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# BACTERICIDAL ACTIVITY OF CONJUGATE OF CAPSULAR POLYSACCHARIDE OF *NEISSERIA MENINGITIDIS* TYPE A WITH RECOMBINANT PROTEIN OF HEPATITE B AS A MENINGOCOCCAL VACCINE CANDIDATE

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## ABSTRACT

Polysaccharide vaccines are effective in individuals from about two Years of age but, as they elicit T-cell independent immunity, they are not effective in younger children. In contrast, polysaccharide-protein conjugates have been shown to be highly immunogenic in infants and induce T-cell dependent immunity. In this study capsular polysaccharide of *Neisseria meningitidis type A* (N.M.A-CPS) was attached to Recombinant protein of Hepatite B (rHbsAg) covalently. *Neisseria meningitidis type A* (CSBPI, G-243) was cultivated in 40 L of modified Frantz medium in bioreactor and by adding cetavlon to liquid culture, the polysaccharide was precipitated, and then purified by cold phenol extraction, alcohol fractionation and ultracentrifuge differentiation methods. HbsAg protein was provided by Pasteur institute of Iran. N.M.A-CPS was conjugated to rHbsAg protein by using of ADH as a spacer, and EDAC as a linker. Immunization was done, choosing 2 groups of rabbits. Pure N.M.A-CPS and conjugate molecule were injected to groups 1 and 2, with a 15-days interval, intramuscularly. The bleeding was performed at days 0, 15, 30, 45 and titers of sera were measured by Serum Bactericidal Assay.

Polysaccharide bactericidal titer on days 15 and 30 and 45 was almost identical and there was no increase in titer. But in the first injection of the conjugate the titer was much more (about twice of purified polysaccharide), and in the second injection even was increased to number 1/64. Results display that conjugated molecules cause more immunity than pure N.M.A-CPS, and can stimulate cell's immunity.

**Keywords: *Neisseria meningitidis* type A, conjugate of N.M.A-CPS-HbsAg, Recombinant protein of hepatite B, Serum Bactericidal Assay**

## INTRODUCTION

The advantage of Capsular polysaccharide vaccine of *Neisseria meningitidis* type A is that the vaccine has specific formulation and has no side effects, and can create effective protection against meningitis, but immune response to the polysaccharide is non-T cell dependent. And memory cells don't exist in immune response so booster dose has no effect in increasing of immune level and antibody titer. Polysaccharide vaccines are effective in individuals from about two years of age but, as they elicit T-cell independent immunity, they are not effective in younger children. In addition, they fail to induce boostable immunity, so to destroy this problem some proteins are used as a carrier, to active both humeral and cellular immune response and to increase the efficiency of the vaccine, and for this purpose we used protein of hepatite B (1-17, 18, 19). Today, it is known that protein carrier molecules lead to activate lymphocytes T (T-helper) toward the molecular structure of polysaccharide after the introduction of conjugated molecule to B lymphocytes. Many bacterial polysaccharides normally don't have active chemical groups such as amino or carboxyl groups and therefore cannot directly be connected to a carrier protein to form covalent bond. In Conjugation with amidation method that is one of the best methods to conjugate polysaccharid compounds to proteins, the Adipic acid dihydrazide (ADH) is used as a spacing molecule and 1-Ethyl-3-(3-dimethylamino-propyl) carbodimide (EDAC) is used as a coupling agent (20- 31). Here we used recombinant protein of hepatite B to conjugate with capsular polysaccaride of *Neisseria meningitidis* type A. Since the animal model is not available for meningococcal infection, bactericidal activity of produced antibodies against the meningococcal is the most important and most accurate test in immunologic evaluation

of meningococcal infections caused by these bacteria in the course of the disease or after vaccination (32-37).

