

The pathological findings of *Rhodococcus equi* infection and its diagnosis with immunoperoxidase technique in foals*

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Summary: Twelve foals with difficulty in respiration that have died during spring-summer months between 2003-2006 years were necropsied. On necropsy, pneumonia was detected in all of the foals. In 5 animals, caseo-necrotic nodules were seen in the lungs. In histopathological examination, purulent bronchopneumonia was noticed. In immunohistochemical staining, *Rhodococcus equi* antigens were determined in the cytoplasm of macrophages and occasionally in neutrophils in lungs and mediastinal lymph nodes by immunoperoxidase technique. No lesions were detected in the intestines confirming that this form of infection is rare compared to the lung form. In the present study, *R. equi* was microbiologically isolated only in two cases. In conclusion, *R. equi* infection was diagnosed in all of the twelve cases, and estimated to be the reason of pneumonia in the foals. It was also shown that immunoperoxidase technique can be successfully used for *R. equi* infection in the field.

Key words: Foal, immunoperoxidase technique, pathology, *Rhodococcus equi*.

Taylarda *Rhodococcus equi* enfeksiyonunun patolojik bulguları ve immunoperoksidaz tekniği ile tanısı

Özet: Klinik olarak solunum güçlüğü bulguları gösteren ve 2003-2006 yıllarının ilkbahar-yaz aylarında ölen 12 tayın nekropsisi yapıldı. On iki tayda da *R. equi*'ye bağlı pnömoni saptandı. Makroskopik olarak; 5 olguda akciğerde tipik kazeonekrotik odaklarla karşılaşıldı. Histopatolojik incelemede; irinli bronkopnömoni tablosu dikkati çekti. *R. equi*'nin, yapılan immunoperoksidaz boyamalarla, akciğer ve mediastinal lenf düğümü kesitlerinde, çoğunlukla makrofajların, bazen de nötrofil lökositlerin sitoplazmasında yerleştiği görüldü. Bağırsaklarda lezyon görülmemesi, bu formun akciğer formuna göre ender şekillendiğini gösterdi. Sadece iki olguda mikrobiyolojik olarak *R. equi* izole edilebildi. Sonuç olarak; çalışmada kullanılan 12 tayda da *R. equi*'nin saptanmış olması, taylarda görülen pnömonilerin çoğunluğundan bu bakterinin sorumlu olduğunu ve ayrıca tanısında immunoperoksidaz tekniğinin başarıyla kullanılabileceğini gösterdi.

Anahtar sözcükler: İmmunoperoksidaz tekniği, patoloji, *Rhodococcus equi*, tay.

Introduction

Rhodococcus equi (*R. equi*) is a facultative intracellular pathogen of macrophages (5, 12, 14). It is an opportunistic pathogen that causes purulent bronchopneumonia, pulmonary abscesses, ulcerative enteritis, and is associated with lymphadenitis in 1 to 6 months old foals (5, 15, 16). The pathogen has also been less frequently associated with osteomyelitis in foals (18). The infection occurs sporadically on some farms and morbidity rate increases as high as 80% at newborns when the immune system is still immature and maternal antibodies have disappeared (19). However, the disease is very rare in adult horses (5, 17).

R. equi is a robust soil organism living widespread in the environment. The organism potentially multiplies wherever there is horse manure. Temperature plays a major role in the growth of *R. equi*, which shows an

optimal growth at 30°C. A direct relationship exists between the number of *R. equi* in the environment of young foals and the number of pneumonia. The pathogen is commonly found in loafing paddocks on horse breeding farms during summer time and reaches to the lung by inhalation (4, 23, 24).

The purpose of this study was to investigate pathological findings of *R. equi* infection, which causes high mortality in foals, and to evaluate the use of immunoperoxidase technique in the diagnosis of the infection.

