Molecular and Cellular Probes

Molecular analysis of *Cryptosporidium* from cattle from five states of Peninsular Malaysia

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1. Introduction

Cryptosporidium species are obligate intracellular protistan parasites that can infect a wide range of animals and human hosts worldwide [1]. Cattle are considered to represent key animal reservoir hosts of *Cryptosporidium*. Importantly, cryptosporidiosis is recognized as one of the major causes of neonatal calf diarrhoea, resulting in weight loss and growth retardation, morbidity and, in severe cases, death, leading to considerable economic losses [2,3]. Furthermore, several foodborne and waterborne outbreaks of human *Cryptosporidium* infections have also been attributed to food produce and water contamination by cattle manure [2,5].

The traditional approach for the diagnosis of infection relies on the microscopic detection of cysts or oocysts in stool samples, but this approach is unable to distinguish the different *Cryptosporidium* taxa based on morphometric or other phenotypic characteristics, due to lack of differentiating morphological features [6,7]. Therefore, molecular tools, such as polymerase chain reaction (PCR)-based methods have been employed, targeting taxonomically informative loci to circumvent this limitation [2,8]. Currently, at least 23 different species of *Cryptosporidium* and more than 70 genotypes have been recognized, with new genotypes continually being identified by molecular means [2]. To date, seven species have been recorded in cattle, which include *C. hominis*, *C. parvum*, *C. bovis*, *C. ryanae*, *C. andersoni* and *C. suis*, as well as two genotypes of *Cryptosporidium* (i.e. pig genotype II and a new *C. suis*-like genotype) [9,10].

In Malaysia, the cattle industry is one of the key components of the agricultural sector, providing gainful employment and producing high quality protein (red meat) and milk for human population. In 2014, Malaysia's ex-farm value of beef was RM 1.25 billion and another RM 150.54 million for milk contributing to a total of 9.12% of the total national output of livestock products [11]. Despite the importance of the cattle industry, there are only two published studies of *Cryptosporidium*/cryptosporidiosis of cattle in Malaysia using microscopic methods [12,13], and two others using molecular techniques [14,15]. Given this lack of information, further studies of different age groups of cattle from different geographical areas are needed to obtain more information on prevalence, distribution and health and economic impact of bovine cryptosporidiosis in Malaysia. Therefore, in the present study, we employed a PCR-based approach targeting genetic markers in the small subunit of ribosomal RNA (*SSU*) and 60 kDa glycoprotein (*gp60*) [16] to genetically characterize *Cryptosporidium* in faecal samples from a cohort of 215 asymptomatic cattle from six farms from five states of Peninsular Malaysia. The aim was to evaluate the species and/or genotypes that they harbour and whether the infections might have zoonotic potential.

2. Materials and methods

2.1. Ethical consideration

The study protocol was approved by the Ethics Committee of the University Malaya Medical Center, Malaysia (MEC Ref. No. 896.36). Permission for the study to be conducted on animal farms was obtained from owners prior to sample collection.

2.2. Faecal sample collection

A total of 215 faecal samples were collected from cattle from six different farms located in east coast (Farm A, Kuantan, Pahang state), northern (farm B, Sungai Siput, Perak state), Central (farm C, Serdang, Selangor state; Farm D, Jerantut, Pahang state) and southern (farm E, Jelai Gemas, Negeri Sembilan state; farm F, Ayer Hitam, Johor state) parts of Peninsular Malaysia (Fig 1). The six farms belong to the Department of Veterinary Services, Ministry of Agriculture and Agro-Based Industry, Malaysia. Faecal samples were collected rectally from individual animals and kept at 2 to 8 ºC immediately after sampling, and frozen at -20 ºC for subsequent DNA isolation and molecular testing.

2.3. Isolation of genomic DNA from faecal samples and PCR amplification

Genomic DNA was isolated from each faecal sample using the PowerSoil DNA Isolation Kit (MoBIO, USA), according to the manufacturer's protocol, and then frozen at -20 ºC until use. Each genomic DNA sample was subjected to nested PCR, for *Cryptosporidium* employing regions (designated p*SSU* and p*gp60*) within the small subunit nuclear ribosomal RNA and 60 kDa glycoprotein genes.

