

Concentration Dependent Antigenic Response to Formalin Inactivated *Streptococcus equi* Isolates in Rabbits

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Abstract

The present study was conducted to evaluate concentration dependent immune response of *Streptococcus equi* in laboratory rabbits to gauge the immunogenic potential of *Streptococcus equi* for the development of future vaccine against strangles in equines. *Streptococcus equi* the causative agent of Strangles in young foals was isolated from the pus of submandibular abscess of strangles affected foals. *Streptococcus equi* was characterized on the basis of morphological, cultural and biochemical on 5% horse serum Sodium azide blood agar base and by Prolax streptococcal latex system and Analytical Profile Index (API) System. Three concentrations of formalized *Streptococcus equi* @ 4×10^7 , 4×10^9 and 4×10^{11} were inoculated in four groups, one group as control negative, each group containing four adult male laboratory rabbits and sera of the inoculated rabbits were subjected to Indirect Heamagglutination Assay by using M-Protein of *Streptococcus equi* as antigen. It was found that the rabbits which were given concentration of *Streptococcus equi* @ 4×10^9 and 4×10^{11} cells per ml showed the highest Indirect Heamagglutination (IHA) antibody titres with GMT as 16.0.

Keywords: Concentration; Antigenic; Response to formalin; *Streptococcus equi*; Rabbits, Strangles vaccine; Indirect heamagglutination assay

Introduction

Strangles (also equine distemper) is a contagious upper respiratory tract infection of young horses and other equines caused by a bacterium, *Streptococcus equi* (not to be confused with *Streptococcus equinus*). Strangles is one of the first diseases in veterinary scientific literature. It has low mortality 1-2% but morbidity goes as high as 100%. It has a great threat to healthy horses kept in stables and horses used for breeding and race purposes [1,2]. *Streptococcus equi* is sensitive to the majority of antibiotics but in vivo treatment gets fail in both early (Before the development of lymph node abscesses) as well as in later stages (After the development of lymph node abscesses) as declared by Harington et al. and Sweeney et al. [3,4]. In early stages, though treatment is capable to clear all the *Streptococcus equi* from animal's body but as the treatment is withdrawn, again animal is caught by the disease because of absence of adequate immunity against *Streptococcus equi*. In later stages when abscessation has been developed then antibiotic provision fails to combat with the infection because of non-availability of sufficient vascularity to the abscess site and ultimately antibiotic remains unable to reach at the site of action and therapeutic levels of the drug cannot be achieved the site of action. Considering the importance of quines and coming to know about the nature of severity of strangles and facts about the antibiotic treatment the only solution of strangles was considered to adopt prophylactic measures in the form of a vaccine development against this disease. In this respect, Bazley in 1940, 1942 and 1943 prepared an effective

vaccine by using young encapsulated cells from rapidly growing cultures of *Streptococcus equi*, which had been heat killed. This vaccine was used extensively in army horses with considerable success. An alum hydroxide adjuvanted vaccine was also prepared by inactivating a *Streptococcus equi* culture with formalin, and adsorbing it with sulphate ions free aluminium hydroxide gel [5,6]. From 1940 to 1985, lot of vaccines came in picture including Strepvax[®], Strep guard[®] and Equibac[®] etc., but failed to provide adequate protective immunity and certain other local reactions were also seen with these vaccines [7]. Though the progress in the development of an effective vaccine against strangles remained slow but efforts could not stop and these efforts included a recombinant *S. equi* hyaluronate associated protein (HAP) [8], a live attenuated intranasal vaccine [9], Equilis Strep E but all the vaccines failed to provide protective immunity. In Pakistan, this disease is a regular visitor among young equine population in spring season and after finding a fact that this disease comes with a combination of two pathogens *S. equi* and *S. equisimilis* [10] a need for bivalent vaccine containing both streptococcal species (*S. equi* and *S. equisimilis*) has been felt but after finding no data regarding immunogenic property of indigenous isolate of *S. equi* and *S. equisimilis* the need of present work has been felt. This paper describes the comparative immune response to various concentrations of formalin-inactivated *S. equi* antigen in laboratory rabbits.

