

Determination of Adsorption Capacity of Alum Hydroxide $\{Al(OH)_3\}$ Gel for *Streptococcus equi sub specie equi* and *Streptococcus dysgalactiae sub species equisimillis*

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Abstract

The present study was conducted to determine the adsorption capacity of Alum Hydroxide gel for *Streptococcus equi* and *Streptococcus equisimillis* in combination. One ml of streptococcal inoculum containing live count of *Streptococcus equi* @ 2×10^9 /ml and *Streptococcus equisimillis* @ 2×10^9 /ml inoculated in each of six Eppendorf tubes containing 0.2 mg, 0.4 mg, 0.6 mg, 0.8 mg, 1.0 mg and 2.0 mgs of autoclaved Aluminium Hydroxide in the form of gel, mixed and centrifuged at 1600 rpm for 15 minutes produced supernatant, which upon streaking and incubation on seven nutrient agar plates including supernatant from 7th control negative Eppendorf tube containing sterilized normal saline produced different number of colonies after adsorbing streptococcal cells depending upon the concentration of $Al(OH)_3$ in gel. 50 colonies were counted from supernatant recovered from Eppendorf containing 0.2 mg of Aluminium Hydroxide, 25 colonies from supernatant over 0.4 mg, 15 colonies from supernatant over 0.6 mg, 10 colonies from supernatant over 0.8 mg and no colony was obtained from supernatants over 1.0 mg and 2.0 mgs of Aluminium Hydroxide while 100 colonies were recovered from control negative eppendorf containing only normal saline without Aluminium Hydroxide. It is concluded that Aluminium Hydroxide as gel should be used as adjuvant @ 1 mg per ml of streptococcal inoculum in a streptococcal vaccine.

Keywords: Adsorption; Capacity; Alum hydroxide gel $\{Al(OH)_3\}$; *Streptococcus equi*; *Subspecies equi*; *Streptococcus dysgalactiae*; *Sub specie equisimillis*

Introduction

Adjuvants also called as immunological adjuvants are needed to improve routing and adaptive immune responses to antigens. Some adjuvants, such as alum, function as delivery systems by generating depots that trap antigens at the injection site, providing a slow release that continues to stimulate the immune system [1]. Alum is the most commonly used adjuvant in human vaccination. It is found in numerous vaccines, including diphtheria-tetanus-pertussis, human papillomavirus, and hepatitis vaccines [2]. Aluminium compounds, including aluminium phosphate ($AlPO_4$), aluminium hydroxide $\{Al(OH)_3\}$ and alum precipitated vaccines, concretely depicted as protein aluminate, are currently the most frequently used adjuvants with human and veterinary vaccines [3,4]. Alum, chemically potassium aluminium sulphate $\{K_2SO_4 \cdot Al_2(SO_4)_3 \cdot 24H_2O\}$ as such cannot be used as an adjuvant because of causing abrupt change in pH and ability of precipitating antigens in the vaccine but it is used along with 1N solution of NaOH to maintain pH. Originally it was used as adjuvant as such in tetanus vaccine [5]. Though there is a continuous research for adjuvants and many adjuvants other than aluminium compounds have been discovered likewise Sepic oil (Montanid) but aluminium compounds will be continued to be used as adjuvants for vaccines for many years due to their good track record of safety, low cost, and adjuvanticity with a variety of antigens [6-8]. As far as the adsorption of antigens on aluminium adjuvants is concerned either during in situ precipitation of aluminium adjuvants or onto preformed aluminium gels it depends upon physical and chemical characteristics of antigen, type of aluminium adjuvant and conditions of adsorption [9-12]. However, certain antigens do not adsorb onto Aluminium hydroxide gel because of the same charge on the adjuvant and antigens [13-15] but this is not

the case with Alum Hydroxide gel and S. In Pakistan, where there is still no suitable vaccine available against strangles for equines and after coming to know about the involvement of *Streptococcus dysgalactiae sub species equisimillis* along with *Streptococcus equi sub specie equi* in the causation of strangles in equines [16] and conducting successful trials regarding the antigenic and concentration dependent immunogenic response of indigenous isolates of *Streptococcus equi sub specie equi* and *Streptococcus dysgalactiae sub species equisimillis* in rabbits [17], the present work has been covered to determine the adsorption of these both streptococci jointly in aluminium hydroxide gel. This work will be helpful in future for the development of aluminium hydroxide adjuvanted vaccine against strangles using indigenous *S.equi* and *S.equisimillis*.

