**Blastocystis** spp. subtype 10 infected beef cattle in Kamal and Socah, Bangkalan, Madura, Indonesia

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**Abstract**

**Background and Aim:** *Blastocystis* spp. is a gastrointestinal parasite that can infect both humans and animals and has the potential to become a zoonotic parasite. This study analyzed a subtype (ST) of *Blastocystis* spp. that had infected beef cattle in Kamal and Socah, Bangkalan, Madura, Indonesia.

**Materials and Methods:** Fresh stool samples were collected from 108 beef cattle at Kamal and Socah, Bangkalan, Madura, Indonesia. *Blastocystis* spp. were detected both morphologically and genetically based on the 18S rRNA gene. The morphology of *Blastocystis* spp. from the stool samples and cultured samples were observed under a light microscope. *Blastocystis* spp. from 20 positive cultures were amplified through polymerase chain reaction, and the resultant sequences were identified by ST.

**Results:** One hundred and eight (100%) fecal samples from the fresh or cultured stools were positive morphologically for *Blastocystis* spp. Molecularly, all 20 of the samples selected for DNA analysis were found to be *Blastocystis* spp. ST 10.

**Conclusion:** Based on morphological and molecular detection, the prevalence of *Blastocystis* spp. infection in beef cattle within Kamal and Socah, Bangkalan, Madura, Indonesia, was high. About 100% were non-zoonotic parasites. This was the first report of *Blastocystis* spp. ST 10 found in infected beef cattle in Kamal and Socah, Bangkalan, Madura, Indonesia.

**Keywords:** beef cattle, *Blastocystis* spp. subtype 10, Madura, Indonesia, zoonotic.

**Introduction**

Madura Island, including the Bangkalan district, is known as a beef cattle production area. The population of beef cattle in Bangkalan was 200,279 heads in 2017 with 5627 heads spread out in the Kamal subdistrict [1]. According to Hariyono et al. [2], beef cattle farming on Madura Island is still traditionally managed. Production of cattle is generally a side business, and the educational background of the farmer is often insufficient terminating at the elementary school level [3]. Thus, inadequate management systems correlate to a high disease burden in livestock, including parasitic diseases [4].

*Blastocystis* spp. is a gastrointestinal parasite found in feces samples from both humans and animals [5], including mammals, birds, amphibians, and reptiles [6]. Several studies have shown that *Blastocystis* infection has the potential to be a zoonotic parasite, as evidenced by the discovery of the same subtype (ST) in both humans and animals [7]. *Blastocystis* is distributed worldwide with varying prevalence in many countries. The prevalence of *Blastocystis* infection in humans within developing countries is significantly higher (50%) than that found in established countries (20%) [8,9]. Furthermore, *Blastocystis* cases in children from Senegal can reach 100% [10]. This difference is due to poor hygiene practices, close animal contact, and consumption of contaminated food or water [11]. *Blastocystis* infection is a waterborne or foodborne disease, and transmission occurs through the fecal–oral route for the infective stage of the cyst form [12,13]. *Blastocystis* isolates from humans are referred to as *Blastocystis hominis* and isolates from animals are generally referred to as *Blastocystis* spp. Further, classification is often based on the type of host [14]. In general, the way to detect *Blastocystis* spp. in the feces is with a direct smear examination using a light microscope or by *in vitro* culture. *Blastocystis* spp. isolates from humans and other animals exhibit similar morphology. There are four morphological forms of *Blastocystis* including vacuolar, granular, amoeboid, or cyst forms [11]. Polymerase chain reaction (PCR) is the most sensitive method for the diagnosis of this parasite [14]. Many
researchers have identified Blastocystis in animals globally. Cattle in Iran were found to be infected with Blastocystis ST 3, 5, and 6 as well as unidentified STs [14]. Furthermore, non-primate animal species in a wildlife park in the UK were found to be positive with six STs: ST1, ST4, ST5, ST10, ST14, and a potentially new ST [15]. In Brazil, from a total of 334 stool samples, 28 different genera of animals were found in six STs: ST1, ST2, ST3, ST4, ST5, and ST8 [16]. However, information about the prevalence of Blastocystis infection in various animals in Indonesia is still very limited. Yoshikawa et al. [17] surveyed Blastocystis STs from humans, domestic pigs, domestic chickens, and wild rodents on Sumba Island, Indonesia, and they found STS 1-3 in children and ST5, ST7, and ST4 in domestic pigs, chickens, and wild rodents, respectively.