Materials and Methods

The study materials were twelve foals suffering from respiratory difficulty and died during spring-summer months in 2003-2006. following routine necropsy, samples of all the organs were fixed in 10%

* This research has been summarized from Ph.D. thesis. It was supported by projects TÜBİTAK (VHAG-2013) and BİYEP (2005K120140-7).

buffered formalin solution, embedded in parafin and cut into 5-6 μ thick sections that were stained with haematoxyline-eosin (HE), Brown-Brenn and Ziehl-Neelsen staining. Then, the sections were dewaxed and rehydrated by routine methods for immunohistochemical staining as follows. The preparations were incubated in 3% H₂O₂ solution for 5 minutes, then incubated with normal goat serum for 20 min at 40°C. The sections were incubated with *anti- Rhodococcus equi* hyperimmune serum diluted 1:300 (AGIDT: 1/200+++) for 1 hour at room temperature (1:300 dilution). The sections were then incubated with streptavidin-peroxidase reagent for 20 min (Dako/Denmark). Colour labeling was developed by a final incubation step using 3-amino-9-ethyl-carbazole (AEC, Dako/Denmark) for 7 minutes. Finally, the sections were counterstained with Mayer's haematoxyline and covered with glycerol-gelatin.

Results

High fever (41°C) (except in case 11), severe coughing and difficulty in breathing (except in case 9) were seen as the general clinical signs in the foals. In case 9 ulceration in cornea and swollen joints were noticed.

Significant pathological changes were observed only in the lungs. Nodules, which were yellowish-white in colour, hard or fluctuant in consistency, about walnut sized, and surrounded by severe and extensive consolidation and atelectatic areas, were detected in lung lobes. On cut surface, the nodules were irregular and well defined and showed white firm or caseous appearance (Figures 1, 2). In four cases (Cases 1, 8, 10, 12), the nodules were located in the cranial lung lobes. In case 7, nodules involved in all lung lobes while in case 2 the nodule was located in the cranio-ventral lobe. A serous

bloody and foamy fluid was seen at bronch and bronchial lumens. In case 9 and 11, hemorrhage and consolidation were the only findings in lungs. There were no lung lesions in cases of 3, 4, 5, and 6.

The mediastinal lymph nodes were swollen and the cut surface showed yellowish-green in colour and creamy appearance (Cases 7, 8, 11) (Figures 9, 10). Moreover, macro-pathologic changes were noted in liver (Cases 8, 11, 12), kidney (Cases 8, 11) and intestines (Case 8).

The macroscopic findings are listed in Table 1.

Histologically, bronchopneumonia was observed in the lungs. Various degrees of hyperemia (Cases 3, 5, 7, 9, 11 and 12) and thrombosis were noted (Case 12). Macrophages and neutrophils, which were observed at alveoli lumens, were seen at bronch and bronchiole and around the blood vessels. The degree of these inflammatory cells which were weak at Cases 3 and 6; were perivascular placement at Case 3. In cases 1, 4, 8 and 12, multinucleated giant cells accompanied inflammatory cell infiltration (Figures 3-6). In some cases (Cases 1, 2, 5 and 12), necrosis of inflammatory cells and alveoli walls were seen. Hyalinization of inflammatory exudate (Case 12) was determined in alveoli lumens. Inter-alveolar septums were dilated with fibrous tissue elements at cases 1, 2 and 4. In some alveoli lumens, pink homogeneous oedema fluid and erythrocytes were observed. Bronchus and bronchioles epitheliums were hyperplastic, and occasionally desquamated in lumens (Cases 5, 8). Peribronchial lymph follicles were hyperplastic in case 4. Oedema (Case 8), macrophage and lymphocyte infiltration, and fibrous tissue proliferation were also seen in pleura (Case 7).

Between the severe inflammatory cell infiltrations and alveoli lumens, immunopositive macrophages and neutrophils were observed (Figures 7, 8).

