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Immune response of novel carrier based vaccine delivery system for Tetanus Toxoid and r-HBsAg as single shot Vaccine

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ABSTRACT

A novel vaccine delivery system was developed for tetanus toxoid and HBsAg by formulating PLGA microspheres and surface modified by Chitosan. The produced microspheres were stabilized with various proteins and based on the results obtained the ideal formulation was optimized. The optimized formulation of TT containing microspheres and HBsAg containing microspheres were further evaluated for its immunological response. The immune response of the TT microspheres PCMS-TT was studied by immunizing guinea pigs with alum adsorbed vaccine (single dose), alum adsorbed vaccine with booster dose, PCMS-TT with trehalose and Mg (OH) $_2$, PCMS-TT with trehalose PCMS-TT with Mg (OH) $_2$. The immune serum of the above antigen was collected and used to treat the toxin induced mice. The PLGA –Chitosan Microspheres containing TT stabilized with trehalose and Mg (OH) $_2$ has produced higher levels of Ig G than the alum adsorbed tetanus vaccine with booster dose. The Immune response of the HBsAg microspheres PCMS-HB was studied by immunizing guinea pigs. The concentration of anti-HBsAg antibody in the collected blood was then determined using the solid phase enzyme linked immunoassay (ELISA). The PLGA –Chitosan Microspheres containing HBsAg stabilized with trehalose and Mg (OH) $_2$ has produced almost equivalent IgG levels of the alum adsorbed hepatitis B vaccine with booster dose.

Keywords: Hepatitis B Vaccine, Chitosan, Single shot vaccine, PLGA microspheres, HBsAg, Enzyme Immuno Assay

INTRODUCTION

One of the critical preclinical evaluations for any new vaccine, adjuvant or delivery system is the study of the immune response in experimental animals using model and novel vaccine antigens. Many PLGA microencapsulated vaccine antigens have been evaluated in a variety of animal models for protection against challenge, antibody responses or cell-mediated immune responses. In this study r-HBsAg was made into PLGA microspheres and the surface charge was changed to cationic by surface modification with chitosan.

Different polymers have been investigated for use in microparticles preparation. In selecting a polymer, it should be biodegradable so that it will not require surgical removal following drug/antigen depletion. Furthermore, it should be non-toxic, heat-stable, and allow for alteration of the antigen release rate[1]. The most studied biodegradable polymers are polylactide (PLA) or poly (DL-lactide-co-glycolide) (PLGA). These polymers degrade *in vivo* to form non-toxic lactic and glycolic acids and enable the rate of antigen release to be altered through varying the poly-lactide to glycolide ratios[2]. Both, *in vitro* and *in vivo* the PLGA copolymer undergoes degradation in an aqueous environment (hydrolytic degradation or biodegradation) through cleavage of its backbone ester linkages. The polymer chains undergo bulk degradation and the degradation occurs at uniform rate throughout the PLGA matrix[3, 4, 5].

The evaluation of controlled-release formulations for protein-based vaccines has become an active area of vaccine pharmaceutical research. Currently, parenteral administration of purified protein-based vaccine antigens involves multiple injections to

achieve an optimum immune response in humans. For example, recombinant hepatitis B surface antigen (HBsAg) adsorbed to aluminum adjuvant is typically administered with multiple injections over many months to obtain a suitable immune response and protection against hepatitis B viral infection in humans. Consequently, for a recombinant vaccine such as HBsAg, the design of an injectable formulation that releases the antigen in a controlled manner over a significant time period to induce long-lasting immunity from a single shot would be a promising improvement in terms of immunization coverage and compliances.

A prime objective in field of vaccination is also the development of non-parenteral immunization regimens, which facilitates the induction of comparable levels of systemic immunity to that elicited by conventional subcutaneous and intramuscular injections. Thus, the overall goal of this study was to test the pharmaceutical feasibility of developing a single shot HBsAg vaccine using PLGA microspheres and also to deliver them through nasal route and compare its mucosal immune responses.

