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Evaluation of immunogenicity of novel multi-epitope subunit vaccines in combination with poly I:C against *Brucella melitensis* and *Brucella abortus* infection

Zohre Sadeghi[a], Mahdi Fasihi-Ramandi[b], Saeid Bouzari[a,*]

[a] *Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran*
[b] *Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran*

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ABSTRACT

Brucellosis is a worldwide zoonotic disease affecting domestic animals and humans. Due to several safety problems associated with live attenuated vaccines (Rev1 and RB51), it is necessary to produce an efficient and safer vaccine against *Brucella.* In this study, we evaluated efficacy of two novel multi-peptide vaccine candidates of FliC, 7α-HSDH, BhuA antigens with and without poly I:C adjuvant. Hence, humoral and cellular immune responses and protective efficacy were determined in immunized mice. Our investigation indicated that multi-epitope antigens showed a significant induction of Th1 immunity with high levels of specific IgG (especially the IgG2a), as well as IFN-γ and IL-2 compared to the control group. The addition of poly I:C to multi-epitope antigens improved the humoral and cellular immune responses. The multi-epitope antigens with and without poly I:C also provided cross protection against *B. melitensis*16M and *B. abortus*544 infections. The present study suggests that the novel multi-epitope vaccine candidates based on B cell, CD4$^+$ and CD8$^+$T-cell epitopes of FliC, 7α-HSDH, BhuA proteins would be potential vaccine candidate against *B. melitensis* and *B. abortus* infections. Furthermore, poly I:C could be considered as a strong Th1-inducing adjuvant in designing vaccine formulation against brucellosis.

1. Introduction

Brucellosis is a worldwide bacterial zoonotic disease caused by *Brucella* spp. Gram-negative facultative intracellular bacteria of the *Brucella* genus classified under alpha-2 Proteobacteria. The genus of *Brucella* consists of more than ten species including: *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis*, *B. neotomae*, *B. microti*, *B. pinnipedialis*, *B. inopinata*, *B. Ceti*, and *B. melitensis*, *B. abortus* and *B. suis* are the most virulent species. Brucellosis is remains as an endemic disease, especially in developing countries that causes abortion and infertility in affected animals, and also undulant fever, endocarditis, arthritis, and several neurological diseases in humans. Humans are mainly infected through direct contact with infected animals or the consumption of unpasteurized dairy products. Therefore, the control of brucellosis in infected animals is essential for its control in humans [1–4]. Vaccine can stimulate the immune responses, so it can be considered as a suggested option in control and prevention of brucellosis. An effective vaccine against brucellosis is required to provide sufficient immunological responses with shift toward Th1 type. It is well known that three mechanisms of the adaptive immune responses are [1] Production of IFN-γ by CD4$^+$ Th1 and CD8$^+$ cytotoxic T lymphocytes (CTL), activates the bactericidal action of the macrophages to limit intracellular *Brucella* survival and replication; [2] Cytotoxic effect of CD8$^+$ CTL to kill infected macrophages; [3] Th1 associated antibodies such as IgG2a isotype to increase opsonization and phagocytosis of *Brucella* [5–7]. At the present, live attenuated vaccine strains such as *B. melitensis* Rev1 and *B. abortus* RB51 are being used for the prophylaxis of brucellosis. However, live attenuated vaccines play an important role in controlling animal brucellosis, they have some disadvantages including abortion in vaccinated pregnant animals, interference with diagnosis of vaccinated animals from infected animals, resistant to the antibiotic and pathogenicity in humans [8,9]. In order to overcome these drawbacks, subunit vaccines should be developed [4,10–16]. New approach based on multiple epitopes of several vaccine antigens used for vaccine development in recent years. Epitope-based vaccines have many advantages, including high specificity, stability in various conditions, safety profile and cost-effective production. In several studies, it has been shown that multi-epitope immunogen containing B- and T-cell epitopes are capable

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* Corresponding author.
E-mail address: bouzari@pasteur.ac.ir (S. Bouzari).

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of inducing protective immunity against various diseases [17–21]. As epitope-based vaccines are more specific and easier to produce, we have focused our study on the identification of multi-epitope vaccine candidates from *Brucella* antigens (flagellin FliC, 7-alpha- hydroxyl steroid dehydrogenase (7α-HSDH) and Heme transporter BhuA) that we introduced as vaccine candidates in previous study (under consideration).

To overcome the low immunogenicity of epitope-based vaccines as a limitation and the induction of desirable immune responses, an adjuvant is required. Polyinosinic: polycytidylic acid (poly I:C), is a synthetic mimic of viral double-stranded RNA (dsRNA) that can ligand to the Toll-like receptor 3 (TLR3) and induce production of Th1 cytokines. This adjuvant stimulates both innate and adaptive immune responses and was used as a Th1-inducing adjuvant for vaccine development [22–24]. In this study, we designed two novel multi-epitope vaccines from FliC, 7α-HSDH and BhuA. The immunogenicity and protective efficacy of designed multi-epitope in combination with poly I:C, adjuvant, were evaluated in mice model.

2. Materials and methods

2.1. Bacterial strains

B. abortus strain 544 and *B. melitensis* strain 16 M (virulent strains) were obtained from the microbial collection, Pasteur Institute of Iran, Karaj, Iran. *B. abortus* RB51, *B. melitensis* Rev.1 (vaccine strains) were obtained from Razi Vaccine and Serum Research Institute, Karaj, Iran. *E. coli* strain BL21 (DE3) (Stratagene, La Jolla, CA) was used for the expression of the recombinant proteins.

