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AN IMMUNOLOGICAL OBSERVATION ON RECOMBINANT HEPATITIS B SURFACE ANTIGEN (RHBSAG) AS A PROTEIN IMMUNOMODULATOR TO CONJUGATE IT WITH VI CAPSULAR POLYSACCHARIDE OF SALMONELLA TYPHI

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ABSTRACT

Typhoid fever is a systematic disease caused by salmonella enterica serotype typhi. This pathogen is exclusive to human being and right now is the most common in developing countries. In recent years multi-resistant salmonella enterica serotype typhi were reported from around the world. The prevalence of these types especially in developing countries increased the mortality rate. Recent prevalence of typhoid fever with multi-resistant antibiotic in Indian subcontinent, Far East and Latin America is the obvious reason. It seems that salmonellas is control can be done solely with general vaccination. The present study aims at covalent binding of Vi polysaccharide of Salmonella typhi to the surface protein of hepatitis B virus in order to add to the immunity caused by Vi polysaccharide. Also, it investigates the level of stimulation of anti Vi IgM, IgG_3, IgG_{2b}, IgG_{2a}, IgG_1, and total IgG, antibodies by conjugated V_i-HbsAG and V_i.

KEYWORDS: HbsAg, Vi Capsular polysaccharide, Salmonella typhi, Typhoid fever

POSITION

These bacteria exist in the nature, and some of them are seen in human or animal gastrointestinal tract. Only a few of them are pathogenic. Some of them are pathogenic for animals but can also cause disease in human beings (2). Different diseases exist in faces of patients and vectors and can cause environmental pollution (1). Salmonella survival in the environment depends on the temperature. Salmonella typhimuriumcan survive two days in 29 ° C and one hour in 60° C.

FORM

Gram-negative bacilli have the features of enterobacteriaceae family. Straight bacilli are 0/7 to 1/5 and 2 to 5 micrometers. None of them produce capsules in 37 ° C but in 20° C and less most of them including salmonella typhi and salmonella paratyphi B produce capsules(1).

BIOCHEMICAL FEATURES

Salmonellas have the general biochemical features of enterobacteriaceae family. Most of strains are mobile and produce hydrogen sulfide from thiosulfate source. These bacteria usually ferment carbohydrates with acid and gas(5). Salmonella typhi, salmonella gallinarum, and rarely variants of other serotypes like salmonella typhimurium and dublin do not create gas. Salmonellas ferment glucose, mannitol, arabinose, sorbitol and dulcitolebut cannot ferment lactose, sucrose, salicin and adonitol (6). All the salmonellas except salmonella typhi and paratyphi A revive citrate as the only carbon source used, and nitrate. In the culture medium sugar, iron, agar, and H_2S are produced. They include negative indole, positive lysinedecarboxylase (except for salmonella paratyphi A), Argininedihydrolase, and positiveornithinedecarboxylase(except for salmonella typhi and gallinarum). Negative urease and gelatin are not melted and ---, lipase, and deoxyribonuclease do not create phenylalanine and tryptophan. MR is positive and VP is negative (7).

Resistance against physical and chemical factors

Organisms ofthe genussalmonella will survive on culture medium for months. They can tolerate above zero densities. It is a good feature considering designing culture medium and isolating these organisms of stool sample. Members of the genus salmonella compared to other intestinal bacteria have remarkable organisms regarding resistance against other physical and chemical factors. In 55° C salmonella is destroyed inan hour, and in 60° C it is destroyed in 15minutes. They are resistant to the cold; and their abilities to grow in -7° C vary based on

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the serotypes. Salmonella grows in pH: 4/5 but its proper pH is 6/5-7/5 (1).

Antigenic structure

The three important antigens of salmonella are O, H, and Vi (1).

O or somatic antigen

These antigens are particular to salmonella organ and are made of phospholipid, protein and polysaccharide. They can tolerate 100° C for a few hours and are resistant to alcohol. Therefore, they are used for producing suspensions made for diagnosing diseases. Today more than 67 types of O antigen are known. They are named by numbers and in each type of salmonella some of them are available. O antigens of salmonella are fixed. Therefore, their classification is based on them. Some of O antigens are the same in several groups (1).

