The Journal of Animal & Plant Sciences, 22(2): 2012, Page: 256-259
ISSN: 1018-7081

COMPARATIVE STUDY OF CULTURE AND PCR FOR DETECTION OF CARRIERS OF *STREPTOCOCCUS EQUI* IN NATURALLY INFECTED MULES IN PAKISTAN

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ABSTRACT

This is the first report of carrier of *Streptococcus equi* (*S. equi*) in naturally infected mules in Pakistan. A total of 20 mules (10 <2 year and 10 of 2-5 years) infected with strangles that remained positive after one week of recovery were selected and monitored for 12 weeks. After the end of 2nd week all mules < 2 years of age were positive but at the end of 3rd to 6th weeks there remained 7, 3, 1 and zero out of 10 on the basis of culture but through PCR, at the end of the 5th week all mules < 2 years of age were positive, but at the end of 6th to 10th weeks there remained 9, 7, 3, 2 and zero out of 10. Similarly the carrier status in 2 and 5 year old mules was also evaluated. All mules were positive up to the 2nd week but at the end of 3rd to 7th weeks there were 6, 4, 2, 1 and zero out of 10 mules on the basis of culture while through PCR, all mules were positive up to 5th week but at the end of 6th to 10th weeks there were 8, 5, 2, 1 and zero. It is concluded that do not mix recovered mules from strangles with healthy equines at least for 9 weeks because the recovered mules remain carriers for prolonged period of time.

Key words: Mules; PCR; *Streptococcus equi*; carrier; Pakistan.

INTRODUCTION

Strangles is an infectious malady of equidae characterized by upper respiratory tract infection, dysponea, anorexia, regional suppurative lymphadenitis and causes high morbidity and low mortality. Once infected, the majorities of animals recover and eliminate *S. equi* over a period of 4–6 weeks. However, in 10% of clinically recovered cases *S. equi* may continue to be shed intermittently for prolonged periods. This carrier status is probably caused by incomplete drainage of exudate from the guttural pouches (empyaema) and/or sinuses following rupture of abscesses formed in the retropharyngeal lymph nodes (Newton *et al.,* 1997).

Carrier animals are considerable hazard to the horse industry. The 2-week quarantine period recommended by some authorities (Reif, 1979; McGee, 1970) might be insufficient to identify such animals since they are often clinically normal, and any signs of respiratory disease they might have are specific. Horses originating in herds in which strangles is enzootic or horses arriving as herd addition with inadequate histories therefore should be isolated while nasopharyngeal specimens are obtained for bacteriologic culture. Because of intermittent nature of shedding of *S. equi* and/or the difficulty of isolating and identifying S. *equi*, at least 3 culture-negative specimens taken several days or even weeks apart should be obtained before declaring an animal free of *S. equi* (George *et al.,* 1983).

The reason for the prolonged shedding of *S. equi* by some horses is not known. Age was not a risk factor for development of strangle in this study, suggesting a uniform exposure history before the arrival of mares onto the farm. This does not eliminate the possibility that carrier mare had some unusual treatment or exposure before being introduced into the farm population (George *et al.,* 1983).

There is considerable wealth of literature is available on *S. equi* in most horse raising communities in the world, but very little information on this pathogen in mules has been published. This study describes the carrier of *S. equi* in mules in Pakistan and will provide baseline data for further work on *S. equi* in mules.

MATERIALS AND METHODS

Twenty mules (10 <2 year and 10 of 2-5 years) infected with strangles from various private/public veterinary hospitals and stud farms located in Sargodha district of Punjab that remained positive after one week of recovery were monitored for 12 weeks. Nasal swab and washes were collected from every mule on weekly basis to study their carrier status through culture and PCR. Samples were processed at microbiology laboratories at the University of Veterinary and Animal Sciences, Lahore, Pakistan and the Gluck Equine Research Center, University of Kentucky, USA.

Isolation of *S. equi* and DNA extraction: Samples were cultured on blood agar plates and incubated at 37°C for 24 h under anaerobic conditions (Jorm, 1990). Typical beta hemolytic streptococci-like colonies were detected on blood agar and identified by characteristic colony morphology, Gram staining, and biochemical tests,

including catalase. Isolates identified as *S. equi* fermented salicin and sucrose, but not lactose, sorbitol, trehalose (Quinn *et al.,* 1994). The DNA from *S. equi* samples was extracted using the Genomic Purification Kit (Fermentas* #K0512). Ten to twenty mg of bacterial (*S. equi*) culture was suspended in a 1.5 ml eppendorf tube containing 200 µl TE buffer and 400 µl of lysis solution, incubated at 65ºC for 5 min in a water bath with slight shaking. Then 600 µl chloroform was added and centrifuged at 10,000 rpm for 2 min. The upper aqueous phase solution containing DNA was transferred to a fresh tube and 800 µl precipitation solutions was added, mixed at room temperature for 1-2 min and centrifuged at 10,000 rpm for 2 min. The supernatant was removed and the DNA pellet dissolved in 100 µl of 1.2M NaCl solution. The pellet was completely dissolved and 300 µl chilled absolute ethanol added to precipitate DNA (10 min at -20ºC) and centrifuged again at 10,000 rpm for 6-8 min. The supernatant was discarded and the pellet washed once with 100 µl 70% ethanol, allowed to dry completely, and dissolved in 100 µl of double distilled deionized water. The DNA samples were allotted the laboratory DNA number and stored at -20°C.

