

## *Theileria annulata* seroprevalence among different cattle breeds in Rajshahi Division, Bangladesh

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**ABSTRACT.** An epidemiological survey of *Theileria annulata* infection was undertaken in a cattle population in Rajshahi Division, Bangladesh. The local cattle breeds from the area (North Bengal Gray and Deshi) and crosses between the local breeds and Holstein cattle were predominantly screened. In total, 192 cattle serum samples were collected in two areas of Rajshahi Division, the Rajshahi District (n=147) and Natore District (n=45). The samples were screened with an enzyme-linked immunosorbent assay using *T. annulata* surface protein (TaSP) as the antigen. The seroprevalence was 80.0% (36/45) in Natore and 20.4% (30/147) in Rajshahi. A logistic regression analysis showed that the sampling location was significantly associated with seropositivity, whereas age, sex and breed were not. Although the logistic regression analysis did not show a linear dependence on age, we considered age-specific seroprevalence separately in the two districts. Seroprevalence did not differ significantly among age categories in the Natore District. In contrast, all the cattle <1 year old in the Rajshahi District were seronegative (11/11). Seroprevalence in the 1- and 2-year-old cattle was significantly lower in the Rajshahi District than in the Natore District. In the older age categories (3, 4 and >5 years), seroprevalence did not differ significantly between the Natore and Rajshahi Districts. These results suggest that the cattle in the Rajshahi District were sporadically exposed to *T. annulata*, whereas most cattle in the Natore District became infected during an early phase of life.

**KEY WORDS:** Bangladesh, seroprevalence, TaSP, *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 North Africa, southern Europe, India, the Middle East and Central Asia [16]. The disease is endemic in cattle populations in many areas, including India and Pakistan [4, 10, 11, 20, 21]. *Theileria annulata* has also been reported in Bangladesh [18]. However, only a few epidemiological studies have been undertaken in Bangladesh, so the prevalence of *T. annulata* in Bangladesh is not yet clear. An epidemiological study in Tunisia revealed two main patterns of disease spread [3]. In the first, almost all the cattle are infected with *T. annulata*, but disease only occurred in young animals. In the second, the frequency of infected individuals was relatively low, but the disease occurred in animals of all ages.

*Theileria annulata* infection in Holstein cattle (*Bos taurus*) causes fatal disease, but it is less pathogenic in indigenous breeds in endemic areas, e.g., Shahiwal cattle (*B. indicus*) in

the Punjab and Kenana cattle (*B. taurus*) in the Sudan [2, 6, 7, 12]. In Bangladesh, the local breeds of *B. indicus* cattle, North Bengal Gray and Deshi, are widely farmed. However, it is not yet known whether these breeds are resistant or susceptible to tropical theileriosis. In this study, we undertook an epidemiological survey of *T. annulata* infection in the northwestern area of Bangladesh.

### MATERIALS AND METHODS

**Study area and sample collection:** The study was undertaken in the suburbs of Rajshahi City and Singra Upazila (a subunit of a district) in the Natore District. Both places are in the Rajshahi Division of Bangladesh. This division is in the midwestern corner of Bangladesh and shares a border with India. In the Rajshahi City area, intensive stock farming is uncommon, and only a few cattle are kept by subsistence farmers using a traditional nonintensive system of animal rearing. We collected 147 serum samples from cattle in four different settlements owned by small-hold farmers in Rajshahi City between November 2012 and December 2014. According to the general inspection by local veterinarians, all examined cattle were asymptomatic. In Singra Upazila, farmers graze a few dozen cattle in one place. We collected 45 serum samples from two different settlements in Singra Upazila in June 2015. Although a few cattle younger than 1 year were grazed with the older cattle in these settlements,

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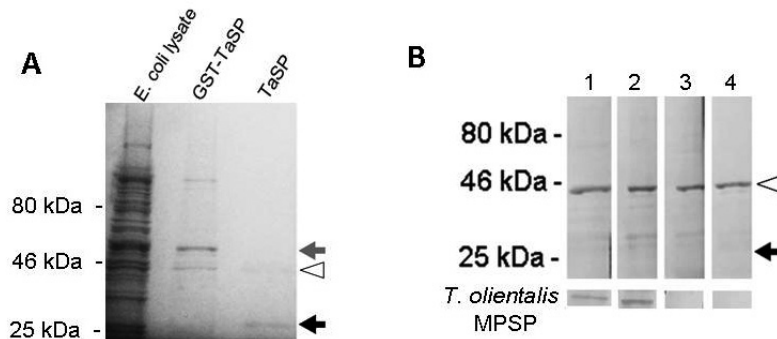