Preparation of Surface-Modified PLGA Microspheres using Chitosan

The surface of the optimized PLGA microspheres was modified as reported by Ravi Kumar *et al*. (2004) with slight modifications[6]. In brief, 800 µl of recombinant HBsAg containing 1.5% w/v trehalose and 2% w/v Mg (OH) $_2$ was suspended in 10 ml of 4% w/v PLGA in dichloromethane and sonicated for 10 seconds in an ice bath (Soniweld, New Delhi, India). To this water-in-oil emulsion, 40 ml of 10% w/v aqueous polyvinyl alcohol containing 0.5% w/v chitosan hydrochloride was added and mixed at high speed with an Ultraturrax T-25 homogenizer (IKA, Germany) for 10 seconds to obtain a W/O/W emulsion. The W/O/W multiple emulsion was poured into 50 ml of 0.3% w/v aqueous polyvinyl alcohol with vigorous stirring for 1 h and the microspheres were collected by centrifugation, washed with distilled water and lyophilized. The sterile working area was regularly monitored by sampling the air and surface of the working area. The sterility tests were performed using fluid thioglycollate medium and soyabean-casein digest medium. The characterization such as size, loading efficiency and *in vitro* release studies of surface modified PLGA microspheres containing recombinant hepatitis B antigen was carried out as per the method previously described by V.Balasubramaniam and K.S. Jaganathan 2019[7]

Immunogenicity Studies of Tetanus Toxoid

The potency of the developed tetanus vaccine (model antigen) was determined by assessing the efficacy of the vaccine to stimulate the production of tetanus antitoxin in guinea pigs. The sera of the guinea pigs were examined for antitoxin by comparing their ability to protect the mice from the paralytic effects of a fixed dose of tetanus toxin (Lp/10) with that of the standard preparation of tetanus antitoxin to give the same protection.

Determination of Lp/10 (Limes Paralyticum/10) Dose of the Test Toxin

The Limes paralyticum/10 (Lp/10) dose of the test toxin is the smallest quantity of the toxin which when mixed with 0.1 units of the standard preparation and injected subcutaneously into mice causes tetanic paralysis within 4 days.

The toxin doses (mixtures) were prepared by adding 2 ml of diluted solution of the TNR preparation into each of five graded volumes of diluted test toxin corresponding to 1.4, 1.5, 1.6, 1.7, 1.8 and 1.9 ml. Their volumes were made upto 5 ml with peptone water, so that 0.5 ml of each mixture contains 0.1 unit of tetanus antitoxin. Mixtures were incubated at room temperature protected from light for 60 min. Then the mice were inoculated with 0.5 ml of mixture/mouse. The injected mice were observed for the following symptoms for 4 days.

Immunization

Male guinea pigs (350-400 g) were used for immunogenicity studies. Animals were housed in groups with free access to food and water (n=10). They were deprived of food 3 h prior to subcutaneous (SC) immunization. The Institutional Animals Ethical Committee of Smt. Sarojini Ramulamma College of Pharmacy has approved the study. The studies were carried out as per the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Dose-dependent response was studied by immunizing guinea pigs with various doses of alum adsorbed TT (0.3, 0.5, 0.7, 0.9 and 1.1 L_f/dose) and the antibody levels were measured after 2 weeks of booster dose as mentioned below. The standard antitoxin was also titrated against Lp/10 dose by toxin neutralization method in various species of mice models such as LACA, Swiss albino and BALB/c. The Lp/10 dose of toxin was mixed with known units of standard antitoxin (1.0 and 2.0 IU/ml) and incubated for 60 min and injected into LACA, Swiss albino and BALB/c mice models. Then the mice were observed for tetanic paralysis.

TT-PLGA microsphere based formulations were diluted with 1.0% w/v solution (sterile) of sodium carboxy methyl cellulose in normal saline individually to get a single dose of 0.5 L_f/ml (1/10 of single human dose) and injected subcutaneously into guinea pigs. The control group received the equivalent dose of alum adsorbed TT and booster dose was given after four weeks of primary immunization. After two weeks of gap following administration of booster, animals were bled by cardiac puncture at 2, 4, 6 and 8 weeks and the serum was separated and used for determination of antibody levels.