Our previous study found that Blastocystis infection in beef cattle within Bangkalan, Madura, Indonesia, was based on the morphology of the organism [18]. Our previous research also found Blastocystis that was morphologically identified in fresh and cultured stool samples from the sugar glider [19].

This study aimed to assess the molecular characteristics of Blastocystis spp. found in beef cattle at Kamal and Socah, Madura. Knowledge of the exact ST of Blastocystis infecting these cities is paramount to design a management program to control this parasite.

Materials and Methods

Ethical approval

Ethical approval for this study was obtained from the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Universitas Airlangga (approval number: 1.K.E. 063.01.2018).

Study period, location and sample collection

This study was conducted between July 2018 and August 2018. Sampling was conducted on July 11, 2018, on beef cattle farms at Kamal and Socah subdistrict, Bangkalan district, Madura. The number of samples was 108 (56 samples from Kamal and 52 from Socah) from 55 farms, and the subject cattle ranged from 0.5 to 7 years of age. The location of farms was in close proximity to the community. The samples consisted of fresh stool collected and stored in labeled sterile containers and then transported in cold condition (8 °C) to the Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga.

Microscopic examination

Parasitological observations were carried out at the Laboratory of the Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Airlangga University. A small amount of feces was diluted with water (1:9) and then was filtered. The fecal sample solution was then centrifuged at 1500 rpm for 5 min, the supernatant was discharged, and the pellets of each sample were smeared onto three glass slides. The first was used to observe the specimen, the second and the third involved the addition of iodine or Giemsa solution around 50 μl (2-3 drops), respectively. Observations were conducted under light microscope 100-400x.

In vitro culture

Approximately 1 g of each fecal sample was inoculated into a sterile conical tube containing 1 ml of simple medium. Each sample was incubated at 37°C for 72 h. The simple medium [6] was comprised 500 ml Ringer’s solution (Otsuka, Indonesia), 0.5 g yeast extract (Merck, Germany), 5 g peptone (Merck, Germany), 20 ml boiled rice water, and 50-100 mg oxytetracycline (Vet-oxy LA, Sanbe, Indonesia). Following 72 h of incubation, the culture was examined for the presence and growth of Blastocystis. Briefly, a little supernatant just above the sediment of the stool was taken and dropped onto a glass object and observed under a light microscope. Positive samples were subsequently maintained by subculturing, and Blastocystis spp. were harvested by removing the culture medium supernatant, which was then centrifuged at 1500 rpm for 10 min. Pellets were removed and resuspended in 1 ml phosphate-buffered saline and stored at −20°C for PCR.

DNA extraction and amplification

Then, 20 positive culture samples (10 samples from Kamal and 10 from Socah) were selected for DNA extraction and amplification. DNA extraction was performed using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. Then, the DNA samples were stored at −20°C for later use. Blastocystis-specific primers b11400 FORC (5’-GGA ATC CTC TTC GAG GGA CAC TAT ACA T-3’) and b11710 REVc (5’-TTA CTA TACATAAAGTATAGGGAAGCTGGAGGCAATA ACAGGTCTGTGATGCCCTTAGATGTCCTGGG [9], and the reference sequence was GGAATCCTCTTAGAGGGACACTA (accession no. KF002562), respectively. Observations were conducted under light microscope 100-400x.
CTGCACGCGCGCGACACTGATTCATTCAAC
AAGTGGCTGAATCGATAGATTTGGCAA
ATCTTTTGAAAATGAATCGTGATGGGG
ATTGATGTCTGTAATAAACGTCATGAACGA
GGAATTCCTAGTAATGCAAGTCATCAACT
TGCGTTGATTACGTCCCTGCCCTTTGTA
CACACCGCCCGTGCACCTACCTACCGATTG
ATGGTCGATGAAACACCTTTGGATTAGTA

Then, each of the positive samples was amplified using seven pairs of STs primers (ST1-7) to determine the ST of *Blastocystis*. This protocol followed the procedure manual from the manufacturer. The DNA from the PCR products with 310 bp was sequenced and then seven pairs of STS primers (ST1-7) were used to identify STs of *Blastocystis* spp.