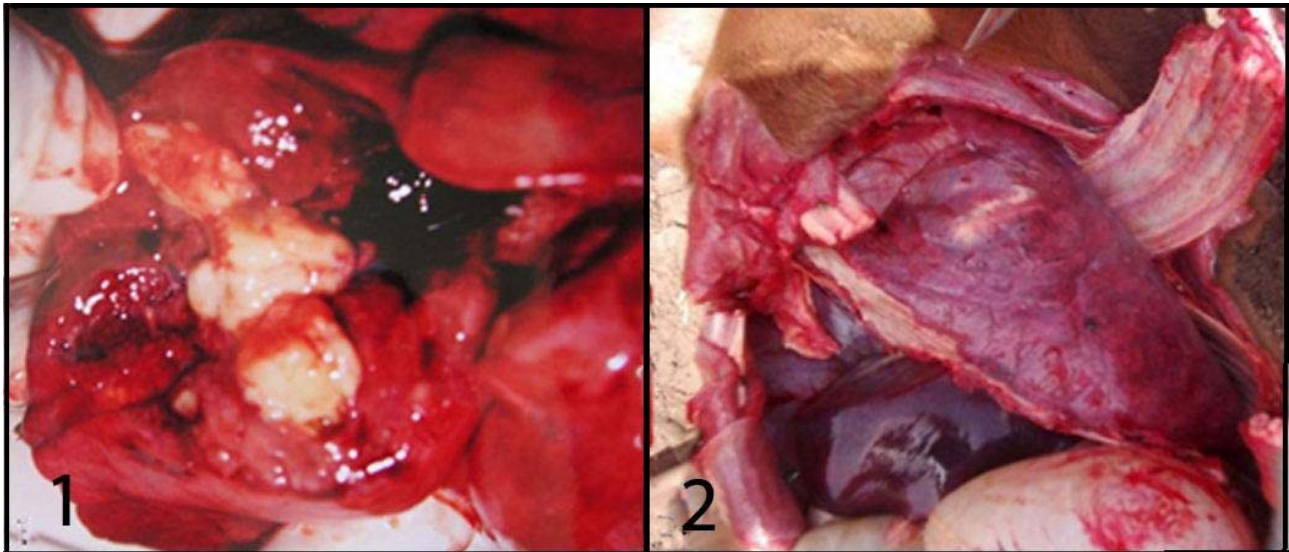


Figure 1. Lung, case number 7; nodule; yellowish-white in colour and fluctuant in consistency.

Şekil 1. Akciğer, olgu no 7; sarımtırak-beyaz renkli, fluktuan kıvamlı nodül.

Figure 2. Lung, case number 10; caseous nodule; hard in consistency, yellowish-white in colour.

Şekil 2. Akciğer, olgu no 10; sert, kolay parçalanabilir kıvamlı, sarımtırak boz beyaz renkte kazeöz nodül.

Table 1. The macroscopic findings of infection.

Tablo 1. Enfeksiyonda gözlenen makroskopik bulgular.

Case number	The localization of nodules/ Other lesions	The consistency and appearance of nodules	The size of nodules	Mediastinal lymph nodes	Intestine	Liver	Kidney
1	Left cranial lobe	Hard in consistency, yellowish-white in colour, caseous nodule	4x3 cm	-	-	-	-
2	Cranioventral lobe		2x3 cm	-	-	-	-
3	-	-		-	-	-	-
4	-	-		-	-	-	-
5	-	-		-	-	-	-
6	-	-		-	-	-	-
7	Diffuse	Hard in consistency, yellowish-white in colour, caseous nodule	Varried between 2x3 cm-4x5.5	Swollen and the cut surface showed yellowish-green in colour and creamy appearance	-	-	-
8	Right cranial lobe	Hard and some areas fluctuant in consistency, white in colour, capsuled with fibrous tissue, caseous nodule	1.5x2 cm and 1x1.5 cm	Swollen and the cut surface showed yellowish-green in colour and creamy appearance	Subserosal damarlar dolgun ve lumeninde sarı sulu renkte içerik ile mukozada hiperemi	Swollen, cut surface was hemorrhagic	Increase in size
9	Petechial hemorrhage areas	-	-	-	-	-	-
10	Right cranial lobe	Hard in consistency, yellowish-white in colour, caseous nodule	2.5x3 cm	-	-	-	-
11	Consolidation at cranial lobes and diffuse petechial hemorrhages	-	-	Swollen and cut surfaces had hemorrhage areas	-	Swollen, cut surface was hemorrhagic	Diffuse, small, white in colour focuses located at cortex
12	Right cranial lobe	Very hard in consistency, white in colour, capsuled with fibrous tissue caseous nodule	3x4 cm	-	-	Swollen, cut surface was hemorrhagic	-

Table 2. The microscopic findings of infection.