**MATERIALS AND METHODS**

To extract capsular polysaccharide of *Neisseria meningitides* type A, standard strain CSBPI, G243 of *Neisseria meningitidis* type A prepared from bacterial vaccines and antigen production-research complex of Pasteur Institute of Iran and was revived. After three hours, culture of these bacteria on Muller-Hinton agar plates was done and was incubated at 37 ° C. Then from this plate single colonies were selected and cultured in two tubes of Muller-Hinton agar and incubated at 37 ° C. Days after it, one tube was used to control, so that the slide was prepared and gram-negative and cocci of *Neisseria meningitides* was confirmed and with mono specific antiserum against *Neisseria meningitidis* type A, serological tests were performed and type A of *Neisseria meningitidis* was confirmed by slide agglutination. With the approval of tests that performed on the first tube, the second tube was used to produce seeds for culture in the fermentor. Purification of capsular polysaccharide in the fermentor was performed according to the following steps: preparation of Franz medium in one liter of distilled water, preparation of dialyzed yeast extract, preparation of seed medium, preparation of fermentor, culture and seed inoculation in to fermentor and final purification of capsular polysaccharide was done according to the following steps: cold phenol extraction, alcohol fractionation and ultracentrifuge differentiation. After extraction, capsular polysaccharide of *Neisseria meningitidis* type A (NMA-CPS) conjugated with recombinant protein of Hepatite B (38, 39-49). Finally, for purification of non-conjugated molecules from conjugated molecules, samples of prepared conjugate were passed from chromatography column and fractions were collected. OD (optical density) of collected fractions at a wavelength of 260 nm was read by spectrophotometer and tubes that have highest absorption were collected as fractions that contain conjugated molecules. A series of quality control tests were performed on capsular polysaccharide and conjugated molecule. To measure the amount of polysaccharide per unit weight of the product, O- acetyl of samples was measured by Hestrin method (1949) and Kabat and Mayer (1971) (50-57). For determining the amount of polysaccharide in conjugated molecule, specific O- acetyl of NMA-CPS was measured. Using pure BSA (bovine serum albumin) as a standard, possible

contamination of the protein in pure polysaccharide was measured by Lowry method (1951). And Protein of conjugate molecule was measured by lowry test too (51, 52, 53, 54). Molecular weight of hepatite B protein and conjugate molecule compared to the standard markers (Sigma) were calculated and evaluated by SDS-PAGE, and Lamyla (1970) in10%, Gel (58, 59). Safe use of NMA-CPS and conjugated molecule in laboratory animals after two pyrogenic tests in rabbits and abnormal toxicity in mice and guinea pigs was confirmed (24). Precipitated reaction of hyper immune antiserum of Purified capsular polysaccharide and conjugated molecule and cell body antiserum of type A was evaluated according to immuno diffiugen gel method in agarose (60, 61). To immunological evaluation and supply of immune serum in this study, 2 Groups of 2 New Zealand white rabbits weighing 1.5 to 2 kg selected. Injection of antigens, the pure capsular polysaccharide and prepared conjugate intramuscularly at days 0, 15 and 30 was done and bleeding at days 0, 15, 30 and 45 to determine the properties of antibodies and serum bactericidal assay test was done. Serum bactericidal assay (SBA) is a laboratory method to evaluate serum bactericidal activity. In fact the bactericidal activity of produced antibodies against meningococcal, is the most important and most accurate test in evaluation of immunological infections caused by these bacteria during infection or after vaccination (55, 56, 57, 58, 59, 60, 61). First we cultured standard strain of *Neisseria meningitidis* type A (CSBPI, G-243) on Mueller Hinton agar, after growth, tubes that contains bacterial colonies were washed by PBS (phosphate buffered saline) with PH 7.2, then 1 ml of the suspension removed and diluted by PBS until the turbidity of number 3 tube of McFarland (dilution of $10^9$ CFU / ml) achieved. Then we prepared $10^3$ CFU / ml dilution of the bacterial sample. Dilution $10^3$ CFU / ml of the sample is accurate for bactericidal assay test (62-68). Then, sera were inactivated by placing them for half an hour in the water bath at 56 ° C. By this work serum complement source destroyed. For each serum sample 8 horizontal wells were considered and for foreign complement supply, 3 weeks baby rabbit blood serum used and then we did the following steps:

