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Prevalence and Antibiogram study of *Rhodococcus equi* in equines of Jammu and Kashmir, India

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The present study was conducted to determine the prevalence of Rhodococcus equi infection in equines of Jammu and Kashmir, India, and evaluate the zoonotic threat posed by this organism to equine owners and tourists. One hundred and forty-one samples (98 samples from adult animals ≥5 years old and 43 samples from foals less than 6 months old) were collected in duplicate from nasopharyngeal tract of equines for isolation and direct PCR. A total of 12 isolates of R. equi were recovered, of which 9 were from foals and 3 from adult animals. Therefore, the present study recorded prevalence rates of 20.93% and 3.06% among foals and adult equines respectively. The prevalence rates were found to be 25.58% and 4.08% by 16S rRNA species-specific PCR among foals and adult animals respectively. Thus, the PCR-based assay was found to be more sensitive and helped in quick detection of R. equi than the culture based method which is time consuming and laborious. However, the culture-based method is still preferred due to some limitations of PCR. The antibiogram of the isolates revealed that erythromycin and rifampicin were the most effective antimicrobials with 100% sensitivity, followed by amoxicillin (66.67%), lincomycin (58.3%) and kanamycin (58.3%). The results also revealed that resistance was highest for penicillin G (50%), followed by kanamycin (25%) and streptomycin (25%).

Key words: *prevalence, Rhodococcus equi, PCR, equine*

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Jammu and Kashmir is a state in India in which equines are found in abundance. They are an important means of generating income for unprivileged rural and semi-urban society, small-farmers and marginal-farmers. Equines including ponies, mules and horses are mainly used by pilgrims and tourists to reach important religious shrines like Mata Vaishno Devi Shrine and the Holy Amaranth Cave located in hilly areas and for recreational activities respectively. Despite their important role in the state, equidae used for this purpose have been subjected to poor management, lack of health care and negative attitudes of the community [4]. As a result, they are carriers of various important pathogenic bacteria, many of which are zoonotic. One of such bacteria is *Rhodococcus equi,* which pose serious threat to pilgrims and tourists using such animals.

R. equi is a Gram-positive, aerobic, non-motile, non-sporulating [5] and metabolically diverse bacterium [10]. It has been recognized as a pulmonary pathogen of horses. It is known for zoonotic infections in foals that are between 1 to 4 months of age [3]. It is ranked among the top most important pathogens in the horse industry especially because of its high prevalence and mortality rate [13]. To the best of the authors' knowledge, very limited work has been done to explore the status of respiratory tract bacterial pathogens in equines of Jammu and Kashmir, India [12].

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Nasal swab samples from 141 equines (98 samples from adult animals ≥5 years old and 43 samples from foals less than 6 months old) having nasal discharge, abnormal lung sounds and fever were collected during August 2010 to September 2011 from various areas of Jammu and Kashmir, India. The samples were immediately transported to the laboratory on ice and processed for isolation of *R. equi*. The swab samples were streaked on *R. equi* selective NANAT media [20] and incubated aerobically at 37°C for 48 hr. Well-isolated colonies were picked and subjected to biochemical tests for *R. equi*.

The isolates suspected of being *R. equi* by colonial morphology and biochemical tests were confirmed by *16S rRNA* species-specific PCR as per the method described by Soedarmanto *et al.* [17] with slight modifications. PCR was also done to detect *R. equi* directly from nasal swab samples directly. The DNA from bacterial cultures was extracted by boiling two to three well-isolated colonies in nuclease-free water for 15 min followed by rapid chilling. The lysate was centrifuged, and the supernatant was used directly as a template for PCR. The nasopharyngeal swabs were soaked in 2 m*l* of PBS and incubated for 10 min at room temperature, vortexed, wrung out, and discarded for DNA extraction from swab samples. The suspension was centrifuged at 6,000 rpm for 10 min, the supernatant was decanted, and the pellet was used for nucleic acid extraction. Nucleic acid was extracted with the help of a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amplifications were performed in 0.2 m*l* thin-walled PCR tubes. The PCR mixture contained a final concentration of 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 3.5 mM $MgCl_2$, 1.0 μM concentrations of each primer and 0.2 mM concentrations of each 2-deoxynucleoside 5-triphosphate and 1.0 U of Taq DNA polymerase. Oligonucleotide primers were procured commercially (Table 1) from Chromous Biotech (Bengaluru, India). The amplification cycle consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles, each consisting of initial denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec which was followed by final extension at 72°C for 5 min. The amplified product was run in 1% agarose gel. The product was visualized in a UV transilluminator and photographed. The PCR-amplified product was purified by using Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced commercially from Chromous Biotech, Bengaluru, India.

The sensitivity/resistance of all *R. equi* isolates was tested on 5% Blood agar by disc diffusion method of Baeur *et al.* [2] using nine antimicrobials, namely, erythromycin, rifampicin, ceftriaxone, lincomycin, amoxicillin, amikacin, kanamycin, streptomycin and nalidixic acid.

