Malays. Appl. Biol. (2017) 46(2): 21–27

PREVALENCE AND MOLECULAR CHARACTERISATION OF *Cryptosporidium* FROM DAIRY CATTLE IN FIVE FARMS IN KUANTAN

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Accepted 7 May 2017, Published online 27 June 2017

ABSTRACT

Cryptosporidium spp. are ubiquitous enteric protozoan parasites that cause diarrhoeal disease known as cryptosporidiosis. This research was conducted to find the prevalence of *Cryptosporidium* from dairy cattle in Kuantan, Pahang, Malaysia and to identify the genotype of *Cryptosporidium* by using 18S rRNA gene. Besides, this study aims to investigate the association between *Cryptosporidium* infection and the age of dairy cattle. A total of 375 stool specimens of dairy cattle were collected and concentrated with formal-ether concentration technique. The *Cryptosporidium* oocysts were detected with modified Ziehl Neelsen staining. *Cryptosporidium* species was identified by nested PCR amplification of 18S rRNA gene. Based on microscopic examination, 16.3% (61/375) dairy cattle were positive for *Cryptosporidium* infection. This research has shown that the highest prevalence of *Cryptosporidium* was recorded in calves with the percentage of 17.4% (12/69), followed by adult cattle and yearling with the percentage of 16.1% (29/180) and 15.9% (20/126), respectively. The findings demonstrated that there was no significant difference ($p > 0.05$) in *Cryptosporidium* infection rates by age. Molecular characterisation revealed that the species of *Cryptosporidium* found in dairy cattle was *Cryptosporidium ryanae.* The present study suggested that proper hygiene practices must be practiced by farmers in order to control the *Cryptosporidium* infection.

Key words: *Cryptosporidium,* age, dairy cattle, prevalence, 18S rRNA

INTRODUCTION

Cryptosporidium spp. are protozoan parasites that cause gastrointestinal infection known as cryptosporidiosis. Since its discovery in 1907, the disease has spread in a broad range of vertebrate hosts including human and livestock (Zhang *et al*., 2013). In Malaysia, cattle farming has been an important part of Malaysian agriculture. Despite the importance of economic importance of cattle at marketing level, many cattle producers overlooked the matter of parasitic infections that usually infect cattle population.

Molecular tools have been established for detection and differentiation of *Cryptosporidium* species/genotypes and subtype (Xiao & Ryan, 2004; Caccio, 2005). Until now, 27 species of *Cryptosporidium* were found worldwide (Ryan *et al*., 2015). Species such as *Cryptosporidium hominis, Cryptosporidium maleangridis, Cryptosporidium felis, Cryptosporidium scrofarum* (formerly pig genotype II)*, Cryptosporidium suis* and *C. suis-like genotype* have been identified in cattle (Trout & Santin, 2008; Abeywardena *et al*., 2015).

The present study was conducted as an extension from the previous study by Hisamuddin *et al.* (2016) which documented the occurrence of *Cryptosporidium* infection in only one dairy cattle farm. Therefore, this study was conducted to find the prevalence of *Cryptosporidium* infection from dairy cattle in five farms in Kuantan, Pahang and to identify the genotype of *Cryptosporidium* by using 18S rRNA gene. Besides, this study aims to

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investigate the association between *Cryptosporidium* infection and the age of dairy cattle.

MATERIALS AND METHODS

Study Area

This cross-sectional study was done in Kuantan, Pahang. Kuantan is located at the east coast of Peninsular Malaysia and the climate is tropical rainforest climate. An average maximum temperature is 31°C and average minimum temperature is 23°C (Malaysian Meteorological Department, 2016). During sampling, the weather condition was hot and humid. The faecal samples of cattle were collected from five (5) different areas in Kuantan; Farm A located at Ulu Lepar (3°43'01.7"N 103°00'41.6"E), Farm B located at Bandar Jaya Gading (3°46'10.7"N 103°09'44.9"E), Farm C located at Bukit Sagu (3°55'22.8"N 103°13'16.0"E), Farm D located at Kampung Pandan (3°47'15.0"N 103°13'33.1"E) and Farm E located at Cherok Paloh (3°36'52.9"N 103°22'32.8"E) (Figure 1). Each farm size is approximately 5 acres. Each farm reared more than 100 dairy cattle. In Farm A and B, approximately 15-20 cattle in barn space with yard while in Farm C, D and E, approximately 20-30 cattle in barn space with yard. The management system of the farms are semi intensive system whereby the animals were brought outside to graze on vast pastures and bushes in the morning and were kept in the barn during night time for security.

