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Bacteriological and Molecular Detection of *Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus* in Equines of Northern India

Irfan AHMAD MIR[1]*, Bablu KUMAR[2], Anil TAKU[1], Farah FARIDI[3], Mohd. ALTAF BHAT[1], Naseer AHMAD BABA[4] and Tahir MAQBOOL[5]

[1] Division of Veterinary Microbiology and Immunology, Faculty of Veterinary Science and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology, Jammu 181102, India
[2] Division of Biological Products, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243122, India
[3] Division of Veterinary Microbiology and Biotechnology, Rajasthan University of Veterinary and Animal Sciences, Bikaner 334001, India
[4] Division of Veterinary Pharmacology & Toxicology, Faculty of Veterinary Science and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology, Jammu 181102, India
[5] Division of Veterinary Public Health & Epidemiology, Sher-e-Kashmir University of Agricultural Sciences & Technology, Srinagar 190001, India

Present study was undertaken to study the prevalence of β-haemolytic streptococci in equine of northern temperate region of Jammu and Kashmir, India. One hundred and forty one samples were collected in duplicate from nasopharyngeal tract of diseased (53) and apparently healthy equine (88) for isolation and direct PCR. A total of 77 isolates of streptococci were recovered from 141 samples with an overall prevalence rate of 54.60%. Out of these 77 isolates, 52 were from diseased and 25 from apparently healthy animals. Of the 77 isolates, 4 were identified as Streptococcus equi subsp. equi, 56 as S. equi subsp. zooepidemicus and 17 as S. dysgalactiae subsp. equisimilis. Thus the overall prevalence of S. equi subsp. equi, S. equi subsp. zooepidemicus and S. dysgalactiae subsp. equisimilis was 2.83, 39.71 and 12.05% respectively. The sensitivity of the PCR for the detection of S. equi species was found higher when attempted from direct swab samples.

Key words: *equine, Streptococcus equi subsp. equi, S. equi subsp. zooepidemicus, S. dysgalactiae subsp. equisimilis*

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Streptococci are an important group of Gram positive cocci bacteria found usually in long chains. They are commonly involved with respiratory tract infections in various animals including humans. In equines, β-haemolytic streptocccci viz. *Streptococcus equi* subsp. *equi*, *Streptococcus equi* subsp. *zooepidemicus* and *Streptococcus dysgalactiae* subsp. *equisimilis* are very important. *S. equi* subsp. *equi* is most notorious agent associated with great economic losses to equine husbandry by affecting the pulmonary functions and reducing their performance [3]. This is the causative agent of strangles, a contagious inflammatory disease of the respiratory tract and associated lymph nodes of equids [9]. *S. equi* subsp. *zooepidemicus* is regarded as archetypal species of the closely related species *S. equi* subsp. *equi* [10]. *S. equi* subsp. *zooepidemicus* is most frequently isolated from cases of equine pneumonia and pleuropneumonia [8]. *S. dysgalactiae* subsp. *equisimilis* is of lesser pathogenic importance and is infrequently associated with lymphadenitis and placentitis in equines [10]. Most of the respiratory tract diseases being contagious, therefore, speed of diagnosis is very important to prevent rapid spread of diseases. Therefore, there is a great demand by clinicians and horse owners for earlier laboratory confirmation. But unfortunately despite the high population of equines and their importance, very little research has been done in India particularly towards standardization of methods for quick diagnosis of respiratory tract infections.

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*Corresponding author. e-mail: mirirfan441@gmail.com

Table 1. List of primers used in the PCR

Primer Name	Test	Nucleotide sequence	Product size (bp)
Sod A Forward	PCR-1	5'-CAGCATTCCTGCTGACATTCGTCAGG-3'	235
Sod A Reverse		5'-CTGACCAGCATTATTCACAACCAGCC-3'	
SeM Forward	PCR-2	5'-TGCATAAAGAAGTTCCTGTC-3'	679
SeM Reverse		5'-GATTCGGTAAGAGCTTGACG-3'	

During the study 141 samples were collected from organised and unorganised sectors, for isolation of β-haemolytic streptococci. Samples were collected from apparently healthy animals (88) and clinical cases (53) showing symptoms like fever, cough, nasal discharge, conjunctivitis, enlargement of submandibular lymph node, and abnormal auscultation of trachea and thoracic cavity.

Samples were collected in duplicate for bacterial isolation and for performing direct PCR. The nasal swab samples were inoculated on Columbia Colistin Nalidixic Acid Agar containing 5% defibrinated blood and incubated aerobically at 37°C for 48 hr. The bacterial isolates were identified employing various cultural, morphological and biochemical tests. Lancefield's serogrouping was done by using commercially available Histrep Latex Agglutination Kit (Himedia, India) as per manufacturer's direction.

Molecular detection was attempted for rapid detection of *S. equi* species. For this purpose two separate PCR mixtures largely based on the previously published work [1] were used with some slight modifications. The DNA template was prepared as per the method [2]. The primers used for these PCRs are shown in Table 1. In brief, PCR-1 was conducted for detection of *S. equi* species on the basis of superoxide dismutase (*sod A*) gene amplification. PCR was carried out in a final reaction volume of 25 *µl* in 0.2 m*l* thin wall sterile and nuclease free PCR tubes (Eppendorf, Germany). The PCR mixture contained a final concentration of 3.5 mM $MgCl_2$, 0.20 mM concentration (each) of dNTPs, 2.5 *µl* of 10× PCR buffer, 1.0 *µ*M of forward and reverse primer, 2.50 *µl* template DNA and 1.0 U of taq DNA polymerase (Chromous Biotech Pvt. Ltd., Bengaluru, India). The amplification cycle consisted of initial denaturation at 94°C for 2 min, followed by 35 cycles, each consisting of denaturation at 94°C for 10 sec, annealing at 70°C for 10 sec and extension at 72˚C for 5 sec which was followed by final extension at 72°C for 5 min. PCR-2 was done for subspecies confirmation on the basis of *SeM* gene amplification, specific for *S. equi* subsp. *equi* and not for *S. equi* subsp. *zooepidemicus.* PCR conditions were kept same except for the annealing temperature which was kept 56˚C.

