Pathogenicity of *Rhodococcus equi* in mice, isolated from environment, human and horse clinical samples[1]

Mateus M. Costa[2], Sérgio A. Machado[3], Cristina C. Krewer[2], Márcia R.S. Ilha[4], Dominguita L. Graça[5], Ana L.M. Guaraldi[6] and Agueda C. Vargas[7*]

ABSTRACT.- Costa M.M., Machado S.A., Krewer C.C., Ilha M.R.S., Graça D.L., Guaraldi A.L.M. & Vargas A.C. 2006. **Pathogenicity of *Rhodococcus equi* in mice, isolated from environment, human and horse clinical samples**. *Pesquisa Veterinária Brasileira 26(3):167-170*. Laboratório de Bacteriologia, Departamento de Medicina Veterinária Preventiva, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Avenida Roraima 1000, Santa Maria, RS 97105-900, Brazil. E-mail: agueda@ccr.ufsm.br

Rhodococcus equi is a facultative intracellular pathogen associated with bronchopneumonia, mesenteric lymphadenitis and enterocolitis in foals. Although *R. equi* is likely to be found in every horse-breeding farm, the clinical disease is unrecognized in most of them. Capsule components, *equi* factor, micolic acid and some products encoded by the large 85-90Kb plasmid were described as virulence factors. However, the pathogenesis of *R. equi* infections and the sensibility of foals are not completely understood. The aim of this study was evaluate the virulence of R. equi isolated from human, horses and environment for mices. Nine strains carrying the 85-90Kb plasmid isolated from foal clinical specimens, one from immunodeficient human patient and six plasmidless strains (four isolated from feces, one from pasture and one from immunodeficient human patient) were inoculated in cyclophosphamide immunossuppressed mice. The pathological changes and viability of *R. equi* cells in the liver of mice was verified after the 3rd, 6th an 10th day after inoculation for horse and environmental isolates and for *R. equi* isolates from human patients on the 1st, 3rd and 6th day. During the necropsy procedures, infiltrate of macrophages and pyogranulomatous lesions were detected after the sixth pos-inoculation day in the liver and spleen. In horse isolates, only plasmid positive strains were virulent, but in human isolates both strains (plasmid positive e plasmid negative) were virulent. Both groups of the immunossupressed mice inoculated with *R. equi* isolated from environment showed pathological changes. All *R. equi* strains were unable to kill non imunossuppressed mice.

INDEX TERMS: *Rhodococcus equi*, mice, virulence, cyclophosphamide.

RESUMO.- [**Patogenicidade em camundongos de isolados clínicos, ambientais e humanos de *Rhodococcus equi*.**] *Rhodococcus equi* é um patógeno intracelular facultativo associado com broncopneumonia, linfadenite mesentérica e enterocolite em potros. Apesar do patógeno ser amplamente distribuído no ambiente equino, a doença não é encontrada em todos os criatórios. Componentes capsulares, "fator equi", ácido micólico e alguns produtos codificados por um grande plasmídeo de 85-90Kb foram descritos como fatores de virulência. Entretanto, a patogênese da infecção e a susceptibilidade dos potros não são completamente entendidas. Nove cepas carreando o plasmídeo e isoladas de potros doentes, um isolado de paciente humano imunossuprimido, e seis cepas sem plasmídeo (4 de fezes, 1 de pastagem e 1 de paciente humano imunossuprimido) foram inoculadas em camundongos imunossuprimidos com ciclofosfa-

mida. As alterações patológicas e a viabilidade das células de *R. equi* no fígado foram verificadas depois do terceiro, sexto e décimo dia após a inoculação para isolados clínicos e depois do primeiro, terceiro e sexto dia para isolados de pacientes humanos. Na necropsia foram detectados infiltrado de macrófagos e lesões piogranulomatosas no fígado e baço dos camundongos após o sexto dia da inoculação. Para isolados clínicos, somente os plasmídeo positivos foram virulentos, mas para os humanos, ambos os isolados (plasmídeo positivos e plasmídeo negativos) foram virulentos. Ambos os grupos de camundongos imunossuprimidos inoculados com isolados ambientais mostraram alterações patológicas. Todos os isolados foram incapazes de matar camundongos imunocompetentes.

TERMOS DE INDEXAÇÃO: *Rhodococcus equi*, camundongos, virulência, ciclofosfamida.

INTRODUCTION

Rhodococcus equi is a pleomorphic coccobacillus gram positive bacteria, usually found in the gastrointestinal tract of horses. It has been recognized as an important pathogen due to reports of 3% of foal deaths widespread in the world (Takai et al. 1993, Sellon et al. 2001). *R. equi* is a major lung pathogen in less than 6-month-old foals with high mortality (Ozgur et al. 2000). The pneumonia induced by *R. equi* may also occur in adult horses, particularly in immunocompromised individuals, when it causes systemic infections (Takai et al. 1996).

