

## Seroprevalence of *Cryptosporidium parvum* infection of dairy cows in three northern provinces of Thailand determined by enzyme-linked immunosorbent assay using recombinant antigen CpP23

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### ABSTRACT

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*Cryptosporidium parvum* is the most frequent parasitic agent that causes diarrhoea in AIDS patients in Thailand. Cryptosporidiosis outbreaks in humans may be attributed to contamination of their drinking water from infected dairy pastures. A 23-kDa glycoprotein of *C. parvum* (CpP23) is a sporozoite surface protein that is geographically conserved among *C. parvum* isolates. This glycoprotein is a potentially useful candidate antigen for the diagnosis of cryptosporidiosis by enzyme-linked immunosorbent assay. Therefore, we investigated the seroprevalence of *C. parvum* infection in dairy cows in northern Thailand using an ELISA based on recombinant CpP23 antigen. Sera were randomly collected from 642 dairy cows of 42 small-holder farmers, which had the top three highest number of the dairy cows' population in Northern Thailand, that included Chiang Mai, Chiang Rai and Lumpang provinces. The overall seroprevalence of the infection was 4.4%, and the seropositive rates for the three provinces were 3.3% in Chiang Mai, 5.1% in Chiang Rai and 3% in Lumpang. These results suggest that cattle could play a role in zoonotic cryptosporidiosis in Thailand.

**Keywords:** *Cryptosporidium parvum*, CpP23, dairy cow, ELISA, Thailand

### INTRODUCTION

*Cryptosporidium parvum* is an obligate intracellular protozoan parasite that mainly infects the gastro-

intestinal tract of a wide range of vertebrates including livestock and humans (Current & Garcia 1991). Infection of immunocompetent humans can induce acute, self-limiting diarrhoea. In contrast, infection of immunodeficient individuals frequently results in persistent, severe and life-threatening diarrhoea (Guerrant 1997). *Cryptosporidium parvum* is the most frequent parasitic agent that causes diarrhoea in AIDS patients in Thailand. Moreover, Manatsathit, Tansupasawasdikul, Wanachiwanawin, Setawarin, Suwanagool, Prakasvejakit, Leelakusolwong, Eampokalap & Kachintorn (1996) reported that *C. parvum* is the most common enteric pathogen contrib-

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uting to 20 % of the causes of chronic diarrhoea in AIDS patients in Thailand.

In the developing world, *Cryptosporidium* constitutes part of a complex group of parasitic, bacterial and viral diseases that leads to an inability of infected individuals to achieve their full potential. This complex group of infections classified by the World Health Organization (WHO) as “neglected diseases”, initiate common diseases associated with poverty (Savioli, Smith & Thompson 2006). Cryptosporidiosis outbreaks in humans are thought to be due to contamination of their drinking water from infected dairy pastures.

In a recent survey in the Nong pho dairy region of central Thailand using *Cryptosporidium*-specific antigen (CSA) it was found that the seroprevalence rate of *Cryptosporidium* infection in dairy cows was 9.4% (Jittapalapong, Pinyopanuwat, Chimnoi, Siripanth & Stich 2006). This finding suggested that the infection could also be endemic in other areas in Thailand where the same study had not been done. Hence, there was need for a further investigation into the distribution of cryptosporidiosis in cattle in these regions.

Furthermore, the diagnosis of *C. parvum* infection relies almost exclusively on the microscopic detection of oocysts in faeces, but this method is relatively time consuming, subjective and unreliable especially when oocysts shedding in the faeces has ceased. Nevertheless, a serological test based on ELISA is capable of partially fulfilling diagnostic requirements because serum antibodies against the parasite persist even after oocysts shedding in faeces has ceased.

A 23-kDa glycoprotein of *C. parvum* (CpP23) is a sporozoite surface protein that is geographically conserved among *C. parvum* isolates. This conserved glycoprotein is potentially a useful candidate antigen for the diagnosis of cryptosporidiosis by ELISA since it is likely to detect *C. parvum* strains in various geographical regions (Perryman, Kapil, Jones & Hunt 1999). Therefore, an ELISA based on the recombinant CpP23 antigen was used in this study to investigate the prevalence of *C. parvum* infection in dairy cows in the northern part of Thailand.

## MATERIALS AND METHODS

### Parasite

*Cryptosporidium parvum* isolate (HNJ-1 strain) was used in this study (Abe, Kimata & Iseki 2002).

