



RESEARCH ARTICLE

**Potency of *Rhodococcus equi* culture filtrate supernatant proteins antigen for skin test in the diagnosis of *Rhodococcus equi* in foals**

Kareema Al-Salihi

**Özet**

**Al-Salihi K.** Taylarda *Rhodococcus equi* tanısı deri testinde *Rhodococcus equi* kültür filtrat supernatant protein antijeninin değerlendirilmesi. **Eurasian J Vet Sci, 2011, 27, 3, 161-165**

**Amaç:** Araştırma *Rhodococcus equi* protein antijenlerinin deri testinin geliştirilmesi için dizayn edildi.

**Gereç ve Yöntem:** *R. equi* ile enfekte 10 adet Arap atı tayına 0.1 mL (12.5 µg/mL) *Rhodococcus antiji* ve 10 adet sağlıklı kontrol grubuna 0.1 mL PBS bacak bölgesine deri içi yolla enjekte edildi. Deri kalınlıkları uygulama öncesi ve sonrası 24, 48 ve 72. saatlerde ölçüldü. Deri biyopsisi uygulama sonrasında 72. saatte yapıldı.

**Bulgular:** Deri reaksiyonları uygulama sonrası 24. saatte hafif, 72. saatte ise yoğun gözlemlendi. Yapılan biyopsilerde makrofaj, nötrofil ve eozinofil gibi yangı hücreleri belirlendi. Kontrol grubu taylarda uygulama sonrası gözle görülen bir reaksiyon gözlenmedi.

**Öneri:** *Rhodococcus*-kültür antijeninin, *R. equi*'ye maruz kalan veya enfekte olan taylarda hastalığın tanısında diagnostik öneminin olabileceği belirlendi. Mevcut araştırmanın sonuçları dikkate alındığında, *R. equi* ile enfekte haralarda deri testinin hastalığın tanısında umut vaat etmektedir.

**Abstract**

**Al-Salihi K.** Potency of *Rhodococcus equi* culture filtrate supernatant proteins antigen for skin test in the diagnosis of *Rhodococcus equi* in foals. **Eurasian J Vet Sci, 2011, 27, 3, 161-165**

**Aim:** This study was designed to developing skin test proteins antigen prepared from *Rhodococcus equi* isolates.

**Materials and Methods:** Ten Arabian foals experimentally infected with *R. equi* were injected intradermally with 0.1 mL (12.5 µg/mL) *Rhodococcus antigen* and 0.1 mL PBS as control each treatment at one side of neck region, while 10 healthy foals had the same treatment and used as control group. Skin thickness was measured prior and at 24, 48 and 72 hours post injection by skin caliber. Skin biopsy was taken from the site of reaction at 72 hours post injection.

**Results:** All infected foals had slightly skin reaction at 24 hours, and maximum skin reaction was determined at 72 hours post inoculation. Inflammatory cells including macrophages, neutrophils and eosinophils were present in the sections prepared from biopsy obtained from positive skin reaction. All control foals revealed no visible reaction in the site of injection.

**Conclusion:** Partially purified *Rhodococcus*-culture antigen is potential diagnostic antigens able to detect foals previously infected or exposed to *R. equi* infection. Our data provide a promising basis for the future development of screening diagnostic skin tests for *R. equi* infection in infected farms.

Department of Veterinary Medicine and Therapeutic, College of Veterinary Medicine, University of Baghdad, Iraq

