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Detection of Rhodococcus equi by PCR from foals and determination of antimicrobial susceptibility

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Abstract: The scope of this research was determination of *Rhodococcus equi* isolates and antimicrobial susceptibilities in nasal swab specimen taken from animals between 0 and 6 months of age period. *R. equi* was identified from 10 isolates (11.1%) by automatic identification device. The verification of isolates were conducted by 16S ribosomal RNA PCR. *VapA* gene presence was detected in two (20%) isolates and multidrug resistance was identified against 11 antimicrobials. The antimicrobial resistance to macrolides and rifampin is arising worldwide since the excessive usage of the chemotherapeutics in various breeding farms just by evaluating monitoring outcomes. These results exhibit the presence of resistant strains among equine populations. The development of fast, economical and highly specific diagnostic technique against the threat of *R. equi* is essensial for better understanding of gene expressions related to resistance and development of effective immunization.

Key words: *Rhodococcus equi*, foal pneumonia, PCR, *vapA*, antibacterial resistance

1. Introduction

Foal pneumonia is a significant cause of malady and deaths in horses. Although various multiple microorganisms causing pneumonia have been identified in horses, *Rhodococcus equi* is regarded as the most important agent of severe pneumonia [1]. The prevalence and lethality rate of *R. equi* pneumonia is frequently high and the veterinary practitioners lack the tools required for effective early diagnosis or prevention. Long-term and high-cost therapies (frequent prophylactic antibiotic use in endemic breeding farms) are the most important obstacles for treatment of *R. equi* pneumonia that cause major economic losses for equine breeding farms [1,2,3]. In addition, in these farms where the disease is endemic, the diagnosis is made mostly by hypothesis of farm managers and veterinarians. This is due to the fact that the tracheobronchial aspiration (TBA) technique [4], which is accepted as the gold standard in the diagnosis, can threaten the health of the foals.

The clinical symptoms are usually seen in foals younger than 4 months in *R. equi* pneumonia [4]. *R.equi* is a gram-positive, facultative intracellular, aerobic, nonmotile, nonspore-forming coccobacilli that causes chronic granulomatous pneumonia and lung abscesses [5]. First isolated by Magnusson in Sweden in 1923, this bacterium spreads worldwide and can often be isolated from soil and miscellaneous environmental specimen [6]. Frequently isolated from cervical lymph nodes of pigs, this bacterium can infect humans and many other mammal species infrequently following immunosuppression. Following infection in these rare cases; lung abscess, granulomatous pneumonia, lymphadenitis (mesenteric, bronchial or cervical lymph nodes), infected wounds and abscesses in different parts of the body is formed [5].

The pathogenicity of *R. equi* depends on the plasmids. Although the functions encoded in plasmids in environmental Rhodococci are generally catabolic, *R. equi* plasmids provide colonization of the host [7,8]. Its pathogenicity and duplication in macrophage cells are due to the existence of a large plasmid of about 85–90 kb [6,9,10]. Pathogenicity is regulated by *vap* (virulence associated proteins) gene located in the PAI (pathogenity island) region acquired by horizontal gene transfer of this plasmid. There are seven virulence associated protein genes (*vapA-B-C-D-E-G-H*) and three truncated virulence associated protein pseudogenes (*vapF-I-X*) [11]. Among these proteins, *vapA* is essential for bacterial pathogenicity in foals [12]. PAI contains a number of non-*vap* genes as well as *vap* genes. Among these genes, the *vir* operon contains *virR* and *virS* regulators that initiate the expression of PAI [13,14]. The virulence plasmid also plays a role in

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host selectivity. To date, 3 host-specific plasmids have been identified. Circular *pvapA* has been associated with horse isolates; circular *pvapB* with porcine isolates and linear plasmid *pvapN* with bovine isolates [12]. The presence of all three types of plasmids in human isolates [15] coincides with the fact that humans are an opportunist host for *R. equi* and that the infection is of zoonotic origin. The scope of this study was to determine the presence of *R. equi* in the foals by conventional and molecular methods and to investigate the antibacterial resistance of the *R. equi*.

2. Materials and methods

Nasal swab specimens were collected from 90 foals in June 2016 from Southern part of Marmara region and Northern part of Aegean region, Turkey. The foals were between 1 month and 6 months of age period from 4 horse farms. The swabs were taken from the left or right ventral meatus of nasal passage. The swab specimens were transferred to laboratory in the cold chain.

