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Original Article

The Pathology of Experimental *Rhoodococcus equi* infection in foals

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Abstract

The pathology of experimental *Rhodococcus equi (R .equi*) infection in 2-8 week-old-foal is studied. For this purpose, twenty foals were divided into three groups, and given *R. equi* intratracheally (1st group), through gastric route (2nd group) and through umbilicus by contamination (3rd group). A control group of foals were given a Phosphate buffered Saline (PBS). Pulmonary and intestinal lesions were seen in foals of all infected groups. Grossly, there were multiple, variable-sized abscesses diffusely scattered throughout the lung parenchyma, in addition to the presence of different stages of pneumonia with variable-sized areas of consolidation and emphysema. Intestinal lesions were evident as engorgement of mesenteric blood vessels, subserosal hemorrhages seen along the intestinal tract especially the small intestine, in addition to enlargement of lymph nodes (mesenteric, bronchial and mediastinal). Some lymph nodes were edematous, have circular foci of caseous necrosis and some of them were filled with yellowish, thick creamy pus.

The microscopic lesions were basically similar in all foals of the experimental groups, but varied depending on the time of death or euthanasia and included: acute pulmonary congestion, acute suppurative broncho-pneumonia, chronic pyogranulomatous pneumonia, and emphysematous and atelectatic area. There were focal necrosis of the pulmonary parenchyma and numerous bacterial colonies seen free or as aggregates within the cytoplasm of many histiocytes. Also there were focal interstitial thickening of the alveolar septae. The pleura and interlobular septae were thickened due to cellular infiltration.

Keywords: *Rhodococcus equi*, foals, histiocytes, umbilicus, pneumonia

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Introduction

Rhodococcus equi is recognized chiefly as a cause of suppurative bronchopneumonia in the foals, but ulcerative entercolitis is another, although less common manifestation of the infection in young horses (Barton and Hughes, 1980; Elissaldi et al., 1980; Meijer and Prescott, 2004; Özsoy, and Haziroglu, 2009). Generalization of infection from the lung occasionally leads to suppurative arthritis, hepatic or splenic abscesses, renal infarcts, vertebral abscesses or hypopyon (Wilson, 1955; Barton and Hughes, 1980). Cellulitis and ulcerative lymphangitis have been reported following skin injury by nematode larvae (Dewes, 1972; Dewes, 1989; Barton and Hughes, 1980; Etherington et al., 1980).
The pulmonary lesions caused by *R.equi* usually take the form of a chronic suppurative bronchopneumonia often with prominent abscesses. The relationship between the development of the intestinal and pulmonary lesions is not fully understood. Many authors consider the intestinal whether clinical or inapparent as usual initiator of the pulmonary abscesses (Bull, 1924; Bain, 1963; Gary Muscatello, 2012; Giguère et al, 2012; Noah Cohen, 2012). It is thought that dissemination of infection from the gut occurs via intestinal lymph nodes to the pulmonary circulation. The distribution of the pulmonary lesions in some naturally-infected foals, which suggests an embolic spread throughout the lungs, has been tendered as supportive evidence for this hypothesis (Bull, 1924; Bain, 1963; Giguère et al, 2012). Barton and Hughes, (1980) suggested that spread of the disease via umbilical route is not an important factor in its pathogenesis, while Martens et al, (1982), suggested that umbilicus is a common route of entry for many pathogens in the neonate. Transmission studies involving umbilical deposition of *R. equi* are not reported to the best of our knowledge. Johnson et al (1983 a, b) described gross and histopathological changes in foals following intrabrochial and intragastric inoculation of *R.equi*. They found that all foals in the intrabronchial group developed a severe bronchopneumonia in the inoculated lung. In 8 to 9 days old lesion, the alveoli were filled with macrophages, neutrophils and multinucleated giant cells and most contained numerous *R. equi*. The few foci of alveolar necrosis were associated with aggregates of bacterial-laden macrophages undergoing degeneration. In addition, there were extensive parenchymal destruction, little fibrous tissue reaction, hyperplastic bronchiolitis, pulmonary edema, perivascular lymphocytic cuffs and a pyogranulomatous lymphadenitis in bronchial lymph nodes. In case of intragastric inoculation, severe ulcerative colitis, typhlitis and lymphadenitis of colonic and cecal nodes were seen.
An aim of the present work was to study the gross and histopathological changes in foals experimentally infected with *R.equi* through different routes (intratracheal, intragastric and navel infections of foals).