Nowadays, *R. equi* has also been increasingly related as opportunistic pathogen of human immunocompromised patients, especially HIV reactor, although infections in immunocompetent hosts may occur (Kedlaya et al. 2001). However, little is known about the characteristics of *R. equi* strains isolated from humans. Cryptic plasmids of various sizes and fimbrial structures were detected frequently suggesting the antibiotic resistance (Nordmann et al. 1994). The majority of clinical isolates of *R. equi* from imunossuppressed human patients did not carry virulence plasmids and were no virulent to mice, suggesting that the pathogenesis of *R. equi* infections in immunossupressed hosts may be different from that which occurs in foals (Meijer et al. 2004).

Despite of the importance of *R. equi* disease, its pathogenesis and the singular sensibility of foals are not completely understood (Takai et al. 1993, Takai et al. 1999). The pathogenicity varies among strains and it is associated with the ability of *R. equi* to resist pulmonary and splenic clearance (Hines et al. 1997, Hondalus et al. 1994). Virulence of *R.. equi* has been related to several microbial factors, as capsular antigens, *equi* factor, micolic acid from cell wall, and the thermoregulated 15-17kDa protein (VAP A) encoded by the 85-90Kb plasmids. Guiguerre et al. (1999) showed that the presence of the 85-90Kb plasmid was essential for *R. equi* disease development among susceptible horses. Strains lacking the 85-90Kb plasmid were less virulent to horses and mice (Takai et al. 1993, Guiguerre et al. 1999). The aim of this study was evaluate the virulence of R. equi isolated from human, horses and environment for mice.

MATERIALS AND METHODS

The pathogenicity test in mice, used eight *Rhodococcus equi* strains isolated from sick foals; four strains from feces, two from soil of pasture in an endemic horse breeding farm; two *R. equi* strains obtained from human imunossupresed patients and *R. equi* ATCC 33701 P+ e 33701 P- (Table 1). For molecular characterization of *Vap* A gene, the *R. equi* isolates were submitted to PCR assays according with methods described previously (Takai et al. 1995). The samples were grown in ovine blood agar and incubated at 37ºC for 48h. Colonies from these cultures were transferred into tubes with BHI (Brain Heart Infusion) and the suspension was adjusted to Mac Farland Standart 1.

Table 1. Virulence of *Rhodococcus equi* strains inoculated in immunossupressed (I) and no immunossupressed (NI) mice

Strain	Source/animal or soil	VAP gene[a]	% mortality	
			I	NI
Control				
ATCC 33701 P+	Control/foal	+	60.0	0.0
ATCC 33701 P-	Control/foal	-	0.0	0.0
Horse clinical and environmental strains				
96/93	Abdominal abscess/ foal	+	50.0	0.0
353/93	Lung abscess/ foal	+	66.7	0.0
389/93	Lung abscess/ foal	+	60.0	0.0
164/94	Lung abscess/ foal	+	33.3	0.0
489/95	Lung abscess/ foal	+	62.5	0.0
557/96	Lung abscess/ foal	+	100.0	0.0
54/97	Lung abscess/ foal	+	100.0	0.0
80/99	Lung abscess/ foal	+	100.0	0.0
NNImagery;	Feces / foal	-	0.0	0.0
NNContarina	Feces / foal	-	0.0	0.0
Telaura	Feces/ dam	-	0.0	0.0
C1 égua	Feces/ dam	-	0.0	0.0
Pasto 1	Pasture/soil	-	0.0	0.0
Human clinical strains				
15/99 H+	Pneumonia/human	+	100.0	0.0
15/99 H-	Pneumonia/human	-	83.3	0.0

[a] + Positive results on PCR, - negative results on PCR.

The imunossupresed murine model described by Multimer & Woolcock (1982) was used in this study. In brief, the imunossuppression in 4 to 5-week-old mice with 20-25g was achieved by intraperitoneal inoculation of cyclophosphamide (80mg/g body weight) on challenge day and the next 3 days. *R. equi* was inoculated into the lateral tail vein and the inoculum range was 1.5×10^6 to 1.25×10^9 cells in 0.2ml of BHI suspension. The animals of the control group were inoculated with bacterial free BHI. Each *R. equi* isolate were inoculated six normal and 12 immunosuppressed mice. The control group treated and untreated with cyclophosphamide consisted of six animals.