2.1. Phenotypic identification

The samples (n = 90) brought to the laboratory were immediately inoculated in *R. equi* selective medium [NANAT: nalidixic acid (20 µg/mL), novobiocin (25 µg/mL), actidione (cycloheximide) (40 µg/mL) and potassium tellurite (0.005%)] and incubated at 37 °C for 48 h. Macroscopic and microscopic morphology of salmon coloured bacterial colonies were examined, and gram staining and biochemical tests (catalase, oxidase, urease, lipase, phosphatase, CAMP with *S. auerus* ATCC 25923) were performed.

2.2. Genotypic identification

2.2.1. Primers

The 16S rRNA primers used in the study were determined as indicated by Mir et al. [16], *vapA* and *vapB* primers by Al-Graibawi and Mohammed [17].

2.2.2. Standard strains

R. equi ATCC 6939 and *E. coli* ATCC 25922 were used as the quality control strains in biochemical and molecular tests.

2.2.3. *Rhodococcus equi* 16S rRNA PCR step

DNA extraction of *R. equi* isolates was performed according to the protocol reported by Mir et al. [16]. The thermal cycling of PCR conditions was as follows: predenaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, 30 s annealing at 56 °C, 30 s extension at 72 °C and 5 min at 72 °C final extension stages. PCR products were electrophoresed in 1% agarose gel and visualised on Vilber Lourmat UV transilluminator (Vilber Lourmat, Collégien, France). The amplicon length of 450 bp was observed for *R. equi*.

2.2.4. *R. equi vapA* and *vapB* gene presence

The molecular characterization of *R. equi* 16S rRNA positive samples were performed by the second PCR specific for *vapA* and *vapB* genes according to the protocol in a previous study [17]. The thermal cycling of PCR conditions was as follows: predenaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, 30 s annealing at 48 °C, 1 min extension at 72 °C and 5 min at 72 °C final extension stages. PCR products were electrophoresed on 2% agarose gel and gel imaging was performed on Vilber Lourmat UV transilluminator. Amplicons were 286 bp for the *R. equi* vapA gene and 477 bp for the vapB gene.

2.3. Determination of antimicrobial susceptibilities of isolates

R. equi colonies were inoculated into tryptic soy broth and incubated at 37 °C for 8 h (1×10^8 cfu/mL) until turbidity reached. Bacteria suspensions were diluted in the ratio of 1:10. Thus, the bacterial inoculum was adjusted to 1×10^7 cfu/mL concentration.

A hundred µL of cation adjusted Mueller–Hinton broth was added to all wells in the microplate, then 100 µL of 256 µg/mL antibiotic solution was added to the first wells and the antibiotic concentration in the first well was adjusted to 128 µg/mL. Dilution was performed by taking 100 µL of this concentration and transferring it to 128 µg/mL. This process was repeated to a well of 0.0625 µg/mL and the last 100 µL was discarded. The final concentrations of antibiotic agents in microplates were between 0.0625–128 µg/mL. Five µL of the bacterial inoculum in a dilution of 1×10^7 cfu/mL was poured all wells. Thus, the final concentration of the bacterial inoculum was adjusted to 5×10^5 cfu/mL. The agar plates were incubated at 35 °C for 18 to 24 h. The lowest antibiotic concentration without growth was recorded as the MIC value [18,19]. Amoxicillin, streptomycin, kanamycin, amoxicillin clavulanic acid, amikacin, clarithromycin, azithromycin, cefoxitin, clindamycin, rifampin, erythromycin (Oxoid Limited, Hampshire, UK) were used to determine the antibiotic susceptibility of the isolates.

3. Results

3.1. Phenotypic findings

Smooth colonies of salmon pink colour were observed in 10 of the nasal swab samples (n = 10) that were grew on NANAT media and incubated at 37 °C for 48 h. All isolates (n = 10) were found as gram-positive, catalase, urease, lipase, phosphatase and CAMP test (*S. aureus* ATCC 25923) positive, and oxidase negative. The isolates were loaded onto BD Phoenix instrument using PMIC/ID-87 panel kits (Becton Dickinson Company, Franklin Lakes, NJ, USA) and all of them were confirmed as *R. equi*.

3.2. Genotypic findings

PCR amplifications of 10 identified 16S rRNA samples were visualized by agarose gel electrophoresis. All *R. equi* isolates (n = 10) were 16S rRNA positive in the 450 bp fragment length as shown in Figure 1.