Materials and methods

Twenty 2-8 week-old- clinically normal Arabian foals were individually housed with their dams. The foals were randomly divided into three groups as follow:

- Group.1 (intratracheal inoculation)

Foals in this experiment were randomly numbered and placed in either infected or control group. Foals, number 1-4 were given $6.5X10^{10}$CFU *R.equi* in 40 ml PBS intratracheally. Foals

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numbered 5-8 were reared in a separate building acted as control and given 40 ml sterial PBS intratracheally.

- Group .2 (intragastric inoculation)

Foals in the second group were randomly divided in either infected or control group. The infected group (9-12) received 6.5X1010CFU *R.equi* in 75 ml PBS by stomach tube daily for five consecutive days. The stomach tube subsequently flushed with 400 ml of tap water before removing through the nose. While control group (13-16) received 75ml of sterile PBS using stomach tube.

- Group .3 (Navel infection)

Two foals (17-18) from the third group were infected through the umbilicus (navel) during the first few hours after birth by bacterial culture. This was done by contaminating the umbilicus of the newborn foal by *R.equi* culture. The other two foals (19-20) were acted as control foals.

Foals showed clinical signs of *R.equi* infection during the course of observation. At the peak of the clinical disease, foals were euthanized by intravenous injection of 10 ml sodium pentobarbital to perform necropsy. Pieces of the following tissue were collected in 10% neutral- buffered formalin for histopathological examinations: lungs, spleen, liver, kidney, brain, lymph nodes (bronchial, mediastainal and mesenteric) and several sites in the small and large intestine. After fixation tissues were processed routinely for paraffin embedding and 4-6 µm thick sections were cut and stained with hematoxylin and eosin. This study was approved by the research committee/College of veterinary medicine / university of Baghdad.

Results

Gross lesions

The most prominent gross lesions seen in foals of the intratracheally-infected group were the followings: In all foals, the eyes had petechial hemorrhages seen in the conjunctivae of both eyes and there was a slight to moderate increase in the amount of synovial fluid of the hip and knee joints (Figure.1). The fluid was serous in nature. The abdominal cavity had hydroperitoneum with a fluid volume ranging from 80-150ml. The fluid was turbid and slightly thick in consistency (Figure. 2). The chest cavity had straw-colored, watery fluid ranging in amount from 15-25 ml, with some fibrin strands seen in it. In two foals No.3, 4 of this group there was an increased amount of serous fluid in the pericardial sac (Figure.3), with the heart of these two foals showing subepicardial petechial hemorrhages in the left ventricle, together with gelatinous atrophy of the subepicardial adipose tissue seen in all foals (Figure.4). Focal subendocardial hemorrhages mostly seen as ecchymoses in the left ventricle were seen in foal No.4. Both lungs of infected foals had multiple abscesses but severity of the lesion seemed to be more extensive in the right lung. Besides, dorsal surface of both lungs was more involved (Figure.5). The left lung of all foals had diffuse consolidation of the caudal lobe together with variable –sized, raised focal emphysematous areas. Two foals (No.1 and 3) showed greyish hepatization while the other foals showed dark red consolidations together with focal petechial hemorrhages seen throughout the dorsal surface and especially the uppermost part of the caudal lobe of left lung. Multiple nodular grayish areas (suppurative foci) were seen extensively scattered through the pulmonary parenchyma of the ventral surface of left lung in all foals. Cut section of these nodules revealed suppurative exudate which was yellowish to greyish in color and creamy in consistency (Figure.6) in two foals

(No.1 and 3) a well- developed suppurative foci (abscesses) were seen since these foci were walled off by fibrous connective tissue capsule. The abscesses were approximately 2mm in diameter, and embedded in the pulmonary parenchyma. In all foals emphysematous areas were seen adjacent to the consolidation areas. In one foal (No.4), cut section of the left caudal lobe revealed a totally consolidated lung tissue with tissue with wet foci from which too much exudate was oozing.

