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Assessment of immunogenicity of alginate microparticle containing *Brucella melitensis* 16M oligo polysaccharide tetanus toxoid conjugate in mouse

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Parivash GHADERINIA[1*], Reza SHAPOURI[2]

[1]*Department of Microbiology, College of Science agriculture and modern technology, Shiraz Branch, Islamic Azad University, Shiraz, IRAN*
[2]*Department of Microbiology, Faculty of Basic and Medical Science, Zanjan Branch, Islamic Azad University, Zanjan, IRAN*
**Corresponding author: Parivash_gh1356@yahoo.com*

Abstract. Brucellosis is an infectious disease caused by *Brucella* bacteria, affecting animals and humans. It is found as an endemic disease in many parts of the world, especially in the Middle East, Central Asian, and South American countries. The objective of this study was to prepare alginate microparticle containing conjugate of *Brucella melitensis* 16 M oligopolysaccharide (OPS) with tetanus toxoid, and to assess its immunogenicity in mouse, in order to find a new method of confronting brucellosis. In this study, we extracted OPS from lipopolysaccharide (LPS) and conjugated it with tetanus toxoid by amidation method. Then, the obtained conjugate was condensed by gel filtration, followed by emulcification method in iso–octane organic phase to prepare alginate microparticles containing the conjugate. In our study, sera titr of antibodies IgM, IgA, IgG_{2a}, IgG_1, IgG_{2b}, IgG_3, and total IgG generated against oligopolysaccharide was performed by ELISA test. The final results, considering the obtained immunogenicity and the increased titer of antibodies IgG1 (19 times), IgG_3 (19 times), IgG (15 times), and IgM (9 times) after injection of produced microparticle in mouse indicates appropriateness of the produced microparticle as a candidate vaccine. Thus, it could be concluded that alginate microparticles containing conjugates of *B.melitensis* 16 M oligopolysaccharide with tetanus toxoid can be proposed as a candidate anti–brucellosis vaccine.

Keyword: *Brucella melitensis*, Microparticle, Tetanus, Alginate, Vaccine.

Introduction

Brucella is a genus of Alpha Proteobacteria that causes infectious disease in mammalians, the disease of which may also be communicable to humans, ruminants and the pigs everywhere in the world are always at risk of catching such disease and therefore, they may be considered as a reservoir of infection and vector of infection to humans. *Brucella* species are different, given the body structure of the animal or human being in which they have been transmitted, and also the physiological ability of that animal or human being and the structural characteristics of cellular surface. Some of the species inside the body of domesticated animals include *B. melitensis* in sheep and goat, *B. abortus* in cow, *B. suis* in pig and *B. ovis* in some sheep. Because of very essential economic role of some of the domesticated ruminants for millions of people, especially in low–income countries, brucellosis is considered as the major factor of economic loss and a huge commercial barrier all over the world.

Furthermore, human brucellosis is a kind of hard and debilitating disease that ultimately leads to disability, and needs to be treated with antibiotic agents. As a result, if it is possible to control and/or eradicate brucellosis, it may be considered as a part of the goals of public health plans in countries confronting such problem [MOZAFARI NOOR *et al.*, 2008].

Brucella is a facultative intracellular parasite which functions specifically inside animal and human cells. It often has a coccobacillar shape and its size is 0.5 to 0.7 × 0.6 to 1.5 µm. The cells are gram–negative, but after gram staining, they often appear to be stained disorderly.

The bacterium is aerobic, non–motile, and not sporogenic. Metabolically, *Brucella* is relatively non–active. Normally, the initial hosts of *B. melitensis* are different goat species, and *B. abortus*

usually causes disease in cows. Although there are several species in family *Brucellaceae*, in fact there is only one species of *B. melitensis* containing several biovars [MALEK NEZHAD *et al.*, 2007, PIRI DOGHAHEH *et al.*, 2008]. Brucellosis leads to abortion in animals such as goat, cow, sheep and pig. Brucellosis in humans presents with fever, severe sweating, weight loss and general malaise.

Other names of brucellosis in animals and humans include abortus fever, contagious abortion, periodic fever; bang disease and malt fever [MOZAFARI NOOR *et al.*, 2008]. Brucellosis is an endemic disease in Iran. Its prevalence among different provinces differs from 0.5 to 10.9 percent [ROBINSON ANDREW *et al.*, 2002].