Tablo 2. Enfeksiyonda gözlenen mikroskopik bulgular.

Case Number	1	2	3	4	5	6	7	8	9	10	11	12
Vascular lesions												
*Hyperemia	-	-	+	-	+	-	+	-	+	-	+	+
*Thrombosis	-	-	-	-	-	-	-	-	-	-	-	+
*Vasculitis	-	-	-	-	-	-	-	-	-	-	-	-
Inflammatory cell infiltration												
*Macrophages	+	+	+	+	+	+	+	+	+	+	+	+
*Neutrophile	+	-	-	-	+	-	+	-	+	-	-	-
*Lymphocyte	+	+	+	+	+	+	+	+	-	+	+	+
*Fibrous tissue	+	+	-	+	-	-	-	-	-	-	-	-
Localization of inflammatory cells												
*Alveoli	+	+	+	+	+	+	+	+	+	+	+	+
*Bronch	+	+	-	-	+	-	+	+	-	+	-	+
*Bronchiol	+	+	-	+	+	-	+	+	+	+	-	+
*Perivascular	-	-	+	-	-	-	+	+	-	-	-	+
Severity												
*Diffuse	+	+	-	+	+	-	+	+	+	+	+	+
*Slight	-	-	+	-	-	+	-	-	-	-	-	-
Necrosis of inflammatory cells	+	+	-	-	+	-	-	-	-	-	-	+
Necrosis of alveoli walls	+	+	-	-	+	-	-	-	-	-	-	+
Giant cell formation	+	-	-	+	-	-	-	+	-	-	-	+
Pleura Inflammation	-	-	-	-	-	-	+	-	-	-	-	-
Oedema	-	-	-	-	-	-	+	+	-	-	-	-