Determination of Protective Antibody Levels[8, 9]

The pooled guinea pig sera from each group were assayed by toxin neutralization method. Briefly, the pooled neat sera was incubated with tetanus toxin (Lp/10 dose) at room temperature for 60 min and injected subcutaneously into LACA mice (n=6 per group) and observed for any paralytic effect upto 4 days. This unit of tetanus antitoxin level was considered as 0.5 IU/ml. The pooled neat sera was also further diluted to get 1.0 IU/ml of antitoxin levels against Lp/10 dose of tetanus toxin level and observed for any tetanic paralytic effect upto 4 days.

Immunogenicity Studies of Recombinant Hepatitis B Antigen

Guinea pigs (350-400 g) were used for *in vivo* studies. Animals were housed in groups with free access to food and water (n=10). They were deprived of food 3 h prior to subcutaneous (SC) immunization. The formulations were prepared by dispersing the microspheres in sterile normal saline containing 1% w/v solution of sodium carboxy methyl cellulose to achieve a single dose of 20 µg/ml[10]. One ml of dosage volume was injected on the back of the guinea pigs. The control group (alum adsorbed vaccine) received 10 µg dose of alum adsorbed HBsAg and booster was given after four weeks of primary immunization. After two weeks of gap following administration of booster, animals were bled by cardiac puncture at 2, 4, 6 and 8 weeks.

The concentration of anti-HBsAg antibody in the collected blood was then determined using the solid phase enzyme linked immunoassay (AUSAB®, Abbott Laboratories, USA) (Nellore *et al.* 1992) (n=6). To signify actual antibody concentration (antibody titre) in mIU/ml, a standard curve was prepared using the calibrated anti-hepatitis B panel provided by Abbott Laboratories. Antibody response was plotted as log of anti-HBsAg antibody titres (mIU/ml) *vs.* time in days[11].

RESULTS AND DISCUSSION

Tetanus Toxoid

The Lp/10 dose of tetanus toxin to be used for the toxin neutralization test was estimated. It was observed that 0.0042 ml of tetanus toxin dose per mouse was the smallest quantity of the toxin which when mixed with 0.1 Unit of the standard antitoxin preparation caused tetanic paralysis within four days and this value was used as the Lp/10 dose and the same dose was used to challenge the animals in the further experiments. After ascertaining the level of Lp/10 value of tetanus toxin, the standard titration conducted done to check the validity of the Lp/10 test using same quantity of the tetanus toxin (0.0042 ml) and different antibody units of standard antitoxin (0.08, 0.09, 0.10, 0.11 and 0.12 IU dose/animal)

Dose-dependent response was studied by immunizing guinea pigs with various doses of alum adsorbed TT (0.3, 0.5, 0.7, 0.9 and 1.1 L_f/dose) and the antibody levels were measured after 2 weeks of booster dose. The results indicated that 0.5 L_f/dose triggered highest levels of antibodies while immunization with >0.5 L_f/dose showed decreasing antibody production. Thus 0.5 L_f/dose was used for the immunization schedule. Further, The Lp/10 dose of toxin was mixed with known units of standard antitoxin (1.0 and 2.0 IU/ml) and incubated for 60 min and injected into LACA, Swiss albino and BALB/c mice models. Subsequently the mice were observed for tetanic paralysis. Results suggested that LACA and Swiss albino mice model responded to real antitoxin levels (1.0 and 2.0 IU/ ml), whereas BALB/c suffered tetanic paralysis even on injecting 2 IU/ ml of tetanus antitoxin (Table 6.4). Therefore LACA and Swiss albino mice models could be utilized for the tetanus toxoid vaccine studies.

