Diagnostic testing patterns of natural Mycobacterium paratuberculosis infection in pygmy goats

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Abstract

Thirteen pygmy goats (Capra hircus) from a herd naturally infected with Mycobacterium avium ss. paratuberculosis (MPTB) were monitored with 4 diagnostic assays for 2 to 15 mo. Cellular and humoral immune responses to the infection were assessed with assays of gamma interferon (IFNγ), serum antibody [enzyme-linked immunosorbent assay (ELISA) and agar gel diffusion (AGID)], and radiometric fecal culture. Microscopic examination and radiometric culture of tissue from 12 sites were performed at necropsy. Goats were considered infected if MPTB was isolated from any tissue sample collected at necropsy. Mycobacterial isolates were confirmed as MPTB with an IS900 polymerase chain reaction assay. Ten goats whose antemortem tests indicated infection carried heavy organism burdens at necropsy, both within and beyond the gastrointestinal system. False-negative ELISA, AGID, and/or culture results were obtained in 5 of the 10 confirmed cases during the study period. In 3 goats with sporadic fecal shedding of MPTB or detectable IFNγ response, or both, no abnormalities were detected at necropsy and no MPTB was isolated from the tissue samples; the antemortem fecal-culture and IFNγ results were thus considered false-positive. Diagnosticians should be alert to the possibility of both false-positive and false-negative test results for Johne's disease in goats. False-positive fecal-culture results may occur when a high prevalence of infection exists in the herd and the premises are likely to be heavily contaminated. The diverse antemortem testing patterns seen in these goats underscore the importance of using varied diagnostic assays serially or in parallel to increase the likelihood of identifying all infected goats.

Résumé

Treize chèvres pygmées (Capra hircus) provenant d’un troupeau naturellement infecté par Mycobacterium avium ss. paratuberculosis (MPTB) ont été surveillées à l’aide de 4 épreuves diagnostiques pendant 2 à 15 mois. Les réponses immunitaires cellulaires et humorales à l’infection ont été évaluées en mesurant l’interféron gamma (IFNγ), les anticorps sériques par ELISA et immunodiffusion en gel (AGID), et culture de feces par radiométrie. L’examen microscopique et la culture par radiométrie de 12 tissus ont été effectués lors de la nécropsie. Les isolats de mycobactérie furent confirmés comme étant MPTB à l’aide d’une réaction d’amplification en chaîne par la polymérase utilisant IS900. Dix chèvres avec des résultats d'épreuve ante-mortem indiquant une infection avaient une charge bactérienne élevée lors de la nécropsie autant au niveau du tractus intra-intestinal qu’extra-intestinal. Des résultats faussement négatifs aux épreuves ELISA, AGID et/ou culture ont été obtenus pour 5 des 10 cas confirmés durant la période d’étude. L’excrétion fécale sporadique de MPTB, la détection d’IFNγ, ou les deux, chez 3 chèvres où aucune anomalie ne fut détectée à la nécropsie et aucun isolé de MPTB ne fut obtenu à partir d’échantillons de tissu ont été considérés comme étant des résultats faux-positifs. Les diagnosticiens devraient être au fait des possibilités de résultats faux-positifs et faux-négatifs pour la maladie de Johne chez les chèvres. Des résultats de culture faussement positifs peuvent survenir lorsque la prévalence d’infection est élevée dans un troupeau et que les lieux risquent d’être fortement contaminés. La diversité des patrons de résultats ante-mortem obtenus chez les chèvres met en évidence l’importance d’utiliser des épreuves diagnostiques variées en série ou en parallèle afin d’augmenter les possibilités de détecter toutes les chèvres infectées.

