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

Sohail Manzoor1,*, Sajjad-un-Rahman2, Muhammad Ashraf3, Syed Abbas Ali4 and Fraz Munir Khan1

1Veterinary Diagnostic Laboratories, North & South Punjab, Pakistan
2Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan
3Remount Veterinary Farms & Corps, Sargodha, Pakistan
4Provincial Diagnostic Laboratory, Lahore, Pakistan

*Corresponding author: Sohail Manzoor, Veterinary Diagnostic Laboratories, North & South Punjab, Pakistan, Tel: +923336503982; E-mail: manzooosohail76@gmail.com

Received date: October 16, 2016, Accepted date: November 07, 2016, Published date: November 20, 2016

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 × 109, 4 × 1010 and 4 × 1011 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 Haemagglutination Assay by using M-Protein of Streptococcus equi as antigen. It was found that the rabbits which were given concentration of Streptococcus equi @ 4 × 109 and 4 × 1011 cells per ml showed the highest indirect Hemagglutination (IHA) antibody titres with GMT as 16.0.

Keywords: Concentration; Antigenic; Response to formalin; Streptococcus equi; Rabbits, Strangles vaccine; Indirect haemagglutination 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 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 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. equisimillis [10] a need for bivalent vaccine containing both streptococcal species (S. equi and S. equisimillis) has been felt but after finding no data regarding immunogenic property of indigenous isolate of S. equi and S. equisimillis 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
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 x 10^7 per ml and 4 x 10^5 per ml and 4 x 10^3 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].

Concentration dependent antigenic response to *S. equi* antigen

Sera samples of rabbits of group A (Inoculated with 1 ml Dose with concentration @ 4 x 10^7 cells/ml per rabbit) was given a higher concentration 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 | 7th Day | 14th Day | 21st 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 Haemagglutination 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 x 10^7 cells/ml.

Group B (Conc @ 4 x 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 | 7th Day | 14th Day | 21st Day |
| B     | 1              | 1:02  | 1:16    | 1:16     | 1:16     |

Concentration dependent antigenic response to *S. equi* antigen

Sera samples of group A (Inoculated with 1 ml Dose with concentration @ 4 x 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].
Table 2: Results of Indirect Hemagglutination Titre (IHA) test at 0.7,14 and 21 day post inoculation in sera of experimental rabbits with dose of 1 ml of Strep. equi @ Conc. of 4 × 10^9 cells/ml

| Group | Sample(Rabbit) | IHA Titres at Post Inoculation Day |
|-------|----------------|-----------------------------------|
|       | 0 Day          | 7th Day                          |
|       | 14th Day       | 21st Day                         |
| C     |                |                                  |
| 1     | 0              | 1:08                             |
|       | 1:08           | 1:16                             |
|       | 1:16           | 1:16                             |
| 2     | 1.02           | 1:08                             |
|       | 1:08           | 1:16                             |
|       | 1:16           | 1:16                             |
| 3     | 1.02           | 1:08                             |
|       | 1:08           | 1:16                             |
|       | 1:16           | 1:16                             |
| 4     | 0              | 1:16                             |
|       | 1:08           | 1:16                             |
|       | 1:16           | 1:16                             |
| GMT   | 1.4            | 9.2                              |
|       | 11.3           | 16.0                             |

Table 3: Results of Indirect Hemagglutination Titre (IHA) test at 0.7,14 and 21 day post inoculation in sera of experimental rabbits with dose of 1 ml of Strep. 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.

Table 4: Results of Indirect Hemagglutination Titre (IHA) test at 0.7,14 and 21 day post inoculation in sera of un-inoculated control rabbits.

Table: Results of Indirect Hemagglutination Titre (IHA) test at 0.7,14 and 21 day post inoculation in sera of experimental rabbits with dose of 1 ml of Strep. equi (Conc. of 4 × 10^9 cells/ml)

Table 4: Results of Indirect Hemagglutination Titre (IHA) test at 0.7,14 and 21 day post inoculation in sera of un-inoculated control rabbits.

Table: Results of Indirect Hemagglutination Titre (IHA) test at 0.7,14 and 21 day post inoculation in sera of experimental rabbits with dose of 1 ml of Strep. equi (Conc. of 4 × 10^9 cells/ml)

Table 4: Results of Indirect Hemagglutination 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 Strep. equi 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 Strep. 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 µm. 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 Proscar, HIP Uric Acid Hydrolysis, Ecsuline Hydrolysis, Pyro glutamic Acid, Alpha Glactose, Beta Galactose, Ribose, Arabinoise, Manitol, Sorbitol, Lactose, Trehalose, Inuline and Rafinose Tests are negative while beta Glucoronidase, Alkaline Phosphatase, Lucien amino peptidase, Arginin Dihydrogenase, Glycogen acidification and beta hemolytic test were positive.

