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MOLECULAR CHARACTERIZATION OF *RHODOCOCCUS EQUI* ISOLATES OF HORSE BREEDING FARMS FROM AN ENDEMIC REGION IN SOUTH OF BRAZIL BY MULTIPLEX PCR

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ABSTRACT

Rhodococcus equi is a gram-positive coco-bacillus and an intracellular opportunistic pathogen which causes pneumonia in foals. It is widely detected in environment and has been isolated from several sources, as soil, feces and gut from health and sick foals. The goal of this study was to characterize the epidemiological status (endemic, sporadic or no infection) of horse breeding farms from Bage County in South of Brazil, using a multiplex PCR. One hundred and eighteen *R. equi* isolates were identified by biochemical tests and submitted to a specie-specific and *vapA* multiplex PCR. These isolates were obtained from: three farms where the *R. equi* infection has been noticed, two farms where the disease has been not reported and one farm where the disease is frequent. All clinical isolates from horse breeding farms where the disease is endemic and/or sporadic were *vapA*-positive. None environmental isolates were *vapA*-positive. In three horse breeding farms with sporadic *R. equi* infection, 11.54% of the isolates from adult horse feces were *vapA*-positive. The multiplex PCR technique has proven to be effective for the molecular and epidemiological characterization of the *R. equi* isolates in horse breeding farms. An important finding in this study was the isolation of *vapA*-positive *R. equi* from adult horse feces, which is an evidence for other routes of dissemination of this pathogen in the farms.

Key-words: *Rhodococcus equi*, Multiplex PCR, bronchopneumonia, epidemiology

INTRODUCTION

Rhodococcus equi causes a very important disease in horses around the world. Virulent *Rhodococcus equi* strains are associated to bronchopneumonia, ulcerative lymphangitis, internal abscess, diarrhea and pleurisy in foals (23). The bacterium is a gram-positive intracellular opportunistic pathogen. It is widely spread in the environment and commonly isolated from soil, feces and gut from healthy and sick animals (8,15,24).

Rhodococcosis is endemic in some farms, sporadic in others and not reported in most of them. The epidemiologic status depends on farm management, foal population density and use of dusty floors in foaling stalls (3). Additionally, environmental issues such as temperature, humidity, dust, and soil pH can influence the development of this infection (12,13). Furthermore, the virulence of *R. equi* strains in a farm, especially those found in the soil and foal feces is the most important concern of this disease (14).

R. equi virulence is associated to the bacterium ability to prevent the phagosome-lysosome fusion and multiplication in macrophages, resisting to clearance by the organism defenses (8). The virulence factor associated to *R. equi* infection in foals is a thermorregulated virulence-associated antigen (VapA), encoded by *vapA* gene, located in the 85-90Kb virulence plasmid (2). According to many researchers the 15 - 17kDa VapA antigen is found in all clinical *R. equi* isolates from foals, as well in some

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environmental samples (4,20). The importance of *vapA* gene in the pathogenesis of *R. equi* infection and its use as an epidemiological marker of bacterium virulence are well established since all strains carrying the *vapA* gene are lethal to mice and foals (4,11,19,21).

The goal of this study was to perform the genotypic characterization of *R. equi* isolates and determine the epidemiological status (endemic, sporadic or no infection) of horse breeding farms from Bage County, RS, Brazil.

MATERIALS AND METHODS

Bacterial Strains

This study was conducted with 118 bacterial isolates previously classified as *R. equi* by biochemical tests (9), collected in six horse breeding farms (Table 1). The strains have been isolated from horse feces (41 from adults and 33 from foals), soil (21), foaling stalls (10) and other domestic animals (3). Other 10 clinical isolates were cultivated from lungs of sick foals. *R. equi* ATCC 33701P+ (*vapA* positive) and *R. equi* ATCC 33701P- (*vapA* negative) were used as controls. The specificity of the PCR technique was evaluated using three *R. equi* related bacterial isolates (*Streptococcus* sp., *Nocardia asteroides* and *Pasteurella multocida*) related to *R. equi.* All isolates were kept lyophilized until submitted to DNA extraction.

DNA extraction

Each isolate was, cultured in 5% ovine blood agar (DIFCO), and transferred to Mueller-Hinton agar (DIFCO) and incubated at 37ºC for 24 hours. The colonies were suspended in 500 µl of milli-Q water and boiled for 10 minutes. The suspension was centrifuged at 13.500 rpm for 3 minutes and 2 µl of the supernatant were used as DNA template for multiplex PCR (16).

