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ISOLATION AND MOLECULAR IDENTIFICATION OF *STREPTOCOCCUS EQUI* SUB SPECIES *EQUI* STRAINS AND VACCINES PREPARATION, EVALUATION OF THEIR EFFICACY IN LABORATORY ANIMALS AND HORSES

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ABSTRACT

The ability of inactivated whole Streptococcus equi cell vaccines to give rise to protective antibody levels was studied in rabbits by serological means. The immunogenicity of vaccines prepared with field isolates was tested in rabbits and mice. Antibody titers were measured by Passive Heam Agglutination (PHA) test. Seven strains of Streptococcus equi subsp. equi were isolated, one classified biochemically as typical and other six as *S.equi subsp*. zooepidemicus. Results of PHA suggested that prepared vaccine from typical field strain give protective antibody titer against typical field strain but not against atypical strain and atypical field vaccine give protective immunity against atypical strains but not against typical field strain and suggested that combined vaccine give immunity against typical and atypical strains.

Keywords:

Streptococcus equi sub species equi, Streptococcus equi subspecies zooepidemicus, PHA test, combined vaccine.

INTRODUCTION

Strangles is one of the most widespread and costly horse disease worldwide and it leads to devastating epidemics in stables where horses are housed. This is an acute, contagious and deadly respiratory tract disease, which typical signs of infection include pyrexia, suppurative, mucopurulent nasal discharge, lymphadenitis and abscessation, often in the lymph nodes of head and neck **(Waller *et al*., 2007).** It occasionally affects other lymph nodes and organs, resulting in a severe stage of the disease called bastard strangles. *S. equi* subspecies *equi* is the causative agent of strangles. Because *S. equi* persists in the environment for only a few weeks, the most important factor for the maintenance of infection is the infected horse **(Sweeney *et al*., 1987)**. Even if generally after 4-6 weeks the infected animals recover from disease

eliminating *S. equi* from their organism, 10% will constitute long-term *S. equi* **carriers**, harboring the microorganism for months. The presence of the pathogen is not detectable in *S. equi* long-term carriers and animals do not show any clinical signs of disease **(Waller *et al*., 2007).** *S. equi* subspecies zooepidemicus cause mimic mild cases of strangles **(Laus *et al*., 2007)**, and the subspecies can also be isolated from horses with confirmed *S. equi* infection **(Webb *et al*., 2013).**Although most horses are colonized by the closely related *S. zooepidemicus*, which shares many cross reactive immunogenic proteins with the clonal *S. equi,* these horses are not protected against strangles. Conversely, strangles vaccines do not protect against respiratory or uterine disease caused by *S. zooepidemicus*. On the other hand, although *S. equi* is sensitive in vitro to some antibiotics, its use is not consensual as most of the treatments are ineffective when external signs of disease are already detectable **(Harrington *et al*.,2002).** Prevention through quarantining and screening is particularly difficult where there is frequent moving and mixing of horses during the breeding season and at racetracks and where strangles outbreaks have not been appropriately investigated and controlled. The use of antibiotics has sparked much controversy. Antibiotics can, in fact, be counter-productive. Antibiotics suppress bacteria for a time, but infection may flare up when the antibiotics are discontinued." Treated horses might become re-infected because they do not develop protective immunity **(Sweeney *et al*., 2005).** Once an abscess forms in the lymph nodes, antibiotics won't penetrate to reach the organism, so when antibiotics are withdrawn, there is recrudescence (reappearance) of disease. **(Sweeney *et al*., 2005).** Studied the serological response of horses following the administration of *Streptococcus equi* vaccine and reported the development of protective levels of antibodies in their sera. Vaccination trials Bryans and Moore further demonstrated the presence of immunity against the disease in vaccinated horses.