Fig. 1. Reactivity against *TaSP* of *T. orientalis* seropositive and negative cattle sera. (A) SDS PAGE of *TaSP* protein expressing *E. coli* lysate, fusion protein with *TaSP* and GST purified using Glutathione Sepharose 4B and *TaSP* protein separated from GST with PreScissionProtease. (B) Western blotting analysis of the separated *TaSP*. Obtained *TaSP* protein was applied for SDS PAGE and reacted with *T. orientalis* seropositive sera (lanes 3 and 4) and seronegative sera (lanes 1 and 2). Gray arrow, black arrow and open arrowhead indicate fusion protein with *TaSP* and GST, *TaSP* and trace amount of contaminated protein.

we only collected serum samples from 1-year-old and older individuals. We obtained the ages of their cattle from the farmers during sample collection. The sex and breed of the sampled cattle were also noted. Blood was drawn from the jugular vein and collected into serum tubes. The tubes were kept in a cooler box with ice bricks and transported to a laboratory at Rajshahi University by road within 5 hr of collection. At the laboratory, the blood samples were centrifuged at  $400 \times g$  for 10 min, and the sera were harvested into labeled vials and stored at  $-20^{\circ}\text{C}$  until analysis. The experiments were approved by Gifu University Animal Care and Use Committee guidelines (Permit number 10066 and 14097).

**Expression of immunogenic protein of *T. orientalis* and *T. annulata*, Western blotting analysis:** The major piroplasm surface protein (MPSP) of *T. orientalis* type 2 strain was expressed using *E. coli* as described previously [22]. The plasmid to express MPSP was kindly provided by Prof. Yokoyama, Obihiro University of Agriculture and Veterinary Medicine (Obihiro, Japan). The part of the *TaSP* protein of *T. annulata* predicted to be highly immunogenic (amino acids 26–157) was expressed as a fusion protein, as described in a previous study [19], with some modification. The gene encoding the protein was synthesized to ensure its codons were those most frequently used by *Escherichia coli*. When the gene was synthesized, *Bam*HI and *Sal*I recognition sites were added to its 5' and 3' termini, respectively. The synthesized gene was inserted into the multiple cloning region of the GST gene fusion vector, pGEX-6P-1 (GE Healthcare UK Ltd., Buckinghamshire, U.K.). *Escherichia coli* strain BL21 was transformed with the resulting plasmid, and the recombinant protein was expressed with standard procedures. The transformed *E. coli* was lysed with B-PER Bacterial Protein Extraction Reagent (Life Technologies Ltd., Palo Alto, CA, U.S.A.), and the fusion protein was purified with Glutathione Sepharose 4B (GE Healthcare UK Ltd.), according to the manufacturers' instructions. The *TaSP* pro-

tein was separated from GST with PreScissionProtease (GE Healthcare UK Ltd.). Although putative molecular weight of the *TaSP* is approximately 14 kDa, SDS-PAGE demonstrated much higher molecular mass of the expressed protein (Fig. 1A) as reported previously [19]. Obtained *T. orientalis* MPSP and *T. annulata* *TaSP* were separated by SDS-PAGE, transferred to Immobilon-P Transfer Membranes (Merck Millipore, Darmstadt, Germany) and reacted with cattle sera corrected in Japan. Reacted protein bands were visualized using horseradish peroxidase conjugated goat anti-bovine total IgG (Bethyl Laboratories, Montgomery, TX, U.S.A.) and Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega, Madison, MD, U.S.A.).

