

## STUDY OF IMMUNOLOGICAL ACTIVITY AGAINST HAEMOPHILUS INFLUENZAE TYPE B PRP- DIPHTHERIA TOXOID CONJUGATE

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### ABSTRACT

*Haemophilus influenzae* is Gram-negative coccobacilli, Obligatory Anaerobic Bacteria without flagellum, without spores, positive catalase, and positive oxidase. Morphologically, polymorphism is well-known, especially in separated bacteria from old cultivars or GSF. They can be observed in different forms including coccoid, coccobacilli, bacilli, or filamentous forms. The main objective by the present study is to conjugate *haemophilus influenzae* type b (Hib) polyribosylphosphate (PRP)-diphtheria toxoid through using different methods. Applied method at the present study would as follows: cultivation of *haemophilus influenzae* in chocolate agar medium with factors X and -V; purification and precipitation of PRP from cultivation liquid through using alcohol precipitation; precipitation by N-Acetyl-N,N,N-trimethylammonium bromide GR (CTAB) Cetavlon; ultracentrifuge and purification by hydroxyapatite; filtering through 22%µm and distribution in sterile vial and lyophilizing it; producing diphtheria toxoid from Razi Factory and dialyzing it with dialyze packet of cutoff 10000 in order to pull out preservative and filtering it to 22%µm and distributing it in sterile vial and then lyophilizing it; conjugation by diphtheria toxoid using ADH and cyanogen bromide as the separating factor and EDAC as the conjugation factor; measuring amount of protein through Lory and Bradford Tests; electrophoresis; measuring amount of polysaccharide through Ribose Test and quellung test; identification of fraction of conjugation and calculation of conjugation output; injection of conjugation and PRP just to several white laboratory rabbits for 2 times with interval of 15 days with aluminum hydroxide adjuvant; taking blood samples and finally investigation of immunology of collected bloods from injection of conjugation and PRP using serum bactericidal test. At the present study, serum titers in serum bactericidal test would be respectively equal to 1.8, 1.16, and 1.32 for the first group; 1.4, 1.8, and 1.16 for the second group; and finally 1.16, 1.32, 1.64 for the third group.

**KEYWORDS:** Immunological evaluation, *Haemophilus influenzae* type b (Hib), Diphtheria toxoid, conjugation, Meningitis, Polysaccharide, Protein carrier, Pathogenic, Pyrogenic

### PATHOGENICITY

Strains without *Haemophilus influenzae* capsule would usually cause infection in previously damaged tissues; for example, inpatients with chronic bronchitis or cystic fibrosis. Cell CF has a key role in connecting these strains to nasopharyngeal mucosa cells.

External membrane proteins are also effective in connection and colonization of the bacteria on the mucosa surfaces. Usually, primary focus of the infection, resulted from strains non-capsular strains, would be appeared in nose and throat; although the infection may be expanded to other parts like sinuses or Eustachian tube in ears. Non-capsular strains would cause usually diseases such as sinusitis, pneumonia, otitis, mastoiditis, conjunctivitis, endometritis, pericarditis, urinary tract infection (UTI), etc.

In capsular strains, especially type b, the main role of the capsule is preventing phagocytosis of Poly Morpho Nuclears and also serum resistance of bacteria. Capsular strains would mainly cause invasive infections in human body such as meningitis, epiglottitis, pneumonia, cellulite, laryngitis, osteomyelitis, septic arthritis, and septicemia.

Primary focus of infection in capsular strains is usually in nasopharynx. The bacteria would be connected to nasopharyngeal mucosa cells by external membrane proteins. The bacteria can enter to blood flow and then to different organs of infants, individuals with immunity disorder, cancer patients, organ recipients, and other similar individuals. The most important infectious disease that may be resulted from such strains is meningitis, in which LOS pieces and bacterial peptidoglycan can lead to

increase in permeability of blood-brain barrier and exacerbate of meninges' inflation.