We added 50 Landa tampon DPBS (phosphate-buffered Dalbecus) to all wells (except vertical first row of wells), then added 100 Landa of inactivated sera in the vertical first row of wells, and then we removed 50 Landa of first well and added to the second well, and dilution done to the end

in each row, 50 Landa of DPBS added to all of the wells then, 50 Landa of bacterial suspension with $10^3$ dilution added and 50 Landa of baby rabbit serum as a complement source added and we inserted plate for 1 hours at the laboratory temperature. After one hour, 50 Landa of each well of the plate removed and transferred to Mueller Hinton agar plates and culture was done. Plates were incubated overnight at 37 ° C. And after that plates removed from the incubator, and the number of colonies of each plate counted and plates that number of colonies in them were 50% or less than the bacterial control plate colonies, serum dilution of them considered as positive titer. We considered 3 well in micro plate as a control: positive and negative and bacterial control.

**RESULTS AND DISCUSSION**

In this study, after the inoculation of 40 ml of modified Franz medium, until the end of the logarithmic phase of growth, all the cultivation parameters in the fermentor controlled

in such a way, cells reached to maximum of their proliferation. Capsular polysaccharide of *Neisseria meningitidis* was purified, and conjugated with recombinant protein of hepatitis B according to the method mentioned in the previous sections. Injection of The final composition of the sample to Sepharose 4B-CL column was done and fractions were collected, then absorbance of fractions at 260 nm wavelength was measured. Fractions that have a higher light absorption at the wavelength of 260 nm were merged together. As seen in the chart below, according to the optical density (OD) obtained from the various fractions, first peak was conjugate molecule. Because molecular weight of glycoprotein conjugate was more than each of its components (protein and polysaccharide), removed out of column faster than the others and non conjugated proteins were removed from the column after that.

In purified capsular polysaccharide type A, amount of O- acetyl in mg weight of capsular polysaccharide was calculated 2.7 micromole and in glycoprotein conjugate, amount of O-acetyl in mg weight of conjugate was calculated 1.10 micromole. By comparing the amount of specific O – acetyl in purified polysaccharide before Conjugation and amount of O- acetyl in glycoprotein conjugate, performance of conjugation according to weight percent for capsular polysaccharide type A, calculated 40.7 percent. We collected and lyophilized fractions that had maximum OD together and then we measured protein levels with Lowry test. OD of peak 1 was 0.04 and by using the

standard curve of protein in Lowry test, protein content was calculated 40 microgram in milliliter and OD of peak 2 was 0.07 and by using standard curve of protein in Lowry test, protein content was calculated 70 microgram in milliliter. The first peak was conjugate peak, which was indicated conjugation of capsular polysaccharide (NMA-CPS) to protein (recombinant protein of hepatite B). And according to the amount of conjugated protein and total protein content performance of conjugation estimated 17.4. Amount of impurities of protein in Capsular polysaccharide was less than 10 milligram per gram polysaccharide so was acceptable. Movement pattern of recombinant protein of hepatite B and conjugated molecule in SDS-PAG electrophoresis10%, compared to standard protein markers was seen in relatively strong bands in the range of protein classes. Movement pattern of Recombinant protein of hepatite B in gel electrophoresis (10%), compared with standard protein markers (Sigma) was observed as a relatively strong bands in the range of 24 kDa. And movement pattern of conjugate molecule (NMA-CPS-HbsAg) in SDS-PAGE electrophoresis 10% compared with standard protein markers (Sigma) was observed as a relatively strong bands in the range of 83 kDa.

