

# Prevalence, Risk Factors and Genotyping of *Cryptosporidium* spp. from Feces of Dairy Cows in Saraburi, Kanchanaburi and Nakhon Pathom Provinces

Tawin Inprankaew<sup>1\*</sup>, Pipat Arunvipas<sup>2</sup>, Nongnuch Pinyopanuwat<sup>1</sup>,  
Wissanuwat Chimnoi<sup>1</sup> and Sathaporn Jittapalapong<sup>1</sup>

---

## ABSTRACT

The aims of this study were to determine the prevalence, risk factors and genotype of *Cryptosporidium* spp. in dairy cows raised in Saraburi, Kanchanaburi and Nakhon Pathom Provinces. A total of 400 fecal samples were randomly collected and examined for *Cryptosporidium* spp. by the DMSO-modified acid-fast stain technique and polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). The prevalence of *Cryptosporidium* infection in dairy cows was 0.5% (2/400) by acid-fast staining and 1.3% (5/400) by PCR-RFLP. Age was the only significant risk factor for infection in dairy cows ( $p < 0.05$ ). All PCR positive samples were *C. parvum* (bovine genotype). The result indicated a potential risk of cryptosporidiosis transmission to humans, particularly to workers in close contact with cows.

**Key words:** *Cryptosporidium*, dairy cows, PCR-RFLP, Thailand

## INTRODUCTION

*Cryptosporidium* is a leading cause of diarrhea in humans, particularly in people who have been immunocompromised, and with AIDS patients, the infection can become fatal. There have been reports of outbreaks of cryptosporidiosis, from the contamination of water supplies in the United States, Canada and Japan that have infected more than 25,000 people, (Roach *et al.*, 1993; Lee *et al.*, 2002; Ochiai *et al.*, 2005). Contamination among animals is likely to be the major cause of infection. Contamination by cryptosporidiosis has also been found in dairy products in Thailand (Jittapalapong *et al.*, 2006).

At present, *Cryptosporidium parvum* is categorized into either a human or an animal genotype. The human genotype is only transmitted from human to human. However, the animal genotype can be transmitted from animal to human, human to animal, and animal to animal. Because of its wide range of transmission possibilities, the animal genotype infects readily and is difficult to prevent and control. Zoonotic evidence has been reported (Qkhuyen and Chappell, 2002). O'Handley *et al.* (1999) studied infection of *Cryptosporidium* in young calves, and found that the infection was the main cause of diarrhea in one-month old calves and that the calves were a reservoir host for transmission to

---

<sup>1</sup> Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand.

<sup>2</sup> Department of Farm Resources and Production Medicine, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand.

\* Corresponding author, e-mail: fvetwti@ku.ac.th

other animals and humans. In Thailand, there has been only limited information available and therefore, it is difficult to develop a proper strategy for a control program; information based on the epidemiology of the disease and the biology of this pathogen are still unclear. Saksirisampant *et al.* (2002) have reported that the prevalence of *Cryptosporidium* in 156 AIDS patients with diarrhea was 12.8%. However, no data are available from a non-clinical population.

The animal genotype of *Cryptosporidium* has been found in AIDS patients (Widmer *et al.*, 2000). There are very few reports of cryptosporidiosis in animals in Thailand and the possibility of infection from animal to human is inconclusive. Therefore, information regarding human and animal cryptosporidiosis is needed.

The diagnosis of cryptosporidiosis is mainly carried out using a staining method (Nizeyi *et al.*, 2002). However, this method requires expert examination to discriminate the tiny protozoan oocysts in the stool. Currently, a molecular technique, such as PCR, has become increasingly popular for diagnosis due to its high specificity and sensitivity. Using this technique will enhance epidemiological study and is beneficial for surveillance.

## MATERIALS AND METHODS

### Animals and sampling

A total of 400 Holstein-Friesian cows were randomly selected from 60 dairy farms in Saraburi, Kanchanaburi and Nakhon Pathom provinces, with 20 dairy farms from each province. Five rectal fecal samples of the cows in each herd

were collected and put into plastic bags (Table 1). All samples were transported in an icebox to the Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok. For screening, the fecal samples were diagnosed for the pathogen using a DMSO-modified acid-fast staining technique (Bronsdon, 1984). For the molecular study, the fecal samples were preserved separately in 2.5% potassium dichromate.

### DMSO-modified acid-fast staining technique

One gram of feces was suspended in 10 ml distilled water in a centrifuge tube. Oocysts were concentrated by centrifugation at 900xg for 5 minutes. One drop of suspension was deposited on a slide, then allowed to air dry before staining using a DMSO-modified acid fast-staining technique as reported by Bronsdon (1984). The slide was examined for *Cryptosporidium* oocysts at 400X and 1,000X magnification under an optical light microscope.