### **IMMUNITY AGAINST INFECTIONS, RESULTED FROM HAEMOPHILUS INFLUENZA**

Presence of antibody against external membrane proteins and capsule plays a key role in preventing localized and disseminated infections. Protective antibody against capsule can be transferred to the infant throughout the placenta and can protect the infant for 6 months after birth day against invasive infections, resulted from Haemophilus influenza. After the mentioned age, protective antibody would be significantly decreased and then infant would become vulnerable against disseminated and invasive infections, especially meningitis. Hence, vaccination is so significant in age range from 2 to 12 months old.

In immunology of infectious diseases, in order to activate T cell, assistant t cell co-receptor (CD4) would identify supplied specific peptide beside the MHC class II and then would transfer identification signal to native T cell. It should be mentioned that 2 signals are required in order to activate T cell. The first signal is same mentioned identification signal. Second signal would be presented through interleukin -2 (IL -2). Through identifying the antigen, T cell would be activated and then would enter to cell cycle. At this stage, IL-2 and receptor can construct it; although in order to continue its activity and increase its clones, the two mentioned components should be connected to each other. In T cell (NFAT), action of IL-2 should be copied, which would be possible through connecting CD28-B7 that both of them are from immunoglobulin super family and are known as Co-stimulation. Clones of T cell would be increased through transferring activity signal of a series of biochemical events inside T cell such as increase in amount of ionized calcium, increase in protein production, and increase in clones.

Assistant T cells would identify complex peptide and MHC on surface of B cell and would activate it against polysaccharide in order to produce antibody.

### **EPIDEMIOLOGY**

Haemophilus influenza is a pathogenic microbe, especially in age range of 3-18 months. At these ages, immune system would not act desirably against independent antigens from thymus like haemophilus

influenza type b polysaccharide. In order to make effective vaccine and consuming it by infants, its capsul antigen would be connected to a protein transporter in conjugated form and then produced vaccine would be applied. Immune function of the mentioned vaccine has been reported to 80% because of imposing effect through T cells against invasive form of Haemophilus influenza. Hence, its application is significant in routine vaccination of infants.

Bacterial meningitis is an important problem in kids and infants. Over the decade, significant advances have been achieved for rapid diagnosis of the meningitis disease such as serologic methods and antigen seeking methods. Etiologic factors of bacterial meningitis are relatively various and most scholars have introduced several factors as the main factors of bacterial meningitis, especially in childhood, including Haemophilus influenza, Neisseriameningitidis, and Streptococcus pneumonia. According to relevant studies, the mentioned factors can be varied based on time, location, and age of patients. Vanger et al (1990) have conducted a study in America and have found that the most common separated bacteria of infants are respectively Streptococcus pneumonia to 40%; Haemophilus influenza type b to 18%; and Neisseriameningitidis to 14%. However, in another study by Ronald Gold et al (1992), they have reported pathogenic factors in 70% of 1-5 children respectively Escherichia coli; Streptococcus beta hemolytic; Haemophilus influenza type b; Neisseriameningitidis; and Streptococcus pneumonia. Legers et al have conducted a wide expanded study on causer factors of bacterial meningitis in some developing countries such as Senegal and Dhaka and have found that Streptococcus pneumonia is the most common causer factor of meningitis in children. In another study on Tehran Hospital during 1990-91, researchers have found that the most common factors of meningitis in children respectively are Neisseriameningitidis; Haemophilus influenza type b; and Streptococcus pneumonia. Moreover, in a similar study by Borjian in Valiasr Hospital in Brojen province Iran, the most common factors have been reported as follows: Haemophilus influenza type b to 36.8%; Streptococcus pneumonia to 26.3%; and Neisseriameningitidis and salmonella paratiphitype A to 10.5%.

