratory of King Chulalongkorn Memorial Hospital in Bangkok for detection of enteric parasites. Of these, 497 were from confirmed HIV/AIDS patients. Isosporiasis was identified in 38 patients by the presence of *I. belli* oocysts in their stool samples: 12 found by direct wet smear method (31.6%), 37 by formalin-ethylacetate sedimentation (97.4%) and 36 by modified kinyoun acid fast stain (94.7%). Isosporiasis patients comprised 35 Thai persons from diverse regions of the country and 3 recent immigrants from Cambodia, Laos, and Pakistan.

Of all *I. belli*-infected patients, 30 were HIV positive, 3

received prolonged corticosteroid therapy for systemic lupus erythematosus or idiopathic thrombocytopenic purpura, and 5 were immunocompetent individuals. More than half of isosporiasis patients were 30–39 years of age and the ratio of males to females 1.38:1. Laboratory tests showed relative eosinophilia (> 4%) in half of HIV-positive cases and all immunocompetent patients but none in the patients receiving prolonged corticosteroid treatment (Table 1). The absolute CD4+ lymphocyte counts in HIV-positive cases were 8–484 cells/μL (mean ± SD = 67.5 ± 57.4 cells/μL). Among HIV-infected cases, concurrent infections with one or more of the following pathogens occurred in 9 patients: herpes simplex virus, herpes zoster virus, *Histoplasma capsulatum*, *Mycobacterium tuberculosis*, *Salmonella* group B, and *Strongyloides stercoralis*.

All HIV-infected individuals developed watery diarrhea ranging from 1 week to 1 year (mean ± SD = 3.3 ± 3.4 months) prior to attending King Chulalongkorn Memorial Hospital. Two patients who received corticosteroids had chronic watery diarrhea and one patient who received this treatment had no symptoms. The clinical profiles of isosporiasis in immunocompetent patients were asymptomatic in two patients, chronic watery diarrhea in two patients, and dyspepsia without diarrhea in one patient. Diarrhea and associated gastrointestinal symptoms resolved within a few days after initiation of treatment with a combination of trimethoprim and sulfamethoxazole. During two months of follow-up, relapse occurred once each in two HIV-infected patients and one immunocompetent patient that was controlled by long-term prophylaxis with trimethoprim and sulfamethoxazole.

**Oocyst morphometry.** The oocyst dimension of each isolate varied from 17 to 37 μm (mean ± SD = 28.3 ± 3.0 μm) in length and from 8 to 21 μm (mean ± SD = 13.5 ± 1.9 μm) in width, and the mean ± SD shape index (length divided by width) was 2.1 ± 0.31 (range = 3–3.3). Although oocysts showed shape and size variations both within and between isolates, the shape indices of all oocysts observed in this study were consistent with that of *I. belli*, being more than 1.2, which was distinct from those for other species of *Isospora* infecting nonhuman mammals (< 1.2).[1]

**Sporulation of oocysts.** We were able to follow oocyst maturation using fresh watery stool samples from two HIV-infected patients and one immunocompetent subject who had not yet taken anti-coccidial drugs. A total of 100 oocysts were observed for each isolate. The average number of oocysts of these samples that underwent complete formation of sporozoites was 27 (range = 20–33). The duration for an oocyst to transform into two sporocysts, each of which contained four sporozoites, ranged from 24 hours to 10 days (mean ± SD = 3.9 ± 3.4 days) (n = 66). Interestingly, *Caryospora*-like oocysts, characterized by oocysts containing one sporocyst that enclosed eight sporozoites, appeared on day 5–14 after incubation. Although this type of oocyst could be found in all three isolates, they occurred at a low frequency (approximately 5%) (Figure 1). The dimensions of *Caryospora*-like oocysts did not differ significantly from those having bisporocysts.

**SSU rRNA, ITS-1, 5.8S rRNA and ITS-2 sequences.** The DNA fragment generated from the secondary PCR encompassing the SSU rRNA, ITS-1, 5.8S rRNA, and ITS-2 genes contained 3,049 basepairs. All isolates yielded single PCR fragments of identical size. Of these, we determined the se-

TABLE 1
Clinical profiles of isosporiasis and morphometry of *Isospora belli* oocysts*

| Patient profiles | n | Age (years) mean ± SD (range) | CD4+ cells/μL mean ± SD (range) | Eosinophils (%) mean ± SD (range) | Oocyst dimensions† Length (μm) mean ± SD (range) | Width (μm) mean ± SD (range) | Shape index mean ± SD (range) |
|---|---|---|---|---|---|---|---|
| HIV infection (18 males, 12 females) | | | | | | | |
| Diarrhea | | | | | | | |
| ≤ 3 weeks | 5 | 35.6 ± 11.0 (23–52) | 104.3 ± 25.7 (89–134) | 5.4 ± 3.6 (0.4–9.1) | 27.2 ± 2.6 (18–33) | 12.8 ± 1.8 (8–19) | 2.2 ± 0.3 (1.3–3.0) |
| > 3 weeks–< 1 year | 22 | 36.7 ± 6.9 (25–50) | 60.4 ± 52.9 (8–480) | 4.5 ± 3.3 (0.1–14.0) | 28.4 ± 2.9 (17–35) | 13.7 ± 1.9 (8–21) | 2.1 ± 0.3 (1.3–3.3) |
| ≥ 1 year | 3 | 27.7 ± 8.3 (21–37) | 80.3 ± 53.6 (25–484) | 3.5 ± 1.8 (2.0–5.5) | 28.4 ± 3.8 (18–34) | 13.8 ± 2.2 (10–19) | 2.1 ± 0.4 (1.4–3.0) |
| Corticosteroid treatment (1 male, 2 females) | | | | | | | |
| No symptom | 1 | 37 | ND | 0.8 | 33.7 ± 2.0 (30–37) | 14.0 ± 1.6 (12–18) | 2.4 ± 0.2 (1.9–2.7) |
| Diarrhea | | | | | | | |
| ≤ 3 weeks | 1 | 51 | ND | 0 | 28.3 ± 2.6 (23–32) | 12.8 ± 1.2 (10–15) | 2.2 ± 0.3 (1.8–3.1) |
| > 3 weeks | 1 | 23 | ND | 1.0 | 27.4 ± 2.1 (23–30) | 13.4 ± 1.3 (11–16) | 2.1 ± 0.3 (1.4–2.6) |
| Immunocompetence (3 males, 2 females) | | | | | | | |
| No symptoms | 1 | 37 | ND | 12.0 | 28.0 ± 1.9 (25–31) | 14.0 ± 1.9 (11–17) | 2.0 ± 0.3 (1.5–2.5) |
| Dyspepsia | 1 | 31 | ND | 11.1 | 30.3 ± 2.6 (25–35) | 13.7 ± 1.2 (12–16) | 2.2 ± 0.3 (1.6–2.5) |
| Diarrhea | | | | | | | |
| < 1 year | 2 | 30.5 ± 2.1 (29–32) | ND | 8.5 ± 0.7 (8.0–9.0) | 27.0 ± 2.4 (20–32) | 12.6 ± 2.2 (9–18) | 2.2 ± 0.4 (1.5–3.1) |
| ≥ 1 year | 1 | 57 | 730 | 16.0 | 28.4 ± 1.4 (26–30) | 15.6 ± 1.7 (12–17) | 1.8 ± 0.2 (1.6–2.3) |

