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# First molecular identification of *Cryptosporidium* by 18S rRNA in goats and association with farm management in Terengganu

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ABSTRACT

**Objective:** To identify the prevalence of *Cryptosporidium* from goats in three types of farm management systems in Terengganu, Malaysia and to determine the *Cryptosporidium* species infecting goats by using 18S rRNA.

**Methods:** A total of 478 fecal samples were randomly collected from goats in three farms; 199 samples were collected from intensive farm, 179 samples from semi-intensive farm and 100 samples from extensive farm. The samples were processed by using formol-ether concentration technique and stained by using modified Ziehl–Neelsen. Positive samples were performed by using nested PCR analysis by using 18S rRNA.

**Results:** Out of 478 goats, 207 (43.3%) were found to be infected with *Cryptosporidium*. Goats reared under the intensive farm management system reported the highest prevalence of infection (49.7%), followed by intensive farm management system (41%) and the lowest prevalence was reported in the goats reared under semi-intensive management system (37.4%).

**Conclusions:** The identified species found in goat was *Cryptosporidium parvum*. Future study on the zoonotic transmission of *Cryptosporidium parvum* in goats needs to be done in order to find the source of transmission of this parasite.

## 1. Introduction

Malaysia is a country that has rapid growth in livestock industry, especially goat. In 2015, the Federation of Livestock Farmer's Association of Malaysia (FLFAM) reported that the population of goats estimated at 439 667 [1]. In addition, most of the gross national income in the country was contributed from goat farming [2]. To date, millions of people and livestock infected with protozoa particularly *Cryptosporidium* [3] and this may result in significant economic losses and health problem worldwide. Since its discovery in 1907, *Cryptosporidium* is a parasite that can infect humans and animals causing from non-symptomatic to chronic gastrointestinal infection [4].

Cryptosporidiosis is related with the clinical symptoms such as severe diarrhea, loss weight, depression and anorexia [5]. Animals infected with *Cryptosporidium* infection can lead to mortality [6]. *Cryptosporidium* can be transmitted through ingestion of infective oocysts (fecal-oral route) via contaminated food, water and pasture [7]. Besides, close proximity between animal handlers and livestock [8], runoff water from livestock production and contaminated water supplies can transmit *Cryptosporidium* infection [7].

Goats are one of the most common animals infected with *Cryptosporidium*. The first study of cryptosporidiosis related to goat was done by Mason *et al.* [9] who found that a 14 days old goat kid in Australia was dead due to diarrhea caused by *Cryptosporidium* after being autopsied. Since then, many studies pertaining to *Cryptosporidium* infection have been reported worldwide in both developed and developing

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countries. Currently, a study done by Diaz *et al.* [10] on 118 goat fecal samples from 23 farms in Spain showed that 74 goats were positive for *Cryptosporidium* with the percentage of 62.7%.

Today, molecular analysis of *Cryptosporidium* isolates from different origin mainly human, animal and environment has been widely used. Latest advancement in molecular identification of *Cryptosporidium* made it feasible to distinguish *Cryptosporidium* oocysts in terms of their species, genotypes and subgenotypes levels [11]. There are various species of *Cryptosporidium* found in goats including *Cryptosporidium andersoni* [12], *Cryptosporidium bovis* like genotypes [13], *Cryptosporidium hominis* [14], *Cryptosporidium parvum* (*C. parvum*) [15], *Cryptosporidium ubiquitum* [16] and *Cryptosporidium xiaoi* [17].

So far, no molecular data concerning goat cryptosporidiosis were conducted in Malaysia. Therefore, it can be said that this is the first molecular study of goat cryptosporidiosis in Malaysia. This study aimed to identify the prevalence of *Cryptosporidium* from goats in three types of farm management systems in Terengganu and to identify the *Cryptosporidium* species infecting goats by using 18S rRNA gene. The findings of the study can contribute to a better understanding of zoonotic transmission of *Cryptosporidium* through phylogenetic analysis.

## 2. Materials and methods

### 2.1. Sample collection

The present study was carried out in the state of Terengganu, Malaysia. A total of 478 goat fecal samples was collected from three different farm management systems in Terengganu from February to November 2015. The farms involved in this study were selected and categorized based on their management systems which are intensive, semi-intensive and extensive. The decision of choosing goat farms was made through consultation from Department of Veterinary Services (DVS), Kuala Terengganu. Fresh fecal samples were collected directly from the rectum of goats with sterile plastic gloves and kept in clean containers. Each goat sample was divided into two containers; one fixed with 10% formalin while the other one without the formalin. All samples were processed and analyzed at the Integrated Centre for Research Animal Care & Use (ICRACU) laboratory, International Islamic University Malaysia (IIUM), Kuantan, Pahang. The samples were preserved in −20 °C until DNA extraction was carried out.