Sample collection and examination

Ethical clearance was obtained from Institutional Animal Care and Use Committee (IACUC), International Islamic University Malaysia (IIUM/220/14/IACUC). 375 faecal samples of cattle were collected between May 2015 and November 2015. Each farm was only visited once and each faecal sample was only collected once from different dairy cattle. The faecal samples were collected according to age group; 69 samples from calves (less than 12 months), 126 samples from yearlings (12 -24 months) and 180 samples from adults (more than 24 months). The fresh faecal samples were collected directly from the rectum using sterile plastic gloves. The faecal samples were concentrated with formal ether concentration technique. *Cryptosporidium* oocysts were detected with Modified Ziehl Neelsen staining. Prior to DNA extraction, *Cryptosporidium* positive samples were stored in 2.5% potassium dichromate at 4°C.

DNA extraction

The positive samples were then washed and centrifuged for five times at 1500 *g* for 10 min at room temperature. Genomic DNA was extracted using QIAamp® Fast DNA Stool Mini Kit (Qiagen, UK) in accordance with manufacturer's protocol with several modifications.

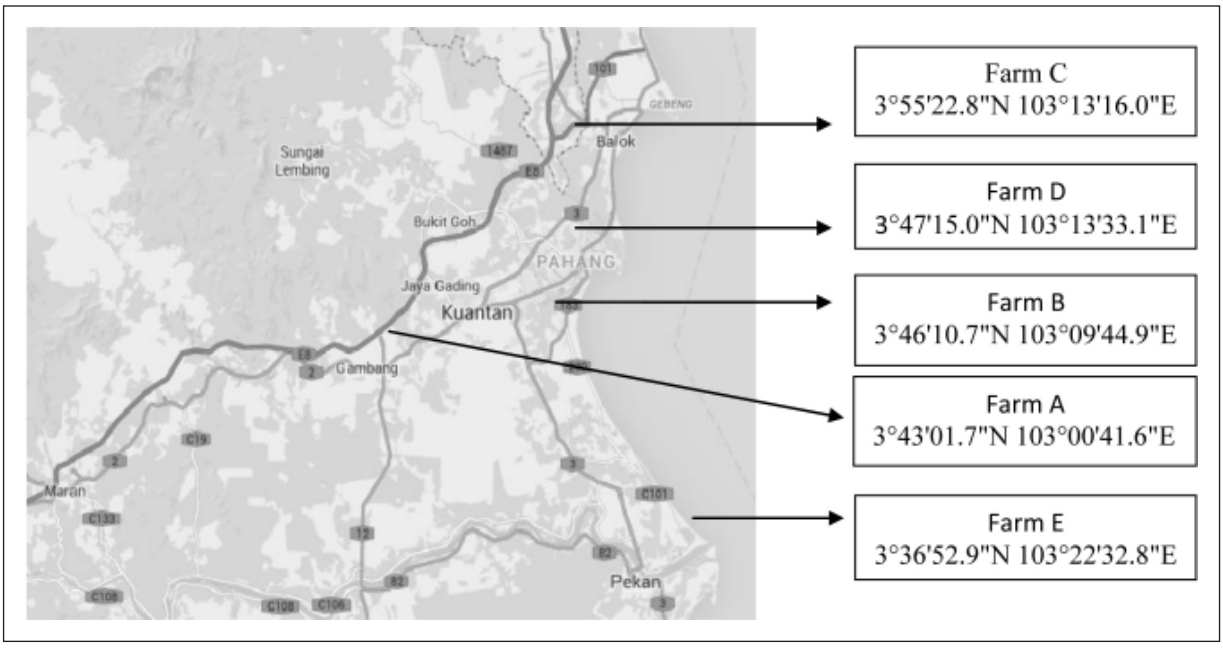

Fig. 1. Location of sampling site of dairy cattle in five farms in Kuantan, Pahang.