## METHODOLOGY

### PRP extraction

#### Alcohol precipitation stage

Bacterial suspension was transferred to 2L containers and was then centrifuged for 1h with 3500rpm. Supernatants were collected and precipitated cells were disposed. Collected supernatants were maintained in the refrigerator for one night under temperature of 4°C. About 9L of supernatants were distributed in 4 alonjes, 3 alonj containing 2.5L supernatant and one alonj containing 1.5L supernatant. Per every 2.5L supernatant, about 6L and 750cc ethanol 96% was added. Then the supernatant was maintained for one night under temperature of 4 °C. During the maintenance time, the PH was regulated on 7.3 and glacial acetic acid was added to it and then pH ratio achieved 6.8. Then, it was maintained again for 24h under temperature of 4°C, so that polysaccharides could be precipitated.

#### Washing by chloro-magnesium

Contents of alonjes were centrifuged for 1h with 3000rpm. Supernatants were disposed and obtained precipitation was maintained. Then, 1.8L chloro-magnesium with density of 0.05 was added to it and was mixed properly, so that sediments could be solved completely. It was then centrifuged for 1h with speed of 3000rpm and sediments were disposed and supernatant was maintained.

#### Treatment by Cetavlon

Collected supernatant was about 2.5L that was mixed with 100cc Cetavlon (sigma) 2% and was maintained for one night under temperature of 4°C. After one night, the solution was centrifuged with speed of 3000rpm. Then, precipitation was maintained and supernatant was disposed.

#### Washing by sodium phosphate

About 1L sodium phosphate 0.7 was added to the sediments and then the solution was centrifuged for 1h with speed of 3500rpm. Then supernatant was separated from sediments and about 930cc was obtained.

#### Repeating alcohol washing

Since at the first step of alcohol washing about 2L and 600cc ethanol was added per liter, at this stage, about 2L and 418cc ethanol 96% was added to supernatant, which was about 930cc. then, the solution

was maintained for 1h under room temperature and then was maintained in the refrigerator for one night.

#### Treatment by hydroxyl apatite

Obtained solution was pulled out of the fridge and centrifuge was conducted for 1h with speed of 3500rpm. Then, about 900cc hydroxyl apatite 1% (Merck) solution was added, so that they could be solved completely. Afterwards, centrifuge was done for 1h in speed of 3500rpm. At the next stage, about 500cc sodium acetate 0.5 was added to obtained sediments from centrifuge and then it was maintained in the fridge for one night. Afterwards, about 5g Sodium deoxycholate was added and was maintained for about 2h in the fridge. Then, solutions were transferred to 50cc falcons and were centrifuged for half an hour with speed of 9000rpm. Then supernatant was collected, filtered, and finally lyophilized.

## QUALITY CONTROL TESTS OF PURE PRP

### Determining humidity ratio

Existed humidity in lyophilized PRP was measured using Colometer Meter Toledo D137 KF through Carl Fischer method. In short, certain amount of pure methanol solvent was added to a vial containing 250mg polysaccharide and was injected by syringe to the machine after that it was solved. According to "WHO" protocol, humidity ratio of final product should be less than weight of dried polysaccharide.

### Protein determination through Lowry method

Using pure BSA (sigma) as the contamination standard, probable protein in pure polysaccharide was measured using Lowry method (1951). The mentioned method includes 2 reactions. In the first reaction, compound of copper ion and protein molecules can produce a protein-copper complex. In the second reaction, acid phosphor Tungstic (APT) and Phosphomolybdic acid from Folin solution can cause specific color in the mentioned complex. In protein-cooper complex, amino acids Tyrosine and tryptophan would be survived and then a blue color would be appeared, which its density is depended on protein percent. Lowry method is a sensitive method and its accuracy is from 1 to 100mg/ML.