Received: 30.04.2011, Accepted: 03.06.2011

\*elsalihi@yahoo.com

Anahtar kelimeler: *Rhodococcus equi*, tay, deri testi

Keywords: *Rhodococcus equi*, foal, skin test

## ► Introduction

*Rhodococcus equi* is a facultative intracellular bacterium with properties similar to those of *Mycobacterium tuberculosis*, a closely related pathogen. Both *R. equi* and *M. tuberculosis* are nocardioform actinomycetes, survive within the phagosomes of macrophages, have characteristic cell walls containing unique lipids, lipoproteins, and glycolipids, and produce pyogranulomatous lesions in the lung (Gansert et al 2003). Whereas *M. tuberculosis* produces tuberculosis in humans, *R. equi* is a common cause of life-threatening pneumonia in young horses. *R. equi* is also an opportunistic pathogen in AIDS patients (Macias et al 1997). *R. equi* is ubiquitous in the equine environment, and virtually all horses are considered exposed, likely in the first few weeks of life. Horses have a distinct age-associated susceptibility, such that equine rhodococcal pneumonia is almost exclusively a disease of foals less than 4 to 5 months of age. Even on farms where infection is endemic, a situation where a significant percentage of foals develop pneumonia and the environmental load of virulent *R. equi* is high, older horses do not manifest with disease. Previous research has provided strong evidence that the resistance of adult horses to *R. equi* challenge reflects acquired immunity and their ability to mount effective recall responses (Hines et al 2001, Hines et al 2003).

Immune adult horses, like humans who have successfully contained *M. tuberculosis* infection, provide a valuable system for understanding the mechanisms by which infection can be controlled and pneumonia prevented. Knowledge of these mechanisms will likely be critical to efforts to design an effective diagnostic skin test antigen like tuberculin and vaccine. Rhodococcal pneumonia is an immunopathologic disease. Adoptive transfer and neutralizing antibody studies have shown that mice that develop Th2 responses, characterized by production of interleukin-4 (IL-4) rather than gamma interferon (IFN- $\gamma$ ), develop characteristic pulmonary lesions (Kanaly et al 1995). In contrast, mice responding to infection with a Th1 response and IFN- $\gamma$  production clear a virulent challenge. As with *M. tuberculosis*, CD8<sup>+</sup> T lymphocytes can also contribute to immune clearance of *R. equi* (Nordmann et al 1992). Even transgenic mice that lack major histocompatibility complex (MHC) class II-restricted CD4<sup>+</sup> T lymphocytes significantly reduced *R. equi* numbers following pulmonary challenge (Kanaly et al 1993).

Over the past 100 years several attempts have been made to develop a skin test antigen (delayed type hypersensitivity reaction DTH) which is sensitive and specific for infectious agent like tuberculin. Tuberculin skin test used to diagnosis of tuberculosis infection. The reaction was discovered in 1882 by Robert Koch, but it wasn't until the 1940s that Landsteiner and Chase proved that the reaction was mediated by the cellular and not the humoral arm of the immune

system. The first described used only the tuberculin antigen (tuberculin reaction), but the definition was later expanded to include cell mediated reactions to other bacterial and viral antigens, responses to pure protein with adjuvant or haptens, and host responses to allograft. The DTH skin test is used to test if prior exposure to an antigen has occurred. When small quantities of antigen are injected dermally, a hallmark response is elicited which includes induration, swelling and monocyctic infiltration into the site of the lesion within 24 to 72 hours. This reaction has been shown to be absolutely dependent on the presence of memory T cells. Both the CD4<sup>+</sup> and CD8<sup>+</sup> fractions of cells have been shown to modulate a response (Black 1999).

Prevention and early detection are the best tools to reducing the incidence and severity of *R. equi* infections in foals. The clinical observation and even serological (AGID and ELISA) are not reliable indicators for detecting *R. equi* infected foals and the only way to definitively diagnose the disease is through culture and cytology which requires approximately two weeks (Giguere et al 2003).

In light of the pressing need for a diagnostic test to evaluate the incidence of *R. equi* infections in foals, this study was designed to developing skin test proteins antigen prepared from three *R. equi* isolates.

## ► Materials and Methods

### • Antigen preparation

Three *R. equi* isolates obtained from a field cases of *R. equi* foal pneumonia, were used in this study. These isolates were previously classified as *R. equi* by biochemical tests and standard identification methods. Three to five colonies from the bacterial isolates on selective medium NANAT were inoculated into trypticase soy broth inoculated for 72 hours at 37 °C for 72 hours. After washing 3 times with PBS, the cultures were harvested by centrifugation at 10.000 Xg for 30 minutes. The sediment was sonicated for 20 minutes in water cooled sonic oscillator at 9 KHz/sec full power. The homogenate was centrifuged twice at 15.000 Xg for 15 minutes each time to remove cellular debris. The supernatants were concentrated by dialysis with sucrose using Spectra/Por 12.000 to 14.000 molecular weight cutoff (MWCO) dialysis tubing (Spectrum, Houston, Tex.). Subsequently, the concentrated supernatants were dialyzed against PBS using a Slide-A-Lyzer (10.000 MWCO, Pierce) and then concentrated using a 10.000 MWCO column (Centriprep 10, Millipore, Bedford, Mass) according to the manufacturer's instructions. Protein concentrations of all antigen preparations were quantitated using the Micro-BCA Protein assay with an albumin standard according to the manufacturer's instructions (Pierce). The prepared antigen were lyophilized and kept until used in -20 °C.

