



NOTE

Parasitology

First Molecular detection of *Theileria annulata* in Bangladesh

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ABSTRACT. In South Asia, *Theileria annulata* is known to be less pathogenic to local breeds of *Bos indicus* cattle comparing to *Bos taurus* cattle and some of mix breeds between them. Seroepidemiological surveys have revealed high sero-prevalence of *T. annulata* in asymptomatic local breeds of cattle in Bangladesh. Therefore, these asymptomatic infection in local breeds can be infectious sources to more sensitive breeds. In this study, 59 bloods of cattle showing no symptom were screened by species specific PCRs for hemato-protozoan parasites, to prove the existence of *T. annulata* parasite in asymptomatic cattle in Bangladesh. The *T. annulata* infection was confirmed along with other parasitic species, and this is the first report of *T. annulata* DNA detection in Bangladesh.

KEY WORDS: asymptomatic, Bangladesh, molecular detection, phylogeny, *Theileria annulata*

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Theileria annulata is a protozoan parasite transmitted by ticks of the genus *Hyalomma* and causes bovine tropical theileriosis in many areas, from the Mediterranean and Middle East area to China, and causes severe economic loss [21]. The clinical signs of tropical theileriosis are lymph node enlargement, anemia and hyperthermia [21]. In South Asia, *T. annulata* infections were identified by species specific polymerase chain reactions (PCR) and/or genes of the detected parasites were physiologically characterized in India, Pakistan and Sri Lanka [3, 5, 8, 11, 17]. In Bangladesh, it has been reported that approximately 22% of asymptomatic cattle showed seropositive for *T. annulata* [15]. We also recently reported that approximately 80 and 20% of asymptomatic cattle in Natore and Rajshahi district, Bangladesh, showed seropositive for *T. annulata* [1]. These reports suggested that asymptomatic infection of *T. annulata* is common among Bangladesh cattle. However, existence of *T. annulata* parasite has not yet been proven based on molecular methods among Bangladesh cattle. In Bangladesh, the local breeds of *Bos indicus* cattle, North Bengal Gray and Deshi, or mix breed between these local breeds and Holstein (*Bos taurus*) are widely used. It is known that *T. annulata* is less pathogenic in local breeds cattle in South Asia [6, 7]. There is a possibility that asymptotically infected local breeds cattle is source of *T. annulata* infection to cause severe tropical theileriosis among Holstein and some of mix breeds, highly productive breeds than local ones, in Bangladesh. Suspected theileriosis of the sensitive cattle are not rare in the local clinical sites (private communication).

Therefore, it is necessary to confirm whether the asymptomatic cattle in Bangladesh are really infected with *T. annulata*. In this study, we identify the infection of *T. annulata* of asymptomatic cattle in Natore district, Bangladesh by PCR and phylogenetic analysis. This is the first report of detection of *T. annulata* DNA in Bangladesh cattle.

To determine whether *T. annulata* infected cattle exists in Bangladesh, blood samples of fifty-nine asymptomatic cattle, 10 local breeding and 49 mix breeding, were randomly collected from a village in Singra Upazila (a subunit of a district) in the Natore

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District, Rajshahi Division of Bangladesh from February to April, 2017. There, farmers graze a few dozen local breeding and mix cattle in one place, and we recently reported that some cattle in the village have antibodies reacting with *T. annulata* surface protein [1]. However, it has not yet confirmed whether these cattle harbor the parasite. The experiments were approved by Gifu University Animal Care and Use Committee guidelines (Permit nos. 10066 and 14097). Total DNA was extracted from each the sample using *Quick-gDNA MiniPrep*TM (Zymo Research, Irvine, CA, U.S.A.) according to the manufacturer's instructions and stored at -20°C until use. The surveillance for *T. annulata* and other hepato-protozoan parasites, *Theileria orientalis*, *Babesia bovis*, *Babesia bigemina*, *Babesia ovata*, *Trypanosoma theileri* and *Trypanosoma evansi*, was performed by using species specific PCR methods described previously (Supplementary Table 1) [2, 4, 10, 12, 13, 16, 18].

