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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Prediction of T cell epitopes of *Brucella abortus* and evaluation of their protective role in mice

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Abstract *Brucellae* are Gram-negative intracellular bacteria that cause an important zoonotic disease called brucellosis. The animal vaccines are available but have disadvantage of causing abortions in a proportion of pregnant animals. The animal vaccines are also pathogenic to humans. Recent trend in vaccine design has shifted to epitope-based vaccines that are safe and specific. In this study, efforts were made to identify MHC-I- and MHC-II-restricted T cell epitopes of *Brucella abortus* and evaluate their vaccine potential in mice. The peptides were designed using online available immunoinformatics tools, and five MHC-I- and one MHC-II-restricted T cell peptides were selected on the basis of their ability to produce interferon gamma (IFN-γ) in in vivo studies. The selected peptides were co-administered with poly DL-lactide-co-glycolide (PLG) microparticles and evaluated for immunogenicity and protection in BALB/c mice. Mice immunized with peptides either entrapped in PLG microparticles (EPLG-Pep) or adsorbed on PLG particles (APLG-Pep) showed significantly higher splenocyte proliferation and IFN-γ generation to all selected peptides than the mice immunized with corresponding irrelevant peptides formulated PLG microparticles or phosphate-buffered saline (PBS). A significant protection compared to PBS control was also observed in EPLG-Pep and APLG-Pep groups. A plasmid DNA vaccine construct (pVaxPep) for peptides encoding DNA sequences was generated and injected to mice by in vivo electroporation. Significant protection was observed (1.66 protection units) when compared with PBS and empty vector control group animals. Overall, the MHC-I and MHC-II peptides identified in this study are immunogenic and protective in mouse model and support the feasibility of peptide-based vaccine for brucellosis.

Keywords *Brucella abortus* · Epitope · DNA vaccine · Immunoinformatics · PLG microparticles

Introduction

Brucellae are Gram-negative intracellular bacteria that cause an important zoonotic disease called brucellosis. The genus *Brucella* includes six classical species namely *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, *Brucella canis*, *Brucella ovi*s and *Brucella neotomae* (Corbel 1997). Some more species viz *Brucella pinnipedialis*, *Brucella ceti*, *Brucella inopinata* and *Brucella microti* were included later on. Brucellosis accounts for more than 500,000 new cases annually. Infection with *B. abortus*, a species that primarily affects bovines, often results in abortions and infertility in domestic and wild mammals (Franco et al. 2007). The persistence of brucellosis in domestic livestock reservoirs remains a continual source of significant numbers of human infections worldwide. Despite regulatory efforts, brucellosis remains endemic in many parts of the world and is re-emerging in many countries.

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* strain Rev.1. The non-smooth strain *B. abortus* RB51 was later

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introduced in some countries. *B. abortus* S19 and *B. melitensis* Rev.1 are proven effective vaccines against *B. abortus* infection in cattle and against *B. melitensis* and *B. ovis* infection in sheep and goats, respectively (Nicoletti 1990). Both vaccines have the disadvantages of causing abortion in a proportion of pregnant animals and of being pathogenic for humans (Perkins et al. 2010; Schurig et al. 1991). Due to the epidemic potential of *Brucella*, absence of a human vaccine, drawback of current vaccine strains in terms of safety, the efficiency of aerosol infection and poor symptomatology of disease, this airborne pathogen is classified as a biosafety level 3 pathogen and considered as a potential biological warfare agent. Humans infected with *B. abortus* are treated with antibiotics such as doxycycline and rifampin; however, the survival of *B. abortus* in macrophages is responsible for the chronic nature of the disease that necessitates a prolonged course of treatment (Hall 1990). Even after treatment, low levels of bacteria have been detected by PCR and relapses have been detected in 5–30 % of cases (Franco et al. 2007; Vrioni et al. 2008).

The Th1 immune response characterized by interferon gamma (IFN-γ) is associated with protection against brucellosis (Murphy et al. 2001; Paranavitana et al. 2005). IFN-γ causes upregulation of macrophage anti-*Brucella* activity (Oliveira et al. 1998) which is the main component of protective response. IFN-γ also induces the expression of many IFN-γ- inducible genes, crucial for the development of innate and adaptive immunity against this pathogen. Clearance of *B. abortus* infection in mice coincided with an increase in IFN-γ-producing CD4+ and CD8+ T cells (Goenka et al. 2011) suggesting the involvement of both types of cells in protection.