Specimens found positive by 16S rRNA PCR were investigated for the *vapA* gene, and the formation of the *vapA* in the 286 bp band gap was detected in 2 (20%) of 10 (isolate no: 53 and 57) samples as shown in Figure 2.

Samples found positive by 16S rRNA PCR were investigated for the *vapB* gene but no positive sample was detected. The PCR results of the isolates are given in Table 1.

The susceptibility of 10 *R. equi* isolates against 11 different antimicrobial agents was determined by microdilution technique. The MIC values of *R. equi* isolates are shown in Table 2.

The MIC values indicating the antibiotic susceptibility of *R. equi* (n = 10) isolates identified in our study showed that the MIC range was ≤0.25 – ≥256. The lowest MIC50 value was 2 µg/mL in erythromycin and the highest MIC90 value was ≥128 µg/mL in kanamycin and amikacin. The antibiogram results of *R. equi* (n = 10) isolates identified according to CLSI standards presented that 80% of the

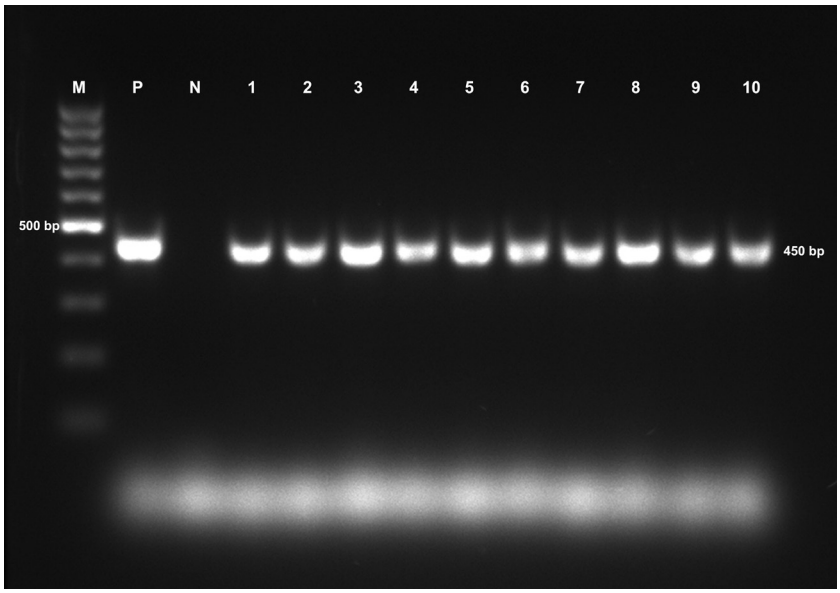

Figure 1. 16S rRNA electrophoresis figure; M: 100bp DNA ladder, P: positive control, *R. equi* ATCC6939, N: negative control (*E. coli* ATCC 25922), 1–10: *R. equi* 16S rRNA positive samples.

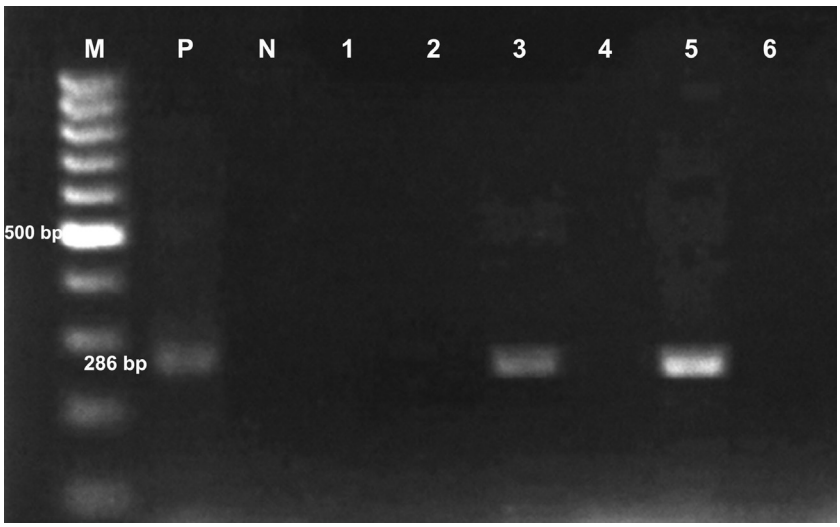

Figure 2. *vapA* electrophoresis figure; M: 100bp DNA ladder, P: positive control, *R. equi* ATCC 6939, N: negative control (*E. coli* ATCC 25922), 3 and 5: *R. equi vapA* positive samples, 1,2,4,6: *R. equi vapA* negative samples.

