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ORIGINAL ARTICLE

Evaluation of PRP anti body *Haemophilus influenza* type b (Hib) response with carrier protein by ELISA

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ABSTRACT
Haemophilus PRP as Immunogenetics structures are considered for design and production of vaccines. Haemophilus influenza PRP in combination with the protein carriers could stimulates immune system to synthesis of high levels of specific IgG molecules against Haemophilus. Haemophilus influenza grown in CV environment then the PRP purified from liquid culture with following procedures: at first it precipitated with ethanol and Stavlon (hexadecyl trimethyl ammonium bromide), the next Ultra centrifugal used to separation and at end the pure hydroxyapatite utilized to purification.Diphtheria toxoid (TD) got form Razi Company as a carrier protein and the proteins and ribose of it measured by Nanodrop spectrophotometer (ND-1000).PRP conjugated with a carrier protein by using ADH and cyanogen bromide as the distancing (or spatial) agents and EDAC used as coupling agent. Using sodium dodecyl sulfate, polyacrylamide gel electrophoresis, gel diffusion assay for antigen - antibody. The rabbits based on in vitro immunization programs were allocated in three groups of 3: Group 1: the rabbits immunized with PRP. Group 2: the rabbits immunized with mixture of PRP and diphtheria toxoid. Group 3: the rabbits immunized with mixture of PRP and complete Freund's adjuvant (CFA). The results showed that the combination of PRP and protein carrier is the highest immunogenic compound that induces higher antibody level in animal models. From present study we concluded that the mixture of PRP and protein carrier has more long-term protective immunity than other compound against Haemophilus and seems that use of these compounds for production of vaccine has beneficial effect.
Keywords: *ELISA, PRP, Carrier protein, Haemophilus influenza*

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INTRODUCTION

According to the World Health Organization (WHO), *Haemophilus influenzae* Type b is Responsible for over 3 million cases of serious disease (meningitis and severe pneumonia) and 380,000 to 700,000 deaths annually in the world [1-4].The disease in more than 95% of cases affects children under 5 years old. Incidence and complications of the disease in developing countries is more than developed countries. This regions also have the highest rate of pathogen carriers (40 percent) and is considered way of spreading the disease [1-4].Several studies have shown that in all of the world's greatest pathogen of pneumonia in children under 5 years old, are Streptococcus pneumoniae and *Haemophilus influenzae* type b. During the past years, several types of vaccines against *Haemophilus influenzae* type b are made [5]. Today, two generations of vaccines are available in markets [1-4] [9].The first generation was polysaccharide vaccines. This generation vaccines had disadvantages that led new vaccines to be made for better output.One of the disadvantages of this generation of vaccines, was age-dependent response to antigen and the lack of a memory response. Also, a large percentage of antibodies that are created by this generation of vaccines is IgM but IgG has more protective role [1, 10].Today, the disease caused by the bacterium *Haemophilus influenzae* Type b is major health problems in the developing and less developed countries [6]. In present study on *Haemophilus influenzae* PRP as a protective antigen antibody, and also with a carrier protein and Freund's complete adjuvant (CFA) is performed by ELISA [7].

MATERIAL AND METHODS

Haemophilus influenzae type BATCC 1623 was prepared from the collection of the Pasteur Institute of Iran and the standard strains of diphtheria toxoid from Razi Institute for Serum Hisarak.

PRP extraction:

Haemophilus influenzae type BATCC 1623 cultured on blood agar medium at 37 ° C for 12 hours then in order to prepare cell seeding, the specific medium for Haemophilus was used. Fermentation process has done for preparing cell mass of *Haemophilus influenzae* according Feikin et al, method (2004) and prepared seeding cells transferred into the 60 liter industrial fermenter tank was inoculated with the culture medium and the cell mass were used to extract PRP [6,7]To extract PRP 35%-37% percent (v / v) formaldehyde solution was used for inactivation the bacteria. Supernatant were collected and 4%concentration of, sodium acetate was added to it, then under cold conditions (4°C) and ethanol was slowly added and PH range changed to 2-6, then refrigerated overnight, centrifuged, and PRP was prepared from crude. After several steps of centrifugation and ethanol precipitation, PRP dissolved in distilled water and hydroxyapatite Add to PRP, the resulting supernatant was passed through a filter and then, PRP purified and lyophilized [11].

