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ORIGINAL ARTICLE

Haemophilus Influenza type b Capsular Polysaccharide Conjugated PRP Compared with Diphtheria Toxoid, Tetanus and *Pseudomonas aeruginosa* Exotoxin using Serum Bactericidal assay

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ABSTRACT

Haemophilus influenza is a common cause of bacterial meningitis in infants and children under 5 years in countries that don't have any vaccination. The capsule of this bacterial is composed of a linear polymer of polyribosyl ribitol phosphate. The goal of this research is to compare the conjugated PRP Haemophilus influenza type b with Diphtheria toxoid (TD), tetanus toxoid (TT) and Pseudomonas aeruginosa exotoxin (ETA) and consider it with different methods. Haemophilus influenza culture in CY environment and PRP purified from fluid culture is done through the precipitation alcohol methods (ethanol precipitation) and precipitation with stavlen (hex Dsyl trimethyl ammonium bromide), ultra-centrifugation and purified by hydroxyapatite prepared toxoid, diphtheria, tetanus and Pseudomonas aeruginosa exotoxin from Hisarak Razi Serum and conjugation with PRP using ADH, cyanogen bromide and EDAC, protein assays, gel diffusion, electrophoresis and injected conjugated proteins and PRPThe number of white rabbits in two consecutive 15-day test with aluminum hydroxide adjuvant and serum samples collected evaluation of serum obtained by immunization injections and PRP conjugated serum bactericidal. In this study, serum bactericidal titer were obtained from group A (from 2/1 to 8/1) and Group (from II 8/1 to 128/).All groups have accepted immune response but second group of antibody titer was very important than other PRP group. It means that conjugation of PRP through these proteins has a reinforcing effect on each other and conjugation can be considered as an option In order to provide vaccine.

Keywords: *Haemophilus influenzae type b - PRP - tetanus toxoid - diphtheria toxoid - Pseudomonas aeruginosa exotoxin – conjugation*

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INTRODUCTION

Gram-negative bacterium Haemophilus influenza is Polymorphism and can be found in the form of kokoid, kokobasil and string. 75% from these bacteria hasn't any capsule while the majority of invasive strains of pathogenic isolated form patient have capsule type B. Haemophilus influenza type b is found normally in the PharynX. About 80% of healthy people are carriers [1]. A wide range of diseases is in humans. Other diseases which this invasive create include Pneumonia, epiglottis, cellulitis and septic arthritis [2]. More than 90% these Infections occur in children under 5 years. In the meantime, infants 6 to 12 months are at risk.

Polysaccharide encapsulated bacteria are a group of Human pathogens. Infection with this organism will lead to significant morbidity and mortality especially in the elderly, infants and people with impaired immune. Immunity against encapsulated bacteria primarily depends on the presence of antibodies against capsular polysaccharides. These antibodies create immunity against encapsulated bacteria through thephagocytosis activation. Antibody response to capsular polysaccharides is defective in infants

and young children. As a result, we can see an increase in developed invasive diseases such as pneumonia, meningitis, otitis media and other bacterial [3].

Polysaccharide vaccine was the first vaccine against Hib which was built in 1980. It was provided just from net PRP. it played an important role in inducing anti-polysaccharide antibodies and protecting people who could react positively to Polysaccharide antigens [4,5].Hib polysaccharide vaccine was effective about 80% in preventing invasive disease caused by Hib in children 14 to 35 months. It didn't have any effects in children less than 18 months who the largest groups were at risk of infection (6). Also, Polysaccharide antibody responses in the elderly, people with liver cirrhosis and diabetes, immunosuppressed patients, the polysaccharide vaccine failed. So, this was caused people to be more caring [7,8]. Because of these problems, Glyco-conjugate vaccines produced in 1998. Then, immunogenic polysaccharides by binding to a protein carrier increased [9]. This approach is used for T cells.