The results of the *in vitro* studies (percent entrapment and *in vitro* release studies) suggested that Trehalose, Gelatin and HP-β-CD were found to be better protein stabilizers with a model antigen (tetanus toxoid). Hence *in vivo* immunogenicity studies by toxin neutralization method was evaluated further to confirm the stabilizing activity. The immunogenicity of the TT-PLGA microspheres was compared with conventional alum adsorbed TT vaccine by using male guinea pigs. Comparisons were made between PLGA microspheres based formulation with protein stabilizers (trehalose, gelatin and HP-β-CD) (single injection), PLGA microspheres based formulation without protein stabilizers (single injection), alum adsorbed TT vaccine (single injection) and alum adsorbed TT vaccine (booster injections). The sera samples from immunized guinea pigs were titrated for tetanus antitoxin against Lp/10 dose by toxin neutralization method as per the method discussed under immunogenicity studies. The sera samples were pooled and examined for tetanus antitoxin against Lp/10 dose of tetanus toxin level and the observations are tabulated. This unit of tetanus antitoxin level was considered as 0.5 IU/ml. The pooled sera were further diluted to get 1.0 IU/ml of antitoxin levels against Lp/10 dose of tetanus toxin level and the observations were tabulated. These results indicated that single injection of PCMS-TT microspheres stabilized with trehalose & gelatin and booster injections of alum adsorbed TT vaccines resulted into an comparable immune response upto 8 weeks after booster immunization, when undiluted pooled sera was used against Lp/10 dose. However, single dose PCMS-TT microspheres stabilized with gelatin did not generate an equal immune response as compared to booster injections of alum vaccines (control) when diluted pooled sera was used against Lp/10 dose (*i.e.*, 1.0 IU/ml) upto 8 weeks after booster immunization. Whereas PCMS-TT microspheres stabilized with trehalose resulted into a comparable or better immune response (in both neat and diluted sera) as against the booster injections of alum vaccines as recorded upto 8 weeks after booster immunization.

The antibody levels of HP-β-CD stabilized PCMS-TT microspheres was found to be less than 0.5 IU/ml after 6 weeks of booster immunization (in both undiluted and diluted sera). Therefore, HP-β-CD could not be considered as an ideal protein stabilizer for PCMS-TT microsphere based formulations. Single injection of alum based TT vaccine and single dose of PCMS-TT microspheres without protein stabilizers showed very poor production of antibody titres. This effect might be attributed to the denaturation of the TT during the process of encapsulation. In conclusion, single dose of PCMS-TT microspheres stabilized with trehalose elicited comparable or better immune responses when compared to alum based TT vaccines (booster doses). The PMS-TT microspheres stabilized with gelatin and HP-β-CD also did not lead to comparable antibody responses. Hence, these trehalose stabilized PCMS-TT microspheres can open the door for the practical development of single-shot vaccines.

Mg $(OH)_2$ could also have acted as additional adjuvant, similar to the well known adjuvant Al $(OH)_3$. Hence, an additional formulation was made. The *in vivo* evaluation of this system did not lead to significant by higher and more prolonged antibody levels (upto 8 weeks after booster immunization) than those measured for the PLGA microspheres without stabilizers, but with $Mg(OH)_2$. These finding revealed that Mg $(OH)_2$ had no additional adjuvant effect and only used to prevent a pH-drop within PLGA microspheres and protein stabilizer (trehalose) could provide stable environment for TT during entrapment and subsequently during its release from the system.

Recombinant Hepatitis B Vaccine

The level of anti-HBsAg antibodies (IgG) recorded for single injection of PCMS-HB-T4 (trehalose stabilized) formulation was 3.2±0.3, 4.6±0.4, 5.1±0.2 and 4.8±0.3 (mIU/ml) on 2, 4, 6 and 8 weeks, respectively while 3.3±0.2, 4.6±0.3, 5.2±0.1 and 4.9±0.4 (mIU/ml) levels of IgG (anti-HBsAg) were recorded on 2, 4, 6 and 8 weeks, respectively for alum adsorbed HBsAg vaccine with booster injections. The IgG level recorded for single injection of PLGAOB4 and PCMS-HB-C4 were however significantly low when compared to PCMS-HB-T4 (single injection) and alum HBsAg vaccine (booster injections). These results suggested that the IgG level was significantly more in case of HBsAg-PLGA microspheres stabilized with trehalose as compared to BSA and HP-β-CD stabilized HBsAg-PLGA microspheres.

