Introduction

*Rhodococcus equi* (*R. equi*) infections are characterized by suppurative pneumonia or ulcerative enteritis in foals and are one of the most serious causes of pneumonia in foals under 6 months of age, which often remain unidentified for weeks or months following infection [1-3, 7, 17]. The infection has been detected by immunohistochemical (IHC) methods in formalin fixed and paraffin embedded sections and found to be highly sensitive and specific when the antibody Mab 10G5 is used [9, 10, 13]. MARIOTTI et al. [10] stated that immunohistochemical tests with Mab 10G5 were highly sensitive and specific and these tests detect both intracellular and extracellular antigens [9, 14]. Moreover some detected antigens (15-17 kDa) are associated with virulence [6, 14]. In a previous study, the sensitivity of the monoclonal antibody in immunocytochemistry was evaluated [12].

The purpose of the study was to retrospectively determine the biological interests in term of *R. equi* infection diagnostic gain and virulence evaluation of histopathology and immunohistochemistry in lungs and in different other tissues.

Material and Methods

**Tissue Samples**

Tissue sections from a foal that died in a stud at the time of study and tissue samples of 8 archived cases were investigated. Anamneses of the animals were given in Table I. After gross examination of recently dead foal, a systemic necropsy was performed. Tissue samples taken from various organs (heart, lungs, liver, spleen, kidneys, lymph nodes, stomach, intestines, thymus, pancreas, bone marrow) were fixed in 10% buffered formalin solution for 24 hours, processed through routine procedures and blocked in paraffin.
HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES

A total of 47 tissue samples (8 from lungs, 7 from heart, liver, spleen and kidneys, 4 from intestines, 3 from lymph nodes and 2 from thymus, 1 from pancreas and from bone marrow) were examined.

For histopathology and immunohistochemistry, 2-3 µm sections from blocks recently prepared and from the department’s archived blocks were cut. For histopathological evaluation, sections were stained with haematoxylin eosin and cover-slipped.

For immunohistochemistry, paraffin sections were collected onto Poly-L-Lysine covered slides. Before used, sections were dewaxed and rehydrated and subjected to antigen retrieval by incubation with heated citrate buffer solution using microwave oven (~70°C, 4x5 minutes, and 20 minutes cooling inside the same solution). Sections were washed three times with phosphate buffered saline (0.1 M PBS pH 7.4), and then incubated with a commercial blocking solution (LabVision, Fremont, California) for 7 minutes. Subsequently the slides were incubated with a monoclonal antibody specific for \textit{R. equi} (clone 10G; Department of Animal Hygiene, School of Veterinary Medicine and Animal Sciences, Kitasato University, Japan) at a dilution of 1:400. After washing again slides three times with PBS, a labelled streptavidin-biotin (LSAB) kit (Dako Universal LSAB2 Kit-HRP; Dako, Glostrup, Denmark) was applied according to the manufacturer’s instructions. The kit contains biotin-labelled affinity-isolated goat anti-rabbit and goat anti-mouse immunoglobulins. Finally, the sections were treated at room temperature for 30 minutes with 3-amino-9-ethylcarbazole (Lab Vision), washed three times with distilled water, counterstained with Mayer’s haematoxylin and cover-slipped under an aqueous mounting medium (ScyTek, Logan, Utah).

Positive control sections were obtained from a seropositive foal with positive cultures from tracheal washes and gross lesions. For negative control, the primary antibody was substituted with PBS.

**Table I:** Anamneses of the horses included in the study for histopathological and immunohistochemical detection of \textit{R. equi}.
Results

Gross examination of the performed necropsy revealed the enlargement of the lungs with darker consolidated areas and small, yellowish foci of abscesses of 0.5-5 cm diameter scatter throughout the whole organ. Mediastinal lymph nodes were also found to be enlarged; the cut surfaces of the organs were gray with necrotic debris in the center. There was a fluid, yellowish content in intestines. The aggregated lymph follicles of the small and large intestines were swollen and reddened. The entire length of the intestinal wall and mucosa were thickened. No other gross pathological alterations were detected in the other tissues.

In lung samples, histopathology revealed pyogranulomatous pneumonia in the necropsied foal. There was oedema and cellular proliferation in alveolar septa and pleura. Desquamations of epithelium in bronchus and mainly in bronchioles were remarkable. Some terminal bronchioles and alveolar spaces were filled with numerous neutrophils, macrophages, necrotic debris, and some multinucleated giant cells. In some areas alveolar and bronchiolar tissues had undergone necrosis and there were various sizes of necrotic foci. Histopathology of the archived lung samples revealed similar lesions with slight differences. A marked fibrosis was evidenced in the lung tissues from the archived cases n°1, 3, 7 and 8. In the archived cases n°2, 7 and 8, the inflammatory cell reaction involved mainly mononuclear cells instead of neutrophils.