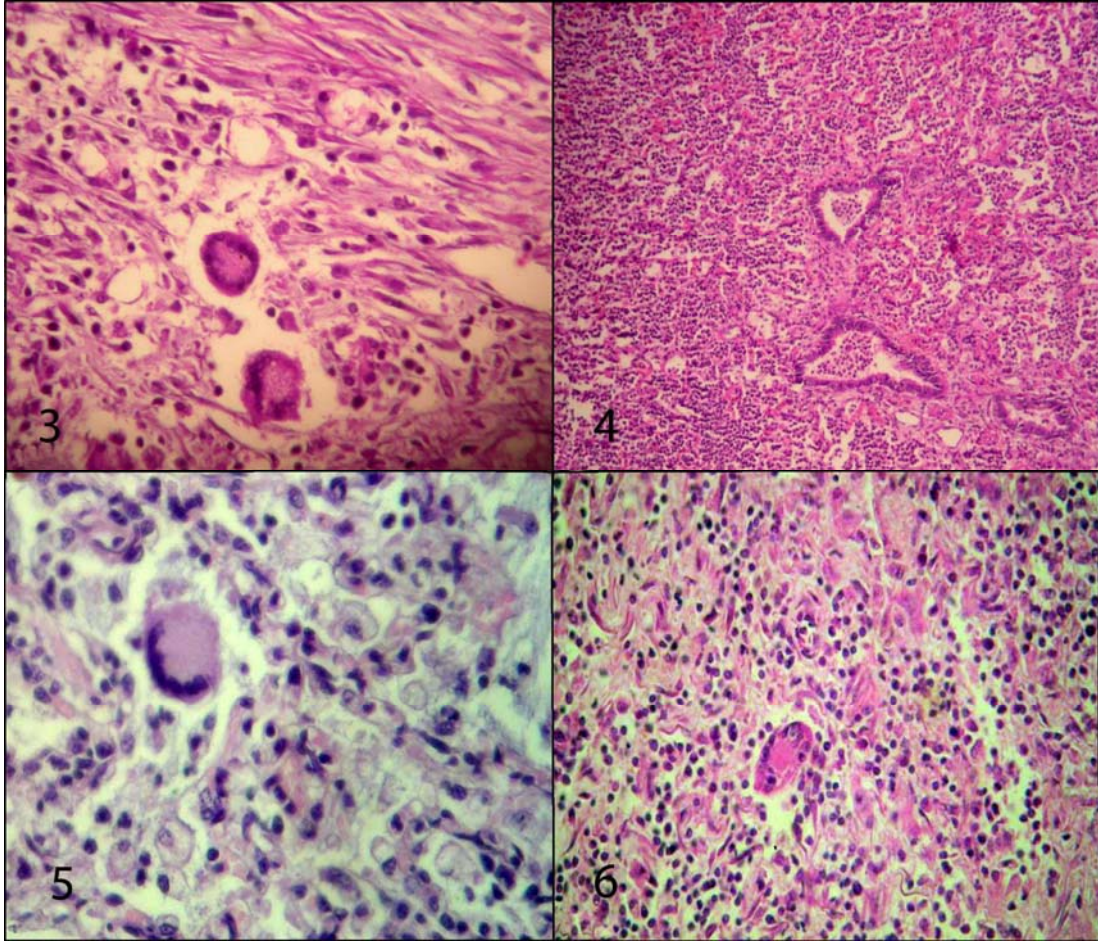


Figure 3. Lung, case number 4; increase of multinucleated giant cell, macrophage, lymphocyte and fibrous tissue, H&E, 400x.

Şekil 3. Akciğer, olgu no 4; çok çekirdekli dev hücreleri, makrofaj, lenfosit ve fibröz doku artışı, HE, 400x.

Figure 4. Lung, case number 5; macrophages and other inflammatory cell infiltration in alveoli and bronchial lumens, H&E, 40x.

Şekil 4. Akciğer, olgu no; 5 alveol ve bronşiol lumenlerinde, makrofaj ve diğer yangısal hücreler, HE, 40x.

Figure 5. Lung, case number 8; multinucleated giant cells, macrophages and lymphocyte infiltration in alveoli lumens and paranchyma, H&E, 400x.

Şekil 5. Akciğer, olgu no 8; alveol lumenlerinde ve paranşimde çok çekirdekli dev hücresi, makrofaj ve lenfosit infiltrasyonu, HE, 400x.

Figure 6. Lung, multinucleated giant cells and mononuclear cell infiltration in alveolar exudate, H&E, 400x

Şekil 6. Akciğer, olgu no 12; alveoler eksudatta çok çekirdekli dev hücresi ve mononükleer hücre infiltrasyonu, HE, 400x.

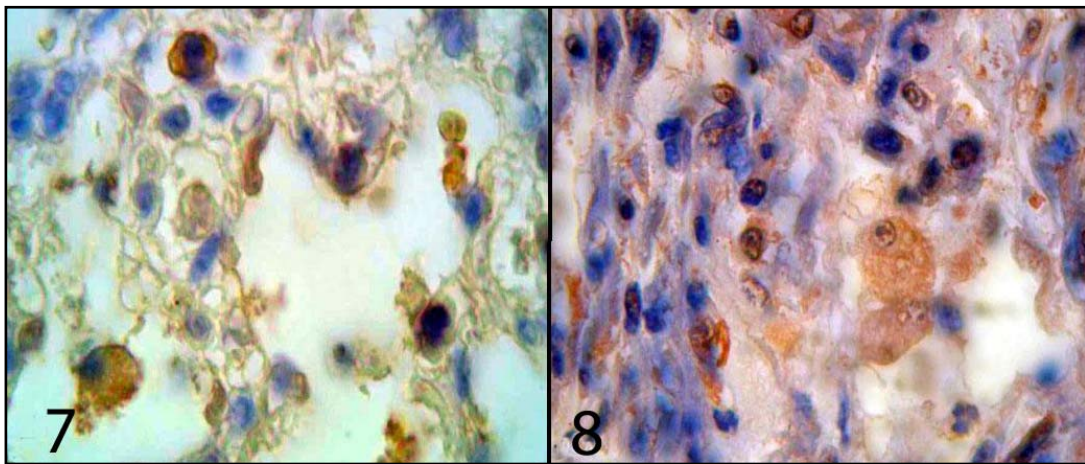


Figure 7. Lung, case number 5; immunopositive staining at macrophages against *R. equi* antiserum, ABC-P, 1000x.