PRP polysaccharide Conjugation to a protein carrier (TD):

Capsular polysaccharide of *Haemophilus influenzae* type b (PRP) was conjugated by using diphtheria toxoid according to [8].

Determination of protein and ribose Concentration:

Protein concentration was measured by using a spectrophotometer (ND-1000) with high repetition strength. Measurement of ribose and PRP was done according to Bial's test [12].

Gel electrophoresis:

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gels was performed on a coverslip and then gel were stained by using Coomassie blue [5].

Immunodiffusion 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 [13].

Immunization Program:

Groups composed of three 6 months New Zealand white rabbits weighing to 2.5 to 3 kg were immunized intramuscularly with three different combinations.

Group 1: 120 mg per ml of purified PRP

Group 2: 120 mg per ml of PRP with an equal volume of complete Freund's adjuvant (CFA)

Group 3: 120 micrograms per milliliter of PRP with the carrier protein (TD)

Injections was performed on 0,7 and 14 days and animals Serum were collected on 0,7, 14 ,21days and was maintained at - 20 degrees [14, 5].

Evaluation of antibody responses:

Haemophilus influenzae type b anti-PRP antibody titer was evaluated ELISA by Microplate with PRP antigen (100 ml in each well) were covered and incubated for 1 h at 37 degrees. Plates in blocking buffer were ghosts (PBS-Casein) for 1 h at 37 ° C. Antiserum at a dilution of 1/1000, 1/10000 and 1/100000 and 100 ml was added to each well and was incubated for 1 h.Three times Washing was performed with phosphate-buffered saline - azide (PBS-azide).Conjugated anti IgG with HRP (Horseradish Peroxidase) at a rate of 100 ml was added to each well, incubated for 30 min and then were washed and substrate solution (chromogen) was added to each well. The optical density (OD) of the samples was measured at 405 nm, with ELISA-reader (15) (16) (9).

PRP-specific IgG titer against *Haemophilus influenzae* type b, after immunization alternating Time with various combinations

first injection	One week after the Second injection	One week after the Third injection	One week after the Antigen
PRP	58478±508	70158±855	132587±2787
PRP + Adj	132789±3078	145652 ±1895	148578±1488
Conjugate	123564 ± 2365	142584±3012	175854±2501
Negative control	3≤	3≤	3≤

Serum titers of immunized animals was significantly greater than the negative control (P <0/05).

RESULTS

Measurement of ribose:

The quality of the extracted polysaccharide is determined by measuring the amount of ribose After charting the standard ribose diagram, the following linear equation was obtained

In this y equation, measured OD is purified PRP.

Amount of ribose per mg of PRP, was calculated 2/7 micrograms per milliliter.

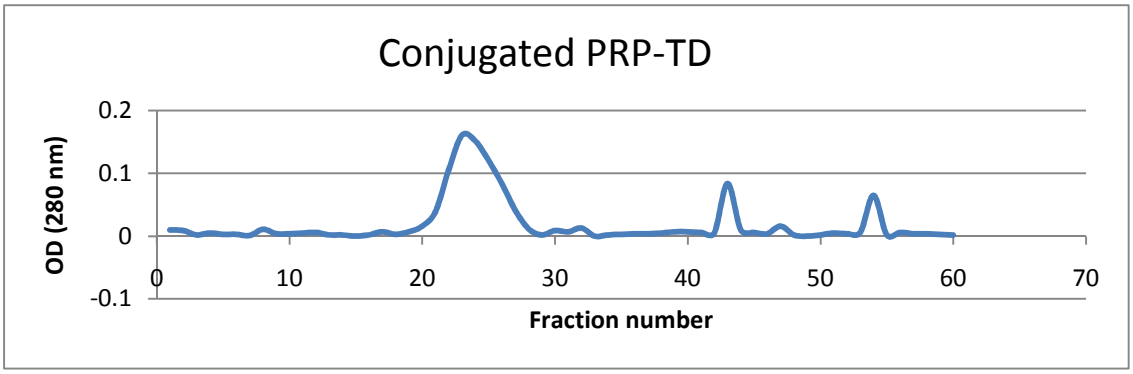
Measurement of protein in PRP-TD:

The amount of protein in conjugated PRP-TD was calculated by using Lowry test and Line equation and standard curve. This amount was calculated 48/51 mg protein per ml of conjugate.

