

Characterization of Strangles-Episodes in Horses Experiencing Post-Vaccinal Reaction

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Abstract: Strangles that is caused by *Streptococcus equi* sp. *equi* is a highly contagious respiratory disease of horses. Prevention of the disease has been attempted using killed, protein extract-based and live genetically modified vaccines. The goal of this study is to characterize post-vaccinal complications following the use of an intranasal vaccine Pinnacle IN™, Fort Dodge Animal Health Animal Health, Overland Park, KS, USA. Horses residing farms in Colorado, Florida, Kansas, Kentucky, Minnesota and Ohio were examined. Fourteen *S. equi* isolates were cultured from various animals. Amplified Fragment Length Polymorphism (AFLP) was performed on the post vaccinal reaction isolates and the vaccine strain. Clinical signs in these post vaccinal reactions episodes included fever, localized neck abscesses or purpura hemorrhagica. DNA analysis showed that all of the post vaccinal reaction isolates that were obtained from various geographic locations were identical to the intranasal vaccine strain. These findings clearly suggest that the horses exhibited various clinical disease that is a result of vaccination.

Key words: *Streptococcus equi*, vaccine, complications, AFLP, disease, animal

INTRODUCTION

Strangles is a highly contagious disease of horses that is manifested by signs of nasal discharges (serous to mucopurulent) and enlargement of lymph nodes in the head and upper neck region (Timoney, 1993). A complicated form of the disease may occur due to the spread of the disease to other organs including the liver, the lungs, the mesenteric lymph nodes and the brain (Sweeney *et al.*, 1987). The impact of the disease is highly significant on the equine industry as a result of its high morbidity that may reach 100%, world wide distribution and expenses associated with management and hygiene procedures to eliminate and control outbreaks.

Streptococcus equi sp. *equi* the causative agent of strangles is a gram-positive organism that positively responds to antibiotic treatment (Hardie, 1986). Development of protective vaccines against strangles has been faced with many obstacles. The use of Bacterin-based vaccine that was heat inactivated was associated with adverse reactions such as inflammation and abscess formation at the site of injection as well as muscle soreness (Bazeley, 1942; Smith, 1994). In addition, a protein extract based vaccines that was aimed to be more effective and cause less vaccine reactions, failed to develop protective mucosal immunity similar to horses recovering from natural infection (Hoffman *et al.*, 1991; Sheoran *et al.*, 1997). To ssssoverride this obstacle a live

attenuated nonencapsulated genetically modified vaccine, Pinnacle IN™ (Fort Dodge, Animal Health, Overland Park, KS, USA) was developed (Walker and Timoney, 2002). This vaccine is administered intranasally so mucosal immune response as well as systemic response are induced.

Following the introduction of the vaccine in 1999 into the market, several incidences of horses experiencing adverse vaccine reactions were observed. *S. equi* was isolated in a pure culture from several episodes of horses with post vaccinal complications. The goal of this research was to describe the clinical impact of the vaccine and to determine whether the vaccine strain, Pinnacle IN™ was genetically similar to those isolates obtained from horses experiencing post vaccinal reactions.

MATERIALS AND METHODS

Horse farms were located in multiple states including Colorado, Florida, Kansas, Kentucky, Minnesota and Ohio (Table 1). Horses were clinically examined by referring veterinarians for possible complications following strangles vaccination. Fourteen *S. equi* isolates were grown from clinical samples that were obtained in the years of 1999-2000. 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 1,500X g for 10 min and the

Table 1: Source of cases, isolates, clinical findings and AFLP subtypes

Case	Isolate	Source	Clinical findings	AFLP subtype
5	5	Minnesota	Fever, localized neck abscess	A
9	9	Minnesota	Fever, localized neck abscess	A
13	13	Minnesota	Fever, depression	A
15	15	Minnesota	Fever, localized neck abscess	A
16	16	Minnesota	Fever, depression	A
23	23	Minnesota	Purpura hemorrhagica	A
24	24	Minnesota	Fever, depression	A
8	8	Colorado	Purpura hemorrhagica	A
17	17	Kansas	Fever, depression	A
11	11	Kentucky	Fever, depression	A
19	19	Kentucky	Fever, localized neck abscess	A
21	21	Kentucky	Fever, depression	A
20	20	Ohio	Fever, depression	A
14	14	Florida	Fever, localized neck abscess	A
Vaccine	SEVS	PinnacleTM	NA	A