Polymerase chain reaction: The two primers were used in the present study. The forward primer of Se 18.7 (5´- AGT TTT AGC CAG TGC AGC AGC) and reverse primer of Se 18.7 (5´- TTA ATT CTC CAG ACT TTT CAA G) similarly the forward primer of EqbE (5´- AAG ATA TAG CAG CAT CGT ATC G) and reverse primer of EqbE (5´- TCT AAA TCT CTA TTA AAT AGC GGT ATA TTG) described (Heather *et al.,* 2008) were used to PCR amplify 481 bp and 130 bp, respectively unique to *S. equi* using ThermoJet Thermocycler. Cycling was as follows: 94°C for five min, 94°C for 1 min, 55°C for 1 min for both primers, and 72°C for 2 min, repeated 30 times; 72°C for ten min; and 4°C final. The PCR products were separated by ethidium bromide agarose gel electrophoresis. Data on comparison of culture and PCR of carrier mules was analyzed by Chi-square test using statistical package for social science (SPSS).

RESULTS AND DISCUSSION

Colony characteristic and beta hemolytic pattern of *S. equi* is shown in fig 1 & 2 whereas PCR of Se 18.7 and EqbE of isolates of *S. equi* generated products of 481 bp and 130 bp is shown in fig 3. Twenty mules (10 < 2 year and 10 between 2 and 5 years of age) remained positive after one week of infection were monitored for 12 weeks to study their carrier status. All mules < 2 years of age after the end of 2nd week were positive but at the end of 3rd to 6th weeks there remained 7, 3, 1 and zero out of 10 on the basis of culture. But when compared the results with PCR, at the end of the 5th week all mules < 2 years of age were positive, but at the end of 6th to 10th weeks there remained 9, 7, 3, 2 and zero out of 10. The carrier status in 2 and 5 year old mules was also evaluated. All mules were positive up to the 2nd week but at the end of 3rd to 7th weeks there were 6, 4, 2, 1 and zero out of 10 mules on the basis of culture. But through PCR, all mules were positive up to 5th week but at the end of 6th to 10th weeks there were 8, 5, 2, 1 and zero. Mules were declared free of infection on the basis of three consecutive negative samples through culture and PCR. Our findings are broadly consistent with the findings of Timoney (1988) who reported that a 6 week course of shedding may be more typical. The organism survives only for a short period in the environment unless protected in moist discharges. Our study also correlates with the study of Timoney and Artiushin (1997) who reported that the sensitivity of PCR appears to be much greater than the culture. Our findings are in agreement with the results of Kahn (2005) who reported that most horses continue to shed organism up to one month following recovery. Three negative nasopharyngeal swabs, at 4-7 days intervals, should be obtained prior to release from quarantine, and a minimum isolation period should be one month. Prolonged bacterial shedding has been identified in a small number of horses. Our results were also broadly consistent with the findings of Sweeny *et al.* (1989) who reported that the shedder state for *S. equi* implies that the equine harbors the *S. equi* organism without manifesting overt clinical signs of strangles. Georage *et al.* (1983) reported that 3 of 20 mares with strangles shed *S. equi* organism for at least 6 weeks after lymph node rupture and a fourth mare never had lymphadenopathy, but on arrival to the herd, it had cultures that were positive for *S. equi* and it continued to shed *S. equi* intermittently over the next 10 months. Before this the longest reported time between the disappearance of clinical signs of strangles and a culture positive specimen of *S. equi* was 4 months. Our study correlates with the finding of Sweeny (1990) who reported that horses with strangles may shed the organism for several weeks following clinical recovery, with one survey detecting the organism for up to 10 month after exposure. Similarly Woolcock (1975) suggested that the clinical disease within a population might be a pre-requisite for development of the shedder state because he was unable to isolate *S. equi* from horses on farms with strangles but without active cases at the time of study. Sweeny *et al.* (1989) also reported failure to isolate *S. equi* from horses which never developed strangles. It suggests that shedders of *S. equi* among horses that never manifest clinical signs of strangles are rare. We believe that *S. equi* shed in nasal secretions in horses recently recovered from strangles is the most likely source of the organism for susceptible horses. Newton *et al.* (2000) identified that after the clinical signs are abolished from the animals, the animal remains in the carrier state and the most predominant site for *S. equi* was the guttural