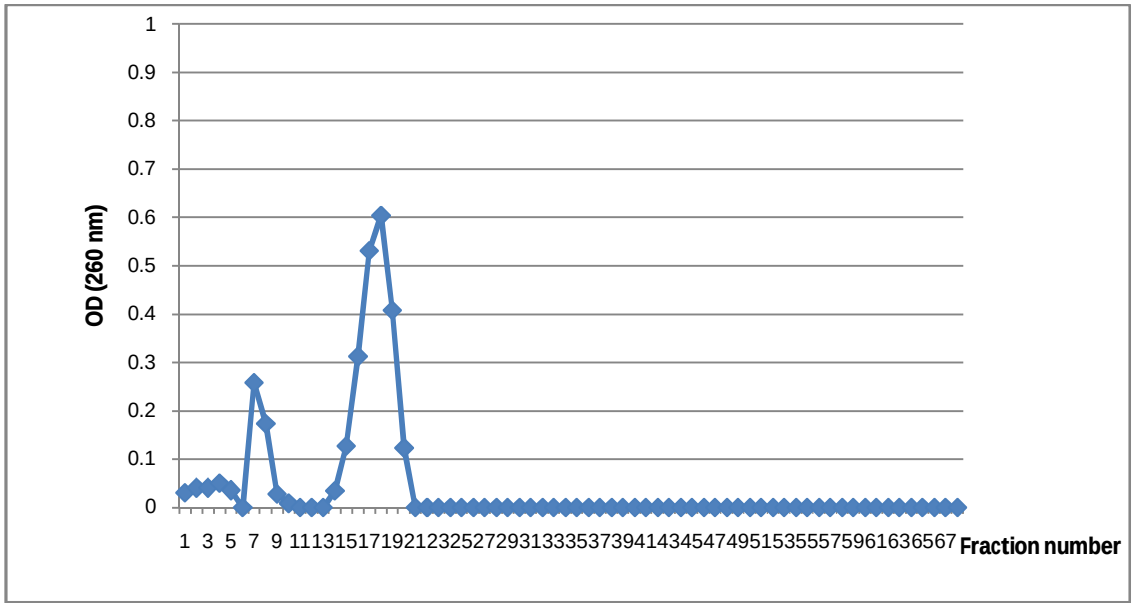
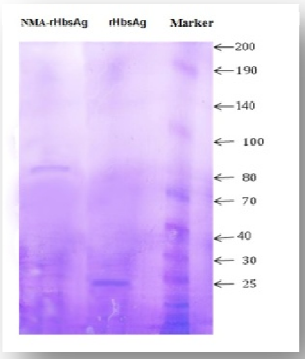



**Diagram 1: absorption spectra of the different fractions obtained from the Sepharose 4B-CL column at a wavelength of 260 nm**

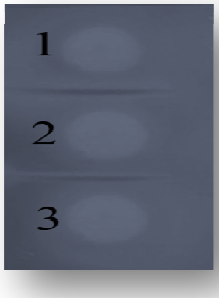



**Column 1: The standard markers**
**Column 2: 24 kDa weight of rHbsAg**
**Column 3: 83 kDa total weight of the NMA-rHbsA**

**Figure 1: rHbsAg and NMA- rHbsAg movement pattern in SDS-PAGE gel test 10%**

Between well number 2, contained pure capsular polysaccharide of *Neisseria meningitidis* Type A and well number 1 contained antiserum against the NMA-CPS in gel, specific sedimentary reaction was created. This phenomenon indicated induced power of NMA-CPS in synthesis of antibodies. Sedimentary reaction between specific antiserum against *Neisseria meningitidis* type A that produced in bacterial vaccine section in Pasteur Institute (standard positive control serum) and NMA-CPS (well No. 2) not only proved specific identity of NMA-CPS in identifying immunoglobulins that have been synthesized against their antigenic characteristics but also expressed resistance of its antigenic properties in various process of extraction and purification.