For human-infective *Cryptosporidium*, primary PCR was carried out using primers gp15-ATG (forward: 5'-ATGAGATTGTCGCCTCATTATC-3') and gp15-STOP (reverse: 5'-TTACAACACGAATAAGGCTGC-3') [17], followed by the secondary reaction to amplify a portion of the *gp60* gene (called p*gp60*; 250-380 bp) using primers gp15-15A (forward: 5'-GCCGTTCCACTCAGAGGAAC-3') and gp15-15E (reverse: 5'-CCACATTACAAATGAAGTGCCGC-3') [18]. Both primary and secondary PCRs were performed in a volume of 50 µl containing 3.0 mM of $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (dNTP), 25 pmol of each oligonucleotide primer and 1.25 U of Go*Taq* (Promega) DNA polymerase in standard PCR buffer (Promega, USA). Primary amplification of p*gp60* utilized the cycling protocol which included an initial cycle of 94 °C for 5 min (initial denaturation), followed by 35 cycles of 94 °C for 30 sec (denaturation), 55 °C for 45 sec (annealing) and 72 °C for 1 min (extension), with a final extension of 72 °C/10 min. From 1 µL of primary amplicon, p*gp60* was amplified using a cycling protocol of 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec, with a final extension at 72 °C for 10 min.

For the amplification of p*SSU* for *Cryptosporidium*, nested PCR was performed in a 50 µL volume containing 2.0 mM of $MgCl_2$, 200 µM of each deoxynucleotide triphosphate

(dNTP), 25 pmol of each oligonucleotide primer and 1.25 U of Mango*Taq* polymerase in a standard buffer (Bioline, USA). Primary reaction was performed using primers XF2 (forward: 5'-GGAAGGGTTGTATTTATTAGATAAAG-3') and XR2 (reverse: 5'-AAGGAGTAAGGAACAACCTCCA-3') [19], followed by a nested amplification of a portion of SSU gene (p*SSU*) using primer set p*SSU*f (forward: 5'-AAAGCTCGTAGTTGGATTTCTGTT-3') and p*SSU*r (reverse: 5'-ACCTCTGACTGTTAAATACRAATGC-3') [20]. Primary amplification was carried out at 94 °C for 5 min (initial denaturation), followed by 30 cycles of 94 °C for 45 sec (denaturation), 45 °C for 2 min (annealing) and 72 °C for 1.5 min (extension), with a final extension of 72 °C for 10 min. From 1µL of primary amplicon, the secondary amplification was performed using a cycling protocol of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 55°C for 30 sec, and 72 °C for 30 sec, with a final extension of 72 °C for 10 min.

2.4. DNA sequence analysis

Secondary PCR products were purified using ExoSAP-IT® (Fermentas, USA), according to the manufacturer's instructions, and then subjected to direct, automated sequencing (BigDye Terminator v.3.1 chemistry, Applied Biosystems, USA) using the forward and reverse primers employed in secondary PCR. The quality of each sequence was assessed based on the corresponding chromatogram, and sequences were compared with reference sequences using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST).

3. Results and discussion

All 215 genomic DNA samples derived from cattle faecal samples from six farms in five different states in Malaysia were subjected to the genetic analysis of *Cryptosporidium*. Although p*gp60* was not amplified from any of the 215 genomic DNA samples tested, the p*SSU* region was amplified from seven, resulting in an overall prevalence of 3.2%. Cattle on four farms (A, C, D and F) were test-positive for *Cryptosporidium*. Among farms, the prevalence ranged from 0% to 10.8% (see Table 1).

Two *Cryptosporidium* species (*C. bovis* and *C. ryanae*) were detected; 2 (0.9%) and 5 (2.3%) samples had *C. bovis* and *C. ryanae*, respectively. No mixed infection was detected in any of the tested samples (see Table 1). *C. bovis* was found only on farm F from Air Hitam, Johor (5.4%) among cattle of 2.5 years old, whereas *C. ryanae* was isolated from cattle of 8 months to 5 years of age on farm A from Kuantan, Pahang (2.7%), farm C from Serdang, Selangor (1.1%), farm D from Jerantut, Pahang (2.9%) and farm F from Air Hitam, Johor (5.4%). The prevalence of *Cryptosporidium* varied from state to state in Peninsular Malaysia. According to farms, farm F had the highest number (i.e., four) of infected cattle. The cattle that harboured *C. bovis* and *C. ryanae* were all ~ 2.5 years of age and of the Mafriwal breed. Farms A, C and D each had one infected animal that was test-positive for *C. ryanae*. The infected cattle on farm A was of the Nellore breed, farm C was of the cross breed of Fresian x Shahiwal, whilst on farm D it was of the Kedah-Kelantan breed.