Several strategies, e.g. subunit recombinant protein vaccines and vector vaccines based on *E. coli*, *Salmonella enterica*, *Ochrobactrum anthropi*, Semliki Forest virus and influenza viruses, are being sought to prevent this disease while avoiding the disadvantages of the currently used live vaccines (Jain et al. 2014a). One strategy for developing safe and efficacious vaccine is immunization with protective T cell epitopes. Determining the epitopes recognized by *Brucella*-specific CD8+ and CD4+ T cells and the *Brucella* genes encoding the protein containing these epitopes can be helpful to establish peptides critical for vaccine development. Since peptides are synthetic, there would be no risk of mutation or reversion and little or no risk of contamination by live bacteria. The synthetic epitope or peptide-based vaccines have been advocated as an attractive approach for prevention or treatment of infectious diseases and malignant disorders (Buteau et al. 2002; Kast and Melief 1991; Rothbard 1987). Epitope-based vaccines have also been suggested as quick method to design and produce vaccines with short turn-around time for biothreat agents (De Groot et al. 2013).

Any invading pathogen or delivered antigen is taken up by antigen-processing cells (APCs) which process it resulting in formation of peptides that are loaded into grooves of newly synthesized MHC class I and class II molecules that then pass to the cell surface (Germain 1994). T cells are stimulated via MHC–peptide complexes and costimulatory molecules. Therefore, combining class I MHC- and class II MHC-restricted peptides in vaccines can increase immunogenic response. In the present work, MHC-I- and MHC-II-restricted T cell peptides of protective proteins or virulence determinants of *B. abortus* were designed and evaluated in vivo for their ability to produce IFN-γ in immunized BALB/c mice spleen cells. We demonstrate the ability of these peptides formulated either with poly DL-lactide-co-glycolide (PLG) microparticles or as plasmid DNA vaccine to confer protection in mouse model.

Materials and methods

Bacterial strains, clones and vectors

B. abortus NCTC 10093 (544) and vaccine strain S19 were obtained from Central Public Health Laboratory, Colindale, London, UK, and Indian Veterinary Research Institute, Izatnagar, UP, India, respectively. *B. abortus* DB79BRAB4, a wild strain, was taken from Defence Research & Development Establishment (DRDE)'s culture collection. *E. coli* host strain DH5α (Novagen, USA) was used for cloning. pVax1 (Invitrogen, USA) eukaryotic expression vector was used for plasmid DNA (pDNA) vaccine preparation. All *Brucella* cultures were grown in Brucella broth (HiMedia, India) at 37 °C with 5 % CO_2 for 2–3 days. For vaccination and challenge, the cultures were suspended in a sterile phosphate-buffered saline (PBS) (0.01 M, pH 7.2), and colony-forming units (CFU) were determined by the plate count method. All live *B. abortus* manipulations were performed in biocontainment safety level 3 (BSL-3) facility.

Animals

Female BALB/c mice (6–8 weeks old) were obtained from the Animal Facility of DRDE and were given water and food ad libitum. The mice were maintained and used in accordance with the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Forests and Environment, Govt. of India. The study had an approved animal protocol from the Institutional Animal Ethics Committee of DRDE, Gwalior (protocol no. MB-06/48/SK). The challenged mice were housed in animal biocontainment safety level 3 (ABSL-3) facility.

Prediction of MHC class I- and class II-restricted T cell epitopes by immunoinformatics tools

Various *Brucella* proteins earlier shown to be either protective proteins or virulence determinants were chosen for designing the peptides (Table 1). The amino acid sequence of these proteins was downloaded from Brucella bioinformatics portal (www.violinet.org). MHC-I-restricted epitopes were designed for H-2D^d, H-2K^d and H-2L^d haplotypes, while MHC-II-restricted epitopes were designed for H-2A^d and H-2E^d haplotypes. MHC-I-restricted epitopes were designed using tools available at Immune Epitope Data Base (IEDB), Rankpep and BIMAS. Lowest Ic50 values (ANN, <500) or top 1 percentile of high binders (consensus) was used as cut-off in IEDB method. Rankpep determines the binding threshold by itself and predicts the epitopes accordingly. Epitopes having highest half-life of dissociation (>100) were chosen by BIMAS. The epitopes picked up by most softwares were shortlisted for synthesis. Rankpep and IEDB tools were used to predict the MHC-II-restricted epitopes using similar cut-offs. Rankpep predicted 9-mer epitopes, whereas IEDB predicted 15-mer epitopes.