In conclusion, the 60th day (*i.e.*, 90th day after primary dose) of serum antibody titres indicated that a single injection of PLGA formulated HBsAg vaccine with trehalose (protein stabilizer) produced almost equivalent immune response when compared with two injections (with booster) of alum adsorbed HBsAg vaccine. Whereas, PLGA formulated HBsAg vaccine without trehalose and a single injection of alum adsorbed HBsAg vaccine did not result in any significant antibody titres. A single injection of a mixture of alum- and PLGA- formulated HBsAg resulted in a good antibody response that mimics multiple injections of alum adsorbed HBsAg vaccine. In contrast, in the present study significant antibody titres were estimated, when a single injection of stabilized HBsAg-PLGA microspheres was administered. If the released HBsAg from the microspheres with stabilizer was not antigenically active, the antibody titre could have been similar to the antibody titre produced by the one shot of the HBsAg-PLGA microspheres without stabilizer or less than the antibody titre produced by the one shot of the alum adsorbed HBsAg vaccine. Hence, it might be concluded that the HBsAg released from the stabilized PLGA microspheres was active enough to match with the two injections of the alum adsorbed HBsAg vaccine in this study. Mg $(OH)_2$ could also have acted as co-adjuvant, similar to the well known adjuvant Al $(OH)_3$. Hence, an additional formulation [HBsAg in PLGA with co-encapsulated trehalose, but without $Mg(OH)_2$] was formulated. The *in vivo* evaluation of this system did not lead to the significant and prolonged antibody levels (upto 8 weeks) than those measured for the PLGA microspheres without trehalose, but with $Mg(OH)_2$. These finding revealed that $Mg(OH)_2$ had no additional adjuvant effect and only could prevent pH-drop within PLGA microspheres and trehalose could provide stable environment for HBsAg during entrapment and subsequently during its release from the system.

CONCLUSION

The immunogenicity of the microspheres containing TT and HBsAg was studied. The surface modified Chitosan-PLGA microspheres stabilized with various stabilizers and antacid were investigated for the immune response. The microspheres stabilized with trehalose and antacid $Mg(OH)_2$ (PCMS-TT-T4 and PCMS-HB-T4) were found to be ideal in the respective *In-Vivo* studies. The PCMS-TT-T4 has shown better effects than the alum adsorbed Tetanus Toxoid and the PCMS-HB-T4 has produced almost equivalent IgG levels of the alum adsorbed hepatitis B vaccine with booster dose. Thus it could me a milestone on the march towards the single dose vaccine for Hepatitis B.

Table 1: IgG levels with TT loaded PLGAMS stabilized with Trehalose (Second week after booster dose) (neat sera)

S.No	Formulation/ Dose	Dilution of Sera	Toxin dose (Lp/10) mL	Observation											
				Day 1			Day 2			Day 3			Day 4		
1	Alum (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
				✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2	Alum (Booster dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
				✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3	PMS-TT-T & $Mg(OH)_2$ (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
				✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
4	PMS-TT & $Mg(OH)_2$ (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	✓	✓	✓	bp	bp	bp	1tp	1tp	1tp	2tp	2tp	2tp
				bp	✓	bp	bp	1tp	1tp	1tp	2tp	2tp	2tp	3tp	D
5	PMS-TT-T (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	✓	✓	✓	✓	✓	✓	bp	bp	Bp	1tp	1tp	1tp
				✓	✓	bp	✓	bp	bp	bp	bp	bp	1tp	2tp	2tp

✓- Normal; bp - Beginning of tetanic paralysis; 1tp – Tetanic paralysis of one limb; 2tp – Tetanic paralysis of two limb; 3tp – Tetanic paralysis of three limb; D - Death due to tetanus

Table 2 IgG levels with TT loaded PLGAMS stabilized with Trehalose (Eighth week after booster dose) (neat sera)

S.No	Formulation/ Dose	Dilution of Sera	Toxin dose (Lp/10) mL	Observation											
				Day 1			Day 2			Day 3			Day 4		
1	Alum (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	D	D	D	-	-	-	-	-	-	-	-	-
				D	D	D	-	-	-	-	-	-	-	-	-