DNA sequence analysis indicated that most sequences obtained were identical to those of reference sequences for *C. bovis* (KM110048) or *C. ryanae* (EU410344) retrieved from the GenBank. However, population variants of *C. ryanae* were detected in two samples, whereby nucleotide sequences from samples A24 (14 months old) and C61 (5 years old) possessed an insertion of T (at one position; no. 61). The p*SSU* sequences were identical to *C. ryanae* from water buffaloes in Egypt (AB777177 and AB777178) [21] and cattle in Australia (KC778535) [22], China (KP7930130) [23], France (GU124627) [24] and Sri Lanka (KF891289) [25]. Genetic variation within *C. ryanae* has also been reported previously in a number of studies [21, 25-27].

Over the years, *Cryptosporidium* has been gaining attention as an important pathogenic enteric protozoan parasite in Malaysia, with high rates of cryptosporidiosis reported in humans, especially in children and AIDS patients [28,29]. However, in contrast to information in humans, there is a paucity of information on the genetic analysis of *Cryptosporidium* spp. of animals, particularly cattle. To date, only two studies have used molecular diagnostic tools to identify *Cryptosporidium* infecting cattle in Malaysia [13,14]. The first data on *Cryptosporidium* genotypes from cattle in Malaysia was reported by Halim and his colleagues in 2008 [13], whereby *C. parvum* was found to be commonest species, followed by *C. ryanae* in 50 diarrhoeic calves of between 1 and 6 months of age from Selangor, Malaysia.

In 2011, a cross-sectional study was conducted by Muhid and his team [14] to determine the prevalence and genotypes of *Cryptosporidium* infections in pre-weaned and post-weaned dairy cattle in Johor, Malaysia. The most prevalent species in pre-weaned calves was *C. parvum*, followed by *C. bovis*, *C. andersoni* and *C. ryanae*. On the other hand, *C. bovis*, *C. andersoni* and *C. ryanae* were detected in the post-weaned calves. The species and genotypes of *Cryptosporidium* infecting cattle are known to vary according to the host age [2] and geographical distribution [30,31]. Given that these two studies focused more on calves aged 1 day to 12 months old from only one particular region of Malaysia, our study has included cattle with a broader age range (i.e. 5 months to 13 years old) sampled from farms located in five states in Peninsular Malaysia to provide a 'broader picture' of *Cryptosporidium* infections in cattle in Malaysia.

The current study has demonstrated a relatively low prevalence of *Cryptosporidium* infection (3.3%) in cattle on the six studied farms. This percentage is lower than those previously reported in studies conducted in Malaysia [13,14], Thailand [32-34], Vietnam [35], Sri Lanka [25] and China [36], which reported of prevalences ranging from 9.4% to 62.1%. In the present study, only *C. bovis* and *C. ryanae* were identified in the cattle, whilst *C. parvum*, the most common zoonotic species in cattle [2,37] was not detected in any of the tested samples. This is due to the age of the cattle tested, as most of the studied cattle (73%) were $\geqslant$ 2 years of age.

Numerous molecular epidemiological studies of *Cryptosporidium* species have shown an age-associated distribution in cattle [38-41]. *C. parvum* usually predominates in pre-weaned calves (< 3 months) with frequent diarrhoea; *C. bovis* and *C. ryanae* are commonly found in post-weaned calves and yearling, whereas *C. andersoni* is mostly identified in older calves and adult cattle [38,42]. Preliminary evidence suggests that *C. bovis* and *C. ryanae* are not associated with any signs of disease [43,44], and this was in agreement with the present study, as the cattle studied were asymptomatic. As the different species have different pathogenicity

in cattle and different infectivity for humans, identifying the factors that contribute to the occurrence of these different species in cattle in future studies is therefore critical to the understanding of economic and public health importance and transmission of cryptosporidiosis in cattle. Future studies should focus on a large-scale study in pre-weaned and weaned calves in rural communities of these regions of Malaysia to assess their zoonotic potential.

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References

[1] U. Ryan, R. Fayer, L. Xiao, *Cryptosporidium* species in humans and animals: current understanding and research needs, Parasitol. 141 (2014) 1667-1685.

