PREVALENCE AND GENOTYPING OF *CRYPTOSPORIDIUM* SPP FROM DAIRY COW FECAL SAMPLES IN WESTERN THAILAND

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Abstract. The aims of this study were to determine the prevalence of *Cryptosporidium* spp in dairy cows in central Thailand and to investigate the genotype of *Cryptosporidium* spp in this population. A total of 200 fecal samples from dairy cows were collected and examined by the acid-fast staining technique and polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). The prevalence of *Cryptosporidium* infection in dairy cows was 7% (95%CI 3.5-10.5) by acid-fast staining, and 15.5% (95%CI 10.5-20.5) by PCR-RFLP. This is the first report of genetic identification of the *C. parvum* bovine genotype in dairy cows in Thailand. PCR-RFLP analysis showed all positive samples were *C. parvum* (bovine genotype). *C. andersoni* was not found in this study. The only significant risk factor for *Cryptosporidium* infection in dairy cows was age. Calves less than 2 months old were more frequently infected by *Cryptosporidium* than others (OR 13.82, 95%CI 3.67-51.97, p=0.001). Cattle may be a potential source of human cryptosporidiosis.

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

INTRODUCTION

Cryptosporidiosis is a widespread parasitic disease caused by obligate and opportunistic parasites of the genus *Cryptosporidium*. At least five genotypes/species of *Cryptosporidium* infect humans: a human genotype (*C. hominis*) found only in humans and 4 zoonotic genotypes: cattle (*C. parvum*), avian (*C. meleagridis*), cat (*C. felis*) and dog (*C. canis*) found mainly in animals. Zoonosis, as defined by the WHO (1979), describes diseases and infections naturally transmitted between vertebrate animals and humans, and includes some protozoa, such as *Cryptosporidium*. In developing regions of the world, *Cryptosporidium* constitutes part of the complex group of parasitic, bacterial and viral diseases that impair the ability to achieve full potential and impairs development and socio-economic improvement. All diseases included in the WHO Neglected Diseases Initiative are linked with poverty. Management of these diseases requires a comprehensive approach; one of these diseases

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is cryptosporidiosis (Savioli *et al*, 2006).

Cryptosporidium spp has been shown to be composed of at least 16 species, including many genotypes (Ryan *et al*, 2004; Xiao *et al*, 2004; Fayer *et al*, 2005; Slapeta, 2005). Cattle have been described as a reservoir for human infection due to cryptosporidiosis by direct contact or through contamination of drinking water resulting in zoonotic transmission (Nagano *et al*, 2007). Four species of *Cryptosporidium* have been identified in cattle: *C. parvum* (bovine genotype) which represents a zoonotic risk, *C. andersoni* which only infect cattle, *C. bovis* and *Cryptosporidium,* a deer-like genotype.

In Thailand, little is known regarding the prevalence, animal health impact, and zoonotic potential of *Cryptosporidium* infections in dairy cows. To date, surveys of cryptosporidiosis in cattle are few. A prevalence of 0.6% was seen using microscopic examination and 9.4% was seen using *Cryptosporidium*-specific antigen (CSA) in the Nong Pho region of central Thailand (Jittapalapong *et al*, 2006). Further information is needed to understand this parasite among dairy cows in Thailand.

The present study was undertaken to determine the prevalence of *Cryptosporidium* spp in dairy cows in western Thailand and to investigate the genotype of *Cryptosporidium* spp in this population.

MATERIALS AND METHODS

Sample size

A total of 200 Holstein-Friesian cows were randomly selected from 34 dairy cow farms in Ratchaburi and Kanchanaburi Provinces. Ages were classified into 4 groups: cows < 2 months old, two months to 1 year old, >1 to 4 years old and > 4 years old (n=50 per group). Farms were classified into 2 types: those with poor management (n=17) and those with acceptable management (n=17) based on conditions, such as the presence of cement floors and bedding, water quality and cleaning and storage of silage grain and hay. This evaluated if the storage clean or not and the water quality was pure or not. Fecal samples were collected from the cows rectum and transported to the Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok in refrigerated ice boxes for screening using acid-fast staining (Grinberg *et al*, 2002) and preserved separately in 20% dimethyl sulfoxide for molecular testing.