Materials and Methods

Isolation and bio characterization of indigenous Strangles streptococcal field isolates

Isolation and bio characterization of bacterial isolates from 70 foals was conducted as mentioned in Buxton & Fraser, 1975 & Cruickshank

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Received January 19, 2017; **Accepted** February 02, 2017; **Published** February 15, 2017

Citation: Manzoor S, Rahman S, Ashraf M, Khan FM, Syed ZH (2017) Determination of Adsorption Capacity of Alum Hydroxide $\{Al(OH)_3\}$ Gel for *Streptococcus equi sub specie equi* and *Streptococcus dysgalactiae sub species equisimillis*. J Antivir Antiretrovir 9: 006-009. doi:10.4172/1948-5964.1000155

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et al. 1975 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 [18] and the purified *S. equi* and *S. equisimillis* isolate was preserved in Trypticase Soy broth [19] containing 20% glycerol and kept at -20°C.

Preparation of formalin-inactivated *Streptococcus equi* and *Streptococcus equisimillis* antigen

Selected colonies of *S. equi* and *S. equisimillis* isolate were inoculated in two 500 ml flasks separately containing Modified Todd-Hewitt broth supplemented with 5% horse serum. Both the flasks were kept on orbital shakers at 60 rpm for 48 h. After that formalin (0.2%) was added in both flasks to kill the *S. equi* and *S. equisimillis* isolate. These bacterial isolates were kept stable for 24 hours for proper action of Formalin. The killed organisms from both flasks were harvested by centrifugation at 6000 xg for 1 h at 4°C. Two washings with sterile PBS (pH 7.2) were done. The pellets from both flasks thus obtained were re-suspended in PBS. The immunogenic concentration of *S. equi* and *S. equisimillis* as declared by Manzoor et al. was adjusted as 2×10^9 per ml and 2×10^9 per ml respectively by spectrophotometer. These preparations were stored at 4°C until utilized. Sterility was checked by streaking a loopful of the killed isolates onto blood agar, MacConkey agar plates and Thioglycolate broth and incubating for 24-48 h at 37°C.

Preparation of alum hydroxide gel

10 per cent solution of Potassium Aluminium Sulphate (Alum) $K_2SO_4 \cdot Al_2(SO_4)_3 \cdot 24H_2O$ was prepared by dissolving 10 g of alum in 1000 ml of distilled water. 1N solution of Sodium Hydroxide (NaOH) was prepared by dissolving 40 g of Sodium Hydroxide with 1000 ml of distilled water. Both solutions were mixed which lead to the formation of 500 ml of white gelatinous precipitate of Alum Hydroxide gel which was stored at 4°C. After overnight storage, supernatant over the gel was discarded and gel was mixed with some quantity of distilled water agitated and placed in refrigerator for 20 minutes. After 20 minutes gel was taken out and supernatant was discarded. This procedure was repeated until the supernatant got free of sulphate ions. Sulphate ions in the supernatant were checked by taking 5 ml of supernatant in a test tube and mixed with 2-3 drops of 1% Silver Nitrate ($AgNO_3$). Supernatant was observed for any change in colour or precipitate formation. Upon cloudy colour change or formation of white precipitate in supernatant the gel was again mixed in distilled water repeating the procedure of sulphate ions washing. Washing of gel was carried until and unless it got free from sulphate ions. The pH of gel was determined and maintained at 7.0 and gel was autoclaved.