[2] L. Xiao, Molecular epidemiology of cryptosporidiosis: An update, Exp. Parasitol. 124 (2010) 80-89.

[3] W. Zhang, R. Wang, F. Yang, L. Zhang, J. Cao, X. Zhang, H. Ling, A. Liu, Y. Shen, Distribution and genetic characterizations of *Cryptosporidium* spp. in pre-weaned dairy calves in Northeastern China's Heilongjiang Province, PLoS One. 8 (2013) e54857.

[4] S. Glaberman, J.E. Moore, C.J. Lowery, R.M. Chalmers, I. Sulaiman, K. Elwin, P.J. Rooney, B.C. Millar, J.S. Dooley, A.A. Lal, L. Xiao, Three drinking-water-associated cryptosporidiosis outbreaks, Northern Ireland, Emerg. Infect. Dis. 8 (2002) 631–633.

[5] B.G. Blackburn, J.M. Mazurek, M. Hlavsa, J. Park, M. Tillapaw, M. Parrish, E. Salehi, W. Franks, E. Koch, F. Smith, L. Xiao, M. Arrowood, V. Hill, A. da Silva, S. Johnston, J.L. Jones, Cryptosporidiosis associated with ozonated apple cider, Emerg. Infect. Dis. 12 (2006) 684-686.

[6] A.R. Jex, H.V. Smith, P.T. Monis, B.E. Campbell, R.B. Gasser, *Cryptosporidium*-biotechnological advances in the detection, diagnosis and analysis of genetic variation, Biotechnol. Adv. 26 (2008) 304-317.

[7] D. Stark, S.E. Al-Qassab, J.L. Barratt, K. Stanley, T. Roberts, D. Marriott, J. Harkness, J.T. Ellis, Evaluation of multiplex tandem real-time PCR for detection of *Cryptosporidium* spp., *Dientamoeba fragilis*, *Entamoeba histolytica*, and *Giardia intestinalis* in clinical stool samples, J. Clin. Microbiol. 49 (2011) 257-262.

[8] M.A. Laxer, B.K. Timblin, R.J. Patel, DNA sequences for the specific detection of *Cryptosporidium parvum* by the polymerase chain reaction, Am. J. Trop. Med. Hyg. 45 (1991) 688-694.

[9] J.M. Trout, M. Santín, Livestock, In: R. Fayer, L. Xiao, (Eds) *Cryptosporidium* and Cryptosporidiosis, CRC Press, Boca Raton, FL, 2008, pp. 451-483.

[10] H. Abeywardena, A.R. Jex, R.B. Gasser, A perspective on *Cryptosporidium* and *Giardia*, with an emphasis on bovines and recent epidemiological findings, Adv. Parasitol. 88 (2015) 243-301.

[11] Department of Veterinary Services, Ministry of Agriculture and Agro-based Industry Malaysia. Malaysia: Ex-Farm Value of Livestock Products (RM Million), 2005-2014, 2014. http://www.dvs.gov.my/documents/10157/d876f4a1-96fd-453c-9672-78b96ff7eccf

[12] P.L. See, Detection of *Cryptosporidium* spp. in cattle farms in Selangor, Bachelor thesis, Universiti Kebangsaan Malaysia, 1997, pp. 35.

[13] S. Farizawati, Y.A.L Lim, R.A. Ahmad, C.T. Fatimah, Y. Siti-Nor, Contribution of cattle farms towards river contamination with *Giardia* cysts and *Cryptosporidium* oocysts in Sungai Langat Basin, Trop. Biomed. 22 (2005) 89-98.

[14] N.A. Halim, J. Plutzer, M.A. Bakheit, P. Karanis, First report of *Cryptosporidium* deer-like genotype in Malaysian cattle, Vet. Parasitol. 152 (2008) 325-329.

[15] A. Muhid, I. Robertson, J. Ng, U. Ryan, Prevalence of and management factors contributing to *Cryptosporidium* sp. infection in pre-weaned and post-weaned calves in Johor, Malaysia, Exp. Parasitol. 127 (2011) 534-538.

.[16] A.R. Jex, R.B. Gasser, Genetic richness and diversity in *Cryptosporidium hominis* and *C. parvum* reveals major knowledge gaps and a need for the application of “next generation” technologies – research review, Biotechnol. Adv. 28 (2010) 17-26.

