Nested Polymerase Chain Reaction and Prenatal Detection of *Mycobacterium avium* Subspecies *paratuberculosis* (Map) in Bovine Allantoic Fluid and Fetuses

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ABSTRACT

Eleven pregnant Holstein cows were subjected to nested polymerase chain reaction (nPCR) testing for evidence of in utero transmission with *Mycobacterium avium* subspecies *paratuberculosis*. Six dams (55%) were shown by nPCR to have positive PCR products in blood and/or milk. Tissues or fluid from fetuses were positive on 36% of the pregnancies; 2 of 4 placentomes tested gave positive PCR reaction products and 1 of 11 (9%) allantoic fluids. All pregnant cows were proven paratuberculosis positive through necropsy and microscopic examination. A percutanous technique on the standing pregnant cow is described for antemortem collection of allantoic fluid for PCR testing.

INTRODUCTION

Paratuberculosis (Johne's disease), an insidious infectious disease of ruminants, occurs worldwide. The disease is caused by *Mycobacterium avium* subspecies p*aratuberculosis* (Map), a facultative intracellular pathogen. It is currently believed that the principal pathway of transmission of Map is the fecal-oral route and that the calf is the most susceptible to such transmission.

Culture studies have shown that between 20% and 40% of fetuses from cows with advanced Johne's disease were infected in utero, compared with 9% of fetuses being culture positive from subclinical cows.[1] From 1 farmed red deer operation in New Zealand, 9/10 fetuses obtained at slaughter were positive for Map on culture of various internal organs and the placentomes.[2] Intrauterine transmission of Map in wild red deer and chamois has been reported from Austria.[3] Because presently there is no possibility to identify such in utero infected

calves prenatally, the decision to keep a calf born to a proven infected dam as replacement is difficult after infection detection in the dam, regardless of the diagnostic method chosen. In a previous report, we described the outcome of detecting in utero transmission of Map in 3 pregnant cows by nested polymerase chain reaction (nPCR) after necropsy.[4] Here we report the feasibility of the nPCR technique for potential application as a prenatal test in the cow via placental fluid collection during late pregnancy.

MATERIALS AND METHODS

Animals

The pregnant cows investigated were all Holsteins and were obtained from 4 dairy herds with a proven history of paratuberculosis. Pregnant animals were selected for studies when they developed clinical signs of paratuberculosis, had positive Map serology results, or had positive Map results on blood nPCR or milk nPCR when still lactating.

Preparation of Animals for Percutaneous Allantoiscentesis

Animals were tied into a headstand. Rectal examination was performed to determine the site of pregnancy in the uterus (right or left uterine horn). Once the site was determined, the hair of the ventral flank of that site above the udder was shaven, and the skin was cleaned with warm water and soap. The prepared area was sterilized with alcohol and iodine. Lidocaine (2%) was applied into the subcutis with a syringe to locally sedate the skin. Fetal fluid was collected by inserting dorso-cranially an 18-gauge spinal needle through the abdominal wall and into the allantoic cavity. Fluid was collected by free flow or aspiration with a 12-mL syringe. Fluid collection was attempted on the standing animals every second day up to 5 times altogether.

Specimen Handling

Blood of the dams was collected onto EDTA-containing vacutainers via coccygeal vein bleeding after rigorous cleaning of the tail skin with cotton soaked in alcohol. The blood samples were subjected to Ficoll-Isopaque™ gradient centrifugation, and monocytes were harvested from the interface. When available, milk was collected into 50-mL sterile centrifuge tubes pooled from all 4 quarters after meticulous cleansing of the teats with cotton soaked in alcohol. Milk samples were subjected to centrifugation at 1,000 g for 15 minutes. After centrifugation, the supernatant was removed and discarded. The remaining samples were washed in PBS 3 times and suspended in 1 mL PBS for cell counting via hemocytometer, resuspended into 100 µL of 0.2N NaOH and boiled at 110°C for 20 minutes to extract crude DNA and centrifuged at 500 g for 3 minutes. Neutralization after NaOH extraction was not attempted. Tissues from fetal liver, spleen, lung, and brain as well as tissues from the placentome were prepared for nPCR by touchpressing on glass slides. After air-drying, 200 µL of 0.2N NaOH were dispersed over tissues, and a sterile razor blade was used to scrape samples from the glass slides. Samples were placed into 1.5-mL centrifuge tubes and boiled at 110°C for 20 minutes. For the PCR reaction, 1 mL of supernatant was chosen. Liquid samples from the allantoic cavity (50 mL) and fetal abomasums (25 mL) were centrifuged for 60 minutes at 1,000 g. Pellets were washed 3× with PBS, suspended in 0.2 NaOH, and boiled as discussed above.

