Upholding the Polyribosyl ribitol phosphate (PRP) content in different stages in manufacturing of Haemophilus influenzae type b (Hib) conjugate vaccine.

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Abstract:

Haemophilus influenzae type b (Hib) causes many severe diseases, including epiglottitis, pneumonia, sepsis, and meningitis (David W McCormick and Elizabeth M Molyneux, 2011). In developed countries, the annual incidence of meningitis caused by bacteria is approximately 5–10 cases per population of 100,000. The Hib conjugate vaccine is considered protective, safe and Effective control (Verma R et al., 2011). Many factors may play a role in the immunogenicity of Hib conjugate vaccines, such as the polysaccharides and proteins carrier used in vaccine construction, as well as the method of conjugation (Heikki Peltola, 2000). A Hib conjugate vaccine has been constructed via chemical synthesis of a Hib saccharide antigen. Two models of carbohydrate-protein conjugate have been established, the single ended model (terminal amination-single method) and cross-linked lattice matrix (dual amination method). HPAEC-PAD (high performance anion-exchange chromatography with pulsed amperometric detection) method was used for free polysaccharide estimation using PRP standard obtained from NIBSC. Maintain the PRP content in different stages are more vital for the effective production of Hib conjugate vaccine.

Key words: *Haemophilus type b* conjugate, Polyribosyl ribitol phosphate (PRP), immunogenicity.

INTRODUCTION

Haemophilus influenzae type B vaccine is a conjugate vaccine developed for the prevention of invasive disease caused by *Haemophilus influenzae* type b bacteria. The Centers for Disease Control and Prevention (CDC) has recommended the use of the Hib vaccine. Due to routine use of the Hib vaccine in the U.S. from 1980 to 1990, the incidence of invasive Hib disease has decreased from 40-100 per 100,000 children down to 1.3 per 100,000. Vaccinations against *Haemophilus influenzae* (Hib) have decreased early childhood meningitis significantly in developed countries and recently in developing countries.

Haemophilus influenzae type b (Hib) is a bacterium responsible for severe pneumonia, meningitis and other invasive diseases almost exclusively in children aged less than 5 years. It is transmitted through the respiratory tract from infected to susceptible individuals (McVernon J et al., 2008).

In 2000, Hib was estimated to have caused two to three million cases of serious disease, notably pneumonia and meningitis, and 386 000 deaths in young children. Hib disease is observed in all parts of the world but is difficult to confirm because it requires prompt laboratory investigation in patients that have not received prior antibiotic treatment.

The vaccine is now used in the routine immunization schedule of more than 100 countries and WHO recommends the use of Hib conjugate vaccines in all countries. The vaccine is available in monovalent presentation or combined with DTP and other vaccine combinations including with hepatitis B and inactivated polio vaccines.

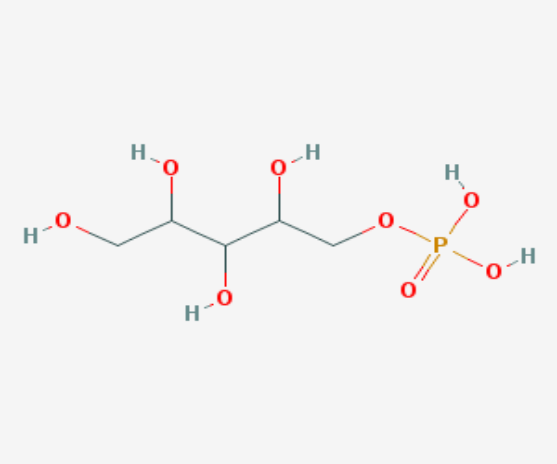
A polyribosylribitol phosphate (polysaccharide)-tetanus protein conjugates vaccine (PRP-T) against *Haemophilus influenzae* type b (Hib) was evaluated for safety and efficacy after vaccination of more than 100,000 infants. No major side effects were attributed to the vaccine. Immunogenicity studies showed an antibody response in 70 % to 100 % of infants after two doses and in 98 % to 100 % of infants after three doses, within the first 6 months of life. Antibodies persisted in 90 % of recipients, in whom significant anamnestic responses developed after a booster dose at 18 months of age. In comparison with other available Hib vaccines, PRP-T induces equal or higher mean titers after three doses. Although licensure of other vaccines interrupted controlled efficacy trials, up to that point five cases of Hib disease in those trials had occurred in placebo recipients, and no Hib disease has been reported in the more than 100,000 vaccinated infants who have received more than one dose of PRP-T. Thus PRP-T combined immunogenicity early in life with induction of immunologic memory (Barbara Bolgiano et al., 2001).