Indirect Hemagglutination (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 strepang 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 strepang vaccine as both concentrations produced similar immune response.

References

1. Sweeney CR, Whitelock RH, Meir DA, Whitehead SO, Barningham SO (1987) Complications associated with S. equi infection on horse stud. J Am Vet Med Asso 191:1446-1448.
2. Goland LC, Hodgson DR, Davis RE, Rawlinson RJ, Collins MB, et al. (1995) Retropharyngeal lymph node infection in horses: 46 cases (1977-1992). Aus Vet J 5: 161-164.

3. Harington DJ, Sutcliffe LC, Chanter N (2002) The molecular basis of Streptococcus equi infection and disease. Microbe Infect 4: 501-510.

4. Sweeney CR, Timmoney JF, Newton JR, Hines MT (2005) Streptococcus equi infections in horses: guidelines for treatment, control, and prevention of strangles. J Vet Intern Med 19: 121-134.

5. Katrinka M, Miutov L, Milic L, Budincevic I (1981) Prophylactic vaccination in the eradication of strangles. Veterinarski Glasnik 34: 1147-1150.

6. Nara PL, Krakowka S, Powers TR, Garg RC (1983) Experimental Streptococcus equi Infection in the horse: Correlation with in vivo and in vitro immune responses. Am J Vet Res 44: 529-534.

7. Mariannet TY (1987) Clinical aspects of S. equi infection. Equine Vet J 19: 158-162.

8. Chanter N, Ward CL, Talbot NC, Flanagan JA, Binns M, et al. (1999) Recombinant Hyaluronate associated protein as a protective immunogen against Streptococcus equi and Streptococcus zooepidemicus challenge in mice. Microb Pathog 27: 133-143.

9. Timmoney W (2002) Consyruction of stable non-mucoid deletion mutant of the Streptococcus equi Pinnacle Vaccine Strain. Vet Microbiol 89: 311-321.

10. Manzoor S, Siddique M, ur-Rahman S, Ashraf M (2008) Occurrence of Lancefield Group C Streptococcal Species in Strangles Cases Of Foals In Punjab, Pakistan. Pak Vet J 28: 17-20.

11. Buxton A, Fraser G (1975) Animals Microbiology. Blackwell Scientific Publication, London 163-177.

12. Cruickshank R, Duguid JP, Marmion BP, Swain RHA (1975) Medical Microbiology-A Practice of Medical Microbiology.

13. Thangavelu CP, Koshi G (1980) Micro-indirect hemagglutination test for detection of antibodies to the Ibc protein of group B Streptococcus. J Clin Microbiol 12: 1-6.

14. Bernstein MT, Stewart JA (1971) Indirect Hemagglutination Test for Detection of Antibodies to Cytomegalovirus. Appl Microbiol 21: 84-89.

15. Corinne R, Sweeney, John F, Timoney J, Newton R, et al. (2005) Streptococcus equi Infections in Horses: Guidelines for Treatment, Control, and Prevention of Strangles. J Vet Intern Med 19: 123-134.

16. Hafez K, El kholy AM, Facklam RR (1981) Extraction of Group A Streptococcal M-Protein with Nitrous Acid. J Clin Microbiol 14: 530-533.

17. Bragh MA (1978) A simple method for recording and analysing serological data. Avian Dis 22: 362-365.

18. Shakoor A, Athar M, Muhammad G, Rahman SU, Butt AA, et al. (2006) Experimental Trials of live attenuated and inactivated Staphylococcus aureus vaccines in rabbits. Pakistan Vet J 26: 51-54.

19. Manzoor S (2009) Development and Evaluation of Strangles vaccine by using indigenous isolates. Pakistan Research Repository.

20. Bazelay PL (1940) Studies with Equine Streptococci I. Experimental immunity to Streptococcus equi. Aus Vet J 16: 243-259.

21. Bazelay PL (1942) Studies with Equine Streptococci. II Vaccination against Strangles. Aus Vet J 18: 14-15.

22. Bazelay PL (1943) Studies with Equine streptococci. III Some relations between virulence of Streptococcus equi and immune response in the host. Asbalian Vet J 19: 62-85.