2.2. In silico design and synthesis of the multi-epitope genes

The full-length protein sequences of flagellin FliC, 7-alpha- hydroxyl steroid dehydrogenase (7α-HSDH) and Heme transporter BhuA of *B. melitensis* and *B. abortus* were obtained from protein databases such as UniProt (http://www.uniprot.org) in FASTA format, and the multiple sequence alignment was performed using Clustal W2 software (http://www.ebi.ac.uk/Tools/clustalw2) to recognize conserved regions in these bacterial species sequences. In order to design multi-epitope immunogen, the FliC, 7α-HSDH and BhuA sequences were submitted to online servers to predict T and B cell epitopes. Several servers such as IEDB (http://tools.iedb.org/main/tcell/), MHCpred (http://www.ddgpharmfac.net/mhcpred/MHCPred/), SYFPEITHI (http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm) and ProPred (http://crdd.osdd.net/raghava/propred1/) were employed to predict potential MHC class I and II epitopes. Prediction of B cell epitopes were carried out using IEDB (http://tools.iedb.org/main/bcell/), ABCpred (http://crdd.osdd.net/raghava/abcpred/) and Bcepred (http://crdd.osdd.net/raghava/bcepred/). The selected amino acid sequences were fused together using appropriate linkers to design two peptide construct. For suitable arrangement of epitopes, different orders of fragments with various linkers were considered. Physico-chemical parameters of designed constructs including molecular weight, theoretical isoelectric point (pI), extinction coefficient, half-life, instability index, grand average of hydropathy (GRAVY) and total number of positive and negative residues were computed using the Expasy ProtParam tool (http://expasy.org/tools/protparam.html). Prediction of secondary and tertiary structures of the multi-epitope proteins were performed using GOR IV (http://expasy.org/tools/gor4.html) and I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER) online web servers respectively. The 3D structure of the proteins were validated using RAMPAGE server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) and demonstrated via Ramachandran plot. Antigenicity of the constructs were determined using VaxiJen server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). Allergenic potential of the constructs were evaluated by Algpred server (www.imtech.res.in/raghava/algpred/). The final constructs sequence were reverse translated into nucleotide sequence and codons were subsequently optimized based on *E. coli* BL21 codon usage by GenScript server (http://www.genscript.com/). At the end, secondary structure of mRNA, was analyzed by Mfold web server (http://unafold.rna.albany.edu/?q = mfold). Chimeric constructs were chemically synthesized by *Biomatik* CO. (Cambridge Ontario, Canada).

2.3. Expression and purification of the recombinant proteins

The synthesized genes were subcloned into the prokaryotic expression vector pET28a and transformed into chemically competent *E. coli* BL21 (DE3) and selected colonies were confirmed by restriction enzyme digestion and sequencing. The transformed bacteria was grown in LB broth containing Kanamycin (50 μg/ml) until OD at 600 nm was reached ~0.5 and expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Thermo Scientific, USA). After centrifugation, the collected cells were treated with lysis buffer (100 mM NaH2PO4, 10 mM Tris–HCL, 8 M urea, pH = 8.0) and followed by sonication on ice in the presence of PMSF (1 mM) as a protease inhibitor. The bacterial lysates was centrifuged and the supernatants was collected and passed through a Ni-NTA affinity column (Qiagen, Hilden, Germany) for protein purification. The purified proteins were dialyzed against phosphate buffered saline (pH 7.2) at 4 °C overnight and concentration of proteins were estimated by Bradford assay. Finally, the purified proteins were analyzed and confirmed by15% SDS-PAGE (Coomassie brilliant blue staining) and western blotting. For western blot, after SDS-PAGE, the separated proteins were transferred on to nitrocellulose membrane using a liquid transfer system (Bio-Rad, USA). After blocking of the membrane with 5% skim milk in PBST (PBS 1% + Tween20), it was washed with PBST. The blocked membrane was incubated with anti-His tag antibody conjugated with horseradish peroxidase (HRP) (Sigma-Aldrich, USA) then membrane was washed in PBST and stained with DAB (Sigma-Aldrich, USA).

2.4. Immunization of mice

Six to eight weeks old female *BALB/c* mice were purchased from Pasteur Institute of Iran. Mice were randomly divided into nine groups (10 mice/group); mice in groups 1–3 were immunized by *poly B*, *poly T* and *poly B + T* without any adjuvant respectively. Mice in groups 4–6 were immunized by *poly B*, *poly T* and *poly B + T* containing poly I:C respectively. Mice in groups 7 and 8 were immunized by Rev1 and RB51 respectively. At the end group ninth received only PBS as negative control. Mice groups were immunized on days of 0, 14 and 28 and sera were collected from all mice at 14, 28 and 42 days after first immunization for further analysis.

2.5. Humoral immune responses

Humoral responses to designed proteins were determined by enzyme-linked immune sorbent assay (ELISA). Briefly, purified proteins (10 μg/ml) were coated in 96-well micro plates (SPL Life Sciences, Korea) and incubated overnight at 4 °C. The plates were washed with wash buffer (PBS containing 0.05% Tween 20) and were blocked using blocking buffer (PBS containing 3%BSA) for 2 h at room temperature. The plates incubated with serial dilutions of serum samples and then washed and incubated with HRP-conjugated goat-anti-mouse antibody (Sigma, USA). After washing, TMB (tetramethylbenzidine) was added and reaction was stopped by stop solution (2 N H2SO4). The absorbance of each well was measured at 450 nm using an ELISA reader. For determination of subclass of serum antibody, specific secondary goat anti-mouse antibodies (Sigma, St. Luis, USA) and then rabbit anti-goat HRP conjugate antibody was added to the wells.

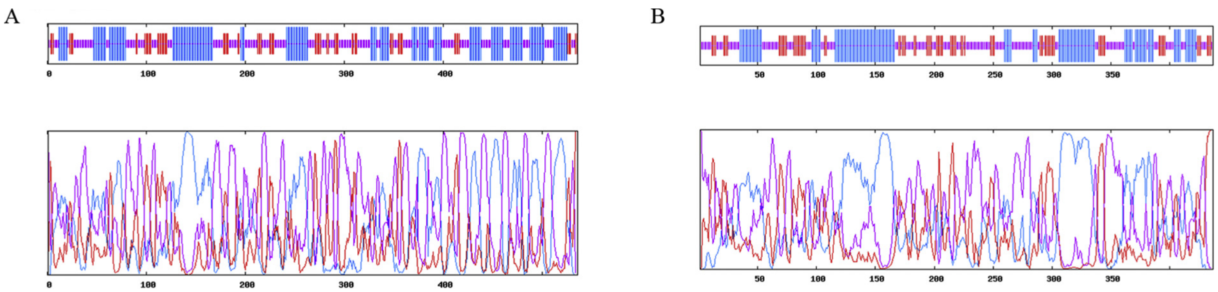

Fig. 1. Graphical results for secondary structure prediction of *poly B* (A) and *poly T* (B) by GOR IV method. Purple, red, and blue colors indicate extended strand, coil, and helix, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.6. Cytokine assay