### Providing required standards

1. Solution A: 20g sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) and 5g double tartarate of sodium-potassium was conveyed to density of 1L using (NaOH).
2. Solution B: 5g cooper sulfate (CuSO) with 5 water molecules (CuSO<sub>4</sub>·5H<sub>2</sub>O) was conveyed to density of 1L using distilled water.
3. Solution C: 50ML of solution A was mixed with 1ML of solution B (while testing).
4. Solution D: Folin-Ciocalteu reagent (FCR) was diluted to ½ ratio using distilled water and then was applied in the test.

## DISCUSSION AND RESULTS

### Purity determination of PRP

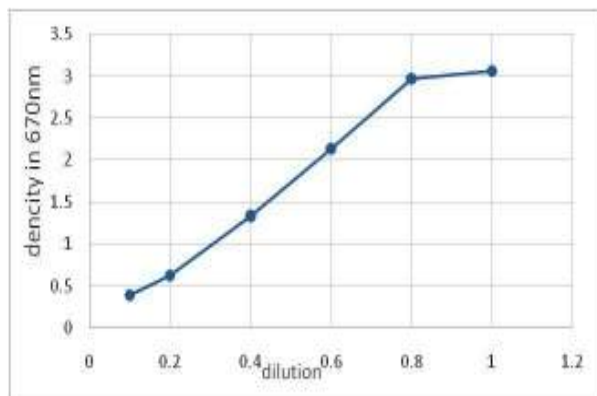
#### Measuring ribose ratio

Quality of extracted polysaccharide would be determined through measuring its ribose ratio. Measurement of ribose in purified PRP has been determined through using the mentioned protocol.

After tracing ribose standard diagram based on figure 1, obtained linear equation would be as follows:  $Y = 0.052X + 0.072$ . In this equation, Y refers to OD of purified PRP, based on which ribose ratio per ML of PRP has been estimated to 8.5mg/ml.

**Table 1: Ribose optical density (OD)**

Ribose density (mg)	OD ratio
6.25	0.394
12.5	0.628
25	1.334
37.5	2.140
50	2.973
62.5	3.06



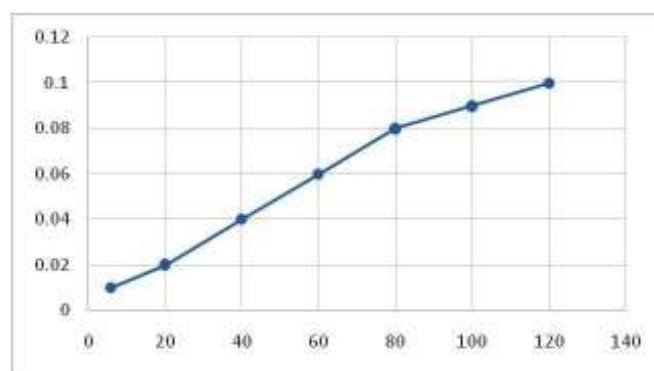
**Figure 1: Ribose standard curve in 670nm**

### Measuring protein in PRP polysaccharide sample

In order to determine existed protein in polysaccharide in purified PRP, Lowry test and also linear equation of  $Y = 0.0008X + 0.0055$  and also standard diagram of protein have been applied. The ratio has been obtained equal to 0.072mg/ml.

### Measuring protein ratio in conjugations PRP-TD

Protein amount in conjugation PRP-TD has been calculated using Lowry test; standard linear equation; and also using protein standard diagram as it is illustrated in figure 2. Obtained value has been equal to 56.589mg/ml protein in conjugation.



