Molecular and Biochemical Detection of *Streptococcus equi* Involved in Strangles in Horses

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Abstract

Strangles is a life threatening highly contagious disease caused by Streptococcus equi (S. equi). The study was designed to quantify the equine infection caused by S. equi in horses. A total of 50 samples were aseptically collected from the abscess of sub-mandibular lymph nodes and analyzed for the presence of S. equi. For conventional diagnosis, morphological and biochemical tests were performed and for advanced molecular detection, PCR technique was adopted for final confirmation. The bacteria caused the haemolysis on blood agar plate, were spherical in shape, appeared as chains on gram staining and only fermented glucose and lactose. PCR results indicated that 9 out of 50 (18%) samples were contaminated with S. equi when spy1258 gene (407bp) was targeted for detection of the said pathogen. The study provides the primary insight of S. equi involved in strangles and will help to timely diagnose and treat the infected horses.

Key words: PCR, Pharyngitis, Pyrexia, Strangles, *Streptococcus equi*

Introduction

Streptococcus equi sub-sp. equi cause the infection strangles that is an acute and contagious disease of upper respiratory tract (Boyle et al 2016). The disease is predominantly occurred in young horses and characterized by rhino-pharyngitis and lymphadenitis of sub-mandibular and retropharyngeal lymph nodes. The bacterium affects the animal by attaching to the tonsillar epithelium at inhalation or ingestion of the infected secretions (Taylor and Wilson 2006). The complications of the infection include empyema of guttural pouches, purpura hemorrhagica and inflammation of muscle cells (Boyle et al 2016). *Streptococcus equi* is associated with a number of diseases which may include mild as impetigo and pharyngotonsillitis to sepsis (Holden et al 2009). *Streptococcus equi* is one of the most common bacterium involved in causing puerperal and neonatal sepsis in horses (Newton et al 2000). The disease could be easily controlled if treated at an early and less severe stage. Sub-clinical and improperly treated horses play an important role in disease transmission as permanent carrier of the bacteria to the healthy susceptible animal populations (Newton et al 2000, Verheyen et al 2000). Control and prevention depend on isolation of affected horses, appropriate sanitation, identification of carriers and judicious use of vaccines.

The diagnosis of the infection is usually made by bacterial culture or could be done by molecular techniques like polymerase chain reaction (PCR). The samples are usually taken from the nasal swabs, aspirates of the nodular abscesses or by the lavage of guttural pouch (Taylor and Wilson 2006). For diagnosis of the disease, blood culture is still considered as gold standard for diagnosis of strangles, although it provides only few positive results especially in early onset of infection (Asmat 2000, Boyle et al 2021). These false negative results may be because of minute quantity of blood used for culture or low quantity of bacterial count present in the sample (Mallicote, 2015). Moreover, the use of antibiotics is continued due to clinical signs and symptoms of the disease and irrational use of antibiotics has severe consequences like immune dys-regulation and negative impact on gut microbes besides risks of pathogens that develop the antibiotic resistance in the animal (Waller, 2014). Hence, to avert the hazardous consequences, accurate, timely and efficient diagnosis of the disease is extremely essential. In this study, the samples were collected from sub-mandibular lymph node abscesses and then subjected to conventional diagnosis and PCR detection.

Methodology

Sample collection

With sterile cotton swab, samples from abscesses of sub-mandibular lymph node from 50 horses were collected from Civil Veterinary Hospitals, Remount Veterinary & Farms Corps and from different private horse stables in and around the Quetta region.

Microbial culture

The collected samples were transferred to nutrient broth for an incubation period of 24 h and then the bacterial growth was transferred to selective media i.e Columbia blood agar. Gram staining was performed for suspected growth to visualize under microscope

Biochemical tests

Series of biochemical tests were performed to identify the *Streptococcus equi*. The tests performed were catalase and oxidase while the sugar (Glucose, Mannitol, Lactose, Xylose and Sorbitol) fermentation tests were performed.

DNA extraction and PCR

Final confirmation of the infection was carried out by performing PCR. The DNA was extracted by boiling method as described earlier (Dunne *et al*., 2013) and the specific pair of primers sequences (Forward-TGGGATTCTGTGCCGATTT and Reverse-ACCCGAAGACACGGTAAA) were used to amplify the target gene (spy1258, 407bp) as mentioned earlier (Holden et al., 2009). The different steps of PCR (25 cycles) used to amplify the target gene were initial denaturation that was done for 5min at 94ºC followed by denaturarion at the same temperature for 45sec, anneling at 53ºC for 45sec, elongation 72ºC for 1min and then final extension at 72ºC for 7min.

Agarose gel electrophoresis

The PCR product was migrated by agarose gel electrophoresis to find out the specific bands as the method described earlier (Ali et al 2014). Briefly, 2% agarose powder was mixed in 100ml of TAE (1%) solution under heating and then solidified in the gel plate after placing a comb for

required number of wells. PCR product (20µl) of each sample is added in a single well of the gel along with positive and negative controls in separate wells and then migrated under electrical potential. The size of the bands in the tested samples was compared with bands of the ladder of known molecular weight (100bp).

Results and Discussion

The collected samples from the abscesses of sub-mandibular lymph nodes were analyzed to isolate *Streptococcus equi*. The first and foremost step was to observe the colony morphology and growth on Columbia blood agar plates.