Two weeks after the last immunization, three mice from each group were sacrificed and their spleens were harvested and homogenized under aseptic conditions. The splenocytes were cultured in RPMI-1640 medium supplemented with penicillin/streptomycin solution and 10% fetal bovine serum (FBS) in the presence of 10 µg/ml of proteins. Positive and negative controls were received phytohemagglutinin-A (PHA) (5 µg/ml, Sigma-Aldrich) and PBS respectively. Cell culture supernatants were collected after 72 h stimulation for measurement of IFN-γ, IL-2 and IL-10 cytokines by sandwich ELISA according to the manufacturer's instructions (Peprotech, Inc. U.K).All assays were performed in triplicate.

2.7. Lymphocyte proliferation assay

As described in the cytokines assay, prepared splenocytes were cultured in RPMI-1640 (Sigma) supplemented with 10% FBS and treated with recombinant proteins (10 µg/ml) at 37 °C and 5% CO2. PHA, (5 µg/ml) and RPMI medium 1640 alone was considered as positive and negative controls, respectively. After 72 h, 20 µl MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 5 mg/ml) was added to each well and the plate incubated for 4 h. After removal of supernatant from each well, the formazan crystals were solubilized using dimethyl sulfoxide (DMSO) (Sigma). The absorbance was measured at 570 nm with an ELISA reader. The stimulation index (SI) was calculated by dividing the mean absorbance of stimulated cells to absorbance of unstimulated cells.

2.8. Protection assay

Six mice from each group were challenged intraperitoneally with 2×10^7 CFU of *B. melitensis*16 M and *B. abortus* 544 after 1 month of the last immunization. Thirty days later, mice were sacrificed and their spleens removed aseptically. Spleens were homogenized in PBS 1×, and each 10-fold serial dilution was cultured on Brucella agar and incubated for 3–4 days at 37 °C. The number of CFU for each spleen sample was counted, and the results were represented as the mean $\log_{10}$ CFU ± SD per group. Units of protection were determined by calculating the difference between the $\log_{10}$ of CFU obtained from experimental group to the $\log_{10}$ of CFU obtained from PBS control group.

2.9. Statistical analysis

Data was analyzed by one-way analysis of variance (ANOVA) and Tukey HSD post hoc test. Data were reported as mean ± standard error of the mean (SEM) and Results with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Design and analysis of the multi-epitope genes

The sequence of FliC, 7α-HSDH and BhuA proteins was retrieved from the Uniprot. Sequence comparison by ClustalW2determined that these proteins were conserved among *B. melitensis* and *B. abortus* strains. After analyzing the results of prediction servers, fragments including more B cell and T CD4$^+$ epitopes (for abbreviation: *poly B*) were selected: FliC (15–46, 130–182, 198–214, 216–255, 246–262a.a), BhuA (60–104, 130–144, 245–279, 570–611,75–89a.a), and 7α-HSDH (118–134, 140–162, 171–185, 194–234, 242–271a.a). For another multi-epitope candidate we selected fragments with more T CD8$^+$ and T CD4$^+$ cell epitopes (for abbreviation: *poly T*): FliC (108-228a.a), BhuA (389-532a.a), and 7α-HSDH (118-234a.a).α-helix forming linker consisting EAAAK repeats and GGSSGG were introduced between fragments for efficient separation. The antigenicity of *poly B* and *poly T* was 0.96 and 0.64, respectively, which was higher than the other orders of the fragments in the chimeric constructs. Alegpred server showed that fusion proteins sequences were not contain experimentally proven IgE epitops. The results of secondary structure prediction indicated that *poly B* and *poly T* consisted of 39.18% and 35.7% alpha helix, 14.74% and 19.91%extended strand, 46.08% and 44.39% random coil respectively without any beta turn in their secondary structures (Fig. 1). The tertiary structure prediction of chimeric proteins was performed by I-TASSER server (Fig. 2). Confidence score (C-score), template modeling (TM-score) and root-mean-square deviation (RMSD) for 3D models were −2.44, 0.43 ± 0.14, 13.4 ± 4.0 Å in *poly B* and − 1.58, 0.52 ± 0.15, 10.7 ± 4.6 Å in *poly T*, respectively. Then, the stereochemical quality of these I-TASSER models were analyzed by Ramachandran plot and the results revealed that 61.5%, 22.1%, 16.3% of residues in *poly B* and 72.4%, 20.5%, 7.1% of residues in *poly T* were located in the favorite, allowed and outlier regions, respectively (Fig. 3). ProtParam results showed that the average molecular weight and pI of *poly B* and *poly T* proteins were 54.3 and 46.3 kDa and 6.29 and 7.22, respectively. The protein's instability index is 39.9 and 25.31 in *poly B* and *poly T*, which classifies these proteins as stable proteins. The aliphatic and GRAVY indexes of *poly B* and *poly T* are calculated 71.05, −0.36 and 69.84, −0.28, respectively. After reverse translation and introduction of necessary elements (restriction enzyme sites and His tag), prediction of mRNA structure was carried out by M fold server. The results of M fold showed that ΔG of the best predicted structures were −592.1 and −497.3 kcal/mol in *poly B* and *poly T* and the 5′ ends of both constructs did not have any stable hairpin or pseudo knot. The *poly B* and *poly T* sequences were submitted to GenBank (accession no. MN125612 and MN125613).

