Parasitology International 67 (2018) 702–705

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Short communication

Prevalence and molecular subtyping of *Blastocystis* from dairy cattle in Kanagawa, Japan

Aya Masuda, Toshiaki Sumiyoshi, Tadatoshi Ohtaki, Jun Matsumoto*

Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-0880, Japan

ARTICLE INFO

Keywords:
Blastocystis
Dairy cattle
Subtypes

ABSTRACT

Blastocystis is an intestinal protist, commonly found in the human population and in a wide range of animals globally. Currently, isolates from mammalian and avian hosts are classified into 17 subtypes (STs) based on phylogeny of the small subunit rRNA gene (SSU rDNA), of which ten (ST1-9, 12) are reported in humans. ST10 is a major ST reported from livestock cattle. However, other STs including ST1, 3, 4, 5, and 6, which have the potential to be transmitted to humans, are also reported from cattle in several countries. Although a survey has been conducted previously in western Japan for livestock cattle, there is no information available regarding other parts of Japan. Therefore, this study surveyed the prevalence of *Blastocystis* and its STs in cattle from Kanagawa prefecture, eastern Japan. Fecal specimens, collected from 133 dairy cattle on four different farms, were subjected to a short-term xenic *in vitro* culture and *Blastocystis* were identified by microscopic examination. Seventy-two cattle were positive for *Blastocystis* (54.1%). Direct sequences for the partial SSU rDNA were obtained for 45 samples. Based on nucleotide sequence homology search and phylogenetic analysis, 44 isolates were identified as ST14 and one as ST10. Our study confirms the presence of these STs in dairy cattle in Japan for the first time. The STs identified here, ST10 and ST14, support previous findings that Bovidae may be the natural host for both STs.

Blastocystis is an intestinal protist, taxonomically placed under Stramenopiles [1], and commonly found in humans and a vast range of animal species. Despite intensive studies in recent years, details including morphology and pathology of this parasite are still largely unknown. Recent molecular techniques have revealed a high genetic diversity of the parasite and isolates from various hosts are classified into different subtypes (STs) based on phylogeny of the small subunit rRNA gene (SSU rDNA). Currently, isolates from mammalian and avian hosts are classified into 17 STs, ten of which (ST1-9, 12) are reported in humans [2, 3]. Although asymptomatic carriage is common, infection with the parasite is also associated with non-specific gastrointestinal symptoms, including diarrhea, nausea, vomiting, abdominal pain, and irritable bowel syndrome [4].

STs that are isolated from humans are also commonly found in various animal groups, such as ST1-3 in non-human primates, ST1 and ST5 in Artiodactyla, and ST6 and ST7 in avian species [5]. This suggests that some STs may have low host specificity and could cause zoonotic transmission. Higher prevalence of the infection of certain STs was reported among animal handlers compared to those who do not have contact with animals [6, 7]. Conversely, there are STs, such as ST10 and ST14, which are commonly found in livestock cattle, but not yet reported in humans [5]. Therefore, understanding ST distribution in animal hosts is crucial as certain STs may be of zoonotic importance. Cattle are suggested to be the natural host for ST10 and ST14, although ST1 also seems to be common and could potentially be transmitted to humans [8]. In Japan, prevalence of *Blastocystis* in livestock cattle in western parts was 71%, and ST1, 3, and 5 were identified among these samples [9–11]. However, the study surveyed only western parts of Japan wherein ten STs (ST8-17) were not identified at that time. Therefore, this study aims to survey the prevalence and ST-identification of *Blastocystis* in livestock cattle in eastern Japan for the first time. These additional data could provide information on host specificity of the parasite and may be of important use for the livestock handlers in this area who are at risk of infection.

A fecal specimen was collected directly from the rectum of 133 dairy cattle between June and December 2017 on four different farms; A, B, C, and D located within a 10-km radius in Kanagawa prefecture, Japan. The number of samples from each farm were 34, 25, 44, and 30,

* Corresponding author at: Laboratory of Medical Zoology, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-0880, Japan.

