Relation between the ELISA optical densities and the histopathological findings in an ovine flock suspected of paratuberculosis

Martínez-Romero G, Estévez-Denaives I and Chávez-Gris G

Departamento de Patología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Ciudad Universitaria 3000, Circuito Exterior, Coyoacán, México, D.F., 04510, Mexico.

Corresponding author: Martínez-Romero G. Phone-Fax: 56-22-58-88. E-mail esthergisela@yahoo.com

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SUMMARY

The relation between the serum ELISA optical densities with the number of mycobacteria per macrophage and the histological lesion were analyzed. A total of 548 adult sheep were tested for serum antibodies to *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) with the absorbed enzyme-linked immunosorbent assay (ELISA). Fifty one animals gave positive results to ELISA; nineteen positive animals and one negative sheep were necropsied. Gross lesions were recorded and tissues from ileocecal valve, ileum, jejunum and mesenteric and ileocecal lymph nodes were fixed in buffered 10% formalin. Paratuberculosis lesions in ileocecal valve, ileum and jejunum were categorised as having focal and diffuse intestinal lesions. The histological lesion of each tissue sample and the mean number of mycobacteria per macrophage were assigned to their respective ELISA optical density subinterval. A clear relation between the ELISA optical density values, the histological findings and the mean number of mycobacteria per macrophage were not determined.

INTRODUCTION

Paratuberculosis (PTB) is a chronic disease of domestic, wild and zoo ruminants, characterized by granulomatous enteritis (1,2,6,7,10,12,14,16,18). The infecting organism, *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) is an acid-fast bacillus (14,17) of 0.5-1.5 μm length (6). The disease occurs throughout the world and results in great economic losses in domestic livestock industries. Paratuberculosis costs in Victorian, Australia dairy and beef industries are $7.5 million per year, and the estimated average annual loss on an infected commercial dairy farm is $2368 (18). PTB-associated economic loss in small ruminants is, in part due, as in cattle, to a decreased milk production. In recent years, PTB has become a public-health concern because of its possible link with Crohn’s disease (14).

After ingestion, the mycobacteria are transcytosed through microfold epithelial cell (M-cells) and are taken up by mononuclear phagocytes in the intestinal mucosa and gut-associated lymphoid organs (GALT) (11). The bacteria are subsequently phagocytosed by subepithelial macrophages. The bacteria are resistant to intracellular degradation, and will slowly replicate in macrophages and stimulate inflammatory and immunological responses (14). A histopathological spectrum related to various immune responses in granulomatous inflammatory reactions has been described for leprosy and was extended to other mycobacterial infections, such as paratuberculosis. This spectrum is defined by the existence of 2 widely differing forms of the disease: a tuberculoid form with strong cell-mediated immune response and lesions characterized by small granulomata with no or few mycobacteria in the lesions; and a lepromatous form, with a strong humoral immune response accompanied by lesions consisting of macrophages full of large numbers of bacilli. Between both extremes of this spectrum are the so-called “borderline forms”, and the individuals manifesting them are those with the most severe clinical signs of disease (15).

The main clinical signs in small ruminants are progressive loss of body condition, weakness (6,14,16,17) and decreased production (13). Intermittent diarrhea can also occur, but is most common in cattle (6,14,17). Susceptibility to infection is highest in animals under 30 days old, but clinical disease does not usually develop in cattle until 2-5 years of age. The major source of infection is feces excreted by infected animals. Fecal contamination of teats and the presence of mycobacteria in colostrum and milk may cause suckling neonatal animals to ingest large doses of organisms; while contaminated pasture, water and...
feed may also be responsible for infection. The long incubation period of the disease allows mycobacterial shedding in feces by animals for up to 18 months before clinical signs are apparent (6). In cattle, the incubation period, assuming infection of neonates, can be up to 14 years (17).

Diagnosis can be difficult on the basis of clinical signs alone and there is no single ante mortem test with absolute sensitivity and specificity (5). The gross and histopathological changes in natural and experimental ovine paratuberculosis have been documented by authors in several countries, sometimes with clinical and serological data (4). The diagnosis of paratuberculosis in sheep can be difficult, particularly for an individual animal. It is important to use a test as a useful tool for seeking evidence of paratuberculosis in live sheep, and therefore is suggested as the basis for control programs. Serological tests for circulating antibody, such as the complement fixation test (CFT), the agar immunodiffusion (AGID) and the absorbed enzyme-linked immunosorbent assay (ELISA) are currently used for cattle. Assessments of these tests have demonstrated high sensitivities when they are used for clinical groups with a heavy mycobacterial infection (5).

In this study, sheep with clinical signs and healthy animals were assessed by the absorbed ELISA. The relations between the serum ELISA optical densities, the number of mycobacteria per macrophage and the pathological findings post mortem were analyzed.

MATERIALS AND METHODS

Sheep. A total of 549 female, adult sheep, between one and four years old were tested for serum antibodies evidence of Map, using the absorbed enzyme-linked immunosorbent assay (ELISA). These sheep had a previous history of severe chronic weight loss; paratuberculosis had been previously diagnosed by use of serological test, pathological studies and isolation.