### • Animals

Twenty clinically normal foals (1 day to 8 week-old) were divided into 4 groups. Group 1 with 4 foals was inoculated with *R. equi* ( $6.5 \times 10^{10}$ ) CFU in 40 mL PBS by intratracheal route. Group 2 with 4 foals was inoculated by intragastric rout using ( $6.5 \times 10^{10}$ ) CFU in 75 mL PBS for 5 consecutive days. Group 3 with 2 foals was inoculated via the umbilicus using bacterial plate and contaminated the navel directly at the time of delivery. Group 4 with 10 foals used as control group. This study was approved by the Ethical and Research Committee of the University of Baghdad.

### • Skin test

On the fourth week after experimental infection, all infected and control foals were injected intradermally with 0.1 mL PBS (as control treatment) and *R. equi* antigen (12.5 µg/mL) in the neck region. Skin thickness was measured prior and at 24, 48 and 72 hours post injection by skin caliber. Skin biopsy was taken from the site of reaction at 72 hours post injection.



Figure 1. Shows positive skin test after 72 hours of intradermal injection with *R. equi* antigen.

### ► Results

All control foals revealed limited negative swelling reaction to *R. equi* antigen at the site of injection with an increase of no more than 2 mm and without clinical signs. Infected foals revealed slightly skin reactivity test at 24 hours post injection. At 48 hours the skin of injected area revealed a positive reaction, which was characterized both indurations (0.4 mm) of the skin and surrounding erythema. At 78 hours the reaction became more advance, (0.69 mm) in addition to diffuse extensive oedema, exudation and pain at the site of injection (Figure 1, Table 1). Skin biopsy from the site of reaction revealed multifocal areas of polymorphonuclear leukocytes (PMNs) predominately neutrophilic granulocytes mixed with few prominent

eosinophils. The centers of these foci were made up of necrotic dermal tissue with free RBCs. Among these cellular infiltrations there were foci of degenerate and necrotic PMNs with stretched basophilia of their nuclear chromatin. Other cellular infiltration included some lymphocyte and macrophage. Similar cell types mostly PMNS were seen around hair follicles but mostly at medium size arteriol. There is evidence of congestion as indicated by the presence of dilated blood vessels. Focal microhemorrhages were also seen (Figure 2).

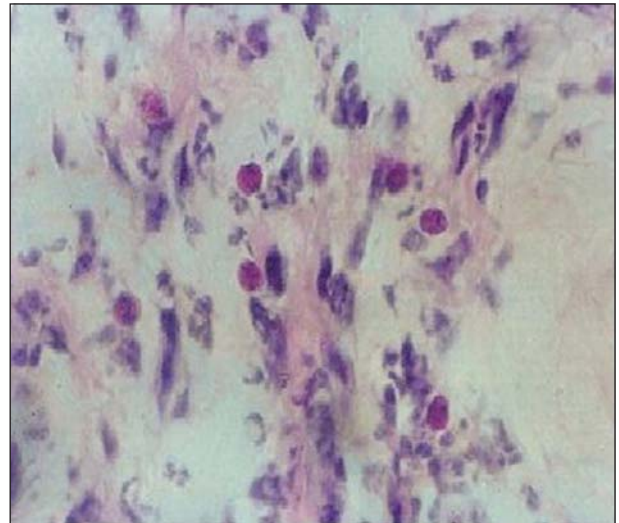


Figure 2. Shows histopathological changes of skin biopsy after 72 hours of intradermal injection with *R. equi* antigen (H&E X400).

### ► Discussion

Delayed type hypersensitivity reaction (DTH) has been a useful diagnostic and epidemiological tool for several infectious agents like tuberculosis and glanders for many years.

