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Isolation of virulent *Rhodococcus equi* from Arabian horses in Şanlıurfa, Turkey

Osman Yaşar TEL, Oktay KESKİN*, Sevil ERDENLİĞ GÜRBİLEK
Department of Microbiology, Faculty of Veterinary Medicine, Harran University, Şanlıurfa, Turkey

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Abstract: The purposes of this study were to investigate plasmid profiles of the isolated virulent (VapA positive) agents by RFLP and to determine the quantitative aspects of *Rhodococcus equi* in the feces of adult horses and foals, as well as in soil. The frequency of detection of *R. equi* and virulent *R. equi* in fecal samples and soil was studied from 20 farms in the Şanlıurfa region. A total of 315 samples were examined. Nasal swabs from 25 foals with pneumonia were also analyzed. *R. equi* was isolated and identified from 263 (83.4%) samples originating from 15 out of 20 farms. From 105 samples each of soil and equine and foal feces, *R. equi* was isolated in 91 (86.6%), 94 (89.5%), and 78 (74.2%) samples, respectively. A total of 789 colonies isolated and identified as *R. equi* were examined by PCR for the VapA gene. As a result of PCR, 32 (4.0%) agents were identified as virulent *R. equi*. All virulent *R. equi* isolates contained 85 kb type-I plasmid. Additionally, *R. equi* could not be isolated from nasal swabs in foals with pneumonia. Because of the existence of virulent *R. equi* in the environment, preventive measures should be taken against the risk of infection.

Key words: Horse, PCR, RFLP, virulence, *Rhodococcus equi*

1. Introduction

Foal pneumonia is a major concern for the equine industry worldwide. *R. equi* is one of the most important bacterial pathogens in young foals, and the incidence of pneumonia due to *R. equi* infection appears to be increasing in all breeds. Infections occur from this organism in foals of less than 6 months of age and cause suppurative bronchopneumonia, lymphadenitis, and enteritis (1,2).

R. equi is commonly cultured from feces of adult horses and foals. Foals with pneumonia are capable of shedding large numbers of the organism into the environment (3,4). *R. equi* can flourish and multiply in soil when environmental conditions are suitable; the greatest number of organisms are recovered from the soil surface (4,5). The prevalence varies widely among farms and years, with rates ranging from 0% to 100% in many enzootic farms where prevalence rates are 13% to 25% (6). The disease caused by *R. equi* is endemic in some farms, sporadic in others, and does not develop in most farms. The virulence of *R. equi* is based on virulence-associated antigens A and B (VapA and VapB) and virulence plasmids. At least 11 virulence plasmid types have been reported (7).

R. equi is a well-described pathogen in foals throughout the world. However, only a small number of investigations have been conducted in Turkey to evaluate the virulence of *R. equi* isolates from feces and soil. Furthermore, little is known about the prevalence of virulence plasmids from Arabian horses in Turkey. The purposes of this study were to investigate plasmid profiles of the isolated virulent (VapA positive) agents by restriction fragment length polymorphism (RFLP) and to determine the quantitative aspects of *R. equi* in feces of adult Arabian horses and foals in the Şanlıurfa region, as well as from soil.

2. Materials and methods

The frequency of detection of *R. equi* and virulent *R. equi* in fecal samples and soil was studied from 20 farms in the Şanlıurfa region. A total of 105 foal fecal samples, 105 horse fecal samples, and 105 soil samples (315 total samples) were examined. Nasal swabs from 25 foals with pneumonia were also tested.

2.1. Soil and fecal samples

Soil and fecal samples were taken from 20 farms in Şanlıurfa. For the selective isolation of *R. equi*, nalidixic acid novobiocin–actidione (cycloheximide)–potassium tellurite (NANAT) medium, described by Woolcock et al. (8), was used. On each farm 2 to 3 teaspoons of surface soil were collected from the paddocks or pastures. Additionally, fresh fecal samples were collected. The samples were collected and processed as previously described by Takai et al. (9). Briefly, samples were placed into sterile tubes. The samples were diluted serially in sterile saline (0.9% NaCl).

* Correspondence: okeskin@harran.edu.tr

Each dilution was inoculated onto 2 plates of NANAT medium, and the plates were incubated at 30 °C for 2 or 3 days. When present, 1 to 10 suspected *R. equi* colonies per soil or fecal specimen were subcultured and identified on the basis of morphological and biochemical characteristics (10).

2.2. Bacterial culture

Nasal swabs obtained from clinical cases were cultured on trypticase soy agar supplemented with 5% sheep blood, and in brain–heart infusion broth with 10% newborn calf serum. All isolates were examined on the basis of described morphological and biochemical characteristics (10).

