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Intrauterine Infection by *Mycobacterium avium* subsp. *paratuberculosis* in buffalo *(Bubalus bubalis)*

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Abstract

This study reports the detection of *Mycobacterium avium* subsp. *paratuberculosis* (Map) in the uterus and fetus of a buffalo (*Bubalus bubalis*). Samples of the buffalo cow's ileum, mesenteric lymph node, uterus and placenta as well as various fetal organs were collected. Quantitative real-time polymerase chain reaction (qPCR), histopathology (hematoxylin-eosin (HE) staining) and Ziehl-Neelsen (ZN) staining were performed. Histopathological analysis revealed lesions consistent with *paratuberculosis* (PTB) in the mesenteric lymph node and small intestine, and ZN staining revealed the presence of acid-alcohol resistant bacilli (AARB). Histological lesions were not identified in fetal tissues. qPCR indicated that uterus, mesenteric lymph node and small intestine samples from the buffalo cow were positive for Map. In contrast, in the fetus, only samples from the digestive tract were positive for Map based on qPCR data. These results indicate that Map is present in various buffalo organs and tissues, including within the reproductive system. Therefore, the intrauterine transmission of Map may occur in buffaloes and could represent an important source of infection.

Keywords: Johne's disease; Diagnostic; Buffaloes; Intrauterine transmission

Introduction

Paratuberculosis (PTB) is caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map), a gram-positive, aerobic, nonsporulating rod that grows slowly in culture. This disease is clinically characterized by chronic intermittent diarrhea, progressive weight loss and decreased production and fertility as well as increased susceptibility to other infections. PTB primarily affects mammals, and domestic and wild ruminants are the most susceptible hosts [1].

The major transmission route for PTB is thefecal-oral route, which involves animals becoming infected by ingesting water or food contaminated with feces from infected animals. The etiological agent of PTB is also secreted in milk and colostrum, which serve as important transmission routes for this disease in a herd [2]. In bovines (*Bos taurus*), Map has been detected in the uterus, male reproductive system, semen, allantoic fluid and fetuses of bovines [3-5]; this bacterium has also been found in the cotyledons and tissues of fetal sheep [6].

PTB has been diagnosed in adult buffaloes in Pakistan [7,8], India [9] and Brazil [10-12]. However, the presence of Map in the buffalo reproductive system or in a buffalo fetus has not previously been reported. Therefore, the objective of the present study was to report the detection of Map in a buffalo (*Bubalus bubalis*) uterus and fetus.

Materials and Methods

The study was conducted on a rural property located along Federal Highway BR 135, Sao Luis, Maranhao, Brazil; at this location, a 10-year-old buffalo cow (*B. bubalis*) at approximately 90 days gestation that presented with progressive weight loss, diarrhea and pronounced physical weakness was evaluated. A clinical suspicion of PTB, in combination with the animal's poor prognosis, suggested that the best option was euthanasia, which would allow for an investigation into the presence of Map and eliminate a possible source of infection for the herd. Other PTB cases had been confirmed on the property in question. Euthanasia was performed in accordance with the recommendations of the National Council of Animal Experimentation Control.

Ileum, mesenteric lymph node, uterus and placenta samples from the buffalo cow and umbilical cord, liver, kidney and digestive tract (pool of rumen, abomasum and small intestine) samples from the fetus were collected during a necropsy. A fragment of each examined tissue was fixed in 10% formalin, and another fragment was individually stored in a 1.5 mL polyethylene tube for qPCR analysis and frozen at -20°C for subsequent processing. These samples were subjected to hematoxylin-eosin (HE) and Ziehl-Neelsen (ZN) staining as well as histopathological analysis. Stool smears with samples collected from the buffalo cow were assessed using ZN staining. Total DNA extraction of organ samples from the buffalo cow and fetus was performed using a Qiagen Kit (QIAamp DNA Mini Kit for tissues) following the manufacturer's recommendations. qPCR amplifications for the IS900 and F57 genes were performed as described by Irenge et al. [13]. The primers F-5′-TGCTGATCGCCTTGCTCA-3′ and R-5′-GGGCCTGATCGGCGATGAT-3′ and the probe S-5′-FAM-CCG GGC AGC GGC TGC TTT ATA TTC-3′-BHQ1 (Sigma, United States) were used for the IS900 gene, whereas the primers F-5′-TTCATCGATACCCAAACTCAGAGA-3′ and R-5′-GTTCGCCGCTTGAATGGT-3′ and the probe S-5′Cy5-TGCCAGCCGCCCACTCGTG-3′-BHQ1) (Sigma, United States) were used for the F57 gene. qPCR samples included 2 µL of DNA, 12.5 µL of RealQ-PCR dUTP-UNG Master Mix (Ampliqon, Denmark), 0.6 pmol/µL of each primer, 0.4 pmol/µL of each probe, 0.5 µL of H_2O and 2 µL of $MgCl_2$ (50 nM) (Invitrogen, United States) in a total reaction volume of 25 µL. The reaction conditions were 50°C for 2 min followed by 95°C for 10 min and 45 cycles of denaturation at 95°C for 15 s and

annealing/extension at 60°C for 1 min [13]. Positive and negative controls were used. The same amplification protocol was utilized for both genes. Each sample was tested in triplicate in a Light Cycler 480 thermo cycler (Roche, Germany), and data analysis was performed using software from the same manufacturer.

Results and Discussion

The buffalo cow exhibited progressive weight loss (with a body condition score of 2 on a scale of 1 to 5); submandibular edema; abdominal and thoracic edemas; and profuse and intermittent chronic diarrhea that was often eliminated in jets (Figure 1A). Mota et al. [5], Yamasaki et al. [1] and Dalto et al. [14] have previously observed the same clinical signs in buffaloes diagnosed with PTB.