(Traduit par Dr Serge Messier)

Introduction

Johne’s disease (paratuberculosis) is a contagious and emaciating gastrointestinal disease of ruminants caused by infection with Mycobacterium avium ss. paratuberculosis (MPTB). Troublesome for the cattle and sheep industries, the disease is also of concern for breeders of other ruminants, such as elk, deer, and goats, as well as for managers of captive wildlife. This infection has been reported in rabbits, fox, horses, pigs, humans, and primates (1,2). Transmission of MPTB has been shown to occur by multiple routes in cattle, including fecal-oral, in utero, and via milk or colostrum (3). This organism is thought to infect animals in the first months of life and elicits a slowly progressive inflammatory response in the gastrointestinal tract that is not clinically evident until months to years later. Clinical signs of this infection are the same as for many other diseases: chronic weight loss and, in some species, unremitting
diarrhea. Diarrhea is usually absent in infected sheep and infrequent in infected goats and deer (4). The organism can disseminate beyond the gastrointestinal tract to other organ systems in advanced cases (5).

The purpose of this study was to track the progression of natural infection in pygmy goats over time using serum antibody, gamma interferon (IFNγ), and fcal-culture assays. This temporal investigation would provide information on the correlations among cellular and humoral responses to MPTB infection and postmortem evidence of infection.

Materials and methods

The source pygmy goat (Capra hircus) herd had been managed as the only hoofstock on a farm for 5 y and averaged 30 adult animals. Kids were born into the herd and raised on the premises. Additions to the herd were made from outside sources as well.

Several goats in the herd were extremely thin, and the owner had noted periodic diarrhea in these animals. One debilitated goat was diagnosed with Johne's disease by histopathologic study [acid-fast bacilli (AFB) were observed in lesions consistent with those of paratuberculosis] and isolation of MPTB by radiometric culture of tissue. Subsequently, the entire herd (30 adults and juveniles) was screened for MPTB infection by radiometric bacterial culture of feces and by enzyme-linked immunosorbent assay (ELISA) for serum antibodies. Thirteen adult goats with test results indicative of infection by either means were moved to an animal research facility at the University of Wisconsin School of Veterinary Medicine and housed indoors, following husbandry guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. Sawdust bedding in their 2 rooms (each 119 ft²) was replaced twice a week, and the rooms were pressure-hosed once a week. On average, each room contained 6 goats. On a monthly basis, fecal and blood samples were collected from each goat until the goat was humanely euthanized. Since the goats were euthanized when it was clinically necessary for or economic reasons after 15 mo of study, the number of samples that could be collected per goat varied, from 2 to 15.

Fecal samples were collected per rectum. The procedure for detection of MPTB in ruminant fecal samples has been reported previously (6). Tissue samples were collected from 12 sites (ileum, jejunum, cecum, colon, lymph nodes [ileocecal, mesenteric, and mediastinal], liver, uterus or testes, lung, muscle, and kidney). They were processed for culture similarly to fecal samples after 3 g of the tissue had been homogenized in 5 mL of sterile phosphate-buffered saline (PBS) with a Stomacher (Tekmar, Cincinnati, Ohio, USA). Mycobacterial isolates were confirmed as MPTB with an IS900 polymerase chain reaction assay.

The cellular immune response to infection with MPTB was assessed by measuring the level of IFNγ produced by peripheral blood mononuclear cells in response to stimulation by purified protein derivative (PPD) antigen from M. avium ss. avium (PPD-A), a mycobacterial antigen proxy for MPTB. The assay has been shown to detect IFNγ from caprine lymphocytes (CSL Ltd., Melbourne, Australia) (7). Blood was collected from the jugular vein into heparin-containing tubes, and aliquots were placed in 4 vials within 8 h. One aliquot was stimulated with PPD-A 0.003 mg/mL; National Veterinary Services Laboratories (NVSL), Ames, Iowa, USA, one with PPD from M. bovis (PPD-B, 0.003 mg/mL; NVSL), and one with phytohemagglutinin antigen (0.0010 mg/mL; Sigma Chemical Company, St. Louis, Missouri, USA) as a positive mitogen control to assess the viability of the lymphocytes. The remaining aliquot served as an unstimulated control; PBS, 10 μL/mL, was added to it. If the positive mitogen control demonstrated that the goat’s lymphocytes were viable, and if the optical density (OD) resulting from PPD-A stimulation minus the OD for the kit control was greater than 0.1 OD units, the result was considered elevated.