(Sigma Biochemicals) with 20% glycerol. Material was frozen at -80°C until analyzed. The Pinnacle INTM vaccine strain was obtained from the pharmacy at the Veterinary Teaching Hospital, University of Minnesota. The vaccine was reconstituted as described by the manufacturer and then the bacterium was growing as of the other post vaccinal isolates. Identity of all *S. equi* isolates was determined by use of a commercially available test (bioMeriase 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% CO₂ for 24 h. DNA was extracted using a DNA extraction kit according to the manufacturer recommendations (Sigma Biochemicals). Finally, purity of the DNA was examined by use of gel electrophoresis in 1.5% agarose gel (Sigma Biochemicals) stained with of ethidium bromide 0.5 µg mL⁻¹ (Sigma Biochemicals) in the gel and in 1X tris-acetate-EDTA buffer. Concentration of DNA was determined by spectrophotometry to ensure high purity and adequate amounts of DNA templates.

Amplified Fragment Length Polymorphism (AFLP): AFLP was performed using the AFLP microbial Fingerprinting kit protocol (Applied Biosystem, Foster City, CA, USA) based on the manufacturer recommendations. *S. equi* DNA was digested with endonuclease enzymes EcoRI and MseI. Following restriction digestion, the restricted fragments were ligated to EcoRI and MseI adapters. The reaction of restriction ligation included 100 ng genomic DNA, 5 U EcoRI, 5 U MseI, 1 U T4 DNA ligase and 0.5 µM of adapter pairs in T4 DNA ligase buffer (50 mM Tris-HCl (pH 7.8) 10 mM DTT, 1 mM ATP, 25 µg BSA) (all New England Inc., Beverly, MA USA). Preselective amplification was performed in a final reaction volume of 20 µL that

contained 4 µL of the product of the restriction ligation reaction, 125 pmol of AFLP preselective primer pairs (EcoRI preselective primer: 5'-GACTGCGTACCAATTC and MseI preselective primer 5'-GATGAGTCCTGAGTAA) and 15 µL AFLP core mix (Applied Biosystem). PCR reaction was performed in a thermal cycler (GenAmp 9800 thermal cycler, Perkin Elmer, Norwalk, CT, USA). The reaction included initial denaturation at 72°C for 2 min, followed by 20 PCR cycles that included denaturation at 94°C for 1 sec, annealing at 56°C for 30 sec and an extension step at 72°C for 2 min. A final extension step at 60°C for 30 min was added. Selective amplification was performed using fluorescent labeled EcoRI selective primer containing an additional A base (FAM-5'-GAC-TGC-GTA-CCA-ATT-CA) (Applied Biosystem). The reaction volume of 10 µL included 1.5 µL of the preselective amplification products, 25 pmol of FAM-EcoRI-A primer and 7.5 µL of AFLP core mix (Applied Biosystem).

Amplification product was aliquoted and 1 µL of the aliquot was mixed with 1 µL of sterile dH₂O and electrophoresed in 5% Long Ranger gels (Applied Biosystem) An ABI Model 377 automated DNA fragment analyzer was used. An internal size standard (GS-500, Rox size standard, Applied Biosystem) was included in each lane to rule out intragel variation. GeneScan (Applied Biosystem) was used to analyze the AFLP fingerprinting results. Following that Genotyper Software (Applied Biosystem) was used to filter and export the data to be suitable for analysis using Molecular Analyst (Bio-Rad, Hercules, CA, USA).

Data analysis: Photographs of gels of AFLP results were analyzed using a computed assisted program (Molecular Analyst, Bio-Rad). 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 (Hunter and Gaston, 1988).

RESULTS

Clinical examination: Clinical examination of the affected horses revealed variable clinical signs that rang between mild disease characterized by fever, depression and localized neck abscess (Fig. 1). Eleven horses were mildly affected. These horses positively responded to antiinflammatory (flunixin meglumine, 0.5 mg kg⁻¹, TID), antibiotic (penicillin 22000, U kg⁻¹, BID) treatment and supportive care (3% povidone-iodine) for localized neck abscesses flush. More severe form of the disease was