* HIV = human immunodeficiency virus; ND = not determined.
† Measurement under 400× magnification from 20 oocysts from each isolate.

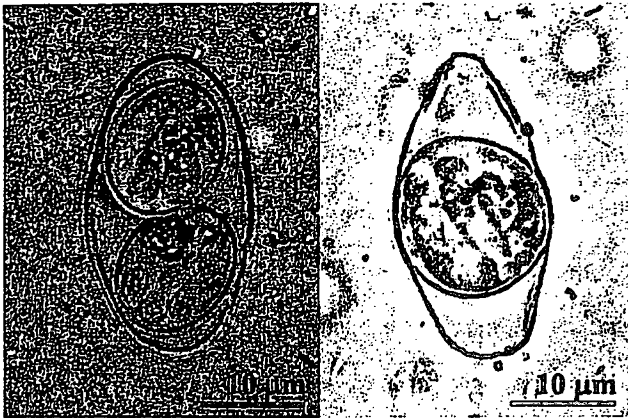


FIGURE 1. Oocysts of *Isospora belli* showing a sporulated oocyst containing two sporocysts, each of which contains 4 sporozoites (left) and a *Caryospora*-like oocyst containing sporozoites enclosed in a single sporocyst (right).

quences of 26 isolates from HIV-infected patients (n = 23), symptomatic immunocompetent individuals (n = 2), and an asymptomatic immunocompetent subject. We did not find any remarkable preponderance toward purine or pyrimidine composition in the region analyzed (% GC = 47.1%). Sequences of the SSU rRNA gene, spanning 1,778 basepairs, of 25 isolates examined were identical with those of strains CI1 and CJLPHD2 (GenBank accession nos. U94787 and AF441289), but differed from the isolate reported by Franzen and others (GenBank accession nos. AF106935) at A679T and A682C.[16] Three additional nucleotide substitutions occurred at T583C, C638A, and G1240T in the isolate from an immunocompetent patient who had multiple relapses.

The 5.8S rRNA, ITS-1, and ITS-2 regions contained 598, 158 and 404 basepairs, respectively. Both the 5.8S rRNA and ITS-2 sequences of *I. belli* showed perfect sequence identity among all isolates examined. Likewise, sequences of the ITS-1 were highly conserved, except for one nucleotide substitution at position 528 with an A to G change (position according to the ITS-1 region). However, there was no association between this nucleotide substitution and clinical severity of the infected individuals. Comparison of the 5.8S rRNA of *I. belli* with those of other coccidian parasites showed a high similarity of sequences (Figure 2). Conversely, the ITS-1 and ITS-2 regions showed extensive sequence diversity.

**Phylogenetic analysis.** The neighbor-joining tree derived from the SSU rRNA sequences confirmed that all isolates of *I. belli* in this study were clustered, which is consistent with a single species (Figure 3). Further analysis using the available complete sequences of the SSU rRNA gene of the genera *Toxoplasma*, *Cryptosporidium*, *Cyclospora*, *Isospora*, *Neospora*, *Eimeria*, and *Hammondia*, which were aligned according to their secondary structure, showed that *I. belli* was more related to *Toxoplasma*, *Neospora*, and *Hammondia* than to *Cyclospora*, *Eimeria*, and *Cryptosporidium*. An identical topology of a phylogenetic tree to that inferred from the SSU rRNA sequences was reaffirmed when the ITS-2 sequences were used for comparison (Figure 4).

## DISCUSSION

We searched for isosporiasis in a large number of patients who submitted their stool samples to King Chulalongkorn Memorial Hospital over a three-year period. The overall prevalence of *I. belli*-positive samples was approximately 0.1% on the basis of total samples examined. The prevalence of isosporiasis among HIV/AIDS patients in this study was 6.0% and decreased to 0.02% in non-HIV-infected cases. However, similar cross-sectional studies in Germany, Haiti, and Venezuela showed a higher prevalence, ranging from 14 to 48%.[4,6,17] The low prevalence of isosporiasis in this study suggested that *I. belli* might not commonly circulate in the Thai population, resulting in a low prevalence of isosporiasis in HIV/AIDS patients in this country. Nevertheless, most isosporiasis patients in Thailand showed immunosuppression, comprising 86.8% of all positive cases.