Identification of various *Brucella* species is accomplished by fermentation reactions, urease production, H_2S, the need for CO_2 for growth, the ability to grow on culture media containing thionine and fushin, and agglutination with specialized antiserums. *Brecella* causes acute and chronic infection of reticuloendothelial system. The key pathogenic characteristics of this microorganism lie in its ability to survive inside non–stimulated macrophages [MOZAFARI NOOR *et al.*, 2008, BUTU, *et al.*, 2014a, PETRACHE, *et al.*, 2014, BUTU, *et al.*, 2014b].

Various species of *Brucella* have been found in most countries worldwide. Currently, there are few reports indicating countries where these bacteria don't exist.

In general, it could be said that this bacterium has an extraordinary variability, and may be studied in a vast spectrum. According to studies, this bacterium and its relevant diseases have been found in almost all of the world, except in only 16 countries [GEORGE FREDRICH, 2002].

Human brucellosis is initiated when people are exposed to animals or animal products [PAKZAD *et al.*, 2007]. In addition, this disease may also be originated form carrying carrion of infected animals or using contaminated milk and dairy products by humans. Transmission of the disease from human to human occurs very rarely [NILDA VENESA and HUMBERTO, 2009].

Vaccination against *Brucella* infections in animals is usually performed by administration of the live attenuated smooth *Brucella* strains: *B. abortus* strain S19 and *B. melitensis* Rev.1. The non–smooth strain *B. abortus* RB51 has recently been introduced in some countries. *B. abortus* S19 and *B. melitensis* Rev.1 can cause brucellosis in humans and are therefore unsuitable for human vaccination.

A variety of live attenuated strains, such as *B. abortus* strain 19BA or *B. abortus* 104M, have been used at some time in the Russia and China, but tend to be reactogenic and of limited efficacy [WHO, 1997]. The objective of this study was to prepare alginate microparticle containing conjugate of *B. melitensis* 16M oligopolysaccharide with tetanus toxoid, and to assess its immunogenicity in mouse, in order to find a new vaccine against brucellosis.

Material and methods

Preparation of Biomass. *B. melitensis* 16 M in was cultivated in Brucella Agar Medium and incubated at 37 °C for 10 days and then, cultivated in Brucella Agar Medium and incubated 37 °C for 7 days [ARENAS–GAMBOA *et al.*, 2011, ZAIDI *et al.*, 2006].

Preparation of precipitate for LPS extraction. Hot phenol was used to extract LPS. In this phase, 220 mL methanol together with 2.5 mL methanol saturated with sodium acetate (222.5 mL solution of methanol and sodium acetate) was first prepared in an Erlenmeyer flask and put in refrigerator at 4° C, to be added later in phenol separation phase.

Then 25 mL distilled water was added to 6.8 grams of sediment resulted from centrifugation, and put it in 66° C water bath for 20 minutes.

After this, 26 mL phenol 90 % was added and the test tube was put in 66° C water bath for 30 minutes. Then, the test tube was put at 4° C on ice for 5–10 minutes and centrifuged it in 2500 rpm for 45 minutes. After completion of centrifuge, 4 phases appeared in the test tube (from bottom: turbid precipitate, phenol phase, white precipitate, and blue phase, accordingly). Then, 222.5 mL of methanol (ethanol) and sodium acetate

solution (that already were prepared and refrigerated) were added to phenol phase and at 4° C, centrifuged at 2500 rpm for 45 minutes, and then, the precipitate was collected. Then, a little distilled water was added to the precipitate, and the volume was increased to 50 mL and laid on a heater–magnet for an hour, so that the heater was off and the magnet was on.

After this time, first 1.5 mL TCA (Trichloro acetic acid) and then, 350 mL pure alcohol or ethyl alcohol 96.6 %, were added and the tube was put in refrigerator for one hour. In this phase, in order to discharge the impurities, supernatant was collected and a dialysis bag was used with cut off 10 Kd in cool water (for 24 hours, and three times replacement, and every time 4 liters of distilled water) for dialysis to take place and therefore, impurities such as phenol and ethanol were completely discharged [ARENAS–GAMBOA *et al.*, 2011, ZAIDI *et al.*, 2006, KASPER *et al.*, 1996].