The right lung was extensively involved. In foal (No.4), the cranial lobe had almost two thirds of its surface area being involved, while the caudal lobe had almost one-half of it being consolidated. The lesions seen in the right lung of two foals (2and 4) were in the form of greyish areas of consolidation, ranging in diameter from 2-8 mm (Figure.7). Also there were dark red, firms, nodular lesion. The cut section of the right caudal lobe revealed foci of consolidation, with meaty to firm consistency dark red color and were well-delineated by a zone of congestion. Occasionally, focal areas of emphysema and atelectasis were also seen. The right lungs of two foals (No.1 and 3) have large foci of atelectasis surrounded by multi focal emphysemateous areas. In addition to the previous lesion, two foals (No.1, and No. 3) had bronchiectasis with the reason for widening was the large amount of suppurative exudate in the airways. In all foals subpleural emphysematous areas were seen on the dorsal aspect of the caudal lobes and some areas of congestion were present. Tracheal mucosa was slightly congested with little amount of mucopurulent exudate seen in the lumen of trachea was severely congested and contained large amount of mucopurulent exudate. Lymph nodes were enlarged in all foals. In three foals (1, 2 and 4) the bronchial, mediastinal and mesenteric lymph nodes were so enlarged, edematous and have ecchymoses in its cortex. Besides, there was no clear demarcation between cortex and medulla. In one foal (No.3) there was suppurative lymphadenitis as evidenced thick greyish to yellow creamy pus (Figure. 8). Mesenteric blood vessels were engorged in all foals and in one foal (1) whitish to grey, nodular to elongated elevations varying in size from 0.3-2.5 cm were seen along the caecum, colon, rectum and mesentery.

Other gross pathological lesions were: congested liver rounded edges and focal areas of whitish to yellowish discoloration in two foals (2 and 4). Kidney was occasionally slightly congested. Adrenal gland had hemorrhages with multifocal greyish areas scattered through its cortex and medulla. The cut section of the foci caused oozing of blood-stained serous fluid. Spleen was slightly enlarged and congested.

All infected foals in the intragastric inoculation appeared in a poor nutritional status, severely emaciated with eyes had bilateral mucopurulent discharge. There was severe hyperemia and petechiation of the conjunctivae. Evidence of dehydration and diarrhea was seen in all foals. There was a serious atrophy of the subcutaneous adipose tissue with severe engorgement of the subcutaneous vessels. All foals showed also the same gross pathological lesions that appeared in the foals of the intratracheal group in addition to attachment of the large intestine and severe engorgement of mesenteric blood vessels (Figure.9). Mesenteric lymph nodes were edematous and approximately four times the normal size. The cecal and colonic lymph nodes contained circular foci of caseous necrosis and some lymph nodes filled with yellowish, thick, creamy pus.Some foals showed 4mm in diameter whitish grey focal area (micro abscesses) on the surface of the liver.

All foals in navel infected group were emaciated with congested mucous membranes and engorged conjunctival capillaries. There was a pale yellowish nasal discharge. Foals also showed hydroperitoneum, congested liver, enlargement of mesenteric lymph nodes,

hydrothorax , hydropericardium and flappy heart . Both lungs showed well-delineated variable sized, multiple abscesses distributed throughout lung parenchyma in addition to other lesions that appeared on the lungs, bronchia intestine, liver and lymph nodes.