Selection of peptides

The predicted epitopes were custom synthesized as peptides and screened for shortlisting by animal experimentation in BALB/c mice. This was carried out by their ability to induce IFN-γ production (peptide immunogenicity) or be immune dominant during natural infection (natural processing). For peptide immunogenicity, a group BALB/c mice (n=5) was immunized subcutaneously with pool of peptides (50 μg each peptide) in PBS emulsified 1:1 with incomplete Freund adjuvant (IFA). The pools of peptides used for immunization in various experiments have been shown in Supplementary data (Table S1). Control mice were injected PBS emulsified in 1:1 IFA. For natural processing, BALB/c mice were injected with 2×10^5 CFU of *B. abortus* strain *544* by intraperitoneal injection, and control mice were injected PBS. The mice were sacrificed on day 10, and the spleen of each mouse was separated. Determination of IFN-γ in spleen culture supernatant was determined by the earlier described method (Kumar et al. 2009). The splenocytes were stimulated with 25 and 50 μg ml^{-1} concentrations of individual peptide for 72 h at 37 °C and 5 % CO_2. Appropriate positive (Con A, 5 μg ml^{-1})

Table 1 Protective proteins and virulence determinants used for designing peptides by various softwares

S. no.	Protective protein/virulence markers (abbreviations)	Accession no.	Reference
1	Bacterioferritin (Bfr)	WP_002966069.1	Al-Mariri et al. (2001)
2	Periplasmic immunogenic protein (BP26)	WP_002964581.1	Yang et al. (2007)
3	Molecular chaperone DnaK (DnaK)	WP_002969217.1	Delpino et al. (2007)
4	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	WP_002964815.1	Rosinha et al. (2002)
5	Invasion protein B (IalB)	WP_004682929.1	Commander et al. (2007)
6	Outer membrane protein 16 (Omp16)	WP_002966947.1	Pasquevich et al. (2009)
7	Outer membrane protein 19 (Omp19)	WP_002964998.1	Pasquevich et al. (2009)
8	Outer membrane protein 25 (Omp25)	WP_004683466.1	Edmonds et al. (2002)
9	Outer membrane protein 31 (Omp31)	WP_002964719.1	Gupta et al. (2007)
10	Brucella lumazine synthase (BLS)	CAA86936.1	Goldbaum et al. (1999)
11	Phospho ribosyl aminoimidazole ribonucleotide synthase (*PurK*)	WP_011382134.1	Hoover et al. (1999)
12	50S subunit of ribosomal protein L7/L12 (L7/L12)	WP_002964371.1	Luo et al. (2006)
13	30S subunit of ribosomal protein S12 (S12)	WP_002964366.1	Cloeckaert et al. (2002)
14	Copper/zinc superoxide dismutase (Cu/Zn SOD)	WP_002972093.1	Vemulapalli et al. (2000)
15	Peptidyl-prolyl *cis-trans* isomerase (SurA)	WP_002966738.1	Delpino et al. (2007)
16	Trigger factor (Tf)	WP_002971016.1	Yang et al. (2007)
17	Transcriptional activator (*vjbR*)	WP_006193251.1	Arenas-Gamboa et al. (2009)
18	Zinc ABC transporter, periplasmic zinc-binding protein (*znuA*)	WP_002966661.1	Yang et al. (2006)
19	Cysteine synthase A (CysK)	WP_002964172.1	Jain et al. (2013)
20	50S subunit of ribosomal protein L9 (L9)	WP_002963608.1	Jain et al. (2014a, b)
21	Dihydrolipoamide succinyltransferase (E2o)	WP_002975967.1	Verma et al. (2012)
22	Hypothetical protein (HP)	WP_002966987.1	Connolly et al. (2006)
23	Outer membrane protein 2b (Omp2b porin)	WP_004689570.1	Connolly et al. (2006)

and negative controls (without antigen) were also included. The supernatant was collected, and level of IFN-γ was measured using commercially available ELISA kits (R & D Systems, USA). For final selection of the above-shortlisted peptides, another round of screening by peptide immunogenicity and natural processing was undertaken. The experiments were same as above except that 100 μg of each peptide was used for mice immunization instead of 50 μg for peptide immunogenicity and booster dose was given at day 7. For natural processing experiment, single dose with field isolate *B. abortus* DB79BRAB4 was administered instead of strain 544. The animals were sacrificed on day 14 for quantification of IFN-γ.