Blood sample collection. The sheep were bled by jugular venepuncture and the serum was separated from the clotted sample and stored at –30 °C, until ELISA was performed.

Absorbed ELISA. The ELISA was performed, using a protoplasmic antigen (PPA-3, Allied Monitor, Fayette, Mo.). Antigen was diluted to a concentration of 0.04 mg/ml in carbonate buffer, and a volume of 100 µl/well was allowed to fix overnight at 4 °C in flat-bottomed microtitration plates. The wells were washed 3 times with a NaCl solution containing 0.05 % Tween 80. Serum samples were absorbed with a suspension of M. phlei antigen in saline solution (dilution 1:2) for 2 h at room temperature. After centrifugation, the supernatant fraction of each sample was recovered and diluted 1:100 in Phosphate Buffered Tween Gelatine solution (PBS-TG); then 100 µl was added to each of 5 wells. The plates were then incubated for 2 h at room temperature. After washing with PBS-TG, a 1:4,500 dilution of a rabbit anti-sheep horseradish peroxidase-labeled IgG was added to each well and the plates were incubated for another 2 h at room temperature. The wells were washed 3 times with PBS-TG. Finally, the plates were incubated for 15 min in the dark at room temperature. The same negative and positive-control sera were included in the test. Absorbance was read at 405 nm, and the optical density (OD) results were transformed to an index value by division of the mean OD for each serum by the mean OD for the positive-control serum in each plate. Results were considered positive when the index value was ≥0.750. This cutoff value was obtained on the basis of results of previous experiments, and considering a sensitivity of 64 % and a specificity of 75 % (8).

Pathologic procedures. Sheep with positive results to ELISA were subjected to a full necropsy. Gross lesions were recorded and samples for histopathological examination were collected from ileocecal valve, ileum, jejunum, and mesenteric and ileocecal lymph nodes. Tissues were fixed in buffered 10 % formalin. Sections were stained with hematoxylin and eosin, and by the Ziehl-Neelsen method. Paratuberculosis lesions in ileocecal valve, ileum and jejunum were categorised as having focal or diffuse histological lesions. From an examination of 200 macrophages in eight fields (40 x) of the mucosa with lesions of each sample tissue, the mean number of mycobacteria per macrophage (100 x) was calculated, and graded as 0 (none), 1 (1-10), 2 (11-60) or 3 (> 60). The ELISA optical density range value, 0.750-1.145, was divided into three equal subintervals 1 (0.750-0.881), 2 (0.881-1.013) and 3 (1.013-1.145). The histological lesion from each tissue sample and the mean number of mycobacteria per macrophage were assigned to their respective subinterval.

RESULTS

Serologic results. Of the 549 sheep tested, 51 (9.3%) had positive results.

Clinical findings. Four of the twenty animals were emaciated, seven were in fair condition and nine sheep were in store condition.

Necropsy and gross lesions. Nineteen positive animals and one negative sheep to ELISA were necropsied. The most severe changes were consistently located in the terminal ileum adjacent to the ileocecal valve. Gross lesions observed in ileum, jejunum, mesenteric and ileocecal lymph nodes are shown in Table 1.
Pathologic findings. Nineteen of 20 sheep showed paratuberculosis lesions which were classified as focal and diffuse lesions. The focal lesions consisted in aggregations of epitheliod cells and macrophages that formed granulomata, located in the tips of villi, and in the interfolicular and basal areas of the ileal Peyer’s patches (Figure 1), accompanied by infiltration of the lamina propria with limphoid cells and variable numbers of eosinophils and neutrophils. There was no associated submucosal or serosal pathology. Diffuse lesions exhibited multifocal aggregations of epithelioid cells and large granulomata in the lamina propria with limphoid cells and variable numbers of eosinophils and neutrophils. There were also characterized by the presence of numerous macrophages and a significant number of Langhans type giant cells, spread diffusely giving a mosaic-like sheet appearance throughout the lamina propria (Figure 2) and submucosa, causing villous fusion. Ziehl-Neelsen staining revealed abundant acid-fast intracellular organisms. Results of the histological lesions are given in Table 2.

Table 2. Pathological findings.

<table>
<thead>
<tr>
<th>Anatomic region</th>
<th>No lesion</th>
<th>Focal</th>
<th>Diffuse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ileocecal valve</td>
<td>1</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Ileum</td>
<td>1</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Jejunum</td>
<td>2</td>
<td>8</td>
<td>10</td>
</tr>
</tbody>
</table>

* Results are expressed as the number of animals in each group (focal, diffuse or no lesion).

Histological findings distribution in ileocecal valve, ileum and jejunum. Diffuse lesions were the most common in the ileocecal valve, ileum and jejunum with 9, 7 and 6 cases respectively. The results are shown in Table 3, 4, and 5.

Presence of acid-fast organisms. In nine sheep the Ziehl-Neelsen staining revealed acid-fast intracellular organisms. Two sheep with focal lesions had mycobacteria.

