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

AFZAN MAT YUSOF\textsuperscript{1,2}\textsuperscript{*} and MUHAMMAD LOKMAN MD ISA\textsuperscript{1,2}

\textsuperscript{1}Department of Basic Medical Sciences, Kulliyyah of Nursing, International Islamic University of Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia
\textsuperscript{2}Integrated Cellular and Molecular Biology Cluster (iMolec), Integrated Centre for Research Animal Care and Use, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia

\textsuperscript{*}Email: afzan@iium.edu.my

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 maleanagridis, 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
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 94°C for 45 s, annealing at 68°C for 45 s and extension at 72°C for 1 min with an initial denaturation at 95°C for 5 min and a final extension at 72°C for 10 min. The secondary PCR had similar cycling conditions except for the annealing temperature which was reduced to 60°C.

Sequencing and phylogenetic analysis

DNA sequencing was carried out by First BASE Laboratories (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>
<thead>
<tr>
<th>Farm</th>
<th>Number of sample (n)</th>
<th>Number of positive sample</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm A</td>
<td>151</td>
<td>24</td>
<td>15.9</td>
</tr>
<tr>
<td>Farm B</td>
<td>67</td>
<td>23</td>
<td>34.3</td>
</tr>
<tr>
<td>Farm C</td>
<td>55</td>
<td>4</td>
<td>7.3</td>
</tr>
<tr>
<td>Farm D</td>
<td>52</td>
<td>10</td>
<td>19.2</td>
</tr>
<tr>
<td>Farm E</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>375</strong></td>
<td><strong>61</strong></td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Fragment length (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>N DIAGF2</td>
<td>5’-CAA TTG GAG GGC AAG TCT TCT GCT TGT GCC AGC-3’</td>
<td>655</td>
<td>Nichols et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>NDIAGR2</td>
<td>5’-CCT TCC TAT GTC TGG ACC TGG TGA GT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary</td>
<td>CPB-DIAGF</td>
<td>5’-AAG CTC GTA GTT GGA TTT CTG-3’</td>
<td>455</td>
<td>Johnson et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>CPB-DIAGR</td>
<td>5’-TAA GGT GCT GAA GGA GTA AGG-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Prevalence and Molecular Characterisation of Cryptosporidium From Dairy Cattle

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

<table>
<thead>
<tr>
<th>Age group</th>
<th>Total sample</th>
<th>Positive</th>
<th>Prevalence (%)</th>
<th>Chi square (df)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calves</td>
<td>69</td>
<td>12</td>
<td>17.4</td>
<td>0.082 (2)</td>
<td>0.960</td>
</tr>
<tr>
<td>Yearling</td>
<td>126</td>
<td>20</td>
<td>15.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>180</td>
<td>29</td>
<td>16.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 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 (Santin 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 (Santin 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 (Santin, 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 (Santin et al., 2004; Fayer et al., 2007; Feng et al., 2007; Langkjaer et al., 2007; Plutzer & Karanis, 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.
REFERENCES