Fig. 1: Localized neck abscess at 6-8 cm depth was most common clinical finding

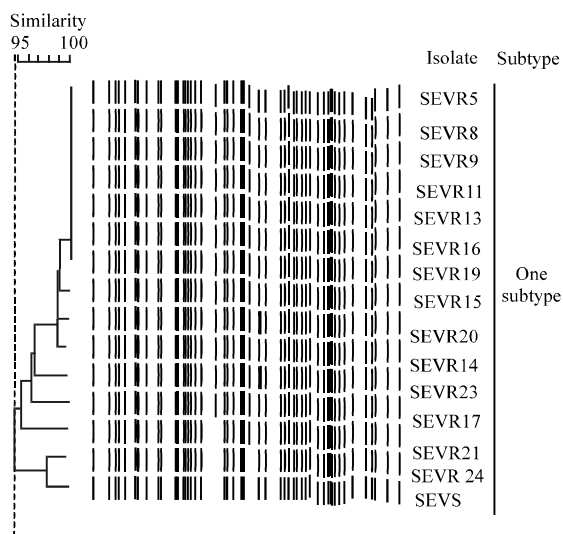


Fig. 2: AFLP detected only one genetic pattern at 95% similarity

hemorrhagica (Table 1). These horses responded to antiinflammatory (dexamethasone 0.1 mg kg^{-1} , BID) and antibiotic (penicillin 22000 U kg^{-1} , BID) treatment.

Amplified Fragment Length Polymorphism (AFLP): The *S. equi* genomic DNA was cut with EcoRI and MseI, amplified with preselective primer pair EcoRI and MseI and then with the selective primers MseI-0 and EcoRI-A. Following the computer analysis using molecular analyst, a dendrogram was generated (Fig. 2). At a cut off point of 95%, there is only one AFLP subtype (Table 1). The post vaccinal reaction isolates and the Pinnacle INTM strain shared at least 95% similarity.

DISCUSSION

The use of antibiotic treatment for strangles is regularly practiced but protection against natural infection

with *S. equi* has been challenging (Sweeny, 2002). The use of whole organism-based vaccine and protein components did not yield satisfactory outcome (Smith, 1994; Hoffman *et al.*, 1991). Since, horses recovering from strangles usually develop mucosal and systemic immunity, therefore the aim of the Pinnacle INTM was to induce maximum protection via local immune response in the upper respiratory mucosa at the entrance site of the *S. equi* as well as a systemic immune response (Sheoran *et al.*, 1997; Walker and Timoney, 2002). Although, adverse vaccine reactions have been reduced, *S. equi* was grown from several horses that experienced post vaccinal complications. These complications were mild in most cases including fever, depression and localized neck abscess. The development of localized neck abscesses may be due to accidental intramuscular injection of the vaccine and or reuse of a contaminated syringe. However, such scenario has been downgraded following accurate questioning of the practicing veterinarians. Other possibilities were contamination of the site of the injection as a result of aerosol spread of the bacteria or hematogenous spread of the bacteria to the inflamed site of intramuscular injection. More severe form of the disease with signs of purpura hemorrhagica was observed in two horses. This variation in the severity of the post vaccinal complications may reveal the difference in the immune experience of horses.

To determine whether these isolates were genetically similar to the strain used in the Pinnacle INTM vaccine, DNA fingerprinting analysis utilizing AFLP was carried out. The AFLP is a powerful typing method that has been used to subtype several bacteria to the strain level (Aats *et al.*, 1998; Gibson *et al.*, 1999; Kokotovic and On, 1999; Vos *et al.*, 1995). In addition, *S. equi* has been differentiated into genetic subtypes using Rep-PCR and pulsed field gel electrophoresis utilizing restriction endonucleases such as NotI has differentiated the bacterium to the strain level (Amonsens *et al.*, 2008; Takai *et al.*, 2000). In this study, AFLP produced high number of bands but they were reproducible over time. This gives the AFLP more advantage to differentiate *S. equi*. However, interestingly the AFLP results showed that all isolates were >95% similar. This included the isolates that were found different from the vaccine strain using rep-PCR in earlier research (Amonsens *et al.*, 2008). Clearly these findings strongly support the earlier rep-PCR work that suggested a single common source which is the Pinnacle INTM vaccine strain to these isolates.

In this study, *S. equi* isolates were obtained from several geographic locations. The use rep-PCR generated several subtypes of *S. equi* isolates from various

geographic sources (Al-Ghamdi *et al.*, 2000). Therefore, the high genetic relationship despite variation in the geographic source of isolates, strongly support a single clone, Pinnacle IN™ vaccine strain being the original source of the isolates.