Out of 141 samples processed for isolation of *R. equi*, 12 isolates were obtained, including 9 from foals less than 6 month old and while 3 from adult animals. Thus the present study recorded prevalence rates of 20.93% and 3.06% among foals and adult equines respectively. The prevalence rates were found to be 25.58% and 4.08% by *16S rRNA* species-specific PCR among foals and adult equines respectively. The positive samples revealed an amplicon of 450 bp (Fig. 1). The sequence of the amplified product was subjected to a nucleotide BLAST search on the NCBI server and showed 100% identity with *R. equi*.

The results concerning *in vitro* antimicrobial susceptibility showed that erythromycin and rifampicin were the most effective antimicrobials with 100% sensitivity observed, among the isolates, followed by amoxicillin (66.67%), lincomycin (58.3%) and kanamycin (58.3%). The results further revealed that resistance was highest for penicillin (50%), followed by streptomycin (25%) and kanamycin (25%).

Rhodococcus equi is an important pathogen resulting in severe bronchopulmonary pneumonia not only in animals but also in humans [11]. Every year, the state of Jammu and Kashmir is visited by thousands of tourists and pilgrims to use equines for recreational and religious purposes. During this time, they come in close contact with these equines and are at risk of contracting infections from them and subsequently spreading them to other parts of the country. High infection rates of this pathogen have been associated with immunocompromised individuals, particularly AIDS patients [15].

The prevalence of *R. equi* recorded in the study is less than that in an earlier report [9]. The low recovery rate of *R. equi* can be attributed to the fact that we did not use the invasive collection method of tracheobronchial aspiration which is regarded as the best method for definitive diagnosis of *R. equi* infection [1, 18]. This method was not selected due to technical difficulty, reluctance by owners and associated life-threatening consequences. Another probable reason is the sporadic nature of the disease [16]. Out of the 12 isolates of *R. equi* recovered during our study, nine were obtained from foals under six months of age, while three isolates from adult horses were recovered from tracheal swabs of three adult mares who had died because of respiratory complications. It seems from this observation that foals are much more prone to *R. equi* infection than adult horses as supported by Muscatello *et al*. [14], who describe *R. equi* as the primary pathogen of foals less than six months of age. The detection rate of *R. equi* by *16S rRNA* PCR was comparatively higher than that by the culture technique. Similar results were found by Vivrette *et al.* [19], who recognized that PCR-based detection of *R. equi* from clinical samples was more sensitive than microbiological culture. However, PCR-based detection is not

Table1. *16S rRNA* gene primers for *R. equi*

Primer	Nucleotide sequence	Product size (bp)
16S rRNA F	5′-GGTCTAATACCGGATATGAGCTCCTGTC 3′	450
16S rRNA R	5′-CGCAAGCTTGGGGTTGAGCCCAA 3′	

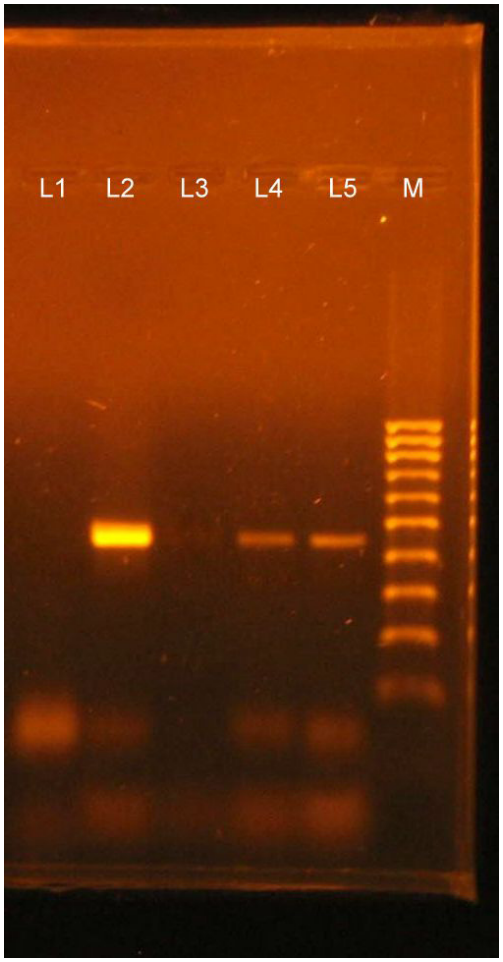

Fig. 1. Amplified product of 450 bp in size from *16S rRNA* species-specific PCR of *R. equi*. L1, Negative Control; L2, Positive control; L3, negative sample; L4 and L5, positive samples; M, 100 bp ladder.

able to distinguish between dead and viable organisms, and it has several other limitations, like false positives due to high sensitivity, cost and the expertise required to perform it. Therefore, the culture-based method is still preferred and remains the gold standard for detection of *R. equi* infection.

Antimicrobial therapy is presently used to reduce the morbidity and mortality of the disease. In our study, erythromycin and rifampicin were found to be the most effective antimicrobials, which is in agreement with the reports of other authors [7, 8]. Non-judicious use of antimicrobials by equine rearers without proper consultations can lead to resistance to various antimicrobials [6]. Although, we did not find any multidrug resistant strains, there is a need for proper judicious use of antimicrobial agents for effective treatment and prevention of emergence of resistant strains.

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