DEVELOPMENT OF STABLE HAEMOPHILUS TYPE b CONJUGATE AND WHOLE CELL PERTUSSIS COMBINED VACCINE

Sujana Prasad Chittineni1*, Satish Chandra Maheshwari2, Lakshmi Narasu Mangamoori3

1,3Department of Biotechnology, Jawaharlal Nehru Technological University, Hyderabad, India.
2Biological E Ltd, Hyderabad, India.

ABSTRACT

Haemophilus type b conjugate and whole cell pertussis liquid vaccines are freeze sensitive and also labile to heat. Freeze sensitivity is one of the major causes for wastage of vaccines during transport and storage in health care centers or pharmacy store. Currently Haemophilus type b conjugate vaccine is available in both liquid and lyophilized forms. Whole cell pertussis vaccines are available only in liquid form, in combination with diphtheria, tetanus, Hepatitis B and Haemophilus type b conjugate. The combination vaccines containing both these antigens are freeze and heat sensitive. Lyophilized Haemophilus type b conjugate vaccine is reconstituted with adjuvant or with DTP-Hepatitis B combination vaccine before injection. Reconstituted vaccines will become freeze and heat sensitive, cannot be stored for longer periods, to be used within 6 hours after reconstitution.

This research work aimed to develop freeze stable and heat stability improved combination vaccine of Haemophilus type b conjugate and whole cell pertussis. Lyophilization is an excellent option to protect vaccine from freezing and heat effects. In general stability of the vaccines in lyophilized state is better compared to liquid state. Lyophilized combined vaccine of Haemophilus type b conjugate and whole cell pertussis vaccines were prepared with novel protective material polaxamer block copolymer as cryo protectant or combination of polaxamer polymer and a sugar. The developed lyophilized vaccines were resistant to freezing and heat stability was enhanced substantially when compared to commercially available vaccines. This lyophilized combination vaccine can be extemporaneously reconstituted before administration using suitable diluent. The lyophilized Haemophilus type
b conjugate and whole cell pertussis combination vaccine can also be reconstituted with Diphtheria, tetanus and hepatitis B vaccines adsorbed to adjuvant or with aluminum phosphate adjuvant before administration. Additionally presence of polaxamer block copolymer gives protection from freezing post reconstitution.

**Key words:** Haemophilus type b conjugate, whole cell pertussis, Freeze stable, Combination vaccine, Polaxamer and Lyophilization.

**INTRODUCTION**
Currently combination vaccines having Haemophilus type b conjugate and whole cell pertussis vaccines available from different manufacturers apart from monovalent lyophilized Haemophilus type b conjugate vaccine. Combination vaccines available are fully liquid vaccines or Hib component in lyophilized form in a separate container. However no lyophilized pertussis vaccine is available in the market. The Haemophilus type b conjugate and whole cell pertussis vaccines were most labile components of currently available combination vaccines. The aim of this work is to develop a formulation that can prevent efficacy loss of Haemophilus type b conjugate and whole cell pertussis vaccine due freezing or heating for sufficient period of time. The available liquid vaccines at present are losing potency or stability on freeze thaw, exposure to heat higher than storage condition. Especially in tropic conditions Haemophilus type b conjugate and whole cell pertussis vaccines can lost efficacy in short time when exposed to temperatures higher than 25°C.

In the past researchers have studied the freeze thaw effect on pertussis and Haemophilus type conjugate vaccines [1, 2, 3, 4, 5, 6, 7]. When DTP vaccines are submitted to freezing at -20°C for 15 days the potency of the pertussis component loses more than 50% of its initial value. Monovalent pertussis vaccines were evidently unstable at 4°C: during storage for 18 months some samples lost 58% to 87% of their initial potency[8]. Pertussis vaccine loses about 0.4% potency per day when exposed to 25°C and about 5% per day when exposed to 35°C. Haemophilus type b when exposed to heat and freeze thaw conditions loses its efficacy through aggregate formation and increase of free polysaccharide[9].

It is well known that numerous chemical and biological systems are unstable when they are in solution or in the presence of not negligible quantities (above 5%) of water. For this reason, they are frequently desiccated in order to ensure their preservation. An increase in water content of the freeze dried products is accompanied by an increase in molecular mobility
which favors chemical reactions.\cite{10} Lyophilization is a process consisting solidifying the aqueous environment by freezing and eliminating subsequently the ice by selective sublimation under a vacuum. By means of this method, various substances and especially biological substances, may be stabilized and preserved for long periods and can be regenerated to a state closely resembling the original condition, simply by rehydrating\cite{11}. So excipients and cryo protectants used in the formulation plays a key role in success of lyophilization process.

Surfactants and non ionic block copolymers at appropriate concentrations can be used as cryo protectants during lyophilization process and can inhibit surface induced denaturation\cite{12}. For some products sugars alone cannot give protection during lyophilization and use of amines along with sugars can lead to cake browning due to denaturation\cite{13}.