### Cloning of CpP23 gene

Purified *C. parvum* oocysts were lysed in 0.1 M Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulphate (SDS), 0.1 M NaCl, and 10 mM EDTA and then treated with proteinase K (100 µg/ml) at 55 °C for 2 h. The genomic DNA pellets were extracted with phenol/chloroform followed by ethanol precipitation. The DNA pellets were dissolved in a TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and used as a template DNA for PCR. The truncated CpP23 gene without sequences encoding a hydrophobic signal peptide and a C-terminus was amplified by PCR using oligonucleotide primers, 5'-ACGGAT-CCAAAATGGGTTGTT-3' and 5'-ACGGATCCT-AATTTAGGCATCA-3', both containing *Bam*HI sites introduced to facilitate cloning. The PCR product was digested with *Bam*HI and then cloned into the *Bam*HI site of the bacterial expression vector, pGEX-4T-3 (Promega, USA). The resulting plasmid was designated as pGEX/CpP23.

### Expression of the CpP23 gene in *Escherichia coli*

The recombinant CpP23 gene was expressed as a glutathione *S*-transferase (GST)-fusion protein (GST-CpP23) in JM109 *E. coli* (Promega, USA) as described by Takashima, Xuan, Kimata, Iseki, Kodama, Nagane, Nagasawa, Matsumoto, Mikami & Otsuka 2003 and then purified.

### ELISA

The ELISA was performed as reported by Bannai, Nishigawa, Seo, Nakamura, Zhang, Kimata, Takashima, Li, Igarashi & Xuan 2006. Briefly, the purified GST-CpP23 was diluted to an optimal concentration (5 µg/ml) in a 50 mM carbonate-bicarbonate buffer (pH 9.6), of which 50 µl were added separately to duplicate wells for each sample. Coated plates were incubated at 4 °C overnight. After the unabsorbed antigen was discarded, the wells were blocked with PBS containing 3% skim milk (blocking solution, 100 µl per well) at 37 °C for 1 h. The plates were then washed once with PBS containing 0.05% Tween 20 (PBS-T). Fifty microlitres of serum diluted in the blocking solution (1:100) were added to each well and incubated at 37 °C for 1 h. After incubation, the wells were washed six times with PBS-T and subsequently incubated with 50 µl of goat anti-bovine IgG-horseradish peroxidase conjugate (ICN Biochemical, USA) (1:4 000) at 37 °C for 1 h. After six washes, 100 µl of substrate solution [0.05% 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sul-

phonic), 0.2 M sodium phosphate, 0.1 M citric acid, 0.003% H<sub>2</sub>O<sub>2</sub>] were added to each well. After 1 h reaction at room temperature, the optimal density (OD) was read at 415 nm by using an MTP-120 ELISA reader (Corona Electric, Japan). The ELISA titre was expressed as the reciprocal of the maximum dilution that showed an ELISA value equal to or greater than 0.1, which is the difference in absorbance between that for the antigen (GST-CpP23) well and that of the control antigen (GST) well.

### Sera

Blood samples ( $n = 642$ ) were collected from the caudal or jugular vein of dairy cows belonging to 42

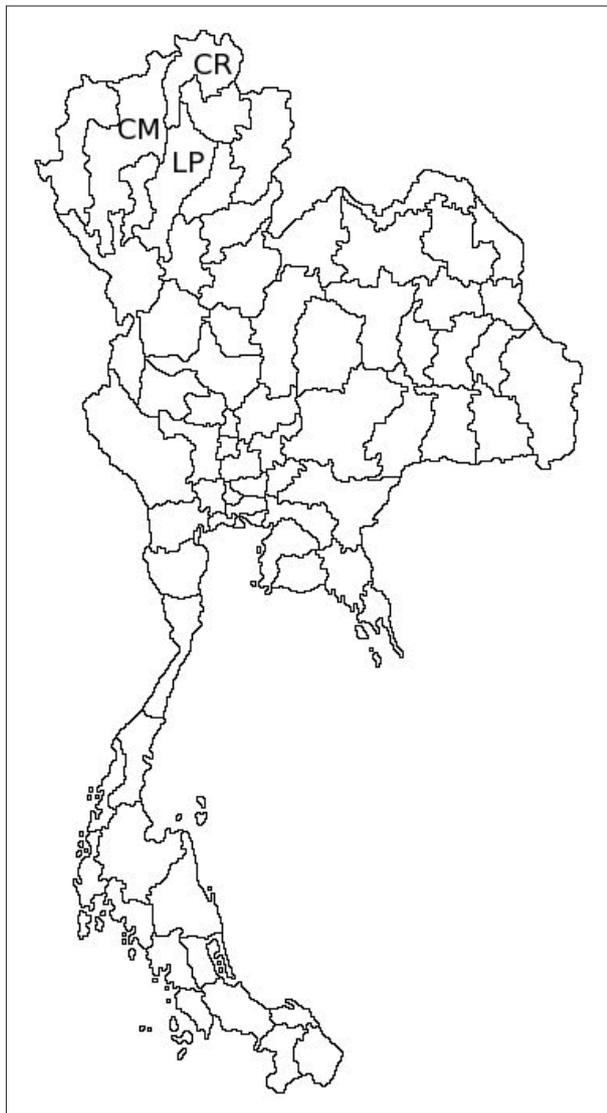


FIG. 1 Map of Thailand showing the location of the three provinces from which blood samples were collected: Chiang Mai Province (CM), Chiang Rai Province (CR) and Lumpang Province (LP)

small-holder farmers of the top three highest number of dairy cow population in Chiang Mai (150 samples), Chiang Rai (392 samples) and Lumpang provinces (100 samples) (Fig. 1). Sera were separated after sedimentation of blood cells and were stored at  $-20^{\circ}\text{C}$  until use.