Şekil 7. Akciğer, olgu no 5; makrofajlarda *R. equi* antiserumuna karşı IP pozitif boyanmalar, ABC-P, 1000x.

Figure 8. Lung, case number 11; immunopositive staining at macrophages against *R. equi* antiserum, ABC-P, 1000x.

Şekil 8. Akciğer, olgu no 11; makrofajlarda *R. equi* antiserumuna karşı IP pozitif boyanmalar, ABC-P, 1000x.

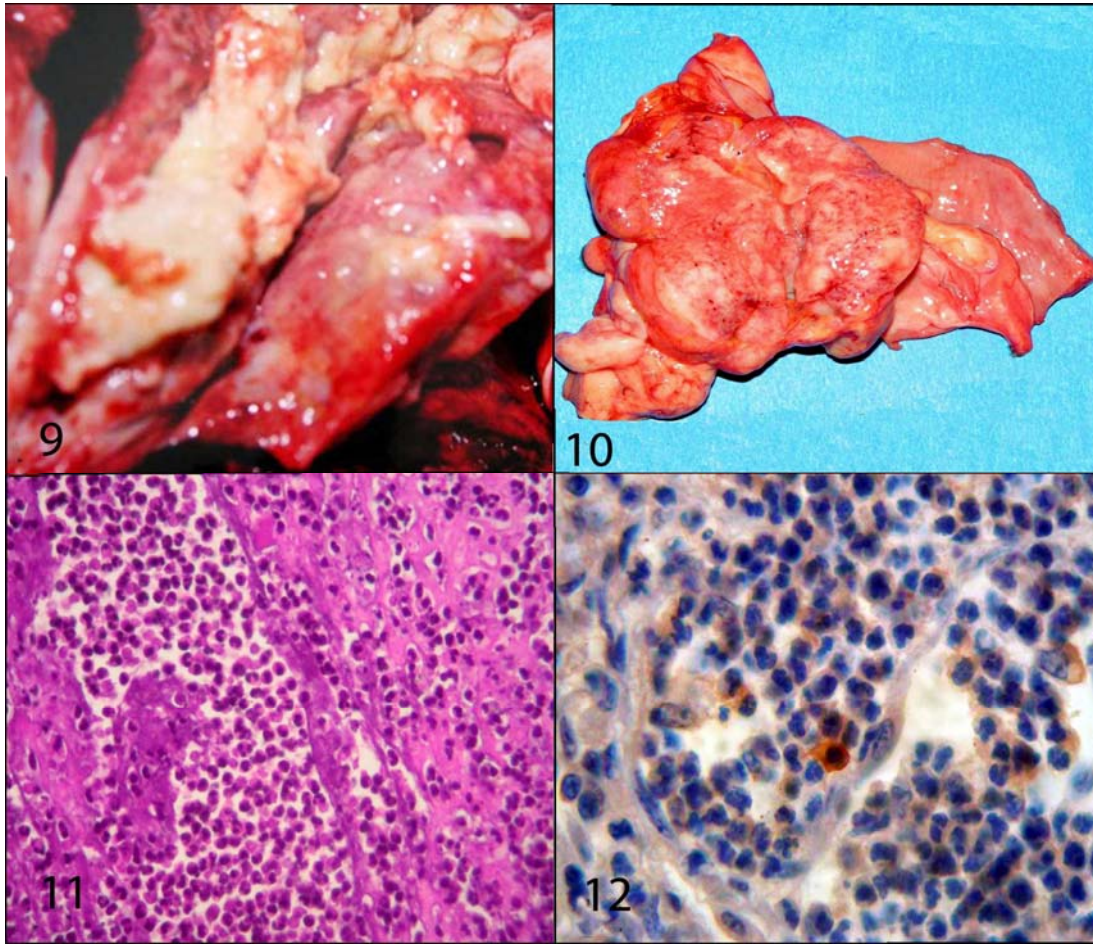


Figure 9. Mediastinal lymph node, case number 7; swollen and the cut surface showed yellowish-green and creamy appearance material.