[17] W.B. Strong, J. Gut, R.G. Nelson, Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products, Infect. Immun. 68 (2000) 4117-4134.

[18] M. Mallon, A. MacLeod, J. Wastling, H. Smith, B. Reilly, A. Trait, Population structures and the role of genetic exchange in the zoonotic pathogen *Cryptosporidium parvum*, J. Mol. Evol. 56 (2003) 407-417.

[19] L. Xiao, U.M. Morgan, J. Limor, A. Escalante, M. Arrowood, W. Shulaw, R.C. Thompson, R. Fayer, A.A. Lal, Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species, Appl. Environ. Microbiol. 65 (1999) 3386-3391.

[20] M.J. Nolan, A.R. Jex, S.R. Haydon, M.A. Stevens, R.B. Gasser, Molecular detection of *Cryptosporidium cuniculus* in rabbits in Australia, Infect. Genet. Evol. 10 (2010) 1179-1187.

[21] S. Amer, S. Zidan, Y. Feng, H. Adamu, N. Li, L. Xiao, Identity and public health potential of *Cryptosporidium* spp. in water buffalo calves, Vet. Parasitol. 191 (2013) 123-127.

[22] H. Abeywardena, A.R. Jex, S.M. Firestone, S. McPhee, N. Driessen, A.V. Koehler, S.R. Haydon, G. von Samson-Himmerlstjerna, M.A. Stevens, R.B. Gasser, Assessing calves as carriers of *Cryptosporidium* and *Giardia* with zoonotic potential on dairy and beef farms within a water catchment area by mutation scanning, Electrophoresis. 34 (2013) 2259-2267.

[23] M. Qi, H. Wang, B. Jing, D. Wang, R. Wang, L. Zhang, Occurrence and molecular identification of *Cryptosporidium* spp. in dairy calves in Xinjiang, Northwestern China, Vet. Parasitol. 212 (2015) 404-407.

[24] J. Follet, K. Guyot, H. Leruste, A. Follet-Dumoulin, O. Hammouma-Ghelboun, G. Certad, E. Dei-Cas, P. Halama, *Cryptosporidium* infection in a veal calf cohort in France: molecular characterization of species in a longitudinal study, Vet. Res. 42 (2011) 116.

[25] H. Abeywardena, A.R. Jex, A.V. Koehler, R.P. Rajapakse, K. Udayawarna, S.R. Haydon, M.A. Stevens, R.B. Gasser, First molecular characterization of *Cryptosporidium* and *Giardia* from bovines (*Bos taurus* and *Bubalus bubalis*) in Sri Lanka: unexpected absence of *C. parvum* from pre-weaned calves, Parasit. Vectors. 7 (2014) 75.

[26] Y. Feng, S.R. Karna, T.K. Dearen, D.K. Singh, L.N. Adhikari, A. Shrestha, L. Xiao, Common occurrence of a unique *Cryptosporidium ryanae* variant in zebu cattle and water buffaloes in the buffer zone of the Chitwan National Park, Nepal, Vet. Parasitol. 185 (2012) 309-314.

[27] F. Murakoshi, L. Xiao, R. Marsubara, R. Sato, Y. Kato, T. Sasaki, Y. Fukuda, C. Tada, Y. Nakai, Molecular characterization of *Cryptosporidium* spp. in grazing beef cattle in