Materials and Methods

Isolation and bio characterization of field isolates

Isolation and bio characterization of bacterial isolates from 70 foals showing sub-mandibular lymph node abscessation suffering from

strangles was conducted as mentioned in Buxton, Fraser et al. and Cruickshank et al. [11,12] in the Institute of Microbiology, University of Agriculture, Faisalabad (Pakistan). The confirmation of Lancefield group C and streptococcal species was made by Prolax[®] streptococcal grouping latex kit and API[®] system [10] and the purified *S. equi* isolate was preserved in Trypticase Soy broth (Difco; Michigan, USA) containing 20% glycerol and kept at -20°C.

Preparation of formalin-inactivated *Streptococcus equi* antigen

Selected *Streptococcus equi* (*S. equi*) isolate was inoculated in 500 ml flask having Modified Todd-Hewitt broth supplemented with 5% horse serum. It was kept on an orbital shaker at 60 rpm for 48 hours. After that formalin (0.2%) was added to kill the *S. equi* isolate. The bacterial isolate was kept stable for 24 hours for proper action of Formalin. The killed organisms were harvested by centrifugation at 6000 xg for 1 hour at 4°C. Two washings with sterile PBS (pH 7.2) were done. The pellet thus obtained was re-suspended in PBS. Three concentrations of *S. equi* were adjusted as 4×10^7 per ml, 4×10^9 per ml and 4×10^{11} per ml by spectrophotometer. These preparations were stored at 4°C until utilized. Sterility was checked by streaking a loopful of the killed *S. equi* onto blood agar, MacConkey agar plates and Thioglycolate broth and incubating for 24-48 hour at 37°C [10].

Antigenic Rresponse to formalin-inactivated *Streptococcus equi* in rabbits

A total of 16 adult healthy rabbits, divided randomly into 4 groups containing 4 rabbits each, were utilized in this study. The rabbits of groups A, B, C and D were used for evaluating the concentration dependent immune response to *S. equi* antigen. The antibody titre was determined by indirect Heamagglutination (IHA) method [13]. Formalised inocula of *S. equi* containing 4×10^7 cells/ml, 4×10^9 per ml and 4×10^{11} per ml were injected subcutaneously in group A, B and C respectively. The rabbits of group D were kept as un-inoculated control. Serum samples were collected at weekly intervals for four consecutive weeks for indirect Heamagglutination (IHA) antibody titres to find out the optimum antigenic concentration/dose for *S. equi* antigen.

Indirect heamagglutination test (IHA)

Indirect heamagglutination test was performed as depicted by Michael et al. [14] but antigen (M-Protein) to be coated on sheep RBC's was extracted from *S. equi* as follows.

Preparation of formalin- inactivated *Streptococcus equi* antigen for IH assay

Antigen to be coated on Sheep RBC's has to be extracted from *S. equi* and the most immunogenic portion of *S. equi* is M-Protein [15] which has been extracted as follows.

Dilute-Nitrous acid extraction of M-protein from *S. equi*

Streptococcal cells from 250 ml Todd-Hewitt broth overnight cultures were collected, washed two times in saline, and then suspended in 0.5 ml of saline. The cell suspension was cooled to 0°C, and 200 µl of 4 M sodium nitrite was also precooled to 0°C, added, and mixed thoroughly. Then 100 µl of glacial acetic acid (room temperature) was added and mixed immediately. The HNO₂ reaction

was allowed to occur for 5 min in an ice bath at 0°C. Nitrous acid reaction was stopped by raising the pH to 7 to 7.4 with 5 M sodium hydroxide. The reaction time was calculated from the time the last drop of acetic acid was added and promptly mixed. Supernatants represented the extracts and were collected at 3,000 rpm for 30 min and tested for precipitation. The extracts to be tested for anti-opsonic properties were dialyzed against 0.01 M phosphate buffer (pH 7 to 7.4) overnight at 4°C by using Spectrapor membranes (6,000 to 8,000 molecular weight cut-off; Spectrum Medical Inc., Los Angeles, Calif.) [16].