Şekil 9. Mediastinal lenf düğümü, olgu no; 7, şişkin, kesit yüzü sarımtırak yeşil renkli ve krema benzeri görünüşte materyal.

Figure 10. Mediastinal lymph node, case number 8; swollen and the cut surface showed yellowish-green in colour and creamy appearance material.

Şekil 10. Mediastinal lenf düğümü, olgu no; 8, şişkin, kesit yüzü sarımtırak yeşil renkli ve krema benzeri görünüşte materyal.

Figure 11. Mediastinal lymph node, case number 7; widen of sinuses, macrophage, neutrophile and lymphocyte cell infiltration was seen in sinus lumens. Lymphoid necrosis was seen, HE, 400x.

Şekil 11. Mediastinal lenf düğümü, olgu no; 7, sinusta genişleme, lümeninde makrofaj, nötrofil ve lenfosit infiltrasyonu. Ayrıca kortekste lenfoid nekrozlar gözlenmekte, HE, 400x.

Figure 12. Mediastinal lymph node, case number 7; IP positive staining at macrophages, ABC-P, 400x.

Şekil 12. Mediastinal lenf düğümü, olgu no; 7, makrofajlarda IP pozitif boyanmalar, ABC-P, 400x.

Table 3. The immunoperoxidase results at lungs and mediastinal lymph nodes.

Tablo 3. Akciğerler ve mediastinal lenf düğümlerinde gözlenen immunoperoksidaz sonuçlar.

Case number	1	2	3	4	5	6	7	8	9	10	11	12
Lungs	++	++	+	+	++	+	+	++	+	+	++	+
Mediastinal lymph nodes	-	-	-	-	-	-	+	-	-	-	-	-

-: Negative +: Weak ++: Strong

Vascular hyperemia and sinusoidal dilatation were observed in mediastinal lymph nodes. Lymphatic follicles were hyperplastic, and infiltrations of macrophage, neutrophile and lymphocyte cells were seen in sinus lumens (Cases 7, 8, 11), and necrosis was noted in Case 7 (Figure 11).

R. equi immunopositive macrophages were seen in medullar sinuses of mediastinal lymph nodes (Case 7) (Figure 12).

Subcut enteritis was observed in the intestine of case 8. In liver, passive hyperemia (Cases 8, 9, 11, 12), degenerative changes (Cases 8, 9, 10, 11, 12) and serous

hepatitis (Cases 9, 12), in rens tubulonephrosis (Cases 8, 10), interstitial nephritis and at glomerular capillaries and at interstitial areas bacterial gatherings were observed (Case 11). The bacterial gatherings were stained blue-purple with Brown-Brenn Gram and Ziehl-Neelsen. The microscopic and immunoperoxidase findings were listed in Tables 2 and 3.

For microbiological examination, tissue samples were taken from cases 8, 10, 11. Only in two cases (Cases 8, 11) *R. equi* was isolated microbiologically.

Discussion and Conclusion

In this study, gross and microscopic findings of *R. equi* infection were evaluated in foals. To determine the presence and localization of *R. equi* antigens, immunoperoxidase technique was used.

Factors such as age, season and sheltering condition are predisposing factors for *R. equi* infection. When the immune system is immature and maternal antibodies have disappeared, the infection causes death about 80% in new born foals (19). Temperature plays a major role in the growth of *R. equi*, as the optimum growth is succeeded at 30°C. A direct relationship between the number of *R. equi* in the environment of young foals and the number of pneumonia exists. The pathogen is commonly found in loafing paddocks on horse breeding farms in summer and reaches to the lung by inhalation (4, 23, 24). In the present study, age of foals varied between 3 days old to 7 months old, and death of foals were seen between the months of April and August.

Clinical findings of high fever (41-41.5 °C), cough and difficult breathing were similar with other reports (1, 15, 22). Infection at distant sites from the respiratory or gastrointestinal tract is likely to be associated with bacteremic dissemination and localization of bacteria at other sites. (2, 5, 15). Ulseration of cornea and swelling at joints were seen before foal 9 was died was also explained with these reports.