pouch. He also reported that the prolonged carrier of *S. equi*, which lasted up to 8 months, was again symmetrical with his study. Our results were also broadly consistent with the findings of Sweeny *et al*. (2005) who reported that healthy horses recovering from recent strangles disease might continue to harbor the *S. equi* after a full clinical recovery. There is evidence that a moderate proportion of horses continue to harbor *S. equi* for several weeks after clinical signs have disappeared, even though the organism is no longer detectable in the majority 4 to 6 weeks after total recovery. A recovered horse may be a potential source of infection for at least 6 weeks after its clinical signs of strangles have resolved. Our results were also in line with the results of Georage *et al*. (1983) who recorded that infected horses can shed *S. equi* at least 4 weeks after the onset of clinical signs and the premises might harbor the organism for a period of one year or longer. Although outbreaks may be initiated by the introduction of clinically normal animals into a herd, diseases may become enzootic on premises resulting in periodic outbreaks when the number of susceptible animals increases.

It is concluded that do not mix recovered animals from strangles with healthy animals at least for 9 weeks because the recovered animals remain carriers for prolonged period of time (6-9 weeks). Periodic shedding of *S. equi* can be a source of infection for susceptible animals.

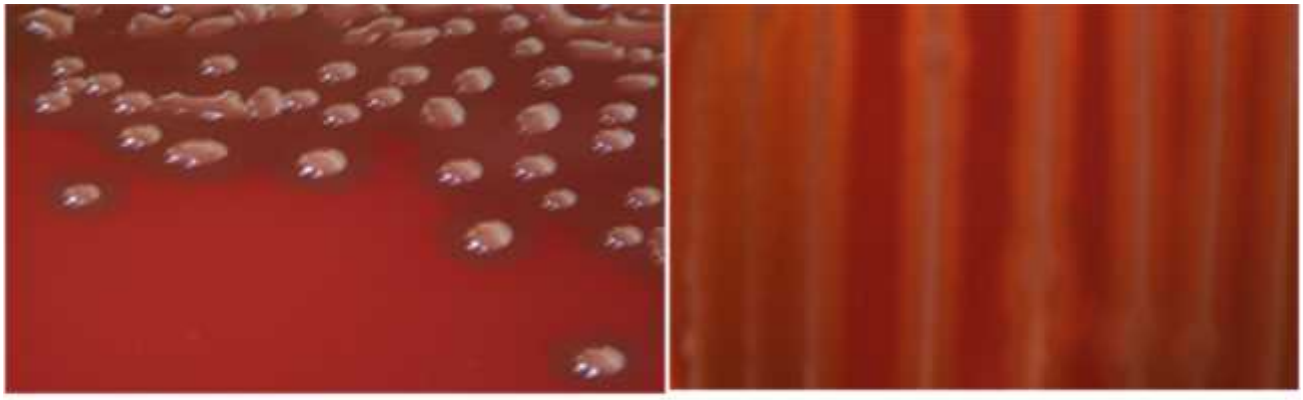

Fig 1: Colony characteristics of *S. equi*

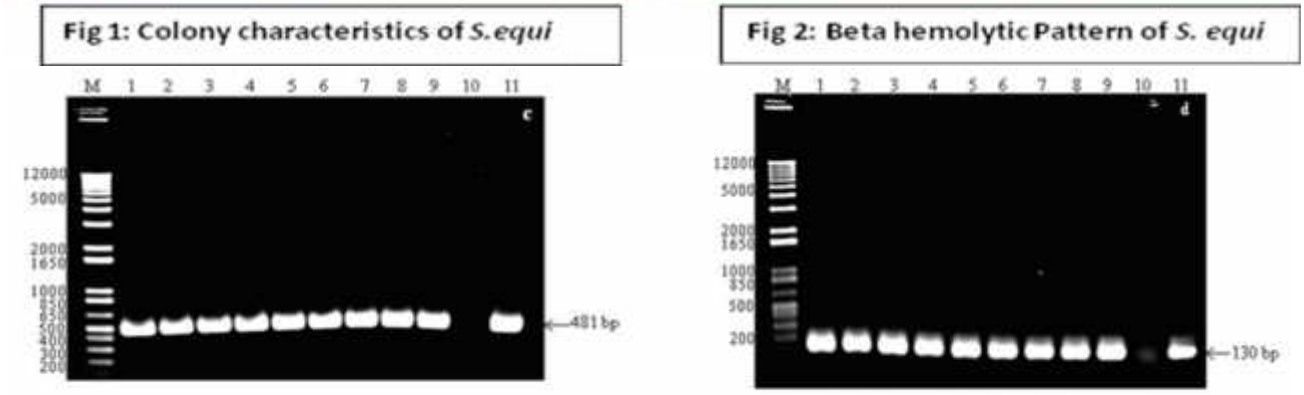

Fig 2: Beta hemolytic Pattern of *S. equi*

Fig. 3: PCR amplification of DNAs from mules isolates of *S. equi Se18.7* (c) and *EqbE* (d). Lanes 1-9 (samples), Lane 10 *S. zooepidemics* W60 (-ve control) and Lane 11 *S. equi* CF32 (+ve control).

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