Preparation of PLG microparticles with adsorbed/entrapped peptides

PLG microparticles for adsorption and entrapment by selected or irrelevant peptides were prepared by a solvent evaporation technique as described by Singh et al. (2004). Briefly, for preparation of PLG microparticles, 10 ml of 6 % *w/v* PLG solution in methylene chloride (Sigma-Aldrich, USA) was homogenized with 2.5 ml PBS using a 10-mm probe (Sonics) for 10 min, thus forming a water-in-oil emulsion. This emulsion was added to 50 ml of triple-distilled water (TDW) containing 6 μg ml^{-1} docusate sodium salt (DSS) and homogenized at very high speed using a homogenizer with a 20-mm probe for 5 min in an ice bath. This resulted in water-in-oil-in-water emulsion that was stirred at 1000 rpm for 12 h at room temperature, and the methylene chloride was allowed to evaporate. The resulting PLG content was lyophilized and weighed. For preparation of PLG microparticles with adsorbed peptides, a suspension containing 15 mg of PLG was incubated with 1 mg of peptide in 1 ml of PBS buffer and left overnight on a rocker at 4 °C. This was followed by centrifugation at 10,000*g* for 10 min. Pellet was re-suspended in PBS, and supernatant was collected to determine the concentration of peptide.

For preparation of PLG microparticles with entrapped peptides, 1 mg of peptide was added in the first step of PLG preparation and the resultant water-in-oil emulsion was added to 50 ml of 10 % *w/v* poly vinyl alcohol. The rest of the procedure was same as described for preparation of PLG microparticles. The microparticles were washed with TDW by centrifugation and lyophilized. The adsorption/entrapment efficiency of peptide to the microparticles was determined by subtracting the calculated peptide amount in supernatant of the final sample from initial concentration used. PLG microparticles were loaded individually with selected or irrelevant peptides and pooled later on. The microparticles were characterized by scanning electron microscopy (Quanta 400 ESEM-EDX, FEI).

Immunization with PLG microparticle formulations

Groups of mice (n=10) were immunized subcutaneously with PLG microparticles containing either adsorbed (APLG-pep) or entrapped peptides (EPLG-pep). Each mouse in these groups received a dose of 25 μg of each peptide on days 0 and 60. Equivalent control groups of animals injected with microparticles of both adsorbed and entrapped types with irrelevant peptides (APLG-NP and EPLG-NP) were included. A group of mice was immunized subcutaneously with the pool of peptides (effective peptide/mouse was 25 μg) with IFA (Pep-Ad) with same immunization schedule. The negative control group animals were injected with PBS on days 0 and 60, and the positive control group of mice (n=6) was inoculated intraperitoneally with *B. abortus* strain S19 vaccine strain (5×10^4 CFU/mouse) on day 60 and used for protection studies.

Splenocyte proliferation and cytokine assays

After 28 days of last immunization (day 88), the spleens from mice were removed aseptically and the single cell suspension of splenocytes was taken for splenocyte proliferation and cytokine assays as described earlier (Kumar et al. 2009). Briefly, the mice were sacrificed and the spleen of each mouse was separated. The splenocytes were suspended in 96-well tissue culture plate ca. 1×10^6 cells ml^{-1} (100 μl $well^{-1}$) along with one volume of homologous peptide (50 μg ml^{-1}) in RPMI 1640 medium. Appropriate positive (Con A, 10 μg ml^{-1}) and negative controls were also included. After 48 h of incubation, 0.2 volume of alamarBlue dye (Biosource, USA) was added to each well and the plate was incubated for further 15–18 h. Experiments were carried out in triplicate wells for each mouse. The reading was taken at 570 and 600 nm, and the results are expressed as mean specific absorbance by subtracting the absorbance at 600 nm from 570-nm absorbance. Supernatants from parallel cultures were harvested after 72 h, and level of IFN-γ was measured using commercially available ELISA kits (R & D Systems, USA).

Generation of 'bead of string'-based vaccine construct

DNA encoding BP238, Zn317, L923, CSA124, E2o343 and Orf136 peptides was custom synthesized without linker in pBluescript SK vector and subcloned into eukaryotic expression vector pVax1. Vaccine-grade pDNA (pVaxPep) was prepared by Qiagen maxi kit as per manufacturer's instruction and was used to immunize BALB/c mice (n=8) by in vivo electroporation by the earlier described method (Jain et al. 2014a). Mice were given two doses of pDNA (100 μg) on days 0

and 28. The control mice were injected with PBS or empty pVax1 vector. A positive control group (n=6) vaccinated intraperitoneally with *B. abortus* strain S19 as described above was also included and was given a single dose on day 28.