In this research, we create covalent conjugate bon via PRP binding to Haemophilus influenza (TD, TT, and ETA). For doing it, we use ADH (adipic acid dihydrazde) and cyanogen bromide as the spacer and EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) as connecting factor. Then, the immunity of the conjugate injected into laboratory animals blood from animal through Serum bactericidal method assess carefully.

MATERIALS AND METHODS

Protein purification and Culture:

In this empirical Study, Haemophilus influenza Type BATCC 1623 was provided by collection of Standard Bacteria of the Pasteur Institute of Iran and (TD, TT.ETA) from Serum Institute mystery Hisarak. In summary, it was cultured in Blood agar medium at 37 ° C for 12 h and then used in Haemophilus specific medium CY in order to cell seeding preparation. Process fermentation was done in order to produce cell mass of Haemophilus influenzae in culture medium containing Soybean pepton (10 g) dextrose (5 g /L) Heminchloride (10 g), NAD, (10 mg) yeast extract (10 ml. l) in 3/7 pH.

Provided cell seeding were inoculated in fermentation tank of 60-liter (b.v.bilthoven unit) containing high- inoculum culture interface. Finally, the resulting cell mass was used for Extracting PRP (7, 8). Inactivation of bacteria was done through 37/0-35/0 percent solution (v / v) and formaldehyde in the field of extracted and purified PRP. Supernatant solution (Supernatant) was the final process of transmission medium. Then 4% sodium acetate was added to it. Under cold conditions (4°C), ethanol was slowly added and its PH reached to6-2. After refrigeration overnight, it was centrifuged and raw PRP was prepared. The obtained raw PRP was dissolved in distilled apirogen water and after removing the waste material, stavlen was added to it. The resulting sediment was dissolved in Sodium chloride 3/0 M solution of water-insoluble substances such as nucleic acids complex - Setavlon was separated from each other [9,10]. Finally, after several centrifugation and sedimentation steps, dissolution in distilled water and adding hydroxyapatite to PRP, The resulting supernatant was passed through a filter mL Poor and after dialysis, PRP purified and lyophilized [11].

Determination of protein Concentration:

Protein concentration was measured by using a spectrophotometer (ND-1000) with high repetition strength [12].

Conjugation of polysaccharide PRP(TD , TT, ETA):

Capsular polysaccharide of Haemophilus influenza type b (PRP) was conjugated by using Amidation way through carbon dioxide diphtheria toxoid, Tetanus toxoid of Pseudomonas aeruginosaetoxin.5 mg PRP solved in2ml saline solution with10/5 PH, then 100 ml cyanogens bromide was added at a concentration of 2.0g/mL acetonitrile, then pH solution shifted by 1Msodium hydroxideto10. 5, the solution was heated for 5min in laboratory temperature. After this period the rate of 70 mg adipic acid hydrazide added to solution, then we set out pH solution with0/01M acid on 5.8. Then solution resulting was maintained overnight at 4 °C.10 mg of proteins separately addedin1ml saline with 5/7 = PH, and derive then drops to ADH-PRP.Thenabout9 mg EDAC was added to the solution and the pH was adjustedto8.5. The mixtures were incubated for 4hours at room temperature and then refrigerated overnight at 4°C. after overnight there salting conjugate was added to dialysis bag with Cut off and a dialysis bagcontaining2.0mM sodium chloride, and a number of magnet placed on Magnetic Stirrers and then refrigerated, and every 8hours, 2.0molar solution of sodium chloride was replaced.In fact, by performing the excess dialysis additional EDAC, ADH protrude out to create additional connections between the PRP and thus prevent protein conjugate PRP-TD, PRP-TTandPRP-ETAobtainedusingsepharose4B-CL chromatography method [13,14].

Gel electrophoresis

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)using12% polyacrylamide gels was performed on a cover slip and then gel were stained by using Coomassie blue [5].

