Original article

CLINICAL AND EXPERIMENTAL VACCINE RESEARCH

Immunogenic characterization of $AlPO_4$ adsorbed Td vaccine and liposome-mediated Td vaccine

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Purpose: The purpose of this study was to compare the antigenic potency and stability of tetanus and diphtheria (Td) vaccines when combined with aluminum phosphate ($AlPO_4$) and liposome adjuvants.

Materials and Methods: *In vitro* and *in vivo* analyses were conducted using the single radial immunodiffusion method and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The Td vaccines were prepared with $AlPO_4$ adsorption and liposome-mediated delivery, and protein antigens were characterized using these methods.

Results: The results revealed that the liposome-mediated Td vaccines exhibited higher immunogenicity compared to the $AlPO_4$-adsorbed Td vaccines. Additionally, the liposome-mediated Td vaccines demonstrated higher stability as native antigens.

Conclusion: This study highlights the importance of utilizing liposome adjuvants in vaccine development. The liposome-mediated Td vaccines showed enhanced immunogenicity and stability, making them a promising approach for improving vaccine efficacy. Understanding and optimizing adjuvant strategies can contribute to the development of effective vaccines against various diseases.

Keywords: Single radial immunodiffusion, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Aluminum phosphate, Liposomes, Tetanus and diphtheria vaccine

Introduction

Multiple combination vaccines have been used globally with specific compositions of toxoid and antigen contents. Diphtheria and tetanus toxoids (DT and TT) have been used since the 1940s to prevent diphtheria and tetanus. More recently, both vaccines have been formulated jointly in diphtheria, pertussis, and tetanus (DTP) combination product. Nevertheless, the tetanus and diphtheria (Td) vaccine is still in use as a booster dose for adults. Td paved the way for the development of different vaccine combinations, which allow multiple toxoids and antigens to be delivered via a single injection. Accordingly, their immunization is attributed to the safety profile of their individual components [1].

Aluminum-precipitated vaccines are widely used with childhood routine vaccines since it was discovered that a suspension of aluminum-precipitated DT had much higher immunogenicity than the soluble toxoid. Glenny [2] proposed that aluminum adjuvants act by depot formation at the site of injection, allowing the slow release of antigen, and thus prolonging the time for interaction between antigen and antigen-pre-

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senting cells and lymphocytes [3].

A key advantage of liposomes, and liposome-based delivery systems in particular, is their versatility and plasticity. Liposomes and liposome-derived nanovesicles such as archaeosomes and virosomes have become important carrier systems and the interest in liposome-based vaccines has markedly increased [4]. Liposome vesicles were previously used for transcutaneous delivery of proteinaceous antigens and were found to increase permeability through transcutaneous immunization [5].

It has been found previously that TT and DT produce better immunogenic effect when the antigens are encapsulated or mixed with liposomes or adsorbed onto aluminum phosphate ($AlPO_4$) [6]. But studies are needed to compare the effectiveness of these adjuvant for Td vaccine as it is more frequently used worldwide. To study and compare the immunogenic potency of Td vaccine used in combination with these two adjuvants, we used single radial immunodiffusion (SRID). Due to its high specificity and specificity, SRID is extensively used for the quantitative estimation of immunogenicity by the measuring antigens. Therefore, allow quantitative determination of both antigen and antibody [7,8].

Apparently, the quality of the immunogenicity as well as the quality of the immune response lead to the higher efficacy of a vaccine, which is characterized by stability and integrity of antigen specially with aluminum salt and emulsions based on squalene oil. To study these characteristics, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) has been frequently used [9].

Effective adjuvants can enhance the antigen-specific immunogenicity as well as the quality of the immune response, which may lead to higher efficacy of the vaccine or reduce the antigen content per vaccine dose (dose sparing). After aluminum salts, the most widely used adjuvants in human vaccines are oil-in-water.

To allow better effectiveness of such vaccine, we tried to investigate the immunogenicity of the formulated Td vaccine using $AlPO_4$ gel-adsorbed Td and liposomes adjuvants. The research was designed as a comparative study of different adjuvants-mediated Td vaccines performed at the Pasteur Institute of India.