## RESULTS AND DISCUSSION

The truncated CpP23 gene without sequences encoding a hydrophobic signal peptide and a C-terminus was inserted into the bacterial expression vector pGEX-4T-3, and expressed as a GST fusion protein (GST-CpP23) in *E. coli*. The GST-CpP23 reacted strongly with sera from *C. parvum*-infected cattle but not with sera from uninfected cattle (data not shown). This result indicated that the GST-CpP23 can be adopted as a useful antigen for serodiagnosis of *C. parvum* infection.

The prevalence of *C. parvum* infection in cattle has been reported in many parts of the world, such as Canada (40.6%) (Trotz Williams, Jarvie, Martin, Leslie & Peregrine 2005), USA (8.7%) (Fayer, Trout & Graczyk 2000), Spain (8.4%) (Castro-Hermida, Almeida, González-Warleta, Correia da Costa, Rumbo-Lorenzo & Mezo 2007), Australia (48%) (Becher, Robertson, Fraser, Palmer & Thompson 2004), Japan (12%) (Sakai, Tsushima, Nagasawa, Ducusin, Tanabe, Uzaka & Sarashina 2003) and Vietnam (33.5%) (Nguyen, Nguyen, Le, Le Hua, Van Nguyen, Honma & Nakai 2007). In Thailand, most investigations have been carried out in humans, but less is known of the infection in animals, particularly in dairy cows, which might be the carrier of the parasite.

The ELISA with GST-CpP23 as antigen was used to investigate the seroprevalence of *C. parvum* infection in dairy cows in the northern part of Thailand. The overall seroprevalence of *C. parvum* infection was 4.4% (28/642). This is lower than the prevalence (9.4%) in Nong Pho dairy areas (the central part of Thailand) as has previously been reported (Jittapalapong *et al.* 2006). The seropositive rates in three provinces were 3.3% (5/150) in Chiang Mai, 5.1% (20/392) in Chiang Rai and 3% (3/100) in Lumpang (Table 1). Chiang Rai Province was the highest endemic area for *C. parvum* infection in this investigation. The numbers of dairy farm harbouring infected cows were from 16 to 42 (38%) and the farm infection prevalence was 37.5% (3/8), 37.9% (11/29) and 40% (2/5) in Chiang Mai, Chiang Rai and Lumpang respectively. A widespread infection rate in dairy cows due to *C. parvum* in the northern

TABLE 1 Seroprevalence of *C. parvum* infection in the dairy cows determined by ELISA

Provinces	<i>C. parvum</i>		
	No. of samples	No. of positive samples	Seroprevalence (%)
Chiang Rai	392	20	5.1
Chiang Mai	150	5	3.3
Lumpang	100	3	3.0
<b>Total</b>	<b>642</b>	<b>28</b>	<b>4.4</b>

TABLE 2 Seroprevalence of *C. parvum* infection in different age groups of the dairy cows or heifers

Provinces	Age (year)	No. of animals	No. of positive serum samples	Seroprevalence (%)
Chiang Mai	< 1	11	0	0
	> 1–5	96	5	5.21
	> 5	43	0	0
Chiang Rai	< 1	0	0	0
	> 1–5	212	9	4.25
	> 5	180	11	6.11
Lumpang	< 1	7	1	14.29
	> 1–5	76	2	2.63
	> 5	17	0	0

region of Thailand was therefore determined in this study.

The seroprevalence of *C. parvum* infection rates varied in different age groups, ranging from 2.63% to 14.29% (Table 2). The age of the animal is one of the most important risk factors associated with cryptosporidiosis. However, the occurrence of *C. parvum* infection in the current study was found in all age groups but no statistically significant differences between the age groups were determined. Nevertheless, it has been shown that asymptomatic adult domestic ruminants, such as dairy cows, sheep and goats may act as carriers and may be a source of infection for younger animals (Fayer *et al.* 2000; Bomfim, Huber, Gomes & Alves 2005).

The current study demonstrated a low prevalence rate of *C. parvum* in dairy cows; however, asymptomatic cattle can serve as important natural reservoirs for this parasite. These data also indicate a potential risk of *C. parvum* transmission to the human population in Thailand and the need for more attention to be paid to its control in dairy cattle because of its zoonotic nature.

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