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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REFERENCES

- Aats, H.J.M., L.A.T. van Lith and J. Keijer, 1998. High-resolution genotyping of *Salmonella* strains by AFLP-fingerprinting. *Let Applied Microbiol.*, 26: 131-135.
- Al-Ghamdi, G.M., V. Kapur, T.R. Ames, D.N. Love, J.F. Timoney and M.A. Mellencamp, 2000. Use of repetitive sequence-based polymerase chain reaction for molecular epidemiologic analysis of *Streptococcus equi* subspecies *equi*. *Am. J. Vet. Res.*, 61: 699-705.
- Amonsén, A., C.J. Gebhart, G.M. Al-Ghamdi, T.R. Ames and V. Kapur, 2008. Use of repetitive sequence-based polymerase chain reaction to characterize *Streptococcus equi* subspecies *Equi* isolates cultured from horses experiencing post-vaccinal reaction. *J. Anim. Vet. Adv.*, 7: 892-896.
- Bazeley, P.L., 1942. Studies with equine streptococci. 3. vaccination against strangles. *Aust. Vet. J.*, 18: 189-194.
- Gibson, J.R., M.A. Ferrus, D. Woodward, J. Xerry and R.J. Owen, 1999. Genetic diversity in *Helicobacter pullorum* from human and poultry sources identified by an amplified fragment length polymorphism technique and pulsed-field gel electrophoresis. *J. Applied Microbiol.*, 87: 602-610.
- Hardie, J.M., 1986. *Genius Streptococcus* Rosenbach 1884, 22AL. In: *Bergey's Manual of Systemic Bacteriology*, Sneath, P.H.A., N.S. Mair, M.E. Sharpe and J.G. Holt (Eds.). Vol. 2. The Williams and Wilkins Co., Baltimore, MD., pp: 1043-1071.
- Hoffman, A.M., H.R. Staempfli, J.F. Prescott and L. Viel, 1991. Field evaluation of a commercial M-protein vaccine against *Streptococcus equi* infection in foals. *Am. J. Vet. Res.*, 52: 589-592.
- Hunter, P.R. and M.A. Gaston, 1988. Numerical index of the discriminatory ability of typing system: An application of Simpsons index of diversity. *J. Clin. Microbiol.*, 26: 2465-2466.
- Kokotovic, B. and S.L. On, 1999. High-resolution genomic fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* by analysis of amplified fragment length polymorphism. *FEMS Microbiol. Let.*, 173: 77-84.
- Sheoran, A.S., B.T. Sponseller, M.A. Holmes and J.F. Timoney, 1997. Serum and mucosal antibody isotype response to M-like protein (SeM) of *Streptococcus equi* in convalescent and vaccinated horses. *Vet. Immunol. Immunopathol.*, 59: 239-251.
- Smith, H., 1994. Reaction to strangles vaccination. *Aust. Vet. J.*, 71: 257-258.
- Sweeney, C.R., R.H. Whitlock, D.A. Meirs, S.C. Whitehead and S.O. Barningham, 1987. Complications associated with *Streptococcus equi* infection on a horse farm. *J. Am. Vet. Med. Assoc.*, 191: 1446-1448.
- Sweeney, C.R., 2002. *Streptococcus equi* Infections (Strangles). In: *Large Animal Internal Medicine*, Smith, B. (Ed.). 3rd Edn., Mosby MT., USA., pp: 504-507.
- Takai, S., T. Anzai, H. Yashiro, C. Ishii, S. Tsubaki, R. Wada and J.F. Timoney, 2000. Detection of DNA restriction fragment polymorphisms in *Streptococcus equi*. *Vet. Rec.*, 146: 159-161.
- Timoney, J.F., 1993. Strangles. *Vet. Clin. North Am. Equine Practice*, 9: 365-374.
- Vos, P., R. Hogers, M. Bleeker, M. Reijans and T.V.D. Lee *et al.*, 1995. Hornes AFLP: A new technique for DNA fingerprinting. *Nucl. Acids Res.*, 23: 4407-4414.
- Walker, J.A. and J.F. Timoney, 2002. Construction of a stable non-mucoid deletion mutant of the *Streptococcus equi* Pinnacle vaccine strain. *Vet. Microbiol.*, 89: 311-321.