3.2. Subcloning, expression and purification of the designed proteins

The synthetic genes were subcloned successfully in to pET28a as an expression vector. The positive colonies were selected on LB containing Kan and existence of fragment in recombinant vectors, were confirmed

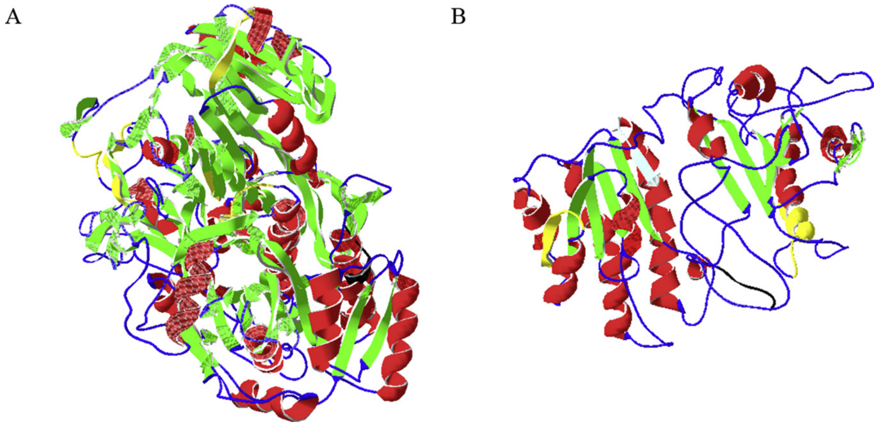

Fig. 2. Prediction of the 3D structure of *poly B* (A) and *poly T* (B) proteins using I-TASSER server.

by double digestion using *Nco I*, *BamHI* and *XhoI* restriction enzymes and sequencing (Fig. 4). Recombinant proteins were successfully expressed in transformed *E. coli* BL21 (DE3). Optimum expression was obtained with 0.5 mM IPTG and incubation time of 5 h at 37 °C. Recombinant proteins were mainly expressed as inclusion body and purified under denaturing condition by Ni-NTA affinity chromatography. The purified proteins were analyzed by SDS-PAGE and western blot (Fig. 5), and the result showed a specific band with a molecular weight of approximately 54 and 44 kDa in *poly B* and *poly T*.

3.3. Humoral immune responses

Specific antibody levels against multi-epitopes were measured by indirect ELISA in different dilutions of serum obtained from immunized mice. A significant increase in specific antibody titer (dilution 1/1000–1/8000) was observed after first immunization in comparison with negative control group (the group receiving PBS) except poly T and poly T + poly I:Cgroups ($P > 0.05$). First immunization elicited weaker antibody titer and the booster doses were effectively increased antibodies. The highest levels of specific antibodies were observed after the second booster (on day 42 of the first immunization), which showed a significant increase compared to first injection (day 14) (Fig. 6); Therefore, based on our results, an effective response was related to third injection and serum from the second booster was studied for IgG subclasses (IgG2a and IgG1). By comparing IgG2a and IgG1levels in immunized mice, our results showed that the immunized mice induced higher IgG2a compared to IgG1 (Fig. 7). Moreover mice groups that immunized with poly B + T had the higher levels of IgG2a in compare to the mice group immunized with *poly B* and *T* alone. Among all immunized groups, a significantly higher IgG2a/IgG1 ratio was detected in groups received poly I:C adjuvant. Since in all vaccinated mice, a high IgG2a to IgG1 ratio was detected, we can say that there is a shift of immune responses toward Th1.

3.4. Cytokine responses

Cell-mediated immune responses of different mice groups were evaluated by cytokine production and lymphocytes proliferation assay. Analysis of IFN-γ, IL-2 and IL-10in the mice groups immunized with poly B, polyT, poly B + T with and without poly I:C, Rev1 and RB51 revealed that the level of IFN-γ in these experimental groups was significantly higher ($P < 0.05$) than control group receiving PBS (Fig. 8A). In addition, there was no significant difference ($P > 0.05$) between groups immunized with *poly B*, polyT, poly B + T. As shown in Fig. 8A, additionof poly I:C to poly B, polyT, poly B + T groups produced statistically higher level of IFN-γ than groups received *poly B*, polyT, poly B + T alone. Although, in comparison to other groups, a significant higher level of IFN-γ was produced in mice received *B. abortus* RB51 and *B. melitensis* Rev1, mice groups received poly B + polyT I:C did not show statistically significant differences with *B. abortus* RB51 and *B. melitensis* Rev1 groups. The level of IL-2 secreted in the spleen cells of immunized mice, revealed similar results to IFN-γ measurements, compared to the negative and positive control groups but mice immunized with *B. melitensis* Rev1 showed higher level of IL-2 production compared to mice immunized with polyB + polyT I:C (Fig. 8B). Analysis of the IL-10 cytokine revealed low level production of IL-10 in the mice groups and did not demonstrate any statistically significant differences between immunized and PBS received mice groups ($P > 0.05$) (Fig. 8C).

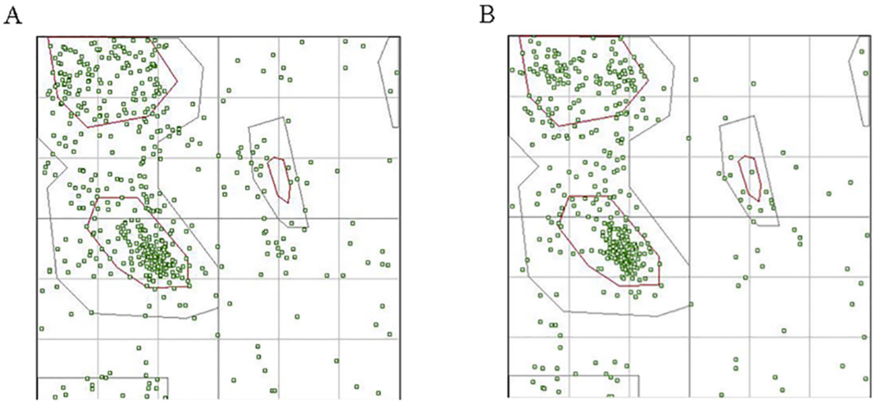

Fig. 3. Validation of *poly B* (A) and *poly T* (B) protein structure by Ramachandran plot.