Despite rapid improvement of symptoms after treatment with oral trimethoprim and sulfamethoxazole, recurrent isosporiasis occurred in three HIV/AIDS patients (approximately 8%) within two months after initiation of treatment. Importantly, approximately half of Haitian patients who received the same or similar treatment had relapses.[5] The duration and frequency of follow-up after treatment may have contributed

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I.belli          AAACTTTCAG CAATGGATGT CTTGGTTCGC GCAACGATGA AGGACGCAGC GAAATGCGAA ACGCAATGTG AATTGCAGAA  80
E.papillata      .......... .......... .....C.... ...T...... .......... .........T .....T.... .........T  80
Cy.cayetanensis  .......... .......... .....C.... ...T...... .......... .........T .....T.... .........T  80
T.gondii         ...T...... .......... .....C.... .......... .......... ...C...... .......... ..........  80
H.heydorni       ...T...... .......... .....C.... .......... .......... ...C...... .......... ..........  80
N.caninum        ...T...... .......... .....C.... .......... .......... ...C...... .......... ..........  80
C.parvum         .C.....A.. T......... .........T. AT........ .......... A..G.....T .A........ ..C....T..  80

I.belli          TTCAGTGAAT CATCAGATTT CTGAACGCAA ATGGCGCTGT GGGG-ATATT CCCTGCAGCA TGTCTGTTTC A-GTGTCTCT 158
E.papillata      .........C .......... .........T .........C T..--GAT.. .......... C......... .-......T. 157
Cy.cayetanensis  .........C .......C.. .........T ..T......C C..CC..... G..G...... C......... .A......T. 160
T.gondii         .......... .......... .......... .....A.CT. ....-..... .T.CTTG.T. C......... .-......T. 158
H.heydorni       .......... .......... .......... .....A.CT. ....-..... .T.CTTG.T. C......... .-......T. 158
N.caninum        .......... .......... .......... ...C.A.CT. ....-..... .T.CTTG.T. C......... .-......T. 158
C.parvum         ...TA....C A.......C. ..C....... ..A..AG.A- --------.. TA.ATT.CT. .A.T.A.A.. .-......A. 151
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FIGURE 2. Alignment of the 5.8S ribosomal RNA sequences of *Isopora belli* with those of other coccidian protozoa. Dots and dashes represent identical residues and deletions, respectively. *E.* = *Eimeria*; *Cy.* = *Cyclospora*; *T.* = *Toxoplasma*; *H.* = *Hammondia*; *N.* = *Neospora*; *C.* = *Cryptosporidium*.

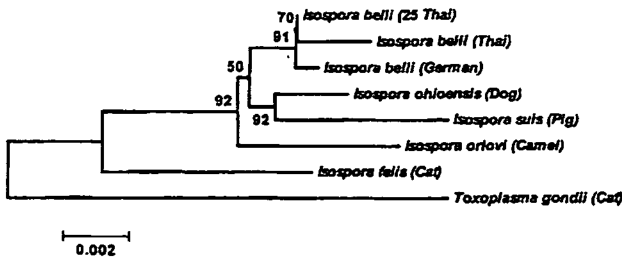


FIGURE 3. Phylogenetic relationships among isolates of *Isospora belli* in this study (DQ060658-83) and those reported by others (U94787 and AF106935) in relation to other nonhuman species of *Isospora* as inferred from small subunit ribosomal RNA sequences and the neighbor-joining method. *Toxoplasma gondii* was used as the outgroup. Bootstrap percentages more than 50% based on 1,000 iterations are shown on the branches. Host origins are shown in parentheses.

to the difference in observed relapsing episodes from each study. However, continuing anti-coccidial prophylaxis will be warranted in certain isosporiasis cases.

The life cycle of *I. belli* depends solely on monoxenous development in a human host without known natural animal reservoirs or paratenic hosts. Both schizogonic and sporogonic development take place intracellularly in epithelium of the small intestine. However, the stages of oocysts that are newly passed in feces contain mostly one sporont, but oocysts with two sporoblasts are occasionally encountered. To observe oocyst development *ex vivo*, we used fresh stool samples without preservatives, such as potassium dichromate, to envisage the fate of oocyst development when excreted into the environment. The duration for complete sporulation of *I. belli* described herein is rather variable. Although we used different experimental conditions from other studies, the earliest appearance of fully sporulated oocyst is similar, i.e., within 24 hours after passage from the host.[2,3]

It is well recognized that mature oocysts of *I. belli* produce two sporocysts, each with four sporozoites. Interestingly, a small percentage of approximately less than 2% of oocysts that underwent sporogonic development contained eight sporozoites in one sporocyst, which is known as *Caryospora*-like, has been reported in *Isospora*, such as *I. canis*, *I. suis*, and *I. rivolta*, that infect other mammals.[18–20] The presence of *Caryospora*-like oocysts of *I. belli* was first described in 1968 by Zaman, who studied sporogonic development of *I. belli* in patients from Singapore.[2] However, no additional studies have confirmed the presence of this stage. In this study, we demonstrated the presence of *Caryospora*-like oocysts in all three isolates examined after an extended period of incubation. Taken together, this stage could be an alternative *ex vivo* development stage of *I. belli*. Factors such as temperature, moisture, level of oxygen, and other unknown conditions could influence the capability and duration required for complete sporulation of this type of oocysts.[1] For instance, the formation of *Caryospora*-like oocysts of the cat isosporan protozoa *I. rivolta* could be induced by brief heating of oocysts to 50°C before incubation. These oocysts remained viable and infectious to cats.[21] Although we did not determine the effect of heat induction on the generation of *Caryospora*-like oocysts in *I. belli*, spontaneous development of this stage occurred during a range of five days to two weeks at ambient temperature (25–30°C). If the viability period of *I. belli* in the environment after complete sporulation was limited, unsynchronized sporulation could extend the period of transmission of this important enteric coccidian protozoa.

Eukaryotic organisms possess the nuclear rRNA genes organized in clusters containing SSU rRNA, of which the 5.8S and 28S subunits are separated by the two ribosomal RNA spacers ITS1 and ITS2. A remarkable feature of these ITS sequences is their high divergence between species, which makes them a suitable marker for speciation and phylogenetic studies. Our analysis of the SSU rRNA, ITS-1, 5.8S rRNA, and ITS-2 regions showed sequence conservation among isolates, except a minimal sequence variation in SSU rDNA and ITS-1. Therefore, cryptic species of *Isospora* infecting patients in this study were unlikely.