Immuno-diffusion test (double diffusion in gel):

Precipitation reaction of PRP antiserum, and TD antiserum and conjugated antiserum was done in the agarose gel diffusion by using gel diffusion method [15,16].

Pyrogen test:

To assess the pyrogenicity test for extracted samples, pharmacopeia way has been done.3 mg / ml conjugated per kilogram body weight of the animals were injectedintravenouslyinto4groups of 3 rabbits and rabbit body temperature eat anytime, until three hours after injection, was examined by rectometer [17].

Toxicity tests:

The test was carried out in mice to determine that the purified conjugate is non-toxic. 4 group's of 5mice were injected intravenously with 100micrograms of conjugate. Mice were cultured for 7daysinnormal condition, and kept for 7daysin terms to determinate injection site side effects, weight loss and mortality [18].

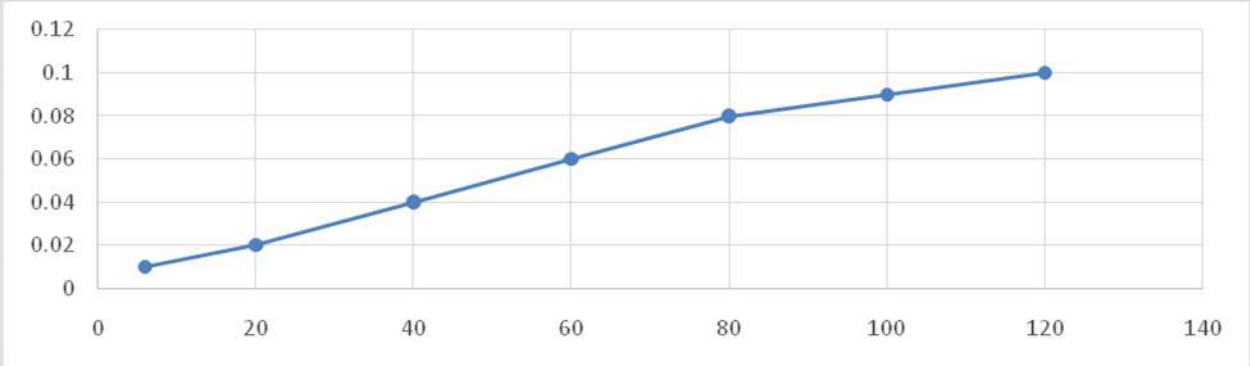
Serum Bactericidal Antibody Assay (SBA):

Haemophilus Influenza b BTCC1623 was used in the SBA for this study. This strain was cultured in brain heart infusion agar (BHI) for 18h. The culture was diluted in Phosphate buffer saline (PBS, pH 7.2) to give approximately 10^3 CFU/ml. The Sera collected from two Haemophilus Influenza negative baby rabbit 3 weeks old was used as the complement resource which was filter sterilized with a 0.22 nm syringe filter, and kept at -20 ºC until use. The sera samples were treated at 56°C for 30 min prior to use to inactivate the complement activity. The bactericidal activity assay was performed in the sterile 96-well polystyrene microplates. The 50 µl heat-inactivated serum samples were serially diluted in PBS, pH7.2 and then 25µl of bacterial suspension and 25µl of baby rabbit complement were added to each well. Control wells included (i) bacteria plus complement; (ii) bacteria plus sample serum; and (iii) bacteria plus PBS. The microplates were incubated at 37°C for 1 h. Before and after the incubation, 10µl from each well was plated onto BHI agar plates, which were incubated for 24h at 37°C. The colonies before (T0) and 1h (T1) after incubation plates were counted. The actual number of CFU per well added before incubation was obtained by multiplying three the average colony count after overnight incubation. The serum bactericidal titer was reported as the reciprocal of the highest serum dilution yielding more than 50% bacterial killing, compared to the number of CFU present before incubation with serum and complement at time zero [19].