H or flagella antigen

These antigens are particular to vibrating strings of salmonella and exist only in mobile types. They are made of protein and are antigenic. They are destroyed by above 60° C temperature, alcohol, and acid. Formalin does not destroy them but fixes the vibrating strings on the surface of bacteria and convers O antigen so that it hinders the agglutination of bacteria with anti O serum. However, it does not hinder their agglutination with anti H serum. So, H suspension is produced by adding formalin (1).

Vi antigen

In some salmonellas like salmonella typhi, salmonella paratyphi A and others, O antigens are covered by another antigen called Vi that is derived from the term virulence and adds to their virulence (1).

Chemical structure of this antigen is similar to O antigen but its residence to heat, acid and phenol is much less. It does not tolerate the heat of more than 100° C for more than a few minutes. This antigen is similar to K antigen in escherichia coli (1).

Some salmonellas -when recently taken from the body- contain Vi antigen and are not agglutinated by anti O serum. Anti Vi antibody cause their agglutination. For destroying this antigen, the bacterium suspension shoud be heated for a few minutes (1).

Resistance to bacteriophage

Some salmonellas can be divided into small classes based on their sensitivity to bacteriophages. Regarding epidemiology, it is of great importance. Since a bacterium type causesepidemic, the source of infection can be determined. Salmonella typhi, when having Vi antigen, is sensitive to too many bacteriophage. They can be classified into more than 80 differentisotypes. Salmonella paratyphi A, B are divided into 10 isotypes and salmonella paratyphi C is divided into 45 isotypes (4).

Polysaccharide-protein conjugated vaccine immunization factors

Polysaccharide chain length

Researches display if a very short or very long polysaccharide molecule is chosen to be used for conjugation, it will be non-immunogenic and will not be able to simulate immune system. Therefore, the chain length of polysaccharide used for conjugation is crucial (4).

Type of connection

For transforming polysaccharides into TD antigens the type of the connection of hapten to carrier molecule should be cholane. Other strong or non-cholane links are not effective. There are several methods for connecting polysaccharide to protein. Three reclamation methods of amination, amidation, and eutrophication are considered as the best methods. Conjugation causes high stability and maintenance of structures of antigenic molecules through one the above-mentioned methods. For this purpose, parameters like pH, temperature, reaction time and chemical solutions should be precisely determined based on the type of hapten and carrier molecule (4).

Carrier molecules

Many bacterial polysaccharides do not have active chemical groups like amino or carboxyl. Therefore, they cannot be directly connected to protein carrier through covalent. In amidation conjugation method –that is one of the best methods of conjugating polysaccharide compounds to protein- adipic acid dihydrazide (ADH) is used as a spacer molecule with six carbons, and 1-etil-3-(3-dimetil amino propil) carbodiimide(EDAC) is used as the coupling agent. EDAC causes the derived covalent binding between polysaccharide adipichydrazide (PS-AH) and protein carrier in the form of amid bond between

hydrazide polysaccharide and carboxyl protein groups. Other adversereactions may happen between amino acid, lysine amino, and carboxyl groups adjacent to a protein molecule or in other protein molecules as cross-linking in or outside the molecules.

METHODOLOGY

Opening the stain

The tag used for Vi capsular polysaccharide was of Ty2 strain and Ty6s variety. The strain is lyophilized and lyophilized ampoule of brain heart infusionbroth (BHI) was used. First lyophilized ampoule was solved in 0/5 milliliter of BHI. Then it was inoculated in 4/5 milliliter of BHI. In the next stage it was kept in 37° C incubator and then the cultivation happened again with 0/5 milliliter of this culture in 4/5 milliliter of BHI. Again 0/5 milliliter of the second culture was inoculated in 4/5 milliliter of BHI and was incubated in 37° C for an hour. In the last stage, 0/1 milliliter of the culture of the third pipe was cultivated as streaks on a plate in agar BHI, and was kept in 37° C incubator for 24hours till single colonies are created. It is cultivated as a slant in two pipes of BHI. A pipe is used for controlling colony regarding being rough or smoothand the other is used as the seed. The next day in order to check the type of single colonyacriflavine method (adding a drop of acriflavine to the suspension) was used. Also, salmonella recognition test using anti serum of mono specific particular to Vi salmonellas was done (8).