**DNA sequencing and phylogenetic tree analyzing**

All PCR products at 310 bp were sent to the First BASE Sdn Bhd Laboratories (Malaysia) through PT. Genetika Science (Indonesia) to be sequenced. All of the nucleotide sequences were aligned using the BioEdit sequence alignment editor (https://bioedit.software.informer.com/download/). Nucleotide sequences were compared to the obtained sequences in the GenBank database using the Basic Local Alignment Search Tool to determine the isolates of *Blastocystis* spp. (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was constructed using neighbor-joining (NJ). Analysis of the 18S ssrRNA for *Blastocystis* spp. was conducted with computer software Molecular Evolutionary Genetic Analysis (MEGA) version 7 (http://www.megasoftware.net).

**Statistical analysis**

The prevalence of *Blastocystis* infection in beef cattle in Kamal and Socah was analyzed through the Chi-square test using SPSS version 20.0, (IBM SPSS Statistics, IBM Corporation, New York, USA). p<0.05 was regarded as statistically significant.

**Results**

The total number of samples was 108 collected from 55 farms of beef cattle. The educational background of most of the farmers was graduated from elementary school. One farmer had 2-5 cattle caged in one small place. The cages were located close to the farmers’ houses. Almost all of the cage floors were soil, and living conditions were dirty, feces piled up in the cage. Grass was the main feed for the cattle in this area, with no existing feedlots.

All of the 108 beef cattle samples (100%) were infected with *Blastocystis* spp., based on the morphology observations of the fresh stool (wet mount) samples and the *in vitro* cultures (Table-1). Twenty samples were selected for further analysis using DNA. The *in vitro* cultures were confirmed for the presence of *Blastocystis* by amplifying the DNA with primers specific to *Blastocystis* spp. and then all of the samples that were molecularly positive were indicated with a band (approximate 310 bp) in the agarose gel (Figure-1); furthermore, seven pairs of STs primers (ST1-7) were used to detect STs in the DNA which could not be detected by the agarose gel electrophoresis.

Using forward and reverse primer, the PCR purified products of both samples from Kamal and Socah were successfully sequenced to identify an ST of *Blastocystis* spp. The sequences for the 20 selected samples were matched with a partial sequence 18S ssrRNA gene of *Blastocystis* spp. ST 10 isolate CA6 under accession no. KC148207.1. Values for maximal score, total score, Corey cover, and percentage identity were 466-523, 466-523, 92-97%, and 97.39-98.52%, respectively (Table-2).

The position of *Blastocystis* spp. from Bangkalan Madura on the phylogenetic tree based on the SSU rRNA gene sequence for *Blastocystis* spp. ST 10 is shown in Figure-2. The rooted NJ tree identified only one clade that corresponded to *Blastocystis* ST 10 in *Bos taurus* from Denmark (FM164412).

**Discussion**

The prevalence of *Blastocystis* infection in beef cattle in Kamal and Socah, Bangkalan, Madura, was

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**Table-1:** Prevalence of *Blastocystis* infection in the examined feces of beef cattle in Bangkalan, Madura.

| Subdistrict | Wet mount number positive/number of sample (%) | Culture number positive/number of Sample (%) | PCR number positive/number of sample (%) |
|-------------|-----------------------------------------------|---------------------------------------------|------------------------------------------|
| Kamal       | 56/56 (100)                                   | 56/56 (100)                                 | 10/10 (100)                              |
| Socah       | 52/52 (100)                                   | 52/52 (100)                                 | 10/10 (100)                              |
| Total       | 108/108 (100)                                 | 108/108 (100)                               | 20/20 (100)                              |

PCR=Polymerase chain reaction

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**Figure-1:** Polymerase chain reaction of DNA for *Blastocystis* spp. from cattle in Kamal and Socah, Madura. M=DNA leader, K−=Negative control, S=Sample.
extremely high, 100%. This condition is related to the management of the farms in Kamal and Socah, especially in terms of the cleanliness of the cages and housing systems. According to Welay et al. [4], insufficient management systems are correlated with a high disease burden in livestock. Blastocystis infection

Table-2: Basic local alignment search tool sequence DNA of Blastocystis spp. in beef cattle from Bangkalan, Madura, compared with the published data on gene bank, partial sequences of 18S ssrRNA genes for Blastocystis spp. ST 10 isolate CA6 under accession no. KC148207.1.