Acid-fast staining technique

One gram of feces was concentrated using the Sheather's sugar flotation technique. One drop from the top of the supernatant was deposited on a slide, let air dry, then stained using an acid-fast staining technique (Grinberg *et al*, 2002). The slide was examined for *Cryptosporidium* oocysts at 400 x and 1,000 x magnification under an optical light microscope.

PCR-RFLP

A nested PCR was used to amplify a 825 bp region of the SSU-rDNA gene using 4 primers as previously described by Xiao *et al* (1999). For restriction fragment analysis, 5 µl of secondary PCR product was digested at 37ºC for 1 hour in 20 µl reaction mixture containing 10 U of *Ssp*I (New England BioLabs, Beverly, MA) or 10 U of *Vsp*I (Gibco BRL, Grand island, NY) and 5 µl of restriction buffer, as recommended by the manufacturers. The digested product was loaded on 2% agarose gel and visualized by ethidium bromide staining. All secondary positive PCR products were sequenced to confirm genotype identification.

Table 1
Age distribution of dairy cows with *Cryptosporidium* infection.

Age	Acid-fast staining technique			PCR-RFLP		
	No. of samples	No. of positive samples	Prevalence (%)	No. of samples	No. of positive samples	Prevalence (%)
< 2 months	50	7	14	50	18	36
2 months-1 year	50	4	8	50	4	8
> 1-4 years	50	3	6	50	9	18
> 4 years	50	0	0	50	0	0
Total	200	14	7	200	31	15.5

Statistical analysis

Descriptive statistics were used to determine the prevalence of cryptosporidiosis using Stata version 8.0.

RESULTS

The prevalence of *Cryptosporidium* in dairy cows was 7% (95%CI 3.5-10.5) by acid-fast staining technique and 15.5% (95%CI 10.5-20.5) by PCR (Table 1). Negative PCR samples were all negative by microscopy. PCR-RFLP analysis revealed in all samples positive for *C. parvum,* when cut by restriction enzyme *(Ssp*I), 3 bands were found at 448 bp, 247 bp and 106 bp, which matched those previously reported by Xiao *et al* (1999). When cut with *Vsp*I enzyme for differentiating the genotype of *C. parvum*, there were 2 bands at 628 bp and 104 bp (Fig 1) which were defined as bovine genotype (Xiao *et al*, 1999). DNA sequencing of the PCR products confirmed all base pairs identical to *C. parvum Ssp*I pattern were *C. parvum*. *C. andersoni* was not found in this study.

The prevalence of *C. parvum* infection varied by age group, ranging from 6% to 14% using the acid-fast staining technique and 8% to 36% using PCR (Table 1). Age was found to be the only significant risk factor for infection in dairy cows. Dairy cows <2 months old were more likely to be infected with *Cryptosporidium* than dairy cows >2 months old (OR 13.82, 95%CI 3.67-51.97, p=0.001). Other risk factors, such as type of farm, were not associated with *C. parvum* infection in this study.

DISCUSSION

Although infections with *Cryptosporidium* spp have been reported in cattle from many parts of the world, the prevalence varies widely, as seen as in Canada (40.6%) (Trotz Williams *et al*, 2005), United States (8.7%) (Fayer *et al*, 2000), Spain (8.4%) (Castro-Hermida *et al*, 2007), Australia (48%) (Becher *et al*, 2004), Japan (12%) (Sakai *et al*, 2003), Vietnam (33.5%) (Nguyen *et al*, 2007) and Malaysia (36%) (Halim *et al*, 2008). Our study found the prevalence of *Cryptosporidium* spp in dairy cows in western Thailand was 7% using the acid-fast staining technique and 15.5% using PCR. This reveals a higher prevalence of *Cryptosporidium* infection in diary cows in Thailand than a previous study (Nuchjangreed *et al*, 2008) using PCR. However, when compared with other countries in the region, the prevalence of