*R. equi*, a gram-positive facultative intracellular pathogen, is the most devastating cause of pneumonia in foals between 3 weeks and 5 months of age. Other less common clinical manifestations of *R. equi* infections in foals include ulcerative enterocolitis, colonic or mesenteric lymphadenopathy, immune-mediated synovitis and uveitis, osteomyelitis, and septic arthritis (Giguere and Prescott 1997). Early recognition of foals with *R. equi* pneumonia prior to development of clinical signs would likely reduce losses and limit the costs associated with long-term therapy of severely affected animals.

*R. equi* pneumonia of foals has traditionally been diagnosed based on culture or PCR amplification of the microorganism from a tracheobronchial aspirate. However, obtaining a tracheobronchial aspirate is not recommended for foals that present with severe respiratory distress, and the technique is not practical for routine diagnosis on large farms where the disease is enzootic and several foals must be sampled. There is

Table 1. Shows the thickness of skin reaction in foals before, and at 48 and 72 hrs after injection with *R. equi* antigen. A. Intratracheal group, B. Intra gastric group, C. Navel infected group.

Foal groups	No of foals	Before test/mm	After 48 hr/mm	After 72 hr/mm
<b>A- Intratracheal group</b>				
Infected foals	4	0.15 (0.11-0.17)	0.42 (0.35-0.47)	0.55 (0.50-0.60)
Control foals	4	0.15 (0.12-0.16)	0.18 (0.17-0.19)	0.15 (0.12-0.16)
<b>B- Intra gastric group</b>				
Infected foals	4	0.10 (0.12-0.15)	0.50 (0.35-0.54)	0.67 (0.50-0.69)
Control foals	4	0.12 (0.10-0.13)	0.19 (0.17-0.18)	0.12 (0.10-0.13)
<b>C- Navel infected foals</b>				
Infected foals	2	0.12 (0.10-0.13)	0.40 (0.35-0.43)	0.54 (0.50-0.55)
Control foals	2	0.11 (0.10-0.12)	0.17 (0.15-0.19)	0.14 (0.11-0.17)

therefore a need for a less invasive and more practical means of diagnosis. Although several assays have been developed to detecting antibody to *R. equi* and are commonly used by practicing veterinarians for the diagnosis of *R. equi* infections, the diagnostic value of these assays has never been assessed in a clinical setting of heavy natural challenge. In the present study, partially purified *Rhodococcus*-culture filtrate supernatant proteins was prepared and injected intradermally as skin test in clinically healthy foals as control group and experimental infected foals with *R. equi*. While all foals in control group revealed negative result, all infected foals revealed positive skin reaction, the reaction characterized by increased in the thickness of skin, pain and inflammation of injected area. The result of this skin test used in this study is similar to the tuberculin reaction when the classic form of DTH is induced by injecting an antigen preparation of *M. tuberculosis* intradermally. If the host has been previously exposed to the bacterium, swelling and induration will result. The histopathological features of biopsy prepared from the site of the positive reaction revealed multifocal areas of polymorphonuclear leukocytes (PMNs) predominately neutrophilic granulocytes mixed with few prominent eosinophils. The centers of these foci were made up of necrotic dermal tissue with free RBCs, this histopathological features is compatible with the features appeared in tuberculin test reported previously. Approximately 4 hours after injection of antigen, neutrophils rapidly accumulate around the post-capillary venules at the injection site. The neutrophil infiltrate rapidly subsides and by about 12 hours the injection site becomes infiltrated with T cells and blood monocytes and some basophils, also organized in perivenular distribution. The endothelial cells lining these venules swell, show increased biosynthetic organelles and become leaky

to plasma macromolecules. Fibrinogen escapes from the blood vessels into the surrounding tissues, where it is converted into fibrin. The deposition of fibrin and a lesser extent accumulation of T cells and monocytes within the extravascular tissue space around the injection site cause the tissue to swell and become indurate. Indurations, the hallmark of DTH, are usually detectable by about 18 hours and maximal by 24 hours in mice, 48 hours in guinea pigs, and 48-72 hours in humans and bovine. The inflammation then subsides. In all species, this reaction is mediated by a mix of CD<sup>4+</sup> and CD<sup>8+</sup> T cells. This may partly be a result of the fact that *Mycobacteria* have both an intracellular and extracellular replication phase (Dannenberg 1991).

