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Testing of immune-stability of Cap-OMP vaccine stored at room temperature in mice

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Abstract
Vaccines are increasingly being used for *Salmonella* Enteritidis and *Salmonella* Typhimurium infection. Outer membrane protein (OMP) plays an important role in induction of immune response in the subject against the organism and can be used as an effective candidate vaccine. OMPs are weak antigen; require adjuvants which act non-specifically to increase the immune response of these weak antigens. Calcium phosphate nanoparticles (CAP) have been widely used as adjuvant given to its ease in large scale production and economical. One of the important aspects of vaccination is the proper maintenance of cold-chain that is one of the greatest issues in the developing countries owing to lack of continuous power supply hence, there is a need for stable vaccine against salmonellosis. In the present study, the stability of *Salmonella* Typhimurium OMP entrapped in calcium phosphate nanoparticle, in terms of immunogenicity, was studied in mice. The CAP-OMP vaccine incubated either for 30 or 60 days in 4°C or 25°C was used to immunize the mice. The CAP-OMP vaccine incubated at 4°C showed significantly higher IgG response than the CAP-OMP vaccine incubated at 25°C. It was observed that CAP-OMP vaccine does not remain stable in room temperature probably due to loss of integrity of antigen that was evident in differential protein band profile in SDS-PAGE.

Keywords: Stability, CAP-OMP, *Salmonella*, incubation, vaccine

Introduction
Salmonellosis is an infection caused by bacteria called *Salmonella* that inhabits in the intestinal tract of animals, including birds and usually transmitted to humans through consumption of foods contaminated with animal faeces. *Salmonella enterica* subsp. *enterica* serovar Typhimurium is the most frequently isolated serovar causing global food- borne outbreaks. Vaccines are increasingly being used for *S.* Enteritidis and *S.* Typhimurium infection. Outer membrane protein (OMP) plays an important role in induction of immune response in the subject against the organism and can be used as an effective candidate vaccine. The OMP of *Salmonella* contains a family of pore farming proteins called porins (Balaji *et al*., 2006) [1]. OMPs are weak immunogen. To be used as effective vaccines, these proteins and peptides require immune-stimulating compounds or adjuvants which act non-specifically to increase the immune response of these weak antigens. He *et al.* (2000, 2002) [3, 4] for the first time showed the significant adjuvanticity of calcium phosphate nanoparticles being complexed with whole capsid proteins of Hepatitis B virus. One of the important aspect of vaccination is the proper maintenance of cold-chain that is one of the greatest issue in the developing countries owing to lack of continuous power supply. He *et al.* (2002) [4] opined that incubation at 37°C, calcium phosphate nanoparticles does not undergo any physic-chemical changes. However, to our knowledge, no works has been carried out on assessment of stability of calcium phosphate nanoparticle adsorbed protein antigen in terms of immunogenicity.
In the present study, the stability, in terms of immunogenicity, of the whole outer membrane protein of *Salmonella* Typhimurium MTCC-98 entrapped in calcium phosphate nanoparticles (CAP-OMP vaccine) was evaluated in mice after a period of incubation for 30 or 60 days at 4°C or 25°C.

Materials and Methods
The Swiss Albino mice were obtained from Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India. The animal experiments were approved by Institutional Animal Ethics Committee of Assam Agricultural University, Khanapara.

The whole outer membrane protein of *Salmonella* Typhimurium was isolated by Cho-Kim *et al*., (1991) [2]. Briefly, the *Salmonella* Typhimurium was grown in BHI broth upto mid-log phase. The cells were harvested by centrifugation at 10,000×g for 10 minutes. The cells were resuspended in HEPES buffer. The cell membrane was broken by ultrasonication. The cell debris was separated by centrifugation at 10,000×g for 10 minutes. The membrane was precipitated by ultracentrifugation at 100,000×g for 1 hour followed by incubation of membrane fraction in 2% sodium lauryl sarcisonate for 1 hour at room temperature (25°C). The suspension was ultracentrifuged again at 100,000×g for 1 hour at 4°C. The pellet of OMP was resuspended in sterile triple glass water.