Reaction to anti serum

For recognizing Vi a drop of the suspension of the strainis added to a drop of Vi mono specific anti serum. 4 agglutination is the sign that the strainis Vi. After these stages, the second pipe which is the seed pipe will be kept for 15days to a month in 4° C fermentor till the time of cultivation.

Providing dialysis

For providing 40 liters of fermentor culture medium, 100grams of yeast extract should be solved in 200 milliliters of distilled water. Since yeast extract has a great amount of polysaccharide and cause interference in isolation of purification of Vi polysaccharide, it should definitely be dialyzed. For this purpose, provided extract went through a dialysis of 24 hours, changing its distilled water every 8 hours in 4° C. for adding other Frantz culture medium components (especially Vi salmonella typhi culture in fermentor) for culture medium of 40 liters 24gram$MgSS_4.7H_2O$ (merck) is solved in 500 milliliters of distilled water. Then we add 200 gram glucose (dextrose) to it and mix it with dialyzed extract. Finally it is sterilized by being passed through a 0/22 nanometer filter.

Table 1: Culture medium synthesis in one liter of distilled water

Substance	Company	Quantity
Glutamicacid	Merck	1/3 gram
Cysteine	Merck	0/02 gram
Na_2HPO_4	Merck	25 grams
KCI	Merck	0/1 gram
NH_4CI	Merck	6 grams
$MgSO_4.7H_2O$	Merck	0/6 gram
Dextrose	Diffco	5 grams
Yeast dialysis extract	Diffco	5 grams

Providing seeds

A pipe of 24 hour culture of Ty6s strain salmonella typhiwashedby normal saline was inoculated in 2 liters of Frantz culture medium in a 5liter gallon. It was incubated in 37° C for 24hours. After the complete growth of the strain in the test tube, we put it in the provided seed culture medium (2gallons containing 2liters of Frantz culture medium). We kept the gallons in 37° C shaking incubator for 18 hours in order to be ready to be inoculated to the fermentor.

Cultivation in fermentor

In this test, contact-flowb.vbilthoven unit system fermentor with the tank of 50liters capacity was used. 40 liters of modified Frantz culture medium without dialyzed yeast extract was provided and put in the fermentor. Then the culture medium was sterilized in two stages of 15minutes and 20minutes in 110° C, the pressure of 0/6 bar, and 1000 rmp. Then the temperature is reduced to 37° C; and dialyzed and filtered yeast extract including $MgSO_4.7H_2O$ and glucose that was previously provided was put into the tank of the fermentorin the sterile conditions. The pH of fermentative environment of the fermentor is regulated as 7/6 in37° C. The Ty6s strain seed that was previously provided was checked for itsgrowth and the probable pollution and then was cultivated for 16hours in fermentor's tank. After inoculation of the seed, each two hours a sample of the culture medium will be provided in order to measure pH

level, absorbance in 640 nm, cell wet weight and sugar level. After 16 hours of seed inoculation, since thecell growth was at its maximum and at the end of logarithmic phase cultivation was stopped and cell suspension is put in the 2liter container and is centrifuged for 60minutes with 3500 rpm till the biomass cell is isolated from fermentative broth. Then we immediately added0/1 % CTAB (cetyltrimethyl ammonium bromide) to the obtained supernatant. Then the supernatant was centrifuged for 60minutes in 4° C with 3500 rpm (Hettich centrifuge model CE-05-BV-I). after the centrifuge supernatants are completely discharged and the sediments are kept in 20° C till the start of the next stage (8).