Genotyping of *Cryptosporidium* based on 18S rRNA

Identification of *Cryptosporidium* spp. was done by using a two-step nested PCR to amplify partial region of the 18S rRNA gene as described by Johnson *et al.* (1995) and Nichols *et al.* (2003). Briefly, two sets of oligonucleotides (primers) with average length of ~20 bases were used for DNA amplifications in this study (Invitrogen, USA). The primers used in primary and secondary PCR, sizes of the expected PCR products and the references are listed in Table 1.

The primary and secondary reactions were performed in a total volume of 50 µL reaction mixture containing 25 µL HotStarTaq® Master Mix Kit (Qiagen, UK), 2.5 µL forward and reverse primers (Invitrogen, USA), 1 µL DNA template, 1 µL MgCl2 (Qiagen, UK) and 18 µL RNAse-free water (Qiagen, UK). The first PCR product was used as template in the secondary PCR. Reagent concentration in the secondary PCR was similar to primary PCR. The thermal cycling conditions for primary PCR was comprised of 35 cycles of denaturation at 94ºC for 45 s, annealing at 68ºC for 45 s and extension at 72ºC for 1 min with an initial denaturation at 95ºC for 5 min and a final extension at 72ºC for 10 min. The secondary PCR had similar cycling conditions except for the annealing temperature which was reduced to 60ºC.

Sequencing and phylogenetic analysis

DNA sequencing was carried out by First BASE (First BASE Laboratories SDN BHD, Malaysia). DNA sequence alignments were checked for sequencing accuracy using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The sequence data was used to run Basic Local Alignment search tool (BLAST) analysis in the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/) to characterize the *Cryptosporidium* positive isolates. The sequenced products were aligned with the sequences available from GenBank™ using Clustal W. Phylogenetic analysis for the partial sequences of 18S rRNA was performed using MEGA6 software (http://www.megasoftware.net/).

Data analysis

The analysis was performed by using Statistical Package for Social Sciences for Windows version 22 (SPSS Inc., Chicago, USA). For descriptive data, rate (percentage) was used to calculate the prevalence of *Cryptosporidium*. Chi square test was used to compare the prevalence of infection between different age group of cattle. In all analysis, P value less than 0.05 was considered as the level of significance.

RESULTS

Table 2 revealed an overall prevalence of *Cryptosporidium* among cattle from five farms in Kuantan. Detection of *Cryptosporidium* from dairy cattle samples by modified Ziehl Neelsen method

Table 1. Primers used in the primary and secondary PCR and the expected sizes of the PCR products

Reaction	Primer name	Primer sequence	Fragment length (bp)	Reference
Primary	N DIAGF2 NDIAGR2	5'-CAA TTG GAG GGC AAG TCT GGT GCC AGC-3' 5'-CCT TCC TAT GTC TGG ACC TGG TGA GT-3'	655	Nichols *et al.* (2003)
Secondary	CPB-DIAGF CPB-DIAGR	5'-AAG CTC GTA GTT GGA TTT CTG-3' 5'-TAA GGT GCT GAA GGA GTA AGG-3'	455	Johnson *et al.* (1995)

Table 2. The overall prevalence of *Cryptosporidium* infections in cattle from five farms in Kuantan

Farm	Number of sample (n)	Number of positive sample	Prevalence (%)
Farm A	151	24	15.9
Farm B	67	23	34.3
Farm C	55	4	7.3
Farm D	52	10	19.2
Farm E	50	0	0
Total	**375**	**61**	