Table 1. PCR results of the *R. equi* isolates.

Specimen no	16S rRNA	*vapA*	*vapB*
20	+	-	-
33	+	-	-
53	+	+	-
56	+	-	-
57	+	+	-
60	+	-	-
61	+	-	-
77	+	-	-
82	+	-	-
90	+	-	-

isolates were resistant to cefoxitin, 70% were resistant to kanamycin, amikacin, clindamycin and rifampin, 60% were resistant to amoxicillin-clavulanic acid, clarithromycin and erythromycin. In conclusion, 60% of *R. equi* (n = 10) isolates were detected 50% susceptible to streptomycin, amoxicillin and erythromycin. The isolates no. 56, 57, 60, and 90 (n = 4) developed multiple antibiotic resistance against 11 antibiotics used in this study.

4. Discussion

R. equi foal pneumonia (REFP), which is widespread all over the world, can be either endemic or sporadic on some farms [5,20]. In farms where REFP is endemic, there are significant differences between the cumulative incidence [21] and it is thought to be between 10% and 20% in 0–6 months foals [22,23].

Clinical signs of REFP have slow progress and they are difficult to detect until development of abscesses in the lungs. Late onset of clinical symptoms may delay prognosis by delaying the diagnosis and treatment. TBA fluid culture method was known as a useful method for diagnosis, however due to the possible antibiotic use or the presence of different pathogenic microorganisms, this method has been reported to be difficult to produce *R. equi* from a single sample [24,25,26]. In a study investigating the results of different sampling techniques, nasal swab PCR (VP) sensitivity, specificity and disease incidence were found to be 50%, 88%, and 18%, respectively for *R. equi* VP [27]. In another study performed with 117 foals to measure the diagnostic value of nasotracheal and transtracheal aspiration techniques, nasotracheal aspiration samples from 96 foals (61%) were obtained from 96 samples, and 14 (66%) of 21 samples received *R. equi* positive culture. In nasal swab cultures performed to evaluate the contamination of nasotracheal aspirate specimens of microorganisms in nasopharynx, 2 of 56 samples were found *R. equi* positive [28]. Since the formerly reported in sufficient activity of TBA fluid culture, nasal swab collection was chosen in this study for more accurate identification. The NANAT selective media was utilized occasionally in experimental research related to *R. equi* [29,30]. The small (1–2 mm diameter), salmon coloured, smooth and bright morphologies of all colonies reproduced overlap with the results of the previous studies in *R. equi* isolation [22,23,31,32].

Table 2. MIC values indicating antibiotic susceptibility of *R. equi* (n = 10) isolates.

Antibiotics	MIC value (µg/mL)													MIC 50 (µg/mL)	MIC 90 (µg/mL)	MIC range (µg/mL)
	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256			
Amoxicillin					1	2	2				4	1		≤4	≥64	1–128
Streptomycin				1	2	1	1			3	2			≤4	64	0.5–64
Kanamycin							1	1		1	4	2	1	≤64	≥128	4–256
Amoxicillin clavulanic acid					2	2					3	3		≤64	128	1–128
Amikacin							2	1			3	3	1	≤64	≥128	4–256
Clarithromycin					1	2	1	2	3	1				≤8	≥16	1–32
Azithromycin					2	1	1	1	2	3				≤8	32	1–32
Cefoxitin					1	1		1	4	2	1			≤16	≥32	1–64
Clindamycin				2		1	1	4	2					≤8	16	1–16
Rifampin				3			1	4	2					≤8	16	1–16
Erythromycin			2		1	2		1	4					≤2	16	0.25–16

In some studies, the 16S rRNA PCR method was found more sensitive than the culture method in the detection of *R. equi* from clinical specimens [16,33]. In another study in which species-specific DNA amplification was performed with similar methodology, 3 and 9 *R. equi* positive results were obtained from 98 nasal swab samples taken from adult horses and 43 nasal swab samples taken from foals, with a prevalence of 4.08% for adult horses and 25.58% for foals, respectively [16]. In our study, the prevalence of *R. equi* in foals was found to be lower, however the results underscore the lack of sensitivity of standard culture techniques for the diagnosis of *R equi*. A major step in understanding the virulence mechanisms of *R. equi* infection has been taken by the discovery of *vap* family genes [34,35]. These genes give *R. equi* the ability to multiply in macrophages and prevent phagosome formation. Although *R. equi* is generally isolated from healthy horses and soil, detection of virulence-related genes is important in characterizing the pathogenicity of *R. equi* infections [36]. Many studies have linked the *vapA* gene with *R. equi* pathogenicity and lethality in foals [34,37,38], and this gene has been used in epidemiological studies of pathogenicity in horses and humans [39].