Final Vi-cps extraction

First the sediment is solved in 0/5 % one molar calcium chloride. Then we added 25% cold ethanol 96% and kept it for an hour in 4° C. After an hour, the above solution was centrifuged for 60minutes in 4° C, the sediments were isolated and then added to absolute cold centrifuged liquid so that there was 80% alcohol in the final volume in order to deposit polysaccharide. Then we kept the solution for 24hours in 4° C and then centrifuged it for 60minutes with 3500 rpm. Obtained deposit is crude polysaccharide and is washedtwo times with ethanol 96%, two times with acetone and two times with diethyl ether. Then it was dried in desiccator containing P_2O_5 and kept in 20° C (8).

Cold phenol extraction

Crude polysaccharide obtained from the previous stages was solved in saturated sodium acetate with density of 20 milligram in milliliter with 1:10 dilution and was shook for threehourstill white hemogen solution is produced. Then the same volume of phenol solution (100grams of phenol crystal in 50 molar 40 milliliter of sodium acetate with pH of 7) was added. After being shook for 5 minutes the above solution was centrifuged for 30minutes in 4° C with 4000 rpm, liquid phase was isolated and extraction is repeated as the above (8).

Ultracentrifuge

First the polysaccharide obtained from phenol extraction was dialyzed during 24 hours with three 0/1 molar calcium chloride changing. Dialyzed solution was centrifuged for 3 hours with 38000 rpm using Beckman centrifuge model L8M. The centrifuged liquid will be kept in 20° C till the next purification stage(8).

Alcohol extraction

80 % absolute ethanol was added to the liquid centrifuge and was kept in 4° C for an hour. The above solution was centrifuged for 30 minutes in 4° C with 4000 rpm. The obtained deposit was washed two times with absolute ethanol and two times with diethyl ether. Pure saccharide is dried in desiccator containingP_2O_5. Pure polysaccharide is solved in a specific amount of progenic solvent and was divided and lyophilized after being sterilized using 0/22 micron fiber in sterile vials. Some samples of crude and pure polysaccharide are provided for control tests (measuring the moisture level, checking protein level using Bradford method, nucleic acid, and measuring total level of carbohydrates using phenol-sulfuric acid method (8).

DISCUSSION AND RESULTS

Measuring protein level in Vi polysaccharide

Protein level of Vi extracted polysaccharide was determined using Lowry assay andY= 0/0008 + 0/0055 line equation. There was 5/5 milligram protein in each gram of dried polysaccharide. According to WHO protocol a acceptable amount of protein in polysaccharide is 10 milligram for each gram of polysaccharide (3).

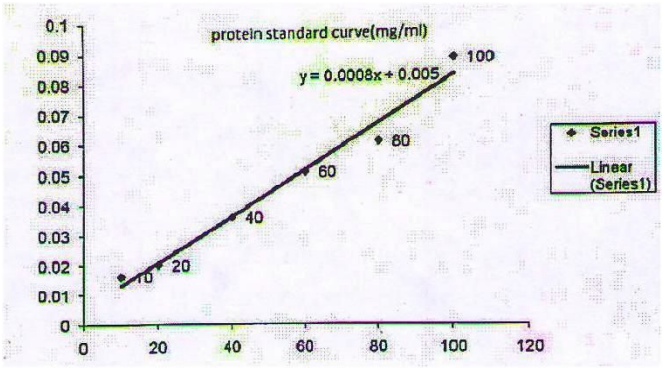

Figure 1: Protein standard curve

Measuring the moisture level in polysaccharide

The moisture level in Vi polysaccharide sample is 2/06% which is less than 2/5 % of the weight of capsular dried polysaccharide. This level of moisture is acceptable according to WHO protocol.

Measuring nucleic acid level in polysaccharide

Nucleic acid level in polysaccharide is measured using UV spectrophotometric method. the level of the

acid was less than 20 microgram in each gram of polysaccharide, and that is acceptable.