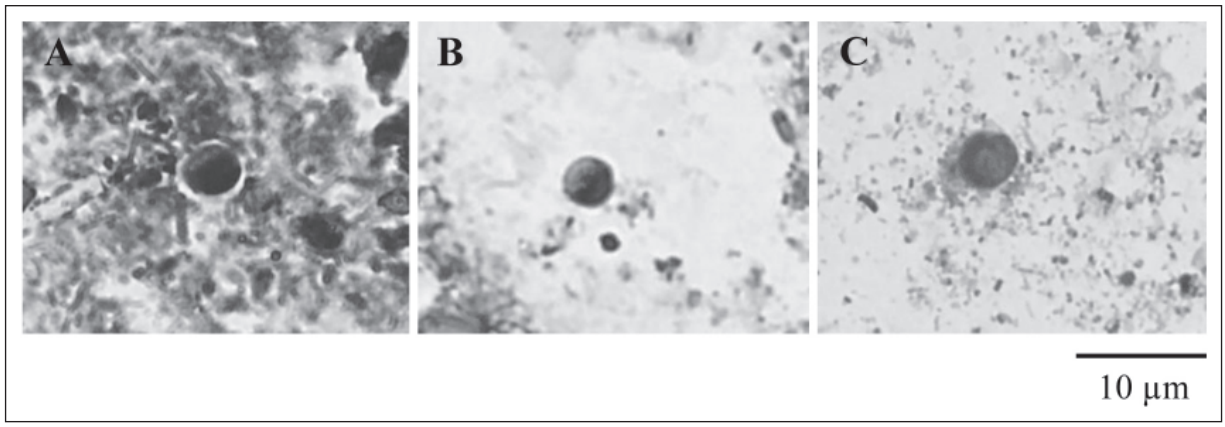

Fig. 2. (A-C) Light microscopy images of *Cryptosporidium* oocysts were stained purplish red using modified Ziehl Neelsen under oil immersion. Oocysts were rounded shape and the size between 4-6 µm. Magnification x1000.

Table 3. The prevalence of *Cryptosporidium* in different age group of cattle

Age group	Total sample	Positive	Prevalence (%)	Chi square (df)	p value
Calves	69	12	17.4		
Yearling	126	20	15.9	0.082 (2)	0.960
Adult	180	29	16.1		

as shown in Figure 2. *Cryptosporidium* oocysts were stained purplish red and the size between 4-6 µm. As shown in Table 2, a total of 16.3% (61/375) cattle were positive for *Cryptosporidium* infection. The result showed that Farm B recorded the highest prevalence with 34.3% (23/67) positive samples, followed by Farm D, Farm A and Farm C with 19.2% (10/52), 15.9% (24/151) and 7.3% (4/55), respectively. There were no positive *Cryptosporidium* cases in Farm E. Overall, there was presence of *Cryptosporidium* in cattle in Kuantan.

The prevalence rate of *Cryptosporidium* infection in three different age groups of cattle consisted of calves, yearling and adult was described in Table 3. The presence of *Cryptosporidium* infection in calves 17.4% (12/69) was the highest compared to 16.1% (29/180) in adult cattle and 15.9% (20/126) in yearling. Nonetheless, there was no significant difference between age and *Cryptosporidium* infection ($p>0.05$). In summary, the highest prevalence of *Cryptosporidium* was recorded in calves, followed by adult cattle and yearling.

From 61 dairy cattle faecal samples, all infected samples were successfully amplified by nested PCR. Based on the analysis of 18S rRNA gene, the primary and secondary PCR products were successfully amplified at 655 bp region and 428 bp region, respectively. Of a total of 61 dairy cattle faecal samples, only 10 samples were submitted for sequencing. DNA sequence analysis of 18S rRNA gene fragments of positive dairy cattle revealed the presence of only one *Cryptosporidium* species, *Cryptosporidium ryanae*. Phylogenetic tree was constructed using sequence from the positive sample (28KP) obtained in this study and 20 sequences representing various *Cryptosporidium* species and genotypes obtained from GenBank database. The unique nucleotide sequence of 18S rRNA gene of *Cryptosporidium* derived from this study has been deposited in the GenBank database under accession no. KU 955862. *Cryptosporidium* species that has been successfully identified and appeared to be the only species in the present study was *C. ryanae.* The inferred phylogenetic tree based on Maximum Parsimony (MP) (Figure 2) was essentially same for branches with high statistical support.

DISCUSSION

The present study showed that 61 from 375 (16.3%) faecal samples were positive for *Cryptosporidium* infection by microscopic observation. The current finding seems to be consistent with the previous local study in Kuantan by Hisamuddin *et al* (2016) who reported 15.89% (24/151) cattle were infected with *Cryptosporidium*. This prevalence is lower than previous local studies conducted in Selangor (Halim *et al*., 2008) and Johor (Muhid *et al*., 2011) with

the percentage of 36% (18/50) and 27.1% (65/240), respectively. The dissimilarities in the prevalence among studies may be due to the differences in sample size, farm management system and host health status.