Final extraction and LPS Condensation. For LPS precipitation, the supernatant was dialyzed and three–fold the volume of the solution, pure cold alcohol or ethyl alcohol 96.6% was added to it. Then, the tube was kept in a refrigerator for 48 hours at 4°C, and then, it was centrifuged at 4° C in 2500 rpm for 45 minutes. Pure alcohol at –20°C or less effectively resulted in LPS precipitation. Then, supernatant was thrown away, and LPS precipitate remained at the tube bottom [ZAIDI *et al.*, 2006, KASPER *et al.*, 1996].

Extraction of OPS from LPS. Extracted LPS was dissolved in Acid Acetic 2 % and the tube was put in autoclave at 120 °C for 10 minutes. Then, dialysis in distilled water, precipitation, and gel filtration chromatography were done [ZAIDI *et al.*, 2006, KASPER *et al.*, 1996].

Conjugation of OPS with TT (Tetanus Toxoid). Conjugation of extracted oligopolysaccharride from LPS to TT was performed by amidation.

In amidation method, bromide cyanogen is used as an inducing factor of amine groups in a polysaccharide molecule, adipic acid dihydrazide (ADH) as a 6–carbon spacer molecule, and 1–ethyl–3 (3–dimethyl amino–propyl) carbodiimide (EDAC) as a linker agent. ADH as a covalence attaches to amine groups of oligopolysaccharide molecule. In this phase, EDAC causes covalence link between ADH–OPS to protein carrier as an amide link between hydrazide polysaccharide with carboxyl groups of TT [ZAIDI *et al.*, 2006, KASPER *et al.*, 1996].

Gel filtration for purification of conjugate molecules. To purify molecules of OPS–TT from non–OPS–TT conjugates, the prepared conjugate was passed through the shaft of sepharose chromatography 4B–CL balanced with 0.2 molar sodium chloride, so that it is separated from polysaccharide and unbound protein. The collected fractions were detected in two wavelengths of 210 nm (for identification of polysaccharides) and 280 nm (for identification of proteins) and then, the tubes containing the highest absorption at both wavelengths were collected as fractions containing conjugated molecules, and mixed. Then, the prepared conjugates were concentrated by ultracentrifuge device and passed through 0.45 filter [ZAIDI *et al.*, 2006, KASPER *et al.*, 1996].

Preparation of Microparticles of OPS–TT Conjugate Containing Alginate. Alginate microparticles were prepared by emulcification in iso–octan organic phase. First, alginate solutions in different concentrations were prepared. Then, 10 mL of the solution containing the conjugate and distilled water was separated and added in drops to 4 g calcium chloride powder 8 % (as a cross–link hemogenizing emulsion) for 3 minutes. Then, 0.2 g sodium alginate and 2 mL iso–octan were added and emulcified for 3 to 6 minutes. Then, 750 lambda of Tween 80 was added and emulcified and at last, calcium chloride 8% was added again [ARENAS–GAMBOA *et al.*, 2011, ZAIDI *et al.*, 2006].

Assesment of serum antibodies against *B. melitensis* 16M OPS. To assess immunogenicity of the prepared conjugate for injection, pure oligopolysaccharride, pure TT, and NS were each injected in 5–6 week and 7–8 week female BALB/c mice intraperitoneally at final volume of 0.5 mL, so that every injected dose contained 2.5

microgram OPS and/or OPS–TT and Micro OPS–TT. The same amount of sterile normal saline was injected to control groups. There were 4 groups for injection: OPS–TT, OPS, TT, and NS, each of which included 15 mice labeled for immunogenicity.

The mice were injected three times in a two–week interval; blood was taken from the mice hearts two weeks after each injection, and the serum was collected. Serum antibody titration of IgM, IgA, IgG_{2a}, IgG_1, IgG_{2b}, IgG_3 and total IgG was achieved by ELISA test. For appropriate serum dilutions, cross table titration "Plate Cheker Board" was used.

In this test, 'Antigen Mediated ELISA' method was used for IgA and IgM antibodies titration, and 'Indirect ELISA' method for IgG Total titration. ELISA test was done in 'triplet' method [ZAIDI *et al.*, 2006, KASPER *et al.*, 1996].

Statistical Tests. Serum titration for each antibody was done in average ± pervasive value. All experiments were done at least in triplicate. Accordingly, to study the differences between serum titers of different groups receiving antigen versus control group, one–way ANOVA test in SPSS software was used.