Hib is predominantly a childhood disease with over 80% of cases worldwide occurring in children aged <5 years. Before the commencement of vaccination, Hib was one of the commonest bacterial causes of pneumonia and meningitis in children aged between 4 and 18 months, with a high case fatality rate the world over.

The type b polysaccharide capsule is attractive as a vaccine antigen since invasive disease is almost exclusively restricted to type b organisms and antipolysaccharide antibodies are important in natural immunity (Fritzell B and Plotkin S, 1992). The induction of anti-PRP antibodies at an age young enough to protect those most at risk of Hib disease has been the goal of vaccine development.

PRP having the different chemical names like Polyribitol phosphate, Polyribosyl ribitol phosphate; Polyribose-ribitol phosphate and 1-o-phosphonopentitol having the Molecular formula of C5H13O8P and molecular weight of 232.125 g/mol. Find the below Figure-1 for its 2 D and 3 D,

Figure-1: 2D and 3D Structure of PRP



2D Structure

3D Structure

Refer the Table-1 for Computed Properties of PRP.

Table-1:

Property Name	Property Value
Molecular Weight	232.125 g/mol
Hydrogen Bond Donor Count	6
Hydrogen Bond Acceptor Count	8
Rotatable Bond Count	6
Complexity	202
Topological Polar Surface Area	148 A^2
Monoisotopic Mass	232.035 g/mol
Exact Mass	232.035 g/mol
XLogP3-AA	-4.1
Compound Is Canonicalized	true
Heavy Atom Count	14
Undefined Atom Stereocenter Count	3
Covalently-Bonded Unit Count	1

MATERIALS AND METHODS

Material

Haemophilus type b conjugate manufactured by conjugating polysaccharide poly ribosyl ribitol phosphate (PRP) with tetanus toxoid in compliance with WHO TRS 897 (WHO Technical Report Series, No. 897, 2000). The *Haemophilus influenzae type b* was grown in synthetic basal media and fermentation media. The fermented *Haemophilus type b* was under go next stage for inactivation and cell separation followed by Purified polysaccharide (PRP) preparation. Purified polysaccharide (PRP) was conjugated with Tetanus Toxoid bulk.

The immune response of children to PRP polysaccharide vaccine is strikingly age related. Young infants respond infrequently and with low antibodies level but the immune responses then improve with age. The limited immunogenicity of the polysaccharide PRP vaccine in infants and young children has led to the development of the Hib protein conjugate vaccine.

Methods

The *Haemophilus influenzae type b* was grown in synthetic basal media, which included general salts like monosodium glutamate, disodium hydrogen phosphate, sodium chloride, potassium chloride, ammonium chloride etc. Supplements included dextrose, magnesium chloride, cysteine; NAD, hemin and low molecular weight diafiltered permeate of yeast extract.

The basal medium was sterilized by autoclaving at 121 ºC under 15 lbs pressure for 20 minutes. Non-autoclavable components were sterile filtered using 0.2 µ filter made up of PES. The final Purified polysaccharide (PRP) were prepared by the below mentioned sequence,

Harvesting and centrifugation followed by inactivation by 0.6 % v/v Formalin and collected the supernatant the concentrate to 18-22 fold using 100 kDa cassette and performed Diafilter 5-6 times with WFI again performed diafiltration of the concentrate using 3-5 volumes of PBS, then the crude polysaccharide Cetavelon precipitation performed after that centrifugation performed then 32% Ethanol precipitation at 2-8° C for 14-18 hrs. the incubation at 2-8° C for 14-18 hrs., after that centrifugation 4500±500 rpm then diafilter with 5 volumes WFI through 100 kDa at 2-8° C and 0.22µ sterile filtration finally the Purified polysaccharide (PRP) preparation.