Determination of antigen adsorption capacity of Alum Hydroxide gel

100 g of Aluminium Hydroxide {Al (OH)₃} were measured as dry matter in 500 ml of gel. One ml of autoclaved gel containing 0.2 g (200 mg) of Aluminium Hydroxide was mixed with 199 ml of sterilized distilled water to get final concentration of gel as 1 mg/ml in 200 ml mixture. A set of seven sterilized Eppendorf tubes was made. Alum hydroxide gel mixture was dispensed in first six tubes at 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, 1.0 ml, and 2.0 ml, respectively. In 7th tube 2 ml of sterilized normal saline was dispensed and this tube was considered as control tube. One ml of a live count of vaccine streptococci 4×10^9 /ml (Containing *S. equi* at 2×10^9 /ml and *S. equisimillis* at 2×10^9 /ml) were mixed in each of all the tubes. All the tubes were shaken gently placed over night, and then centrifuged at 1600 rpm for 15 minutes.

Supernatant was procured in separate sterilized Eppendorf tubes. The unabsorbed streptococci (Antigen) in the supernatant of all the seven test tubes were measured by live count as follows,

- Inoculum from the supernatant over each of seven tubes was cultured on seven nutrient agar plates separately.
- Plates were incubated at 37°C for 24 h
- After 24 h incubation, live count on the nutrient agar surface in the form of colonies was made with the help of colony counter
- After live count, 1 mg of autoclaved alum hydroxide gel was recommended for the newly developed bivalent strangles vaccine (Aluminium hydroxide adjuvanted vaccine), as adjuvant.

Results

Calculations regarding Al-Hydrogel and Alum Hydroxide {Al (OH)₃} distribution in Eppendorf tubes

- 10% Solution of Alum in 1000 ml of Distilled Water + 1 N Solution of NaOH in 1000 ml of Distilled water makes = 500ml of Alum Hydroxide gel
- Dry weight of 500 ml of Alum = 100 grams of Alum Hydroxide {Al (OH)₃} Hydroxide gel
- 1 ml of Alum Hydroxide gel contained = 0.2 grams (200/ mgs) of Al (OH)₃
- 1 ml of Alum Hydroxide gel mixed in = 200 ml of mixture contains 0.2g of Al (OH)₃ 199 ml of Distilled water
- 0.2 ml of Mixture contains = 0.001 ml of gel and 0.2 mg Al (OH)₃
- 0.4 ml of Mixture contains = 0.002 ml of gel and 0.4 mg Al (OH)₃
- 0.6 ml of Mixture contains = 0.003 ml of gel and 0.6 mg Al (OH)₃
- 0.8 ml of Mixture contains = 0.004 ml of gel and 0.8 mg Al (OH)₃
- 1.0 ml of Mixture contains = 0.005 ml of gel and 1.0 mg Al (OH)₃
- 2.0 ml of Mixture contains = 0.01 ml of gel and 1.0 mg Al (OH)₃

Discussion

In alum adsorbed vaccines the immunogenicity of antigens adsorbed onto aluminium adjuvants appears to depend on the degree of antigen adsorption and the dose of adjuvant [20-23]. Despite these controversies and uncertainty about precise mechanism of action of aluminium adjuvants, adsorption is still considered to be a very important parameter for the function of aluminium adjuvants [7]. Thus, measuring the degree of adsorption is one of the parameters that can be controlled in the formulation process during manufacture of aluminium adsorbed vaccines to achieve consistency in production. [24]. Degree of adsorption can be measured by simply centrifuging the adsorbed vaccine and assaying the supernatant for total protein (by Lowry assay, BCA assay, or other protein assay), antigenicity (flocculation), or polysaccharide (by anthrone or some specific sugar assay) depending upon the nature of the antigen [25,26].

The ultimate objective of this study is to determine the streptococcal adsorption capacity of different concentrations of Alum Hydroxide gel for *S. equi* and *S. equisimillis*. Sulphate ions were removed from alum hydroxide gel and after complete removal of sulphate ions NaOH drops were used to maintain the pH as depicted by Lindblad et al. and Gupta et al. [7,27]. To minimize variations, a specific preparation (Alhydrogel[®], aluminium hydroxide, from Superfos Biosector, Vedbaek, Denmark)