**Enzyme-linked immunosorbent assay (ELISA):** A 96-well ELISA plate (Iwaki Ltd., Chiba, Japan) was coated with recombinant *TaSP* diluted with carbonate buffer (pH 9.6) to a concentration of  $2.5 \mu\text{g/ml}$ . An aliquot ( $50 \mu\text{l}$ ) was pipetted into each well and incubated at  $4^{\circ}\text{C}$  overnight. After the wells were blocked with 3% skim milk in phosphate-buffered saline (PBS-SM) for 1 hr at  $37^{\circ}\text{C}$ , the plates were washed with washing solution (PBS containing 0.05% Tween 20). The serum samples were diluted 1:100 with PBS-SM, and an aliquot ( $50 \mu\text{l}$ ) was added to each well. The plates were incubated at  $37^{\circ}\text{C}$  for 1 hr. After the plates were washed six times with washing solution, they were incubated with horseradish-peroxidase-conjugated anti-bovine IgG antibody (ORB16576, Biorbyt Ltd., Cambridge, U.K.) at  $37^{\circ}\text{C}$  for 1 hr. After the plates were washed six times with washing solution, colorimetric reactions were performed by adding  $50 \mu\text{l}$  portions of the substrate Sure Blue TMB 1-Well Microwell Peroxidase (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, U.S.A.). After incubation at room temperature for 15 min, the reaction was stopped by adding  $50 \mu\text{l}$  of 1 N HCl, and the absorbance at 450 nm ( $A_{450}$ ) was measured in each well. Thirty-seven cattle serum samples collected in a *T. annulata*-free area (Japan) were used as the

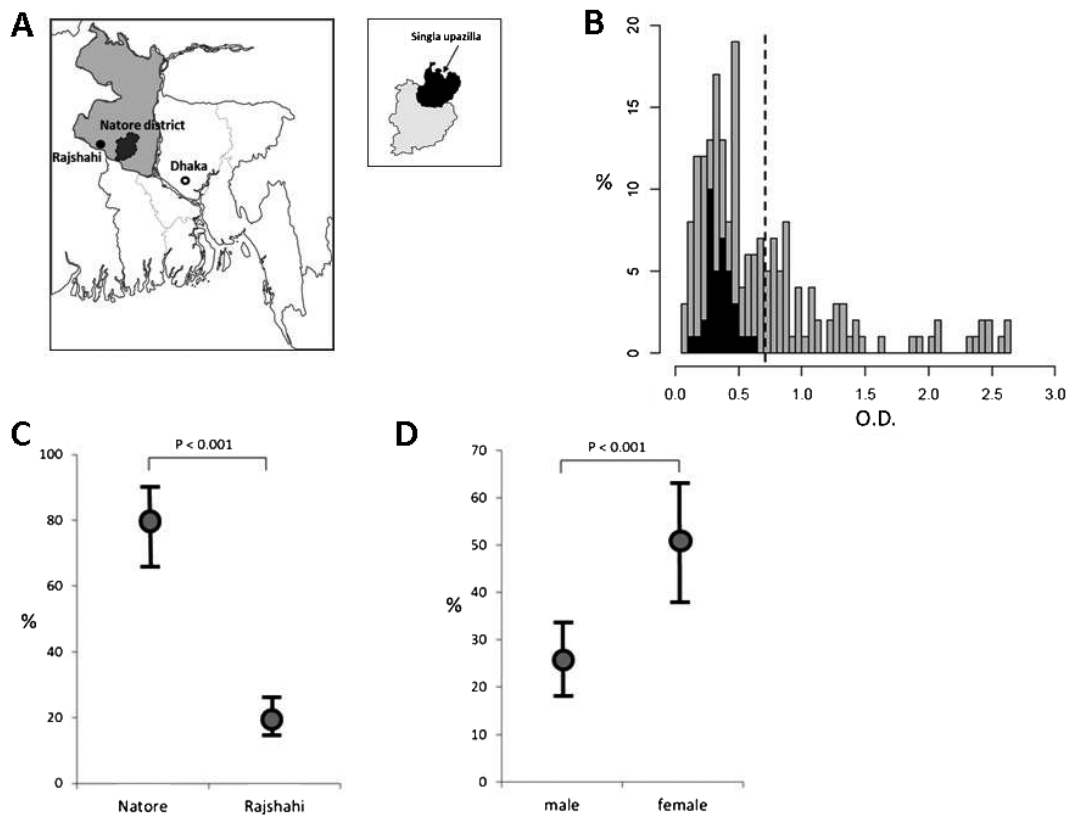


Fig. 2. Seroprevalence of *T. annulata* in cattle in Bangladesh. (A) Location of the study areas. Gray and deep-gray areas on the map show Rajshahi Division and the Natore District, respectively. Inserted map shows the Natore District. The black area indicates Singra Upazila in the Natore District. (B) Distribution of absorbance values on the anti-*TaSP* ELISA. Black and gray bars indicate Japanese and Bangladesh cattle data, respectively (n=37 and 192, respectively). Dotted line indicates the calculated cut-off value. (C, D) Seroprevalence in the two sampling areas and sexes. 95% CIs of seroprevalence are also indicated.

negative controls. The cut-off value for positivity was calculated as the mean value plus three standard deviations (mean + 3SD) of the negative control sera.