Results

The study was undertaken to evaluate the immune response of formalin inactivated *S. equi* in rabbits. The *S. equi* was isolated on the basis of cultural, morphological and bio chemical characteristics. The isolates of *S. equi* were G+Ve, Cocci, non-motile and arranged in chains. On blood agar it produced translucent round beta haemolytic colonies.

Bio chemically the isolates were catalase negative and API number was 0461007 which means Voges Proscaur, HIP Uric Acid Hydrolysis, Esculine Hydrolysis, Pyroglutamic Acid, Alpha Galactose, Beta Galactose, Ribose, Arabinose, Mannitol, Sorbitol, Lactose, Trehalose, Inuline and Raffinose Tests are negative while Beta Glucuronidase, Alkaline Phosphatase, Leucine amino peptidase, Arginin Dihydrogenase, Glycogen acidification and beta hemolytic tests were positive.

Concentration dependent antigenic response to *S. equi* antigen

Sera samples of rabbits of group A (Inoculated with 1 ml Dose with concentration @ 4×10^7 cells/ml per rabbit) indicated progressive increase in GMT with maximum value of 11.3 at 21st day as shown in Table 1. GMT (Geometric Mean Titre) was calculated as depicted by Brugh [17].

Group	Sample (Rabbit)	IHA Titres at Post Inoculation Day			
		0 Day	7 th Day	14 th Day	21 st Day
A	1	1:02	1:04	1:16	1:04
	2	0	1:16	1:16	1:16
	3	1:02	1:04	1:08	1:16
	4	0	1:16	1:04	1:16
	GMT	1.4	8	9.2	11.3

Table 1: Results of Indirect Heamagglutination Titre (IHA) test at 0,7,14 and 21 day post inoculation in sera of experimental rabbits with dose of 1 ml of *Streptococcus equi* @ Conc. of 4×10^7 cells/ml

Group B (Conc @ 4×10^9 /ml per rabbit) was given a higher concentration than Group A and it showed increase in GMT with maximum value of 16.0 at 21st day as shown in Table 2.

Group	Sample (Rabbit)	IHA Titres at Post Inoculation Day			
		0 Day	7 th Day	14 th Day	21 st Day
B	1	1:02	1:16	1:16	1:16

	2	1:02	1:08	1:08	1:16
	3	0	1:08	1:16	1:16
	4	1:02	1:08	1:08	1:16
	GMT	1.6	9.2	11.3	16

Table 2: Results of Indirect Heamagglutination Titre (IHA) test at 0,7,14 and 21 day post inoculation in sera of experimental rabbits with dose of 1 ml of *Streptococcus equi* @ Conc. of 4×10^9 cells/ml)

Group	Sample(Rabbit)	IHA Titres at Post Inoculation Day			
		0 Day	7 th Day	14 th Day	21 st Day
C	1	0	1:08	1:16	1:16
	2	1:02	1:08	1:16	1:16
	3	1:02	1:08	1:08	1:16
	4	0	1:16	1:08	1:16
	GMT	1.4	9.2	11.3	16.0

Table 3: Results of Indirect Heamagglutination Titre (IHA) test at 0,7,14 and 21 day post inoculation in sera of experimental rabbits with dose of 1 ml of *Streptococcus equi* (Conc. of 4×10^{11} cells/ml)

Group C (Concentration @ 4×10^{11} /ml per rabbit) was given a higher dose than Group B and it showed increase in GMT with maximum value of 16.0 at 21st day again as shown in Table 3, which indicated a positive concentration dependent antigenic response of *S. equi* whereas rabbits of group D showed no increase in titres as shown Table 4.