Statistical test was used to assess LSD and *P* value <0.01 [ZAIDI *et al.*, 2006, KASPER *et al.*, 1996].

Results and discussion

IgA, IgM and IgG. In these kinds of antibody, two weeks after the first injection, antibody titration shows an increase in groups vaccinated with conjugates of antigen TT and microparticle alginate OPS–TT, but the increase is not considerable.

Given the statistical results of one–way ANOVA test with *P*<0/01, a meaningful difference is observed in IgA antibody titration in vaccinated group with microparticle alginate OPS–TT solution, two weeks after the 1st, 2nd, and 3rd injections versus control group (Table 1).

According to this table, IgM antibody titration against OPS and microparticle alginate OPS–TT shows an increase.

In the group vaccinated with microparticle solution, an increase is observed from 3100 to 27000 in the third injection compared with the first one.

According to the results shown in the table using SPSS and Tukey (one–way ANOVA) test with *P*<0/01, a meaningful difference is observed in titration of all antigens injected in vaccinated groups with microparticle solution and the group vaccinated with OPS antigen two weeks after 1st, 2nd, and 3rd injections versus control group (Table 1).

A proportional increase is observed in IgG antibody titration against OPS during three intervals of injection, but in groups vaccinated with microparticle alginate OPS–TT, serum antibody titration shows an increase from 3000 to 45000 from the first injection to the third injection.

According to results shown in table, using SPSS software and LSD (one–way ANOVA) test with *P*<0/01, a meaningful difference is observed in titration of all antigens injected in vaccinated groups with microparticle solution and the group vaccinated with OPS antigen two weeks after 1st, 2nd, and 3rd injections versus control group (Table 1).

Table 1.

IgA, IgM and IgG titers

Antigen	Titer of sera (OD unit) (mean) After 1 injection			After 2 injections			After 3 injections		
	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG
Micro	68	3100	3000	200	26000	31000	301	27000	45000
OPS	39	220	205	100	1000	2000	155	1000	2400
TT	0	0	0	0	0	0	0	0	0
Neg. control	0	0	0	0	0	0	0	0	0

IgG_1, IgG_{2a}, IgG_{2b} and IgG_3. An increase is observed from 1100 in the first injection to 20000 in the third injection in the titrations.

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Given the results using SPSS software with Tukey (one–way ANOVA) test with *P*<0/01, the titration of all injected antigens versus control group shows a meaningful difference (Table 2).

IgG_{2a} antibody titration versus microparticle does not show a considerable increase, but after the 2nd and 3rd injections in groups vaccinated with conjugate solution, titration in 3rd injection shows an increase from 400 to 1300 compared with 1st injection.

Given the results using SPSS software with Tukey (one–way ANOVA) test with *P*<0/01, the titration of this antibody two weeks after the 1st, 2nd and 3rd injections versus control group shows a meaningful difference (Table 2).

Table 2.

IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 titers

Antigen	Titer of sera (OD unit) (mean) After 1 injection				After 2 injections				After 3 injections			
	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3
Micro	1100	400	400	1000	14000	1000	990	12000	20000	1300	1280	19000
OPS	102	46	40	100	950	100	100	960	1000	120	115	980
TT	0	0	0	0	0	0	0	0	0	0	0	0
Neg. control	0	0	0	0	0	0	0	0	0	0	0	0

As shown in the table 2, IgG_{2b} serum antibody titration in the group vaccinated with microparticle solution of conjugated alginate OPS *B. melitensis* 16M with TT, has increased from 400 to 1280 in three doses.

Hence, given the results using SPSS software with Tukey (one–way ANOVA) test with *P*<0/01, the titration of IgG_{2b} in the two groups shows a meaningful difference (Table 2).

IgG_3 serum antibody titration in the vaccinated group with microparticle solution has increased from 1000 to 19000 in three doses. Given the results using SPSS software with Tukey (one–way ANOVA) test with *P*<0/01, the titration of IgG_3 in the two groups shows a meaningful difference (Table 2).

In figure 1 comparison of OPS in sera after 3 injections and in figure 2 comparisons of Micro OPS–TT in sera after 3 injections are showed.