Experiment 1

The first experimental was performed with 14 hrs. incubation (2-8° C) period for 32% Ethanol precipitation step. Growth of Influenzae type b strain shall be stopped by adding formalin to the culture medium. Capsular polysaccharides shall be precipitated by adding cetrimide to the culture medium supernatant obtained by centrifugation and Influenzae type b polysaccharides are obtained by treating with phenol and ethanol for purification (Porter Anderson and David H Smith, 1977). After the Cetavelon precipitation step centrifugation was performed then 32% Ethanol precipitation at 2-8° C for 14 hrs. incubated for proper precipitation. The next step incubated the solution at 2-8° C for 14-18 hrs., after that centrifuged 4500±500 rpm then diafiltered

with 5 volumes WFI through 100 kDa at 2-8° C. Final step was 0.22µ sterile filtration then obtained sterile Purified polysaccharide (PRP). This PRP content was more important for the Hib vaccine production. This was conjugated with Tetanus toxoid for producing Hib vaccine. In the 14 hrs. of 32% Ethanol precipitation the suitable and sufficient amount of the precipitation was available.

The samples are collected in different stages to check the PRP concentration as below (Tabel-2),

Table-2: Sample collection stage & Sample details:

Sample Collection Stage	Sample details
After Crude polysaccharide Cetavelon Precipitation	Clarified Supernatant
After 75 % Ethanol	Clarified Supernatant
After 32 % Ethanol	Clarified Supernatant
After Diafilter with 5 volumes WFI	Final PRP Purified content

Experiment 2

The second experimental was performed with 10 hrs. incubation (2-8° C) period for 32% Ethanol precipitation step. In this experimental the incubation period was reduced by 4 hrs. This reduced timing will increase the production rate of Hib vaccine. The time reduction was considered without affecting the PRP content in the final purification stage. It was maintained with the reduced time reduction incubation period. After the Cetavelon precipitation step centrifugation was performed then 32% Ethanol precipitation at 2-8° C for 10 hrs. incubated for proper precipitation. The next step incubated the solution at 2-8° C for 14-18 hrs., after that centrifuged 4500±500 rpm then diafiltered with 5 volumes WFI through 100 kDa at 2-8° C.

Final step was 0.22µ sterile filtration then obtained sterile Purified polysaccharide (PRP). This PRP content was more important for the Hib vaccine production. This was conjugated with Tetanus toxoid for producing Hib vaccine. In the 10 hrs. of 32% Ethanol precipitation the suitable and sufficient amount of the precipitation was available. The samples are collected in different stages to check the PRP concentration with 10 hrs. incubation period of 32% Ethanol precipitation at 2-8° C mentioned in the Table-2,

Experiment 3

The third experimental was performed with 7 hrs. incubation period (2-8° C) for 32% Ethanol precipitation step. In this experimental the incubation period was reduced by another 3 hrs. This reduced timing will increase the production rate of Hib vaccine. The time reduction was considered without affecting the PRP content in the final purification stage. It was maintained with the reduced time reduction incubation period. After the Cetavelon precipitation step centrifugation was performed then 32% Ethanol precipitation at 2-8° C for 10 hrs. incubated for proper precipitation. The next step incubated the solution at 2-8° C for 14-18 hrs., after that centrifuged 4500±500 rpm then diafiltered with 5 volumes WFI through 100 kDa at 2-8° C. Final step was 0.22µ sterile filtration then obtained sterile Purified polysaccharide (PRP). This PRP content was more important for the Hib vaccine production. This was conjugated with Tetanus toxoid for producing Hib

vaccine. In the 10 hrs. of 32% Ethanol precipitation the suitable and sufficient amount of the precipitation was available. The samples are collected in different stages to check the PRP concentration with 7 hrs. incubation period of 32% Ethanol precipitation at 2-8° C mentioned in the Table-2. It was clear that the reduction in the incubation time was higher than necessary based on use of the present trend.

Testing methods

The purified PRP content was tested for different step for parameters like PRP content and phosphorus content. Fermentation of *Haemophilus influenzae type b*, was carried out and analyzed the samples at different stages of purification to keep the in-process controls and check the quality of the final purified PRP sample from three different experiments. The final purified PRP polysaccharide bulk was analyzed and stored at – 20 °C.

