Original Research

Biochemical Characterization and Molecular Detection of *Streptococcus pluranimalium*

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Abstract

In present study, ninety-six equine nasal swab samples were subjected for isolation of Streptococcus spp. Four isolates of Streptococcus pluranimalium were isolated and identified by different biochemical testing. The isolates were further confirmed as S.pluranimalium by PCR amplification of 1481 base pair16S rRNA product and subsequent sequence analysis. BLAST1 analysis against the Gen Bank database identified a closest match to S. pluranimalium.

Key words: 16S rRNA, *S. pluranimalium*, Polymerase Chain Reaction

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Introduction

The genus *Streptococcus* encompasses a broad range of Gram-positive, catalase-negative chain forming coccus shaped organisms. The genus *Streptococcus* comprises a large number of Gram-positive cocci, non-motile and chain-forming bacteria, including both harmless commensals and formidable pathogens (Facklamm, 2002; Krzyściak *et al.*, 2013). The important bacterial agents associated with respiratory tract diseases of equines include *Streptococcus equi* subsp. *equi* (*S. equi* subsp. *equi*), the causative agent of equine strangles; *Streptococcus equi* subsp. *zooepidemicus* (*S. equi* subsp. *zooepidemicus*), the cause of respiratory infections and suppurative diseases in many animal species especially horses. Besides these various other opportunistic pathogens are found associated with respiratory tract diseases there by increasing the severity of the infection. The important ones include *Streptococcus equismilis, Streptococcus pneumonaie, Staphylococcus aureus, Corynebacterium sp., Pasteurella and Actinomyces sp.* In animals, the main habitats of streptococci are the skin and mucous membranes of the respiratory gastrointestinal and

urogenital tracts (Hirsh and Biberstein, 2004). In addition streptococcus species have been associated with infections causing growth depression and increased mortality without obvious clinical signs (Chadfield *et al.*, 2004).

Streptococcus pluranimalium was first described by Devriese *et al.* (1999) for isolates recovered from several species of domestic animals, including cattle (genital tract, tonsils, and subclinical mastitis), canaries (crop, respiratory tract), a goat (tonsil) and a cat (tonsil). The organism has been associated with meningoventriculitis in a calf, probably as a sequel to septicaemia and endocarditis and septicaemia in broiler chickens (Hedegaard *et al.,* 2009). Probably, it is the first time that this organism is reported from equines in (Jammu and Kashmir State) of India. The present study was conducted to isolate bacterial agents from diseased and apparently healthy equines in R.S. Pura and Katra regions of Jammu, Jammu and Kashmir.

Material and Methods

A total of 96 nasopharyngeal swab samples were collected from both clinically affected and apparently healthy equines. The nasal samples were taken for the isolation of other respiratory organisms causing upper respiratory diseases irrespective of the situation that *S. pluranimalium* could be isolated from these samples. After collection, samples were immediately transported to the laboratory on ice for further processing.

Isolation and Biochemical Characterization of Bacteria

Nasal swab samples were inoculated in Brain Heart Infusion broth for enrichment at 37ºC for 4 hrs. From these enrichment broths, samples were inoculated on Blood agar plates (containing 5% sheep blood) and Columbia Nalidixic agar plates and incubated at 37ºC for 24 hrs for the isolation of streptococci. The bacterial isolates which showed smooth translucent, shiny colonies with zones of α-haemolysis, were selected for further processing. Pure colonies of bacteria were obtained by sub-culturing (2-3 times) on the 5% sheep blood agar. The bacterial isolates were presumptively identified based on color and status of haemolysis and Gram's staining. Further differentiation was done on the basis of various biochemical tests. All the Streptococcal isolates obtained were subjected to biochemical characterization using Histrep Identification Kit (KB 005A, Himedia, Mumbai, India). The kit contains 12 biochemical tests which include Voges-Proskauer, Esculin hydrolysis, Pyrolidonyl Acrylamidase Test, ONGP (O-nitro phenyl-β-d-galactopyranoside), arginine utilization, and fermentation of seven sugars *viz.* glucose, lactose, arabinose, sucrose, sorbitol, mannitol and raffinose. The results were interpreted as per the manufacturer's instructions.