Whereas there was no histopathological alterations in the liver samples from the necropsied foal, parenchyma degeneration, oedema, infiltrates of mononuclear inflammatory cells around hepatic triads and increase in the number of bile ducts and hyperplasia in bile epithelium were detected in all the archived cases. Besides, in the cases n°6 and 7, haemorrhages and necrotic foci were observed in parenchyma.

In intestinal sections from the necropsied case, marked hyperaemia and haemorrhages were observed. There were desquamation of epithelium and atrophy in some villi. Abundant infiltrations of inflammatory cells which were dominant of plasma cells in submucosa layer between glands were determined. In archived cases besides these findings, hyperplasia of lymphoid nodules was remarkable. In the case n°6, gland atrophy and fibrosis with histiocytes and fibroblasts were found in mucosal layer.

The histopathological findings in lymphoid tissue samples from archived cases and from the dead foal were similar. The most remarkable changes observed in the spleen were the depletion of germinal centers. Reticular cells filled the area of missing lymphocytes and there was hyalinization in some germinal centers. In some sections there were remarkable karyorrhexis and karyolysis in lymphoid cells. Besides, inflammatory cell infiltrations in red and white pulp, distinctive haemosiderin infiltrations in some animals and reticular hyperplasia were observed. In the thymus sample from the dead foal, hyperaemia, haemorrhages and lymphoid hyperplasia were evident. Germinal centers were hyperplastic in lymph nodes, and abundant endothelial cells were found in their center, sinusoids were filled with macrophages and inflammatory cells (predominantly plasma cells).

Kidneys preserved broadly their histological structures. In two archived cases (archived cases n°3 and 4), slight mononuclear cells infiltrations were noted in inter-tubular areas.

In stomach, pancreas and bone marrow samples, no conspicuous histopathological changes were seen.
Immunohistochemical labelling of the lung sections revealed the presence of *R. equi* antigen in the cytoplasm of alveolar macrophages (figure 1A), neutrophils and multinucleated giant cells. All lung tissues except one archived sample (archived case n°1) were immunopositive. Besides, in sections where fibrosis was observed in parenchyma, positive cytoplasm labelling of histiocytes, fibroblasts and endothelial cells of veins were observed (figures 1B and 1C). In the archived diaphragm sample n°8, only fibroblasts, endothelial cells and few macrophages were positively stained (figure 1D).

![Figure 3: Thymus. Immunopositive intracytoplasmic staining in macrophages (white arrows) and endothelial cells (black arrows), LSAB (labelled streptavidin-biotin revelation).](image)

In liver, positive reactions were evidenced in hepatocytes and in Kupffer cells only in tissue sample from the archived case n°6 (figure 2). Endothelial cells and macrophages were also positively stained for *R. equi* in lymphoid tissues (lymph nodes and thymuses) (figures 3A and 3B). In intestine tissues, background staining especially in goblet cells even in negative control tissues did not allow conclusive *R. equi* evidence in any sample. No positive reactions were found in stomach, pancreas and bone marrow sections.

**Discussion**

The macroscopic and microscopic alterations seen in this study were typical of the disease caused by *R. equi* infections in foals [11, 15] and when compared with immunohistochemistry included archived tissue samples, the observed pathological changes were not differential but sufficient for the diagnosis of *R. equi*.

FREESTONE et al. [4] reported that *R. equi* associated pneumonia can be encountered in adult horses as a result of acquired immunodeficiency. Compatible with this finding we thought that positive reaction in the lung tissues of the 9 years old horse (archived case n°8) resulted from an acquired immunodeficiency. In this horse immunopositive staining were observed generally in endothelial cells and fibroblasts. FREESTONE et al. [5] reported the first hepatitis and cholangitis associated to *R. equi* infection in 1989 and KARADAS et al. [8] reported subcapsular haemorrhages, centro-lobular necrosis and mononuclear cell infiltrations in the liver tissues from 2 foals with *R. equi* infection. These reports were in agreement with the liver lesions and immunopositive reactions of hepatocytes and Kupffer cells observed in a foal (archived case n°6). It would be stated that *R. equi* is the direct causative agent of hepatitis, resulting from bacteraemia or ascending infection via the bile ducts from the intestines [5]. ÖZSOY and HAZIROĞLU [11] reported immunopositive macrophages in the medullar sinuses of mediastinal lymph nodes. Positive staining in the cytoplasm of free macrophages, in addition to immunopositive staining of endothelial cells of the lymphoid tissues without any pathological alterations was also observed in this study. This finding confirms the opinion that fixed macrophages are able of destroying *R. equi* presented to them via the bloodstream [16].

As a conclusion, as reported previously the monoclonal Mab 10G5 antibody was found to be highly sensitive and specific in paraffin blocked and embedded tissues [9, 10, 13]. In addition, immunohistochemistry has allowed evidencing virulent *R. equi* agents in various tissues of horses including lungs, liver and lymphoid tissues (lymph nodes, thymus and spleen) and was in concordance with histopathological findings except in one case (archived case n°1).

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