Identification of *Mycobacterium avium* subspecies *paratuberculosis* by Polymerase Chain Reaction in Blood and Semen of a Bull with Clinical Paratuberculosis

Claus D. Buergelt, DVM, PhD[a]
G. Arthur Donovan, DVM, MS[b]
Joseph E. Williams, BS[a]

University of Florida, College of Veterinary Medicine
[a]*Department of Pathobiology*
[b]*Department of Large Animal Clinical Sciences, Gainesville, FL 32611*

KEY WORDS: Paratuberculosis, *Mycobacterium avium* subsp *paratuberculosis*, bull, blood, semen, nested polymerase chain reaction

ABSTRACT

Mycobacterium avium subspecies *paratuberculosis* was identified by nested polymerase chain reaction in the blood and semen of a 4-year-old Holstein breeding bull that exhibited clinical signs of paratuberculosis and was serologically positive for the organism by agar gel immunodiffusion and ELISA, although three fecal cultures were negative for the organism. The animal subsequently died, but a necropsy could not be performed for collection of tissues for histopathologic confirmation of the presence of the organism. *M. avium* subsp *paratuberculosis* may disseminate hematogenously to the male reproductive tract and semen as an extraintestinal site and agent reservoir.

INTRODUCTION

Paratuberculosis (Johne's disease) caused by *Mycobacterium avium* subspecies *paratuberculosis*, a facultative intracellular, acid-fast bacillus, affects ruminants worldwide. Control of the infectious disease is hampered by unreliable diagnostic methods, particularly as they relate to the detection of the organism in subclinically infected animals. Clinical signs exhibited by some infected animals are typically characterized by protracted diarrhea and weight loss. Serologic tests, such as ELISA, agar gel immunodiffusion (AGID) test, and fecal culture are recommended to confirm the diagnosis of paratuberculosis in a clinically affected animal or in an infected herd.

The disease is principally confined to the small intestinal tract and its draining lymph nodes. Infection may disseminate to extraintestinal sites as evidenced by successful cultural isolation of the organism from milk, fetus, lung, and semen.[1] Unlike the intestinal tract, these other organs do not elicit a typical inflammatory response to the presence of the organism. Earlier reports describe the isolation of *M. avium* subsp *paratuberculosis* from male accessory genital organs and semen in bulls[2,3] and from semen in rams.[4]

The present article describes the diagnosis of paratuberculosis in a clinically affected bull and identification of the organism in blood and semen samples by nested polymerase chain reaction (nPCR).

MATERIALS AND METHODS

Animal

A 4-year-old Holstein pedigreed bull that was used with 12 other bulls for semen collection and artificial insemination in a herd of 3,600 Holstein dairy cows was observed to have severe protracted diarrhea and weight loss despite a healthy appetite. Evaluations for endoparasites and *Salmonella* pathogens were negative. Standard bovine vaccinations, including infectious bovine rhinotracheitis (IBR), bovine viral diarrhea (BVD), parainfluenza$_3$ (PI_3), bovine respiratory syncytial virus (BRSV), multivalent *Clostridium* bacterin/toxoid, and multivalent *Leptospira* were current. The bull tested negative for BVD virus via immunohistochemistry of the ear skin. Serum samples from the animal were submitted to a laboratory for serology testing for paratuberculosis. Fecal samples were cultured, and whole blood and semen were subjected to nPCR analysis. The bull died shortly after the last round of sampling and was buried on the premises. A necropsy could not be performed for collection of tissues for histopathologic confirmation of the presence of the organism.

Fecal Culture

Isolation of *M. avium* subsp *paratuberculosis* was accomplished using Herrold's egg-yolk method with sedimentation. Briefly, 1 gram of fecal matter was suspended in 40 ml of sterile distilled water and shaken for 30 minutes. After allowing the sample to precipitate for 30 minutes, 5 ml of supernatant were transferred into 35 ml of 0.3% benzalkonium chloride and allowed to stand undisturbed for 24 hours. An aliquot (0.2 ml) of the sediment was distributed over each of four medium slants. Three slants contained mycobactin, and one was prepared without mycobactin. Three fecal samples were processed 4 weeks apart, and results were evaluated after 6 months of incubation.