Nested Polymerase Chain Reaction

An nPCR developed in our laboratory was used for identifying Map in blood monocytes and milk macrophages. Briefly, for the first reaction, primers P90,P91 were chosen, and primers J1,J2 were designed for the second reaction. After DNA extraction, 1 µL of the lysates were submitted for PCR. A protocol of 35 cycles of 30 seconds at 94°C, 15 seconds at 58°C, and 60 seconds at 72°C was followed for simple PCR. For the nested PCR, a program of 36 cycles with 30 seconds at 94°C, 15 seconds at 63°C, and

60 seconds at 72°C was applied . A commercial reaction mix (Eppendorf Hotmaster Mix, Westbury, NY) was used according to the company's specification. A volume of 10 µL of the PCR reaction products was run on 1.5% agarose gel by electrophoresis in TAE running buffer (Continental Lab Products, San Diego, Calif). DNA extracted from Map laboratory strain No. 295 was used as positive control for primers P90,P91 and J1,J2 (nested control). Sterile water was used as negative control for the PCR assay. Gel inspection was performed using ultraviolet light and recorded with a computerized digital camera (UVP Transilluminator System, Upland, Calif).

To avoid false-positive results, tubes containing blood, milk, and fetal fluid samples were washed with alcohol before processing samples. Samples were initially processed in a sterile hood and then in 2 separated workstations, cleaned each time for each test with alcohol. Needles, syringe holders, and gloves were changed with each animal and for each step of the procedure.

ELISA

The ELISA originally developed by W.D. Richard (Allied Laboratories, Ames, Iowa) was performed with crude, soluble protoplasmic antigen (Allied Monitor, Fayette, Missouri). The test sera were preabsorbed with *Mycobacterium phlei*. Results were calculated for wavelength readings (OD at 405 nm) of triplicates and recorded as negative (<1.5 OD), suspicious (1.5 to 1.9 OD), and positive (>2.0 OD).

Agar-Gel Immunodiffusion Test

The same crude antigen that was used for the ELISA was selected for the agar-gel immunodiffusion test. The central well was loaded with 35 µL of antigen. The peripheral wells were inoculated with 45 µL of test sera. A reference serum from a proven positive paratuberculous cow was used as positive control. Final readings were performed after 48 hours.

Histopathology

Tissue sections collected after a complete necropsy were fixed in 10% buffered formalin, paraffinized, cut on a microtome, stained with hematoxylin-eosin (H&E) and acid-fast stains (Fite's), and examined under a light microscope to qualitatively determine the extent of the granulomatous inflammation and the number of acid-fast bacilli.

RESULTS

The average age of the cows was 5.6 years (Table 1). Necropsy confirmed that all cows had paratuberculosis characterized by granulomatous enteritis and mesenteric lymphadenitis and the presence of intralesional acid-fast bacilli. All were pregnant, with cow #10 having twins. The average gestational stage was 6.4 months (Table 1). The sex of the fetuses was male in 7 and female

Table 1. Signalement and Gestational Period of Pregnant Cows.

Number	Breed	Age (years)	Gestation (months)
1	Holstein	5	6
2	Holstein	5	6
3	Holstein	6	4
4	Holstein	6	5
5	Holstein	7	6
6	Holstein	3.5	6
7	Holstein	6	7
8	Holstein	6	8
9	Holstein	6	8
10	Holstein	8	8
11	Holstein	4	7

in 5 animals. All but 1 animal had a positive ELISA reading and 8 of 11 animals were positive on agar gel immunodiffusion (Table 2). Blood nPCR showed 4 of 11 cows positive and 2 of 9 cows had a positive milk nPCR reading. Fecal culture was not attempted. The placentome when subjected to nPCR was positive in 2 of 4 tissues examined (Table 3, Figure 1). In cow #9, the placentome showed an individual cluster of acid-fast bacilli on touch preparation examined under the light microscope

Table 2. Fecal, Serological, and PCR Results of Pregnant Animals.

Number	Fecal Culture	AGID	ELISA	Blood PCR	Milk PCR
1	ND	+	-	-	+
2	ND	+	+	-	-
3	ND	+	+	+	ND
4	ND	+	+	-	ND
5	ND	-	+	-	+
6	ND	+	+	+	-
7	ND	+	+	+	-
8	ND	+	+	+	-
9	ND	-	+	-	-
10	ND	+	+	-	-
11	ND	-	+	-	-

AGID = agar-gel immunodiffusion test; ND = not done.

Figure 1. Gel electrophoresis of J1,J2 amplification products of intestinal lymph nodes and placentome from cow # 9. Lanes 1,2: positive DNA controls from lab strain No. 295; lane 3: ileo-cecal lymph node, dam; lane 6: mesenteric lymph node, dam; lane 8: placentome; lanes 14,15: negative controls; lane 16: molecular markers.