**Figure 2: Protein standard curve**

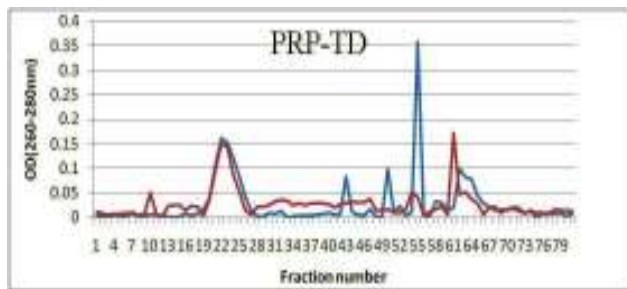
### Measuring PRP polysaccharide ratio in conjugation

In order to measure PRP polysaccharide ratio, ribose measuring test has been applied, since 40 to 41% of PRP's weight has been composed of ribose (Crisel et al, 1986). According to obtained linear equation at the previous section and also according to figure 1, ribose ratio has been calculated per ml in conjugation. The value has been equal to 6mg/ml. since every 1mg of ribose is equal to 2.55mg PRP, calculated PRP value per ml of conjugation has been equal to 0.2mg.

### Polyribosylphosphate (PRP)-diphtheria toxoid (TD) conjugation

After producing PRP-TD conjugation, in order to purify conjugated molecules from non-conjugated molecules, the conjugation has been transferred throughout the chromatography column 4B-CL through using filtration method. Then OD of different fractions was read along waves of 260 and 280nm. As it is obvious in figure 3, the first peak is related to PRP-TD conjugation, which shows conjugation of PRP polysaccharide capsule with protein carrier (diphtheria

toxoid). Second and third peaks illustrate also non-conjugated proteins.



**Figure 3: OD curve of conjugation fractions in 280 and 260nm**

**Conjugation’s output**

Through calculating ratio of conjugated protein to overall protein in PRP-TD conjugation, output of conjugation has been obtained to 46.36%.

**Results of pyrogenicity test of PRP and PRP-TD conjugation**

PRP polysaccharide and PRP-TD conjugation in 3 rabbits have been less than 1.4 and less than 0.6 in every rabbit. Hence, according to table 2, PRP samples and provided conjugation were free from injective and pyrogenic materials.

**Table 2: Results of perogenicity test**

Experimental material	Number of rabbits	Injection	Amount	Mean value of body temperature rise	Result
PRP	3	IV	0.025mg	Negative	Non-pyrogenic
PRP-TD conjugation	2	IV	0.1ml	Negative	Non-pyrogenic

**Toxicity test**

In order to investigate toxicity of produced conjugation, samples were injected to 5 laboratory

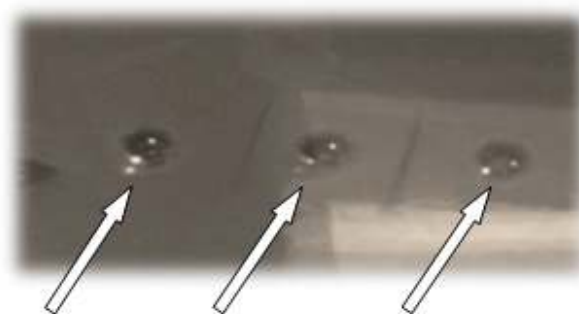
mice and they were under investigation for 7 days. As it is obvious from table 3, lack of decrease in weight and mortality of mice indicates that the conjugation has been nontoxic.

**Table 3: Results of toxicity test**

Experimental materials	No of mice	Injection	Amount	Weight reduction	Duration	Result
PRP-TD conjugation	5	IM	100mg	Negative	7 days	Nontoxic

**Gel diffusion test**

Relevant results of dual diffusion of antigen and antibody test in agar gel have been illustrated in figure 4. The test has been conducted in order to investigate validity of connection between PRP polysaccharide antigen and tetanus-diphtheria toxoid. The mentioned test has been also conducted in order to ensure that antigenic structures of polysaccharide and protein have remained non-manipulated and undestroyed after conjugation. As it is obvious in figure 4, precipitation lines have been formed as a result of interactions between antigen and antibody. This would indicate that produced conjugation molecule is able to interact with anti-PRP and anti-TD.