As shown in Table 1, thirty-nine samples out of the fifty-nine showed positive for *T. orientalis* (66.1%). Among them, one sample obtained from a mix breeding cattle showed positive for *T. annulata* also, suggesting that the asymptomatic cattle was co-infected with both the *Theileria* species. No other sample showed positive for *T. annulata*. Two cattle showed positive for *B. bovis*, and three cattle showed for *T. theileri*. No one showed positive for *B. bigemina*, *B. ovata*, and *T. evansi* (Table 1). The prevalence of *T. orientalis* was remarkably high comparing with other species, and this result is similar to that of a previous molecular based survey on tick-borne pathogens using cattle blood samples in Mymensingh, another area of Bangladesh [14].

To confirm the *T. annulata* infection, the specimen showing double positive for *T. orientalis* and *T. annulata* was further analyzed. The partial nucleotides sequences of mitochondrial cytochrome *c* oxidase subunit 3 gene (*cox3*) were amplified by PCR with primers: CO3-seq-F (5'-CCA AAT MAT TTC TAC AAA ATG CC-3') and CO3-seq-R (5'-GTA AAT CAA CAT TTA YTA TAT GGA AC-3'), which had been designed as primers for universal *Theileria cox3* based on the referenced sequences [9]. 18S ribosomal region and Tams1 gene are known as potential targets for genotyping of *Theileria* and/or *Babesia* species. However, 18S is too conserving for molecular identification in *Theileria* and *Babesia* species and Tams1 gene of *T. orientalis* has never analyzed. On the other hand, the nucleotide sequences of *cox3* have analyzed in many bovine *Theileria* and *Babesia* species, and the nucleotide diversity is enough level for phylogenetic analysis [9]. So, we used the *cox3* region to confirm *T. annulata* infection.

The PCR was performed with the Tks Gflex DNA Polymerase, and the thermal cycling conditions for the amplification consisted of an initial denaturation step at 94°C for 1 min; followed by 30 cycles at 98°C for 10 sec, 55°C for 30 sec, and 68°C for 1 min. The PCR product was blunted and 5'-phosphorylated by using Mighty Cloning Reagent Set Blunt End (Takara Bio Inc., Kusatsu, Japan) prior to cloning into pUC118 vector. And then, ten plasmid clones were isolated, and the sequences of their insertions were determined using ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The ten plasmid clones yielded seven novel haplotypes Bd1, Bd2 and Bd4 to Bd8, four clones showed Bd2 while one clone each showed the other six haplotypes. These haplotypes were registered in Genbank (accession nos., LC472488, LC472489 and LC485174 to LC485178). For the phylogenetic analysis, the two haplotypes were aligned along with those of *Theileria* and *Babesia* species [9]. In the phylogenetic tree, Bd1 constructed a clade with that of referenced *T. annulata* with high bootstrap values, while Bd2 constructed another clade with that of the referenced *T. orientalis* (Fig. 1). In addition, the similarity between the Bd1 and that of the referenced *T. annulata* was considered to be intraspecific level (99.1%). These results demonstrate that the one asymptomatic cattle was co-infected with *T. annulata* and *T. orientalis*. As far as we know, this is the first molecular based evidence of *T. annulata* infection in Bangladesh. In addition to the specimen, partial *cox3* sequences of two *T. orientalis* only positive individuals were sequenced directly. The sequences of them yielded Bd2 and a novel haplotype Bd3 (accession no. LC472490). In the phylogenetic tree, Bd3 constructed a clade along with Bd2 and that of the referenced *Th. orientalis* (Fig. 1). It was confirmed that these cattle were infected with *T. orientalis*.