RESULTS

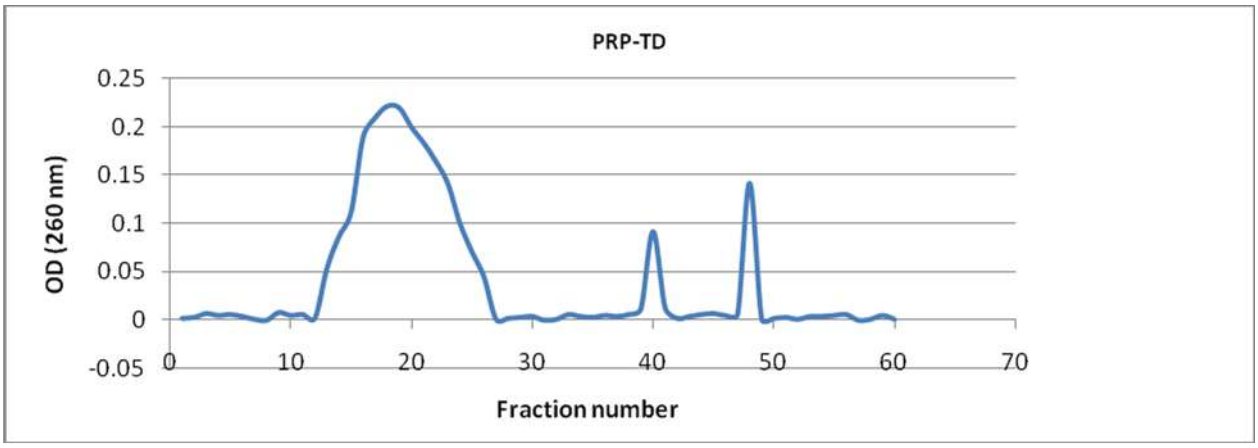
Measuring the amount of protein conjugated PRP-TD, PRP-TT, PRP-ETA:

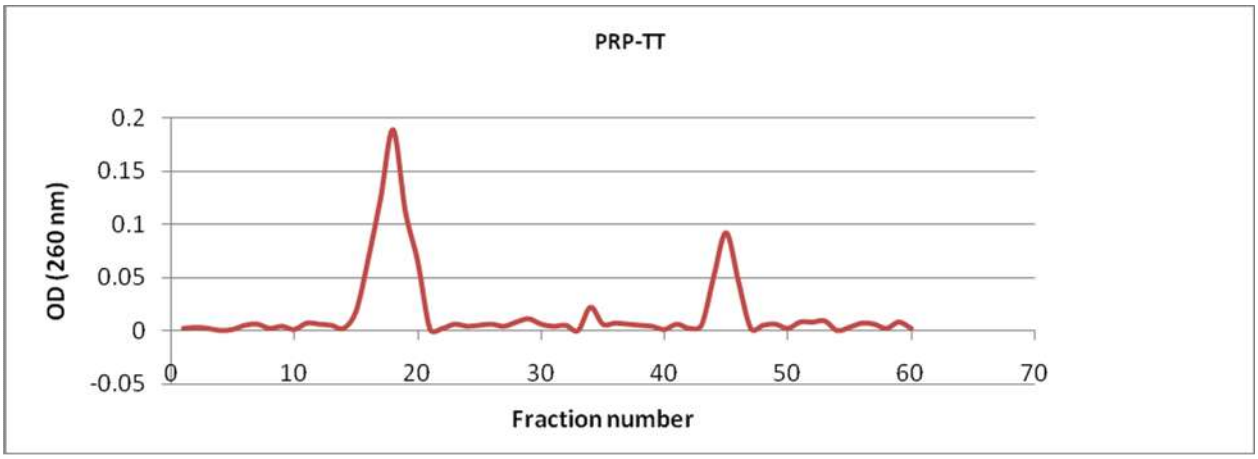
The amount of protein in the conjugate was determined by the Lowry test chart (1), the standard graph and linear equation. These rates were calculated respectively 278/48 - 486/44- 524/43 and mg protein per ml of conjugation.