Protection assay

Protection studies were carried out by previously described method (Jain et al. 2014b). Briefly, 30 days after last injection (day 90 for PLG groups and day 58 for DNA vaccine groups),

Table 2 MHC-I-restricted T cell epitopes shortlisted for in vivo studies

S. no	Target protein	Name peptide	Peptide sequence	Allele	IEDB (Ann)	IEDB (Con)	Rankpep	BIMAS	Score
1	BP26	Bp238	SYNVSVNVV	H-2K^d	7.2+	0.2+	19.367+	720 +	4
2	BP26	Bp108	VYPDDKNNL	H-2K^d	–	–	17.348+	4147+	2
3	Gap	Gap196	LYRARAAAL	H-2K^d	161.6+	0.40+	14.709+	2400+	4
4	Rpsl	Rps94	RYHIIRGVL	H-2K^d	11.5+	0.80+	18.849+	2400+	4
5	vjbR	vjb67	YYRNDTSAI	H-2K^d	14.6+	0.10+	14.861+	2880+	4
6	znuA	Zn316	LYPQLIRNL	H-2K^d	–	–	21.705+	2400+	2
7	Omp16	Mp87	RYPQYSITI	H-2K^d	–	–	22.931+	2000+	2
8	Gap	Gap71	YGPIKVHAV	H-2D^d	–	0.30+	–	600+	2
9	Omp19	Mp120	AGPLRCPGE	H-2D^d	167.7+	0.30+	–	–	2
10	znuA	Zn82	AGPSMETFL	H-2D^d	162.4+	0.60+	–	200+	3
11	znuA	Zn312	DGPDLYPQL	H-2D^d	276.4+	0.10+	–	576+	3
12	Omp19	Mp79	LPPASAPDL	H-2L^d	3.9+	0.10+	24.666+	225+	4
13	Gap	Gap237	TPNVSVVDL	H-2L^d	30.8+	0.25+	25.251+	25.251+	4
14	PurK	Pur71	LPPPAALEI	H-2L^d	5.4+	0.55+	–	–	2
15	znuA	Zn221	KPVKDKPFI	H-2L^d	241.0+	0.60+	–	138.24–	3
16	Tig	Tig128	LPAIEVKDF	H-2L^d	–	0.95+	–	300+	2
17	*BP26*	*Bp122*	*TGYSVSTSL*	*H-2K^d*	–	*NC*	8.64	*NC*	–
18	*znuA*	*Zn317*	*YPQLIRNLA*	*H-2L^d*	–	*NC*	7.641	*NC*	–
19	Bp26	Bp8	FLAASFSTI	H-2K^d	14.9+	0.10+	–	–	2
20	DnaK	Dna563	AIAALKTSL	H-2K^d	122.9+	0.30+	–	–	2
21	Gap	Gap181	SYTGDQPTL	H-2K^d	31.1+	0.80+	–	2400+	3
22	Gap	Gap133	VYGVNNDKL	H-2K^d	–	–	17.979+	2400+	2
23	Omp31	Mp174	AYGKVKTSL	H-2K^d	7.0+	0.10+	–	4147.2+	3
24	Omp31	Mp5	KGTVMKTAL	H-2K^d	248.1+	0.50+	–	–	2
25	Tig	Tig397	RYPGQEKEI	H-2K^d	–	–	17.296+	2000+	2
26	Omp25	Mp130	VMPYLTAGI	H-2D^d	80.4+	0.20+	–	–	2
27	SodC	Sod32	TGPGKEVGT	H-2D^d	313.1+	0.20+	–	100+	3
28	IalB	Ial150	QPVAFKISL	H-2L^d	355+	0.95+	–	–	2
29	Omp16	Mp10	SPIAIALFM	H-2L^d	3.5+	0.10+	–	150+	3
30	Omp19	Mp121	GPLRCPGEL	H-2L^d	68.3+	0.70+	–	150+	3
31	Omp25	Mp15	LPFSATAFA	H-2L^d	44.4+	0.65+	–	–	2
32	PurK	Pur56	VPVSAADKL	H-2L^d	5.5+	0.30+	–	150+	3
33	Omp19	Mp60	FPNAPSTDM	H-2L^d	11.6+	0.10+	–	150+	3
34	Bfr	Bfr75	SPLRIGQNV	H-2L^d	150.6+	1.00+	–	–	2
35	Hp	Hp65	TGPDKAPFT	H-2D^d	–	0.2+	–	240+	2
36	L9	L923	DGYARNFLL	H-2D^d	–	0.4+	–	120 +	2
37	CSA	CSA124	NYVRLSGRL	H-2K^d	139+	0.6+	14.58+	4800+	4
38	O2b	O2b90	NYAANNSGV	H-2K^d	45+	0.3+	19.25+	720+	4
39	E2o	E2o343	APQSGILGM	H-2L^d	206+	0.6+	25.7+	292+	4
40	*Bp26*	*Bp219*	*RTMLAAAPD*	*H-2D^d*	*37,782.0*	99 *NC*	*−16.3 NC*	*NC*	–
41	*SodC*	*Sod89*	*AGGHYDPGN*	*H-2K^d*	37,789.0	99 *NC*	*−11.5 NC*	*NC*	–

