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Use of Repetitive Sequence-Based Polymerase Chain Reaction to Characterize *Streptococcus equi* Subspecies *Equi* Isolates Cultured from Horses Experiencing Post-Vaccinal Reaction

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Abstract: Strangles caused by *Streptococcus equi* subspecies *equi* is a continuous challenge to horse owners and practitioners. This led to great effort in developing vaccines against this disease. The goal of this study was to characterize *S. equi* isolates obtained from horses suffering from post vaccinal reactions following the use of an intranasal vaccine Pinnacle IN™, Fort Dodge Animal Health. Fifteen *S. equi* isolates obtained from several distinct post vaccinal reaction episodes in Colorado, Florida, Kansas, Kentucky, Minnesota and Ohio, USA. Clinical signs in these post vaccinal reactions episodes included fever, localized neck abscesses, strangles, or purpura hemorrhagica. *S. equi* was cultured from neck abscesses and/or lymph nodes. Repetitive element based polymerase chain reaction (rep-PCR) was performed on the post vaccinal reaction isolates and the vaccine strain. DNA analysis showed that all of the post vaccinal reaction isolates except one obtained from various geographic locations were identical to the intranasal vaccine strain. One isolate was found to be genetically distinct from the intranasal vaccine strain using the rep-PCR. These findings clearly suggest that the horses exhibited complication as a result of vaccination except in one incidence. Whether these complications occurred from a return to virulence by the organism, immune reaction of the host or inappropriate administration of the vaccine is unclear.

Key words: *S. equi*, vaccine, strain, Rep-PCR, horse

INTRODUCTION

Streptococcus equi subspecies *equi* causes strangles a highly contagious disease of horses (Timoney, 2004). The disease is characterized by signs of nasal discharges and enlargement of lymph nodes in the head and upper neck region. On occasion, the disease may spread infiltrating other organs including the liver, the lungs, the mesenteric lymph nodes and the brain (Sweeny *et al*., 1987). The disease significantly impact the equine industry because of its high morbidity that may reach 100%, world wide distribution and expenses associated with management and hygiene procedures to eliminate and control outbreaks.

Despite the fact that vaccination of horses against strangle has been practiced for decades, results of vaccination were not rewarding. Bacterin-based vaccine, that was heat inactivated, was used in Australia in the 1940s (Bazely, 1942) and later in the US in the 1960s. Vaccinated horses developed adverse reaction such as inflammation and abscess formation at the site of injection as well as muscle soreness. Evaluation of protein extract based vaccines that were developed in the 1970-80 indicated they were more effective and cause less vaccine reactions. However, vaccinated horses failed to develop protective mucosal immunity similar to horses recovering from natural infection (Sheoran *et al*., 1997). To overcome this obstacle a live attenuated non-encapsulated genetically modified vaccine, Pinnacle IN™ (Fort Dodge, Animal Health, Overland Park, KS, USA) was developed. This vaccine is administered intranasaly, in 2 doses and at an interval of one to two weeks. Therefore, it is aimed to induce mucosal immune response as well as systemic response.

Since, the vaccine has been introduced into the market in the 1998, we have observed horses developing adverse reactions to the vaccine. *S. equi* was isolated in a pure culture from several episodes of horses with post

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vaccinal complications. Our goal was to determine whether the vaccine strain, Pinnacle IN™, was genetically similar to those isolates obtained from horses experiencing post vaccinal reactions. To achieve this goal, DNA fingerprinting was performed utilizing multiple DNA testing.

MATERIALS AND METHODS

Sample population: Fifteen *S. equi* isolates were obtained from referring veterinarians in Colorado, Florida, Kansas, Kentucky, Minnesota and Ohio (Table 1). On receipt, a single colony of bacteria was grown in brain-heart infusion broth (Sigma Biochemicals, St Louis, MO, USA) at 37°C for 24 h. The media was centrifuged at 1,500× g for 10 min and the pellet was resuspended in one ml of Todd Hewitt broth (Sigma Biochemicals, St Louis, MO, USA) with 20% glycerol. Material was frozen at -80°C until analyzed. The Pinnacle IN™ vaccine strain was obtained from the pharmacy at the Veterinary Teaching Hospital, University of Minnesota, MN, USA. The vaccine was reconstituted as described by the manufacturer and then the bacterium was growing as of the other post vaccinal isolates.

Identification of bacterial isolates: Identity of all *S. equi* isolates was determined by use of a commercially available test bioMeriease Vitak Inc, Hazelwood, MO, USA). Only isolates that had excellent identification were chosen for genetic investigation.