Controlling O-acetyl level in Vi polysaccharide

The O-acetyl in polysaccharide is an index for measuring Vi-CPS on quality and quantity grounds and is calculated using Hestrinmethodand Y= 0/656 X + 0/0042 line equation. Its amount was calculated as 4/7 millimolefor each polysaccharide gram (3).

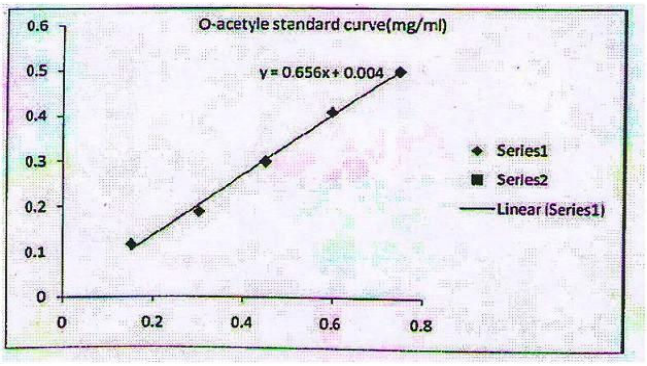

Figure 2: O-acetyl standard curve

Measuring O-acetyl level in Vi-HBs Agconjugates

The O-acetyl level in Vi-HBsAg conjugates is calucalted using Hestrin test and Y= 0/656 X + 0/0042 line equation. It is 31/148 micro mole.

Measuring total carbohydrate level in Vi-HBsAg conjugates

Total carbohydrate level in Vi-HBsAg conjugates is calculated using phenol-sulfuric acid method and Y= 0/0115 X + 0/0249 line equation.

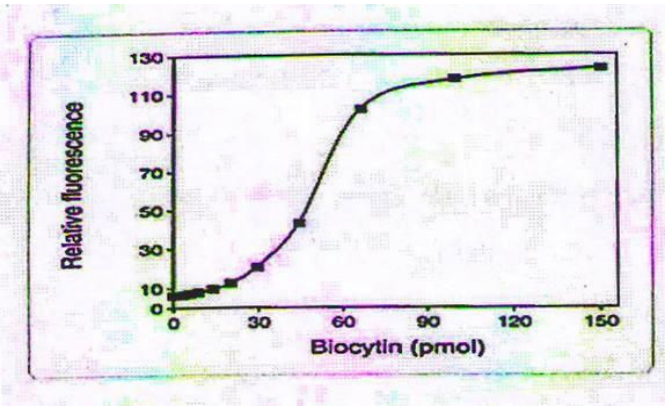

Figure 3: Total carbohydrate standard curve

Vi-CPS conjugation with HBsAg

For purifying Vi-HBsAg conjugates we passed it through sepharose column using gel filteration method. Then the absorbance of fractions was calculated in the wavelength of 280nanometer. As it is observed first peak is related to Vi-HBsAg conjugate and displays conjuction of Vi and Vi-HBsAg. The second peak shows non-conjugate protein.

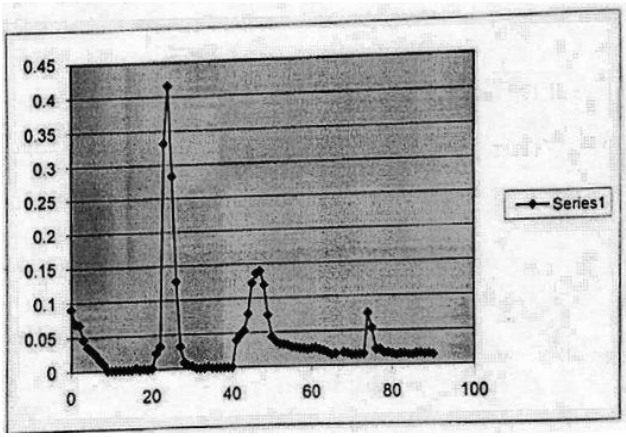

Figure 4: Vi-HBsAg conjugate fractions' OD curve

Vi-HBsAg conjugation efficiency

Comparing conjugate protein and primary protein in conjugated Vi-HbsAg molecule the conjugation efficiency is calculated as 76%.