NC Negative control peptides (shown in italics)

six immunized mice from all groups were challenged with 2×10^5 CFU mouse^{-1} of *B. abortus* strain 544 by intraperitoneal injection. The challenged animals were sacrificed 30 days after being challenged by cervical dislocation, and their spleens were removed aseptically. The numbers of colonies were counted after 48 h, and the results were expressed as the mean log CFU spleen^{-1}±SD per group. Units of protection were obtained by subtracting the mean log CFU spleen^{-1} of the vaccinated group from the mean log CFU spleen^{-1} of the control (PBS immunized) group.

Statistical analysis

The cytokine, splenocyte proliferation and protection study data of all experiments was analyzed statistically by one-way ANOVA followed by Tukey's multiple comparison test (GraphPad Prism 6.0 USA). Peptide immunogenicity and natural processing data was analyzed by Student's *t* test between peptide and PBS groups. Difference between two groups ($p<0.05$ or lesser) was considered as significant.

Table 3 MHC-II-restricted T cell peptides shortlisted for in vivo studies

S. no	Protein	Name peptide	Peptide sequence	Haplotype	Software	Score
1	DnaK	Dna137	QAVITVPAY	H-2A^d	Rankpep	21.507
2	IalB	Ial57	QEQSSAQAG	H-2A^d	Rankpep	20.004
3	Omp16	Mp111	LGQRRAAAT	H-2A^d	Rankpep	15.721
4	Omp19	Mp69	QGYRAGPLR	H-2A^d	Rankpep	15.757
5	Omp31	Mp193	AGNSKTKAG	H-2A^d	Rankpep	16.928
6	Orf	Orf136	KGVEAAHAA	H-2A^d	Rankpep	28.829
7	PurK	Pur203	AAISVQTAE	H-2A^d	Rankpep	20.366
8	Rpil	Rpil30	WGVSAAAPV	H-2A^d	Rankpep	20.941
9	CSA	Csa250	FSYQIPDAE	H-2A^d	Rankpep	15.908
10	HP	Hp55	PAAQAAPAS	H-2A^d	Rankpep	20.757
11	IalB	Ial151	PVAFKISLK	H-2E^d	Rankpep	20.719
12	Rpsl	Rpsl108	KNRKQRRSK	H-2E^d	Rankpep	24.309
13	*DnaK*	*Dna238*	*VEYLVAEFKKESGID*	*H-2A^d*	*IEDB*	22,772
14	*Bfr*	*Bfr148*	*RYGQLNAAP*	*H-2E^d*	*Rankpep*	*3.106*
15	Bp26	Bp171	EARKRAVAN	H-2A^d	Rankpep	21.482
16	SodC	Sod59	LHFKVNMEK	H-2E^d	Rankpep	24.414
17	SodC	Sod92	HYDPGNTHH	H-2E^d	Rankpep	23.997
18	SurA	Sur304	KYVQELREK	H-2E^d	Rankpep	21.571
19	DnaK	Dna144	AVITVPAYFNDAQRQ	H-2E^d	IEDB	14.2
20	Rpsl	Rpsl43	VYTTTPKKPNSALRK	H-2E^d	IEDB	21.2
21	L9	L937	PQGKALRANEANKKK	H-2E^d	IEDB	37.01
22	DnaK	Dna430	DNQSAVTIRVFQGER	H-2A^d	IEDB	7.7
23	Omp25	Mp129	GYDLNPVMPYLTAGI	H-2A^d	IEDB	9.8
24	Omp31	Mp160	RLGFTPTERLMVYGT	H-2A^d	IEDB	3.7
25	Omp31	Mp25	FTSSAMAADIIVAEP	H-2A^d	IEDB	5.6
26	PurK	Pur23	AGCPAAQVANRQIVA	H-2A^d	IEDB	10.4
27	SurA	Sur136	KYIMVQMGWGRLVSA	H-2A^d	IEDB	0.82
28	znuA	Znu258	SPEKAPGAARIQQIH	H-2A^d	IEDB	4.0
29	CSA	Csa73	EGTAGNTGIGLTMVA	H-2A^d	IEDB	64.69
30	CSA	Csa294	LGPGHTIVTVLCDYG	H-2A^d	IEDB	37.88
31	HP	Hp332	WFGAVANRIVATENK	H-2A^d	IEDB	14.64
32	Bfr	Bfr134	LETQLDLLAKIGGER	H-2A^d	IEDB	16.09
33	vjbR	Vjb30	GDCTKVNSADLTVLM	H-2A^d	IEDB	12.7
34	Bfr	Bfr132	DFLETQLDLLAKIGG	H-2A^d	IEDB	544.3
35	*PurK*	*Pur24*	*VANRQIVAA*	*H-2E^d*	*Rankpep*	*3.754*
36	*Omp31*	*Mp238*	*YIDEENVNINMENKV*	*H-2A^d*	*IEDB*	*50,000*