### 2.2. Modified Ziehl–Neelsen staining

The samples preserved in 10% formalin and processed by using formal-ether concentration technique prior to stain with modified Ziehl–Neelsen. The slides were examined microscopically under oil immersion (magnification ×1 000) for the detection of *Cryptosporidium* oocyst.

### 2.3. DNA extraction

Positive fecal samples confirmed by modified Ziehl–Neelsen staining were kept in 2.5% potassium dichromate. The samples were then washed and centrifuged for five times at 1 500 r/min for 10 min at room temperature. Genomic DNA was extracted using QIAamp® Fast DNA Stool Mini Kit (Hilden, Germany) per manufacturer's protocol. The concentration of DNA was measured by Thermo Scientific Nanodrop 2000® spectrophotometer.

### 2.4. Nested PCR analysis

A nested set of primers was used to amplify a partial region of the 18S rRNA gene of *Cryptosporidium*. Forward and reverse primers, namely N-DIAG-F2 (CAA TTG GAG GGC AAG TCT GGT GCC AGC) and N-DIAG-R2 (CCT TCC TAT GTC TGG ACC TGG TGA GT) have been used in primary PCR reaction to amplify approximately 655 bp target DNA fragments [18]. In addition, secondary PCR reaction has been performed by using forward primer CPB-DIAG-F (AAG CTC GTA GTT GGA TTT CTG) and reverse primer CPB-DIAG-R (TAA GGT GCT GAA GGA GTA AGG) in order to amplify approximately 435 bp target DNA fragments [19]. For the primary round of amplification, an initial activation step at 95 °C for 5 min, followed by 35 cycles of amplification (94 °C for 45 s, 68 °C for 45 s and 72 °C for 1 min) and a final extension step of 72 °C for 10 min for total of 50 μL reactions. The same conditions were followed for the secondary round of amplification, except that the annealing temperature was reduced to 60 °C. Amplified DNA was analyzed by 1.2% agarose gel electrophoresis.

### 2.5. DNA sequencing and phylogenetic analysis

The secondary amplified product was sent to First BASE Lab for DNA purification and sequencing. The sequence data was used to conduct BLAST analysis in the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) to illustrate the *Cryptosporidium* positive isolates. The sequenced products were aligned to sequences available from GenBank™ using Clustal W. Phylogenetic and molecular evolutionary analyses were made using MEGA6.

## 3. Results

### 3.1. Microscopic identification

A total of 478 goat fecal specimens was collected and examined for cryptosporidiosis. The prevalence rate in this study shows that 207 (43.4%) goats were positive for *Cryptosporidium* infection (Table 1). The data indicated that among 207 positive cases, 49.7% (99/199) were found in goats reared under intensive farm management system, 41.0% (41/100) were found in goats reared under intensive farm management system and the remaining 37.4% (67/179) were found in goats reared under semi-intensive farm management system. This finding was based on microscopic examination by Modified Ziehl–Neelsen staining.

**Table 1**
The overall prevalence of *Cryptosporidium* infections in goats from three farm management systems in Terengganu.

| Type of farm management | Number of sample (*n*) | Number of positive sample (*n*) | Percentage of infection (%) |
|---|---|---|---|
| Intensive | 199 | 99 | 49.7 |
| Semi-intensive | 179 | 67 | 37.4 |
| Extensive | 100 | 41 | 41.0 |
| Total | 478 | 207 | 43.3 |