2.3. Virulence assays

Virulence-associated protein antigen in plasmids in *R. equi* isolated from foals, horse feces, and soil were identified by use of described polymerase chain reaction (PCR) techniques. Plasmid DNA was extracted using a commercially available plasmid preparation kit (GeneJET Plasmid Miniprep Kit, Fermentas, Lithuania). Primers for PCR were synthesized. Primer 1 (5'-GACTCTTCACAAGACGGT-3') corresponded to positions 6–23 of the gene, while primer 2 (5'-TAGGCGTTGTGCCAGCTA-3') corresponded to the antisense strand at positions 569–552 (11).

PCR amplification was performed with 10 µL of plasmid-DNA preparation in a 50-µL reaction volume containing 10 mM Tris–HCl (pH 8.3 at 25 °C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleotide triphosphates, 1 µM each primer, and 2.5 U Taq DNA polymerase (Fermentas, Lithuania). The samples were subjected to 30 cycles of amplification. The cycling conditions were as follows: denaturation, 90 s at 94 °C; primer annealing, 60 s at 55 °C; and extension, 2 min at 72 °C.

After amplification, 10 µL of the reaction mixture was electrophoresed on a 1% agarose gel, and DNA fragments were identified by ultraviolet fluorescence after staining with ethidium bromide.

2.4. Analysis of plasmid DNA

Plasmid DNAs were analyzed by RFLP using BamHI, EcoRI, EcoT22I, and HindIII restriction endonucleases for estimation of plasmid sizes (12). Samples of the plasmid preparations were separated on 0.7% agarose gels at approximately 5 V/cm for 2 h.

3. Results

A total of 315 samples (soil, feces) taken from 20 farms were examined for *R. equi* isolation. *R. equi* was isolated and identified from 263 (83.4%) samples originating from 15 of the 20 farms. From 105 samples each of soil, equine feces, and foal feces, *R. equi* was isolated in 91 (86.6%), 94 (89.5%), and 78 (74.2%) samples, respectively. As a result of bacteriologic examination of colonies, a total of 263 colonies from soil, 266 colonies from equine feces, and 260 colonies from foal feces samples were identified as *R. equi*. Viability counts of *R. equi* from soil in the environment ranged from 1×10^2 to 5.7×10^4 CFU/g of soil. *R. equi* was enumerated from fecal samples collected from horses and foals as 1.5×10^2–6.8×10^5 and 1.3×10^2–5×10^6 CFU/g feces, respectively. A total of 789 colonies that were isolated and identified as *R. equi* were examined by PCR for the VapA gene. A total of 32 (4.0%) isolates were determined to be virulent *R. equi* (Figure 1). The rates of virulent *R. equi* in soil and horse and foal feces were 3.0%, 3.3%, and 5.7%, respectively (Table).

RFLP analysis results of both plasmid profiles of our virulent isolate and the reference *R. equi* strain by EcoRI were found to be similar. Virulent *R. equi* isolates were determined to contain 85 kb type-I plasmid by RFLP (Figure 2). On the other hand, *R. equi* could not be isolated from nasal swabs in foals with pneumonia. Restriction endonucleases BamHI, EcoT221, and HindIII digestion patterns of plasmids were found to be similar to those obtained by Takai et al. (12) (data not shown). The resulting digestion fragments were faint, and therefore they failed to be shown.

4. Discussion

Many different organisms may cause respiratory disease, but *R. equi* is considered the most common cause of severe pneumonia in foals. *R. equi* is a soil organism that is widespread in the feces of herbivores, especially in horses, and in their environment (1,13,14). *R. equi* has been isolated from feces and soil at various isolation rates (4,14–16). *R. equi* culture positivity rates were reported as 68%–86% (16), 86% (17), and 95% (15) from soil samples. On the other hand, Takai et al. reported 72%–92% culture positivity for *R. equi* in equine feces (9). Our

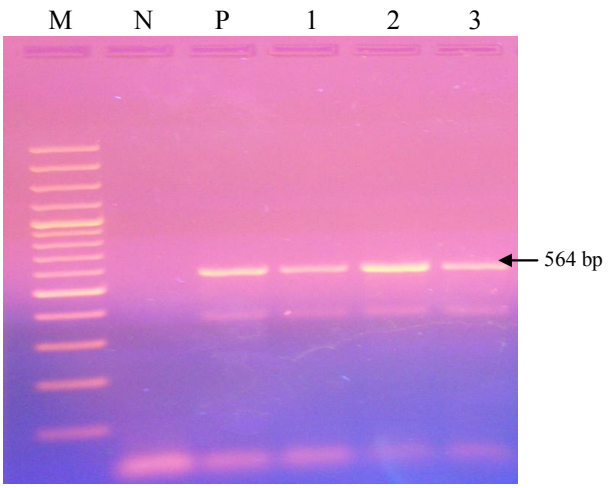

Figure 1. Gel image of VapA gene. M: Marker; N: Negative control; P: Positive control (ATCC 33701); 1, 2, 3: Positive samples.

Table. Isolation of *R. equi* from soil and feces samples.