NC Negative control peptides (shown in italics)

Results

In silico prediction of MHC-I- and MHC-II-restricted T cell epitopes of *B. abortus* proteins

A total of 93 MHC-I-restricted epitopes were predicted, out of which 37 highly scored binding epitopes by maximum number of prediction tools were custom synthesized for in vivo experiments (Table 2). Epitopes predicted by single tool were not considered. Four negative control (NC) epitopes having very low binding values were also included. By Rankpep, 118 9-mer MHC-II epitopes were predicted, while by IEDB, about 80–100 15-mer epitopes were predicted for each protein. Since no rational criterion to shortlist the peptide synthesis was available, hence, we chose to randomly select 32 peptides for in vivo screening. Here, also, four peptides having very low binding values were also included as NC (Table 3).

Determining the peptide immunogenicity in mice

The predicted epitopes were screened on the basis of their ability to induce IFN-γ production in mice (peptide immunogenicity) or their ability to get naturally processed for IFN-γ production during infection with live culture of *B. abortus* (natural processing). Peptides with vaccine potential were expected to be positive for both peptide immunogenicity and natural processing.

Twelve MHC-I-restricted peptides, e.g. Bp238, Zn316, Gap71, Pur71, Zn317, Dna563, Mp130, Hp65, L923, CSA124, O2b90 and E2o343, were found to release significant amount of IFN-γ in both peptide immunogenicity and natural processing (Fig. 1a, b). Surprisingly, the predicted low affinity binder, Zn317, which was included as NC, produced high level of IFN-γ. To further validate the results, the above-shortlisted MHC-I peptides were taken for second round of in vivo screening. Seven of the 12 peptides induced IFN-γ (Fig. 3a) and were taken further for natural processing experiment.