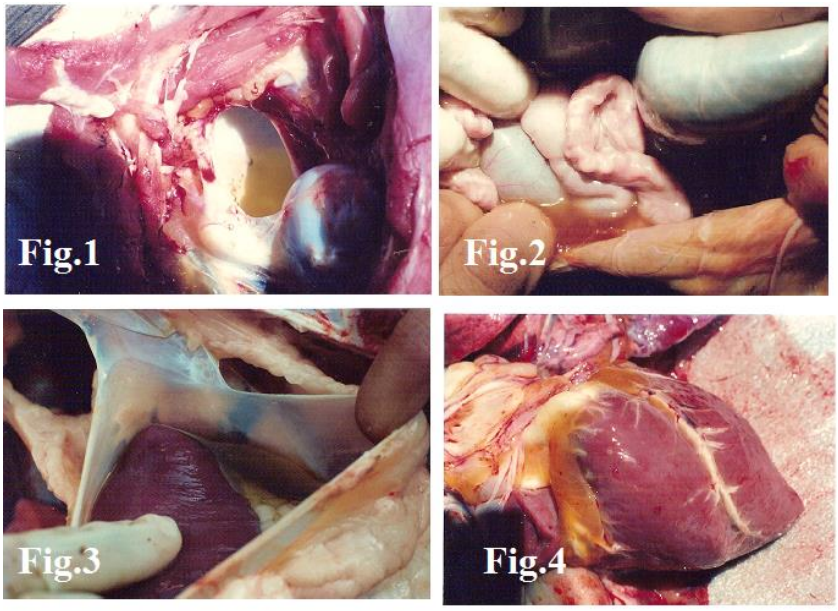

Figure.1: Shows the increase in the amount of synovial fluid of the hip joint.
Figure.2: shows the hydroperitoneum with a fluid volume ranging from 80-150ml.
Figure.3 shows the hydropericardium
Figure.4: Shows subepicardial petechial hemorrhages in the left ventricle, together with gelatinous atrophy of the subepicardial adipose tissue.

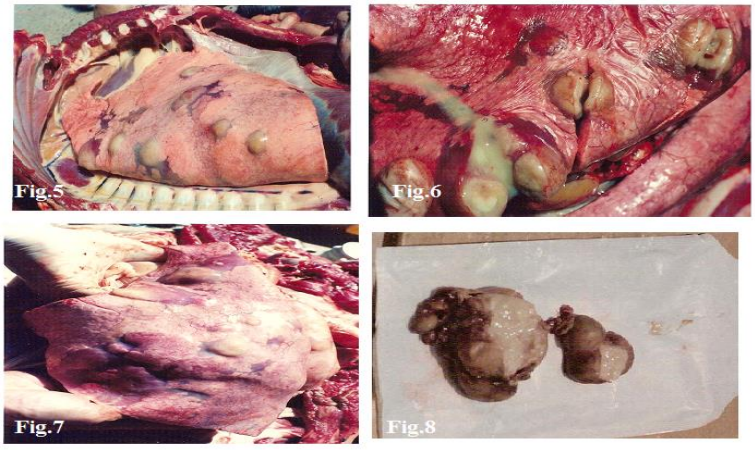

Figure.5: Shows multiple abscesses distributed on the surface of the lungs

Figure.6: shows the cut section of the nodules that revealed suppurative exudate which was yellowish to greyish in color and creamy in consistency
Figure.7: Shows greyish areas of consolidation, focal areas of emphysema and atelectasis
Figure.8: Shows suppurative adenitis that contained thick greyish to yellowish creamy pus

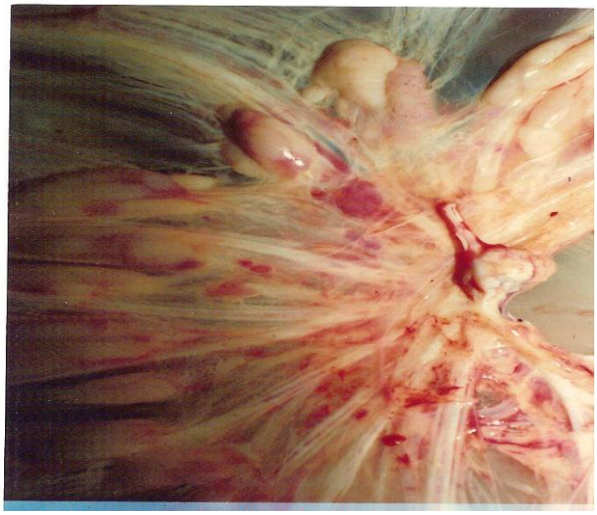

Figure.9: Shows severe engorgement of mesenteric blood vessels in addition to enlargement of mesenteric lymph nodes.