Agar Gel Immunodiffusion and ELISA Testing

The AGID test was used to detect antibodies produced in response to infection. For this test, serum from the test animal was placed in a well in the agar and *M. avium* subsp *paratuberculosis* antigen was placed in a nearby well. When the two test components passively diffuse out of the well into the agar, the components bind and form an antigen-antibody complex that precipitates in the agar for samples that contain antibodies to *M. avium* subsp *paratuberculosis* antigen. A crude protoplasmic antigen was used for the test, and a serum sample from a cow confirmed to have Johne's disease was added as a control. Samples were evaluated after 48 hours.

The ELISA assay was performed with the same crude, soluble protoplasmic antigen. The test was performed as originally developed.[2] The sera were preabsorbed with *Mycobacterium phlei.* ELISA results were calculated from optical density (OD) readings of triplicate samples at 405 nm and recorded as negative (OD <1.5), suspicious (OD 1.5–1.9), low positive (OD 2.0–2.5) or high positive (OD >2.5).

Polymerase Chain Reaction

PCR was performed on blood monocytes and semen was tested by nPCR with two sets of primers that specifically target 417 base pair [bp] (P90/P91) and 333 bp (J1/J2) of the insertion sequence IS900. The nPCR was developed to increase the sensitivity when no PCR products were visualized on electropherograms after probing only with a simple set of primers (P90/P91).

Blood samples collected in tubes containing EDTA were processed by Ficoll-Isopaque gradient centrifugation, and monocytes were harvested from the interface. Two semen samples collected from a straw (0.5 ml) were centrifuged at 3,000 rpm for 5 minutes. One microliter of sedi-

Table 1. Results of Agar Gel Immunodiffusion (AGID), ELISA, Fecal Culture, and Nested Polymerase Chain Reaction (nPCR) Tests Performed for a Holstein Bull Showing Clinical Signs of *Mycobacterium avium* subsp *paratuberculosis*

					nPCR	
Sample	Date	AGID	ELISA (OD)†	Fecal Culture	P90/P91 (first primer)	J1/J2 (second primer)
Serum	1/11/02	Negative	Negative	ND	ND	ND
Serum	11/4/02	Positive	4.6	ND	ND	ND
Serum	11/9/02	Negative	3.8	Negative	ND	ND
Serum	12/7/02	Positive	3.5	Negative	ND	ND
Serum	1/27/03	Negative	4.0	Negative	Negative	+B
Serum	2/7/03	Negative	4.0	ND	ND	ND
Semen	1/27/02	ND	ND	ND	Positive	Positive
Semen	3/17/03	ND	ND	ND	Negative	Negative

*Tests were conducted on whole blood.
†Values greater than 2.0 indicate positive test reaction.
+B = positive amplicon in blood; ND = not done.

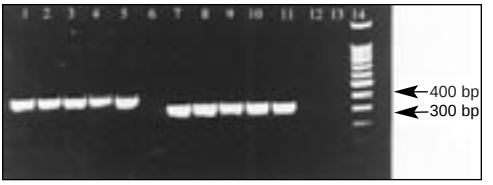

Figure 1. Gel electrophoresis of IS900 amplification products of bull semen. Lane 1: DNA extraction from laboratory strain of *Mycobacterium avium* subsp *paratuberculosis*. Lanes 2–5 are reaction products with primers P90/P91 only. Lanes 7–13 show the addition of primers J1/J2 to the nested polymerase chain reaction. Lane 2: semen sediment; lane 3: 200 µl fluid; lane 4: 50 µl fluid; lane 5: 10 µl fluid; lane 6: negative control; lanes 7 and 8: positive controls; lane 9: 200 µl fluid; lane 10: 50 µl fluid, lane 11: 10 µl fluid; lanes 12 and 13: negative controls.

ment and 200 µl, 50 µl, or 10 µl of supernatant were added to 0.2% sodium hydroxide, boiled at 110˚C for 20 minutes to extract DNA and recentrifuged at 3,000 rpm for 3 minutes before analysis by PCR. DNA extracted from a lab strain of *M. avium* subsp *paratuberculosis* was used as a positive control; distilled water was used as negative control. A semen sample from one unrelated bull that was collected and handled as described above was used as negative control.