The gel double immune diffusion test based on dual release of NMA-CPS and HbsAg and glycoprotein conjugate against induced antibody in serum of animal that was secured against conjugated macromolecule, was performed. The results showed that all three products had reacted with the antibody produced in secured animal, So that their specific sedimentary bond between them that designed by the model, was created. In this model, it can be concluded that the glycoprotein conjugate was able to synthesis specific antibody and this antibody had the ability to react with each component of conjugated macromolecule.




**Well No. 1: antiserum agains capsular polysaccharide *of Neisseria meningitidis* type A**
**Well No.2: pure capsular polysaccharide of *Neisseria meningitidis* type A**
**Well No. 3: mono specific antiserum against *Neisseria meningitidis* type A produced in bacterial vaccine section of Pasteur Institute (standard positive control serum)** **Figure 2: gel diffusion test**

**Well No 1: conjugate**
**Well No 2: antibody against conjugate**
**Well No. 3. Antibodiy against recombinant protein of hepatite B**
**Well No 4: antibody against capsular polysaccharide of *Neisseria meningitidis* type A**
**Well No. 5: capsular polysaccharide of *Neisseria meningitidis* type C (negative control)**

**Figure 3: gel Immunodiffusion Test**

To see the absence of fever-causing impurities in glycoprotein conjugate and capsular polysaccharide, pyrogeny test was done in rabbits. With intravenous injection of 0.025 microgram in milliliter of capsular polysaccharide and conjugated macromolecule per kg weight of rabbit's body, abnormal rise in the animal's body heat temperature not seen. And with abnormal toxicity test in mice and pigs absence of any type of poison in samples was proved. Intraperitoneal injection of 500 microgram of the conjugate and purified NMA-CPS to a group of 5 pigs and intraperitoneal injection of 100 microgram of these to a group of 5 mice within 7 days in good condition, not only didn't cause death or animal body weight loss, but also after the autopsy, tissue damage at the injection site or any internal organs not seen. Since serum bactericidal activity is the most important test in immunological evaluation of meningococcal vaccines, this test was done on animal serum samples secured with NMA-CPS-HbsAg and pure NMA-CPS against standard strain CSBPI, G -243 type A and hepatite B protein. According to tables and graphs, the results showed that the ability to induce synthesis of bactericidal antibodies in secured animal after the first injection and the second and third booster injection at 15-day intervals induced the synthesis of high levels of bactericidal antibodies in this Group, against *Neisseria meningitidis* type A and HbsAg. In this test, serum dilution comparing with bacterial control plate, killed more than 50 percent colonies, considered as a positive titer and colonies number in bacterial control plate was 190 colonies. By comparing of serum bactericidal titer, bactericidal titer of pure NMA-CPS was positive until 1/8 titer and booster dose injection with pure NMA-CPS didn't have any effect in rising of bactericidal titer. Bactericidal titer of conjugate in first injection was 1/64 and this amount not only was more than bactericidal titer of pure NMA-CPS in this injection, but also with

injection of booster dose, bactericidal titer raised to 1/128. These results showed that conjugate macromolecule in first stage created more immunity comparing with polysaccharide and in the second stage of injection this titer was significantly higher and that was for immune response associated with T cell (cell immunity). Finally, it can be concluded that the conjugate molecule in a booster dose can create better safety and immunity.

**Table 1: bactericidal titer of IgG produced against NMA-CPS and NMA-CPS-HbsAg on days of 15, 30, and 45**

| | Dilution | 1 | $\frac{1}{2}$ | $\frac{1}{4}$ | $\frac{1}{8}$ | $\frac{1}{16}$ | $\frac{1}{32}$ | $\frac{1}{64}$ | $\frac{1}{128}$ |
|---|---|---|---|---|---|---|---|---|---|
| Colony numbers | N.M.A-CPS day of 15 | 45 | 50 | 65 | 77 | 120 | 146 | 173 | 209 |
| | N.M.A-CPS day of 30 | 42 | 47 | 63 | 73 | 114 | 135 | 163 | 190 |
| | N.M.A-CPS day of 45 | 35 | 37 | 55 | 75 | 110 | 127 | 153 | 181 |
| | N.M.A-CPS-HbsAg day of 15 | 6 | 8 | 15 | 36 | 50 | 63 | 80 | 100 |
| | N.M.A-CPS-HbsAg day of 30 | 5 | 6 | 9 | 26 | 42 | 47 | 71 | 98 |
| | N.M.A-CPS-HbsAg day of 45 | 1 | 3 | 5 | 5 | 18 | 23 | 37 | 60 |