Microscopical findings

The microscopic appearance of lung lesions was similar in animals of the experimental group with slight differences. The most salient features of the lesions were briefly as follows: Acute suppurative bronchopneumonia was diagnosed and characterized by cellular filling of the alveolar spaces. The inflammatory cells present were PMNS and MN-cell type. Occasionally minimal amount of proteinaceous seepage was seen in the alveolar lumen together with some fibrinous networks (Figure. 10) .Lesion of the airways was characterized by degenerative changes of its mucosa with loss of cilia, focal loss of epithelial lining, migration of acute inflammatory cells toward the lumen and presence of suppurative exudate in their lumen. Multiple, variable- sized abscesses were seen in various stages of development but usually with no complete fibrous tissue encapsulation but definitely a starting to well-developed fibroplasia was seen adjacent to a pool of necrotic and living inflammatory cells (PMNS) (Figure.11). Morphological diagnosis of the more advanced lesion was chronic suppurative granulomatous pneumonia ¨pyogranulmatous response¨ (Figure.12 A&B), which was characterized by a diffuse mononuclear, polymorphonuclear cellular infiltration predominantly neutrophils and histiocytes together with lymphocytes, plasma cells and fibroblasts. Numerous multinucleated giant cell formations were also seen, these giant cells were variable in size and the number of their nuclei, associated with these lesions was focal areas of necrosis of the pulmonary parenchyma with numerous dots of phagocytized bacteria seen free or within cytoplasm of many inflammatory cells as basophilic granular structures (Figure.13&14). Also there were multifocal areas of emphysema and atelectasis and acute pulmonary congestion characterized by severe dilatation of the alveolar capillaries and occasionally frank hemorrhages were seen. Focal interstitial thickening of the alveolar wall,

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Pleura and interlobular septae thickening were seen due to cellular infiltrations, exudation and congested blood vessel.

Small intestine of all foals revealed acute necrotizing enteritis with necrotic eosinophilic masses, congestion and edema of the submucosa of ileum together with mild to moderate cellular infiltration predominantly eosinophils and lymphocytes. There was focal soughing of the superfacial epithelium of the gastric mucosa with slight MN type cells infiltration including macrophages, plasma cells and few lymphocytes were seen in the lamina proparia. Submucosa had edema and numerous MN cells mainly macrophages but with some eosinophils. Adrenal glands showed vacuolation of cells of the zona reticularis. The cells were swollen and had fine vacuolation together with degenerative changes of their nuclei (karyorrhexis). Spleen showed congestion and focal mild hemosiderosis. Kidneys showed acute mild tubular degeneration. Liver had focal MN cellular infiltrations in the portal areas and in the lobular parenchyma, in addition to acute hepatocellular degeneration with eosinophilic infiltrations (chronic necrotizing hepatitis).

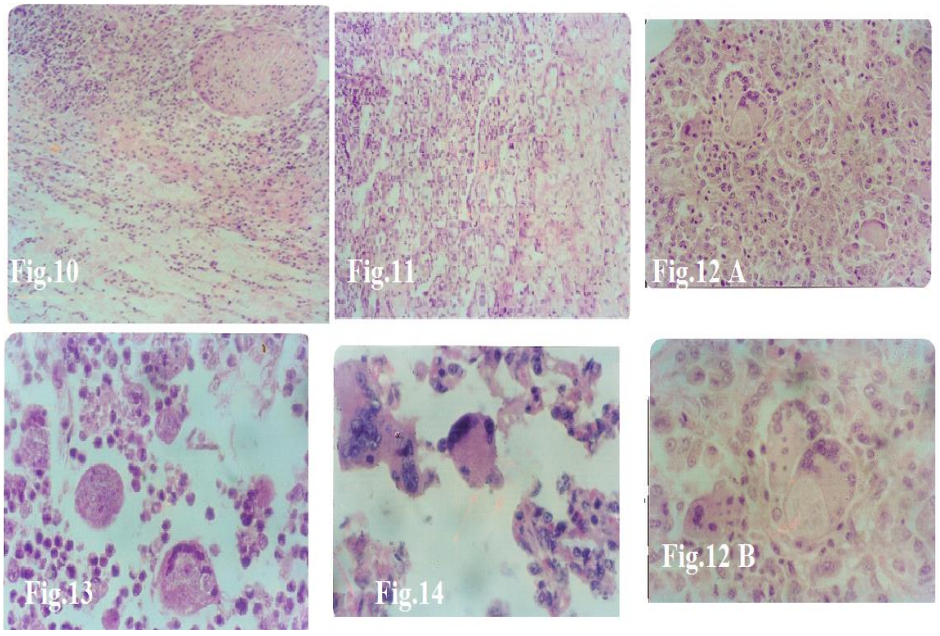

Figure.10: Shows lung of an intratracheal infected foal. Cellular filling of the alveolar space (PMNs and MN-cell type) with minimal amount of proteinaceous seepage together with an intravascular fibrinous network (H&E X100)