The pathological changes and viability of *R. equi* cells in the liver of mice was verified after the 3rd, 6th and 10th day after bacterial inoculation of *R. equi* isolates from horse clinical samples, horse feces and soil of pasture; and 1st, 4th and 6th day after inoculation of *R. equi* isolates from human patients. The animals were sacrificed, necropsied and examined to observe macroscopic changes, and liver fragments were removed for histopathological analysis and enumeration of *R. equi* CFU/g in the hepatic content of inoculated mice. Twenty-five microliters of hepatic content was streaked in two plates of ovine blood agar and kept for 48h at 37ºC. Blood samples were collected from the control group at 4th and 10th day after inoculation to make a blood leukocyte count. The results were analyzed by the chi (χ^2) test.

RESULTS

Results in Table 1 demonstrate that 81.5% of cyclophosphamide treated mice died due to equine *Rhodococcus equi* isolates infection. In necropsy, since the sixth day after inoculation with virulent equine strains was observed lesions in the liver and spleen in cyclophosphamide treated mice. The same lesions were present in inoculated mice with human isolates. The histopathological analysis of the liver presented multifocal granulomatous hepatitis. Histopathological changes were characterized by infiltrate of macrophages and pyogranulomatous lesions, but avirulent strains caused weak hepatic lesions without granuloma formation. The evaluation of *R. equi* counts (CFU) in the hepatic tissue showed differences among cyclophosphamide treated and untreated mice. Clearance of *R. equi* cells from the hepatic tissue was reduced among cyclophosphamide treated mice. Differences in the results of bacterial survival assays were statistically significant ($p < 0.0001$) and proved a high susceptibility of immunocompromised hosts to *R. equi* infection. Strains isolated from foal clinical specimens, all carrying the 85-90 Kb virulence plasmid were able to persist longer in the hepatic tissues of cyclophosphamide treated mice. In contrast to the ATCC 33701P+, ATCC 33701P- and human isolates results; data demonstrate that the CFU number of clinical and environmental horse isolates increased in the 6th day after bacterial challenge. However, in the 10th experimental day, the *R. equi* ATTC and horse isolates were reduced in the liver of inoculated mice. Human *R. equi* isolates show a increasing in bacterial number at the 10th after inoculation.

DISCUSSION

Death of cyclophosphamide treated mice occurred only when challenged with 85-90 Kb plasmid-positive isolates, although human *Rhodococcus equi* isolates killed mice independent of the presence of the virulence plasmid. This date is different from previous reports, where the human *R. equi* isolates were only virulent to mice when carrying virulence plasmid (Meijer et al. 2004).

Liver and spleen lesions in mice, inoculated with virulent *R. equi*, may be caused by a diffuse proliferation of macrophages and epithelioid cells, as well as hepatic necrosis due to thrombosis and pyogranulomatous lesions (Madarame et al. 1997). Avirulent strains may cause weak hepatic lesions without granuloma formation (Madarame et al. 1997, Guiguerre et al. 1999).

The ability of *R. equi* to cause infection and death in foals and mice experimentally inoculated was related to survive and multiply within macrophages (Takai et al. 1993, Madarame et al. 1997, Takai et al. 1999). The animals with a sound immune system did not die naturally, independently of the presence of virulence plasmids and of the bacterial number in their hepatic tissue. Many authors also suggest the presence of *R. equi* in inoculated animals without pathological manifestations (Multimer & Woolcock 1982, Yager et al. 1991, Lazzari et al. 1997). Previous investigations demonstrated *R. equi* lethality in immuno-competent mice (Takai et al. 1999). Our data demonstrated the effect of immunodeficiency on resistance to *R. equi* infection in mice. The cyclophosphamide helped human and foal clinical *R.*

equi isolates to turn virulent to Swiss mice. However the mechanism which cyclophosphamide induces immuno-suppression and increase of mortality rates to infected mice remains unknown. Data demonstrated that the cyclophos-phamide inhibited neutrophil production in mice, as previously observed in dogs (Madarame et al. 1997). Results of this paper support the findings in literature where increased lethality of *R. equi* in mice was associated with immunosuppression (Bowles et al. 1989a,b, Yager et al. 1991, Multimer & Woolcock 1992, Hondalus & Mussel 1994). Further studies are necessary to determine the cyclophosphamide effect in phagocytosis process.

This study showed that *R. equi* containing 85-90 Kb plasmids such as the ATCC 33701 P+ strain, were virulent to foal and mice. Only cyclophosphamide treated mice died due to *R. equi* infection. However, death of cyclophosphamide treated mice was related with the presence of the 85-90 Kb virulence plasmid just in *R. equi* strains obtained from foals. On the other hand, *R. equi* strains isolated form HIV reactors were virulent to immunossupressed mice independent of the presence of 85-90Kb plasmid. The majority of *R. equi* clinical isolates from patients with or without AIDS did not carry virulence plasmid and were avirulent to mice (Takai et al. 1999).