**Table 2: bactericidal titer of IgG produced against NMA-CPS-HbsAg on days of 15, 30 and 45**

| | Dilution | 1 | $\frac{1}{2}$ | $\frac{1}{4}$ | $\frac{1}{8}$ | $\frac{1}{16}$ | $\frac{1}{32}$ | $\frac{1}{64}$ | $\frac{1}{128}$ |
|---|---|---|---|---|---|---|---|---|---|
| Colony numbers | N.M.A-CPS day of 15 | (+) | (+) | (+) | (+) | (-) | (-) | (-) | (-) |
| | N.M.A-CPS day of 30 | (+) | (+) | (+) | (+) | (-) | (-) | (-) | (-) |
| | N.M.A-CPS day of 45 | (+) | (+) | (+) | (+) | (-) | (-) | (-) | (-) |
| | N.M.A-CPS-HbsAg day of 15 | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (-) |
| | N.M.A-CPS-HbsAg day of 30 | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (-) |
| | N.M.A-CPS-HbsAg day of 45 | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) |

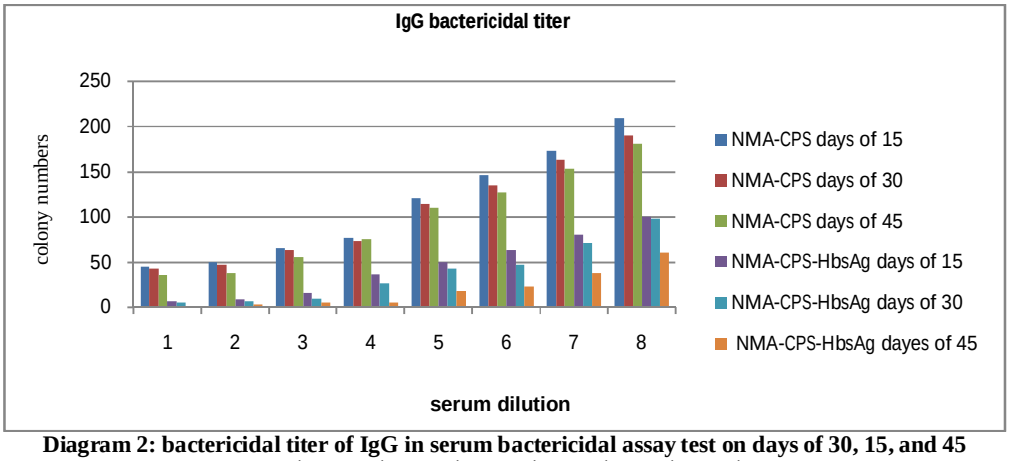



**Diagram 2: bactericidal titer of IgG in serum bactericidal assay test on days of 30, 15, and 45**

**Dilutions: $(\frac{1}{128})$ 8 – $(\frac{1}{64})$ 7 – $(\frac{1}{32})$ 6 – $(\frac{1}{16})$ 5 – $(\frac{1}{8})$ 4 – $(\frac{1}{4})$ 3 – $(\frac{1}{2})$ 2 – (1) 1**