Preparation of calcium phosphate nanoparticle adsorbed outer membrane protein vaccine (CAP-OMP)

The CAP-OMP was prepared as per the method described by He *et al*. (2002) [4]. Briefly, 1 mg of the OMP was added to a stirring conical flask followed by addition of 7.5 ml of 12.5 mM of calcium chloride and 12.5 mM sodium phosphate (dibasic) in the presence of 1.5 ml of 15.6 mM sodium citrate. The solution was stirred for one hour. The suspension was coated with cellobiose followed by addition of 4 mg of OMP. The resultant CAP-OMP was lyophilized. The total protein (entrapped protein plus the protein coated outside the nanoparticle by cellobiose) of calcium phosphate nanoparticle was estimated by modified Lowry's method (Stoscheck, 1990) [8].

Incubation of CAP-OMP and animal experiment

The CAP-OMP was incubated either in 25°C or in 4°C for 30 or 60 days. The CAP-OMP was dissolved in 0.1M PBS immediately after the end of incubation and was injected to mice at the rate of 50µg per mice. The control CAP-OMP was injected immediately after preparation. The booster dose (50µg per mice) was administered on 14 day post primary immunization.

Group of mice immunized with CAP-OMP and condition of incubation was mentioned table 1.

Table 1: Condition of incubation of cap-omp vaccine

Group of mice (n=6)	Storage conditions of vaccine that was injected to mice subcutaneously	Dose per mice (Primary or Booster dose)
I	Phosphate buffered saline.	100µl
II	CAP-OMP Vaccine stored in 4ºC for 30 days	50µg
III	CAP-OMP Vaccine stored in 4ºC for 60 days	50µg
IV	CAP-OMP Vaccine stored in 25ºC for 30 days	50µg
V	CAP-OMP Vaccine stored in 25ºC for 60 days	50µg
VI	CAP-OMP Vaccine of zero days of preparation (no incubation)	50µg

The blood samples were collected from tail vein of mice on 0 day before immunization, 7th, 14th, 21th and 28th days post primary immunization. After separation of serum, the humoral immune response of all the experimental groups was determined using indirect Elisa.

Determination of integrity of proteins entrapped in calcium phosphate nanoparticle

The integrity of entrapped protein in CAP-OMP was determined by SDS-PAGE. Briefly, the CAP-OMP was dissolved in 2×SDS PAGE gel loading buffer and was run in 12% SDS-PAGE. The molecular weights of separated bands of protein was determined by comparing the migration of protein bands with that of standard protein marker.

Statistical Analysis

All the data were expressed in terms of mean ± standard error of mean. The difference between the groups was analyzed by two factors ANOVA. The post-hoc multiple comparison of the mean at 95% family-wise confidence level was carried using Tukey's HSD test. The p-value less than 0.05 were considered statistically significant. All of the analysis was carried out in statistical software program R (version 3.4.1).

Results and Discussion

The antibody response of the mice vaccinated with calcium phosphate nanoparticles (CAP-OMP) vaccine formulation was determined by indirect ELISA. The table 2 represents values of mean antibody response of the vaccinated mice up to 30 days post-booster vaccination. The mean IgG response of group VI vaccinated with CAP-OMP vaccine (no incubation) significantly increased up to 14 days. Following booster vaccination on 14th day, it was increased significantly up to 21 days which was maintained up to 28th day. The mean IgG response of group II vaccinated with CAP-OMP vaccine (stored at 4° for 30 day) was maintained up to 14 days. Following booster vaccination on 14th day, it was increased significantly up to 28 days post-primary immunization. The mean IgG response of group III vaccinated with CAP-OMP vaccine (stored at 4°C for 60 days) significantly increased up to 14th day post-primary vaccination. Following booster vaccination on 14th day, it was increased significantly up to 28th day post-primary immunization. The mean IgG response of group IV vaccinated with CAP-OMP vaccine (stored at 25°C for 30 days) was maintained without significant changes up to 14 days. Following booster vaccination on 14th days, it increased significantly up to 28th day post-primary immunization. The mean IgG response of group V vaccinated with CAP-OMP vaccine (stored at 25°C for 60 days) was maintained up to 14 days. Following booster vaccination on 14th day, it increased significantly up to 28th day post-primary immunization.