Eppendorf Tube No.	Quantity of Alum hydroxide gel mixture (ml)	Alum Hydroxide gel Quantity in mixture (ml)	Amount of Alum hydroxide {Al (OH) ₃ } in Alum Hydroxide gel (mg)	Count of <i>S. equi</i> + <i>S. equisimillis</i> /ml inoculated in Alum Hydroxide gel mixture	No. of colonies from supernatant over Alum Hydroxide gel mixture
T ₁	0.2	0.001	0.2	(2 × 10 ⁹ /ml)+(2 × 0 ⁹ /ml)	50
T ₂	0.4	0.002	0.4	(2 × 10 ⁹ /ml)+(2 × 10 ⁹ /ml)	25
T ₃	0.6	0.003	0.6	(2 × 10 ⁹ /ml)+(2 × 10 ⁹ /ml)	15
T ₄	0.8	0.004	0.8	(2 × 10 ⁹ /ml)+(2 × 10 ⁹ /ml)	10
T ₅	1.0	0.005	1.0	(2 × 10 ⁹ /ml)+(2 × 10 ⁹ /ml)	0
T ₆	2.0	1.000	2.0	(2 × 10 ⁹ /ml)+(2 × 10 ⁹ /ml)	0
T ₇ (Control)	Not contained	Normal Saline	Not contained	((2 × 10 ⁹ /ml)+ (2 × 10 ⁹ /ml)	100

Table 1: Streptococcal adsorption capacity of various concentrations of alum hydroxide gel

was selected as a scientific standard for evaluation of our aluminium hydroxide adjuvant [28]. It was found that 0.001 ml quantity of gel containing 0.2 mg of Al (OH)₃ adsorbed minimum quantity of streptococci with maximum retention of unadsorbed streptococcal cells, producing 50 colonies as compared to 0.004 ml quantity of gel containing 0.8 mg of Al(OH)₃ with minimum retention of streptococcal cells producing only 10 colonies while 0.005 ml and 1.0 ml quantity of gel containing 1.0 mg and 2.0 mgs of Al (OH)₃ respectively adsorbed all the inoculated (4 × 10⁹) cells as depicted in Table 1. In the control negative tube all the streptococcal cells remained un-adsorbed and 100 colonies were produced from the supernatant. From this experimentation it can be concluded that 0.005 ml of alum hydroxide gel containing 1.0 mg of Al (OH)₃ should be preferred over 1.0 ml of gel containing 2.0 mg of Al (OH)₃ because of same adsorption capacity and getting more economical. Moreover the amount of adjuvant is also very important because a fact has been proved in animal studies according to which as the amount of aluminium adjuvant was increased, the adjuvant effect increased, but only to a certain concentration after which, the adjuvant effect declined [24-26,29]. The reasons for this optimum concentration of adjuvant are unknown. It is speculated that a certain minimum amount of aluminium compound is necessary to form a depot at the site of injection or to optimally stimulate macrophages [7]. Excessive amounts of aluminium compounds may suppress immunity by covering the antigen completely with mineral compounds [14,15] or through toxicity to macrophages [9]. After coming to know this fact, 1 mg of aluminium hydroxide in 0.005 ml of aluminium hydroxide gel was considered sufficient and more than 1 mg of aluminium hydroxide was avoided though also adsorbed the immunogenic count of streptococcal cells of *S. equi* and *S. equisimillis*. The usual dose of aluminium used for human vaccines is around 0.5 mg. The upper allowable limits of aluminium adjuvants for injection in humans is 1.25 mg as per WHO regulations [30] and 0.85 to 1.25 mg as per United States Food and Drug Administration guidelines [19] and our findings exactly fall in this range of USA Food and Drug administration.

Conclusion

The preparation of *S. equi* @ 2 × 10⁹/ml and *S. equisimillis* @ 2 × 10⁹/ml was completely adsorbed in 0.005 ml gel containing 1.0 mg of aluminium hydroxide.

Acknowledgment

Authors are highly thank full to RV & FC, Pakistan personals for their cooperation in sampling of Strangles affected cases of young foals in all the equine rearing districts of Punjab, Pakistan including Faisalabad, Okara and Sargodha w.e.f January 2003 to December

2004 and import of laboratory consumables e. g Prolax® streptococcal grouping latex kit and API® system and other essential glassware.

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