The caprine serum antibody ELISA was an adaptation of the MPTB Antibody Test Kit licensed by the US Department of Agriculture for use in cattle (IDEXX Laboratories, Inc., Westbrook, Maine, USA). An anti-goat/sheep immunoglobulin (Sigma) conjugated with horseradish peroxidase according to the instructions of the supplier (Zymed Laboratories, South San Francisco, California, USA) was substituted for the conjugate provided in the kit. The assay was performed according to the manufacturer’s instructions, the substitute conjugate being used at a dilution of approximately 1:12,000. The positive control was ELISA positive serum from a goat confirmed to have been infected by MPTB by isolation of the organism from multiple tissues. The ELISA OD₆₃₀ₐ₅₄₇ data were reported as an S/P ratio (sample — negative control/positive control — negative control).

The agar gel immunodiffusion (AGID) assay (ImmuCell, Portland, Maine, USA) was performed according to the manufacturer’s specifications, with the use of serum that had been frozen at −70°C.

A complete necropsy was performed for each goat. Sections of the same tissues collected for radiometric culture were also processed and stained with Ziehl–Neelsen’s method and with hematoxylin and eosin for microscopic examination. A scoring system (0–4) was used to express the relative number of AFB in the tissue samples.

Results

Goats were considered infected if MPTB was isolated by radiometric culture from any tissue collected at necropsy. Of the 13 goats, 10 (goats A–J) met the case definition for infection; Figure 1 shows the temporal patterns of test results for these goats. Figure 2 shows the temporal patterns for the 3 goats (K–M) that did not meet the case definition.

The typical clinical presentation was progressive weight loss despite a good appetite and adequate rations, rough hair coat with mild alopecia distinct from normal seasonal hair loss, plus weakness and an inability to rise in the goats with advanced disease. Diarrhea was rarely observed. At necropsy there was neither gross nor histopathologic evidence of other health factors that might have contributed to the goats’ condition, such as secondary malnutrition, gastrointestinal parasites, caprine arthritis-encephalitis (CAE), or chronic infection with other bacteria, such as Corynebacterium pseudotuberculosis.

All 10 infected goats had elevated ELISA results at some point during the study period. Three (G, H, and J) showed a declining S/P value in the months before necropsy. ELISA values near those of the kit negative control for 5 infected goats (C, F, G, H, and J) during the study. All serum samples for the 3 noninfected goats
Figure 1. Assay results for the 10 goats meeting the case definition of infection with *Mycobacterium avium* ss. *paratuberculosis*. OD — optical density; S/P — for results of the enzyme-linked immunosorbent assay (ELISA) (see text for explanation); squares — ELISA values; diamonds — values for gamma interferon; asterisks — data not available. The circled months are those in which fecal shedding was detected.
produced ELISA results bordering the S/P values for the kit negative control. AGID assays performed on frozen serum samples first produced a visible precipitation line either with the first monthly sample that had an S/P value above 0.25 (6 goats) or with the sample obtained 1 mo after the first sample that had an S/P value above S/P 0.25 (4 goats). If subsequent ELISA values were above 0.25, AGID precipitation lines were visible for the same sample.

Of the 10 infected goats, 7 (B-H) produced elevated amounts of IFNγ at least once during the study period. Goat C showed 2 IFNγ peaks; antibody levels rose during the decline after the 2nd peak. This pattern was recorded for goat F as well, although less IFNγ was produced over a shorter period in the 2nd peak. Initially elevated and then slowly declining IFNγ values were recorded concurrently with elevated antibody values for goat B. Of the 3 noninfected goats, 1 (K) had elevated levels of IFNγ in 5 consecutive monthly samples; the next 5 consecutive monthly samples were negative for IFNγ. The other 2 noninfected goats were consistently IFNγ-negative.

During the study, MPTB was isolated from fecal samples by radiometric culture at least once in all 10 cases. It was the only mycobacterial species isolated from any fecal sample collected. In 8 cases (A-F, H, and J), fecal shedding of the organism was detected in 3 or more consecutive monthly samples. In 4 cases (C, D, F, and G), fecal-culture results were negative at least once. The organism was also isolated at least once from fecal pellets from the 3 goats not meeting the case definition and was recovered from a fecal sample collected at birth from a kid (not part of the study group) born to an infected doe.