Figure.11: lung section shows suppurative exudate (right side) was developed fibroplasia. (H&E X100)

Figure.12: shows chronic suppurative granulomatous pneumonia ،،pyogranulmatous response،، (A.H&E X200, B. H&E X400)

Figure.13: Lung of a foal from intratracheal inoculation group.Numerous dots of phagocytized bacteria seen within cytoplasm of histiocyte. (H&E X400).

Figure.14. shows multinucleated giant cells.

Discussion

The pneumonia associated with *R. equi* infection in the foal is described most commonly as a suppurative bronchopneumonia with prominent abscess formation (Bain, 1963; Barton and hughes 1980; Barton and hughes 1984; Elissalde et al 1980;Meijer and Prescott, 2004; Özsoy, and Haziroglu, 2009). In this study all foals in the intratracheal group developed severe and extensive suppurative bronchopneumonia with large areas of caseous necrosis. The encountered lesions resembled those of natural infection of foal in which the disease could run a more fulminating course than usual, besides, this result is in agreement with previous experimental work (Johnson et al, 1983 a; Perkins,et al 2001: Jacks, et al 2007). The high concentration of bacteria in the inoculum and the manner of deposition (directly into the lung) no doubt contributed to the extensive tissue destruction. Histologically, lung lesions were characterized by massive cellular infiltration into the alveolar space. The majority of these cells were large macrophages, most probably representing macrophages both from resident proliferating alveolar macrophages and from inflammatory monocytes recruited from the circulating blood.

Multinucleated giant cells were a prominent component of the cellular exudate, even in the early lesions. These polykaryons were thought to be inflammatory cells of monocyte-macrophage lineage rather than syncytial giant cells of epithelial origin. One of the mechanisms of formation of inflammatory polykaryons is simultaneous endocytosis, where by several macrophages attempting to phagocytize the same particles and fuse in the process (Chambers, 1977). Inflammatory polykaryons once formed have a low phagocytic capability (Chambers,1978). Since most of the multinucleated giant cells in the *R. equi* experimental lesions (including our findings), had large phagocytic vacuoles containing numerous bacteria, it is likely that simultaneous endocytosis was the mechanism for their formation.

The presence of giant cells, as well as the predominance of macrophages among, the other things has established the lesions of *R.equi* as granulomatous in nature (Sippel et al, 1968; Reuss, et al 2009). Microscopic intestinal lesions typical of *R. equi* infection were present in all foals, intestinal lesions often accompanied pulmonary ones in the naturally- occurring diseases although the foal may not develop diarrhea (Hutchins et al, 1980). It has been suggested that the intestinal lesions develop from swallowing infectious expectorate. The naturally occurring *R. equi* entercolitis, in which there is no accompanying pneumonia (Bull, 1924; Sippel, 1968; Cimprich and Rooney 1977). In this study we produced typical intestinal lesions, in foals given the organism on five consecutive days. A single dose of the organism was not effective, emphasizing the dose dependence of lesion generation. The interval between infection and development of enteric lesions was approximately three weeks.

A single dose of organism was insufficient to alter the balance of the normal flora or to breach the intestinal epithelium in sufficient numbers to cause gross lesions, although microscopic lesions were seen in foals after infection. During the period of establishment of *R. equi* in the normal foal intestinal tract, transient microscopic and sometimes macroscopic lesions may develop in the mucosa and in the draining lymph nodes (Hutchins, et al, 1980: Jacks, et al 2007). Such macroscopic lesions are occasionally described as incidental necropsy findings in foals which die of other causes (Mahaffey, 1962). Whether foals develop severe lesions and the associated clinical sign (diarrhea) probably depends upon the size of the bacterial challenge and individual foal factors, including the animal's immunological status (Woolcock et al., 1980; Perkins, et al 2001: Jacks, et al 2007). The gross lesions in experimental foals were typical of those seen in naturally- infected foals described in the literature. The earliest microscopic lesion in the intestinal tract of the experimental foals indicated that *R.equi* penetrates the specialized epithelium lying over the payer's patches. This route of entry has