E-mail addresses: aya.masuda@nihon-u.ac.jp (A. Masuda), sumiyoshi.toshiaki@nihon-u.ac.jp (T. Sumiyoshi), ohtaki.tadatoshi@nihon-u.ac.jp (T. Ohtaki), matsumoto.jun82@nihon-u.ac.jp (J. Matsumoto).

https://doi.org/10.1016/j.parint.2018.07.005
Received 13 March 2018; Received in revised form 4 July 2018; Accepted 4 July 2018

respectively. Cattle breeds examined here were predominantly Holstein (120/133, 90.2%). However, farms B and D also had a few Jersey, Brown Swiss, and Ayrshire breeds. Cattle were housed in a tie stall barn for farms B and C, and a free stall paddock for farm D, whereas both a tie stall and a free barn paddock were used for farm A. All of the cattle examined here were above 22 months of age and the average age on sampling was 51.5 months (SD = 23.0). None of the cattle exhibited clinical signs. Cattle in farms A, C, and D were routinely treated with Ivermectin Pour-On. All four farms had cattle (92/133, 69.2%) that were previously reared or grazed on other farms in the Kanagawa prefecture or in other prefectures including, Hokkaido, Gunma, Shizuoka, and Nagano. The average time cattle spent on these other farms was 18.2 months (SD = 9.5).

Fresh fecal samples were collected and subjected to short-term xenic *in vitro* culture using modified Tanabe-Chiba medium with 10% horse serum at 37 °C [12]. Following 3 days of culture, sediments were subjected to microscopic examination at 200× magnification. Samples negative for *Blastocystis* were re-examined after 2 more days of culture and defined negative when *Blastocystis* were not observed on either of these occasions [9]. Positive samples were sub-cultured in diphasic agar-slant medium prepared with modified Tanabe-Chiba medium with 10% horse serum [12] for 5 to 10 days at 37 °C. Sub-cultured samples were washed with PBS three times, and the genomic DNA of the parasite was extracted using DNAzol reagent (Invitrogen Life Technologies, USA) according to the manufacturer's protocol. Partial (320–342 bp) SSU rDNA were amplified using primers BL18SPPF1: 5′-AGTAGTCATACGCTCGTCTCAAA-3′ and BL18SR2PP: 5′-TCTTCGTT ACCCGTTACTGC-3′ [13]. The primer set used in this study is reported to be sufficient enough for differentiating *Blastocystis* STs, except for ST11 and ST12 for which only the partial sequences are currently available [5, 13]. PCR was performed in a 50 μL volume, including 1 μL of DNA template, 1× Ex Taq Buffer, 1.25 U of Takara Ex Taq (Takara Bio Inc., Japan), 0.2 mM dNTP mixture, and 0.2 μM primers. The thermal profile was conducted according to the manufacturer's recommended protocol with 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s. Amplified products were visualized on a 1.5% agarose gel. Positive PCR products were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany) according to the manufacturer's protocol, and directly sequenced on both strands using the same primers used for the PCR in a sequencing facility (FASMAC Co., Ltd., Japan). Sequences were compared with *Blastocystis* homologous sequences available from GenBank (National Centre for Biotechnology Information) using the nucleotide Basic Local Alignment Search Tool program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Subtypes were determined by the exact match or identity ≥98% against all known mammalian and avian *Blastocystis* subtypes, with a query coverage of ≥98%. Additionally, sequences obtained in this study were aligned with 29 sequences of known *Blastocystis* subtypes from ST1 to ST10 and ST13 to ST17, available from GenBank, using MUSCLE implemented in MEGA7 [14]. Reference sequence of ST11 and ST12 were not included because the domains used in this study were not available for these sequences [5]. The phylogenetic relationships of *Blastocystis* isolates were inferred by the Maximum Likelihoods (ML) method using MEGA7. The Tamura 3-parameter model incorporating a gamma distribution and invariant sites was selected as a substitution model based on the Bayesian information criterion. A phylogenetic tree was obtained after bootstrap analysis with 1000 replications. The sequences obtained in this study were deposited in GenBank under accession numbers LC388696 to LC388740.