PRP Conjugation to carrier protein (TD):

After preparation of conjugated PRP-TD In order to purified non-conjugated molecules from conjugated molecules, it passed through a gel filtration chromatography column (4B-CL)and the optical density was read at 280 nm.

As shown in the diagram (3)



The first peak is showing the conjugated PRP-TD and represents that PRP capsular polysaccharide conjugated to a carrier protein (TD) The second and third peaks represent non-conjugated proteins.

Gel diffusion tests

Results of double diffusion antigen and antibody test in agarose gel is shown in Fig (1)

This test represent that PRP polysaccharide antigen bind to protein carrier (TD) As shown in the fig (1), These results suggest that conjugated molecule is capable of reacting with anti-PRP and anti- (TD).

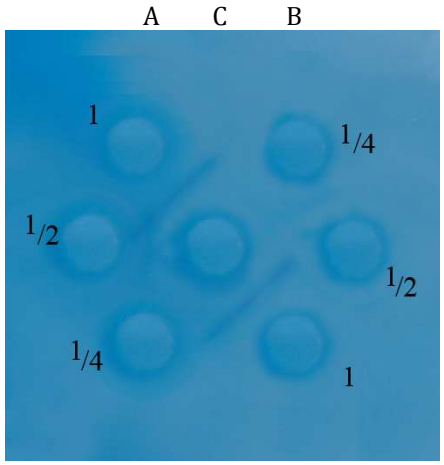


Figure 1. Gel diffusion test

Column A: Dilution 1. (TD), Column B: Dilution 1. (PRP), Column C: (Conjugate)

Electrophoresis result:

A single Conjugated band was created in the area of 150 kD and the fig(2) is confirmed PRP molecules.

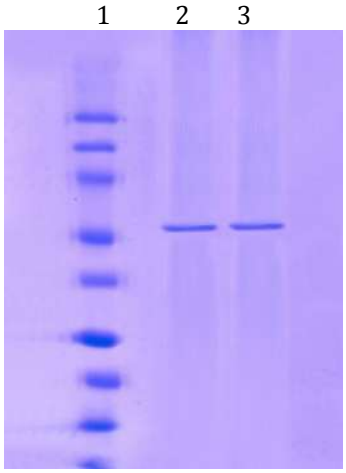


Figure 2. Patterns of SDS-PAGE electrophoresis of PRP with diphtheria toxoid
Column 1: standard, high molecular weight markers, Columns 2 and 3: conjugate PRP-TD with a molecular weight of 150 KD

ELISA result:
Specific antibody response to Haemophilus influenzae PRP was measured by ELISA. IgG antibody on days 0, 7, 14 and 21 after the first injection were determined and the optical density were expressed. Non immunological rabbit serum was used as negative control. The data obtained were analyzed statistically by ELISA-reader devices (Table 1).

Discussion
ELISA analysis of immunogenicity in the rabbit serum by combination of protein carriers with Haemophilus influenzae PRP is induced highest humoral response in a week after the third injection (17). In addition, groups of immunized rabbits with combination of PRP with a carrier protein and CFA with PRP antibody titers was significantly higher than injected group with PRP. Therefore, these two compounds are introduced as the most immunogenic composition (18). Although the difference between IgG titer in two groups carrier protein with PRP and CFA was not significant ($P < 0.05$), but both groups compared with animals without carrier protein with PRP and CFA, significantly showed a higher grade ($P < 0.05$) (19). High levels of IgG titer against Haemophilus influenzae compared with the control group, showed accuracy of immunogenicity Haemophilus influenzae PRP and PRP purification method (20). As a result carrier protein are strong antigens and can cause memory cells, long-term safety and no toxic to people and they are finally safe.When the carrier protein is conjugated to PRP in order to stimulate the immune system, would be more effective. Therefore, as a result it can be suitable candidate for development of vaccines for prevention of Haemophilus influenza (21).

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