Estimation Polyribosyl Ribitol Phosphate (PRP) content in the different samples:

Prepared Orcinol reagent, 10 mM Stock Standard Ribose solution and 0.2 mM working standard ribose solution. Then prepared a series of test tubes with standard ribose solution such that the concentration of ribose is 0.75 µg, 1.5 µg, 3 µg, 6 µg and 9 µg. An appropriate dilution of the sample to be tested such that the concentration after dilution falls in the range of ribose standards are prepared. Added Orcinol reagent to all the tubes and kept the tubes in water bath at 90 °C for 20 minutes. Cool the tubes to room temperature and read the absorbance of the samples at 670 nm in a UV-Vis spectrophotometer. Plotted a graph taking concentration at the X- Axis and Absorbance at the Y-axis to check the linearity of the curve and accuracy of the experiment conducted. Calculated the amount of PRP of the sample by using formulae 1:

Formulae -1: Estimation of PRP Content:

$$\text{PRP content in µg/mL} = \frac{\text{Average Test OD}}{\text{Average Standard OD}} \text{ X } \frac{\text{Weighed quantity of Ribose}}{\text{Dissolved volume in mL}} \text{ X}$$

$$\text{Working stock dilution} = \frac{\text{Mol. Wt. of PRP}}{\text{Mol Wt. of Ribose}} \text{ X } \frac{\text{Avg. Vol. of Std. in µl}}{\text{Avg. Vol. of sample in µl}} \text{ X } 1000 \text{ X } \text{Sample dilution}$$

Estimation of Phosphorus content in the final sample

Totally taken 24 borosilicate tubes with the size of 10 X 75 mm. Label them as B1, B2, 2, 2, 2, 3, 3, 3, 4, 4, 4, 5, 5, 5, 6, 6, 6, 7, 7, 7, T1, T1, T2 and T2 respectively. Dilution of the test sample such that the concentration of PRP in the sample is below 1.25 mg/mL. Then introduced 100 µl of various concentrations of standards and test samples in triplicates in all the respectively labelled tubes. Added 60 µl of conc. sulfuric acid to all the tubes. Kept all the tubes in a Borosil glass beaker and place the beaker in the vacuum oven at 160°C for 30 minutes. Removed the beaker, containing tubes, from the Vacuum oven. Kept the heating block tube stand on the hot plate at 165 °C so that the temperature of the tube stand reaches 160 °C. Kept all the tubes at 160

°C in heating block tube stand, kept on hot plate, for 1 hour. Allowed the tubes to cool down for few minutes. Added 30 µl of 70% Perchloric acid solution to each tube. Again, kept all the tubes at 160 °C in heating block tube stand, kept on hot plate, for 1.5 hour. Taken out all the tubes and allow them to cool down. Added 2.5 mL WFI to each tube and mix gently by vortexing and then pipetting the solution in and out thrice. Transfer 1 mL from each tube to another clean test tube, labelled respectively. Prepared working reagent afresh and add 1 mL of it into blank, standards and test samples. Mixed the solution by vortexing the incubated the tubes at 37 °C for 2 hours. Measured the absorbance at 820 nm using VIS-Spectrophotometer. Use blank to set auto zero. Generate a graph by plotting standard concentrations on X-axis and corresponding mean. absorbance values on Y-axis, and determine slope, Y-intercept and correlation coefficient values. Calculate the Phosphorous concentration of the test sample using formulae – 2:

Formulae - 2: Estimation of Phosphorus Concentration:

$$\textbf{Phosphorous concentration in µg/mL} = \frac{\mathbf{F_{dil}\ X\ \ A_{820} - b}}{m}$$

Where
F_{dil} = Dilution fold of the test sample
A_{820} = Mean absorbance of the test sample
b = y-intercept
m = Slope

% Phosphorus in PRP sample (on wet basis) as follows:

$$\%\ \text{Phosphorus in PRP} = \frac{\text{Conc. of Phosphorus in mg/mL X } 100}{\text{Conc. of PRP in mg/mL}}$$

RESULTS

For all 3 different incubation period for 32% Ethanol precipitation the PRP content and Phosphorus concentration looks goods.