Methods:

Bacteriological examination

Samples for bacteriological examination were collected from nasal cavities of horses using sterile swabs (NS) taken from distant part of nasal cavities after careful cleaning of nares and anterior part of nasal mucosa with disinfectant. All samples were collected in dublicate and processed bacterial isolation as soon as possible usually for 0.5 up to 4 hours after collection. At least two cultures were made for each specimen. Samples were streaked into blood agar. All samples incubated into 5 % (v/v) Co_2 at 37ºC for 24-48h.Typical β hemolytic Streptococci

like colonies were detected on blood agar and identified by characteristic colony morphology, gram staining and biochemical test including catalase. Isolates identified as *S. equi* fermented sucrose and salicin but not lactose, sorbitol, trehalose. Isolates identified as *S. zooepidemicus* given the same biochemical results but fermented lactose, sorbitol **(Quinn *et al.,* 1994).**

Polymerase Chain Reaction (PCR):

DNA extraction performed according to method described previously with some modification **(Alber *et al.,* 2004)** as shown in (Table 1).

Table (1): primer sequences for seM and sodA gene amplification were described.

primer	Nucleotide sequence(5' - 3')	Annealing Temperature	Product Size
Streptococcus equi species Se M PCR 1	**TGCATAAAGAAGTTCCTGTC** **GATTCGGTAAGACCTTGACG**	**72ºC**	**677bp**
Streptococcus Zooepidemicus Sod A PCR2	**CAGCATTCCTGCTGACATTCGTCAGG** **CTGACCAGCCTTATTCACAACCAGCC**	**56ºC**	**235bp**

The two separate PCR mixtures based on previously described **(Alber *et al.*, 2004)** with some modification**.** The program for PCR1 included a denaturation step of 94°C for 2 min and then subjected to 35 cycles of amplification, each consisting of (denaturation at 94°C for 10 seconds, annealing at 70°C for 10 seconds and extension at 72˚C for 5 seconds which was followed by final extension at 72°C for 5 min). PCR-2 conditions were kept same except for the annealing temperature which was kept 56˚C. The expected bands of amplicons were 235bp in *S. equi sub. Zooepidemicus* and 677bp *in S. equi sub. equi* .PCR products were visualized in a 2%gel at 80V.

Vaccine Preparation: (Srivastava and Barnum, 1981).

Three vaccines prepared by master seed bacteria

1- *Streptococcus equi sub spp equi* vaccine

2- *Streptococcus equi sub spp zooepidemicus* vaccine

3- Combined vaccine from these two subspecies.

Methods of culture and preparation of antigen (Srivastava and Barnum, 1981).

Tests for safety, sterility and purity were performed following CFR 9, § 113.100 (CFR 9, 1996).

Immunization of animals:

-Isogenic Balb-c (two months-old) mice vaccination (Moraes *et al.*, 2009)

Isogenic Balb-c two months-old mice were randomly divided in 3 groups with eight mice each (5 vaccinated and 3 control).

The animals of each group were inoculated subcutaneously with $1/20^{th}$ of a horse dose (CFR 9, 1996) of the respective vaccine, on days 0 and 14 of the experiment. Blood samples were collected from each animal at 0, 14, 28 days after the application of the first dose of vaccine (dpi).

- **Rabbits (Srivastava and Barnum, 1981).**
- **Horses (Srivastava and Barnum, 1981).**

MEASUREMENT OF IMMUNE RESPONSE:

The following serological tests were used.

- **Passive haemagglutination (PHA) TEST (Srivastava and Barnum, 1981).**

i) Antigen preparation Purified M protein of *Streptococcus equi sub equi* strain and M like protein of *Streptococcus equi sub zooepidemicus* was extracted from washed 18 hour old cells of *S. equi* by the method of enzymatic digestion and detergent extraction **(Brawn *et al*., 1996).**

ii) The sera were first diluted1/ 10 and then two fold dilutions of the test sera.

The reciprocal of the highest serum dilution showing a clear matt formation was considered the PHA antibody titre.

The titers are expressed as 10 x log2.

RESULTS

Results of isolation and identification:

Results of bacterial culture obtained from nasal swabs of diseased horses were typical β-hemolytic colonies detected on Edward's media and pure colonies stained by Gram's stain and examined microscopically shown Gram positive cocci appeared in long chains and catalase test was negative.