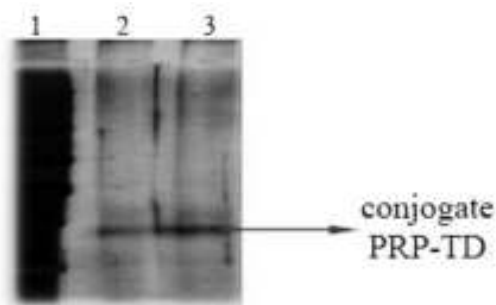
Anti- PRPConjugate Anti-TD

**Figure 4: dual diffusion test of gel**

**Mid hole: PRP-TD conjugation; right-hand hole: PRP; and left-hand hole: anti-TD**

**Electrophoresis results**

First hole (1) contains marker molecule and holes 2 and 3 contain conjugation molecules. Provided single band in 150KD in figure 5 has confirmed PRP-TD conjugation molecule.



**Figure 5: confirmation of conjugation molecule by electrophoresis**

Hole N o.1 contains marker molecular and holes 2 and 3 contain conjugation. As it is obvious in figure 5, a single-band has been appeared in 150KD distance.

**Serum bactericidal test results**

Humoral immune of PRP-TD conjugation and pure PRP has been studied using serum bactericidal test and opsonization activities against organism. In this test,

**Table 4: obtained results from bactericidal test**

Duration	1	2	3	4	5	6	7	8
PRP 15days	+	+	+	+	-	-	-	-
PRP 45days	+	+	+	+	+	-	-	-
Conjugation 15days	+	+	+	+	+	+	-	-
Conjugation 45days	+	+	+	+	+	+	+	+
haemophilus influenza	+	+	-	-	-	-	-	-
Negative control	-	-	-	-	-	-	-	-

**CONCLUSION**

In haemophilus influenza, more than 3million people suffer from the mentioned disease and annually, 400-700 thousand people would die as a result of such disease. In America, 12-20 thousand people would suffer from the disease. Outbreak of the disease is in winter and early days of spring and can be transmitted through droplets and respiratory secretions. Over the past years, a series of antibiotics were applied in order to prevent such disease and the method was successful from 5-10%. Through this, a series of people were survived; although many serious defects were appeared such as disorder in

dilution of the serum, which has killed 50% of colonies comparing to bacterial control plate, has been considered as the positive titer.

**Obtained results from counting colonies**

Number of colonies in plate, resulted from bacterial suspension cultivation with dilution of 10<sup>3</sup> IUCFU/ml, has been equal to 375. Through counting colonies of haemophilus influenza type b (Hib) on plates and also comparing them with bacterial control plate, obtained results would be as follows:

Through comparing titer of bacterial killing in serums, it was found that titer of bacterial killing of pure PRP has become positive to titer  $\frac{1}{16}$  and has had bacterial killing effect. Injection of the mentioned dose with PRP has had also no effect on enhancing bacterial titer.

Titer of bacteria killing in PRP-TD conjugation in the first injection has been equal to  $\frac{1}{32}$ . The value has been not only more than titer of bacterial killing in pure PRP, but also through injecting the mentioned dose, titer of bacterial killing has been increased to  $\frac{1}{128}$ .

their sight, hearing, and also dementia disorder. Ampicillin was selected antibiotic in treating meningitis. The most important problem with using such drugs is that, haemophilus influenza would become resistant against ampicillin and other antibiotics and would make the action complicated. On the other hand, using such antibiotics is problematic, since they would not pass throughout the brain-blood barrier easily.

Clearly, prevention is better than treatment. CDC is Center for Disease Control, which has offered using Hib vaccines in order to prevent outbreak of such diseases. America has applied the vaccine during 1980-

1990 and has observed that number of survived people has achieved to 40-100.100000 from 1.3100000 people. At early 1990, the vaccine has been offered for children below 5 years old. In 2003, 92% of population in developed countries has become vaccinated against Hib; while mean value of vaccination in developing countries has been equal to 42%.