DNA extraction: A lawn of bacteria was grown on brain-heart infusion agar plates at 37°C with 5% CO_2 for 24 h. DNA was extracted using a DNA extraction kit according to the manufacturer recommendations (Sigma Biochemicals, St Louis, MO, USA). Finally, purity of the DNA was examined by use of gel electrophoresis in 1.5% agarose gel (Sigma Biochemicals, St Louis, MO, USA) stained with 0.5 μg of ethidium bromide (Sigma Biochemicals, St Louis, MO, USA) mL^{-1} in the gel and in 1× tris-acetate-EDTA buffer. Concentration of DNA was determined by spectrophotometry to ensure high purity and adequate amounts of DNA templates.

Repetitive element based polymerase chain reaction (REP-PCR): The rep-PCR was performed, using primers ERIC1R (5'-ATG-TAA-GCT-CCT-GGG-GAT-TCA-C-3') and ERIC2 (5'-AAG-TAA-GTG-ACT-GGG-GTG-AGC-G-3') (Genosys, The Woodlands, TX, USA) as well as REP-primers REP1R (5'-III-ICG-ICG-ICA-TCI-GGC-3') and REP2I (5'-ICG-ICT-TAT-CIG-GCC-TAC-3') (Genosys, The Woodlands, TX, USA) as described elsewhere (Versalovic *et al*., 1991). The reaction mixture (25 μL) consisted of 100 ng of purified genomic DNA, 1 μM of each primer, 1X PCR-buffer II, 200 μM of each of dATP, dCTP, dGTP and dTTP, 3 mM $MgCl_2$ and 1 U Taq DNA polymerase and A thermal cycler was used to perform the reaction (all Perkin Elmer, Norwalk, CT, USA). In every rep-PCR experiment, a negative control sample containing all the reagents but none of the genomic DNA template was included. The PCR included 35 cycles (denaturation step at 94°C for 30 sec, annealing step at 51°C for 3 min and extension step at 72°C for 2 min). These cycles were preceded by a denaturation step at 94°C for 5 min. At the end of PCR, an additional extension step at 72°C for 7 min was added.

Table 1: ERIC and REP PCR subtypes of vaccine reaction isolates and the vaccine strain with refrence to the geographic source of isolates

Isolate	Source	ERIC subtype	REP subtype
5	Minnesota	A	A
9	Minnesota	A	A
13	Minnesota	A	A
15	Minnesota	A	A
16	Minnesota	B	B
23	Minnesota	A	A
24	Minnesota	A	A
8	Colorado	A	A
17	Kansas	A	A
11	Kentucky	A	A
19	Kentucky	A	A
21	Kentucky	A	A
20	Ohio	A	A
14	Florida	A	A
SEVS	PinnacleTM	A	A

Gel electrophoresis: Eight microliters of the rep-PCR product was mixed with 2 μL of loading buffer (0.2% Orange G in 50% glycerol), then examined on 1.5% agarose gel stained with 0.5 μg of ethidium bromide mL^{-1} in the gel and in 1×tris-acetate-EDTA buffer. For each gel electrophoresis that was conducted, a 100-base pair (bp) DNA marker (Roche Diagnostics Corp., Indianapolis, IN, USA) was used as a size marker as well as a reference for the normalization of each gel. The product was electrophoresed at 85 V for 2 h. Each gel was photographed under UV light, using a gel documentation system (Bio-Rad Hercules, CA, USA). Banding pattern of DNA fingerprinting was used to determine similarities or differences among *S. equi* isolates.

Data analysis: Photographs of gels of rep-PCR products were analyzed using a computed assisted program (Molecular Analyst, Bio-Rad Hercules, CA, USA). Dice coefficient was used to analyze the fingerprinting pattern and a dendrogram was calculated by use of the unweighted-pair group method, using average linkage (Sneath and Sokal, 1973; Hunter and Gaston, 1988).

RESULTS

Rep-PCR utilizing ERIC primers produced a band pattern that ranged from 10 to 12 bands (Fig. 1). The PCR product ranged in size from 100-2000 bp. Following the analysis of the band patterns with Molecular Analyst a dendrogram was generated (Fig. 2). Eight post-vaccinal reaction isolates were 100% identical to the vaccine strain. Two other isolates (SEVR5 and 14) were 95% similar to the vaccine strain. SEVR20 isolate was less than 90% similar to the vaccine strain while SEVR16 isolate was less than 85% similar to the vaccine strain. At 85% similarity cut off point, all post vaccinal reaction isolates but one (SEVR16) were genetically similar to the vaccine strain.

The use of REP primers produced a band pattern that ranged from 5-6 bands (Fig. 3). The PCR product ranged in size from 300-2000 bp. Following the analysis of the band patterns with Molecular Analyst a dendrogram was generated (Fig. 4). All the post-vaccinal reaction isolates were 100% identical to the vaccine strain except one isolate (SEVR16). This isolate shared around 90% similarity to the vaccine strain.