Group	Sample(Rabbit)	IHA Titres at Post Inoculation Day			
		0 Day	7 th Day	14 th Day	21 st Day
C	1	0	01:04	01:02	1:02
	2	1:02	0	0	1:02
	3	1:02	0	0	0
	4	0	0	0	0
	GMT	1.4	1.4	1.2	1.4

Table 4: Results of Indirect Heamagglutination Titre (IHA) test at 0,7,14 and 21 day post inoculation in sera of un-inoculated control rabbits.

Discussion

The ultimate objective of such studies is to evaluate the immunity of causative agent of Strangles in equines which are the actual host of this disease. However preliminary trials are pre-requisites before its final commencement in Horses. That is why the present study was conducted in laboratory rabbits. *S. equi* is a causative agent of Strangles in young foals as already the similar kind of study has been elaborated by A. Shakoor et al. and Sohail Manzoor [18,19]. The establishment of infection not only depends upon the toxigenic capacity of the infecting bacteria but the environmental temperature is also involved e.g this

disease is most prevalent in spring season as compared to any season. The usual media for *Streptococcus* specie is blood agar but Sodium Azide @0.2% was used as cross contaminant to avoid the growth of *Staphylococcus* and to enhance the growth of *Streptococcus equi* 5% horse serum was used. The positive pus samples along with scrapings of submandibular lymph node abscesses were streaked on Sodium Azide Blood agar base supplemented with 5% horse serum. This medium is highly selective for *S. equi* as Sodium Azide is cross contaminant and allows only G+Ve Catalase-Ve [10]. *S. equi* isolate was selected after studying the morphological and bio-chemical characteristics of the isolates. The selected isolates of *S. equi* when subjected to morphological and cultural examination, all showed as gram-positive cocci arranged in chains. The size of organism ranged between 0.5-1.5 μ m. These variants were non-motile and non-spore bearing. These isolated variants produced transparent, moist and dew drop like colonies on blood agar with beta haemolysis around colonies. The colony size ranged between 1-2 mm after 48 hour incubation.

The selected isolates were catalase negative, Voges Proscaur, HIP Uric Acid Hydrolysis, Esculine Hydrolysis, Pyro glutamic Acid, Alpha Galactose, Beta Galactose, Ribose, Arabinose, Manitol, Sorbitol, Lactose, Trehalose, Inuline and Rafinose Tests are negative while Beta Glucuronidase, Alkaline Phosphatase, Lucien amino peptidase, Arginin Dihydrogenase, Glycogen acidification and beta hemolytic test were positive.

Indirect Heamagglutination (IHA) assay was used for the determination of concentration dependent immunity of *S. equi*. A total of 16 healthy rabbits divided randomly into 4 groups, containing 4 rabbits, each were utilized in this study. The rabbits of groups A, B, C and D were used for evaluating the concentration dependent immune response.

Groups A, B and C resulted in progressive increase in titres with maximum value at day 21 with GMT 11.3, 16.0 and 16.0 respectively. While sera of group D (negative control) showed no increase in titres. This indicated a positive progressive concentration dependent antigenic response of *S. equi* isolate [20]. This is pertinent to mention here that concentration of *S. equi* at 4×10^9 /ml and 4×10^{11} /ml produced equal immune response depicting that in future vaccine concentration of *S. equi* at 4×10^9 /ml should be preferred over 4×10^{11} /ml [21]. This indicated that the selected field isolate of *S. equi* is antigenic in nature and it produced concentration dependent immune response in laboratory animals, therefore indigenous isolate of *S. equi* at 4×10^9 /ml is recommended for the preparation of successful strangles vaccine [22].

Conclusion

The preparation of *S. equi* antigen showed antigenic response in the experimental animals (rabbits) and was found safe and no untoward reaction was observed in any rabbit. The antigenic response to the preparation of *S. equi* was found concentration/dose dependent. *S. equi* concentration at 4×10^9 /ml should be preferred over 4×10^{11} /ml in future strangles vaccine as both concentrations produced similar immune response.

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