**Data analysis:** The data were analyzed with R [14]. The 95% confidence interval (CI) of the seropositive rate was calculated using an exact method, assuming a binomial distribution. The statistical significance of differences in seroprevalence between the sexes (male and female) and locations (Rajshahi and Natore) was determined with Fisher's exact test. Fisher's exact test was also used to determine whether the sex and breed ratios between the samples collected in Rajshahi and Natore were similar. Logistic regression was used to assess age, sex, location and breed as risk factors for seropositivity. To check for multicollinearity, the variance inflation factor (VIF) was calculated using the R DAAG package [9]. Since none of the VIF values exceeded 10 and the mean VIF of the model was <6, we concluded that there was no multi-collinearity problem. Using the model as the initial model, backward elimination was applied. The predictive performance and model fit were assessed using the area under the receiver operating characteristic (ROC) curve (AUC) and Akaike's information criterion (AIC). AUC was

calculated with the R pROC package [15].

## RESULTS

A total of 192 cattle were sampled in the suburbs of Rajshahi City and Singra Upazila in the Natore District of Rajshahi Division, Bangladesh (Fig. 2A), and the sera were screened with an ELISA using *TaSP* as antigen. The sampled cattle were local breeds (North Bengal Gray and Deshi), Pakistan-derived dairy cattle (Sahiwal), India-derived Zebu or crossbred cattle (Table 1). A cut-off value was determined as the mean + 3SD of 37 control sera collected in a *T. annulata*-free country, Japan (OD=0.6774). Regardless of reactivity against a *T. orientalis* antigen, MPSP, any Japanese cattle serum did not react with *TaSP* (Fig. 1B, black arrow). It indicates that *TaSP* is not cross reacted with serum of *T. orientalis* infected cattle serum, which is common in Japan. Therefore, we concluded that cattle serum collected in Japan can be used as a negative control regardless of *T. orientalis* infection. When we produced and purified *TaSP*, trace amount of contaminated protein remained (Fig. 1A). The contaminated protein reacted with all examined cattle sera

Table 1. Cattle breeds in Natore and Rajshahi

	Natore	Rajshahi
NBG <sup>a</sup> )	15.9% (7)	15.0% (22)
Deshi	70.5% (31)	37.9% (55)
Zebu <sup>b</sup> )	0.0% (0)	4.1% (6)
Sahiwal	2.3% (1)	0.0% (0)
Mix	11.4% (5)	42.8% (62)

a) North Bengal Gray, b) Indian derived Zebu. Percentage of each breed in Natore and Rajshahi. Percentage of each breed in Natore and Rajshahi.

in a similar manner (Fig. 1B, open arrowhead). Therefore, although the trace amount of contaminated protein might increase back ground level in ELISA, we considered it could not be a reason for false-positive. Under our criteria, 66 cattle were considered seropositive (Fig. 2B), and the seroprevalence was 34.4% (95% CI: 27.7–41.6). In the suburbs of Rajshahi City, 30 of 147 cattle (20.4%, 95% CI: 14.2–27.8) were seropositive, and in Singra Upazila, 36 of 45 cattle (80.0%, 95% CI: 65.4–90.4) were seropositive. Thus, the seroprevalence differed significantly at the two locations (Fig. 2C). We recorded the age, sex and breed of each animal during sample collection. However, two samples obtained in the Rajshahi City area and one sample obtained in the suburbs of Natore town lacked these data. Therefore, we excluded those three samples from further analysis. As shown in Fig. 2D, male cattle showed significantly lower seroprevalence (25.4%, 95% CI: 18.0–34.1) than female cattle (50.5%, 95% CI: 38.2–63.2). However, farmers in Bangladesh tend to keep female cattle for more years than male cattle. Age and sex could confound the outcome, because age is associated with exposures to the parasite.