been demonstrated for other enteric pathogens, such as Salmonella (Carter and Collins, 1974), and would explain the predilection of the *R. equi* lesion for the gut-associated lymphoid tissue. Within the payer's patches, failure of the phagocytes to eliminate the organism generates the typical pyogranulomatous inflammatory response described in the natural disease (Elissalde et al, 1980; Falcon, et al 1985; Zink, et al 1986; Takai, et al 2000; Noah, 2012). Villous atrophy has been described in the small intestine of both acute and chronic cases of naturally-occurring *R.equi* enteritis (Cimprich and Rooney, 1977). Microcopic lesions in the mesenteric lymph nodes from the natural disease were also pyogranulomatous and usually so extensive that the architecture of the node is obliterated. Where the structure of the node has not been destroyed, the microscopic picture suggests that viable *R. equi* are transported to the node within phagocytic cells and are arrested in the interfollicular cortex, where the earliest pyogranulomatous lesions occurred.

Foals in the navel- infected group showed both pulmonary and enteric lesions. The umbilicus is a common route of entry for many pathogens in the neonate. However, transmission studies involving umbilical deposition of *R. equi* have not been reported (Martens et al, 1982). The development of pulmonary and enteric lesions in the navel- infected foal in this study means that this route is an important one in the development of the disease. Comparisons have been done between the microscopic lesions of *R. equi* and human mycobacterial infections, particularly lepromatous leprosy (Sippel et al 1968; Elissalde et al , 1980; Gansert et al 2003). *R. equi* furthermore, causes a lymphoadenitis in cattle and pigs which is indistinguishable grossly and microscopically from tuberculosis (Densen and Mandell 1980). The presence of mycolic acids in the cell wall of *R. equi*, Mycobacterium , and Nocardia spp (Barton and Hughes ,1980; Prescott,1991; Gansert et al 2003) may account for the similarity of tissue response which is typical of a facultative intracellular parasite. Facultative intracellular parasites are characterized by their ability to survive and to multiply within a phagocytic cell by evading the usual destruction that follows upon ingestion (Densen and Mandell 1980; Jacks, et al 2007). The suggestion has been made that *R. equi* behaves in such a manner (Hutchins et al, 1980). In the experimentally induced lesions, the bacteria are strongly cell associated. Indeed that's what we encountered, since few bacteria appeared to be multiplying freely in the alveolar space or in the necrotic material and many of them were intracellular. The bacteria retain their Gram-positive staining characteristics within the phagocytic vacuoles indicating that the cell wall is undamaged. Furthermore, the earliest indication of necrosis in the lung parenchyma in the experimentally-induced lesions was associated with groups of degenerate macrophages which primarily contained many bacteria. It is likely that the bacteria were responsible for the degeneration of the phagocytes. The histopathology of advanced natural and experimental *R. equi* lung lesions showed caseous necrosis as the predominant lesion. The tissue destruction may be due to either to bacterial, toxins, products of the inflammatory response, or to a combination of both factors. One potential *R. equi* toxin is the phospholipase C which could acts synergistically with a product of *C. pseudotuberculosis* to lyse sheep erythrocytes (Bernheimer et al, 1980; Prescott, 1991). It is doubtful, however, that this toxin is important in vivo, lysosomal enzyme, chiefly acid hydrolases, which have the capacity to degrade a wide range of natural tissue substrates probably are responsible for much of the tissue destruction in *R.equi* lesions. The enzymes can diffuse into the tissue destruction in *R.equi* lesions. The enzymes can diffuse into the tissue, not only from disintegrating macrophages and neutrophils but also from live cells engaged in phagocytosis.

In conclusion, this study approved that experimental infection of foals by *R. equi* ,via intratracheal, intragastric and navel infection lead to develop Pulmonary and intestinal lesions in foals of all infected groups.

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