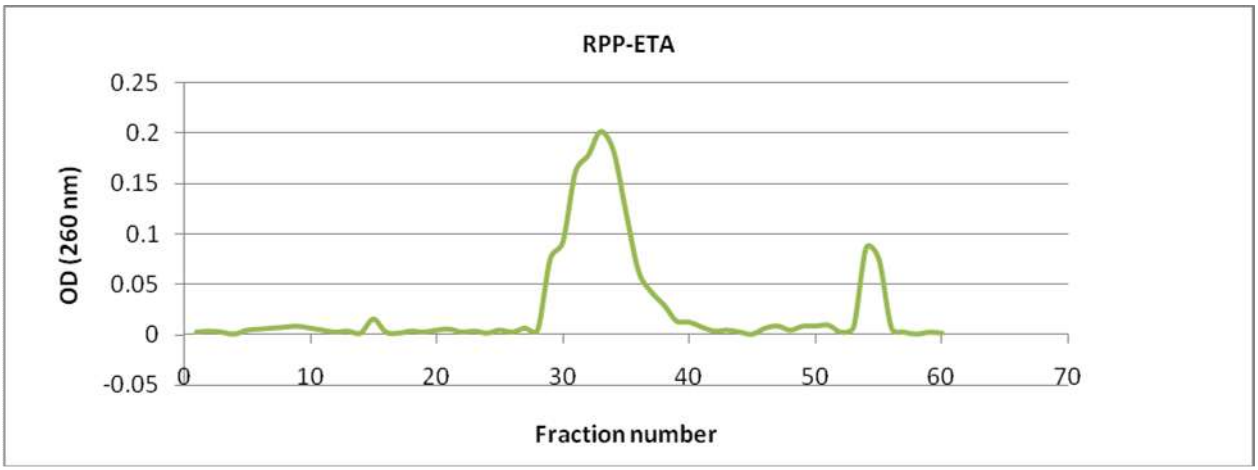


PRP Conjugation with (TD,TT,EXO):

After preparation of conjugation in order to Purification of conjugated or non-conjugated molecules, it was passed from 4B-CL column chromatography through Gel filtration method and fraction of light absorbance read at a wavelength of 260 nm. As shown in chart 2, red line is related to the PRP-TT conjugate and the green line is corresponded to the conjugate PRP-EXO.







Gel diffusion tests:

Results of a double diffusion test antigen and antibody in agarose gel is shown in picture. This test was conducted to evaluate the PRP polysaccharide antigen binding to the carrier protein (TD, TT, and ETA) and after conjugation, antigenic structures of molecules and antigenic polysaccharides and proteins remained. As shown in figure (1), antigen-antibody reactions in sedimentary lines have been established. These results show that conjugated molecule can react with PRP antibody and anti-toxoid.

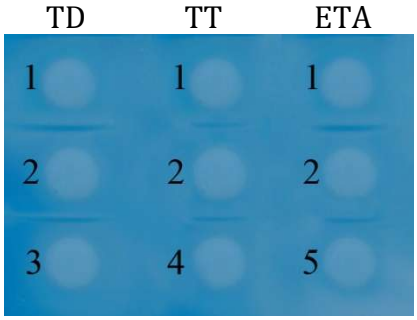


Figure (1): gel diffusion test
Column (TD): 1) antibody PRP-TD, 2) PRP-TD, 3)PRP antibody
Column (TT): 1) antibody PRP-TT, 2) PRP-TT, 3)PRP antibody
Column (ETA): 1) antibody PRP-ETA, 2) PRP-ETA, 3)PRP antibody

Electrophoresis results:

It is noticeable in Figure (2) SDS-PAGE electrophoresis pattern of motion PRP is 10percent compared with standard protein markers (Sigma) as a relatively strong b and was observed the range of18kDa.