| Sample | Characteristics of cattle | Accession number of sample | Accession number reference | Maximal score | Total score | Corey cover (%) | Identity (%) |
|--------|--------------------------|----------------------------|---------------------------|---------------|-------------|----------------|--------------|
| Kamal 1 | Female 7 years, No diarrhea | MN606117 | KC148207.1 | 473 | 473 | 92 | 98.51 |
| Kamal 2 | Male 3 years, Diarrhea | MN606118 | KC148207.1 | 477 | 477 | 92 | 98.52 |
| Kamal 3 | Male 10 months, Diarrhea | MN606119 | KC148207.1 | 477 | 477 | 93 | 98.18 |
| Kamal 4 | Male 1.5 years, No diarrhea | MN606120 | KC148207.1 | 477 | 477 | 93 | 98.18 |
| Kamal 5 | Male 3 years, No diarrhea | MN606121 | KC148207.1 | 477 | 477 | 93 | 98.18 |
| Kamal 6 | Male 6 months, No diarrhea | MN606122 | KC148207.1 | 477 | 477 | 93 | 98.18 |
| Kamal 7 | Male 1.5 years, No diarrhea | MN606123 | KC148207.1 | 477 | 477 | 93 | 98.18 |
| Kamal 8 | Male 2.5 years, Diarrhea | MN606124 | KC148207.1 | 466 | 466 | 94 | 97.45 |
| Kamal 9 | Female 5 years, No diarrhea | MN606125 | KC148207.1 | 473 | 473 | 92 | 98.51 |
| Kamal 10 | Female 6 years, No diarrhea | MN606126 | KC148207.1 | 479 | 479 | 94 | 98.18 |
| Socah 1 | Female 1 year, No diarrhea | MN606127 | KC148207.1 | 472 | 472 | 93 | 97.81 |
| Socah 2 | Male 7 months, No diarrhea | MN606128 | KC148207.1 | 479 | 479 | 93 | 98.18 |
| Socah 3 | Female 7 years, No diarrhea | MN606130 | KC148207.1 | 475 | 475 | 93 | 98.52 |
| Socah 4 | Female 3 years, Diarrhea | MN606131 | KC148207.1 | 477 | 477 | 93 | 98.52 |
| Socah 5 | Female 3 years, No diarrhea | MN606132 | KC148207.1 | 472 | 472 | 94 | 97.82 |
| Socah 6 | Male 2 years, No diarrhea | MN606133 | KC148207.1 | 477 | 477 | 92 | 98.52 |
| Socah 7 | Female 5 months, Diarrhea | MN606134 | KC148207.1 | 479 | 479 | 93 | 98.18 |
| Socah 8 | Female 1 year, No diarrhea | MN606135 | KC148207.1 | 472 | 472 | 93 | 98.52 |
| Socah 9 | Female 7 years, No diarrhea | MN606136 | KC148207.1 | 523 | 523 | 97 | 97.39 |
| Socah 10 | Female 7 years, Diarrhea | MN606137 | KC148207.1 | 523 | 523 | 97 | 97.39 |

Figure-2: Evolutionary relationships for taxa of Blastocystis spp. ST 10. The evolutionary history was inferred using the neighbor-joining method (bootstrap 1000). The optimal tree with the sum of branch length=2.1354045 is shown (next to the branches). The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. This analysis involved 28 nucleotide sequences. Codon positions included were 1st, 2nd, and 3rd non-coding. All positions with gaps or missing data were eliminated. There were a total of 241 positions in the final dataset. Evolutionary analyses were conducted in Molecular Evolutionary Genetic Analysis 7.
is a waterborne or foodborne disease transmitted by the fecal-oral route [11-13]. When cattle are kept in the same cage, transmission occurs between the cows. Moreover, the cage floor is ground, so the conditions are dirty with no place to graze for food.

Compared to epidemiological studies from several other countries, the prevalence of Blastocystis spp. infection in cattle in Kamal and Socah, Madura, was the highest at 100%. Research conducted in other countries found the following results for the prevalence of 1.8% in Spain [20], 19.15% in the USA [21], 34.5% in Malaysia [22], 9.6% in Iran [14], 9.6% in China [23], and 50% in Thailand [24]. Our previous study showed that the prevalence of Blastocystis infections in sugar gliders from Surabaya was also very high (100%) [19]. Yoshikawa et al. [17] reported that the prevalence of Blastocystis infections in animals from Sumba, Indonesia, depended on the genus of the animals such as pigs, chickens, or wild rodents and the results were 87.1%, 34.2%, and 13.0%, respectively.