Of the four farms sampled, all had cattle positive for *Blastocystis* (72/133, 54.1%), which was confirmed by microscopic examination. Vacuolar forms of the parasites with a size range from 5 to 50 μm were observed in positive samples under light microscopy. Prevalence of farm A, B, C, and D was 27/34 (79.4%), 11/25 (44.0%), 21/44 (47.7%), and 13/30 (43.3%), respectively (Table 1). The number of *Blastocystis*-positive cattle that previously reared or grazed on other farms are also shown in Table 1. For molecular subtyping, SSU rDNA PCR were performed for 57 positive isolates (27, 11, 6, and 13 samples from farm A, B, C, and D, respectively), and a PCR product size of 320–342 bp was observed on a 1.5% agarose gel for all isolates. Direct sequences for the SSU rDNA were obtained for 45 samples. Based on nucleotide sequence homology search, 44 isolates belonged to ST14 and one isolate from farm A to ST10. Similarly, 44 isolates were grouped with the reference sequences for ST14 and one with ST10 in the phylogenetic analysis of SSU rDNA sequences using the ML method (Fig. 1).

Table 1
Number of cattle confirmed positive for *Blastocystis* by microscopic examination.

Farm	Number of cattle positive/examined			
		Number of cattle previously reared or grazed on other farms		
	Total	Hokkaido	Others [a]	None [b]
A	27/34 (79.4%)	8/10	13/15	6/9
B	11/25 (44.0%)	7/12	1/2	3/11
C	21/44 (47.7%)	20/43	0	1/1
D	13/30 (43.3%)	3/7	2/3	8/20
Total	72/133 (54.1%)	38/72	16/20	18/41

[a] Others include farms in Kanagawa, Gunma, Shizuoka and Nagano prefectures, which are the Kanto and Chubu regions in Honshu.
[b] No experience of being reared/grazed on other farms.

This study reports the presence of *Blastocystis* infection among dairy cattle in eastern Japan for the first time. We reported a prevalence (54.1%) that was lower than the study conducted in western Japan (71.0%) [9], but higher than those in China (9.5%, 10.3%) [15, 16], Libya (41.7%), UK (22.6%) [17], and USA (19.0%) [18]. Most of the sub-cultured isolates in the present study were identified as ST14, except one isolate from farm A, which was identified as ST10. Both STs were previously reported in cattle outside Japan, ST10 being the dominant ST in countries including China, Denmark, Libya, UK, and USA, whereas ST14 is reported less frequently [16]. Although this study is the first to report these two STs from cattle in Japan, ST14 has been detected from Hokkaido sika deer (*Cervus nippon yesoensis*) recently (Dr. Shinya Fukumoto, personal communication) where they are known to feed on grasslands [19]. More than half of the *Blastocystis*-positive cattle in this study were previously reared on farms in Hokkaido, and there is a possibility of ST14 being transmitted between cattle and wild sika deer, although further investigation is required to confirm this. Remaining *Blastocystis*-positive cattle in this study, mainly from farm A, were born and/or grazed on other farms in Kanagawa or other prefectures including Gunma, Shizuoka, or Nagano, which are in the Kanto and Chubu regions. As a result of this frequent transfer of the animals, it is suggested that the infections of ST14 and possibly ST10 are already widespread and well established among cattle in these areas.

Although mixed infections of ST10 and ST14 have been reported in cattle previously [18], we did not confirm any mixed infection in the present study. Detecting mixed ST infection can be problematic as there is a possibility of dominant ST masking the presence of other STs [17]. We cannot be certain whether ST identified here was the only ST present or was just dominant enough over other STs to be identified in a mixed infection. The short-term xenic *in vitro* culture method used in this study is suggested to prevent differential outgrowth of STs [17], but the method also has the possibility to create cultural conditions favoring the growth of certain STs. Furthermore, identifying STs in mixed infection using direct sequencing can be an issue due to mixed signals on chromatograms. ST-specific primers have been developed for ST1 to ST9, enabling the identification of mixed infection in humans [20]. However, primers for ST10 to ST17 are not yet available which