In 1985, several vaccines were applied in U.S for 18 years old children and above it. The vaccines were not applicable for children below 18 years old; although children in this age range are the main victims. PRP is a kind of polysaccharide that same as other ones, immune response against it is depended on T cells. In order to remove it, suitable proteins or carriers should be applied and immune system of T cells should be activated same as Humoral immune system. Today, designing macromolecules of conjugation is one of the most advanced microbial-biotechnological domains in order to produce biochemical products.

Marshall et al (2008) injected Hib – HBV-DTPa vaccines in mixed form and separately to 360 healthy infants of 2, 4, and 6 months. One month after the first vaccination, antibody titer against HBV was equal to 98%; 94-97% for Hib; and was equal to 68% for DTPa vaccine.

Dr. Ahmadi and Dr. Tabarraei et al (2007) have conjugated Vi-CPS to BSA. They applied EDAC in order to conduct conjugation. Obtained conjugations were injected to 3 groups of rabbits in every group 3 rabbits from New Zealand. Obtained results from the study indicated about 30times enhancement of antibody titer, comparing to Vi-CPS and was also consistent with the mentioned results.

Moharrar (2010-2011), following advices of Dr. Ahmadi and Dr. Shapoori has conducted a study in this regard. In this study, he has conjugated Vi-CPS with tetanus toxoid through amidation method. In order to ensure about proper conjugation among molecules and avoid basic changes in 3-d and antigenic structures of molecules, gel diffusion test was applied. Moreover, in order to investigate induction value of serum titer of antibodies, ELISA test was applied and obtained results indicated that all receptor groups of conjugated vaccine were significantly different from control group in terms of antibody titer.

In another study by Cui et al (2004), capsular polysaccharide of optical Salmonella enterica was conjugated with diphtheria toxoid as protein carrier. They have also achieved previous results and it was found that titer of IgG antibody against Vi in mice by Vi-CPS-DT has been significantly more than antibody titer of Vi-CPS separately. Obtained results from the study have been also consistent with obtained results from the present study.

Relevant studies have indicated that PRP is a desirable immunity factor against meningitis; although it can't provide stable immune as it is unable to motivate cell immune system. However, when the factor I conjugated with a protein carrier, it would gain such capability. At the present study, first capsular polysaccharide of PRP has been extracted from ATCC 1623 of haemophilus influenza. Then, the factor has been conjugated to surface protein of diphtheria-toxoid as the protein carrier using ADH and EDAC. The, 4B-CL column has been applied in order to purify conjugated and non-conjugated molecules. Afterwards, 2 injections were done on white rabbit from New Zealand with 2-weeks interval. Then, blood sampling was done for 4 times in 0, 5, 30, and 45min durations.

According to obtained information about conjugation of capsular polysaccharide of haemophilus influenza and also application of different carriers in order to conjugate the mentioned polysaccharide and also investigate its immunity, there is no desirable work in Iran. The only work in this regard has been working on bacterial vaccine sector and production of antigen of Iran Institutopastor in order to purify, produce, and immunological evaluate of PRP polysaccharide. Moreover, in order to conjugate other polysaccharides (especially optical salmonella VI capsular polysaccharide and Neisseriameningitidis type A and C capsular polysaccharide with exotoxin protein A Pseudomonas aeruginosa- tetanus toxoid and recombinant protein of hepatitis type B), some works have been conducted in university and research levels. Based on obtained results and amount of antibody titer of the protein, it can be a desirable protein carrier for PRP and also can provide suitable immune.

Obtained results from the present study indicate that there are better immune conjugated molecules than PRP, which can motivate cell immune. This would lead to reduction of injection repetitions and also reduction of

costs. Moreover, surface protein of diphtheria can act as a desirable protein carrier.