Seroepidemiological surveys had suggested that asymptomatic infection of *T. annulata* is common, and it might be an infectious source to other host animals in Bangladesh [1]. However, existence of *T. annulata* in asymptomatic cattle had not been proven yet. In this study, we detected the *T. annulata* DNA in the asymptomatic cattle in Bangladesh. In Bangladesh, not only local breeds (*B. indicus*) but also Holstein (*B. taurus*) and mixed breeds between them are widely used. In addition, *T. annulata* is known to be less pathogenic to the local breeds, and the asymptomatic infected cattle can be an infectious source to severe theileriosis in Holstein and some susceptible mixed breeds [6, 7]. Therefore, to control tropical theileriosis caused by *T. annulata* in Bangladesh, further PCR based investigation are required to reveal current *T. annulata* infection even in asymptomatic cattle.

Table 1. The polymerase chain reactions (PCR) diagnose of hemato-protozoan parasites in asymptomatic cattle

No. samples	<i>Theileria orientalis</i>	<i>T. annulata</i>	<i>Babesia bovis</i>	<i>B. bigemina</i>	<i>B. ovata</i>	<i>Tripanosoma theileri</i>	<i>T. evansi</i>
34 ^{a)}	+	-	-	-	-	-	-
1 ^{b)}	+	+	-	-	-	-	-
1	+	-	+	-	-	-	-
3	+	-	-	-	-	+	-
1	+	-	N.A.	N.A.	N.A.	N.A.	N.A.
1	-	-	+	-	-	+	-
1	-	-	-	-	-	+	-
16	-	-	-	-	-	-	-
1	-	-	N.A.	N.A.	N.A.	N.A.	N.A.
Total	59	39	1	2	0	5	0

a) The *cox3* haplotypes of two specimens were used in the phylogenetic tree after sequenced directly. b) The *cox3* haplotypes were determined after cloning and used in the phylogenetic tree. +: Positive, -: Negative, N.A.: not analyzed.

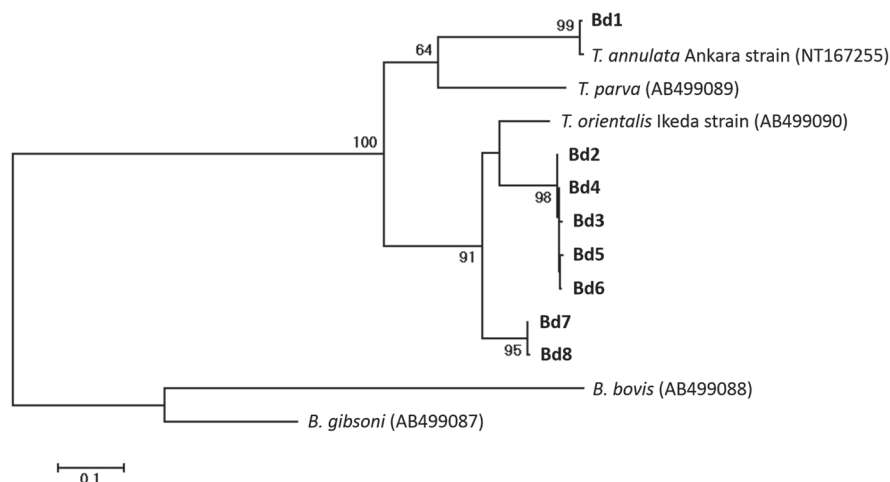


Fig. 1. Phylogenetic tree of *Theileria* spp. based on partial nucleotide sequences of mitochondrial *cox1* gene (453 bp). The tree was constructed by neighbor-joining method using Tamura 3 parameter model with gamma distribution (0.84) in MEGA version 6.06 [19, 20], and node support was calculated by 1,000 bootstrap replications. Current haplotypes are shown in boldface. The sequences of *Theileria annulata* (Genbank accession no. NT167255), *T. parva* (AB499089), *T. orientalis* (AB499090) were used as referenced sequences, and those of *Babesia bovis* (AB499088) and *B. gibsoni* (AB499087) were used as outer species [9].

