Determination of Adsorption Capacity of Alum Hydroxide \{Al (OH)\}_3 Gel for \textit{Streptococcus equi} sub specie equi and \textit{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 \textit{Streptococcus equi} and \textit{Streptococcus dysgalactiae} in combination. One ml of streptococcal inoculum containing live count of \textit{Streptococcus equi} @ 2 × 10^9/ml and \textit{Streptococcus dysgalactiae} @ 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)\textsubscript{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 ependorf 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; \textit{Streptococcus equi} Subspecies equi; \textit{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 (Al PO\textsubscript{4}), aluminium hydroxide (Al(OH)\textsubscript{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\textsubscript{2}SO\textsubscript{4}·Al(SO\textsubscript{4})\cdot24H\textsubscript{2}O\} 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\textsuperscript{	extregistered}) but aluminium compounds will be helpful in future for the development of aluminium hydroxide adjuvanted vaccine against strangles using indigenous \textit{S.equi} and \textit{S.equisimillis}.

Materials and Methods

Isolation and bio characterization of indigenous Strangles streptococcal field isolates

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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 Prolex’ 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-Hewlett 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 x g 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 x 10^9/ ml and 2 x 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) K2SO4 . AL2 (SO4)3 . 24H2O 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 2-1 drops of 1% Silver Nitrate (AgNO3). Supernatant was observed for any change in colour or precipitate formation. Upon cloudy colour formation or formation of white precipitate in supernatant the gel was again mixed in distilled 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 m) 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 x 10^9/ml (Containing S. equi at 2 x 10^9/ml and S. equisimillis at 2 x 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 adjuvant vaccine), as adjuvant.

Results

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

i) 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

ii) Dry weight of 500 ml of Alum = 100 grams of Alum Hydroxide [Al (OH)3] Hydroxide gel

iii) 1 ml of Alum Hydroxide gel contained = 0.2 grams (200/ mgs) of Al(OH)3

iv) 1 ml of Alum Hydroxide gel mixed in = 200 ml of mixture contains 0.2g of Al(OH)3, 199 ml of Distilled water

v) 0.2 ml of Mixture contains = 0.001 ml of gel and 0.2 mg Al (OH)3

vi) 0.4 ml of Mixture contains = 0.002 ml of gel and 0.4 mg Al (OH)3

vii) 0.6 ml of Mixture contains = 0.003 ml of gel and 0.6 mg Al (OH)3

viii) 0.8 ml of Mixture contains = 0.004 ml of gel and 0.8 mg Al (OH)3

ix) 1.0 ml of Mixture contains = 0.005 ml of gel and 1.0 mg Al (OH)3

x) 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)
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 unabsorbed streptococcal streptococci with maximum retention of unabsorbed streptococcal streptococci. It can be concluded that 0.005 ml of alum hydroxide 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 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 unabsorbed streptococcal streptococci with maximum retention of unabsorbed streptococcal streptococci. 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, 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 metal 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.

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

<table>
<thead>
<tr>
<th>Eppendorf Tube No.</th>
<th>Quantity of Alum hydroxide gel mixture (ml)</th>
<th>Alum Hydroxide gel d Quantity in mixture (ml)</th>
<th>Amount of Alum hydroxide (Al(OH)₃) in Alum Hydroxide gel (mg)</th>
<th>Count of S. equi + S. equisimillis/ml inoculated in Alum Hydroxide gel mixture</th>
<th>No. of colonies from supernatant over Alum Hydroxide gel mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₁</td>
<td>0.2</td>
<td>0.001</td>
<td>0.2</td>
<td>(2 × 10⁹/ml) + (2 × 10⁹/ml)</td>
<td>50</td>
</tr>
<tr>
<td>T₂</td>
<td>0.4</td>
<td>0.002</td>
<td>0.4</td>
<td>(2 × 10⁹/ml) + (2 × 10⁹/ml)</td>
<td>25</td>
</tr>
<tr>
<td>T₃</td>
<td>0.6</td>
<td>0.003</td>
<td>0.6</td>
<td>(2 × 10⁹/ml) + (2 × 10⁹/ml)</td>
<td>15</td>
</tr>
<tr>
<td>T₄</td>
<td>0.8</td>
<td>0.004</td>
<td>0.8</td>
<td>(2 × 10⁹/ml) + (2 × 10⁹/ml)</td>
<td>10</td>
</tr>
<tr>
<td>T₅</td>
<td>1.0</td>
<td>0.005</td>
<td>1.0</td>
<td>(2 × 10⁹/ml) + (2 × 10⁹/ml)</td>
<td>0</td>
</tr>
<tr>
<td>T₆</td>
<td>2.0</td>
<td>1.000</td>
<td>2.0</td>
<td>(2 × 10⁹/ml) + (2 × 10⁹/ml)</td>
<td>0</td>
</tr>
<tr>
<td>T₇ (Control)</td>
<td>Not contained</td>
<td>Normal Saline</td>
<td>Not contained</td>
<td>(2 × 10⁹/ml) + (2 × 10⁹/ml)</td>
<td>100</td>
</tr>
</tbody>
</table>

### 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.

### Acknowledgments

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### References