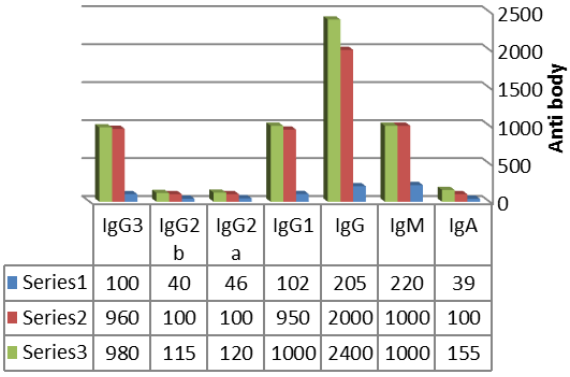

Figure 1. Compare OPS in sera after 3 injections

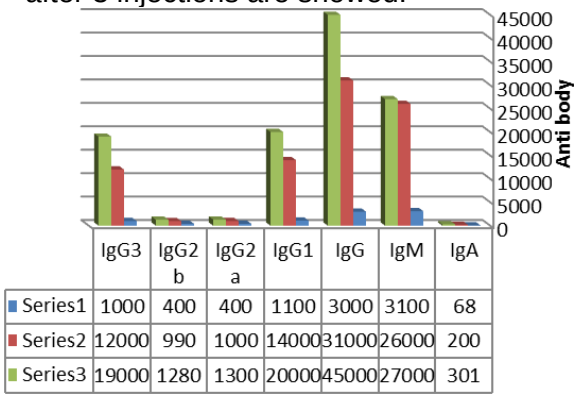

Figure 2. Compare Micro OPS–TT in sera after 3 injections

The final results, considering the obtained immunogenicity and the increased titer of antibodies IgG_1 (19 times), IgG_3 (19 times), IgG (15 times), and IgM (9 times) after injection of produced microparticle in mouse indicates appropriateness of the produced microparticle as a candidate vaccine (Table 3).

The first glycoconjugate vaccine for use in humans, a *Haemophilus influenzae* type b (Hib) conjugate, was licensed in the USA in 1987 and shortly thereafter was introduced into the US infant immunization schedule.

The success of the Hib conjugate vaccines resulted in reducing the incidence of invasive Hib disease in childhood. Infections due to several other

bacterial pathogens are theoretically preventable by this type of vaccine.

Conjugates based on the OPS of the lipopolysaccharide of gram–negative organisms such as *Pseudomonas aeruginosa* and *E. coli* have shown some promise in phase II studies [ZAIDI *et al.*, 2006, CRYZ *et al.*, 1986], and *Staphylococcus aureus* capsular PS protein vaccines are also in trials, although predominantly in veterinary practice [KASPER *et al.*, 1996].

The mucosal administration of conjugate PS antigens either associated with or directly conjugated to protein biological mucosal adjuvants is also an intriguing possibility for the future.

The reasons behind choosing tetanus toxoid and its conjugate with *B. melitensis* 16M–OPS are preparing an efficient immunogenic compound against *B. melitensis* 16M–OPS disease, implementation of protein properties of tetanus TT as a carrier for oligopolysaccharide molecule, and using adjuvant characteristics of *B. melitensis* 16M–OPS to induce higher immunity in comparison to TT.

Therefore, proven the efficiency of this conjugate compound against *B. melitensis* and tetanus, this conjugate can be introduced as a candidate vaccine against both diseases.

In the present study, ADH and EDAC were used as spacer molecule and linker agent, respectively.

Table 3.

The serum titer of IgG after 1st, 2nd and 3rd injection.

Titer of sera (OD unit) (mean)				
Antibody	Antigen	2weeks after first injection	2 week after second injection	2 week after third injection
IgG Total	Microparticle OPS – TT	*§3000	*§31000	*§45000
	OPS	*§205	*§2000	*§2400
	TT	0	0	0
	Neg.Control	0	0	0
IgG_1	Microparticle OPS – TT	*§1100	*§14000	*§2000
	OPS	*§102	*§950	*§1000
	TT	0	0	0
	Neg.Control	0	0	0
IgG_{2a}	Microparticle OPS – TT	*§400	*§1000	*§1300
	OPS	*§46	*§100	*§120
	TT	0	0	0
	Neg.Control	0	0	0
IgG_{2b}	Microparticle OPS – TT	*§400	*§990	*§1280
	OPS	*§40	*§100	*§115
	TT	0	0	0
	Neg.Control	0	0	0
IgG_3	Microparticle OPS – TT	*§1000	*§12000	*§19000
	OPS	*§100	*§960	*§980
	TT	0	0	0
	Neg.Control	0	0	0
IgA	Microparticle OPS – TT	*§68	*§200	*§301
	OPS	*§39	*§100	*§155
	TT	0	0	0
	Neg.Control	0	0	0
IgM	Microparticle OPS – TT	*§3100	*§2600	*§27000
	OPS	*§220	*§1000	*§1000
	TT	0	0	0
	Neg.Control	0	0	0