Phylogenetic relationships inferred from the SSU rRNA gene showed that *Isospora* is more closely related to the genera *Neospora* and *Toxoplasma* than to the genera *Eimeria* and *Cyclospora*, which is consistent with analysis by Franzen and others.[16] Likewise, the phylogenetic tree derived from the informative sites of ITS-2 based on secondary structure alignment yielded results similar to those obtained from the SSU rRNA sequence, which supports the value of ITS-2 data for tracing evolutionary relationship of coccidian protozoa as those used in analysis of other organisms.[22–24]

In conclusion, unlike *Cryptosporidium* infecting humans that comprises both zoonotic and anthroponotic species, our study, on the basis of morphometric and molecular evidence, showed that human isosporiasis is caused by one species of *I. belli*. Although isosporiasis is more common among immunocompromised patients than immunocompetent hosts, it seems likely that severity of infections does not simply depend on the immune status of infected individuals because some immunocompetent patients exhibit chronic debilitating illness with multiple recurrent prolonged diarrheal episodes.[1,8] Whether strain difference in *I. belli* could contribute to disease severity will require further investigation. In this regard, analysis of yet unknown polymorphic genetic markers in *I. belli* would be essential.

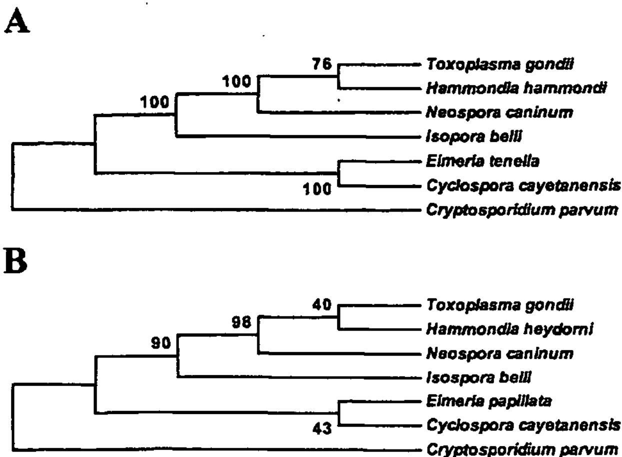


FIGURE 4. Topology of neighbor-joining trees derived from the small subunit ribosomal RNA (**A**) and the internal transcribed spacer 2 region (**B**) of *Isospora belli* and other coccidian protozoa using the Kimura-2 parameter. Bootstrap values based on 1,000 iterations are indicated on the branches.

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Authors' addresses: Somchai Jongwutiwes, Chaturong Putaporntip, and Malee Charoenkorn, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Rama 4 Road, Pathumwan, Bangkok 10330, Thailand. Takuya Iwasaki, Department of Laboratory Investigation, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 8528523, Japan; and Division of Laboratory Medicine, National Iwate Hospital, Iwate 0210056, Japan.Takuro Endo, Department of Parasitology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan.

Reprint requests: Somchai Jongwutiwes, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand, Telephone: 66-2-256-4000 extension 3685, Fax 66-2-252-4963, E-mail: fmedsjw@md2.md.chula.ac.th.

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Short communication

# Prevalence and first genetic identification of *Cryptosporidium* spp. in cattle in central Viet Nam

Sam Thi Nguyen [b], Duc Tan Nguyen [b], Duc Quyet Le [b], Luc Ngoc Le Hua [b], Thoai Van Nguyen [b], Hajime Honma [a], Yutaka Nakai [a,*]

[a] *Laboratory of Animal Health and Management, Graduate School of Agricultural Science, Tohoku University, 232-3 Yomogida, Naruko-Onsen, Osaki, Miyagi 989-6711, Japan*
[b] *Department of Parasitology, Central Viet Nam Veterinary Institute, km4 Dong De Street, Nha Trang, Viet Nam*

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**Abstract**

We investigated the prevalence of *Cryptosporidium* infection in relation to age and clinical status in cattle in the central region of Viet Nam. A total of 266 fecal samples from diarrheic and non-diarrheic cattle were examined by the modified Ziehl-Neelsen staining method. Prevalence of *Cryptosporidium parvum* type infections, those of the *Cryptosporidium andersoni* type, and mixed infection of both types was 33.5% (89/266), 5.6% (15/266), and 3.4% (9/266), respectively. The infection rate of 44.3% (35/79) of *C. parvum* in calves less than 6 months old was significantly higher than that of 28.9% (54/187) in cattle greater than 6 months old ($P < 0.01$). Although no *C. andersoni* oocysts were detected in calves less than 3 months old, no significant difference was observed between the age groups in the prevalence of *C. andersoni* infection and mixed infection. The percentage of diarrheic and non-diarrheic cattle identified to be shedding *C. parvum* oocysts was 46.5% (74/159) and 14.0% (15/107), respectively ($P < 0.0001$). The risk of diarrhea was 1.7 times greater in *C. parvum*-infected calves than in their non-infected counterparts. DNA sequences of 18S rRNA genes of *C. parvum* type and *C. andersoni* type indicated that they were *C. parvum* bovine genotype and *C. andersoni*, respectively. This is the first genetic identification of *C. parvum* bovine genotype and *C. andersoni* from cattle in Viet Nam.
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*Keywords:* *Cryptosporidium*; Cattle; Prevalence; Genetic identification; Viet Nam

## 1. Introduction

*Cryptosporidium* is a protozoan genera that belongs to the phylum *Apicomplexa*, class *Sporozoasida*, subclass *Coccidiasina*, order *Eucoccidiorida*, suborder *Eimeriorina* and family *Cryptosporidiidae* (Sterling and Arrowood, 1993; OIE, 2004). *Cryptosporidium* species have been reported to be major pathogens of diarrhea in humans and many species of livestock such as cattle, sheep, goat, pig, horse, and birds (Fayer et al., 1997).