After DNA extraction, lysates were subjected to PCR with a protocol of 35 cycles of 30 seconds at 94˚C, at 58˚C for 15 seconds and at 72˚C for 60 seconds. Samples were probed with primers P90, P91 and J1, J2.

RESULTS

Fecal cultures were negative for *M. avium* subsp *paratuberculosis* after 6 months for all three samples, suggesting either the animal was not shedding the organism or the result was a false-negative due to the relative lack of sensitivity for the selected method. Serum samples tested positive by AGID on two occasions, and a high ELISA titer (OD = 3.5–4.6) was obtained on four occasions (Table 1). PCR testing was positive for the organism in blood samples only when nPCR was used, and semen samples were positive on one of two tests performed (Table 1). Positive bands at the expected locations were obtained with primers P90/P91 and J1/J2 (Figure 1). The control semen samples from one unrelated bull were negative for the organism by nPCR assay.

DISCUSSION

The nPCR reaction was not used for fecal samples because the performance of this test has been reported to be less reliable than other fecal culture techniques due to inhibitory fecal factors.[5] Despite a negative fecal culture for this animal and the absence of tissues and organs for necropsy examination, results from serologic tests, the appearance of clinical signs typical of paratuberculosis, and the knowledge that paratuberculosis existed in the herd made this bull a viable candidate for the diagnosis of Johne's dis-

ease. The test results described may not provide sufficient proof of the presence of Johne's disease in this bull, but they are highly suggestive of the infection. The accuracy of the diagnosis was best reflected by a positive AGID reaction on two occasions, a test that is known to have a high level of specificity. However, AGID has a lower sensitivity in cattle and its use is primarily restricted to verifying a diagnosis for animals that are demonstrating clinical signs characteristic of Johne's disease.

The nPCR assay was developed with the specific aim to identify the presence of *M. avium* subsp *paratuberculosis* in extraintestinal sites in clinically and subclinically infected animals for which serology and fecal culture do not perform to satisfaction. In a pilot study of 46 subclinically infected, lactating Holstein cows from a herd with confirmed paratuberculosis, nPCR yielded a total of 24 (52%) animals positive for the organism in milk or blood samples or both. Conversely, 18 (39%) positive or suspicious animals were detected by ELISA testing. Several positive samples were identified by nPCR in subgroups of animals that had been deemed negative for antibodies by ELISA, suggesting nPCR is a more sensitive indicator of early infection.

PCR is a tool to amplify DNA specific to mammalian and prokaryotic cells. In the case of *M. avium* subsp *paratuberculosis*, the claimed species-specific insertion sequence IS900 is probed for with primers P90/P91 to yield a reaction product of a 413 bp.[6] The second set of primers (J1/J2), was designed to be used in a nPCR assay. It spans a 333-bp region within the P90/P91 region. The advantage of nPCR analysis is that it allows further amplification of the signal already amplified by the first pair of primers, particularly when the first signals are weak or invisible on agar gel. PCR assays for IS900 have been reported to be capable of detecting infections with as few as 10^4 colony forming units per gram of feces in shedding cows.[5] This is suggestive of a large number of bacilli in the semen of this bull, since the first set of primers already produced positive amplicons. It could not be determined by culture whether the detection of amplicons in the semen and blood of this bull represented viable *M. avium* subsp *paratuberculosis* organisms.