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REFERENCES

1. Ali, M. W., Alauddin, M., Azad, M. T., Hasan, M. A., Appiah-Kwarteng, C., Takasu, M., Baba, M., Kitoh, K., Rahman, M. and Takashima, Y. 2016. *Theileria annulata* seroprevalence among different cattle breeds in Rajshahi Division, Bangladesh. *J. Vet. Med. Sci.* **78**: 1577–1582. [Medline] [CrossRef]
2. Artama, W. T., Agey, M. W. and Donelson, J. E. 1992. DNA comparisons of *Trypanosoma evansi* (Indonesia) and *Trypanosoma brucei* spp. *Parasitology* **104**: 67–74. [Medline] [CrossRef]
3. Dandasena, D., Bhandari, V., Sreenivasamurthy, G. S., Murthy, S., Roy, S., Bhanot, V., Arora, J. S., Singh, S. and Sharma, P. 2018. A Real-Time PCR based assay for determining parasite to host ratio and parasitaemia in the clinical samples of bovine Theileriosis. *Sci. Rep.* **8**: 15441. [Medline] [CrossRef]
4. Figueroa, J. V., Chieves, L. P., Johnson, G. S. and Buening, G. M. 1993. Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood. *Vet. Parasitol.* **50**: 69–81. [Medline] [CrossRef]
5. George, N., Bhandari, V., Reddy, D. P. and Sharma, P. 2015. Molecular and Phylogenetic analysis revealed new genotypes of *Theileria annulata* parasites from India. *Parasit. Vectors* **8**: 468. [Medline] [CrossRef]
6. Glass, E. J. and Jensen, K. 2007. Resistance and susceptibility to a protozoan parasite of cattle--gene expression differences in macrophages from different breeds of cattle. *Vet. Immunol. Immunopathol.* **120**: 20–30. [Medline] [CrossRef]
7. Glass, E. J., Preston, P. M., Springbett, A., Craigmile, S., Kirvar, E., Wilkie, G. and Brown, C. G. 2005. *Bos taurus* and *Bos indicus* (Sahiwal) calves respond differently to infection with *Theileria annulata* and produce markedly different levels of acute phase proteins. *Int. J. Parasitol.* **35**: 337–347. [Medline] [CrossRef]
8. Hassan, M. A., Liu, J., Sajid, M. S., Mahmood, A., Zhao, S., Abbas, Q., Guan, G., Yin, H. and Luo, J. 2018. Molecular detection of *Theileria annulata* in cattle from different regions of punjab, Pakistan, by using recombinase polymerase amplification and polymerase chain reaction. *J. Parasitol.* **104**: 196–201. [Medline] [CrossRef]
9. Hikosaka, K., Watanabe, Y., Tsuji, N., Kita, K., Kishine, H., Arisue, N., Palacpac, N. M., Kawazu, S., Sawai, H., Horii, T., Igarashi, I. and Tanabe, K. 2010. Divergence of the mitochondrial genome structure in the apicomplexan parasites, *Babesia* and *Theileria*. *Mol. Biol. Evol.* **27**: 1107–1116. [Medline] [CrossRef]
10. Kirvar, E., Ilhan, T., Katzer, F., Hooshmand-Rad, P., Zweggarth, E., Gerstenberg, C., Phipps, P. and Brown, C. G. D. 2000. Detection of *Theileria annulata* in cattle and vector ticks by PCR using the Tams1 gene sequences. *Parasitology* **120**: 245–254. [Medline] [CrossRef]
11. Kolte, S. W., Lacombe, S. D., Jadhao, S. G., Magar, S. P., Warthi, G., Kurkure, N. V., Glass, E. J. and Shiels, B. R. 2017. PCR diagnosis of tick-borne pathogens in Maharashtra state, India indicates fitness cost associated with carrier infections is greater for crossbred than native cattle breeds. *PLoS One* **12**: e0174595. [Medline] [CrossRef]
12. Ota, N., Mizuno, D., Kuboki, N., Igarashi, I., Nakamura, Y., Yamashina, H., Hanzaike, T., Fujii, K., Onoe, S., Hata, H., Kondo, S., Matsui, S., Koga, M., Matsumoto, K., Inokuma, H. and Yokoyama, N. 2009. Epidemiological survey of *Theileria orientalis* infection in grazing cattle in the eastern part of Hokkaido, Japan. *J. Vet. Med. Sci.* **71**: 937–944. [Medline] [CrossRef]
13. Rodrigues, A. C., Garcia, H. A., Ortiz, P. A., Cortez, A. P., Martinkovic, F., Paiva, F., Batista, J. S., Minervino, A. H., Campaner, M., Pral, E. M., Alfieri, S. C. and Teixeira, M. M. 2010. Cysteine proteases of *Trypanosoma (Megatrypanum) theileri*: cathepsin L-like gene sequences as targets for