Materials and Methods

Pre-prepared formulated Td vaccine with aluminum or liposome adjuvants was subjected to a SRID assay and SDS-PAGE to determine the stability and integrity of the vaccine.

Table 1. Ingredients for formulation of Td vaccine

Ingredients	Volume (mL)
Tetanus toxoid	2.32
Diphtheria toxoid	0.31
Aluminum phosphate gel	19.02
Thiomersal	2.00
Normal saline	176.35
Total volume	200.00

Td, tetanus and diphtheria.

Preparation of Td vaccine with aluminum phosphate gel

The ingredients of the vaccine to be formulated were calculated in the expected volume as shown in Table 1 and finally blended under sterile conditions as previously described [10]. Finally, the components were added to these volumes and the Td vaccine is formulated and preserved at 2°C–8°C. sterility of the had been confirmed by incubating random samples in alternate thioglycollate medium and soybean casein digest medium at 25°C and 37°C for 14 days.

Estimation of aluminum content

This was carried out in a pre-formed gel by titration. The gel samples were diluted in the ratio of 1:10 and 2 mL of diluted sample was transferred into a 250 mL conical flask to which 1 mL of concentrated sulphuric acid was added and boiled. After cooling, 10 mL of water was added and boiled.

The solution was allowed to cool and two drops of 0.1% methyl orange were added and the solution turned pinkish red in color. It was neutralized by dropwise addition of 50% sodium hydroxide till a yellow-colored point was reached. And 25 mL of ethylenediaminetetraacetic acid and 10 mL of acetate buffer were further added.

The contents were boiled gently for 3 minutes and 1 mL of 1-(2-pyridylazo)-2-naphthol (PAN) indicator (0.1% in 95% ethanol) was added. The hot solution was titrated against copper sulphate solution. The endpoint was the appearance of a purple-brown color. The total aluminum content in a formulated bulk should be less than 1.25 mg/single human dose when formulated with $AlPO_4$ or aluminum hydroxide.

$$\text{Amount of aluminum} = \frac{(\text{blank titre-test titre}) \times \text{normality of aluminium}}{\text{volume of sample taken}}$$

$$= \frac{(24.7-20.3) \times 0.2698}{2 \times 2}$$

$$= 0.2967$$

Calculation for aluminum content

The permissible range of aluminum content should always be less than 0.5 mg/mL according to the previous study [11], to avoid the neurotoxic activity of aluminum. The scientific evidence shows that the higher level can lead to neurological diseases such as dementia, autism, and Parkinson's disease.

Preparation of Td vaccine using antigen loaded liposomes

Liposomes were prepared by hand shaking method with slight modifications. Briefly, SPC, and CH (7%:3%, weight/weight) were dissolved in 5 mL diethyl ether and allowed to water bath at 35°C for 30 minutes to remove any residual ether. A thin lipid layer was formed and phosphate buffer saline containing Td vaccine was then added.

The mixture was kept in a shaker at 34°C for 1 hour, followed by overnight incubation at 4°C. After incubation, the mixture was well shaken and sonicated for 21 minutes at 50% amplitude to reduce the size of the liposomes. This process resulted in the formation of a liquid suspension. To hydrate the vesicles before lyophilization, 3 mL of phosphate-buffered saline (PBS) was added to the suspension.

Test for specific toxicity

The purpose of the specific toxicity test for the Td vaccine with $AlPO_4$ gel is to confirm freedom from residual toxin and reversion to toxicity in final bulk vaccines and/or bulk purified toxoids. The tests for specific toxicity or toxicity reversal are usually performed in guinea pigs or mice by subcutaneous injection. The test was carried out according to CPCA guidelines and approval number 66/1999/CPCSEA. Five guinea pigs were subcutaneously injected with 0.5 mL of formulated Td vaccine, both with and without $AlPO_4$ or liposomes (one animal received the vaccine without adjuvant, and two animals were administered each adjuvant). The animals were then observed for 42 days. The weight of the animal was followed and recorded weekly.