Multiplex PCR

The primers used in this study for amplification of 16S ribosomal RNA and *vapA* genes fragments were respectively: RG-Forward (CGT CTA ATA CCG GAT ATG AGC TCC TGT C); RG-Reverse (CGC AAG CTT GGG GTT GAG CCC CAA) (1) and *vapA*-Forward (GAC TCT TCA CAA GAC GGT); *vapA*-Reverse (TAG GCG TTG TGC CAG CTA) (19). The conditions tested for multiplex PCR standardization were adapted from Takai *et al.* (19), with adjustments on primers and magnesium chloride concentrations, as well as temperature for amplification. All conditions were tested three times. Samples were submitted to 35 cycles (30 s at 94ºC, 30 s at 56ºC and 90 s at 72ºC) followed by 10 minutes at 72ºC. The products (7 µl) were applied to a 1.5% agarosis gel and submitted to electrophoresis per 30 minutes at 100 V. After stained with ethidium bromide, the bands were visualized in ultraviolet light.

The *vapA* and 16S ribosomal RNA amplified fragments were purified from agarosis gel with polietileneglicol (16) and submitted to sequencing in an automatic DNA sequencer (MEGABACE 1000) at the Molecular Biology Laboratory, Federal University of Santa Maria.

RESULTS

In this study, the best PCR amplifying condition was achieved using 30 pmol of RG primers, 90 pmol of *vapA* primers and 2.5 mM of $MgCl_2$, showing both for 16S ribosomal RNA (468 pb) and *vapA* (564 pb) genes fragments amplification (Fig. 1). The length of the DNA fragments amplified by multiplex PCR were in accordance with previous results (2,18). The identity of the PCR products was sustained by DNA sequencing analysis. *N. asteroides, Streptococcus* sp. and *P. multocida,* tested by the PCR assay presented no amplification, validating the multiplex PCR specificity.

The multiplex PCR results are shown in Table 1 and Fig. 1. Ten (100%) isolates obtained from sick foals and seven (6.54%) from adults and foals feces were positive for both *vapA* and 16S ribosomal RNA, denoting the virulence potential of these strains to susceptible foals. Most pathogenic isolates were obtained

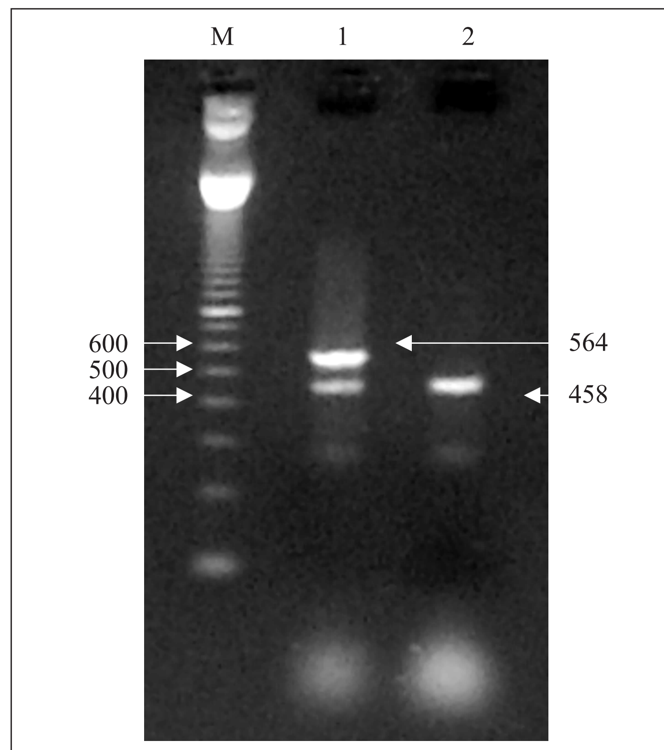

Figure 1. Multiplex PCR amplification of both 16S rDNA and *vapA* gene fragments. L: Mollecular ladder (Ladder 100 pb Ludwigbiotec), 1: ATCC 33701P+, 2: ATCC 33701P-. Fragments sizes are indicated in base pairs (pb).

Table 1. Molecular characterization of *R. equi* isolated from horse breeding farms in Bagé, RS, Brazil, by multiplex PCR for 16S ribosomal RNA and *vapA* gene fragments.