There are four distinct morphological forms for Blastocystis spp. including vacuolar, granular, amoeboid, and cyst forms [11]. Since these parasites are pleomorphic organisms, they can be confused with other organisms, in fecal samples in the absence of staining [25]. Therefore, this study used wet mount, iodine, and Giemsa staining. Our study failed to find the amoeboid form, while the other three forms, vacuolar, granular, and cyst, were isolated. However, this was an expected result as Ahmed and Karanis [26] found that the amoeboid form is rarely reported.

PCR is the most sensitive method for Blastocystis diagnosis [14]. According to Badparva et al. [14], using the primers b11400 FORC and b11710 REVC, the DNA sequence size for the genus Blastocystis was 310 bp, which was in accordance with the results of this study, and convinced us that the organism found in the cattle feces samples was a genus of Blastocystis. In the current study, all of the 20 DNA samples (100%) were positive for this parasite. However, this study failed to determine Blastocystis ST using the STS primers (ST1-7). This suggested that Blastocystis ST from Kamal and Socah did not belong to Blastocystis STs 1-7. Furthermore, this research did not use barcode regions to detect the ST because extraction of the DNA was conducted using cultures of Blastocystis. This was because, according to research conducted by Stensvold [27], the barcoding PCR had higher applicability and sensitivity for fresh direct stool samples, while the STs primers generally worked better for DNA extracted from Blastocystis cultures.

Since the STs primer could identify STs of Blastocystis samples, we sequenced the PCR product. All of 20 selected samples were confirmed to be Blastocystis spp. ST10. The similarity percentages for the isolated Blastocystis spp. ST10 CA6 (accession no. KC148207.1) sequences were 97.39-98.52%. The results in the present study were highly similar to research by Santin et al. [28] that reported that their cattle samples were also only infected with ST10. Blastocystis spp. ST10 was also found in several other countries, and it was a predominant ST in cattle from Denmark [29], the USA [21], the UK, Libya [30], in China [23], in Thailand [28], and in Lebanon [31]. Therefore, the findings of this research reinforced the hypothesis by Cian et al. [5] that Bovidae may be natural hosts of Blastocystis spp. ST10. Their research showed that various Bovidae in a French Zoo were infected by ST10 and ST14. In Indonesia, Yoshikawa et al. [17] reported that a pig, chicken, and wild rodent in Sumba were infected with Blastocystis ST 1, 2, 4, and 7. This is the first report of Blastocystis ST 10 infection in cattle from Indonesia.

All of 20 DNA samples were located in the same clade with Blastocystis ST 10 from B. taurus from Denmark (FM164412). However, this was a different clade of Blastocystis ST10 from other ruminants, including Budorcas taxicolor from China (KY823348.1), Taurotragus oryx from China (KY823348.1), Camelus dromedarius from Libya (KC148207.1), and Bos grunniens from China (KY823393.1).

Although Blastocystis spp. ST10 is not considered a zoonotic organism, the existence of a high prevalence of ST10 in Bangkalan, Madura, in such close proximity to humans creates a potential for future transmission. Moreover, it poses a risk for other types of livestock and pets in Madura as a potential source of transmission. Domesticated animals that have already been reported to be infected by ST10 included sheep in China [32] and goats in Thailand [24], China [33], and Turkey [34]. Noradilah et al. [35] also reported Blastocystis spp. ST10 in dogs, goats, and birds in Malaysia.

**Conclusion**

Beef cattle in Kamal and Socah, Bangkalan, Madura, Indonesia, were infected by Blastocystis spp. ST10. This finding added to the literature that Blastocystis ST 10 is a predominant ST in cattle and it was the first report of Blastocystis ST 10 infection in cattle in Indonesia. Moreover, the prevalence found in this study was significantly higher than in other countries.

**Authors’ Contributions**

LTS, YS, ES, PH, and NDRL designed the concept for this research and scientific paper. All authors conducted the research. LTS and YS collected samples from the fields. LTS, YS, and NDRL conducted the laboratory work. All authors participated in the draft and revision of the manuscript and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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