In each of the 10 cases MPTB was recovered from at least 1 tissue (Table I). It was the only mycobacterial species isolated from any tissue sample. The organism was recovered from extraintestinal tissues, such as hindlimb muscle, uterus, testes, lung, and kidney and was cultured from the hepatic tissue of 7 of the 10 infected goats. It was not isolated from any of the tissues sampled from 3 goats; thus, these animals were not considered to have true infection. The bacilli were isolated from the intestinal contents and intestinal tissue of a mid-gestation fetus sampled at necropsy of an infected doe.

Gross pathological abnormalities in the 10 cases varied in extent and degree but were characterized by emaciation, loss of fat stores, corded mesenteric lymphatics, and mildly to moderately enlarged mesenteric lymph nodes with pale, nodular foci. Corrugation and thickening was noted in the distal gastrointestinal tract, but to a lesser extent than is

Table I. Frequency of isolation of Mycobacterium avium ss. paratuberculosis (MPTB) and histopathologic lesions, by tissue site, for the 10 goats meeting the case definition of infection with this organism

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Isolation of MPTB</th>
<th>Histopathologic lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ileum</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Jejunum</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Cecum</td>
<td>6</td>
<td>10</td>
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<tr>
<td>Colon</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Ileocecal nodes</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Mesenteric nodes</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Mediastinal nodes</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Liver</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Uterus</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Testes</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Muscle</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Kidney</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
usually seen in a comparably clinically affected bovid. Gross findings
in the 3 noninfected goats were within normal limits.

The frequency, by tissue, of histopathologic findings consistent
with Johne's disease is shown in Table I. Each of the 10 goats with
histopathologic evidence of paratuberculosis in the gastrointestinal
tract also exhibited granulomatous hepatitis with rare AFB. In the
10 cases, the presence of AFB in at least 1 tissue was scored as 3. These
10 goats had a primarily lymphocytic and plasmacytic, and to a
lesser extent histiocytic, inflammatory infiltrate in the ileum, jejunum,
cecum, and liver. Although primarily observed within the superficial
(villar) lamina propria, the infiltrate occasionally extended into an
dematous submucosa. Six of the 10 goats exhibited a similar infiltrate
in the colon. Focal accumulations of macrophages contained rare to
numerous intracytoplasmic AFB, depending on the tissue site and
animal. Granulomatous lymphadenitis was present in mesenteric
or colic lymph nodes or both tissues. Giant cells were rarely identified
in any of the tissues examined histologically. Granulomatous lesions
were noted in the liver of all 10 goats. Microscopic findings in the 3 non-
infected goats were within normal limits.

Discussion

Paratuberculosis is a slowly developing disease that elicits cellular
and humoral immunologic responses at different phases of the
infection. The purpose of this study was to track the progress of
naturally occurring MPTB infection in pygmy goats using serologic,
IFNγ and fecal-culture assays. The test results presented diverse
diagnostic patterns, demonstrating the possibility of both
false-positive and false-negative antemortem results during
surveillance for Johne's disease in a heavily infected herd.

Since the goats were naturally rather than experimentally infected,
each goat was likely at a different phase of MPTB infection during the
study period, as would occur in any goat herd seen in veterinary
practice. This factor may have contributed to the variety in diagnostic
patterns, from what may be considered typical (as in goat F: early
IFNγ spike; subsequently climbing antibody values; and multiple,
although intermittent, positive fecal-culture results) to a more
unusual pattern (as in goat M: no detectable IFNγ or antibody but
multiple positive fecal-culture results). Other factors likely to have
contributed to the diversity of diagnostic pattern include differences
in age at infection, MPTB dose received and age at receipt, immuno-
logic status, and perhaps individual genetic susceptibility, as has
been seen in other mycobacterial infections (8).