Source	Number of farms	Number of samples	Number of positive cultures (isolation rate %)	Number of isolates	Viability counts per gram of sample (range)	Mean Number of *R. equi* per gram of sample	Number of virulent *R. equi* (%)	85 kb type I plasmid
Soil	20	105	91 (86.6)	263	1×10^2–5.7×10^4	1×10^4	8 (3.0)	8
Horse feces		105	94 (89.5)	266	1.5×10^2–6.8×10^5	7×10^4	9 (3.3)	9
Foal feces		105	78 (74.2)	260	1.3×10^2–5×10^6	28×10^4	15 (5.7)	15
Total	20	315	263 (83.4)	789	-		32 (4.0)	32

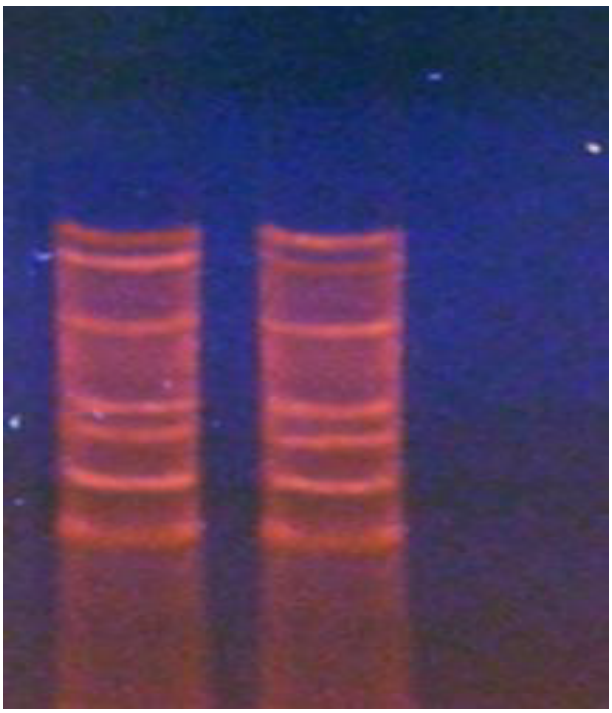

Figure 2. EcoRI restriction fragments of the 2 plasmids types of virulent *R. equi* isolates. Lane 1: strain ATCC 33701, (85-kb type I); Lane 2: strain isolated from samples (85-kb type I).

results were similar to those obtained by several other researchers.

R. equi can be isolated from the feces of adult horses at the range of 10^2–10^3 CFU/g of feces. It was isolated from the feces of foals in the range 10^4–10^5 CFU/g of feces or higher (7), which was similar to our findings. In addition, a pneumonic foal with the disease can constantly shed VapA positive *R. equi* in its feces at over 10^6 CFU/g of feces (6). In this study, *R. equi* was isolated from 15/20 farms. A total of 789 colonies were tested for the presence of the VapA gene, and 32 (4.0%) isolates were determined as virulent *R. equi*. The rates of virulent *R. equi* in soil and horse and foal feces were 3.0%, 3.3%, and 5.7%, respectively. Takai et al. (15,18) reported that the rates of virulent *R. equi* in soil and feces had ranges of 0%–12.9% and 0.5%–8.1%, respectively.

Some researchers (16,19) have reported a higher *R. equi* isolation rate in tracheal wash fluids than in nasal swabs. Sellon et al. (20) isolated *R. equi* in tracheal wash fluids from 10 (18.9%) of 54 foals with pneumonia. Different physical and environmental factors like age, season, housing conditions, immune status, heat, humidity, dust, and soil pH affect the emergence of *R. equi* infection (6,16,21,22). In the current study, *R. equi* could not be isolated from nasal swabs in foals with pneumonia. The failure in isolation of *R. equi* might result from sample differences, sample size and count, and other factors affecting emergence of disease.

Plasmid–RFLP analysis of virulent *R. equi* is a useful tool to describe molecular epidemiology around the world. Recent studies have demonstrated geographic differences in the distributions of the virulence-associated plasmids found in the Americas, Europe, Australia, Africa, Korea, and Japan (23,24). Most of the clinical isolates from the Americas, Australia, and Europe contain 85-kb type I or 87-kb type I plasmids (12,25–28). The 85-kb type II plasmid is found only in French isolates, and the 85-kb type III and type IV plasmids are found only in isolates from the United States (12,15,29). In the present study, virulent *R. equi* isolates contained 85 kb type-I plasmid, similar to those found in Europe.

As a result, 4.0% of *R. equi* isolates from soil and feces were VapA-positive and contained 85 kb type-I plasmid.

R. equi was not isolated from nasal samples in pneumonic foals. However, preventive measures should be taken against the risk of the disease, because of the existence of virulent *R. equi* in the environment. More extensive studies investigating *R. equi* in foals should also be performed.

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