The result of skin test in our study is in agreement with previous result showed that cell-mediated immunity plays an important role in *R. equi* infection. In addition the result in agreement with the fact that *R. equi* shares the lipid-rich cell wall envelope characteristic of the mycolata, including *M. tuberculosis*, as well as the ability of pathogenic members of this group to survive within macrophages. The positive result of skin test in this study is in agreement also with result of others (Wilson 1955, Ellenberger et al 1984). Wilson (1955) observed positive reaction at 18-24 hours post injection using a filtrate of a killed culture of organism isolated from natural cases. He concluded that there was an association between a positive skin test in a mare and infection in her foal. Other researcher (Ellenberger et al 1984) used skin test for quantitative measurement of delayed-type hypersensitivity reaction develops in experimentally infected animals. They found that positive test reaction occurred at 48 to 72 hours after intradermal injection with *R. equi* extracts.

## ► Conclusions

We conclude that partially purified *Rhodococcus*-culture antigen is a potential diagnostic antigen able to detect foals previously infected or exposed to *R. equi* infection. We demonstrate for the first time the utility of partially purified *Rhodococcus*-culture antigen as promising skin test antigens for *R. equi* pneumonia in foals. Our data provide a promising basis for the future development of skin tests for *R. equi* infection in infected farms.

## ► References

- Black CA, 1999. Delayed type hypersensitivity: Current theories with an historic perspective. *Dermatology Online Journal*, 5, 7.
- Dannenberg JAM, 1991. Delayed-type hypersensitivity and cell-mediated immunity in the pathogenesis of tuberculosis. *Immunol Today*, 12, 228-33.
- Ellenberger MA, Kaeberle ML, Roth JA, 1984. Equine cell-mediated immune response to *Rhodococcus* (*Corynebacterium*) *equi*. *Am J Vet Res*, 45, 2424
- Gansert JL, Kiessler V, Engele M, Wittke F, Rollinghoff M, Krensky AM, Porcelli SA, Modlin RL, Stenger S, 2003. Human NKT cells express granulysin and exhibit antimycobacterial activity. *J Immunol*, 170, 3154-3161.
- Giguere S, Prescott JF, 1997. Clinical manifestations, diagnosis, treatment, and prevention of *Rhodococcus equi* infections in foals. *Vet Microbiol*, 56, 313-334.
- Hines MT, Paasch KM, Alperin DC, Palmer GH, Westhoff NC, Hines SA, 2001. Immunity to *Rhodococcus equi*: antigen-specific recall responses in the lungs of adult horses. *Vet Immunol Immunopathol*, 79, 101-114.
- Hines SA, Stone DM, Hines MT, Alperin DC, Knowles DP, Norton LK, Hamilton MJ, Davis WC, McGuire TC, 2003. Clearance of virulent but not avirulent *Rhodococcus equi* from the lungs of adult horses is associated with intracytoplasmic gamma interferon production by CD4 and CD8 T lymphocytes. *Clin Diagn Lab Immunol*, 10, 208-215.
- Kanally ST, Hines SA, Palmer GH, 1995. Cytokine modulation alters pulmonary clearance of *Rhodococcus equi* and development of granulomatous pneumonia. *Infect Immun*, 63, 3037-3041.
- Kanally ST, Hines SA, Palmer GH, 1993. Failure of pulmonary clearance of *Rhodococcus equi* infection in CD4+ T-lymphocyte-deficient transgenic mice. *Infect Immun*, 61, 4929-4932.
- Macias J, Pineda JA, Borderas F, Gallardo FA, Delgado J, Leal M, Sanchez-Quijano A, Lissen E, 1997. *Rhodococcus* or mycobacterium? An example of misdiagnosis in HIV infection. *AIDS*, 11, 253-254.
- Nordmann P, Ronco E, Nauciel C, 1992. Role of T-lymphocyte subsets in *Rhodococcus equi* infection. *Infect Immun*, 60, 2748-2752.
- Giguere S, Hernandez J, Gaskin J, Prescott JF, Takai S, Miller C, 2003. Performance of five serological assays for diagnosis of *Rhodococcus equi* pneumonia in foals. *Clin Diagn Lab Immunol*, 10, 241-245.
- Wilson MM, 1955. A study of *Corynebacterium equi* infection in a study of Thoroughbred horses in Victoria. *Aust Vet J*, 31, 175-181.