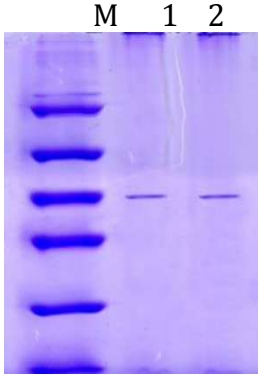


Fig (2). Pattern of PRP gel electrophoresis in 10%SDS-PAGE
Column M: The standard molecular weight markers of 50kDa
Columns1and2: total weight of 18Kda polysaccharide PRP

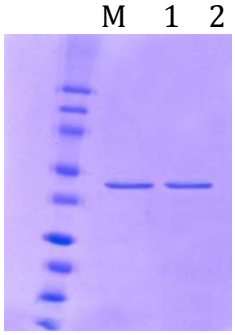


Fig (3). Pattern of PRP-TD gel electrophoresis in 10%SDS-PAGE
Column M: The standard molecular weight markers of 150kDa
Columns1and2: total weight of85kDaconjugatePRP-TD

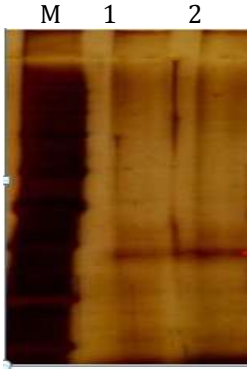


Fig (4). Pattern of PRP-TTgel electrophoresis in 10%SDS-PAGE
Column M: The standard molecular weight markers of 150kDa
Columns1and2: total weight of87kDaconjugatePRP-TT

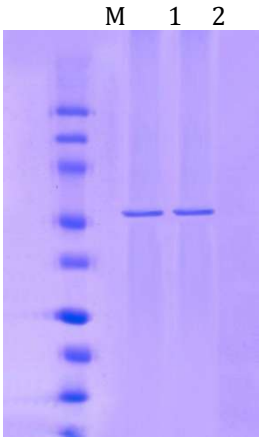


Fig (5). Pattern of PRP-ETA gel electrophoresis in 10%SDS-PAGE
ColumnM: The standard molecular weight markers of 150kDa
Columns1and2: total weight of85kDaconjugatePRP-ETA

PRP Conjugate efficiency:
PRP-TT, 5/45 and PRP-ETA, 2/43 were calculated by determining Conjugated to protein ratio to all protein PRP-TD, 3/46.

The result of PRP pyrogenic test and tested conjugation:
PRP polysaccharide and conjugate under test in all 4 groups of 3 rabbits was less than ¼ and in each rabbit was less than 0/6. So, PRP samples provided conjugate didn't have any febrile materials and were Injectable.

Table (1) Test results Pyrogen

Number of rabbits		Injection	value	the mean increase in body temperature	Result
PRP	3	Venous	0.025µg	negative	Non-pyrogenic
PRP-TD	3	Venous	0.1 µg	negative	Non-pyrogenic
PRP-TT	3	Venous	0.1 µg	negative	Non-pyrogenic
PRP-EXO	3	Venous	0.1 µg	negative	Non-pyrogenic

Toxicity tests:
To investigate toxic or nontoxic specimen prepared conjugate samples into 4groups of 5 animals injected to mice and for 7dayswere studied and the lack of weight loss and mortality in mice, indicate that conjugate is non-toxic.