Single radial immunodiffusion

SRID is commonly used for the quantitative estimation of antigens. The antigen-antibody precipitation is made sensitive by the incorporation of antiserum in the agarose and the antigen is then allowed to diffuse from wells cut in the gel in which the antiserum is uniformly disturbed. However, as antigen diffuses from the well, the antigen-antibody complex reacts with more amount of antibody resulting in a lattice that precipitates to form a precipitin ring. Thus, by running a range of known antigen concentrations on the gel and by measuring the diameters of their precipitin rings, a calibration graph can be plotted. Antigen concentration of unknown samples, run on the same gel can be found by measuring the diameter of precipitin rings and extra plotting the value on the calibration graph [8].

The test has been performed as originally described [12]. One mL of antiserum (diphtheria and tetanus antitoxin) was added to 15 mL+15 mL of agarose solution added to two different SRID plates. Then, mixed by gentle swirling for a uniform distribution of antibodies. Agarose solution containing the antiserum was poured onto a grease-free glass plate and set on a horizontal surface. It was left undisturbed to form a gel. A gel puncher was used for wells formation on the template. 15 μL of the given standard antigens and Test antigens were added to the wells. The gel plate was kept in a moist chamber and incubated overnight at room temperature. Then Coomassie blue stain solution was added and kept in a shaker for few hours. The decolorizer solution was added to remove stain residue in the gel and kept for an hour. Then it was kept overnight for drying.

The diameter of the ring precipitin was measured and plotted on a calibrated graph of the diameter of the zone (y-axis) versus the concentration of antigen (x-axis) on a semi-log graph sheet. The concentration of antigen in the test samples was determined and tabulated.

Conductivity measurement

$AlPO_4$-adsorbed Td and liposome-mediated Td to determine the stability of both. The test was performed using the Thermo Scientific Orion 3-Star Benchtop Conductivity Meter (Thermo Scientific Orion, Waltham, MA, USA), which provides reliable and accurate conductivity measurement in μs/cm. The principle of the instrument is depending on the measurement of ions that can conduct electric current so that the current that passes through the cell's solution is measured.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE is a common method used to separate proteins with molecular masses between 5 and 250 kDa sodium dodecyl sulfate (SDS) and polyacrylamide gel allows disassembled the influence of structure and charge, and proteins to be separated solely on the basis of differences in their molecular weight [13,14].

Characterization of the integrity of protein antigens is an essential component of any vaccine development program.

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Even more important when adjuvants are added to the vaccine preparation since antigen integrity or stability could potentially be affected by the presence of an adjuvant and vice versa [11].

We used this assay in order to characterize the protein separation pattern and staining. The glass slides were thoroughly cleaned, dried, and fixed in the frame without any leakage. The gel was prepared as follows.

The separating gel (12%) was prepared, and $AlPO_4$-adsorbed Td, liposome-mediated Td, adjuvant-free Td, or molecular weight marker was added to the gel. And then, electrophoresis was performed as previously described [15]. Briefly, 20 μg protein samples were mixed with sample loading dye (β-mercaptoethanol) in an approximately 1:1 ratio. Denaturation of protein samples was achieved by heating at 95°C for 15 minutes in a water bath. The samples were loaded into the 7-well using a sample loading guide. Samples loaded along with a gel marker (bromophenol blue) were loaded onto the gel. Electrophoresis was running first at 80 V (240 mA) for 30 minutes, then at 120 V (240 mA) for about 60 minutes, until the tracking dye reached the bottom of the gel.

The gel was carefully removed from the glass slides and kept in silver nitrate staining solution for about 30 minutes with mild shaking at the room temperature. The stained gel was then reveled using sodium carbonate and Formaldehyde solution to visualize the bands, the staining was disrupted by adding 50% acetic acid.

Statistical analysis

GraphPad Prism ver. 9.0.0 (244; GraphPad Inc., San Diego, CA, USA) and Microsoft Excel 2016 (Microsoft Corp., Redmond, WA, USA) software were used. Comparison of data was performed as described in the respective figure legends. All p-values of 0.05 or less were considered as significant (significant values with *p≤0.05, **p≤0.01, ***p≤0.001, and ****p≤0.0001).