Results of biochemical identification of *Streptococcus equi* species:

- One strain was negative for lactose, sorbitol fermentation.
- Four strains were positive for lactose, sorbitol fermentation.

Table (1): Results of isolation trials from horses.

Type and no of samples	No of isolates	Species
Six samples of pus from ripened abcessiated submandibularlymph node in 6 foals	**4**	***S.equi* subspecies *zooepidemicus***
One nasal Swab from young horse	**1**	***S.equi* subspecies *equi***

Results of molecular examination (PCR):

-Four stains which were lactose, sorbitol fermenter showed Typical PCR product of *S.equi* subsp. zooepidemicus with size of 235pb specific for Sod a gene.

-One strain which was non-lactose, sorbitol fermentor showed Typical PCR products of *S. equi* subsp. *equi PCR* products of 677pb specific for SeM gene.

- Two other strains showed no PCR product with two primers specific for the two Subspecies.

Quality control on the prepared Vaccine:

Purity:

Gram' s stained films of the three vaccinal formulations were examined microscopically. Only Gram positive cocci arranged in chains were seen with no other contaminants either with gram positive or other forms of gram positive bacteria.

Results of sterility test:

No evidence of any bacterial (aerobic or anaerobic contaminants) or fungal growth has been detected even after prolonged incubation (14 days).

Results of safety test:

Mice inoculation test.

No deaths, local or general reaction were observed in mice injected in either vaccinal preparation up to 1 month of injection.

Results of evaluation of the protective efficacy of the prepared vaccine in rabbit with PHA test:

Nine rabbits divided into three groups each group contain 3 (two vaccinated and one control).

Two rabbits of each group were inoculated intramuscularly on three occasions with 1 mL of respective *S. equi* vaccines at ten day intervals. Sera collected before and at various periods after the vaccination were stored frozen at-20°C until tested. The collected sera from Rabbits were first diluted1/ 10 and then two fold dilutions of the test sera. The reciprocal of the highest serum dilution showing a clear matt formation was considered the PHA antibody titre. The titers are expressed as 10 x log2.

Table (2): Anti *S. equi* Titer as Measured by Passive Haemagglutination Test in immunized Rabbits with three formulations of vaccines using M protein of *S. equi* sub. Equi and *S.equi* sub. *Zooepidemicus*.

Time of Sera Collection	PHA
One week after 1st immunization series	**1/160**
One week after 2nd immunization series	**1/640**
One week after 3rd immunization series	**1/2560**
One month after 3rd Immunization	**1/5120**

DISCUSSION

Streptococcus equi subsp*ecies equi* was isolated from only one horse with typical signs of Strangles, stressing the difficulty to reach an etiological diagnosis of the disease. Atypical strains **(Grant *et al.,* 1993)** that found in most of Strangles cases were recovered from three foals and three young horses belonging to two herds. They fermented at least one carbohydrate three of the four atypical strains fermented carbohydrates only in media containing serum, in agreement with previous results of **Timony and Mukhatar (1993).** Previous studies have suggested that immunity in horses against strangles is mainly due to the presence of humoral antibodies produced as a result of immunization or natural infection **(Srivastava and Barnum, 1981).** Thus detection of these antibodies are considered useful in determining the immune status of horses. In this work, all the rabbits that received two or three inoculations of the vaccine showed the development of passive haemagglutinating

antibodies and antibodies that induce the formation of long chains **(Srivastava and Barnum, 1981).** The development of serological response in foals was gradual and was recorded over an extended duration after vaccination. Maximum antibody levels in most of the groups were recorded at 28 days' post vaccination **(Srivastava and Barnum, 1981).** All types of vaccines were immunogenic, although those prepared with typical strains, induced very low seroconversions. The highest seroconversion was induced by an atypical strain. But vaccines from typical *S. equi* strains not protect against atypical *S. equi infection* and vice versa **(Moraes *et al.*, 2009).**

CONCLUSION

Combined vaccines between typical and atypical strains considered immunogenically protected from typical and atypical strains infection.

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