**DISCUSSION**

The amount of protein in the prepared conjugate was determined by Lowry test and according to the amount of protein in conjugate and amount of total protein, Conjugation efficiency by weight of protein was calculated17.4%. Conjugation efficiency by weight of protein in studies of Chu and shnyrson and colleagues, was estimated 30 to 60 percent. Therefore our results with the results obtained by the researchers to some extent corresponded. By comparing the amount of specific O - acetyl in purified polysaccharide before conjugation and specific O - acetyl of polysaccharide in glycoprotein conjugate, conjugation efficiency based on weight percentage for capsular polysaccharide type A, was estimated 40.7 percent. And this efficiency in studies of jean and colleagues for capsular polysaccharide type A in conjugate was about 40% by weight percentage of polysaccharide (65). Immunodiffusion results showed that antigenic structure of capsular polysaccharide of *Neisseria meningitidis* type A and recombinant protein of hepatite B not destroyed during conjugation and able to react with related antibodies and also according to the resulting conjugate, isolated by using of Sepharose 4B-CL chromatography column from the non-conjugated polysaccharide and protein molecules, it can be concluded that the two molecules are covalently linked together. Double immunodiffusion test results showed that antibodies induced against a covalent conjugate in serum of animal, in addition to react with the conjugate, have the ability to react with both component of conjugated macromolecule. By analyzing these results, it can be said that the antigenic structure of the epitopes in capsular polysaccharide of *Neisseria meningitidis* type A and recombinant protein of hepatite B not damaged during conjugation and this conjugate as an effective immunogen against both component worked. It was also confirmed that the electrophoretic analysis in electrophoresis test that was done on conjugated macromolecule, a strong band in the range of 83 kDa was observed. In order to determine titer of antibody and humeral immunity of conjugate, immunization of rabbits with conjugate and pure NMA-CPS was done. To observe the effect of booster dose, immunization was performed in 2 stages. The second phase, carried out 15 days after the first injection, the serum bactericidal assay was used for the purpose. The results in serum bactericidal assay (SBA) showed that second injection of NMA-CPS-HbsAg and pure NMA-CPS, increasing antibody titer

compared to the first injection. The second injection of conjugate created higher titer of antibody than second injection of NMA-CPS and the first injection of conjugate that indicated effect of booster dose in increasing of immune response in conjugate injection and showed that conjugation of capsular polysaccharide of *Neisseria meningitidis* type A with recombinant protein of hepatite B due to Tcell- dependent state in the pure capsular polysaccharide and also stimulated production of the memory cells. So booster dose had effect in increasing of safety level and titer of antibody that were seen in results of serum bactericidal assy. So this test and other tests that had been done showed that conjugated macromolecule induced higher titer of immunity than the pure polysaccharide and can be used as a bivalent immunogen. By comparing the serums bactericidal titer, was found that bactericidal titer of pure NMA-CPS was positive until 1/8 and had a bactericidal effect, and booster dose injection of NMA-CPS didn't have any effect in rising of bactericidal titer. Bactericidal titer of conjugate in the first injection was 1/64. That this amount not only was higher than the bactericidal titer of pure NMA-CPS, but also by a booster dose injection bactericidal titer was increased to about 1/128. These results indicated that conjugate created more immunity in the first stage than pure polysaccharide, and by the second injection (Booster Dose), the titer was significantly higher than pure polysaccharide, that was for Tcell-dependent immune response (cellular immunity).

**CONCLUSION**

Finally, it can be concluded that conjugate molecule in booster dose can provide a better level of safety. In this project we wanted to see whether conjugation of a capsular polysaccharide with low molecular weight protein such as hepatite B protein with a molecular weight of 24 kDa can make good immunogenicity and this conjugate can be used as a bivalent suitable immunogen. In fact, in this project we want to conjugate capsular polysaccharide of *Neisseria meningitidis* type A to recombinant protein of hepatite B with covalently bond to provide one immunogen that immune response toward it be T-cell dependent (provide full safety) and be used to product bivalent vaccine, considering the results and titer of antibodies that were obtained from tests performed, this protein can be a good carrier protein for NMA-CPS and provide appropriate safety.

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