Rabbit pyrogen test

Provided conjugate went through rabitpyrogen test. After 24 hours three rabbits went through temperature increase that is less than 1/4. Provided conjugate sample do not contain pyrogenicsubstancesand can be injected.

Toxicity testing

Samples were injected to 5 mice and 2 guinea pigs in order to examine whether provided conjugate molecules are toxic or not. They were studied for 7days. Mortality and weight loss was not observed. So, it was concluded that conjugate samples were non-toxic and can be injected.

Sterility testing

In this test, conjugate samples were cultivated in thioglycolate, nutrient agar, and sabouraud dextrose agar, in aerobic and anaerobic conditions. Examining bacterial cultures after 24 and 48 hours, and examining fungal environment after 72 hours of heat rate displayed that microorganisms did not grow on mentioned mediums and samples are sterile.

Gel double immunodiffusion test

This test is done in order to check the connection between Vi polysaccharide antigen and Vi-HBsAg. The other aim was to see whether antigenic structures of the molecules will remain healthy and antigenic after the conjugation. For this purpose, precipitation lines caused by antigen reactions to antibodies are examined. Results showed that provided conjugate molecule can react to anti serum Vi polysaccharide gen and tetanus antitoxin, and producing precipitation lines.

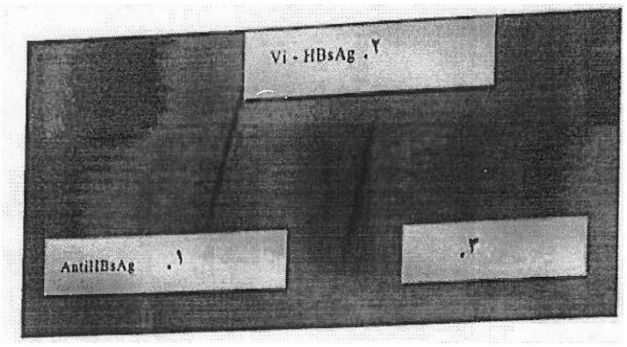

Figure 5: Gel double immunodiffusion test for ALG-TT, Sink 1 amtiHBsAg, sink 2 Vi-HBsAg, sink 3 anti Vi

Results of obtained antibody titers for each group in ELISA

The titer level of IgG3, IgG2b, IgG1, IgM and IgG are shown in the following table in OD unit. According to Turkey test results, considering p<0/01, injected antigen titers (Vi-HBsAg) were significant two weeks after the first and the second injection compared to the control group.

Table 2: Produced IgG titer level against Vi for two weeks after first, second and third injections

Antigen	Titer of sera (OD unit) mean ± SD		
	2weeks After first injection	After 2weeks Second injection	After 2weeks Third injection
Vi	110 * ±6	406 * ± 10	430 * ±11
Vi-HBsAg	391* ±11	1200 * ±12	1905* ±14
Neg.control	0/03 ± 0/1	0/01 ± 0/1	0/01±0/1

It shows Vi-HBsAgsignificance compared to Vi and control group (p<0/01).

Table 3: Produced IgM titer level against Vi for two weeks after first, second and third injections

Antigen	Titer of sera (OD unit) mean ± SD		
	2weeks After first injection	After 2weeks Second injection	After 2weeks Third injection
Vi	122 * ±6	240 * ± 10	260 * ±11
Vi-HBsAg	140* ±11	310 * ±12	760* ±14
Neg.control	0/03 ± 0/1	0/01 ± 0/1	0/01±0/1

It shows Vi-HBsAg significance compared to Vi and control group (p<0/01).