### PCR-RFLP

A nested PCR was used to amplify the 825 bp region of the SSU-rDNA gene using four primers under conditions as previously described by Xiao *et al.* (1999). For restriction fragment analyses, 5 µl of the secondary PCR products were digested at 37°C for 1 h in 20 µl reaction mixtures containing 10 U of *SspI* (New England BioLabs, Beverly, Mass) or 10 U of *VspI* (Gibco BRL, Grand Island, New York) and 5 µl of restriction buffer, as recommended by the manufacturer (New England BioLabs, Beverly, Mass and Gibco BRL, Grand Island, New York). The digested products were loaded on 2% agarose gels and visualized

**Table 1** Samples and sources of dairy cows in the study.

	Saraburi province	Kanchanaburi province	Nakhon Pathom province	Total
Calf (< 3month)	29	39	32	100
Dairy cow	100	100	100	300
Total	129	139	132	400

by ethidium bromide staining.

### Statistical analysis

Uni-variate risk factors for the prevalence of *Cryptosporidium* in dairy cows and host, behavioral and environmental factors were initially analyzed using SPSS version 14.0. Only variables significant at  $p \leq 0.25$  in the uni-variate analyses were considered eligible for multivariate risk factor analysis (SPSS version 14.0).

## RESULTS AND DISCUSSION

### Prevalence of *Cryptosporidium* spp.

The overall prevalence of *Cryptosporidium* infection in dairy cows from three provinces in Thailand was 0.5% (2/400) by DMSO-modified acid-fast stain and 1.25% (5/400) by PCR-RFLP (Figure 1).

### Risk factor analysis in association with *Cryptosporidium* spp. infection

Age was the only risk factor for *Cryptosporidium* infection in dairy cows. Calves

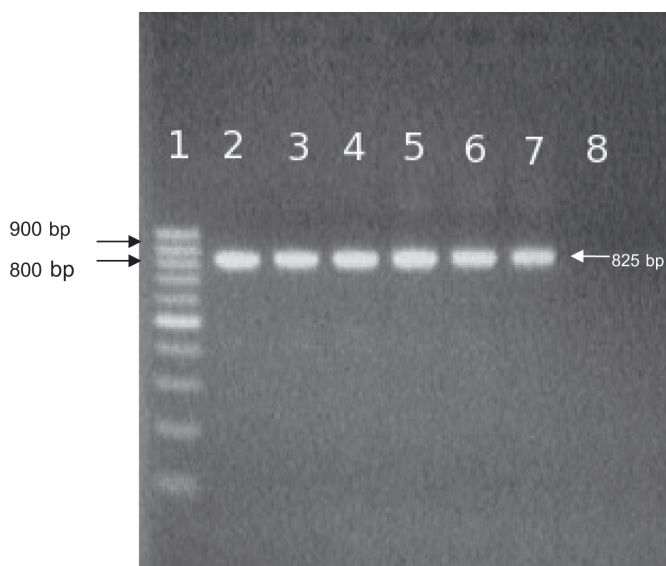
less than three months old were more likely to be infected with *Cryptosporidium* than adult dairy cows (OR= 13.82, 95% confidence intervals = 3.67 to 51.97%,  $p=0.001$ ).

### Genotyping of *Cryptosporidium* spp.

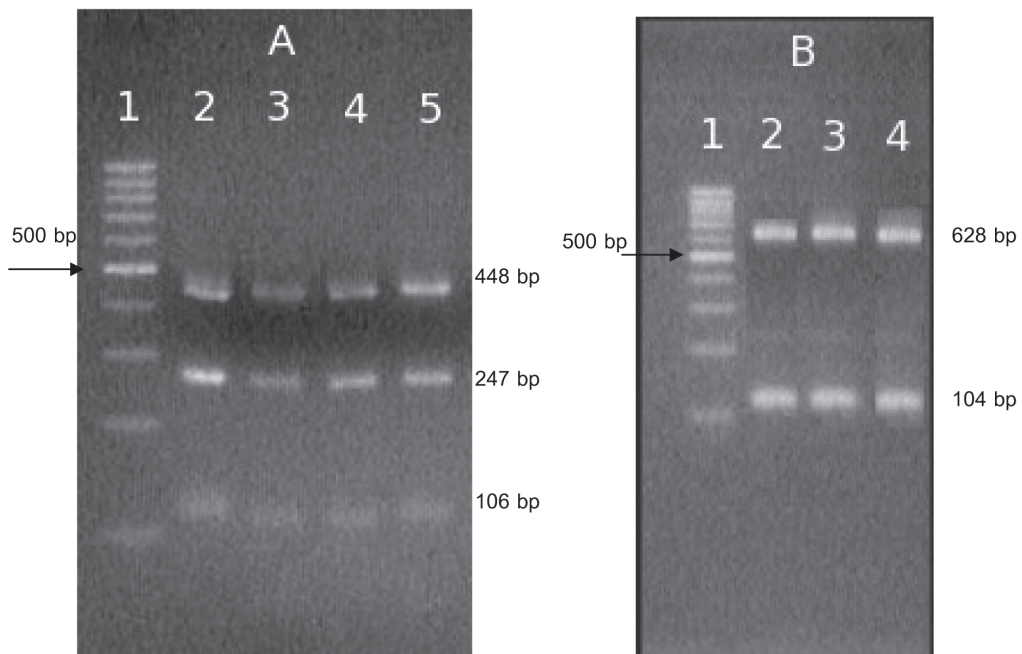
PCR-RFLP analysis showed that all positive samples were *C. parvum* when cut from PCR products using a restriction enzyme (*SspI*). Three bands were found at 448 bp, 247 bp and 106 bp (Figure 2A) which were matched with those previously reported by Xiao *et al.* (1999). On the other hand, when cut with *VspI* enzyme to differentiate the genotype of *C. parvum*, there were two bands at 628 bp and 104 bp (Figure 2B,) which were defined as the bovine genotype. However, *C. andersoni* was not found in this study.