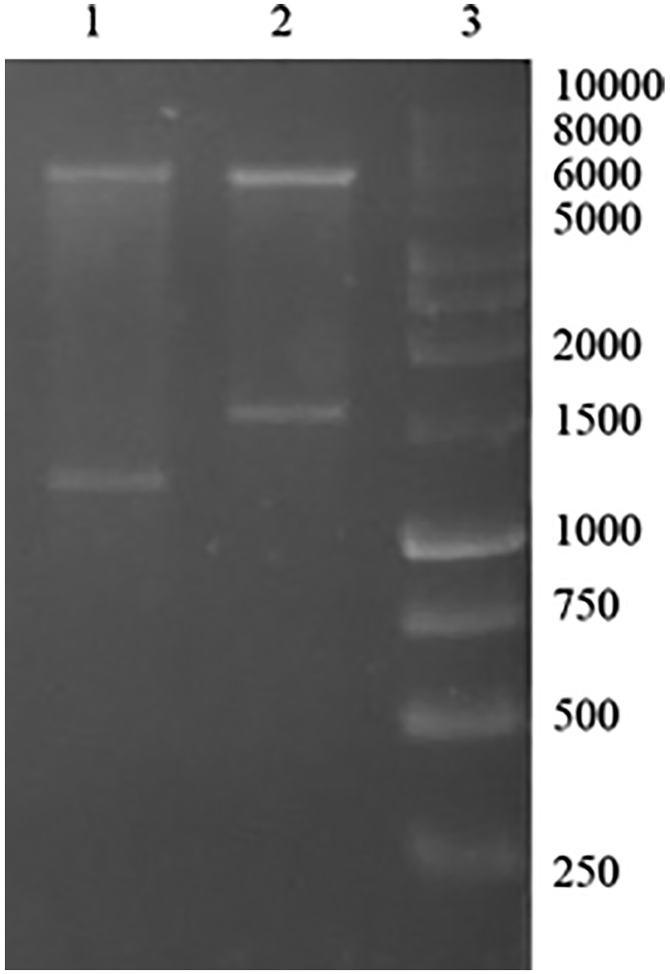

Fig. 4. Double digestion of the recombinant vectors using *NcoI*, *BamHI* and *XhoI* restriction enzymes. Lane 1, Double digestion of the pET28a-*poly T*; lane 2, Double digestion of the pET28a-*poly B*; lane 3, 1 kb DNA size marker.

3.5. Proliferation assay

Lymphocytes proliferation assay as an indicator of cell immune responses were evaluated through MTT assay. As shown in Fig. 9, stimulation index (SI) of all experimental groups were higher than negative control group. Mice groups that immunized with poly B, polyT, poly B + T plus poly I:C adjuvant after stimulation with purified proteins indicated significantly higher stimulation index than mice immunized *with* poly B, polyT, poly B + T without adjuvant. The SI of all experimental groups that immunized with poly B, polyT, poly B + T without adjuvant in different stimulation condition was significantly lower than positive control groups (Rev1 and RB51), although in adjuvanted groups similar proliferative response observed compared to Rev1 and RB51.

3.6. Protection assay

Efficacy of multi-epitopes to protect against *B. melitensis*16M and *B. abortus* 544 were evaluated after an experimental challenge. Different immunized groups exhibited a significant degree of protection compared to negative control mice that received PBS ($P < 0.05$). *Poly B*, *polyT*, *poly B + T* gave 0.49, 0.61 and 0.66 log units of protection against *B. melitensis* and 0.58, 0.74 and 0.74 log$_{10}$ units of protection against *B. abortus*, respectively ($P < 0.05$). Immunization with these multi-epitopes plus poly I:C induced a significant higher degree of protection than multi-epitopes only groups. *Poly B* + poly I:C, *Poly T* + poly I:C and *Poly B + T* + poly I:C conferred 1.19, 1.37 and 1.57 log$_{10}$ units of protection against *B. melitensis* and 0.9, 1.25 and 1.4 log$_{10}$ units of protection against *B. abortus*, respectively ($P < 0.05$). Protection induced by *Poly B*, *poly T*, *poly B + T* immunized groups was lower than that induced by live attenuated *B. melitensis* Rev1 (1.78 log$_{10}$ unit of protection) and *B. abortus* RB51 (1.6 log$_{10}$ unit of protection) control vaccines (Table 2). Mice immunized with multi-epitopes plus poly I:C had not statistically significant differences with control vaccines groups ($P > 0.05$).

4. Discussion

Epitope-based vaccines represent a new type of vaccines which specifically stimulates immunity against the selected epitopes. Multi-epitope vaccines consisting of T-cell and B-cell epitopes that are designed by immunoinformatics tools are capable of inducing immune responses against different bacterial pathogens such as *H. pylori and S. aureus* [20,25]. Escalona et al. designed a multi-epitope DNA vaccine from Cu—Zn superoxide dismutase and Open Reading Frame of *B. abortus* that elicits humoral and cellular protective immunity against *B. abortus* [26]. In another study Yin et al., developed a multi-epitope subunit vaccine from OMP16, OMP2b, OMP31, and BP26 that conferred high levels of protection in mice against *B. melitensis* [21]. Previously, we have shown that three *Brucella* antigens i.e. BhuA, 7α-HSDH or FliC and *co*-administration of these proteins with poly (I:C) can greatly increase the immune responses against *B. melitensis* and *B. abortus* in BALB/c mice (manuscript under consideration). In the present work, we designed two novel multi-epitope vaccine that might be more likely to develop as an effective vaccine against *B. melitensis* and *B. abortus.* Since T CD4$^+$cells are crucial in protection against *Brucella* infection, it is essential that both multi-epitope vaccines candidates comprise CD4$^+$ T cell epitopes to improve the vaccine efficacy. A multi-epitope vaccine candidate called the *poly B* is composed of more B cell and T CD4$^+$ epitopes and the other multi-epitope vaccine candidate, called the *poly T*, is composed of more T CD8$^+$ cell and T CD4$^+$ epitopes.