The results demonstrated that the samples contained *S. equi* caused haemolysis when grown on blood agar plates (Figure 1A). Columbia blood agar is a differential media for specific bacteria as the pathogens having hemolytic properties will lead to lysis of the blood cells present in the growth media (Sweeney et al 2005). Hence, the haemolytic enzyme harboured by *S. equi* lead to of the blood cells. The bacterial growth on blood agar plates was Gram stained and hence, *S. equi* appeared as cocci or chains of bacterial cells were observed under microscope (Figure 1B). The microscopic results supported the preliminary results of bacterial growth on blood agar plates (Lawrence et al 1985).

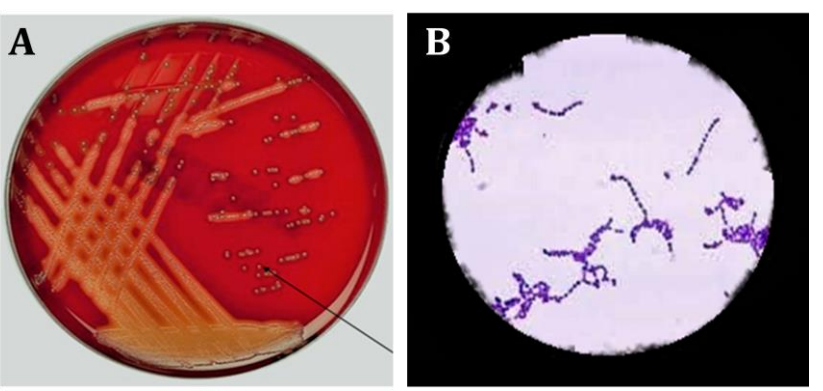

Figure 1: Typical colonies of *S. equi* caused the haemolysis on blood agar plate (A) and Gram positive bacterial cells appeared as spherical/cocci in shape in the form of chains under light microscope (B).

This growth pattern (cocci and haemolysis) provides a primary insight about the detection of the bacterium, therefore a further series of biochemical tests were conducted for definitive diagnosis. The results of biochemical tests performed against the isolated pathogens from the collected samples provided further insight about the bacteria involved. As expected, S. equi was not able to ferment sorbitol, xylose and mannitol (Pusterla et al 2018). However, glucose and lactose were fermented successfully by the isolated pathogen (Table 1) and these fermentation patterns further indicated the presence of S. equi in the infected samples as desribed previosuly (Lawrence et al 1985).

Table 1: Biochemical tests performed to identify *S. equi*

Tests	**Biochemical tests**	**Presence of bacteria**
IMViC tests	Indole	- ve
	MR	+ ve
	VP	- ve
	Citrate	- ve
Sugar fermantation tests	Glucose	+ ve
	Lactose	+ ve
	Fructose	--
	Mannitol	- ve
	Xylose	- ve
	Sorbitol	- ve
Other biochemical tests	Catalase	- ve
	Oxidase	- ve
	DNase	--
	Coagulase	--

The final confirmation of isolated bacterial growth was performed by PCR test. The specific gene (*spy*1258*)* of *S. equi* (Waller 2022) was targeted and successfully amplified (Figure 2). S. equi was detected only in 9 (18%) samples out of 50 analyzed samples of sub-mandibular lymph node abcesses. Hence, it was found that S. equi is the most common pathogen to be involved in strangles in horses.

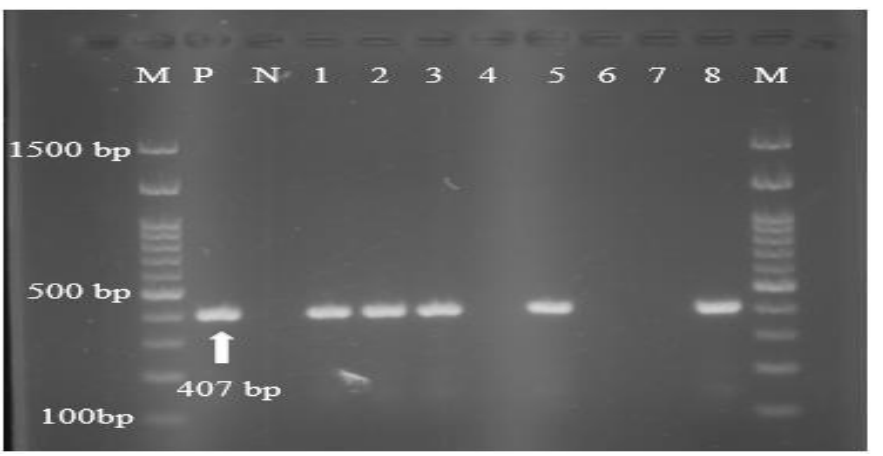

Figure 2: Agarose **gel electrophoresis presenting the amplification fragment** of *Streptococcus equi* (407bp). The lanes 1 to 8 are the tested samples wherein 1, 2, 3, 5 and 8 represent the positive results. M, P and N represent the molecular weight marker, positive and negative controls, respectively.

Conclusion

In developing countries, use of antibiotics is irrational and the horses are not the exception in this regard. However, antibiotic therapy in horses suffering from strangles is observed to inhibit the development of immunity upto required level on exposure to *S. equi* (Basile et al 2018, Pringle et al 2020). They are often treated with antibiotics based on clinical signs and symptoms and no proper diagnostic tests are performed or the performed tests are not that much efficient and accurate (Pusterla et al 2011, Timoney et al 2008). This study provides the significant insight in Balochistan province especially in Quetta regarding the *S. equi* infection in horses. Furthermore, it also elaborates the efficient detection process which will help to make the accurate diagnosis timely. PCR based

detection of pathogens involved is reliable, efficient and accurate and it can help to prescribe the best fit medicine not only to control the infection but also minimize the risk of antibiotic resistance development.

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