Japan, Vet. Parasitol. 187 (2012) 123-128.
[28] Y.A.L Lim, R.A. Ahmad, H.V. Smith, Current status and future trends in *Cryptosporidium* and *Giardia* epidemiology in Malaysia, J. Water Health. 6 (2008) 239-254.
[29] Y.A.L. Lim, A.R. Jex, H.V. Smith, R.B. Gasser, Cryptosporidiosis in Southeast Asia: What's out There? Adv. Parasitol. 71 (2010) 1-31.
[30] Y. Feng, Y. Ortega, G. He, P. Das, M. Xu, X. Zhang, R. Fayer, W. Gatei, V. Cama, L Xiao,. Wide geographic distribution of *Cryptosporidium bovis* and the deer-like genotype in bovine, Vet. Parasitol. 144 (2007) 1-9.
[31] J. Ng, R. Yang, S. McCarthy, C. Gordon, N. Hijjawi, U. Ryan, Molecular characterization of *Cryptosporidium* and *Giardia* in pre-weaned calves in Western Australia and New South Wales, Vet. Parasitol. 176 (2011) 145-150.
[32] S. Jittapalapong, N. Pinyopanuwat, W. Chimnoi, C. Siripanth, R.W. Stich, Prevalence of *Cryptosporidium* among dairy cows in Thailand, Ann. N. Y. Acad. Sci. 1081 (2006) 328-335.
[33] C. Nuchjangreed, K. Boonrod, J. Ongerth, P. Karanis, Prevalence and molecular characterization of human and bovine *Cryptosporidium* isolates in Thailand, Parasitol. Res. 103 (2008) 1347-1353.
[34] T. Inpankaew, T. Jiyipong, N. Pinyopanuwat, W. Chimnoi, R.C. Thompson, S. Jittapalapong, Prevalence and genotyping of *Cryptosporidium* spp. from dairy cow faecal samples in western Thailand, Southeast. Asian. J. Trop. Med. Public. Health. 41 (2010) 770-775.
[35] S.T. Nguyen, D.T. Nguyen, D.Q. Lee, L.N. Le Hua, T. Van Nguyen, H. Honma, Y. Nakai, Prevalence and first genetic identification of *Cryptosporidium* spp. in cattle in central Viet Nam, Vet. Parasitol. 150 (2007) 357-361.
[36] J. Ma, P. Li, X. Zhao, H. Xu, W. Wu, Y. Wang, Y. Guo, L. Wang, Y. Feng, L. Xiao, Occurrence and molecular characterization of *Cryptosporidium* spp. and *Enterocytozoon bieneusi* in dairy cattle, beef cattle and water buffaloes in China, Vet. Parasitol. 207 (2015) 220-227.
[37] L. Xiao, Y. Feng, Zoonotic cryptosporidiosis, FEMS. Immunol. Med. Microbiol. 52 (2008) 309-323.
[38] M. Santín, J.M. Trout, L. Xiao, L. Zhou, E. Greiner, R. Fayer, Prevalence and age-related variation of *Cryptosporidium* species and genotypes in dairy calves, Vet. Parasitol. 122 (2004) 103-117.
[39] R. Fayer, M. Santín, J.M. Trout, E. Greiner, Prevalence of species and genotypes of *Cryptosporidium* found in 1-2 year-old dairy cattle in the eastern United States, Vet. Parasitol. 135 (2006) 105-112.
[40] M. Kváč, M. Kouba, J. Vitovec, Age-related and housing-dependence of *Cryptosporidium* infection of calves from dairy and beef herds in South Bohemia, Czech Republic, Vet. Parasitol. 137 (2006) 202-209.
[41] R. Fayer, M. Santín, J.M. Trout, Prevalence of *Cryptosporidium* species and genotypes in mature dairy cattle on farms in eastern United States compared with younger cattle from the same locations, Vet. Parasitol. 145 (2007) 260-266.
[42] G. Robinson, A.L. Thomas, R.G. Daniel, S.J. Hadfield, K. Elwin, R.M. Chalmers,

Sample prevalence and molecular characterization of *Cryptosporidium andersoni* within a dairy herd in the United Kingdom, Vet. Parasitol. 142 (2006) 163-167.

[43] R. Fayer, M. Santín, L. Xiao, *Cryptosporidium bovis* n. sp. (Apicomplexa: Cryptosporidiidae) in cattle (*Bos taurus*), J. Parasitol. 91 (2005) 624-629.

[44] R. Fayer, M. Santín, J.M. Trout, *Cryptosporidium ryanae* n. sp. (Apicomplexa: Cryptosporidiidae) in cattle (*Bos Taurus*), Vet. Parasitol. 156 (2008) 191-198.