- phylogenetic analysis, genotyping diagnosis. *Parasitol. Int.* **59**: 318–325. [[Medline](#)] [[CrossRef](#)]
14. Roy, B. C., Krücken, J., Ahmed, J. S., Majumder, S., Baumann, M. P., Clausen, P. H. and Nijhof, A. M. 2018. Molecular identification of tick-borne pathogens infecting cattle in Mymensingh district of Bangladesh reveals emerging species of *Anaplasma* and *Babesia*. *Transbound. Emerg. Dis.* **65**: e231–e242. [[Medline](#)] [[CrossRef](#)]
 15. Samad, A., Dhar, S. and Gautam, O. P. 1983. Prevalence of *Theileria annulata* infection among cattle of Bangladesh. *Indian J. Parasitol.* **7**: 61–63.
 16. Sivakumar, T., Altangerel, K., Battsetseg, B., Battur, B., Aboulaila, M., Munkhjargal, T., Yoshinari, T., Yokoyama, N. and Igarashi, I. 2012. Genetic detection of *Babesia bigemina* from Mongolian cattle using apical membrane antigen-1 gene-based PCR assay. *Vet. Parasitol.* **187**: 17–22. [[Medline](#)] [[CrossRef](#)]
 17. Sivakumar, T., Kothalawala, H., Abeyratne, S. A., Vimalakumar, S. C., Meewewa, A. S., Hadirampela, D. T., Puvirajan, T., Sukumar, S., Kuleswarakumar, K., Chandrasiri, A. D., Igarashi, I. and Yokoyama, N. 2012. A PCR-based survey of selected *Babesia* and *Theileria* parasites in cattle in Sri Lanka. *Vet. Parasitol.* **190**: 263–267. [[Medline](#)] [[CrossRef](#)]
 18. Sivakumar, T., Tagawa, M., Yoshinari, T., Ybañez, A. P., Igarashi, I., Ikehara, Y., Hata, H., Kondo, S., Matsumoto, K., Inokuma, H. and Yokoyama, N. 2012. PCR detection of *Babesia ovata* from cattle reared in Japan and clinical significance of coinfection with *Theileria orientalis*. *J. Clin. Microbiol.* **50**: 2111–2113. [[Medline](#)] [[CrossRef](#)]
 19. Tamura, K. and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **10**: 512–526. [[Medline](#)]
 20. Tamura, K., Stecher, G., Peterson, D., Filipiński, A. and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30**: 2725–2729. [[Medline](#)] [[CrossRef](#)]
 21. Weir, W., Karagenc, T., Baird, M., Tait, A. and Shiels, B. R. 2010. Evolution and diversity of secretome genes in the apicomplexan parasite *Theileria annulata*. *BMC Genomics* **11**: 42. [[Medline](#)] [[CrossRef](#)]