Post-vaccinal reactions isolates from various geographic sources were located in one rep-PCR subtype with the exception of isolate 16 (Table 1). This isolate was obtained from Minnesota.

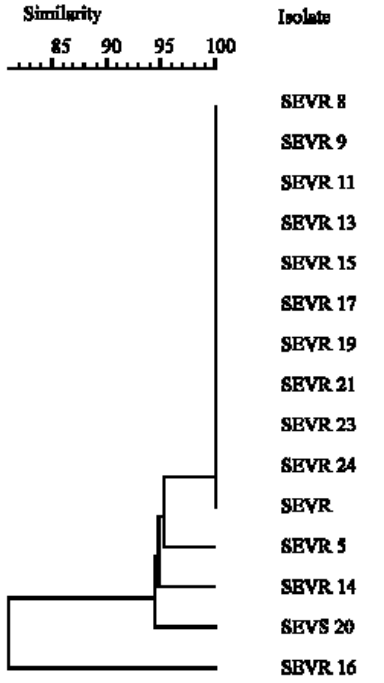

Fig. 2: Computer generated dendrogram showing 95% similarity between all vaccine reaction isolates except (SEVRS 16) and the vaccine strain using ERIC primers

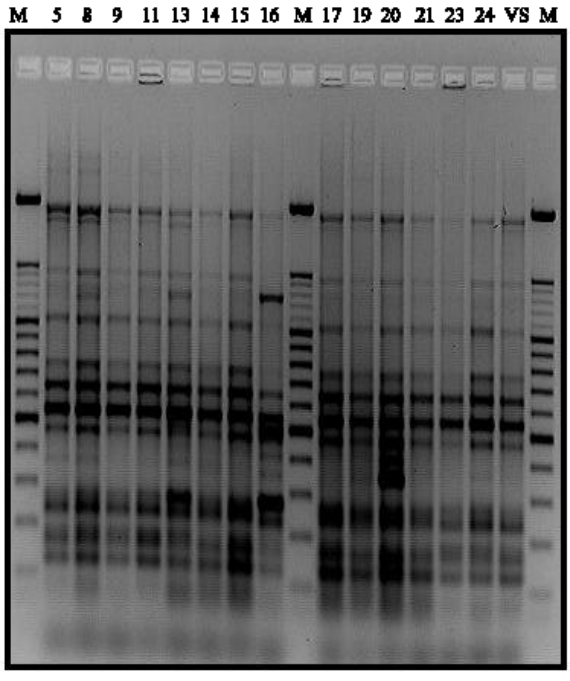

Fig. 1: Gel image of amplication using ERIC primers of SEVRS (5, 8, 9, 11, 13, 14, 15, 16, 17, 19, 20, 21, 23, 24) isolates and the vaccine strain (VS), M: 100 bp marker

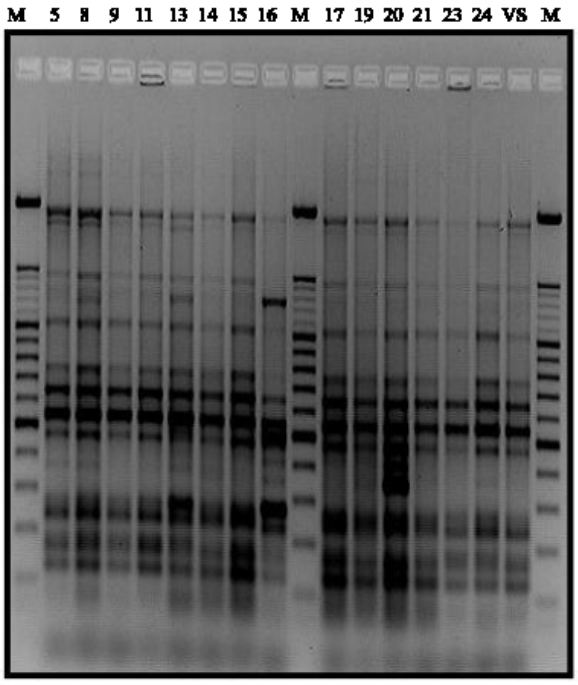

Fig. 3: Gel image of amplication using ERIC primers of SEVRS (5, 8, 9, 11, 13, 14, 15, 16, 17, 19, 20, 21, 23, 24) isolates and the vaccine strain (VS), M: 100 bp marker