As mentioned earlier, epitope-based vaccines have many advantages in comparison to other type of vaccines but one of the most important drawbacks associated with these vaccines is their low immunogenicity

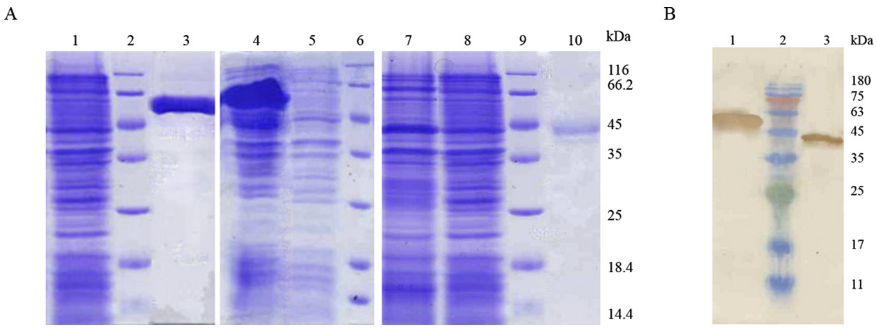

Fig. 5. SDS-PAGE and western blot analysis of *poly B* and *poly T*. (A) Expression analysis of proteins. Lane 1: induced *poly B* protein; lane 2, 4 and 8: uninduced cell lysates; lane 3, 5 and 9: Molecular Weight Marker; lane 6: purified *poly B* protein; lane 7: induced *poly T* protein; lane 10: purified *poly T* protein. (B) Western blot analysis of constructs. lane1: *poly B*; Lane 2: Molecular Weight Marker; lane 3: *poly T*.

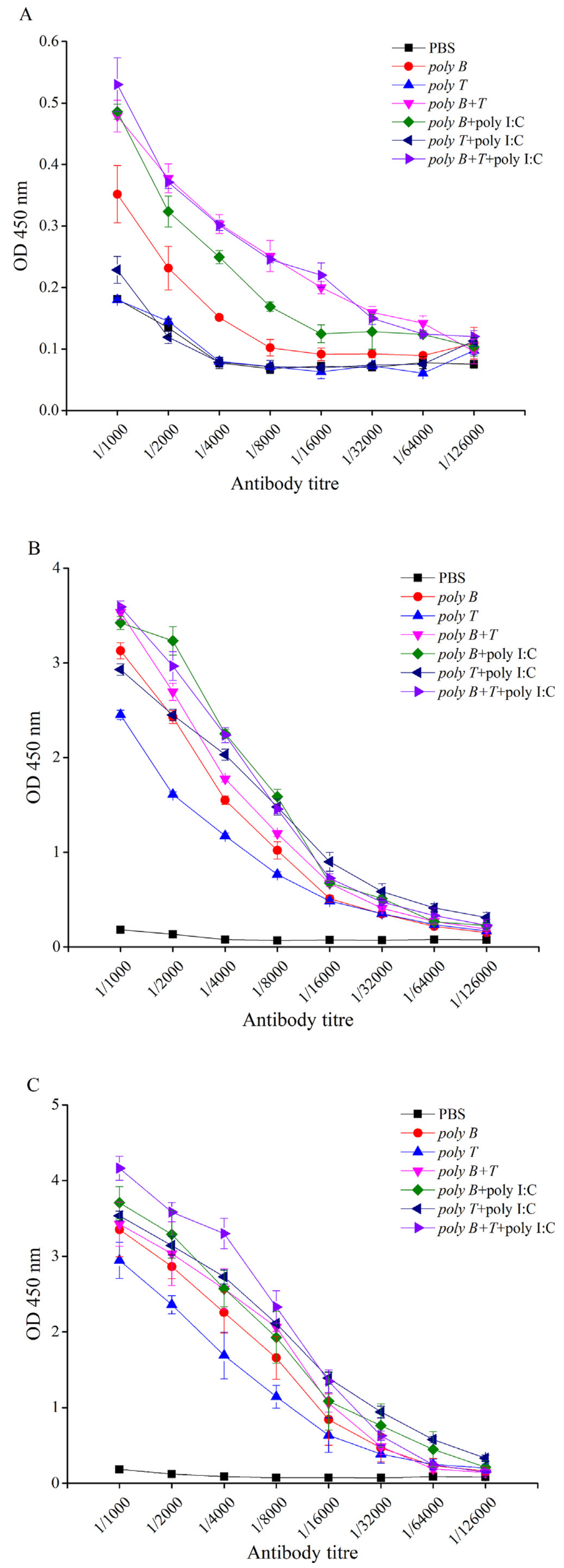

Fig. 6. The level of specific antibody in different dilution of sera after immunization with *poly B*, *poly T*, and *poly B + T* with and without poly I:C. (A) After the first immunization. (B) After the second immunization. (C) After third immunization. Data represents Mean of the OD450 ± SD from serum samples of experimental groups.

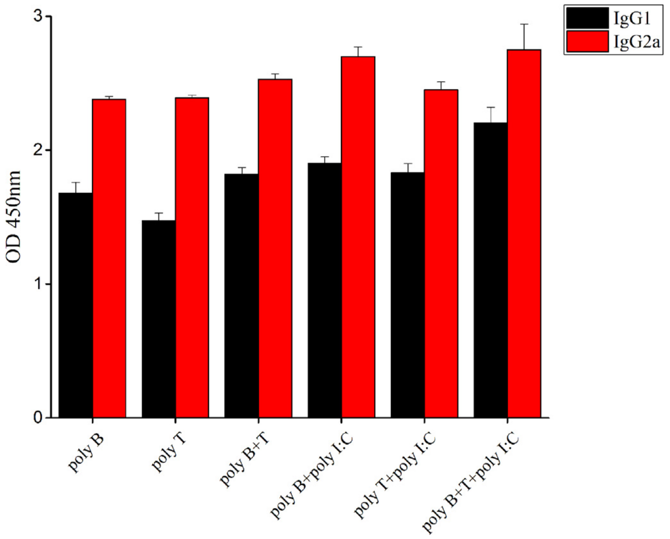

Fig. 7. IgG subclasses in serum of mice immunized with different treatments. Data represents Mean of the OD450 ± SD from serum samples of experimental groups. Statistically significant differences with control group (PBS) was indicated by *($P < 0.05$).