MATERIALS AND METHODS
Material
Haemophilus type b conjugate manufactured by conjugating polysaccharide poly ribosyl ribitol phosphate (PRP) with tetanus toxoidin compliance with WHO TRS 897 and was obtained from Biological E Ltd. Whole cell pertussis antigen was prepared by growing two strains (134 and 509) of Bordetella pertussis individually using liquid medium in fermentor, heat inactivated and mixed together. Whole cell pertussis antigen bivalent was obtained from Biological E ltd. Two nonionic block copolymers from polxamer series (polxamer 407, was purchased from Sigma and polaxamer 188 was purchased from HiMedia Laboratories Pvt. Ltd) were selected based on their suitability as pharmaceutical excipients. Sucrose and tris were purchased from Merck. All the chemicals used for testing were of analytical grade and purchased from commercial suppliers.

Methods
Stock solutions of polaxamer at 10% W/V concentration prepared in cold water for injection and kept at 2-8°C overnight for complete dissolution. These stock solutions were sterilized by filtration through 0.2µ filter. Tris buffer 20 mM was prepared by dissolving 2.4g of tris base in 1 liter of water for injection and adjustment of pH to 6.5 with hydrochloric acid.

Formulation and Lyophilization process
In first step pH of the adjusted whole cell pertussis antigen is adjusted to 5.7 to 6.7 using tris buffer and cooled to 4°C. Sufficient quantity of polaxamer stock solution is added and mixed
thoroughly by stirring using magnetic stirrer at 150 rpm to attain homogeneity. Then required quantity of haemophilus type b conjugate maintained at 2-8°C is added and stirred for 20 minutes. Final volume was made up with water for injection. This formulated combination vaccine 2.5 mL was filled in to 10 mL glass vials, half stoppered with slotted rubber closures and subjected to lyophilization using a Virtis lyophilizer under controlled lyophilization cycle. After completion of lyophilization cycle vials were stoppered completely under vacuum.

Four formulations having the following compositions gave elegant and intact cake and in formulations having polaxamer above 3% proper cake formation was not observed.

Formulation1: 30 IOU/mL pertussis antigen, 20µg per mL haemophilus type b conjugate and polaxamer 188 2% W/V. Formulation2: 30 IOU/mL pertussis antigen, 20µg per mL haemophilus type b conjugate, polaxamer 188 1% W/V and sucrose 1% W/V. Formulation3: 30 IOU/mL pertussis antigen, 20µg per mL haemophilus type b conjugate and polaxamer 407 2% W/V. Formulation4: 30 IOU/mL pertussis antigen, 20µg per mL haemophilus type b conjugate, polaxamer 407 1% W/V and sucrose 1% W/V.

In all four formulations tris buffer was used to adjust the pH of pertussis antigen. The lyophilization cycle parameters comprises of preliminary freezing was done at temperature below -40°C for about 3 hours and primary drying was carried out for about 40 hours at temperature between -50°C and 20°C. secondary drying was carried out at temperature above or equal to 25°C for about 6 hours to achieve moisture below 3%. Vaccum of 200µbar used for primary drying and 20µbar for secondary drying.

These four formulations were tested for different quality parameters required for vaccine to declare as safe and potent. All 4 formulations were subjected for stability analysis at real time conditions and accelerated conditions. Tests involved animals were performed only on formulation 3 for stability studies. Animal usage for the experiments was approved by institutional animal ethics committee of Biological E Ltd as per CPCSEA guidelines.

**Testing methods**

The lyophilized combination vaccine comprising Haemophilus type b and whole cell pertussis vaccine was tested for different quality parameters like appearance, moisture content, pH, pertussis specific toxicity, pertussis potency, free polysaccharide for Haemophilus type b conjugate, Haemophilus type b conjugate immunogenicity and
abnormal toxicity. Appearance was tested visually, moisture content was tested by Karl Fisher titration method using Mettler auto titrator as per Indian Pharmacopoeia\(^{[19]}\), pH was tested by potentiometric method using Thermo Orion pH meter having glass electrode at 20 to 25\(^{\circ}\)C\(^{[17]}\). Pertussis specific toxicity was tested by mouse weight gain test in mice and pertussis potency was tested by intra-cerebral mice challenge assay (kendrik method)\(^{[14]}\).

HPAEC-PAD (high performance anion-exchange chromatography with pulsed amperometric detection) method was used for free polysaccharide estimation using PRP standard obtained from NIBSC. The prepared lyophilized vaccine reconstituted with water for injection prior to free polysaccharide separation from conjugated polysaccharide. Alkaline hydrolysis of the standard and vaccine samples was performed using sodium hydroxide. C4 cartridge column was used for separating free from total polysaccharide prior to hydrolysis.

A Dionex ion chromatography system with Dionex PA-10 column with AminoTrap and PA-10 guard column was used.\(^{[15]}\) Hib immunogenicity was tested in mice using saline as control and sera was titrated using 96 well plastic plates having modified surface to bind polysaccharide in presence of N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride cross linker as per method described else were.\(^{[16]}\) Abnormal toxicity was performed as per Indian Pharmacopoeia\(^{[19]}\).