R.equi core genomes were described in 2016 [40]. *VapA* is an important immunity inducing protein and a study stated that *vapA* and bacterin hyperimmune serum administration in mares' foals effectively protected foals against *R. equi* challenge in Turkey [41]. Another vaccine research conducted in Turkey reported that bacterin + *vapA* + IMS combined vaccines were found useful to protect mice against *R. equi* infection [42]. In our study, 2 of 10 samples with 16S rRNA species identification were found to be *vapA* positive and virulent *R. equi* incidence was calculated as 20%. In our study, *vapB* gene was not detected in PCR amplification in 10 samples that were identified by molecular method. In a study performed earlier in Marmara region of Turkey, 2 animals perished on 2 distinct stud farms independent with the serological screening research, and virulent *R. equi* was identified from lesions of the six dead foals. The entire isolates had R. equi specific antigens virulence plasmids [43]. The data represented that virulent *R. equi* was prevalent in Thoroughbred foals in the Marmara Region as indicated in this study. In a recent study conducted in Şanlıurfa province of Turkey, 4.0% of *R. equi* isolates from fecal and environmental samples were found *vapA*-positive and expressed 85 kb type-I plasmid from feces of the foals [44]. However, *R. equi* could not be detected from nasal samples in contrary to this research.

Although most of the *R. equi* strains sampled from the foals were greatly susceptible to macrolides and rifampin, strains with resistance to both classes of antibiotic have been reported. The use of rifampin alone is not recommended as it will accelerate resistance formation [37,45]. Resistance development is associated with mutation in the *rpoB* gene encoding the RNA polymerase ß-subunit [46,47]. In another study, the prevalence of resistance to erythromycin and rifampin was found to be 4% in samples collected from foals in the states of Florida and Texas for 10 years. The mortality rate of foals infected with resistant strains was found 7 times higher [47]. In addition, the same study suggested that some strains of *R. equi* sensitive to macrolides were misclassified as resistant.

Resistant strains have been reported due to the widespread use of macrolides and rifampin in foals with subclinical disease [48,49]. Burton et al. [50] found macrolide and rifampin-resistant isolates in 14 of 38 samples collected from a farm in the state of Kentucky. In the same study, the incidence of resistance was found to be 24% in foals that did not begin treatment and 62% in treated foals. In a study conducted by Riesenberg et al. [51] with 200 *R. equi* isolates, 3% of *R. equi* samples were found resistant against rifampin. The treatment of foals with diarrhoea during macrolide therapy or of foals infected with macrolide-resistant strains is controversial due to the deficient availability of alternative antimicrobials. Macrolide and rifampin resistant *R. equi* strains were found to be sensitive to fluoroquinolones, aminoglycosides, oxazolidinones and glycopeptide antibacterials in vitro [47, 52]. In a study, 18 of the 24 isolates were also susceptible to chloramphenicol, tetracycline and trimethoprim sulfamethoxazole [47].

The reports about recommended antimicrobials for the therapy of foals infected with macrolides and rifampin-susceptible strains are deficient. Despite the lack of data, many different classes of antimicrobials have been used to cure *R. equi* infections in foals. Anecdotal information is available that oral doxycycline treats *R. equi* pneumonia in foals when used together with rifampin. Doxycycline and rifampin show a strong synergistic effect against *R. equi* especially in in vitro conditions [53]. In our study, one of the two *vapA* positive isolates was resistant to 11 antibiotics and the other isolate was resistant to seven antibiotics. The presence of clarithromycin, azithromycin and rifampin resistance in isolates is consistent with previous studies [48–50].

Considering the results of this study, the presence of *R. equi* has been revealed among horse population in the North Aegean and Marmara regions, Turkey. Further epidemiological studies are needed to determine virulent plasmids and antibiotic resistance profiles by analyzing virulent *R. equi* in other regions of horse farms of our country. Identification of the infection in horses and the correct use of appropriate antibiotics will benefit the breeding and the national economy. For this reason, it is recommended that veterinarians and farm administrations should confirm

the *R. equi* diagnosis especially by molecular methods, if possible, from nasal swab before applying treatment to foals that do not show clinical pneumonia, and make a detailed examination of the clinical picture of the foal.

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