The main routes of infection are the respiratory and alimentary tracts. Inhalation of the organism is probably is the main route of exposure in all foals (22). Zink et al. (1986) reported that 96 % of pneumonias observed in foals which were died due to *R. equi* infection (27) and this infection accounted for 45 % of all foals with pneumonia (25). The lesions are usually more extensive in the right lung than the left (7). Similar to the report of Hillidge (1986) (7), most of the lesions in cases 8, 10 and 12 were located in the right lung in the present study. Although congestion and consolidation was the only findings in cases 9 and 11, in some cases (Cases 3, 4, 5, 6) no macroscopic findings were observed. These findings thought that at peracute form of infection death

was occurred quickly. The isolation of *R. equi* from case 11 is supported this opinion.

The macroscopic appearance of caseous nodules in lungs and the swollen mediastinal lymph nodes, as well as morphological features in the cut surfaces of nodules and lymph nodes were similar with previous reports (6, 11, 14).

Infiltration of numerous bacteria laden macrophages and neutrophils with multinucleated giant cells and necrosis of these inflammatory cells were seen in lungs. The microscopic appearance of lungs seemed as in previous reports (6, 11, 20, 21). In mediastinal lymph nodes, inflammatory cell infiltration and necrosis of lymphoid follicles were also observed similarly to the previous reports (8,14).

R. equi induced enterocolitis has been described in a few case reports. The findings of this form ulcerative enteritis and associated with lymphadenitis (3, 5, 10, 15, 16) were not seen in the present study. Only subacute enteritis was observed in case 8. No findings any lesions in intestine confirmed that this form of the infection is rarely encountered compared to the lung form.

When *R. equi* agents spread from lungs, pathological lesions may form in parenchymal organs such as liver and kidney (9). In this study, gross and histopathologic changes were seen in liver (Cases 8, 11, 12) and kidney (Cases 8, 11)

Morphological identification of *R. equi* in tissue samples is difficult, since spesific histochemical stains are not available. Gram and ZN stains can be regarded only as diagnostic aids. But immunohistochemical tests which are highly sensitive and specific, detect intracellular and extracellular antigen (13). The immunohistechemical method is relatively inexpensive and, unlike PCR, does not require special laboratories or specialist personel. In contrast to indirect immunofluorescence (IIF), immunohistochemical preparations can be examined with a simple light microscope and can be stored for extended periods, and immunohistochemistry enables the study of cell and tissue morphology at the same time (20). *R. equi* has been detected by immunohistochemical methods, using polyclonal and monoclonal antibodies in formalin-fixed and parafin-embedded sections. *R. equi* antigens were demonstrated with immunoperoxidase technique usually in cytoplasm of macrophages and rarely in cytoplasm neutrophils and giant cells at lung and mediastinal lymph node sections (8, 13, 14, 20, 21, 26). In the present study the *R. equi* antigens were located in the center of suppurative inflammation, in the bronchiole lumina, and in the cytoplasm of macrophages and rarely neutrophils. In the mediastinal lymph nodes near the necrotic lenfoid follicles, *R. equi* positive immunostained few macrophages were seen (Case 7).

In this study, presence of macrophages, some of them were bacteria laden, within the lesions could be explained by role of these cells as the main defender against agents that are inhaled as previously reported (5, 8, 13, 24).

In conclusion, *R. equi* infection was diagnosed in twelve foals. It was concluded that *R. equi* might be one of the most important factor in foal pneumonias. We have shown that immunoperoxidase technique could be successfully used as a diagnostic mean of the infection on the field cases.

Acknowledgements

I'm grateful to Professor Doctor Yakut ÖZGÜR (University of İstanbul, Faculty of Veterinary Medicine, Department of Microbiology) for assurance of virulent *R. equi* which was containing an 85 kb type I plasmid, was used for preparing immunization antigen.

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Geliş tarihi: 13.05.2008 / Kabul tarihi: 07.07.2008

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