[17–19]. To overcome this drawback, adjuvants are added to multi-epitope vaccines to improve the immunogenicity of vaccine constructs and modulate it toward the desired immune responses. Toll-like receptors (TLRs) are conserved receptors which recognized pathogen-associated molecular patterns (PAMPs) and activates immune responses. In recent years, many studies have shown that TLR agonists can be successful adjuvants and some of them have been used in approved vaccines [27–30]. Poly I:C, as a TLR3 agonist, is able to efficiently stimulate both innate and adaptive immune responses [22–24]. In the present study to enhance immunogenicity of multi-epitope vaccine candidate, we used poly I:C as a strong Th1-inducing adjuvant.

Efficient immune responses against intracellular microorganism such as *Brucella* are predominantly mediated by cell-mediated immunity including T helper 1 (Th1) and cytotoxic T lymphocyte (CTL) immune responses [5–7]. Results from splenocyte proliferation assay showed significant levels of proliferation in response to stimulation with *Poly B*, *Poly T* and *Poly B + T*, which could be further increased in multi-epitops + poly I:C immunized mice (Fig. 9). Significant levels of proliferative response to designed multi-epitops in Rev1 and RB51 vaccinated mice revealed that the live attenuated vaccine stimulated immune responses against designed multi-epitops. Th1 cytokines (especially IFN-γ) activates the bactericidal function of macrophages and skewing antibody responses to protective IgG2a. We observed increased levels of Th1 cytokines (IFN-γ and IL-2) in immunized mice (Fig. 8). Our results were in accordance with the report of Yin et al., that suggested multi-epitope subunit construct from OMP16, OMP2b, OMP31, and BP26 induced high level of IFN-γ cytokine [21]. Furthermore, there was no significant differences in the levels of IFN-γ and IL-2 between groups immunized with *poly B*, *poly T* and *poly B + T*, which all contained T $CD4^+$. These results are in accordance with observations made by other authors suggesting that $CD4^+$ T lymphocytes are the major producers of IFNγ than other cell subsets such as $CD8^+$ T lymphocytes [31–33]. In addition, splenocytes from mice immunized with multi-epitopes + poly I:C produced higher levels of IFN-γ and IL-2 than mice injected with multi-epitopes alone. These observations were

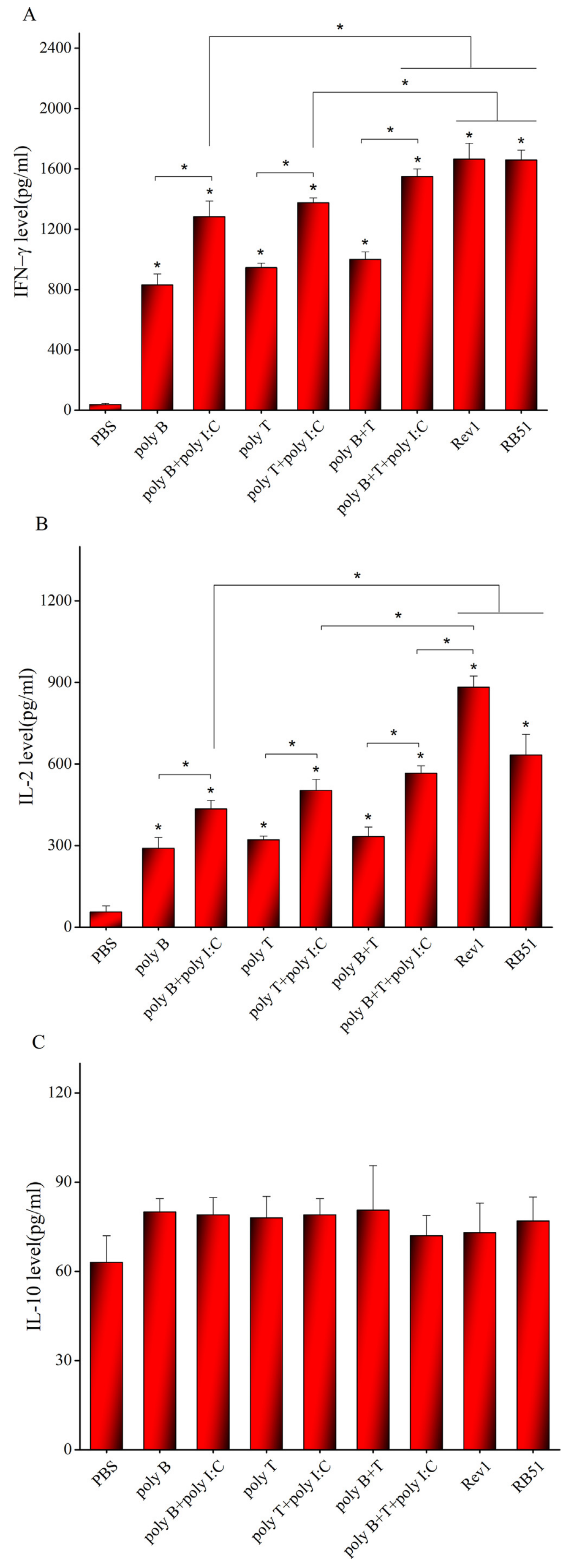

Fig. 8. Cytokines production by splenocytes of immunized mice. (A) Level of IFN-γ production. (B) Level of IL-2 production. (C) Level of IL-10 production. Each value represents the mean ± SD of three individual mice from each group with three repeats. Statistically significant differences between experimental groups were indicated by *(P < 0.05).

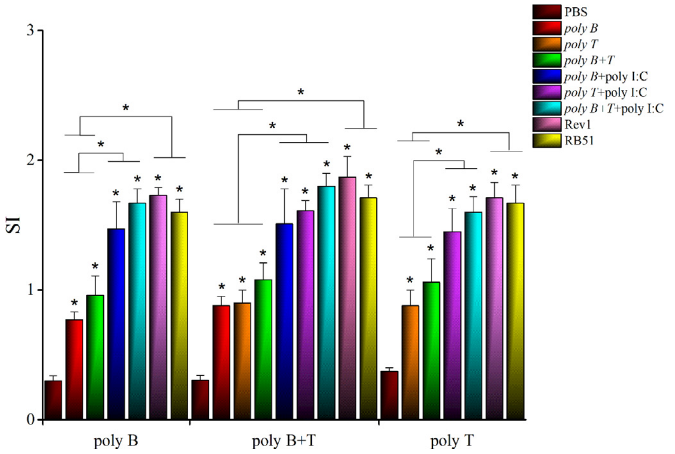

Fig. 9. Lymphocyte proliferation assay of splenocytes from different mice groups. Splenocytes from immunized mice were stimulated with *poly B*, *polyT*, *poly B + T* and the proliferative response was determined by MTT assay. The data are the mean SI ± SD of three individual mice from each group with three repeats. Statistically significant difference between experimental groups ($P < 0.05$) is indicated by an asterisk.