Farm	Disease Occurrence	Source	Total	PCR results positive	
				16 S rDNA	*vapA*
1	Sporadic	Soil	5	5	0
		Adults equine feces	8	8	1
		Foal feces	9	9	0
		Foaling Stalls	2	2	0
		Bovine feces	1	1	0
		Chicken feces	1	1	0
			26		
2	Sporadic	Soil	3	3	0
		Adults equine feces	10	10	1
		Foal feces	4	4	0
		Foaling Stalls	1	1	0
		Clinical samples	1	1	1
			19		
3	Sporadic	Soil	4	4	0
		Adults equine feces	8	8	1
		Foals feces	8	8	0
		Foaling Stalls	1	1	0
			21		
4	Endemic	Soil	3	3	0
		Adults equine feces	11	11	3
		Foals feces	6	6	1
		Foaling Stalls	4	4	0
		Clinical samples	9	9	9
			33		
5	Not reported	Soil	2	2	0
		Adults equine feces	3	3	0
		Foals feces	4	4	0
		Foaling Stalls	2	2	0
		Bovine Feces	1	1	0
			12		
6	Not reported	Soil	4	4	0
		Adults equine feces	1	1	0
		Foal feces	2	2	0
			7		
Total of *R. equi* isolates			**118**		

from the same endemic horse breeding farm (Fig. 2): Virulent *R. equi* were isolated from nine (90%) sick foals and from four (66.6%) samples of faecal matter from healthy adults and foals. Other two virulent isolates (one from sick foal and one from adult animal feces) were obtained in two other farms (1 and 2), which report sporadic occurrence of Rhodococosis. Virulent *R. equi* strains were isolated in adult horses feces from farms 1, 2, 3 and 4 (Fig. 2).

DISCUSSION

The multiplex PCR technique described in this study has proven to be effective for the *R. equi* molecular characterization. Additionally, the epidemiological status (endemic, sporadic or no infection) of horse breeding farms from Bage County, RS, Brazil (Table 1 and Fig. 2) could be confirmed using both