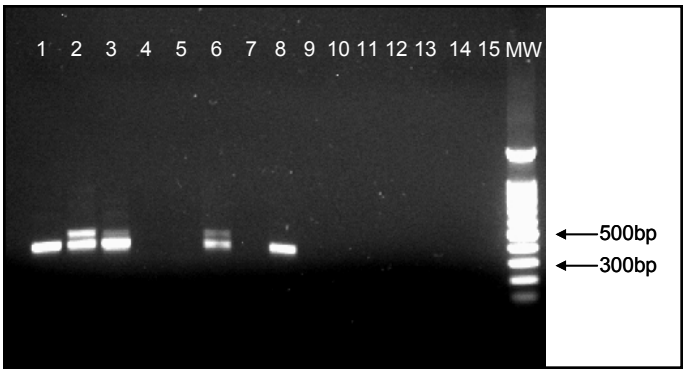
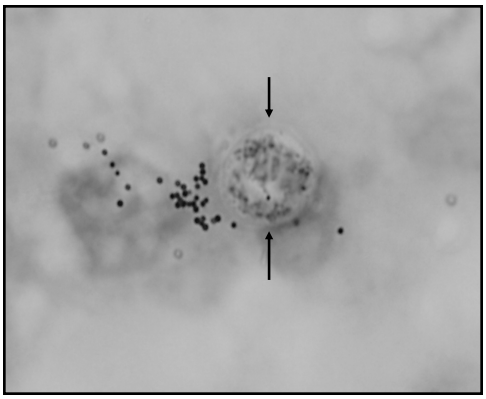


Figure 2. Touch imprint of placentome from cow # 9 stained with acid-fast stain. Arrows denote presence of bacilli in trophoblast. Oil-immersion, ×100.



(Figure 2). There was no evidence of a granulomatous inflammatory response in the placentome tissue. Allantoic fluid was positive on nPCR in 1 of 11 animals (Table 3, Figure 3). Fetal tissue had positive nPCR reaction products in 3 of 11 spleens tested, in 2 of 11 livers tested , in 1 of 11 brains tested, and in the abomasal fluid of 1 of 10 samples tested (Table 3). When the fetal tissues were examined microscopically, they showed no evidence of an inflammatory response or evidence of acid-fast bacilli.

DISCUSSION

The detection of Map DNA by nested PCR in fetal fluids and various fetal tissues supports the concept that Johne's disease is a disseminated infectious disease with in utero transmission as a possibility to be considered in addition to the traditional fecal-oral route. This additional pathway of transmission should be considered in the epidemiology and control of the disease through testing and culling. The management is often reluctant to remove the offspring together with infected dams from the herd. The diagnosis of prenatal infection via allantoiscentesis is considered a possibility to help along this decision process.

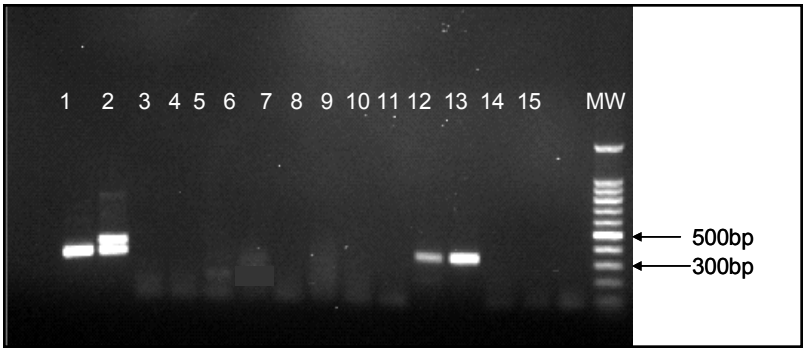
The results from a small population of 11 pregnant infected cows demonstrate that Map is transmitted transplacentally, even though bacterial DNA may not appear very often in placental fluid. This may be the result of a low number of bacilli in comparison with the large volume of placental fluid during late pregnancy or the low number of phagocytes in the fluid to be used by the

Table 3. PCR Products in Fetal Fluid and Tissues.

Number	Placentome	Placental Fluid	Fetus				
			Liver	Spleen	Lung	Brain	Abomasum
1	ND	-	-	-	-	-	-
2	ND	-	-	-	ND	-	-
3	ND	-	-	+	ND	-	-
4	ND	-	-	-	ND	-	-
5	ND	-	+	-	ND	-	-
6	ND	+	-	-	ND	-	+
7	+	-	-	-	ND	-	-
8	ND	-	-	+	-	+	-
9	+	-	-	-	ND	-	-
10	-	-	-	-	ND	-	-
11	-	-	+	+	ND	-	ND

ND = not done.