Table 4: Produced IgG_3 titer level against Vi for two weeks after first, second and third injections

Antigen	Titer of sera (OD unit) mean ± SD		
	2weeks After first injection	After 2weeks Second injection	After 2weeks Third injection
Vi	75 * ±6	180 * ± 10	206 * ±11
Vi-HBsAg	84* ±11	418 * ±12	976* ±14
Neg.control	0/03 ± 0/1	0/01 ± 0/1	0/01±0/1

It shows Vi-HBsAg significance compared to Vi and control group (p<0/01).

Table 5: Produced IgG_3 titer level against Vi for two weeks after first, second and third injections

Antigen	Titer of sera (OD unit) mean ± SD		
	2weeks After first injection	After 2weeks Second injection	After 2weeks Third injection
Vi	75 * ±6	180 * ± 10	206 * ±11
Vi-HBsAg	84* ±11	418 * ±12	976* ±14
Neg.control	0/03 ± 0/1	0/01 ± 0/1	0/01±0/1

It shows Vi-HBsAg significance compared to Vi and control group (p<0/01).

Table 6: Produced IgG_1 titer level against Vi for two weeks after first, second and third injections

Antigen	Titer of sera (OD unit) mean ± SD		
	2weeks After first injection	After 2weeks Second injection	After 2weeks Third injection
Vi	73 * ±6	146 * ± 10	150 * ±11
Vi-HBsAg	116* ±11	450 * ±12	1108* ±14
Neg.control	0/03 ± 0/1	0/01 ± 0/1	0/01±0/1

It shows Vi-HBsAg significance compared to Vi and control group (p<0/01).

Table 7: Produced IgG_{2a} titer level against Vi for two weeks after first, second and third injections

Antigen	Titer of sera (OD unit) mean ± SD		
	2weeks After first injection	After 2weeks Second injection	After 2weeks Third injection
Vi	29 * ±6	61 * ± 10	82 * ±11
Vi-HBsAg	46* ±11	99 * ±12	130* ±14
Neg.control	0/03 ± 0/1	0/01 ± 0/1	0/01±0/1

It shows Vi-HBsAg significance compared to Vi and control group (p<0/01).

Table 8: Produced IgG_{2b} titer level against Vi for two weeks after first, second and third injections

Antigen	Titer of sera (OD unit) mean ± SD		
	2weeks After first injection	After 2weeks Second injection	After 2weeks Third injection
Vi	29 * ±6	40 * ± 10	49 * ±11
Vi-HBsAg	39* ±11	80 * ±12	112* ±14
Neg.control	0/03 ± 0/1	0/01 ± 0/1	0/01±0/1

It shows Vi-HBsAg significance compared to Vi and control group (p<0/01).

CONCLUSION

Results display that conjugate molecules cause more immunity than Vi-CPS and can stimulate cell's immunity. This causes reduction of the times of injection and expenses. Also, surface protein of hepatitis B virus can act as an acceptable protein carrier.

REFERENCES

Wolfgang, K. J., Joklik, D. P., Willett, H. P., & Amos, D. B. (2003). Zinsser microbiology. (M. K.Rahimi, Trans). Tehran: Aige.

ZahraiSalehi, T. (1999).Salmonella. Tehran: University of Tehran Publication.

NikkhahBahrami, L. (2010). Investigation on HBsAg as a protein immunomodulator in order to be conjugated to Vi capsular polysaccharide of salmonella typhi

Robinson, A., Hudson, M. J. Crange, M. P. (2000). Vaccine protocols (2nded).

Peter Borriello, S. Murray, P. R., Funke, G. D. (2006). Microbiology and Microbial Infections in Topply& Wilson, London U.K.

Palmer, S. R., Rowe, B. (1986). Trends in salmonella infections. PHLS Microbial Dig.

Le Minor, Le Minor S,Grimont PAD. (1985). Rapport quadriennal du centra national des salmonella surl'origine et al repartition en serotypes des souchesisolees en France continentale au cours des annees 1980 a 1983, Rev EphidemiolSantePublique.

Gheradi M. M., Garcia, V. E. (1993). Protective capacity of a temperature sensitive mutant of S. typhia after oral and intragastricinoculation in murine model.Vaccine.