The most important and interesting finding in this study was that the prevalence of *Cryptosporidium* infection in calves was the highest compared to adult cattle and yearling. The high prevalence of *Cryptosporidium* in young calves compared to adult cattle has been reported by other studies (Santín *et al*., 2008; Al- zubaidi, 2012; Ouchene *et al*., 2014). This finding is most likely due to the young neonates' developing immune system as suggested by Maurya *et al.* (2013). Young animals can become more vulnerable to infection and disease, while adults are usually resistant or appeared asymptomatic (O'Donoghue, 1995). In addition, our study found no significant difference between age group and *Cryptosporidium* infection. Similarly, study by Chen & Huang (2012) found no significant association between age and prevalence rate of *Cryptosporidium*. However, several other studies have found a link between age and the presence of *Cryptosporidium* infection (Santín *et al*., 2004; Silverlas *et al*., 2010; Maikai *et al*., 2011; Zhao *et al*., 2013).

Molecular tools have made a significant contribution which enables the identification and validation of the existence of multiple species of *Cryptosporidium*. The nested PCR protocol used in this study was modified from Nichols *et al.* (2003) and Johnson *et al.* (1995). This protocol has been reported to be sensitive and able to amplify nested PCR products of 18S rRNA gene that were found in stool specimens (Nichols *et al.*, 2003). The 18S rRNA gene is useful because it contains combination of regions that vary between species and conserved within the *Cryptosporidium* genus whereby specific primers that target most species could be designed (Silverlas *et al.,* 2010). Many studies conducted on *Cryptosporidium* have suggested that cattle were usually infected with *C. parvum*, *C. andersoni*, *C. bovis* and *C. ryanae* (Xiao, 2010; Maikai *et al*., 2011; Tomazic *et al*., 2013; Huang *et al*., 2014). In this present study, the sequencing results showed that *C. ryanae* as the only species detected in dairy cattle.

Cryptosporidium ryanae, previously known as the deer-like genotype, have been found in dairy and beef cattle globally (Fayer *et al*., 2008). The prepatent period for *C. ryanae* is 11 days (Fayer *et al.*, 2008) and it localizes in the small intestine. *Cryptosporidium* can cause a wide range of clinical signs that can vary from asymptomatic to serious infection and eventually, death. For *C. ryanae*, no histological information and subclinical pathology has been reported so far (Santín, 2013). In a study conducted by Fayer *et al* (2008), they found that calves infected with *C. ryanae* appeared to be asymptomatic.

The finding of this study is in agreement with a recent local study in Kuantan in which *C. ryanae* has been identified as the species found in cattle (Yap *et al*., 2016). In contrast to *C. ryanae*, *C. bovis* was found in cattle in a previous study conducted in Ayer Hitam, Johor (Yap *et al*., 2016). The occurrence of *C. ryanae* in cattle has also been reported in many countries such as Australia, Brazil, China, Canada, Denmark, Hungary, Japan, Kenya, Nepal, Nigeria, Northern Ireland, United Kingdom and United States (Santín *et al*., 2004; Fayer *et al*., 2007; Feng *et al*., 2007; Langkjaer *et al*., 2007; Plutzer & Karanis, 2007; Thompson *et al*., 2007; Feltus *et al*., 2008; Szonyi *et al*., 2008; Amer *et al*., 2009; Brook *et al*., 2009; Ayinmode *et al*., 2010; Dixon *et al*., 2011; Meireles *et al*., 2011; Waldron *et al*., 2011).

CONCLUSION

The conclusion that can be drawn from the study is the prevalence of *Cryptosporidium* infection in dairy cattle from Kuantan is 16.3% and calves had the highest rate of infection. Regarding the species of *Cryptosporidium*, the species detected was *C. ryanae.* Good management practices and proper hygiene must be practised by the farmers to reduce *Cryptosporidium* infection. As mentioned by Majewska *et al.* (2000) there is a possibility that poor hygiene practised by the farms may influence the exposure of the animals to *Cryptosporidium* infection. Hence, this study suggested veterinary health agencies should organize a systematic awareness program especially for farmers to give an insight on the appropriate care of the animals for both maximum production and safety of mankind. The results of the study will be useful to make a better animal management practices and improve hygiene practices in order to decrease the infection among cattle which indirectly help to reduce economic losses due to this parasitic infection.

ACKNOWLEDGEMENTS

The authors would like to express the gratitude and appreciation to the officers from the Department of Veterinary Services, Kuantan Malaysia and the farmers for assisting and supporting in various ways throughout the study. The study was funded by IIUM Research Initiative Grant (RIGS) no. 16-301-0465.

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