The mean IgG response of vaccine stored at 4°C was significantly higher than that of the one stored at 25°C. On the other hand, storage at 4°C was not found to alter the immuno-potential of the vaccine as evident from the lack of significant differences in IgG response of the former from that of the vaccine used for immunization immediately after preparation. As a whole, there was significant difference among all the groups except groups.

Table 2: Represents values of mean antibody response of the vaccinated mice up to 30 days post-booster vaccination.

Days	Days post primary vaccination				
	0	7	14	21	28
Group	Mean± SE	Mean± SE	Mean± SE	Mean± SE	Mean ± SE
Group VI	0.00	$0.09^{a;\ I} \pm 0.0021$	$0.19^{a;\ II} \pm 0.0025$	$0.23^{a;\ III} \pm 0.0023$	$0.24^{a;\ III} \pm 0.012$
Group II	0.000	$0.14^{b;\ I} \pm 0.0009$	$0.17^{a,b;\ I} \pm 0.0038$	$0.26^{a;\ II} \pm 0.0033$	$0.24^{a;\ III} \pm 0.01$
Group III	0.00	$0.12^{b;\ I} \pm 0.0037$	$0.16^{b;\ II} \pm 0.002$	$0.25^{a;\ III} \pm 0.0093$	$0.28^{b;\ IV} \pm 0.0035$
Group IV	0.0001	$0.08^{a;\ I,III} \pm 0.0027$	$0.054^{c;\ I} \pm 0.0007$	$0.10^{b;\ III} \pm 0.0053$	$0.14^{c;\ II} \pm 0.0035$
Group V	0.00	$0.08^{a;\ I} \pm 0.0040$	$0.083^{d;\ I} \pm 0.0032$	$0.16^{c;\ II} \pm 0.0022$	$0.13^{c;\ III} \pm 0.0086$

Different alphabets within a column indicate statistically significant difference between groups and different roman numbers within a row indicate statistically significant difference within a group at different time intervals.
Group VI (CAP-OMP vaccine zero days of preparation)
Group II (CAP-OMP vaccine stored in 4^0C for 30 days)
Group III (CAP-OMP vaccine stored in 4^0C for 60 days)
Group IV (CAP-OMP vaccine stored in 25^0C for 30 days)
Group V (CAP-OMP vaccine stored in 25^0C for 60 days).

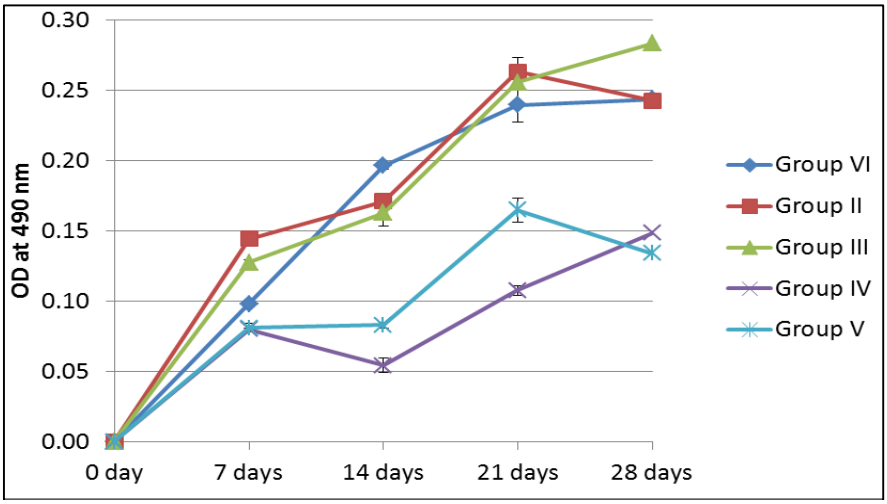

Fig 1: Graphical presentation of igg response against cap-omp vaccine

He *et al.* (2000) [3] analyzed CAP with subunit vaccine glycoproteins against HSV-2 virus at 4°C, room temperature, 37°C, and 50° C for a 6-month period and observed no significant changes in pH, size, or surface morphology. In the present study antibody response decreased against CAP-OMP vaccine which incubated at 25° C for 30 and 60 days. Differences in bio-physical properties of sub - unit vaccine were used with CAP could be the possible reason.