In cattle, at least two species of *Cryptosporidium* are commonly detected: the intestinal species *C. parvum*, which is the most common causative agent of cryptosporidiosis in pre-weaned calves, and the abomasal species *C. andersoni*, which is less pathogenic (Xiao et al., 2004; Santin et al., 2004). Most recently, the intestinal species *C. bovis* has been identified and considered to be the predominant species in post-weaned calves (Santin et al., 2004; Fayer et al., 2006). Genetically *C. parvum* is divided into two groups: the human genotype 1 is restricted to humans, while the bovine genotype 2 shows widespread distribution in that

* Corresponding author. Tel.: +81 229 84 7391; fax: +81 229 84 7391.
*E-mail address:* nakai@bios.tohoku.ac.jp (Y. Nakai).

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it infected cattle, humans, and mice (Thompson, 2003; Xiao et al., 2004). However, they are now known as *C. hominis* and *C. parvum*, respectively. Due to its zoonotic nature, this parasite is considered to be a major cause of public health problems and has been extensively studied in many countries such as the U.S. (Atwill, 1997; Fayer et al., 2005), Japan (Nakai et al., 2004; Koyama et al., 2005), and Taiwan (Watanabe et al., 2005).

In Viet Nam, cattle are the major meat-producing animals and the most common domestic animal, and they play an important role in the economics of Viet Nam. Diarrhea remains a common calf disease and is responsible for approximately 5.1% of the total mortality rate in certain regions. Nevertheless, only limited information is available on diarrhea due to cryptosporidiosis in Viet Nam. *Cryptosporidium* spp. remain an unknown cause of diarrhea of cattle in Viet Nam. Therefore, it is important to understand the prevalence of bovine cryptosporidiosis in order to screen *Cryptosporidium* species/genotypes harboring in cattle for the control of cryptosporidiosis in humans and animals.

In this study, the prevalence of *Cryptosporidium* infection was surveyed in cattle in Viet Nam, and the isolates were genetically identified.

## 2. Materials and methods

During the period from March 2005 to May 2006, a total of 266 bovine fecal samples with and without diarrhea were collected from three different provinces in the central region of Viet Nam—Quang Ngai, Khanh Hoa, and Dac Lac. Approximately 20 g of rectal feces were obtained directly from each animal. One portion (5 g) of each sample was stored at 4 °C, and the remaining portion was stored in 2.5% potassium dichromate at 4 °C until use. The age and clinical status of the cattle as well as the date and place of collection were recorded. The cattle were divided into different age groups: less than 3 months old, from 3 to 6 months old, greater than 6 to 12 months old, and greater than 12 months old.

*Cryptosporidium* infection was diagnosed by the modified Ziehl-Neelsen (mZN) staining method, using direct fecal smears that were prepared and stained according to the instructions of the Organisation of International Epidemiology (OIE, 2004). The stained smears were observed microscopically under 400× or 1000× magnification. The *Cryptosporidium* oocysts were visualized as bright red round bodies against a pale green background, containing elongated naked sporozoites. The diameter of the oocysts was measured at 1000× magnification. *Cryptosporidium* infection was scored positive or negative based on the presence or absence of the oocyst in the examined samples.

From *Cryptosporidium*-positive fecal samples, single oocysts of large and small size were separately selected under a differential interference contrast microscope (DIC, Nomarski technique) using glass capillary pipettes and subsequently transferred to PCR tubes. After five cycles of freezing and thawing, followed by boiling for 10 min, nested-PCR was performed for amplifying a fragment of the 18S rRNA gene (approximately 1540 bp) by using the primer set mentioned by Xiao et al. (1999). The DNA were sequenced using the ABI Prism Big Dye Terminator Cycle sequencing kit (Applied Biosystems, CA, U.S.A.) on an ABI Prism 310 genetic analyzer automated sequencer according to the manufacturer's instructions.

### 2.1. Statistical analysis

Statistical analysis was carried out using Fisher's exact test. Probability values of $P < 0.05$ were considered significant.

## 3. Results

Of the 266 bovine fecal samples, 95 (35.7%) were positive for *Cryptosporidium* oocysts. Unstained oocysts were round, colorless, and contained naked sporozoites, whereas the stained oocysts appeared bright red round to ovoid bodies against a pale green background.

Two different diameter sizes of oocysts were noted in the samples. The small oocysts ranged from 4.5 to 5.5 μm with a mean diameter size of 4.81 ± 0.30 μm ($n = 30$), whereas the larger ones ranged from 6.5 to 8.0 μm with a mean diameter size of 6.91 ± 0.49 μm ($n = 30$). Based on the size, the oocysts were identified as the *C. parvum* type and *C. andersoni* type, respectively. The prevalence of two types of *Cryptosporidium* oocysts from diarrheic and non-diarrheic cattle in different age groups has been shown in Tables 1–3.

Table 1 shows that the *C. parvum* type was detected in 89 of 266 cattle examined, and the prevalence was thus 33.5%. In relation to age, the *C. parvum* type was found in 35 (44.3%) of 79 calves less than 6 months old and 54 (28.9%) of 187 cattle greater than 6 months old ($P < 0.01$). The *C. parvum* type was detected in 74 (46.5%) of 159 diarrheic cattle and in 15 (14.0%) of 107 non-diarrheic cattle ($P < 0.0001$). Additionally, 74 out of 89 positive cattle and 85 out of 177 negative ones

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Table 1
Prevalence of *Cryptosporidium parvum*-type oocysts in cattle based on age-group classification

| Age (months) | No. of positive/no. of examined (%) | | |
|---|---|---|---|
| | Diarrheic cattle | Non-diarrheic cattle | Total |
| <3 | 14/20 (70%) | 1/11 (9.1%) | 15/31 (48.4%) |
| 3–6 | 15/26 (57.7%) | 5/22 (22.7%) | 20/48 (41.7%) |
| >6–12 | 18/41 (43.9%) | 3/37 (8.1%) | 21/78 (26.9%) |
| >12 | 27/72 (37.5%) | 6/37 (16.2%) | 33/109 (30.3%) |
| | 74/159 (46.5%) | 15/107 (14.0%) | 89/266 (33.5%) |