ZN staining of stool smears revealed the presence of acid-alcohol resistant bacilli (AARB) (Figure 1B). Histological examinations of buffalo cow samples found lesions compatible with PTB in the mesenteric lymph node and small intestine, and AARB were observed when these tissues were assessed by ZN staining. Similar results were reported by Khan et al. [15], Mota et al. [12], Yamasaki et al. [5] and Dalto et al. [14], who used ZN staining and/or PCR to observe AARB in buffaloes with PTB. No histological lesions were identified in fetal tissues. One explanation of this phenomenon is that animals only express clinical signs as adults; therefore, PTB-associated lesions are not observed in young animals [6,16].

qPCR data indicated that uterus, mesenteric lymph node and small intestine tissue samples from the buffalo cow were positive for Map but that only digestive tract tissues from the fetus were positive for Map. In buffalo cow and fetus tissue samples, the IS900 and F57 genes were both amplified by qPCR. A major difference between Map and other bacteria of the M. avium complex is that the Map genome includes 14 to 18 copies of the IS900 insertion element, which is the DNA fragment most commonly used for molecular diagnostic techniques. The F57 gene has also been used for the detection of M. avium [12,14,17]. In this study, the etiological agent could be detected using both IS900 and F57.

In Brazil, Silva et al. [5] reported the presence of AARB in the spleen of a fetus from a cow with clinical signs of PTB. Whittington and Windsor [14] detected intrauterine infections in cattle with PTB and determined that affected fetuses express clinical signs as adults. Those authors stated that knowledge of this form of vertical transmission is critical for the implementation of control and prophylaxis measures in the herd [13]. Seitz et al. [4] detected Map in the uteri and fetuses of female cattle positive for PTB by culture in Herrold's medium. Buergelt et al. [1] utilized nested PCR to detect Map in the allantoic fluid and fetuses of cattle. Using bacterial culture, Lambeth et al. [6] detected Map in the cotyledons and tissues of fetal sheep.

No bacterial culture was performed in the present study. PTB diagnosis via PCR amplifications that use IS900 produced satisfactory and rapid results. In addition, the use of this region is recommended by the World Organization for Animal Health. Ravva et al. [16] demonstrated that this region is specific to Map and can be used to differentiate Map from other bacteria of the M. avium complex. Intrauterine infection of bovines in advanced stages of the disease may occur when pregnant cows carry large quantities of microorganisms, and anergia allows for the migration of infected macrophages to other organs through the lymphatic system [15].

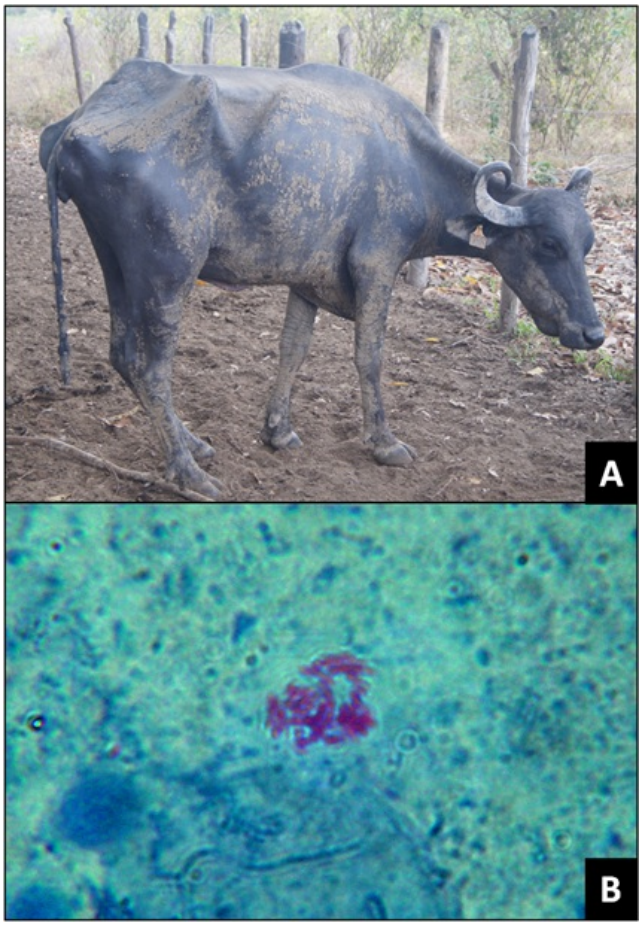

Figure 1: Paratuberculosis in a buffalo. A. Buffalo cow with a body condition score of 2 and submandibular edema. B. Presence of acid-alcohol resistant bacilli in a buffalo cow stool smear subjected to Ziehl-Neelsen staining.

Siva kumar et al. [8], Khan et al. [13], Mota et al. [11], Yamasaki et al. [1] and Dalto et al. [10] used ZN staining and PCR to examine intestinal tissues and mesenteric lymph nodes from buffaloes positive for PTB. Siva kumar et al. [8] and Dalto et al. [10] also performed bacterial culture and immunohistochemistry, respectively. However, none of the aforementioned authors investigated the presence of Map in the uteri or fetuses of buffaloes or assessed potential intrauterine transmission.

The present study describes the detection of Map in the uterus and fetus of a buffalo (B. bubalis). The study results indicate that Map is present in different organs and tissues of buffaloes, including the reproductive system, and that intrauterine transmission could be an important infection route in the epidemiology of PTB within buffalo production systems.

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