Mean number of acid-fast organisms per macrophage. Sheep with diffuse lesions had the highest mean number of mycobacteria in ileocaecal valve, ileum and jejunum. Data are summarized in Tables 6, 7, 8.
**DISCUSSION**

One of twenty animals had no gross or histologic lesions. The most severe gross lesions were observed in four of twenty animals, particularly in the terminal ileum, as well as in the jejunum, showing diffuse lesions. The mesenteric lymph nodes were enlarged with a nodular aspect. The segmental distribution observed in some animals has been reported previously and may be explained by the fact that the first lesions develop in, or in close proximity to, Peyer’s patches, while areas remote from these are only involved when lesions become confluent. The manner in which the lesions have also been seen to increase in severity and extent from the duodenum to the ileocecal valve suggests that this relationship with lymphoid tissue is of importance (3). The lesions were most marked in the terminal ileum, the apparent site of primary infection (4).

Three of four animals emaciated had diffuse lesions. Nevertheless, the other animal presented focal lesions, for which reason we attributed the emaciation to an intestinal parasitosis (*Moenzia* sp). In the other 16 animals, the clinical condition was different and did not coincide with the gross and histopathological lesions. There is little correlation between the severity of the clinical syndrome and the severity of the lesions. Many animals allowed to die have gross and microscopic lesions so slight that they would be easily missed unless specifically sought; on the other hand, severe lesions can be found in animals which appear relatively healthy (9).

The seronegative animals (3/20) showed diffuse lesions and were in a fair condition. A possible explanation for the false negative results in animals with diffuse lesions could be an humoral anergy, considering the severity of the lesions, or an imperfect sensitivity of the serological test. The reason for imperfect sensitivity of serological test, even in late stages of the disease, is the variability of immune response of individual. A significant proportion of clinically affected sheep confirmed to have well-developed histological lesions have negative results in serological tests, particularly those with paucibacillary lesions (17). The false positive result observed in just one animal could be due to a cross reactivity to other mycobacterial species or other genus such as *Corynebacterium pseudotuberculosis* (15).

In the present study the diffuse lesions were the most common. In accordance with the results obtained in the histopathological study, we observed a variation in the distribution of the histological lesions showing a decrease of the number of cases, among the subinterval 0.750-0.881, 0.881-1.013 and 1.013-1.145. In spite of this, in the last subinterval, where we thought the animals must have a higher antibody concentration, the only animal there showed diffuse lesions. In the light of the immunological and histopathological spectrum of paratuberculosis, it would seem possible that the range of lesions were related to an immune response (15), however, we have too few observations. The diffuse lesions, associated with a strong humoral response, in ileocecal valve, ileum and jejunum, were distributed in the three subintervals, which explains why we did not appreciate an increased tendency in the subintervals with higher optical density values. The distribution of the diffuse lesions did not show a tendency in anyone of the subintervals, so we inferred that it could be a fluctuation of the circulating antibodies.

Other infiltrating cells that we observed, included lymphoid cells, eosinophils and neutrophils. Two sheep with focal lesions had mycobacteria. Nine of twenty sheep had acid-fast bacilli in macrophages without showing an increased tendency to do so in any subinterval. Mycobacteria were always most numerous in the intestinal mucosa. This reflects the localization of lesions in paratuberculosis and suggests a particular susceptibility of the intestine to infection (4). The higher mean number of bacilli

| Table 6. Mean number of acid-fast organisms determined in ileocecal valve. |
|--------------------------|--------------------------|--------------------------|
| ELISA optical density subintervals | Histological lesion | (0.750-0.881) | (0.881-1.013) | (1.013-1.145) |
| Focal | 3.37 | 0.5 | 7.1 |
| Diffuse | 89.6 | 2.25 | 6.4 |

| Table 7. Mean number of acid-fast organisms determined in ileum. |
|--------------------------|--------------------------|--------------------------|
| ELISA optical density subintervals | Histological lesion | (0.750-0.881) | (0.881-1.013) | (1.013-1.145) |
| Focal | 82.5 | 0.5 | 80.37 |
| Diffuse | 76.6 | 2.5 | 120.25 |

| Table 8. Mean number of acid-fast organisms determined in jejunum. |
|--------------------------|--------------------------|--------------------------|
| ELISA optical density subintervals | Histological lesion | (0.750-0.881) | (0.881-1.013) | (1.013-1.145) |
| Focal | 32.75 | 0.5 | 3.4 |
| Diffuse | 92.5 | 27.81 | 97.87 |
per macrophage was observed in the diffuse lesions. Nevertheless, it was shown a slightly increase of the mean number of bacilli per macrophage from subinterval 0.750-0.881 to the last one, we could not determine a possible relationship between this two variables, because there was only one animal.

CONCLUSIONS

All the pathological forms could probably be the result of the various immunologic mechanisms operating in mycobacterial granulomatous inflammatory responses, although the fact that they might represent different stages in the evolution of the disease cannot be completely excluded (15). The fact that we have not observed a clear relationship between the ELISA optical density values with the pathological findings and the mean number of mycobacteria per macrophage could be attributed to the different courses of the disease, which could influence the histopathological findings and serological results. However, to confirm or to reject this affirmation it is necessary to carry out more researches in a greater number of animals positive to ELISA.

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