### 3.2. Nested PCR amplification of 18S rRNA gene

All positive fecal samples were successfully amplified by nested PCR. All samples produced amplification product of approximately ~655 bp and ~435 bp for primary and secondary reaction, respectively. The positive samples were sequenced and searched using BLAST. The finding shows that the species of *Cryptosporidium* detected in goats was *C. parvum*. The inferred phylogenetic tree based on maximum parsimony (MP) was essentially same for branches with high statistical support. MP tree placed isolate 23MB that was collected from goat stool together with *C. parvum*. This grouping was further confirmed with BLAST search where isolate 23MB was 98% similar to *C. parvum* (Accession number: KF128754). 23MB isolate was grouped in the same recent common ancestor at the same sister taxa with KF128754 (*C. parvum*) of 98% similarity even with slightly lower statistical probability of bootstrap value. However, this isolate was also grouped with other same species in other clade that has common ancestor with the high and significant bootstrap value of 83% such as DQ060424 (*C. parvum*) and AF115377 (*C. parvum*). In addition, KF128754 with 23MB were also grouped with AY030084 (*C. parvum*) in the same small clade. At common ancestor of 55 percent statistically inferred, AY030086 (*C. parvum*) was also grouped with 23MB isolate even it was rather distant in genetic relatedness of the same partial nucleotide sequence of 18S ribosomal RNA gene. Other species such as *C. suis*, *C. meleagridis* and *C. ubiquitum* also showed the genetic relatedness due to have the shared sequences of 18S ribosomal RNA gene even the common presence of polymorphic sequence of nearly few alternating nucleotide bases along with 23MB isolate sequence. On the other hand, JX312812 (*E. tenella*) was made to be an outgroup to root the inferred phylogenetic tree and to show far genetic relatedness due to different genus and species. Nevertheless, *E. tenella* was commonly used for exhibiting the pattern of lineage relatedness.

## 4. Discussion

A cross sectional study was conducted from February to November 2015 on goats from three types of farm management systems in Terengganu, Malaysia. The nested PCR protocol used in this study was modified by Nichols *et al.* [18] from a previous protocol developed by Johnson *et al.* [19]. This protocol produced a 435 bp fragment which is the same as previous studies that have been reported approximately 435 bp in genotyping of *Cryptosporidium*. The overall findings from this study showed that out of 478 goats, 207 (43.4%) were infected with *Cryptosporidium* infections. This rate of *Cryptosporidium* infection was comparatively lower than the study conducted in Spain [10] which recorded higher prevalence of cryptosporidiosis with the percentage of 62.7% (74/118). However, many studies in other developing countries reported that low occurrence of cryptosporidiosis in goats which is less than 30%. The studies conducted in Bangladesh [17], Ethiopia [20], Iran [21] and Nigeria [22] reported that the prevalence of *Cryptosporidium* in goats was 15.0% (15/100), 11.5% (7/61), 18.86% (66/350) and 24.0% (36/150), respectively. According to Kakar and Kakarsulemankhel [23], the variation in prevalence among different studies may be due to geo-climatic surrounding, sample size, management system and seasonal variation.

The results of the study showed that goats reared under intensive farm management system were significantly ($P < 0.05$) most susceptible to *Cryptosporidium* infection which occurred at 49.7% (99/199). A similar finding from other livestock was reported by Geurden *et al.* [24] in Zambia showed that 42.8% (107/250) of dairy calves raised in intensive systems were highly infected with *Cryptosporidium* than extensive systems. In this study, the highest infection rate of *Cryptosporidium* in goats was under intensive farm management system than other farm management systems. This could be due to high stocking rate of goats under intensive farm management system. The goats under intensive farm management system were occupied with 10–15 heads per shed, which can cause overcrowding. Overcrowding increased the chance of goats to transmit infection from one to another through skin contact with the infected goat and through ingestion of contaminated food and water [25].

So far, there is no molecular characterization study of goat cryptosporidiosis in Malaysia. To date, the only molecular study conducted in goats was on giardiasis [26]. The finding of this study shows that *C. parvum* was detected in goats in Terengganu, Malaysia. Therefore, this is the first molecular study that successfully amplified *Cryptosporidium* species in goats in Malaysia. There have been a few studies in Malaysia that found *C. parvum* but in different hosts like human [27,28], avian [29] and cattle [30].

Molecular analyses have proved that *C. parvum* had infected goats especially goat kids [10,31]. *Cryptosporidium* species have been identified in goats from various countries like Belgium [24], China [32], Egypt [33], India [34], Papua New Guinea [14], Philippines [8] and Spain [10], and the studies have showed that *C. parvum* was predominant or the only species identified in goats. *C. parvum* is known as the most common zoonotic parasite infecting humans and ruminants [35].

As a conclusion, there was an occurrence of *Cryptosporidium* infections in goats in Terengganu whereby the overall prevalence of infection was 43.4%. Among three farm management systems, the occurrence of cryptosporidiosis was predominant in goats under intensive farm management system with the percentage of 49.7%. This first molecular identification revealed that *Cryptosporidium* species in goats were *C. parvum*. However, further study should be conducted on larger samples from different locations to achieve more precise data on *Cryptosporidium* infection in goats. Besides, future work is needed to identify the transmission dynamics of zoonotic potential of *C. parvum* from goats to humans by taking human samples especially goat handlers or farmers.

## Conflict of interest statement

We declare that we have no conflict of interest.

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