Institutional review board statement

The animal study protocol was approved by the Institutional Review Board of Committee for the Purpose of Control and Supervision of Experiments on Animals and Prevention of Animal Cruelty CPCA, New Delhi state, Government of India (approval no., 66/1999/CPCSEA).

Results

Visual appearance

The prepared liposome-mediated Td vaccine was visually examined for quality of production and freedom of contaminated particles and as shown in Fig. 1.

Scanning electron microscope

The morphology of the liposome-mediated Td was analyzed by scanning electron microscope and vesicle size and shape were also examined. The rough surface of the vesicle was due to the freeze-drying. The analyzed morphology is included in Fig. 2.

Test for specific toxicity

The weight gain of the guinea pigs is tabulated as shown in Fig. 3. Both adjuvants vaccine combination was found safe and nontoxic to the animals, though the liposome-mediated Td

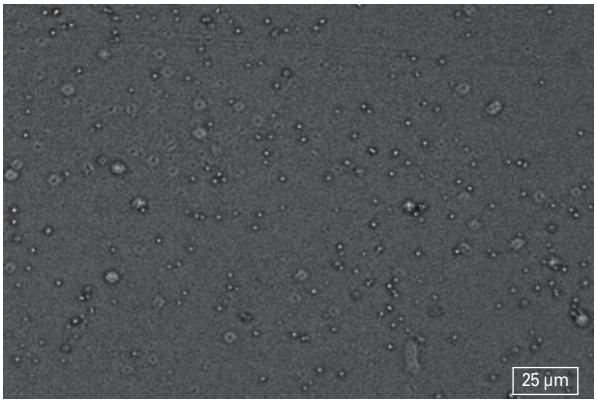

Fig. 1. Liposome-mediated tetanus and diphtheria (Td) vaccine visualized by optical phase contrast microscope. The vesicular structures appear as bright small dots measuring around 0.3 μm and less.

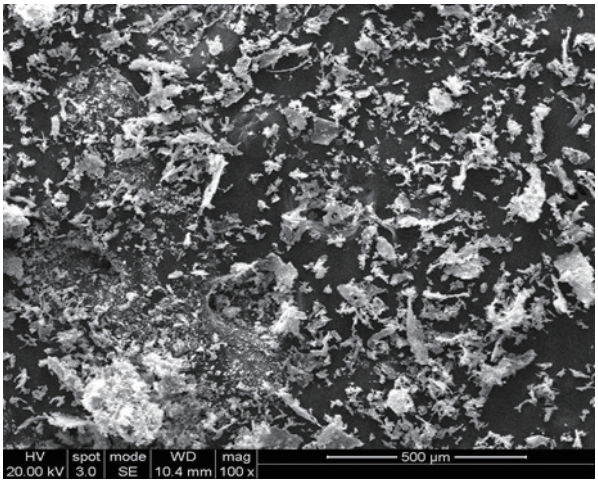

Fig. 2. Liposome-mediated tetanus and diphtheria (Td) vaccine visualized by scanning electron microscope. The vesicles appear intact and has rough surface, due to freeze-drying.