2	Alum (Booster dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
				✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3	PMS-TT-T & $Mg(OH)_2$ (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
				✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
4	PMS-TT & $Mg(OH)_2$ (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	D	D	D	-	-	-	-	-	-	-	-	-
				D	D	D	-	-	-	-	-	-	-	-	-
5	PMS-TT-T (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	D	D	D	-	-	-	-	-	-	-	-	-
				D	D	D	-	-	-	-	-	-	-	-	-

✓- *Normal; bp - Beginning of tetanic paralysis; 1tp – Tetanic paralysis of one limb; 2tp – Tetanic paralysis of two limb; 3tp – Tetanic paralysis of three limb; D - Death due to tetanus*

Table 3 IgG levels with TT loaded PLGAMS stabilized with Trehalose (Second week after booster dose) (Diluted sera: 1 ml pooled sera and 1 mL normal saline)

S.No	Formulation/ Dose	Dilution of Sera	Toxin dose (Lp/10) mL	Observation											
				Day 1			Day 2			Day 3			Day 4		
1	Alum (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	D	D	D	-	-	-	-	-	-	-	-	-
				D	D	D	-	-	-	-	-	-	-	-	-
2	Alum (Booster dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
				✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3	PMS-TT-T & $Mg(OH)_2$ (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
				✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
4	PMS-TT & $Mg(OH)_2$ (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	D	D	D	-	-	-	-	-	-	-	-	-
				D	D	D	-	-	-	-	-	-	-	-	-
5	PMS-TT-T (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	D	D	D	-	-	-	-	-	-	-	-	-
				D	D	D	-	-	-	-	-	-	-	-	-

✓- *Normal; bp - Beginning of tetanic paralysis; 1tp – Tetanic paralysis of one limb; 2tp – Tetanic paralysis of two limb; 3tp – Tetanic paralysis of three limb; D - Death due to tetanus*

Table 4 IgG levels with TT loaded PLGAMS stabilized with Trehalose (Eighth week after booster dose) (Diluted sera: 1 ml pooled sera and 1 mL normal saline)

S.No	Formulation/ Dose	Dilution of Sera	Toxin dose (Lp/10) mL	Observation											
				Day 1			Day 2			Day 3			Day 4		
1	Alum (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	D	D	D	-	-	-	-	-	-	-	-	-
				D	D	D	-	-	-	-	-	-	-	-	-
2	Alum (Booster dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
				✓	✓	✓	✓	✓	✓	✓	✓		✓		
												1tp		1tp	2tp
3	PMS-TT-T & $Mg(OH)_2$ (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
				✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
4	PMS-TT & $Mg(OH)_2$ (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	D	D	D	-	-	-	-	-	-	-	-	-
				D	D	D	-	-	-	-	-	-	-	-	-
5	PMS-TT-T (Single dose)	2 ml pooled sera + 1.7 ml toxin + 1.3 ml peptone water	0.0042	D	D	D	-	-	-	-	-	-	-	-	-
				D	D	D	-	-	-	-	-	-	-	-	-

✓- *Normal; bp - Beginning of tetanic paralysis; 1tp – Tetanic paralysis of one limb; 2tp – Tetanic paralysis of two limb; 3tp – Tetanic paralysis of three limb; D - Death due to tetanus*

Table 5: IgG levels with HBsAg loaded PLGAMS stabilized with Trehalose (1.5% w/v)

S.No	Formulation/ Dose	Log anti-HBsAg (mIU/mL)*			
		2 Weeks	4 Weeks	6 Weeks	8 Weeks
1	Alum (Single dose)	2.1±0.3	2.5±0.2	2.4±0.2	2.4±0.3
2	Alum (Booster dose)	3.3±0.2	4.6±0.3	5.2±0.1	4.9±0.4
3	PCMS-HB-T4 & $Mg(OH)_2$ (Single dose)	3.2±0.3	4.6±0.4	5.1±0.2	4.8±0.3
4	PCMS-HB & $Mg(OH)_2$ (Single dose)	1.5±0.4	1.7±0.2	1.2±0.3	1.0±0.2
5	PCMS-HB-T4 (Single dose)	2.1±0.2	1.8±0.3	1.3±0.4	1.1±0.3

*All values are expressed as mean ± S.D. (n=6)

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