Table 2
Prevalence of *Cryptosporidium andersoni*-type oocysts in cattle based on age-group classification

| Age (months) | No. of positive/no. of examined (%) | | |
|---|---|---|---|
| | Diarrheic cattle | Non-diarrheic cattle | Total |
| <3 | 0/20 (0%) | 0/11 (0%) | 0/31 (0%) |
| 3–6 | 0/26 (0%) | 3/22 (13.6%) | 3/48 (6.3%) |
| >6–12 | 0/41 (0%) | 2/37 (5.4%) | 2/78 (2.6%) |
| >12 | 4/72 (5.6%) | 6/37 (16.2%) | 10/109 (9.2%) |
| | 4/159 (2.5%) | 11/107 (10.3%) | 15/266 (5.6%) |

Table 3
Prevalence of mixed infection of the *C. parvum*- and *C. andersoni*-type oocysts in cattle based on age-group classification

| Age (months) | No. of positive/no. of examined (%) | | |
|---|---|---|---|
| | Diarrheic cattle | Non-diarrheic cattle | Total |
| <3 | 0/20 (0%) | 0/11 (0%) | 0/31 (0%) |
| 3–6 | 0/26 (0%) | 1/22 (4.5%) | 1/48 (2.1%) |
| >6–12 | 0/41 (0%) | 1/37 (2.7%) | 1/78 (1.3%) |
| >12 | 4/72 (5.6%) | 3/37 (8.1%) | 7/109 (6.4%) |
| | 4/159 (2.5%) | 5/107 (4.7%) | 9/266 (3.4%) |

*Note*: The individuals with mixed infections are grouped under cattle that were positive for *C. parvum* or *C. andersoni* infection, as shown in Tables 1 and 2.

showed diarrhea. The odds ratio was 1.7 (74:89/85:177) with 95% confidence intervals 1.17–2.46.

The prevalence of the *C. andersoni* type in cattle is shown in Table 2. Among the 266 cattle examined, 15 were positive for this type, and the prevalence was thus 5.6%. No oocysts of the *C. andersoni* type were found in any of the cattle aged less than 3 months, as well as in any of the diarrheic ones with the exception of the mixed infection case. No significant difference was observed in the prevalence among different age groups.

Mixed infection of the *C. parvum* and *C. andersoni* types is shown in Table 3. Of the 266 cattle examined, 9 were infected with both types of oocysts, and the infection rate was 3.4%. There was no significant difference regarding the prevalence of mixed infection in different ages. Both types of oocysts were detected in the same individual in 4 (2.5%) of 159 diarrheic cattle and in 5 (4.7%) of 107 non-diarrheic cattle. There was no significant difference between the diarrheic and non-diarrheic cattle ($P > 0.05$).

The DNA sequence of the 18S rRNA gene of one of the samples positive for the *C. parvum* type showed 100% similarity with the *C. parvum* HNJ-1 strain (Satoh et al., 2005) and other bovine genotypes of *C. parvum* (AF093490, AF093493, AF108864, AF164102, AF164856, AF161857, AF161858, L16996, and AF093494). Two samples positive for the *C. andersoni* type showed high similarity, 99.7% and 99.6%, to *C. andersoni* (AB089285) and *C. andersoni* strain bjcm (AY954885), respectively. Although their DNA sequences were not completely identical to the published sequences of *C. andersoni*, they were in the same cluster in the phylogenetic tree (data not shown).

## 4. Discussion

The results of oocyst measurement in association with the morphological characterisation described in previous studies (Lindsay et al., 2000; Koyama et al., 2005) revealed that cattle in Viet Nam harbor at least two *Cryptosporidium* species: the small oocysts (mean size: 4.81 ± 0.30 μm), which may be *C. parvum*, and the large ones (mean size: 6.91 ± 0.49 μm), which may be *C. andersoni*.

The overall prevalence of *Cryptosporidium* spp. from cattle in Viet Nam was 35.7%. Regarding specific species, we found that the prevalence of *C. parvum* in cattle was 33.5%. This is higher than the percentage (20.6%) reported by Sevinc et al. (2003) from calves in Turkey using the same method, but lower than that found in pre-weaned calves in Canada (Trotz Williams et al., 2005) and the United States (Fayer et al., 2005), which were 40.6% and 85%, respectively, using PCR techniques. The superior infection rate of *C. parvum*, i.e., 33.5%, as compared to that of *C. andersoni* (5.6%) appears to reflect the dominance of *C. parvum*. Overall, many cattle are carriers for *C. parvum* in Viet Nam, and asymptomatic cattle can serve as an important natural reservoir for this species. Due to its zoonotic nature, more attention should be paid to this parasite for the control of bovine and human cryptosporidiosis.

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As mentioned by Xiao et al. (2004), host age is an important factor that influences the pathogenicity of *Cryptosporidium*. Our study revealed that although *C. parvum* was observed among all age groups, the prevalence of this species in calves less than 6 months old is significantly higher than that in cattle more than 6 months old. This finding is in agreement with previous studies reporting on the relationship of host age with the susceptibility to this parasite (Majewska et al., 2000; Maddox et al., 2006). Additionally, in our study, *C. parvum* accounted for 46.5% of the diarrheic cattle and 14.0% of non-diarrheic cattle. This showed that diarrheic cattle showed significantly higher infection prevalence than their non-diarrheic counterparts, which indicated a strong correlation between infection with *C. parvum* and the occurrence of diarrhea in calves. This correlation was also confirmed by many research investigators such as Atwill (1997), Sevinc et al. (2003), and Trotz Williams et al. (2005). The comparison based on the odds ratio suggested that the risk of diarrhea in calves infected with *C. parvum* was 1.7 times greater than that in the non-infected ones. This phenomenon is remarkably demonstrated in calves less than 3 months old because 70.0% of the diarrheic calves examined in this age group were infected with *C. parvum*. This validated the relationship between host age and susceptibility to the parasite.