Therefore, we performed a logistic regression analysis to separately evaluate age, sex, location and breed as risk factors for seropositivity. As shown in Table 2, neither age nor sex nor breed was a significant predictor of seropositivity

(95% CI of the AUC of the model was 0.72–0.87). Only the location of sampling was significantly associated with seropositivity, and the odds ratio between the suburbs of Rajshahi City and Singra Upazila in the Natore District was 11.92 (95% CI: 4.63–30.72). Although backward elimination was applied to the initial model, no marked improvement in the predictive performance or in the model fit was observed (not shown). As the logistic analysis predicted, the differences in seroprevalence between sexes, breeds and age categories within each sampling location were not significant (Fig. 3A–C). Although the logistic regression analysis did not show a linear dependence on age, in order to consider a non-linear dependence, age-specific seroprevalence separately in the two districts was compared. The 1- and 2-year-old cattle in Rajshahi City showed significantly lower seroprevalence than those in Natore. All the sampled cattle younger than 1 year in Rajshahi City were seronegative (11/11). In contrast, older cattle (3, 4 and >5 years) showed higher seroprevalence in both locations, and the difference in seroprevalence between Rajshahi and Natore for each category was not significant (Fig. 3C).

## DISCUSSION

In this study, we surveyed *T. annulata* infection among cattle in Rajshahi Division, Bangladesh, and found a high seroprevalence in asymptomatic cattle in the Singra Upazila of the Natore District. Seroprevalence was significantly higher in that area than in the Rajshahi city area. The young cattle in the Rajshahi City area showed particularly low seroprevalence. In contrast, the cattle in Singra Upazila of Natore showed high seroprevalence, regardless of age. These results suggest that the cattle in Rajshahi City were sporadically exposed to *T. annulata*, whereas the majority of cattle in Natore City became infected during an early phase of life. This difference may be attributable to the differences

Table 2. Result of logistic regression analysis

		Result of logistic regression analysis	Odds ratio (95% CI)	P
Age	< 1 Year	0 (0–28.5)		
	1 Year	30.1 (20.0–42.0)		
	2 Years	32.7 (19.9–47.5)		
	3 Years	55.6 (35.3–74.5)	1.13 <sup>a</sup> ) (0.88–1.46)	0.34
	4 Years	33.3 (11.8–61.6)		
	> 5 Years	50.0 (23.0–77.0)		
Sex	Male	25.4 (18.0–34.1)	-	-
	Female	50.7 (38.2–63.2)	1.27 (0.55–2.96)	0.58
Location	Rajshahi	20.7 (14.4–28.2)	-	-
	Natore	79.5 (64.7–90.2)	11.92 (4.63–30.72)	<0.001
Breed	NBG <sup>b</sup> )	24.1 (10.3–43.5)	-	-
	Deshi	41.9 (31.3–53.0)	2.07 (0.68–6.36)	0.20
	Zebu <sup>c</sup> )	16.7 (0.4–64.1)	1.24 (0.11–13.64)	0.86
	Sahiwal	100 (2.5–100)	e <sup>14.2</sup> (0.00–inf.)	0.99
	Mix	26.9 (16.8–39.1)	1.89 (0.57–6.22)	0.3

a) Odds ratio per year, b) North Bengal Gray, c) Indian derived Zebu.