During our study, 77 isolates of β-haemolytic streptococci were recovered from 141 samples, of which 52 were isolated from diseased animals and 25 from apparently healthy animals. From 77 isolates, 4 were identified as *S. equi* subsp. *equi* (all from diseased cases), 56 as *S. equi* subsp. *zooepidemicus* (37 from diseased cases and 19 from apparently healthy) and 17 as *S. dysgalactiae* subsp. *equisimilis* (11 from diseased and 6 from apparently healthy). The present study revealed sample-wise prevalence of *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus* and *S. dysgalactiae* subsp. *equisimilis* among diseased animals to be 7.5, 69.81 and 20.75% respectively whereas 0, 21.59 and 6.8% respectively among apparently healthy animals. Thus the overall prevalence of *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus* and *S. dysgalactiae* subsp. *equisimilis* was 2.83, 39.71 and 12.05% respectively. Isolation rate of *S. equi* subsp. *equi* was very low with a prevalence of 2.83%. Several reasons could be suggested for such negative results. Most obvious reason seems that sampling was done only from nasopharyngeal tract and not from guttural pouch, where this pathogen resides even after disappearance of clinical symptoms of disease, thereby resulting in negative results [7]. *S. equi* subsp. *zooepidemicus* was recovered with a high prevalence rate (39.71%) from the upper respiratory tract of equines which is also reported by Malik and Kalra [6]. Similar findings had been reported by Jannatabadi *et al.* who got 25 isolates of *S. equi* subsp. *zooepidemicus* from 30 cases of respiratory diseases of equines [4]. Further analysis of data revealed that out of a total 56 isolates, 37 isolates out of 53 samples were from diseased whereas only 19 isolates out of 88 samples were from apparently healthy animals. Thus it seems from frequent recovery of *S. equi* subsp. *zooepidemicus* from cases of respiratory diseases that it is involved in causing strangles like diseases besides *S. equi* subsp. *equi* [5]. *S. dysgalactiae* subsp. *equisimilis* was obtained at a rate of 20.75% from diseased animals compared to 6.8% from apparently healthier animals. Most of *S. dysgalactiae* subsp. *equisimilis* isolates were recovered along with other types of *Streptococcus*. The high recovery rate of *S. dysgalactiae* subsp. *equisimilis* from the diseased as compared to apparently healthier animals could imply their pathogenic effect on the equine in our study.

Out of 53 samples from diseased animals, subjected to species specific *sod A* PCR (PCR 1) for detection of *S. equi* species directly from the samples, 48 (90.56%) samples were found to be positive as they showed an amplicon of 235 bp (Fig. 1) confirming presence of *S. equi* species.

Out of these 53 samples, only 6 samples were found positive for *S. equi* subsp. *equi* as they showed an amplicon of 679 bp (Fig. 1) in *SeM* PCR (PCR 2) while as the rest 42 samples were concluded to be *S. equi* subsp. *zooepidemicus* on exclusion basis. Thus the prevalence of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* directly by PCR among diseased animals was 11.32 and 79.24% respectively. From 88 samples of apparently healthy animals, 22 were positive in PCR 1. Out of these 22 positive samples, none was found positive for *S. equi* subsp. *equi* in *SeM* PCR. Thus all 22 *Sod A* positive samples were confirmed to be *S. equi* subsp. *zooepidemicus* on exclusion basis. Therfore, the prevalence of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* directly by PCR among apparently healthy animals was 0 and 25% respectively. Hence overall prevalence of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* was 4.25 and 45.39% respectively directly by PCR of the samples.

The relatively high detection rate of the *Sod A* PCR as compared to culture suggests more sensitivity of this technique in our study. Since the diagnosis of *S. equi* species on the basis of isolation is difficult and time consuming process, not only because of the slow-growing and fastidious nature of this facultative anaerobe, but also because of the overgrowth of large number of different bacteria comprising the upper respiratory tract microflora, therefore we suggest *Sod A* based PCR assay should be used for rapid identification of *S. equi* species. Similarly, sensitivity of *SeM* PCR was more than cultural method which is in consistent with the findings reported by Timoney and Artiuschin [11].

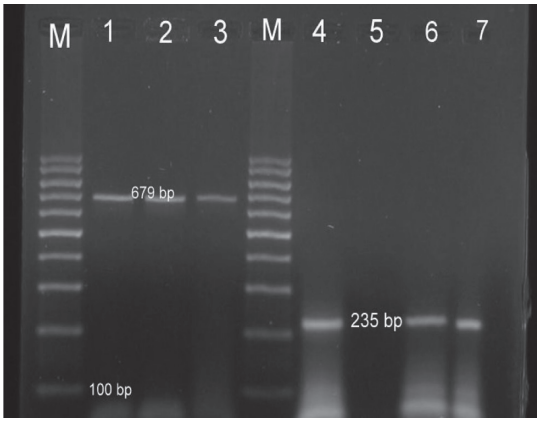

Fig. 1. PCR amplified product of *Sod A* gene (235 bp) for *S. equi* species and *SeM* gene (679 bp) for *S. equi* subsp. *equi*. Lane M: 100 bp ladder, Lane 1–3: Positive samples of *S. equi* subsp. *equi* (679 bp); Lane 4, 6 & 7: Positive samples of *S. equi* species (235 bp); lane 5: negative sample.

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