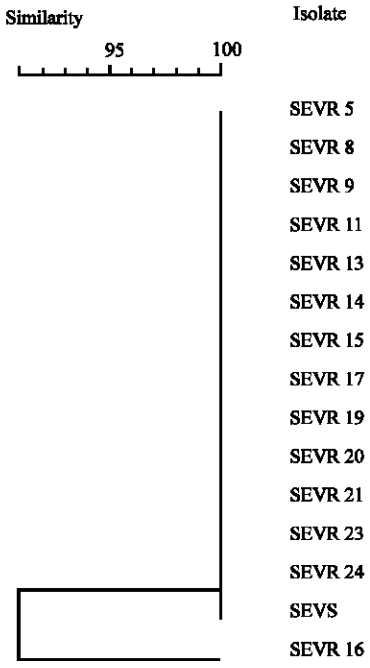

Fig. 4: Computer generated dendrogram showing 100% similarity between all vaccine reaction isolates except (SEVRS 16) and the vaccine strain using PER primers

DISCUSSION

The use of the Pinnacle IN ™ was aimed to induce local immune response in the upper respiratory mucosa at the entrance site of the *S. equi* as well as a systemic immune response. Therefore maximum protection is provided to horses against natural exposure to the disease. Although adverse vaccine reactions have been reduced, *S. equi* was grown from several horses that experienced post vaccinal complication. These horses were located on several farms spread in scattered geographic locations in the USA. To determine whether these isolates were genetically similar to the strain used in the Pinnacle IN ™ vaccine, DNA fingerprinting analysis utilizing rep-PCR was carried out. Rep-PCR using ERIC primers has been used successfully to differentiate *S equi* to the strain level (Al-Ghamdi *et al*., 2000). In addition the use of pulsed field gel electrophoresis utilizing restriction endonucleases such as *Not*I has shown that *S. equi* can be differentiated to the strain level (Takai, 2000). In this study, rep-PCR using ERIC primers differentiated the sample group into 2 subtypes at 85% cut off point. The first subtype included all the post vaccinal reaction isolates, except one isolate and the Pinnacle IN ™ strain. The second subtype included one post vaccinal reaction isolate. Eight isolates were a 100% genetically identical and they were a 100% identical to the Pinaccal IN™ vaccine strain. Two isolates were at least 95% similar to the vaccine strain and two isolates shared almost 95% similarity to the vaccine strain. Finally one isolate was almost 90% similar to the vaccine strain. This suggests that these isolates originated from a single common source which is the Pinnacle IN ™ vaccine strain.

In this research, ERIC-PCR was chosen as our reference test since it has been shown to have strong discrimination power to differentiate *S. equi* isolates. However, additional tests were performed to further confirm our findings. Rep-PCR utilizing REP primers, although produced only half the number of the bands produced by the ERIC primers, showed high reproducibility. In this work the use of REP primers indicated that all isolates except one single isolate, were a 100% identical to the Pinnacle IN ™ vaccine strain. Clearly the REP primers showed agreement with the ERIC primers. AFLP is a very powerful technique that is used to differentiate bacterial isolates to the strain level (Vos, 1995). Despite the fact that this test has not been used to examine wild isolates of *S. equi* before, it revealed higher number of bands than the number of bands produced by rep-PCR (Data not shown). However, interestingly the AFLP results showed that all isolates were over 95% similar. This included the isolates that were found different from the vaccine strain using rep-PCR.

The use of rep-PCR to test the genetic relationship between *S. equi* isolates obtained from various geographic locations showed high genetic diversity. Numerous rep-PCR subtypes were produced (Al-Ghamdi, 2000). In this study, *S. equi* isolates were obtained from several geographic locations. Nevertheless, a high similarity existed between all isolates. The two isolates that showed slight genetic differences from the vaccine strain were obtained from Minnesota and Ohio. On the other hand isolates obtained from Florida, Kentucky, Colorado and several isolates from Minnesota were clearly identical to the vaccine strain. Therefore, such genetic similarity despite variation in the geographic source of isolates, strongly support a common clone, Pinnacle IN™ vaccine strain, being the original source of the majority of isolates.

The pathogenesis of the post vaccinal complication is unknown. Previous reports described complications associated with vaccines against strangles (Smith, 1994). Nonetheless finding a scenario that might explain the complications following the Pinnacle IN™ vaccines might be difficult. Accidental intramuscular injection of the vaccine and or reuse of a contaminated syringe were suggested. However, practitioners involved in the post vaccinal reaction episodes have ruled out any inappropriate administration. Other possible explanation

was contamination of the site of the injection as a result of aerosal spread of the bacteria. In addition hematogenous spread of the bacteria to the inflammed site of intramuscular injection can not be ruled out. Finally, retain of the virulence of the organism and or variations in the immune response of horses to the vaccine are factors that remain to be examined. All these scenarios remain speculations and the exact mechanism of the complications is not clear.

CONCLUSION

The vaccine strain, Pinnacle IN™, is responsible for the complications following vaccination. Careful handling of the vaccine by the practitioner cannot be over emphasized.

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