On the other hand, *C. andersoni* was detected in 5.6% of all the cattle examined. This value is slightly higher than the 1.5% reported from adult cattle in Japan, using a gene sequence (Koyama et al., 2005) but lower than the 16% detected within a dairy herd in the United Kingdom by using immunofluorescence microscopy (Robinson et al., 2006). Xiao et al. (2004) mentioned that *C. andersoni* infection was found only in cattle, bactrian camels and sheep, and that the infectivity of this species was poor in both bovine and nonbovine hosts. These characterisations accompanied by the narrow range of its host species probably explain the low prevalence of this parasite. In addition, there were no *C. andersoni* oocysts found in any of the cattle less than 3 months old, supporting other reports that chronic *C. andersoni* infection usually occurred in adult cattle with no clinical symptoms (Fayer et al., 1997; Thompson, 2003). However, since *C. andersoni* caused gastritis, reduced milk yield and poor weight gain (Anderson, 1998), the pathogenicity of this organism should be clarified.

Infections of the mixed type involving *C. parvum* and *C. andersoni* constituted 3.4%. Excluding calves less than 3 months old, this mixed infection was observed in all other remaining age groups. Among nine positive cattle, four were associated with diarrhea, and it is difficult to determine whether this diarrhea was caused by *C. parvum*, *C. andersoni*, or other enteric pathogens. Watanabe et al. (2005) detected two groups of *Cryptosporidium* oocysts with different diameters in individual cattle of the same type in Taiwan by using the mZN method. The effects of mixed infection caused by bovine cryptosporidia should be investigated.

DNA sequencing of the 18S rRNA gene identified one sample of the *C. parvum*-type oocysts as *C. parvum* (the bovine genotype), and two samples of the *C. andersoni* type as *C. andersoni*. This is the first genetic identification of *C. parvum* bovine genotype and *C. andersoni* in cattle in Viet Nam.

In this study, the high prevalence of *C. parvum* (33.5%) suggests a potential risk of cryptosporidiosis transmission to the human population in Viet Nam. Further investigations may be needed to clarify the current situation of cryptosporidiosis in human as well as in livestock in Viet Nam.

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# Discrimination of *Cryptosporidium* species by denaturing gradient gel electrophoresis

**Masaaki Satoh**[1] **and Yutaka Nakai**[1, 2]

(1) Laboratory of Animal Health and Management, Graduate School of Agricultural Science, Tohoku University, Naruko-Onsen, Osaki City Miyagi, 989-6711, Japan

(2) Laboratory of Animal Health and Management, Graduate School of Agricultural Science, Tohoku University, Naruko-Onsen, Osaki City Miyagi, 989-6811, Japan

**Yutaka Nakai**
**Email:**

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**Abstract** Denaturing gradient gel electrophoresis (DGGE) was used for the discrimination of three species and one genotype of the protozoan parasite *Cryptosporidium*: the *C. parvum*, *C. andersoni*, *C. muris*, and *C. muris* Japanese field mouse genotype. A set of primers specific for the 18S rRNA gene of *Cryptosporidium* was used in the DGGE; consequently, the four strains showed different banding patterns. This is a potentially convenient and precise method for the discrimination of *Cryptosporidium* spp.

# Introduction

The apicomplexan parasite *Cryptosporidium* infects the microvillous border of the epithelium of the gastrointestinal or respiratory tract in a wide range of vertebrate hosts, including humans. To date, 16 *Cryptosporidium* species have been defined as valid species (Ryan et al. ; Xiao et al. ; Fayer et al. ; Sunnotel et al. ). Among these species, *C. parvum* is associated with human cryptosporidiosis. Whereas, 25 genotypes of *C. parvum* were reported (Xiao et al. ); *C. hominis* and *C. bovis*, which were defined as genotype I and genotype B of *C. parvum*, respectively, are now considered as a new species of *Cryptosporidium* (Morgan-Ryan et al. ; Fayer et al. ). Furthermore, because a number of *Cryptosporidium* spp., including *C. parvum* and *C. muris*, have been isolated from a variety of animal species (Xiao et al. ) and environmental samples, effective and convenient molecular tools for their identification are required.

Molecular tools have been developed to identify and validate the genetic characteristics of *Cryptosporidium* (Widmer et al. ). Among the polymerase chain reaction (PCR)-based techniques, the following have been used for the discrimination of species and genotypes: PCR-restriction fragment length (PCR-RFLP; Awad-el-Kariem et al. ; Spano et al. ; Sulaiman et al. ), random amplification of polymorphic DNA-PCR (RAPD-PCR; Morgan et al. ), single-strand conformation (SSCP; Gasser et al. ),

heteroduplex mobility assay (HMA; Leoni et al. ), and sequence-based characterization of species (Pedraza-Diaz et al. ; Xiao et al. ).

Denaturing gradient gel electrophoresis (DGGE) is an electrophoresis method based on differences in the melting behaviour of double-stranded DNA fragments. The electrophoresis takes place in a vertically placed polyacrylamide gel in a gradient of denaturants. As the concentration of the denaturants is gradually increased, the double-stranded DNA fragments in the solution display a distinct pattern of melting behaviour. It is well known that the composition of the base in the DNA fragment affects its temperature of melting (*T*m), which is the temperature at which the double-stranded DNA fragment dissociates (Myers et al. ). Thus, the DGGE method can separate DNA molecules with minor differences in their nucleotide sequences (Aldridge et al. ; Muyzer et al. ).

The purpose of this study is to apply the DGGE method to detect polymorphism in *Cryptosporidium* spp. by using genus-specific primers against the variable region of the 18S rRNA gene.