*indicant of meaningful difference in comparison with control group (*P*<0/01) in each bloodletting.
§ indicant of meaningful difference in comparison with similar group in three of bloodletting.

Noting that ADH is a nucleophilic molecule, it binds covalently to amine groups of alginate molecule. EDAC causes covalent binding of adipic dihydrazide–polysaccharide to protein carrier (TT) between (Micro OPS–TT) and

carboxyl groups of the protein. Conjugation via amidation makes OPS bind to carrier molecule at several sites, a status called latticed structure.

The investigations showed that amidation method produces one of the most permanent types of conjugate molecules [RAD, 2009]. In 1984, Cryz and collab. observed a remarkable boost in the resultant immunity after using polysaccharide and LPS along with other *P. aeruginosa* antigens such as toxin A, toxoids, or detoxified proteases in patients suffering from burns [CRYZ *et al*., 1984].

They found that *P. aeruginosa* LPS produced a high protection against *Pseudomonas* infections and immunization with LPS caused increased bacterial clearance and prevention from bacteremia. Because of toxicity, lipopolysaccharide could not be used as a suitable vaccine. Therefore, to resolve this problem, the toxic compartment of LPS (Lipid A) might be removed and the remaining polysaccharide could be applied as a vaccine.

But, unfortunately the side chain of remaining polysaccharide has low immunogenicity and is unable to induce a proper immune response. As a result, both these molecules are useless for vaccine production [NASSAJI, 2005, RODINO, *et al.,* 2014, BUTU, *et al.,* 2014c].

During 1986, Furer and coworkers bound covalently the polysaccharide derived from immunotype 5 of *P. aeruginosa* to Toxin A via amidation reaction with ADH as spacer molecule. The prepared vaccine included 27.5 % polysaccharide and 72.5 % Toxin A. This conjugate was injected to mice and the anti–polysaccharide antibody titer was assessed through ELIISA method.

In this study, anti–conjugated–polysaccharide IgG titer was higher than non–conjugated polysaccharide and the injection of booster dose caused an increase in IgG titer [MURRAY *et al.,* 2009].

In an investigation in 1986 by Sadov and collab. covalent OPS derived from LPS of serotypes 1, 2, 3, 4, 5, 6, 11, and 12 was conjugated to Toxin A. ADH was used as spacer molecule to facilitate conjugation.

The produced vaccine contained 37 % OPS and 63 % Toxin A, inducing protective immune responses, especially via IgG titer increase in mice, which probably was due to production of latticed structures and the implementation of ADH [SADOV et al., 1986, MURRAY *et al.,* 2009, WALKER, 2008, SAMFIRA, *et al.,* 2015, BUTNARIU, *et al.,* 2015].

In a study in 2007, Shapouri and colleagues conjugated *B. abortus* oligopolysaccharide with TT and estimated its anti–tetanus antibody induction level compared to pure tetanus toxoid. The conjugated vaccine was prepared via amidation and adjuvant properties of *B. abortus* OPS were assed and the resultant anti–tetanus immune response evoked by conjugated vaccine (OPS–) was compared with pure tetanus toxoid. Results obviously showed elevated antibody titers induced by conjugated OPS–TT vaccine in comparison with pure TT, indicating adjuvant characteristics of *B. abortus* OPS for tetanus toxoid [GEORGE FREDRICH, 2002 BUTNARIU, 2014, PENTEA, *et al.,* 2015].