Table (2): Test results Toxicity

Number of rabbits		Injection	value	Weight loss	Time	Result
PRP	5	Intraperitoneal	100µg	negative	7- day	Non-toxic
PRP-TD	5	Intraperitoneal	100 µg	negative	7- day	Non-toxic
PRP-TT	5	Intraperitoneal	100 µg	negative	7- day	Non-toxic
PRP-EXO	5	Intraperitoneal	100 µg	negative	7- day	Non-toxic

Serum bactericidal results:
Humoral and PRP conjugate net is studied using a serum bactericidal and opsonization activities the organisms. In this test;Serum concentrations compared with the control plate bacterial colonies that have killed more than 50% can be considered Positive titer.The results of the counting of colonies in control plates showed that the number of colonies on culture plates with serial dilutions of bacterial suspension IUCFU / ml 3 10 is 327. The following results were obtained by examining and counting the number of

colonies of Haemophilus influenza type b and its effects on Plate and its comparison with the control bacterial plate.Bacterial killing titer in first group PRP till 1/8 titer was positive. it had the effect of bacterial killing. Injecting dose PRP didn't have any effects on increasing bacterial killing.

The level of bacterial killing activity in group 2 in the first injection was 1/8 ≤1/32. It was not only more than bacterial killing activity in net PRP; but also, it increased via injecting the bacterial killingdose and reached to the 1/64≤1/128.

Table (3) Comparison of serum bactericidal assay

Serum dilution Serumsamples1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
PRP, 15 day	+	+	-	-	-	-	-	-
PRP, 45 day	+	+	+	+	-	-	-	-
PRP- TD , 15 day	+	+	+	+	+	-	-	-
PRP- TD , 45 day	+	+	+	+	+	+	+	-
PRP- TT , 15 day	+	+	+	+	+	+	-	-
PRP- TT , 45 day	+	+	+	+	+	+	+	+
PRP- ETA , 15 day	+	+	+	+	-	-	-	-
PRP- ETA , 45 day	+	+	+	+	+	+	+	-
Negative control	-	-	-	-	-	-	-	-

DISCUSSION

Borgone and his colleagues [20] said that Vaccination with purified capsular polysaccharide was successful to some extent. This kind of vaccination weren't inducing Protective antibody responses in children under 2 years. Because of these problems, glycoconjugate vaccines were developed in 1988. immunogenisation polysaccharides increased by covalent binding to a protein carrier [25]. It approach use for T cells.

The first commercial conjugate was polymer of polyribosyl ribitol phosphate with Diphtheria toxoid (TD) Capsular polysaccharide (PRP) was bound to diphtheria toxoid via a C6 spacer to DVD Adipine acid hydrazide (ADH) (27). ADH-derived diphtheria toxoid conjugated in the presence of EDAC (cyanylation conjugation) to activated polysaccharide. But in this process, the bindings were unstable and resulting conjugation was problematic in dissolution. This method was modified by Cho et al. ADH SP User was added to Polysaccharide. Then, ADH-derived diphtheria toxoid conjugated in the presence of EDAC (cyanylation conjugation) to activated polysaccharide (karbodimid reactivity). These changes were increased conjugation efficiency and Conjugate solubility in water [21]. Some of protein carries is used in clinical evaluation of conjugate vaccines. Proteins like Tetanus toxoid and diphtheria toxoid are the first proteins which are used as a carrier. Since immunity is created against diphtheria and tetanus by vaccination (CRM197).Toxin is non-toxic mutant of diphtheria which doesn't have chemical detoxification. It is used widely as carrier in conjugate vaccines [22]. The protein of Nisseria meningitidis outer membrane (OMP) is used for in conjugate vaccines. Today, researchers are trying to increase the adsorption of antigens through Immune cells in the field of more effective vaccines [23].

The results showed that extracted and purified PRP done properly and PRP were active and immunologicals was created. Also, they showed that there is a meaningful difference between groups which has received conjugated PRP-TD, PRP-TT and PRP-EXO. Also, there is a difference in serum titer IGS anti-PRP between days 15 and 45 after the first injection. Binding PRP to toxoid as a protein carrier and conjugate preparation can increase PRP antigen immunogenicity [24]. Also, it can increase conversion of antigen-independent and dependent to thymus. So, we conclude based on the obtained results of Serum bactericidal that Serum bactericidal is a suitable method for determining antibody titer and evaluating immune responses. This method not only shows the antigen titer but also the immunogene titer [26].

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