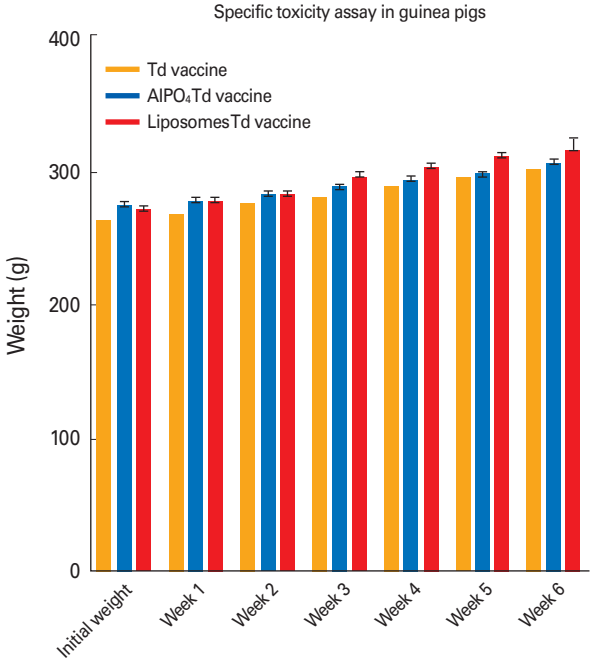

Fig. 3. Chart for the weight of the guinea pigs. All vaccine combinations were administered subcutaneously to the animals. No significant weight loss was observed, indicating that all combination were not toxic to the animals. Although the absence of significance a slight tendency to gain more weight can be observed in case of liposome-mediated tetanus and diphtheria (Td) vaccine. $AlPO_4$, aluminum phosphate.

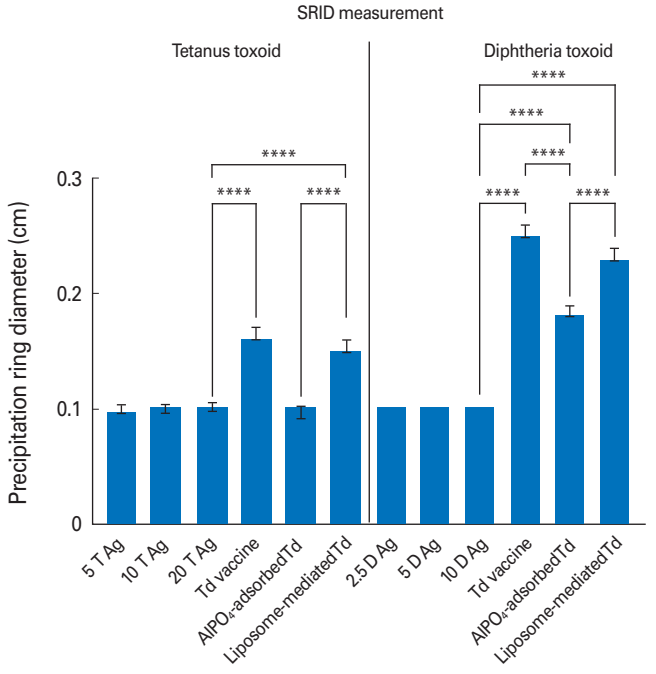

Fig. 4. Precipitation ring diameter from single radial immunodiffusion (SRID) for diphtheria toxoid and tetanus toxoid. Data were analyzed using using analysis of variance multiple comparison test. Data represent mean±standard error of mean of three independent measurement. Ag, antigen; Td, tetanus and diphtheria; $AlPO_4$, aluminum phosphate. ****$p \leq 0.0001$.