Figure 3. Gel electrophoresis of J1,J2 amplification products of allantoic fluid from cow # 6. Lanes 1,2: positive DNA controls from lab strain No. 295; lanes 12,13 :allantoic fluid; lanes 14,15: controls; lane 16: molecular markers.



bacilli for replication. The successful isolation of Map DNA in the allantoic fluid at necropsy in a previous cow at 2 months of gestation might reflect this volume disadvantage.[4] While the technique of percutaneous allantoiscentesis on the standing animal in late gestation can be easily performed technically, it may have the disadvantage to dilute the few bacilli shed from the fetus within the fluid at the time when this technique is attempted during late pregnancy. The presence of PCR inhibitory substances in the allantoic fluid might have also influenced the DNA amplification outcome. The identification of acid-fast bacilli in 1 randomly collected placentome by light microscopy is additional proof of transplacental transmission of Map.

Application of bovine fetal fluid collection is a relatively unexplored scenario in bovine medicine.[5-9] It has been sporadically reported in the literature mainly for the purpose of gender determination of the unborn calf.[7] Our experience and that of others[5] have been that the technique can be easily performed on the standing animal after head restraint and local sedation of the skin at the application site. The animals rarely resist the procedure. When done sterilely, the technique does not result in peritonitis and adhesions as demonstrated in those animals at necropsy where the procedure was performed several times (eg, every second day over a 10-day period of time). The animals tolerated the procedure well. Prophylactic treatment with antibiotics was not attempted. Sporadic abortion did not occur in the 11 animals where this technique was performed. The anatomic location of the fetus deep and ventral within the caudal abdomen 7 months into gestation allows for the amenability of the procedure chosen.[10] The insertion site of the needle hits the allantoic sac ventrally close to the fetal extremities. The volume of fluid recovered was at least 50 mL with each attempt and was considered adequate to run the nested PCR. This volume can be

easily obtained in late gestation as the fetal fluid at that time is estimated to be 2.5 L in the amnion and 9 L in the allantoic cavity.[9]

The allantoic cavity stores the waste products of the fetal kidneys.[9] The bovine allantoic fluid is watery, amber, and contains albumin, fructose, and urea.[10] The bovine amniotic fluid is mucoid in nature, and contains protein, fructose, fat, and salts.[10] Fetal urine contribution to allantoic fluid diminishes at term. The source of the mucoid fluid is fetal saliva and secretions of the nasopharynx, but also fetal urine and secretions of the amniotic epithelium. Permeability of the membranes to various solutes affects fetal fluid composition and volume. Response of the membranes to hormones such as progesterone, estrogen, and prolactin affects fetal fluids as well.

Cultural isolation of Map from fetuses has been reported from the United Kingdom and United States.[1] From a small collection of pregnant infected cows sent to slaughter, 4 of 9 fetuses from first to third trimester of gestation were culture positive in various organs such as liver, lung, intestine, brain, spleen, kidneys, and placenta, and in fetal fluids such as abomasal contents and amnion (Buergelt, PhD, Thesis, Ithaca, NY,1976). A previous identification of Map DNA by nPCR from 3 fetuses of infected dams was reported from brain, liver, and lung.[4] A tissue reaction such as inflammation to the presence of organisms was not noticed in all fetuses of various gestations upon microscopic examination. It appears that fetuses are immunotolerant to the virulence of Map bacilli, regardless of gestational age.

The PCR is a tool to amplify DNA specific to mammalian and prokaryotic cells to the point of being able to be demonstrated by various detection methods. In the case of Map, the species-specific insertion sequence IS 900 is probed for with primers P90,P91 to yield reaction products at 413 bp.[11] The second set of primers, J1,J2, was designed to be used in an nPCR assay. It spans a 333-bp region within the P90,P91 region.[4] An nPCR is considered a further amplification of the signal already amplified, but undetectable by visualization, increasing the specificity and sensitivity of the test system. In terms of predicting the presence of in utero infection with Map, the nPCR was thought to have a place as prenatal test.

In summary, it is technically possible to obtain allantoic fluid for prenatal Map infection testing and easily performed aseptically in late pregnancy (>7 months) without inducing peritonitis or abortion. The flank procedure cannot be done in earlier pregnancy due to the unavailability of the pregnant uterus in the ventral and lateral region of the abdomen. The positive nPCR in the allantoic fluid of only 1/11 animals (9% success) is disappointing. More late-term pregnant animals should be tested to yield a data base. The detection of Map DNA in various fetal tissues (45%) supports the concept of transuterine transmission of Map in addition to the oral-fecal or milk route of transmission and should be of concern to the management of disease control by test and cull of only infected adult animals.

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