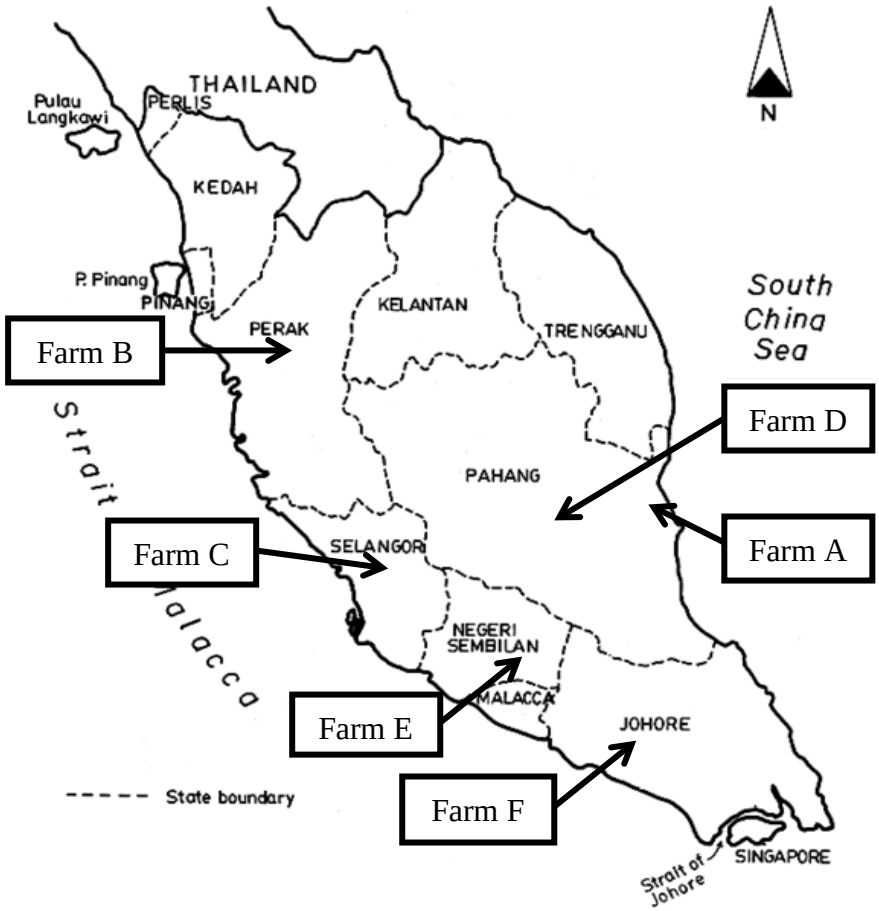

Figure 1. Locations of farms A-F in Peninsular Malaysia from which samples were collected from cattle for testing of *Cryptosporidium* infection.

Table 1. Prevalence of *Cryptosporidium bovis* and *Cryptosporidium ryanae* infections among cattle collected from six farms located in five states in Peninsular Malaysia.

Farm	Location	Total no.	*Cryptosporidium* occurence	
			C. bovis	*C. ryanae*
			No. test-positive (%)	No. test-positive (%)
A	Kuantan, Pahang	37	0 (0.0%)	1 (2.7%)
B	Sungai Siput, Perak	4	0 (0.0%)	0 (0.0%)
C	Serdang Selangor	88	0 (0.0%)	1 (1.1%)
D	Jerantut, Pahang	35	0 (0.0%)	1 (2.9%)
E	Jelai Gemas, Negeri Sembilan	14	0 (0.0%)	0 (0.0%)
F	Ayer Hitam, Johor	37	2 (5.4%)	2 (5.4%)
	Total	215	2 (0.9%)	5 (2.3%)

Table 2. Cattle information (including sample code, age, sex, breed, body weight (kg) as well as collection venue) pertaining to faecal DNA samples that were test-positive for *Cryptosporidium* by PCR-based analyses of particular gene loci (p*SSU*) as well as the species of protist identified, based on the direct sequencing of amplicons, and respective GenBank accession numbers of the sequences determined.

Sample code	Farm	Collection venue	Age	Sex	Breed	Body weight (kg)	Species identified	GenBank accession no.
A24	A	Kuantan, Pahang	14 months	Male	Nellore	196	*C. ryanae*	XXXXXX
C61	C	Serdang, Selangor	5 years	Female	Fresian x Shahiwal	429	*C. ryanae*	XXXXXX
D17	D	Jerantut, Pahang	8 months	Male	Kedah-Kelantan	105	*C. ryanae*	XXXXXX
F17	F	Ayer Hitam, Johor	2.5 years	Female	Mafriwal	160	*C. ryanae*	XXXXXX
F26	F	Ayer Hitam, Johor	2.5 years	Male	Mafriwal	151	*C. ryanae*	XXXXXX
F29	F	Ayer Hitam, Johor	2.5 years	Male	Mafriwal	172	*C. bovis*	XXXXXX
F32	F	Ayer Hitam, Johor	2.5 years	Male	Mafriwal	191	*C. bovis*	XXXXXX