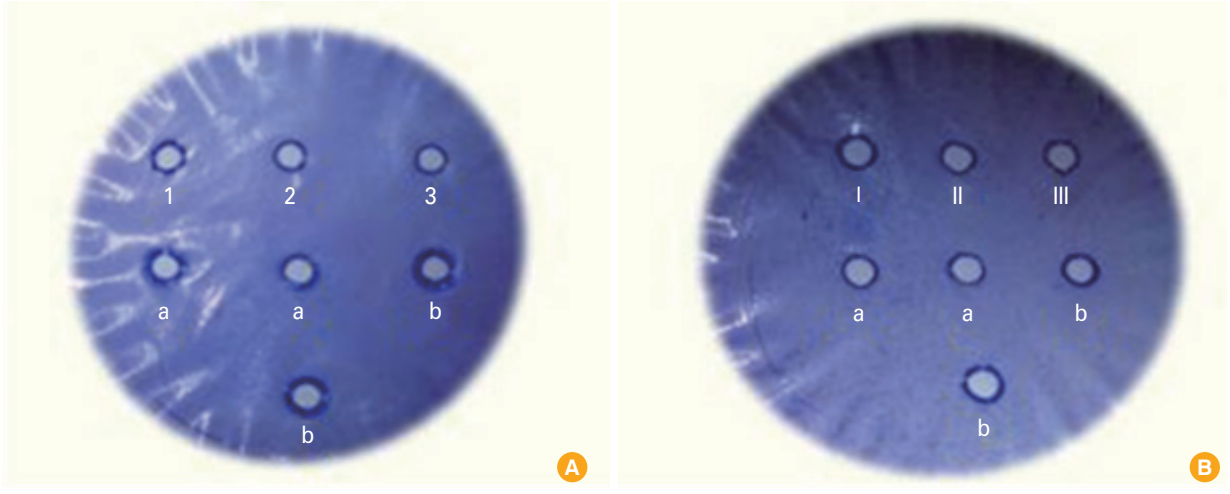

Fig. 5. Single radial immunodiffusion for diphtheria toxoid depicted in **(A)** and tetanus toxoid depicted in **(B)**. A bigger diameter of the precipitation zone can be observed visually around liposome-mediated tetanus and diphtheria (Td) vaccine wells b in both toxoids. a, aluminum phosphate ($AlPO_4$) gel-adsorbed Td; b, liposomes mediated Td; 1, diphtheria antigen (Ag) 2.5 Lf/mL; 2, diphtheria Ag 5 Lf/mL; 3, diphtheria Ag 10 Lf/mL; I, tetanus Ag 5 Lf/mL; II, tetanus Ag 10 Lf/mL; III, tetanus Ag 20 Lf/mL; Lf, limit of flocculation.

vaccine was less toxic than the $AlPO_4$ gel-adsorbed Td vaccine as the extent of weight increase was more with guinea pig administered with liposome-mediated Td vaccine when compared to the guinea pig administered with $AlPO_4$ mediated Td vaccine. These findings support previous results in mice for TT and DT vaccines [6], it might explain previous studies [16,17], where immunogenicity in humans had been found to be moderate when Td vaccine delivered alone or with alumi-

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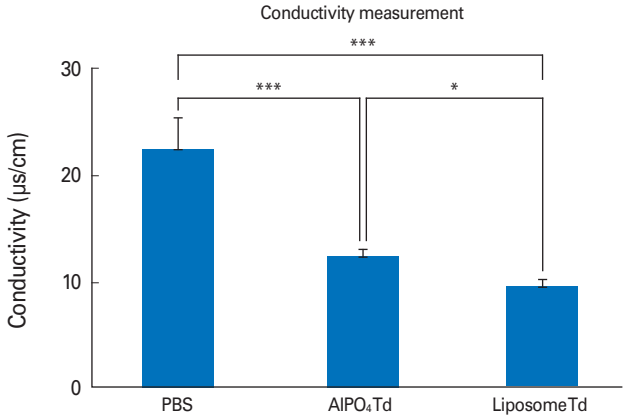

Fig. 6. Conductivity of aluminum phosphate ($AlPO_4$) gel-adsorbed tetanus and diphtheria (Td) vaccine were compared to liposome-mediated Td vaccine. Phosphate-buffered saline (PBS) was used as a control, both adjuvant-containing vaccines had significantly lower conductivity than PBS and the liposomes vaccine had lower conductivity when compared to $AlPO_4$ Td vaccine. Data were analyzed using analysis of variance multiple comparison test. Data represent mean±standard error of mean of three independent measurement. *$p \leq 0.05$. ***$p \leq 0.001$.

num hydroxide, whereas liposome formulation enhanced the T helper type 1-mediated response and generated higher antibody titers.

Nevertheless, further repetition of this assay by the same or different approaches is necessary for granite safety. The results from the SRID assay were measured and statistically analyzed for significance as shown in Fig. 4.

Single radial immunodiffusion

The antigen and antibody interaction was confirmed by the presence of rings of precipitation, which has a diameter in the range of 0.1–0.3 cm as shown in Fig. 5. The results from the SRID assay were measured and analyzed statistically for significance as shown in Fig. 4. Precipitation diameter size is higher in liposome-mediated Td than the $AlPO_4$-adsorbed Td, suggesting that the immunochemical reaction is higher in liposome-mediated Td and thus immunogenicity will be higher for liposome-mediated Td than the other.