The 10 goats meeting the case definition displayed pathological
findings in keeping with what is termed a TH2 or lepromatous pattern
of disease: frequent fecal shedding of the organism plus a heavy MPTB
tissue burden. Elevated antibody values were recorded for 7 of
these goats for a number of months, another facet of the TH2 pattern.
Based on the finding in this study of evidence of infection (AFB plus
tissue lesions and/or isolation of the organism) in hepatic tissue in all
10 cases, it may be useful to sample the liver postmortem for evidence
of Johne's disease in goats in addition to the sites usually recom-
ended (ileum and mesenteric lymph nodes).

The 3 goats not meeting the case definition either were never
infected, or were infected but cleared the infection, or were infected
but had such slight and focal evidence at necropsy that it could not
be found with the methods employed. One of these goats (K) dis-
played some characteristics of the tuberculous form of MPTB infec-
tion: repeatedly elevated IFNγ values in response to PPD-A stim-
ulation and sporadic low-level shedding of MPTB. These findings
are in keeping with those for 2 experimentally infected goats with
antemortem IFNγ production in response to PPD-A stimulation that
were free of signs of active infection at necropsy (9). Positive IFNγ
test results have also been reported in sheep without histopathologic
evidence of infection that came from infected flocks (10). It is pos-
sible that the cell-mediated response, as indicated by the detectable
IFNγ released from peripheral blood mononuclear cells, may have
arrested the progress of the infection during the study period.

It was interesting to note the relationship between the IFNγ and
antibody responses in the 2 goats not producing antibody at the start
of the study (C and F). In both cases, a 2-peak IFNγ production
pattern was seen. During the 2nd peak, antibody production began,
and it continued as IFNγ production declined. This overlap does not
mirror what has been reported in cattle: that the cellular immune
response precedes the humoral reaction to infection, described as the
TH1–TH2 shift (11). Even less similar were the findings for goat B:
4 mo of concurrently elevated values for IFNγ and antibody.

The lack of specificity frequently reported with assays using
mycobacterial PPD preparations (12) was found in this study as well.
For virtually all samples, the same interpretation of the IFNγ assay
was reached whether the peripheral blood mononuclear cells were
stimulated by PPD-B (data not shown) or by PPD-A. Since MPTB
was the only mycobacterial organism isolated from any fecal or tissue
sample, however, we believe that the cellular and humoral immune
responses seen in these goats were stimulated by that particular
mycobacterium.

The antemortem isolation of MPTB from fecal pellets taken from the
goats with no postmortem evidence of infection despite intensive
scrutiny raises the possibility that positive fecal-culture results may
not always indicate true infection. If these 3 goats were truly free of
infection, the antemortem fecal-culture results must be considered false-
positive. Goats can be coprophagic, and since these adult animals were
living among infected animals (first in a large outdoor pen and then
in an indoor enclosure), it is possible that the positive fecal-culture
results could be explained by "pass-through", as opposed to true
infection. Herds with a high prevalence of Johne's disease are likely
to be managed on highly contaminated premises; the diagnostic
specificity of fecal culture in this situation may be less than 100%.

Isolation of MPTB from neonatal feces as well as from the
intestinal contents and intestinal tissue of a mid-gestation fetus
supports the hypothesis that in utero infection by MPTB can occur
in goats. Thus, accurate record-keeping is important in goat
husbandry to enable culling or close monitoring of potentially
infected offspring of any doe with a diagnosis of the disease. Even
if the kid escapes in utero infection, transmission of the organism
through milk from an infected doe or through contact with
contaminated manure may occur.

Although the ELISA is reported to be a more sensitive method of
antibody detection than the AGID in cattle (13), the 2 assays
performed similarly during this study. Perhaps once these goats
began to produce antibody the amount rose so precipitously that it
swamped any difference in detection sensitivity between the 2 tests.
Serum samples would need to be collected more often than monthly to test this hypothesis.

The pygmy goat species represents an instructive naturally susceptible animal model for further study of the immunopathological events occurring during the course of MPTB infection. The species displays immunologic responses that vary in character, extent, and duration. This variability, however, can confound attempts to control the infection in a herd if a single diagnostic assay is used in a test-and-cull program. Serial or parallel testing with assays aimed at different aspects of the infection (organism shedding and cellular or humoral response) can increase the likelihood of identifying infected goats in a herd with previously confirmed cases of Johne’s disease.

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**References**