Comparing stability of omp after incubation

For better understanding of the stability of the protein after incubation, SDS-PAGE (12%) was run for all proteins of CAP-OMP vaccine stored at different temperature and banding pattern obtained for each of them was analysed. CAP-OMP vaccine stored at 25°C for 30 and 60 days did not show the 29.64 KDa protein band but this band was present in the CAP-OMP vaccine stored at 4°C for 30 and 60 days.

OMP = outer membrane protein
VI = CAP-OMP vaccine at zero day of preparation
II = CAP-OMP vaccine stored in 4°C for 30 days
III = CAP-OMP vaccine stored in 4°C for 60 days
IV = CAP-OMP vaccine stored in 25°C for 30 days
V = CAP-OMP vaccine stored in 25°C for 60 days

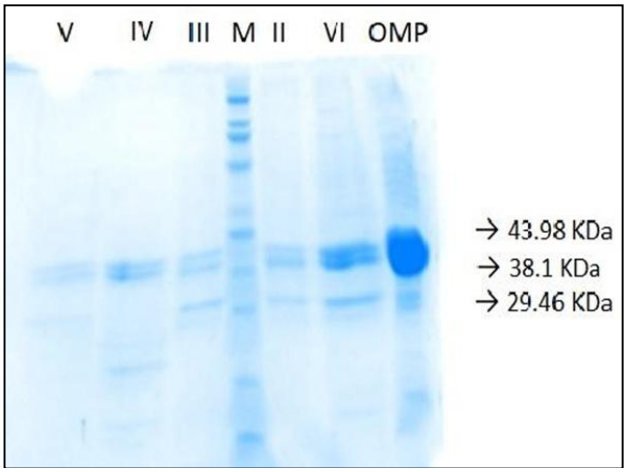

Fig 2: Coomassie brilliant blue stained sds-page (12%) profile of outer membrane proteins of *salmonella* typhimurium after incubation at different temperatures along with standard mw marker

Sing *et al*. (2018) [7] reported that the outer membrane proteins of *Salmonella* Typhimurium with molecular weight 43.98, 38.1, 29.46 KDa are immunogenic. This could justify the lesser immune response observed in the present study in the group of mice vaccinated with CAP-OMP vaccine stored at 25°C for 60 days.

Mei *et al.* (2002) [5] reported that the TT conjugates can remain immunogenic at higher storage temperatures (up to 55°C for 5 weeks) and are more thermo-stable. Similarly, the antibody response against the carrier protein TT was almost unchanged when the vaccines were exposed to freeze-thawing cycles, stored at 23°C or 37°C. Storage of the Hib–TT vaccines at high temperature (55°C) lowered the anti-TT response but again this was not significantly different ($P>0.05$) from those stored at 4°C. These results are in contrast to the result of the present study where the low molecular weight protein got lost on storage at 25°C.

Conclusion

A review of available literature revealed that till date no study has been conducted to assess the stability of vaccines based on calcium phosphate nanoparticle adjuvanted OMP of *Salmonella* Typhimurium at room temperature. In the present study, the stability of CAP-OMP vaccine kept at 4°C or 25°C for 30 or 60 days was assessed. The CAP- OMP vaccine incubated at 4°C showed significantly higher IgG response than that of CAP- OMP vaccine incubated at 25°C. The SDS-Page was run for all CAP – OMP vaccine following incubation at different temperatures. The results showed that CAP- OMP vaccine stored at 25°C lost a 29.46 KDa protein band that was reported to be immunogenic (Sing, 2018) [7]. It was observed that CAP-OMP vaccine does not remain stable in room temperature probably due to loss of integrity of antigen that was evident in differential protein band profile in SDS-Page.

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