The SRID test results clearly show that liposomes, unlike $AlPO_4$, are not influencing antigen-antibody interaction the results are also suggestive of inhibiting factors in $AlPO_4$ when used in Td that reduce the protectiveness against tetanus, these results need to be confirmed by quantifying the specific antigen and antibody ration using more sensitive and specific test line enzyme-linked immunosorbent assay.

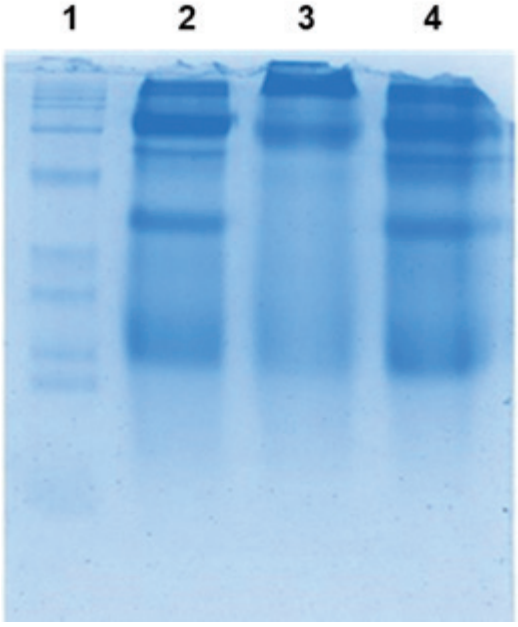

Fig. 7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the test samples. Similar band distribution pattern of the antigens found in the original tetanus and diphtheria (Td) vaccine were maintained in liposome-mediated Td vaccine. Lane 1, ladder; lane 2, Td+liposome; lane 3, Td+aluminum phosphate ($AlPO_4$) gel; lane 4, Td.

Conductivity measurement

When compared to the standard PBS used, both liposome- and aluminum-mediated Td vaccines have lower conductivities, indicating their stability. Three measurements were performed at room temperature (approximately 25°C); the results were expressed as a mean±standard deviation in Fig. 6.

The lower conductivity signifies the fewer free ions on the surface of the molecules, indicating higher stability. Liposome-mediated Td vaccine showed higher stability than the $AlPO_4$ gel-adsorbed Td vaccine due to its lower conductivity compared to the other.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

It is one of the easiest and fastest characterization methods for proteins. This method allows the determination of the molecular weight of the protein of interest; thus, the degradation of the protein's primary structure can be monitored [11].

SDS-PAGE was performed to determine the stability and integrity of antigens during the preparation of liposomal systems. SDS-PAGE was performed for liposomal formulation and a soluble mixture of antigens to characterize them and to have an insight into the effect of preparation conditions on the integrity of the antigen. The vertical gel electrophoresis under denaturing conditions (0.1% SDS) separates proteins based on

their molecular weight or size, as they moved towards the anode. The integrity of the antigen was maintained during the liposome preparation as no band was observed for the degradation product of the antigen. The bands of the encapsulated antigens were found to be similar to those of the native antigens (Fig. 7).

Discussion

The findings accumulated from this study go in line with previous studies, which together suggest and encourage the inclusion of liposomes as a promising adjuvant [6], nevertheless, the limited study traced their use in the Td vaccine previously. The total advantages of using liposomes as vaccine adjuvants are more than the factors limiting the use of liposomes as vaccine adjuvants. Therefore, liposomes can be effectively used as an alternative to aluminum adjuvants. Liposomes serve as an alternate adjuvant in vaccine delivery as they had the capacity to provide both cell-mediated and humoral immunity and had multiple routes of administration of vaccines. Liposome-mediated Td has a promising immunogenicity because it induces both innate and cell-mediated immunity and it curtailed the need for repetitive booster dose administration because of the memory induced by immunity cells. Furthermore, examining different liposomes' lipid compositions would also be crucial to achieving better effectiveness [18]. On the other hand, aluminum salts were known for their promising safety against various diseases by providing humoral immunity with a further benefit of low cost in the formulation of vaccines. To that end, further studies should be conducted on combining aluminum and liposomes adjuvants as one modified or upgraded adjuvant due to the beneficial properties of both substances and to investigate the immunogenicity and boost the level of both adjuvants.

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