The identification of *M. avium* subsp *paratuberculosis* DNA in semen from this bull was not unexpected in the setting of dissemination of the organism during infection. It has been reported that such dissemination takes place in clinically affected animals up to 35% into milk[7] and up to 40% in utero to the fetus.[8] A recent study using nPCR demonstrated the serial dissemination of *M. avium* subsp *paratuberculosis* into extraintestinal tissues such as blood, milk, and liver in three clinically affected animals with a diagnostic sensitivity of 40%, 96%, and 93%, respectively.[9] These results suggest that hematogenous episodes of dissemination to milk and liver had most likely occurred through circulating monocytes. On one occasion, the bull in this report was shown to have a positive amplicon in blood leukocytes.

The few early reports of cultural isolation of *M. avium* subsp *paratuberculosis* from ruminant semen were overshadowed by the suspicion of contamination from feces. Positive cultural isolation of the organism from the reproductive tract (i.e., seminal vesicles and bulbourethral glands) obtained at slaughter supported the likelihood that the organisms can transfer to the reproductive organs and particularly to the semen. Any stored semen should be destroyed as a precaution once the donor is diagnosed with Johne's disease. The demonstration of the organism in semen may constitute a threat to the artificial insemination industry as a potential route to transmit paratuberculosis into a herd that has a negative disease status.[10] Natural transmission from the semen to cows via the vaginal route has not been documented. Experimentally, *M. avium* subsp *paratuberculosis* has been inoculated into the uterus of several cows.[11] In that study, the organ-

isms were recovered by culture from the uterus of cows necropsied 1 to 14 day after inoculation, but not in specimens taken 3 to 4 weeks after intrauterine inoculation, suggesting the improbability that semen is infective to the developing fetus. The first reported case of intrauterine *M. avium* subsp *paratuberculosis* transmission from a bovine embryo recipient to the resultant calf might have occurred hematogenously.[12]

REFERENCES

1. Sweeney RW: Transmission of paratuberculosis. *Vet Clin North Am* 1996; 12:305–312.
2. Larsen AB, Kopecky KE: *Mycobacterium paratuberculosis* in reproductive organs and semen of bulls. *Am J Vet Res* 1970; 31:255–171.
3. Larsen AB, Stahlheim, OHV, Hughes DE, et al: *Mycobacterium paratuberculosis* in the semen and genital organs of a semen donor bull. *JAVMA* 1981; 179:169–171.
4. Eppleston J, Whittington RY: Isolation of *Mycobacterium avium* subsp *paratuberculosis* from the semen of rams with clinical Johne's disease. *Aust Vet J* 2001; 79:776–777.
5. Whipple DL, Kapke PA, Andersen PR: Comparison of a commercial DNA probe test and the cultivation procedures for detection of *Mycobacterium paratuberculosis* in bovine feces. *J Vet Diagn Invest* 1992; 4:23–27.
6. Vary PH, Andersen PR, Green E, et al.: Use of highly specific DNA probe and polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. *J Clin Microbiol* 1990; 28:933–937.
7. Giese SB, Ahrens P: Isolation of *Mycobacterium avium* subsp. *paratuberculosis* in milk from clinically affected cows by PCR and culture. *Vet Microbiol* 2000; 20:291–297.
8. Seitz SE, Heider LE, Hueston WD, et al.: Bovine fetal infection with *Mycobacterium paratuberculosis. JAVMA* 1989; 194:1423–1426.
9. Barrington GM, Gay JM, Eriks IS, et al: Temporal patterns of diagnostic results in serial samples from cattle with advanced paratuberculosis infections. *J Vet Diagn Invest* 2003; 15:195–200.
10. Philpott M: The dangers of disease transmission by artificial insemination and embryo transfer. *Br Vet J* 1993; 149:339–369.
11. Merkal RS, Miller JM, Hintz AM, et al: Intrauterine inoculation of *Mycobacterium paratuberculosis* into guinea pigs and cattle. *Am J Vet Res* 1982; 43:676–678.
12. Manning EJB, Augenstein M, Collins MT, et al.: Case report–Johne's disease: The recipient risk. *Bovine Pract* 2003; 37:20–22.