In another work by Shapouri and collab. detoxified lipopolysaccharide conjugate and O–polysaccharide of *B. abortus* were used with TT as brucellosis vaccine and *B. abortus* S99 was the antigen source. LPS was extracted through hot phenol method and detoxified and OPS was extracted from LPS by acetic acid. The conjugate was prepared via amidation and pyrogenesis tests in rabbit confirmed non–pyrogenic effect with injection of 10 micrograms [SHAPOURI *et al*., 2007, ALVANDI, 2013].

Six–carbon ADH spacer molecule was also used in the present study for better conjugation of OPS alginate–microparticles, resulting in increased permanency and immunogenicity of the conjugated molecule by preserving the immunogenic sites of antigen molecules. Instead of Toxin A, which is not permissible as a vaccine, TT was used as carrier protein.

Like the investigations mentioned above, in the present survey, antibody titers in conjugated status were higher than pure alginate and injection of booster dose led in increased antibody titers.

As well as using ADH as spacer molecule, EDAC was employed as linker agent and TT as carrier protein (instead of Toxin A).

In comparison with pure oligopolysaccharide, a significant increase in serum IgG titer produced against oligopolysaccharide alginate–microparticle in conjugated state was observed with injection dose of 2.5 micrograms with three injections in BALB/c mice at 14–day intervals.

TT protein carrier was used in this investigation for conjugation and in three injections, total IgG (and its subtypes) titer against Micro–OPS TT in conjugated state showed a notable increase in comparison with pure OPS.

In 1988, a conjugate of O–polysaccharide and Exotoxin A of *P. aeruginosa* was prepared. ADH was used as spacer molecule and EDAC as linker agent for conjugation of O–polysaccharide with Exotoxin A.

This conjugate increased anti–LPS and anti–Exotoxin A antibody titer as much as the former conjugate, but its immune permanency was much higher than the former conjugate.

But minor side effects including loss of appetite, headache, and swollen injection site were also detectable in some volunteers [ALIZADEH, 2011, BUTNARIU, 2012, CERNEA, *et al.,* 2015].

Different experiments by other researchers during the recent years have proven the effectiveness of these vaccines.

Therefore, the produced conjugated vaccine can be introduced as a proper candidate vaccine against brucellosis and tetanus. In the present article, ADH was employed as spacer molecule and EDAC played the role of linker agent.

As a nucleophilic molecule, ADH binds covalently to amine groups of alginate molecule.

EDAC causes covalent binding of adipic dihydrazide–polysaccharide to protein carrier (tetanus toxoid) between (Micro OPS–TT) and carboxyl groups of protein. Amidation–mediated conjugation makes ops bind to carrier molecule at several sites and this produces a latticed structure [RAD, 2009, BUTNARIU and GIUCH 2011, BUTU, *et al.,* 2015].

In 2014, Ommi and Alvandi conducted a research about immunogenicity of *B. abortus* alginate and detoxified LPS conjugated with tetanus toxoid in BALB/c mice. In that study, alginate was extracted before conjugation with detoxified TT and LPS was extracted by hot phenol method and detoxified.

Then, alginate and LPS were conjugated with tetanus toxoid via amidation method. Antibody titers against these two polysaccharide antigens were assessed and interpreted.

Total serum IgG (IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3) produced against conjugated alginate and LPS showed a mean 4.4–fold increase in all three injections [RAD, 2009, PISHVA et al., 2007].

In the present work with TT as carrier protein for conjugation with alginate–containing B. *melitensis* 16M oligopolysaccharide, the efficiency of this vaccine in properly increasing serum antibody titers up to almost 20 folds and their durable immunity in rat model was documented.

The reasons for using TT were its permissibility for vaccine production and human administration, its easy preparation and production in Iran, and the documented effectiveness of this protein carrier in all experiments of vaccine preparation during recent years.

Conclusions

According to the results obtained in this study, it can be concluded that conjugation of TT as a protein carrier, with microparticle alginate oligopolysaccharide *B. melitensis* 16M by amidation method, led to production of a vaccine with a high potential for stable and long–term protection in comparison with pure OPS, and caused activation of T–lymphocytes (T–helper) and creates immune memory through stimulation of T cells.

In addition, the prepared microparticle induces stable epitops, which by trapping the conjugate, present OPS–TT antigen in a better way to the body immune system.

Furthermore, titration of extracted antibodies against Micro OPS–TT leads in about a 20–fold increase, making it a suitable candidate against brucellosis and tetanus.

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