confirmed by lymphocytes proliferation assay results that poly I:C in combination with multi-epitopes played an important role in inducing cellular immune responses. According to Golshani et al., that indicated rL7/L12 + rTOmp31 + rOmp2b formulated in poly I:C stimulated high levels of IFN-γ and IL-2 in immunized mice [34]. We also detected low levels of Th2 (IL-10) cytokine in all immunized mice. Although IL-10 is generally considered a regulatory cytokine that down-regulate protective immunity during primary *Brucella* infection, many study recently revealed that the concomitant induction of IL-10 after *Brucella* vaccination did not reduce the degree of protection against *Brucella* challenge [10,35,36].

In this study, it was found that multi-epitopes with and without the use of adjuvant could elicit specific antibodies responses (Fig. 6). In accordance with this data Yin et al., indicated that multi-epitope construct from *Brucella* antigens (OMP16, OMP2b, OMP31, and BP26) successfully induced an increased level of IgG [21]. Th1 associated antibodies, such as IgG2a, are generated in humoral immune response against *Brucella*, enhance opsonization and phagocytosis of *Brucella* [37–40].All immunized mice produced significant levels of IgG2a (Th1 response) and IgG1 (Th2 response) with shifted responses to IgG2a production (Fig. 7). In addition the ratios of IgG2a/IgG1 approximately, was equal or > 1 in all groups. Due to the importance of Th1 in immune responses against *Brucella*, in designed multi-epitopes, we put $CD4^+$T-cell epitopes in two constructs and these constructs shifted humoral responses to IgG2a (Th1 associated antibody)production. Moreover, in all immunized groups, a significantly higher IgG2a/IgG1 ratio was detected in groups received poly I:C adjuvant. Our results are in accordance with observations made by Tewari et al., suggesting that poly I:C inducing potent antibody and Th1 responses to *P. falciparum* circum sporozoite protein (CSP) and αDEC-CSP [41]. Abouie Mehrizi et al., showed that rPfTRAP formulated with poly I:C elicited a higher ratio of IgG2a/IgG1 responses than rPfTRAP + CFA/IFA [42]. In another study Cao et al., demonstrated that poly I:C combined with multi-epitope vaccine elicited Foot and mouth disease virus (FMDV)-specific neutralizing antibodies and total IgG antibodies in pig [43].

According to the challenge results, designed multi-epitopes in the

Table 1
Protection against *B. melitensis* 16 M and *B. abortus* 544 challenge in immunized mice.

Vaccine	Log_{10} CFU of *B. melitensis* 16 M in spleen	Units of protection	Log_{10} CFU of *B. abortus* 544 in spleen	Units of protection
PBS	5.79 ± 0.17	0	5.37 ± 0.15	0
Poly B	5.3 ± 0.13*	0.49	4.79 ± 0.05*	0.58
Poly T	5.18 ± 0.18*	0.61	4.63 ± 0.2*	0.74
Poly B + T	5.13 ± 0.17*	0.66	4.63 ± 0.19*	0.74
Poly B + poly I:C	4.6 ± 0.18*	1.19	4.47 ± 0.11*	0.9
Poly T + poly I:C	4.42 ± 0.07*	1.37	4.12 ± 0.22*	1.25
Poly B + T + poly I:C	4.22 ± 0.19*	1.57	3.97 ± 0.2*	1.4
B. melitensis Rev1	4.01 ± 0.2*	1.78	–	–
B. abortus RB51	–	–	3.77 ± 0.34*	1.6

* Significantly different compared to the control groups ($P < 0.05$).

presence or absence of poly I:C adjuvant conferred significant levels of protection in mice against *B. melitensis*16M and *B. abortus*544 infections as compared to PBS group (Table.1). Vaccination with these multi-epitopes plus poly I:C provided comparatively better protection than multi-epitopes only groups. On the other hand, *poly B*, *poly T* and *poly B + T* groups induced less protection than live attenuated *B. melitensis* Rev1 and *B. abortus* RB51 vaccine groups. However *poly B* + poly I:C, *poly T* + poly I:C and *poly B + T* + poly I:C offered a protection level similar to a live attenuated *B. melitensis* Rev1 and *B. abortus* RB51 vaccine groups and these formulation conferred cross protection against both *B. melitensis* and *B. abortus*. This finding was in agreement with a previous study that found *Brucella* cocktail protein vaccine (rL7/L12 + rTOmp31 + rSOmp2b) in poly I:C induced cross protection against *B. melitensis* and *B. abortus* infections [34]. In accordance with this data Cao et al., reported that multi-epitope + poly I:C improved protection against virulent foot and mouth disease virus challenge [43]. Escalona et al., also showed multi epitope DNA vaccine induced a statistically significant level of protection against *B. abortus* 2308 in *BALB/c* mice [26]. Although with the new vaccine strategy (epitope based vaccine) we were able to achieve a better humoral and cellular immune response from a full-length antigen, we did not see important differences in the amount of protection, and this may be possible through the use of different adjuvant compounds, so in later studies, we will focus on the use of different adjuvants.

5. Conclusion

In the present study, we designed and constructed two novel multi-peptide vaccine candidates based on B cell and $CD4^+$ and $CD8^+$T-cell epitopes of FliC, 7α-HSDH, BhuA proteins and their effectiveness were assessed with and without poly I:C as a strong Th1-inducing adjuvant. Our result indicated these multi-epitope proteins combined with poly I:C could generate an effective vaccine for induction of immune responses and protection against *B. melitensis* and *B. abortus* infection in mice. Further studies focusing on increasing protective efficacy of these designed novel multi-peptide vaccines candidate are underway.

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Declaration of competing interest

The authors declare no conflicts of interest.

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