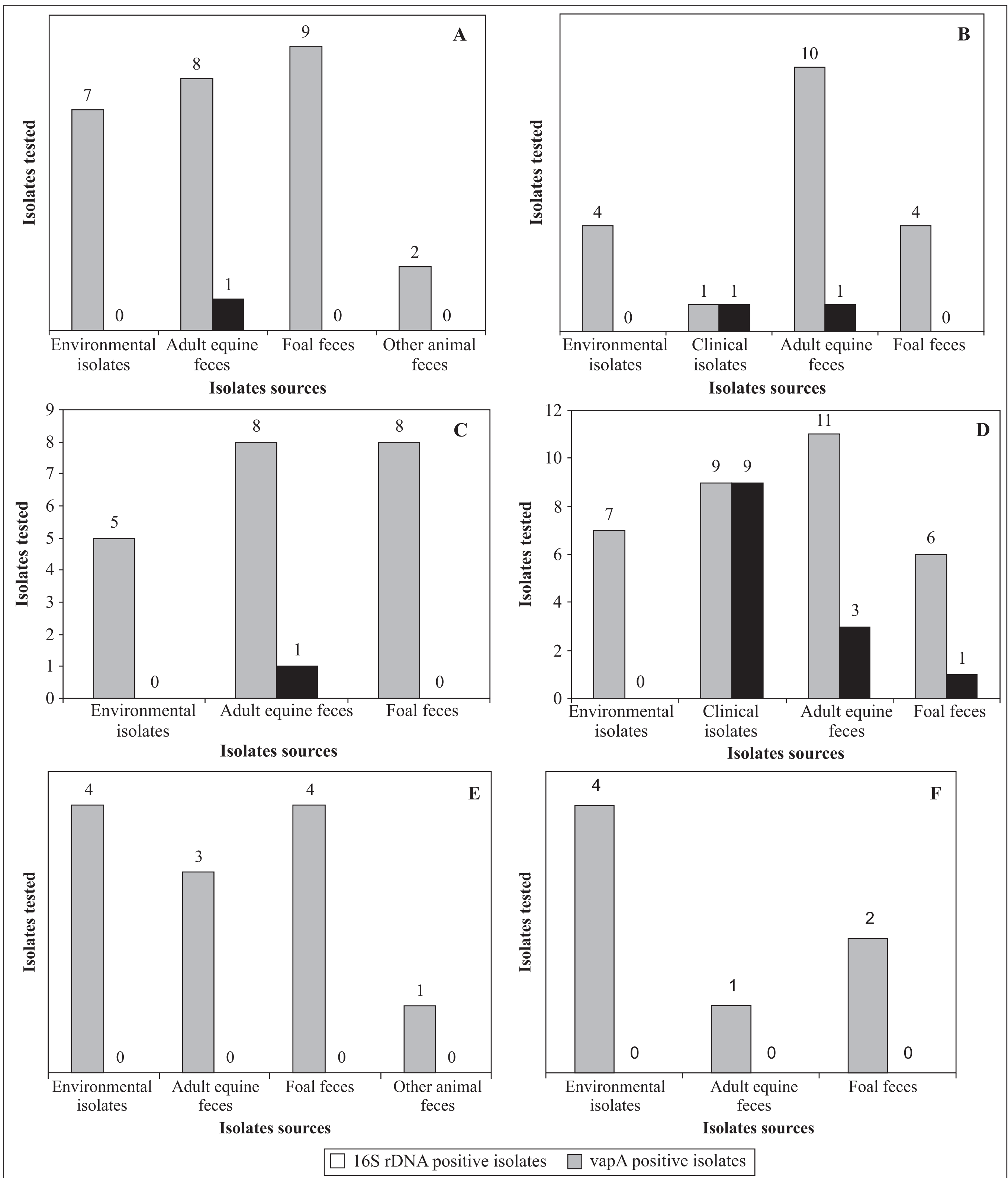

Figure 2. Prevalence of *R. equi vapA*-positive isolates in different horse breeding farms in Bage County, RS . A: Farm 1, B: Farm 2, C: Farm 3, D: Farm 4, E: Farm 5, F: Farm 6.

environment (soil and foaling stalls) and horse samples (from sick and healthy animals).

The virulence-associated antigens (VapA) and plasmids are widely used as epidemiological markers of *R. equi* virulence for foal and experimentally infected mice (6,22). The relationship between the presence of *vapA* gene and the lethality of *R. equi* to susceptible foals and mice was reported by many researchers (19,21,25). In the present study, most virulent *R. equi* isolates were from the endemic horse breeding farm. This can be explained by the higher soil contamination, coming mainly from diarrheic feces from ill animals (12,18).

The *R. equi* isolation from horse breeding farms, where occurrence of disease, is sporadic indicate that adoption of prophylactic measures to decrease the incidence of this infection is required (12,19). The multiplex PCR was also important to determine the sources of infection and the importance of virulent isolates on epidemiological aspects of Rodococosis in horse breeding farms from Bage, RS, Brazil. The usefulness of *vapA* gene or virulence plasmid to determine the risk of *R. equi* infection was demonstrated by Takai *et al.* (21).

The molecular characterization of *R. equi* in this study indicated the occurrence of *vapA*-positive isolates in stools of animals, with no clinical signs associated to *R. equi* infection. In this way virulent *R. equi* were found in adult horse feces, confirming that the prevalence of *R. equi* infection is associated with the presence and number of virulent microorganisms in the environment and animal feces (12,17). Takai *et al.* (21) reported that sick foals are the major source of virulent bacteria for the environment, since *R. equi* is a teluric bacterium that can multiply both in soil and gastrointestinal tract, supporting a cycle involving horses and their environment (9, 14). Halbert *et al.* (7) demonstrated that 97% of *R. equi* isolates from foals were *vapA*-positive, suggesting that these animals are important for the transmission of this infection. In contrast, this study demonstrates that not only foals, but healthy adult horses can carry virulent *R. equi* in their feces and spread them in their environment, indicating that these animals play an important role as a source of contamination of susceptible foals. Future studies are needed to evaluate the potential of multiplication of *R. equi* in the intestinal tract of adult horses.

RESUMO

PCR multiplex para caracterização molecular de isolados de *Rhodococcus equi* provenientes de haras de uma região endêmica no Sul do Brasil

Rhodococcus equi é um coco-bacilo gram positivo que causa pneumonia em potros. Trata-se de um patógeno oportunista amplamente detectado no ambiente e isolado de várias fontes, como solo, fezes e intestino de potros doentes e sadios. O presente estudo visa caracterizar a situação epidemiológica de criatórios eqüinos da região de Bagé, RS, Brasil, pela técnica de PCR multiplex. Cento e dezoito isolados de *R. equi* foram identificados por testes bioquímicos e, posteriormente, submetidos a um PCR multiplex para caracterização da espécie e da presença do gene *vapA*. Estes isolados eram provenientes de três haras com histórico da doença, dois haras onde não havia casos da doença e uma propriedade onde a infecção por *R. equi* é relatada frequentemente. Todos os isolados clínicos provenientes de haras onde a doença é endêmica e/ou esporádica foram *vapA* positivos. Nenhum isolado ambiental foi *vapA* positivo. Nos três haras onde a doença é esporádica, 11,54% dos isolados de fezes de eqüinos adultos foram positivos para o gene *vapA*. A técnica de PCR multiplex mostrou-se efetiva para caracterização epidemiológica e molecular dos criatórios equinos, estando de acordo com o histórico da propriedade. Um fato relevante demonstrado pela aplicação desta técnica foi a detecção de *R. equi vapA* positivo nas fezes de eqüinos adultos. Esta observação pode pressupor que haja outras vias de disseminação da bactéria dentro de uma propriedade.

Palavras-Chave: *Rhodococcus equi*, PCR multiplex, broncopneumonia, epidemiologia

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