## REFERENCES

- Crisel, R. W., Baker, R. S., and Dorman, D. E. (1975) Capsular polymer of *Haemophilus influenzae*, type b. I. Structural characterization of the capsular polymer of strain Eagan. *J. Biol. Chem.* **250**, 4926–4930
- Pfeiffer, R. (1892) Vorlaufige mitteilungen uber die erreger der *influenzaa*. *Dtsch. Med, Wochenschr.* **18**, 28–34
- Pittman, M. (1931) Variation and type specificity in the bacterial species of *Haemophilus influenzae*. *J. Exp. Med.* **53**, 471–492
- Alexander, H. E. (1965) The *Haemophilus* group, in *Bacterial and Mycotic Infections of Man* (Dabos, R. J. and Hirsch, J. G., eds.), Pitman Medical Publishing Co., Ltd., London.
- Turk, D. C. and May, J. R. (1967) *Haemophilus influenzae, Its Clinical Importance*. The English Universities Press, Ltd., London
- Fothergill, L. D. and Wright, J. (1933) Influenza meningitis. The relation of age incidence to the bactericidal power of blood against the casual organism, *J. Immunol.* **24**, 273–284
- Taylor, H. G., Mills, E. L., Ciampi, A., et al. (1990) the sequelae of *Haemophilus influenzae* meningitis in school-age children. *N. Engl. J. Med.* **323**, 1657–1663
- Haggerty, R. J. and Ziai, M. (1964) Bacterial meningitis in infancy and childhood, in *Advances in Pediatrics* (Levine S. Z., ed.), Year Book Medical Publishers, Chicago
- Crook, W. G., Clanton, B. R., and Hodes, H. L. (1949). *Haemophilus influenzae* meningitis: observations on the treatment of 110 cases. *Pediatrics* **4**, 643–659
- Katz, S. L. (1966) the prevention of mental retardation through control of infectious diseases. *U. S. Public Health Service Publication 1692*, 318
- Sell, S. H. (1970) the clinical importance of *Haemophilus influenzae* infections in children. *Pediatr. Clin. N. Am.* **17**, 415–426
- Sproles, E. T., Azerrad, J., Williamson, C., and Merrill, R. E. (1969) Meningitis due to *Haemophilus influenzae*: long-term sequelae. *J. Pediatr.* **75**, 782–788
- American Academy of Pediatrics, Committee on Infectious Diseases. (1975) *Ampicillin resistant strains of Haemophilus influenzae type B*. *Pediatrics*, **55**, 145–146
- Booy, R. and Moxon, E. R. (1991) Immunisation of infants against *Haemophilus influenzae* type b in the UK. *Arch Dis Child* **66**, 1251–1254
- Jenner, B. M., Williamson, G., and Luppino, M. (1990) fatal meningitis caused by chloramphenicol-resistant *Haemophilus influenzae*. *Med. J. Austral.* **152**, 335–336
- Fleischmann, R. D., Adams, M. D., White, O., et al. (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science.* **269**, 496–512
- Davis, B. D., Dulbecco, R., Eisen, H. N., Ginsberg, H. S., and Wood, W. B., Jr. (1973) *The Haemophilus-Bordetella group*. *Microbiology*, Harper & Row Publishers, Inc., New York, pp. 792–800
- Barenkamp, S. J, Munson, R. S., Jr., and Granoff, D. M. (1981) Subtyping isolates of *Haemophilus influenzae* type b by outer-membrane protein profiles. *J. Infect Dis.* **143**, 668–676
- Loeb, M. R. and Smith, D. H. (1980) Outer membrane protein composition in disease isolates of *Haemophilus influenzae*: pathogenic and epidemiological implications. *Infect. Immun.* **30**, 709–717
- Van Alphen, L., Riemens, T., Poolman, J., and Zanen, H. C. (1983) Characteristics of major outer membrane proteins of *Haemophilus influenzae*. *J. Bacteriol.* **155**, 878–885
- Inzana, T. J. (1983) Electrophoretic heterogeneity and interstrain variation of the lipopolysaccharide of *Haemophilus influenzae*. *J. Infect. Dis.* **148**, 492–499