REFERENCES

Bowles P.M., Woolcock J.B. & Multimer M.D. 1989a. The effect of immunosuppression on resistance to *Rhodococcus equi* in mice. Vet. Immunol. Immunopat. 22:369-378.

Bowles P.M., Woolcock J.B. & Multimer M.D. 1989b. Early events associated with experimental infection of the murine lung with *Rhodococcus equi*. J. Comp. Pat. 101:11-420.

Guigerre S., Hondalus M.K., Yager J.A., Darrah P., Moser D.M. & Prescott J.F. 1999. Role of the 85 Kb plasmid and plasmid encoded virulence protein A in intracellular survival and virulence of *Rhodococcus equi*. Infect. Imun. 67:3548-3557.

Hines S.A., Kanaly S.T., Byrne B.A. & Palmer, G.H. 1997. Immunity to *Rhodococcus equi*. Vet. Microbiol. 56:177-185.

Hondalus M.K., & Mossel D.M. 1994. Survival and replication of *Rhodococcus equi* in macrophages. Infect. Immun. 62:4167-4175.

Kedlaya I., Ing M.B. & Wong, S.S. 2001. *R. equi* infections in immuno-competent hosts: case report and review. Clin. Infect. Dis. 32:39-47.

Lazzari A., Vargas A.C., Dutra V., Araujo L.A., Castagna L. & Costa M.M. 1997. Pathogenicity of clinical and environmental *Rhodococcus equi* isolates in mice. Vet. Téc. 4:24-30.

Madarame H., Takai S., Matsumoto C., Minamiyama K., Sasaki Y., Tsubaki S., Hasegawa Y. & Nakane A. 1997. Virulent and avirulent *Rhodococcus equi* infection in T-cell deficient athymic nude mice: pathologic, bacteriologic and immunologic responses. FEMS Immunol. Med. Microbiol. 17:251-262.

Martens J.G., Martens R.J. & Renshaw H.W. 1988. *Rhodococcus (Coryne-bacterium) equi*: Bactericidal capacity of neutrophils from neonatal and adult horse. Am. J. Vet. Res. 49:295-299.

Meijer W.G. & Prescott J.F. 2004. *Rhodococcus equi*. Vet Res. 35(4):383-96.

Multimer M.D. & Woolcock J.B. 1982. Experimental *Corynebacterium equi* infection in mice. J. Rep. Fert. 32:469-472.

Nordmann P., Zinzendorf N., Keller M., Lair I., Ronco E. & Guenounou M. 1994. Interaction of virulent and non-virulent *Rhodococcus equi* human

isolates with phagocytes, fibroblast- and epithelial-derived cells. FEMS Immunol Med. Microbiol. 9(3):199-205.

Ozgur Y., Ikiz S., Carioglu B., Ligas A. & Takai S. 2000. Two cases of dead foals associated with R. equi pneumonia in Turkey. J. Eq. Sci. 11:1-5.

Sellon D.C., Besser T.E., Vivrette S.L. & Mcconnico R.S. 2001. Comparison of nucleic acid amplification, serology and microbiological culture of diagnosis of R. equi pneumonia in foals. J. Clin. Microbiol. 39:1289-1293.

Takai S., Ikeda T., Sasaki Y., Watanabe,Y., Ozawa T., Tsubaki S. & Sekizaki T. 1995. Identification of virulent Rhodococcus equi by amplification of gene coding for 15-17 kDa antigen. J. Clin. Microbiol. 33:1624-1627.

Takai S., Anzai T., Sasaki Y., Tsubaki S. & Kamada M. 1993. Virulence of Rhodococcus equi isolated from lesions of infected foals. Bull. Eq. Res. Inst. 30:9-14.

Takai S., Fukunaga N., Ochiai S., Sakai T., Sasaki Y. & Tsubaki S. 1996. Isolation of virulent and intermediately virulent Rhodococcus equi from soil and sand on parks and yards in Japan. J. Vet. Med. Sci. 58:669-672.

Takai S., Kobayashi C., Murakami K., Sasaki Y. & Tsubaki S. 1999. Live virulent Rhodococcus equi, rather than killed and avirulent, elicits protective immunity to R. equi infection in mice. FEMS Immunol. Med. Microbiol. 24:1-9.

Yager J.A., Prescott C.A., Kramar D.P., Hannah H., Balson G.A. & Croy B.A. 1991. The effect of experimental infection with Rhodococcus equi on immunodeficient mice. Vet. Microbiol. 28:363-376.