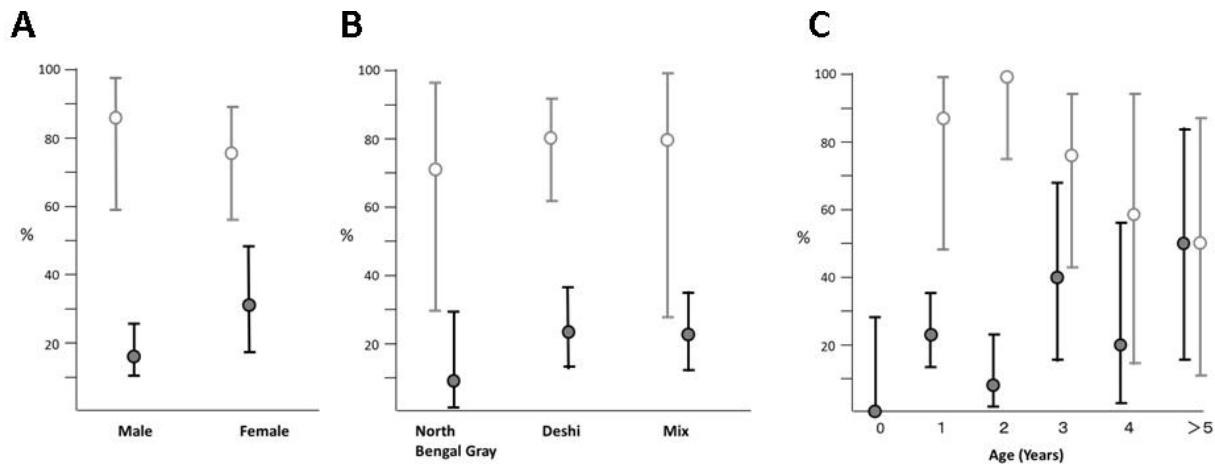


Fig. 3. Seroprevalence of *T. annulata* in cattle in Natore (open circles) and Rajshahi (solid circles). 95% CIs of seroprevalence are also indicated. Seroprevalence patterns by sex (A), breed (B) and age categories (C).

in the rearing styles at these two locations. In the Rajshahi City area, intensive stock farming is uncommon, and only a few cattle are kept in farmhouse premises. In contrast, in the suburbs of Natore, farmers send a few dozen cattle to graze in one place, so it is possible that many cattle become infected during communal grazing.

The differences in the rearing systems and in the prevalence of ticks in these two areas may be the risk factors underlying the significant difference in the prevalence of theileriosis. In Singra in the Natore District, farmers graze their cattle in groups of 10–30 on wet, low-lying pasture, especially near the banks of rivers, where the chance of tick infestation and disease transmission is elevated. In contrast, the farmers of Rajshahi city harvest grass from the pastures to supply their cattle, so there is less chance of disease because the cattle are not exposed to ticks on open grazing land. There is also some degree of variation in the weather patterns of these two areas. The environment of the Natore area is subhumid and semiarid, which favors ticks more than the hot and humid environment of the Rajshahi City area. In this study, we could not consider seasonal difference, because the sampling period in Rajshahi city and Singra Upazila was different. To consider seasonal difference of seroconversion, a further study is necessary.

Tropical theileriosis is sometimes confused with other diseases that also cause anemia and/or jaundice. Recently, serodiagnosis systems have been developed to detect *T. annulata* infection [1, 13, 17]. These serodiagnostic techniques can be very useful tools for the differential diagnosis of tropical theileriosis. The amino acid sequence of *TaSP* used in this study is derived from a parasite strain isolated in Turkey [19]. To improve specificity and sensitivity of ELISA for detection of *T. annulata* infected cattle in Bangladesh, it is necessary to isolate *T. annulata* strains in this area and decide *TaSP* amino acid sequence of local strains. However, because the seroprevalence of *T. annulata* is high among asymptomatic cattle in Rajshahi Division, animals with anemia and/or jaundice caused by other diseases, including

babesiosis, anaplasmosis or leptospirosis, will frequently show positive results in a serodiagnosis system for *T. annulata* infection. Therefore, using a serodiagnosis system to detect tropical theileriosis in the veterinary clinical context may not be realistic in Rajshahi Division, Bangladesh. The establishment of a cheap and simple way to distinguish acute *T. annulata* infection from asymptomatic persistent infection must be developed for the appropriate treatment of tropical theileriosis in this area.

*Theileria annulata* induces severe inflammation in susceptible cattle breeds, with high mortality. However, it has been shown that the parasite does not induce severe symptoms in resistant breeds [5–8, 12]. In this study, we have demonstrated that most cattle of the local Bangladeshi *B. indicus* breeds, North Bengal Gray and Deshi, were seropositive, but asymptomatic in Natore. This strongly suggests that North Bengal Gray and Deshi cattle are resistant to tropical theileriosis. Cattle crossbred from Holstein and the local in Bangladesh breeds also showed no symptoms. Therefore, the crossbred cattle raised in these areas may have inherited resistance from the local breeds.

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