PCR Based Detection of Streptococcal Isolates

All Streptococcal isolates identified by culture and biochemical tests were subjected to16S rRNA genus

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specific PCR for confirmation as Streptococci. For preparation of DNA template, isolated bacterial colonies were suspended in 300 µl of distilled water in 2ml eppendorf tubes. Tubes were then boiled for 15 minutes, so that the bacterial colony is completely dispersed followed by chilling for 15 minutes (snap and chill method). The DNA were further stored at -20˚C till further use. The 16S rRNA gene was amplified using conventional PCR with published primers (Marin *et al.,* 2011) fD1 (forward, 5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (reverse, 5'-ACGGCTACCTTGTTACGACTT-3'). The PCR reaction was made in 25µl of a reaction mixture consisting of 0.5µl of 25mM of each primer, 0.5µl of 10mMdeoxynucleoside triphosphates, 2.5µl of 2.5mM $MgCl_2$, 2.5µl of 10xTaq buffer, 2.5 units of Taq DNA polymerase and 2µl of DNA. The thermal cycler condition was initial denaturation at 94ºC for 5 min and 35 cycles of denaturation at 94ºC for 1 min, annealing at 60ºC for 1 min and extension at 72ºC for 1.5 min, followed by a final extension step of 72ºC for 10 min as per Marin *et al.* (2011).Confirmed streptococcal isolates in PCR were further subjected to sequencing from external source. BLAST1 analysis against the GenBank database identified a closest match to *S. pluranimalium* (>99% sequence identity).

Results and Discussion

Forty streptococcal isolates were revealed on the basis of their colony characteristics such as smooth translucent, shiny colonies with zones of α-haemolysis and positive in gram stain. Single colonies were harvested for biochemical characterization using the HiStrep identification kit (Himedia, India).The organism was negative for Voges – Proskauer reaction, lactose, PYR, ONPG, arabinose, sorbitol, mannitol and raffinose while others arginine, sucrose and glucose were found positive. Four isolates of *Streptococcus pluranimalium* were identified based on Histrep identification kit. All isolates identified as *S. pluranimalium* were strongly α-haemolytic on bovine blood agar plates, while isolates of enterococcus spp and other streptococcal organisms produced weaker and greener alpha haemolysis. The isolates were identified as *S. pluranimaiium* but could not be differentiated reliably from *Streptococcus acidominimus*, *enterococcus* spp by phenotypic characterization using commercial kits routinely used in veterinary laboratories

All isolates were positive for 1481 base pair gene specific 16S rRNA PCR product in 16SrRNA PCR (Fig.1). In 16sRNA sequencing isolates were found similarity with *Streptococcus pluranimalium*. BLAST1 analysis against the GenBank database identified a closest match to *S. pluranimalium* (99% sequence identity).

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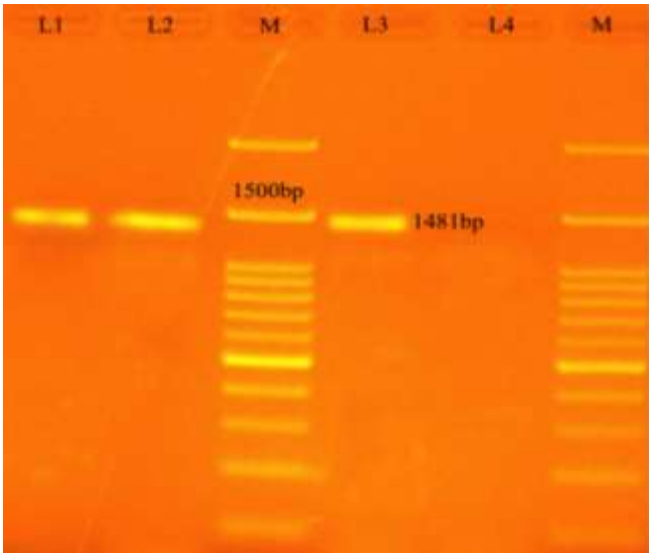

Fig. 1: Polymerase chain reaction amplified products of three isolates. (Lane-1 isolates1, Lane-2 isolate 2, DNA Marker (M), Lane-3 isolate 3, Lane-4 negative control and M (DNA marker)

S. pluranimalium is an emerging pathogen, but the mechanisms of its pathogenesis are largely unknown. Kalhoro *et al.* (2015) showed that the mouse model could be used to assess the virulence of the bacterium. They reported that the pathological findings in the brain showed that *S. pluranimalium* is capable of inducing brain damage by migrating into the brain by crossing the blood–brain barrier (BBB).

Conclusion

Complete understanding of the pathogenesis of *S. pluranimalium* should lead to the development of novel strategies to prevent the infection of this bacterium. The 16S rRNA gene sequences of the bacterial isolate were found 99 % identical to the sequence of *S. pluranimalium*. The 16sRNA nucleotide sequence of 1481 bp of *Streptococcus pluranimalium* isolate was submitted in NCBI genebank with (Accession No. KY020401.1). Guccione *et al.* (2016) isolated the *Streptococcus pluranimalium* from Mediterranean buffaloes in Italy and sequenced 1420bp product which shows 99% identity with this isolate. The isolates showed 99% identity with nucleotide sequences of other strains from different parts of world such as Italy, Denmark, Thailand and Brazil.

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