PRP in different stages like Clarified Supernatant, permeate sample, 75 % Ethanol, 32 % Ethanol and final Purified PRP measured by Orcinol Method and Phosphorus content in Final Purified PRP. The observations for the different experiments carried out to calculate PRP concentration in different samples found below Table – 3 & Figure - 2:

Table -3: PRP Concentration Experiments wise:

Sample No.	Sample Details	PRP Concentration		
		Exp. 1 (mg/mL)	Exp. 2 (mg/mL)	Exp. 3 (mg/mL)
1	Clarified Supernatant	0.3335	0.3214	0.3420
2	75 % Ethanol	2.17	2.18	2.19
3	32 % Ethanol	1.76	1.64	1.79
4	Final PRP Purified	2.219	2.306	2.247

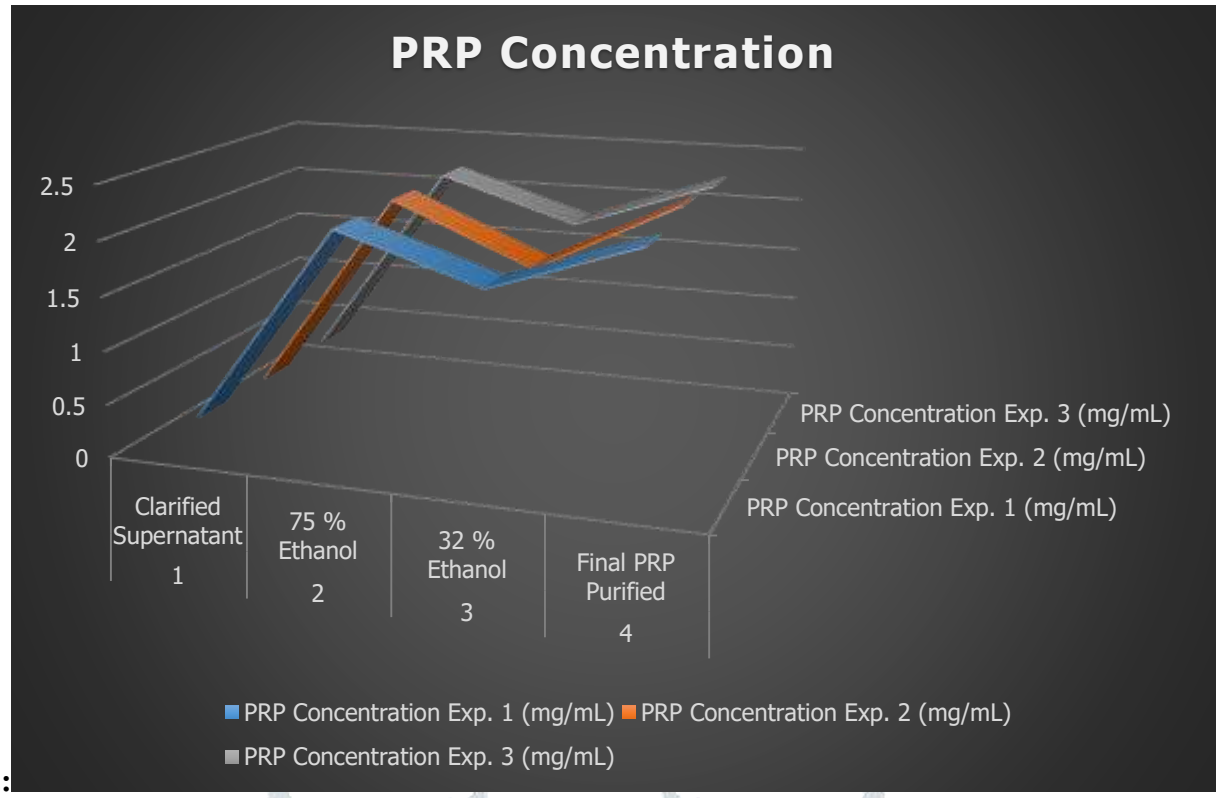


Figure -2:

The observations for the different experiments for the phosphorus estimation found below Table – 4 & Figure - 3:

Table -4: Phosphorus Concentration Experiments wise:

Sample No.	Sample Details	Phosphorus Concentration		
		Exp. 1 (µg/mL)	Exp. 2 (µg /mL)	Exp. 3 (µg /mL)
1	Final PRP Purified	157	159	158

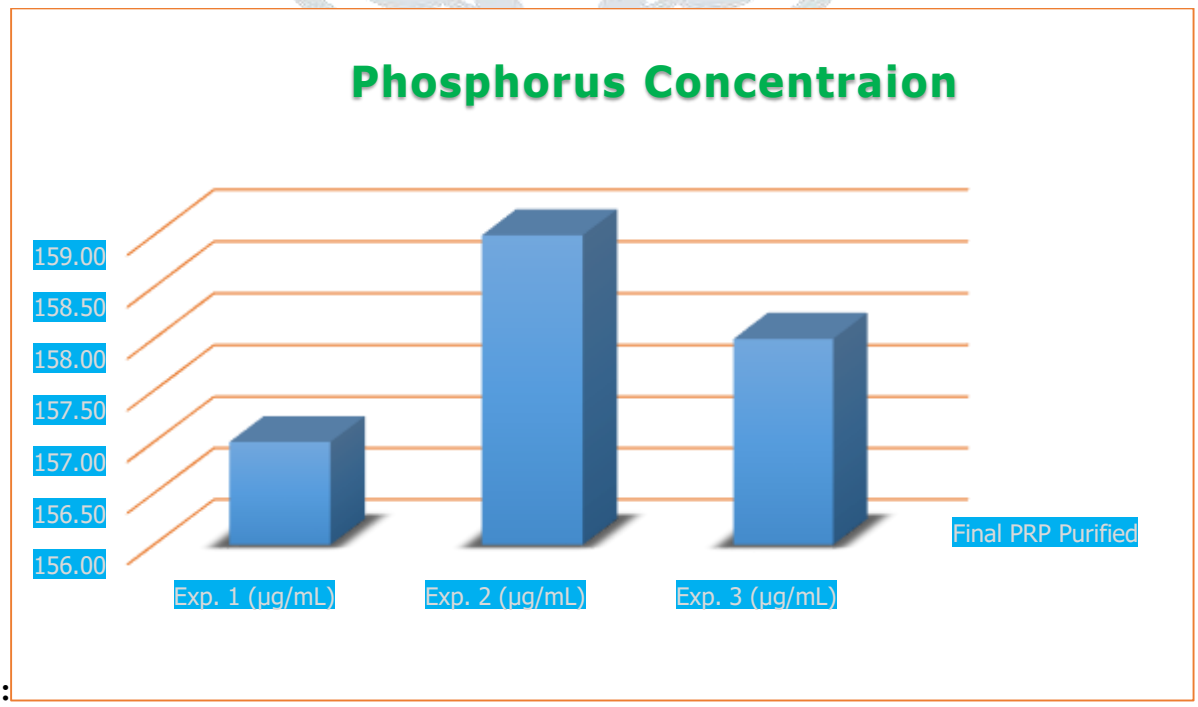


Figure – 3:

DISCUSSION

Three Experiments were giving good PRP and Phosphorus content after final purified PRP stage. In the Experiment 1 with 14 hrs. incubation in 2-8° C period for 32% Ethanol precipitation step the results for PRP concentration found for Clarified supernatant step was 0.3335 mg/mL, 75% Ethanol step it was 2.17 mg/mL, 32% Ethanol step it was 1.76 mg/mL and final Purified PRP it was 2.219 mg/mL (Refer Table-3 and Figure-2). Here the 14 hrs. incubation period for 32% Ethanol precipitation step not impacted the PRP content. In the second experiment with 10 hrs. incubation in 2-8° C period for 32% Ethanol precipitation step the results for PRP concentration found for Clarified supernatant step was 0.3214 mg/mL, 75% Ethanol step it was 2.18 mg/mL, 32% Ethanol step it was 1.64 mg/mL and final Purified PRP it was 2.306 mg/mL (Refer Table-3 and Figure-2). Here the time reduction of 4 hrs. in the incubation period for 32% Ethanol precipitation step not obstructed the PRP content. We observed the good results in the PRP concentration, sufficient for the production of purified PRP in the final step. In the third experiment with 8 hrs. incubation in 2-8° C period for 32% Ethanol precipitation step the results for PRP concentration found for Clarified supernatant step was 0.3420 mg/mL, 75% Ethanol step it was 2.19 mg/mL, 32% Ethanol step it was 1.79 mg/mL and final Purified PRP it was 2.247 mg/mL (Refer Table-3 and Figure-2). Here the time reduction of 7 hrs. in the incubation period for 32% Ethanol precipitation step not clogged the PRP content. Found the almost equal in the other experiments results. The time reduction of around 7 hrs. will impact the production rate and targets. All the three experiments the phosphorus concentration in the Final PRP purified was 157 µg/mL for Exp.1, 159 µg/mL for Exp.2 and 158 µg/mL for Exp.3 measured. The time reduction in the incubation period in 2-8° C for 32% Ethanol precipitation not affect the phosphorus concentration.