## CONCLUSION

The results showed an overall prevalence of 0.5% *Cryptosporidium* infection in dairy cows from western Thailand by the acid-fast staining technique and 1.3 % by PCR-RFLP. Age was the



**Figure 1** Detection of *Cryptosporidium* spp. by nested PCR. Lane 1 = molecular weight marker, lane 2-6 = positive samples from nested PCR, lane 7 = positive control, lane 8 = negative control.



**Figure 2** Molecular diagnosis of *Cryptosporidium* parasites by a nested PCR-RFLP procedure based on SSU rRNA gene sequences: (A) Species diagnosis of *Cryptosporidium* parasites by *SspI* digestion of the nested PCR products, with lane 1 = molecular weight marker; lanes 2-4 = positive samples from nested PCR; and lane 5 = positive control. (B) Differentiation of two genotypes of *C. parvum* by *VspI* digestion, with lane 1 = molecular weight marker; lanes 2-3, = positive samples from nested PCR; and lane 4, positive control.

only significant risk factor for *Cryptosporidium* infection in this study. Genotype analysis of *Cryptosporidium* in this study found that all samples were *C. parvum* (bovine genotype) and could transmit to humans. *C. parvum* (bovine genotype) was the only positive genotype found in all positives and was likely to transmit to humans.

#### ACKNOWLEDGEMENTS

The authors gratefully acknowledge the collection of field samples by the sixth-year students of the Faculty of Veterinary Medicine, Kasetsart University and also would like to thank all dairy farmers who co-operated with this project.

The project was financially supported by the Intervet Research Fund and the Faculty Research Committee, Faculty of Veterinary Medicine, Kasetsart University.

#### LITERATURE CITED

- Bronsdon, M.A. 1984. Rapid Dimethyl Sulphoxide-Modified Acid-Fast Stain of *Cryptosporidium* Oocyst in Stool Specimens. *J. Clin. Microbiol.* 19: 952-953.
- Jittapalapong, S., N. Pinyopanuwat, W. Chimnoi, C. Siripanth and R.W. Stich. 2006. Prevalence of *Cryptosporidium* among dairy cows in Thailand. *Ann. N. Y. Acad. Sci.* 1081: 328-335.

- Lee, S.M., D.A. Levy, G.F. Craun, M.J. Beach and R.L. Calderon. 2002. Surveillance for waterborne-disease outbreaks United States, 1999-2000. **MMWR. Surveill. Summ.** 51: 1-47.
- Nizeyi, J.B., D. Sebunya and A.J. Dasilva. 2002. Cryptosporidiosis in people sharing habitats with free ranging mountain Gorillas (*Gorilla gorilla beringei*), Uganda. **Am.J. Trop. Med. Hyg.** 66: 442-444.
- Ochiai, Y., C. Takada and M. Hosaka. 2005. Detection and discrimination of *Cryptosporidium parvum* and *C. hominis* in water samples by immunomagnetic separation-PCR. **Appl. Environ. Microbiol.** 71: 898-903.
- O'Handley, R.M., C. Cockwill, T.A. McAllister, M. Jelinski, D.W. Morck and M.E. Olson. 1999. Duration of naturally acquired giardiasis and cryptosporidiosis in dairy calves and their association with diarrhea. **J. Am. Vet. Med. Assoc.** 214: 391-396.
- Qkhuysen, P.C. and C.L. Chappell. 2002. *Cryptosporidium* virulence determinants-are we there yet?. **Inter. J. Parasitol.** 32: 517-525.
- Roach, P.D., M.E. Olson, G. Whitley and P.M. Wallis. 1993. Waterborne *Giardia* cysts and *Cryptosporidium* oocysts in the Yukon, Canada. **Appl. Environ. Microbiol.** 59: 67-73.
- Saksirisampant, W., B. Eampokalap, M. Rattanasrithong, S. Likanonsakul, V. Wiwanitkit, A. Nasingkarn and N. Denmasae. 2002. A prevalence of *Cryptosporidium* infections among Thai HIV-infected patients. **J. Med. Assoc. Thai.** 85 Suppl 1: S424-S428.
- Widmer, G., E.A. Covey and B. Stein. 2000. Host cell apoptosis impairs *Cryptosporidium parvum* development in vitro. **J. Parasitol.** 86: 922-928.
- Xiao, L., L. Escalante and C. Yang. 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the Small-Subunit rDNA gene locus. **Appl. Environ. Microbiol.** 65: 1578-1583.