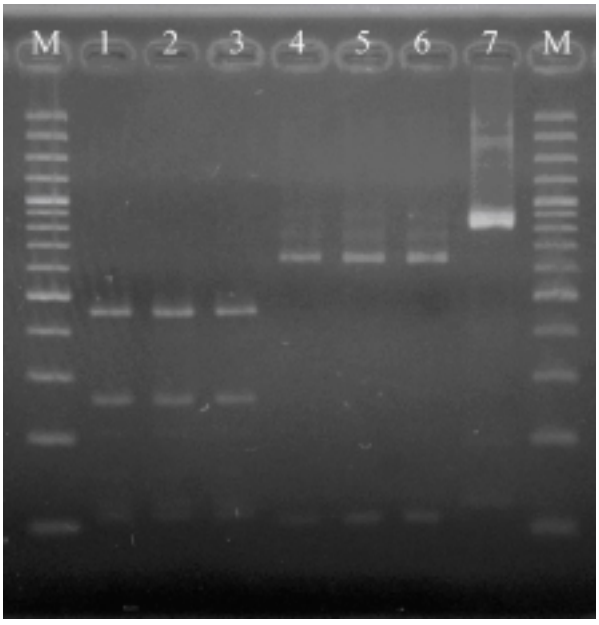

Fig 1–Nested PCR-RFLP based on SSU rRNA gene sequences for identification of *Cryptosporidium* genotypes. Lane M is the molecular weight maker, lanes 1-3 are *C. parvum* with *Ssp*I digestion, lanes 4-6 are *C. parvum* (bovine genotype) with *Vsp*I digestion and lane 7 is an uncut positive control.

C. parvum infection in our study was lower than in Vietnam (33.5%) (Nguyen *et al*, 2007) and Malaysia (36%) (Halim *et al*, 2008).

Molecular characterization of *Cryptosporidium* has given rise to a clearer epidemiological picture, with better information about the zoonotic potential and transmission mechanisms; therefore, SSU-rDNA nested PCR has been used to identify the prevalence of *Cryptosporidium* infection and characterize the genotype of this parasite worldwide (Xiao and Feng, 2008). The current study suggests PCR can be as a method to screen for cryptosporidiosis in Thailand.

This is the first genetic identification of *C. parvum* bovine genotype in dairy cows in Thailand. Other studies have found *C. parvum* as the predominant species infecting humans in Thailand (Tiangtip and Jongwutiwes, 2002). Studies of HIV-infected patients in Thailand with chronic diarrhea found 50% of cryptosporidiosis cases were due to *C. parvum* human genotype following by *C. meleagridis* (20.6%), *C. parvum* bovine genotype (14.7%), *C. felis* (8.8%) and *C. canis* (5.9%) (Gatei *et al*, 2002). *C. parvum* bovine genotype, a common cause of cryptosporidiosis among HIV patients in Thailand, was found to be the main infection in dairy cows in this study. Dairy cows may serve as a reservoir of *C. parvum* and should be considered as a potential source of food and water contamination. *C. parvum* is an important zoonotic species in dairy cows in Thailand.

Xiao *et al* (2004) found host age is an important factor that influences the pathogenicity of *Cryptosporidium*, with young animals being more susceptible to infection than adults. Asymptomatic adult domestic ruminants may act as healthy carriers and may be a source of infection for young animals (Fayer *et al*, 2000; Bomfim *et al*, 2005). Our study found the only significant risk factor for *Cryptosporidium* infection in dairy cows was age. Calves less than 2 months old were more frequently infected by *Cryptosporidium* than others (OR 13.82, 95%CI 3.67-51.97, p=0.001).

Cattle are the most important reservoir for outbreaks of *C. parvum* infection in humans (Hunter and Thompson, 2005). Epidemiological studies also indicate direct contact with farm animals is associated with an increased risk of *C. parvum* infection in humans (Hunter *et al*, 2004).

People who handle cattle frequently, such as dairy farm workers, have higher rates of infection and exposure to *C. parvum*, than those who do not have contact with cattle (Lengerich *et al*, 1993; Siwila *et al*, 2007).

This study showed a high prevalence of *C. parvum* (15.5%) in healthy adult dairy cows. However, asymptomatic cattle can serve as an important natural reservoir for this parasite and can suggests a potential risk of cryptosporidiosis transmission to humans from animal sources in Thailand. Further studies are needed to clarify the epidemiology of cryptosporidiosis in humans and livestock in other parts of Thailand. More attention should be given to the control of bovine and human cryptosporidiosis due to the zoonotic nature of this parasite.

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