CONCLUSIONS

Improvement of the PRP content and Phosphors concentration in the final purified PRP found good. In the incubation of 2-8° C period for 32% Ethanol precipitation step the results for PRP concentration found satisfactory for all the three experiments. Time reduction in the incubation period not affected the concentration of PRP and Phosphorus concentration. By changing in incubation period, the the PRP content and phosphorus content was unchanged. It shows the positive result at the end of experiments. Conjugate vaccines utilize PRP usually purified from H. influenzae type b organisms grown in a bioreactor, and chemically linking them to various carrier proteins by the method of conjugation with or without the use of a spacer molecule. These conjugates are able to increase the Immunogenicity of PRP by recruitment of T helper cells (T-cell dependent immune response) resulting in heightened anti-PRP antibody titres (Yeh Chen Lee et al., 2008). In this study, our attempt to evaluate the potential for reduction in the incubation of 2-8° C period for 32% Ethanol precipitation step was satisfied with PRP content and Phosphorus concentration in the final PRP.

REFERENCES

1. World Health Organization. The effects of freezing on the appearance, potency, and toxicity of adsorbed and unadsorbed DTP vaccines. Weekly Epidemiological Record 1980;55:385-92.
2. Recommendations for the production and control of *Haemophilus influenzae* type b conjugate vaccines, Annex 1 in: WHO Expert Committee on Biological Standardization. Forty-ninth report. Geneva, World Health Organization, 2000 (WHO Technical Report Series, No. 897).
3. McVernon J, Ramsay ME, McLean AR. Understanding the impact of Hib conjugate vaccine on transmission, immunity and disease in the United Kingdom. Epidemiol. Infect. 2008; 136: 800-812.
4. Yeh Chen Lee, Dominic F Kelly, Ly-Mee Yu, Mary PE Slack, Robert Booy, Paul T Heath, Claire-Anne Siegrist, Richard E Moxon, Andrew J Pollard. *Haemophilus influenzae* Type b Vaccine failure in children is associated with inadequate production of High-Quality Antibody. Clinical Infectious Diseases 2008; 46: 186-192.
5. David W McCormick, Elizabeth M Molyneux. Bacterial Meningitis and *Haemophilus influenzae* Type b Conjugate Vaccine, Malawi. Emerging Infectious Diseases 2011; 17(4): 688-690.
6. Fritzell B, Plotkin S. Efficacy and safety of a *Haemophilus influenzae* Type b capsular polysaccharide-tetanus protein conjugate vaccine. J Pediatr. 1992; 121(3): 355-362.
7. Verma R, Khanna P, Chawala S, Bairwa M, Prinja S, Rajput M. *Haemophilus influenzae* Type b (Hib) vaccine: An effective control strategy in India. Hum Vaccin. 2011; 7(11): 1158-1160.
8. Heikki Peltola. Worldwide *Haemophilus influenzae* Type b disease at the beginning of the 21st century: Global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates. Clin. Microbiol. Rev. 2000; 13(2): 302-317.
9. Porter Anderson, David H Smith. Isolation of the capsular polysaccharide for culture supernatant of *Haemophilus influenzae* type b. Infection and Immunity 1977; 2: 472-477.