**Stability studies**

The lyophilized combined vaccine formulations were subjected for stability at stress and real time conditions as per ICH guidelines\(^{[20]}\). Stress study was conducted for 6 months at 37\(^{\circ}\)C and real time stability study was conducted for 36 months at 2-8\(^{\circ}\)C. Animal experiments were carried out only on formulation 3 to reduce the animal usage.

**RESULTS**

For all 4 formulations elegant and intact cake was obtained after lyophilization (Fig.1b)
Post reconstitution solution appearance was homogeneous, vaccine was resuspended easily within 30 seconds and no clumps were observed. (Fig.1a)
Fig. 1a: Picture of reconstituted lyophilized vial of formulation 3 with water for injection, Fig. 1b: Picture of lyophilized vial of formulation 3.

Moisture content was less than 5% in all the formulations when tested by karlfisher titration method. Specific toxicity test for pertussis component by mouse weight gain test showed good weight gain (>60%) against control mice for all the four formulations. More than 4 IU potency was obtained for pertussis component when tested in mice by intra cerebral challenge method and complied with requirements [15, 19].

Estimated free PRP (unconjugated polysaccharide) was less than 20% when tested using HPAEC-PAD method after lyophilization and complied with monovalent haemophilus type b conjugate vaccine requirement [14, 19]. About 3 to 5% raise in free PRP was observed during lyophilization.

![Fig 2: Chromatogram showing free PRP peak in HPAEC-PAD analysis.](image)

100% sero conversion was obtained in all the formulations for Haemophilus type b conjugate immunogenicity. All the four formulations passed for abnormal toxicity. Post reconstitution appearance was good for all four formulations at both real time and stress conditions at all
time points. Results of key stability parameters like haemophilus type b conjugate free PRP of all four formulations and pertussis potency (only for formulation 3), at real time and accelerated conditions were shown in fig3, 4, 5 and 6. Hib immunogenicity sero conversion was 100% for formulation 3 at all time points of real time and accelerated conditions.

Fig.3: %Free PRP at real time stability conditions

Fig.4: Potency of whole cell pertussis at real time stability conditions for formulation 3

Fig.5: %Free PRP at stress stability conditions
DISCUSSION

Nonionic block copolymers polaxamer 407 found suitable as protective agent for lyophilization of haemophilus type b conjugate and whole cell pertussis. Block copolymers at concentrations below 3% found suitable in lyophilization process. Polxamer 188 also found suitable and formulations showed similar results compared to formulations made with polaxamer 407. (Data not shown) Low concentrations of polaxamer (1%) in combination with low concentrations of sugars like sucrose (1%) also can be used to prepare lyophilized formulations of whole cell pertussis and haemophilus type b conjugate. Developed formulations were gave good cake and devoid of aggregation of dried product. Whole cell pertussis and media components presents in the whole cell pertussis bulk helps in formation of good cake size. Lyophilization cycle time can be shortened (to about 48 hours) using block copolymers or combination of block copolymer and sugars. Reduced cycle times reduce the cost of lyophilization as well. Homogenization of whole cell pertussis in presence of polaxamer block copolymer can eliminate clumps and can protect from stirring stress.

The developed lyophilized haemophilus type b conjugate and whole cell pertussis combination vaccine test results meets the requirement for individual components defined in Indian Pharmacopoeia, WHO TRS 897 and WHO TRS 941[19, 14, 15]. In both real time and stress stability studies minimal increase in free PRP ("Fig 3", "Fig 5") and minimal reduction in pertussis potency from initial value ("Fig 4", "Fig 6") was observed in the formulations studied. Free PRP rise was less than 10% in 6 months at 37°C and 36 months at 2-8°C. Pertussis potency drop was less than 2 IU in 6 months at 37°C and 36 months at 2-8°C. Free
PRP below 10% after lyophilization can be achieved by selecting the bulks of haemophilus type b conjugate with free PRP near to 5%.

The developed lyophilized haemophilus type b conjugate and whole cell pertussis combination vaccine can be reconstituted before administration with suitable diluent like water for injection or aluminum phosphate adjuvant or aluminum adjuvanted vaccines like Tetanus, Diphtheria and Hepatitis B.

CONCLUSIONS
Combined haemophilus type b conjugate and whole cell vaccine lyophilized was successfully developed using polxamer block copolymer single component as formulation excipient or combination of polaxamer block copolymer and sugar. Improvement of the stability and increasing the shelf life of the combination vaccines containing haemophilus type b conjugate and whole cell vaccines achieved by development of lyophilized whole cell pertussis and Haemophilus type b conjugate combined vaccine. Separation both these components for extemporaneous reconstitution and improved heat stability brings advantage for development of stable multivalent vaccines outside the cold chain. Long shelf life (minimum 3 years) of lyophilized whole cell pertussis and Haemophilus type b conjugate combined vaccine is suitable for stock piling for mass immunizations. Because of vaccine in lyophilized form resistant to freezing was achieved. The lyophilized whole cell pertussis and haemophilus type b conjugate combined vaccine can be used for immunization of children or adults not received pertussis and haemophilus vaccination.

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