# Materials and methods

As reference strains, we used oocysts of *C. parvum* HNJ-1 (Masuda et al. ), *C. muris* RN66 (Iseki ), *C. andersoni* Kawatabi strain (Satoh et al. ), and *C. muris* Japanese field mouse genotype (Hikosaka and Nakai ). These oocysts had been passaged and maintained in the severe combined immunodeficiency (SCID) mice in our laboratory, and the oocysts were purified from the fecal sample of the experimental infected SCID mice using sugar floatation method (Nakai et al. ).

DNA from these samples was extracted by using the MagExtractor genome (Toyobo, Osaka, Japan) after five rounds of freezing and thawing of the $10^3$ oocysts. PCR amplification was carried out to develop a novel DGGE technique. A set of primers (forward primer 5′-AGGATTGACAGATTGA-3′ and reverse primer 5′-ACAGACCTGTTATTGCCT-3′) was constructed to amplify a 212-bp fragment of the 18S rRNA gene corresponding to nucleotides 1181 to 1392 in the sequence of the *C. andersoni* registered in GenBank (accession no. AF093496, defined previously as calf genotype of *C. muris*). The nucleotide sequence of the forward primer contained a 40-base GC clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′) at its 5′- end for stabilizing the melting behaviour of the DNA fragments. For effective PCR-RFLP discrimination of the sequences obtained by PCR amplifications in each strain, we performed a restriction endonuclease analysis by using NEBcutter version 2.0 (website of New England Biolabs, ). The sequence information was obtained from the sequences registered in GenBank (*C. muris* RN66, accession no. AB089284; *C. andersoni*, AB089285; *C. muris* Japanese field mouse genotype, AY642591; and *C. parvum* HNJ-1, AB089290). In this analysis, *C. parvum* was distinguishable from *C. andersoni* and two genotypes of *C. muris* by using SspI and Apo I. In contrast, there is no alternative restriction enzyme site for PCR-RFLP among *C. andersoni*, *C. muris* RN66, and *C. muris* Japanese field mouse genotype. Thus, *C. andersoni* and the two genotypes of *C. muris* cannot be differentiated by using PCR-RFLP (Fig. ).

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C.muris         AAGGATTGACAGATTGATAGCTCTTTCTTGATTTTATGGGTGGTGGTGCATGGCCGTTCT
C.m.genotype    AAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCT
C.andersoni     AAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCT
C.parvum        AAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCT

C.muris         TAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAA
C.m.genotype    TAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAA
C.andersoni     TAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAA
C.parvum        TAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAA

C.muris         ATAGGTAATAGAAAT-TTATTTTTCTATATTATCTTCTTAGAGGGACTTTGCGTGTCTAA
C.m.genotype    ATAGGTAATAGAAAT-TT-TTTTTCTATATTATCTTCTTAGAGGGACTTTGCGTGTCTAA
C.andersoni     ATAGGTAATAGAAAT-TT-TATTTCTATCTTATCTTCTTAGAGGGACTTTGCGTGCCTAA
C.parvum        ATAGACATAAGAAATATTATATTTTTTATCTGTCTTCTTAGAGGGACTTTGTATGTTTAA

C.muris         CGCGAGGAAGTTTGAGGCAATAACAGGTCTGTGA
C.m.genotype    CGCGAGGAAGTTTGAGGCAATAACAGGTCTGTGA
C.andersoni     CGCGAGGAAGTTTGAGGCAATAACAGGTCTGTGA
C.parvum        TACAGGGAAGTTTTAGGCAATAACAGGTCTGTGA
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**Fig. 1** Alignment of the region of 18S rRNA amplified in this study of *C. parvum* HNJ-1, *C. andersoni* Kawatabi strain, *C. muris* RN66 (*C. muris*), and *C. muris* Japanese field mouse genotype (*C.m.genotype*). *Underlined* sequences of each strains indicate pairs of primers used in this study. *Bold* "A" in the sequence of *C. muris* RN66 indicates the difference compared with that of *C. muris* Japanese field mouse genotype. *Box* across two genotypes of *C. muris* and *C. andersoni* indicates Apo I site for discrimination with *C. parvum* HNJ-1, and *arrow* indicates Ssp I site for discrimination *C. parvum* with two genotypes of *C. muris* and *C. andersoni*

The PCR amplicons were analyzed by electrophoresis in 1.5% (wt/vol) agarose gels. DGGE was performed using the Dcode universal mutation detection system (Bio-Rad Laboratories) with 10% (wt/vol) acrylamide gels (in 0.5× TAE containing 20-mM Tris acetate, pH 7.8; 10-mM sodium acetate; and 0.5-mM disodium ethylenediaminetetraacetic acid) containing a linear chemical gradient. The gels were prepared from 10% (wt/vol) acrylamide stock solutions (acrylamide-*N*,*N*-methylene-bisacrylamide, 37:1) containing 0 and 100% denaturants [7-M urea and 10% (vol/vol) formamide, deionized with AG 501-X8 mixed-bed resin (Bio-Rad Laboratories)]. The gels were run for 300 min at 60°C and 130 V, and the band patterns were detected by staining with ethidium bromide and visualized under UV light.

The fragments obtained by DGGE were subjected to gel electrophoretic separation and were eluted by using a cellulose dialysis membrane. The eluted fragments were amplified using the same set of primers but without the GC-rich clamp described previously. These products were purified using MagExtractor-PCR and gel clean up (Toyobo, Osaka, Japan) according to the manufacturer's instructions and sequenced using the ABI Prism BigDye terminator cycle sequencing FS ready reaction kit (Applied Biosystems Japan, Tokyo, Japan) on an automated sequencer (ABI 310).

# Results and discussion

When a gradient of denatured solution ranging from 20 to 50% was used in the DGGE gel, three *Cryptosporidium* species and one genotype showed distinctly different banding patterns (Fig. ). The nucleotide sequences of the fragments of each *Cryptosporidium* species amplified by PCR coincided with those reported previously (data not shown).