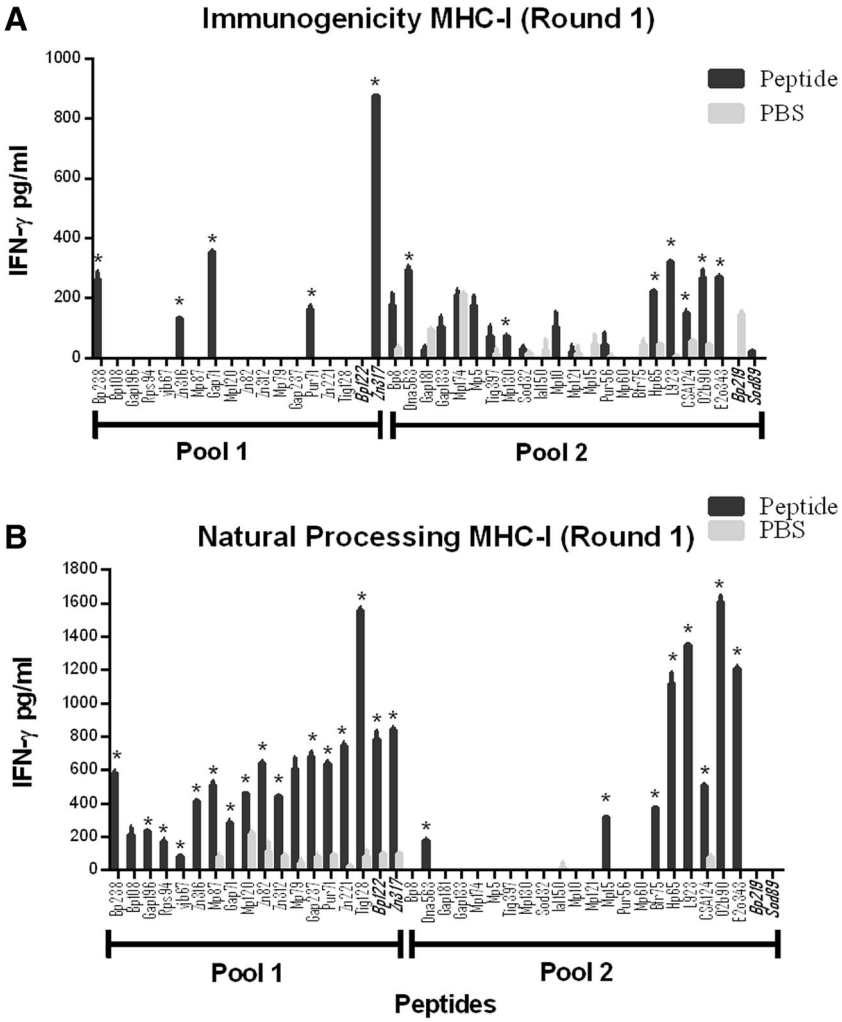

Fig. 1 In vivo peptide screening (round 1) for MHC-I-restricted T cell peptides by peptide immunogenicity (**a**) and natural processing (**b**). Mice were injected with pool of peptides as shown. Release of IFN-γ in culture supernatant of splenocytes was determined after stimulation with individual peptide of the pool. Significant differences of comparison with PBS group were determined by Student's *t* test and are indicated by *asterisk* ($p<0.05$). Results shown at stimulating concentration of 50 μg ml^{-1} of peptide

MHC-II-restricted peptides were relatively poor produces of IFN-γ. Only three peptides Orf136, Hp55 and Rpsl108 produced statistically significant amount of IFN-γ in peptide immunogenicity and natural processing (Fig. 2a, b). In the second round, only one peptide, Orf136, was found immunogenic (Fig. 3b). All seven MHC-I and one MHC-II shortlisted peptides were tested by natural processing, and five MHC-I and one MHC-II peptides were selected for studying the vaccine potential in mice (Fig. 3c, Table 4). Some of the peptides that did not release IFN-γ in immunogenicity or natural processing experiments on repeated occasions were randomly selected as irrelevant peptides (as NC) for immune response and protection studies (Table S2).

Evaluation of epitope-based vaccine formulated in PLG microparticles

PLG microparticles were prepared as described in "Materials and methods" section, and their sizes were determined by scanning electron microscope (Fig. S1). Size range of microparticles of adsorbed type (APLG) with or without peptide was found out to be 0.25–1.25 or 0.3–1.0 μm, respectively. PLG microparticles with entrapped (EPLG) peptides were of size range of 0.3–1.21 μm. Adsorption and entrapment efficiency of PLG microparticles were determined to be 89.6 %.

Mice were immunized on day 0 and day 60 with APLG-pep/EPLG-Pep formulations. Splenocytes from immunized mice were prepared 28 days after the last dose to investigate the cellular immune response. Splenocytes from EPLG-pep-, APLG-pep- and Pep-Ad-vaccinated mice had significant proliferative response to all peptides when compared with APLG-NP, EPLG-NP and PBS control groups ($p<0.0001$). No proliferation occurred in cultures of PBS group mice by the selected peptides and of EPLG-NP (except vjbR30) and APLG-NP group mice by irrelevant peptides. The proliferative response to most peptides in EPLG-pep or APLG-Pep groups was higher than in the Pep-Ad group (Fig. 4a). The ConA mitogen was able to induce spleen cell proliferation in all cultures non-specifically.

Release of IFN-γ in the culture supernatants of spleen cells of immunized mice was evaluated by ELISA. A significant ($p<0.0001$) production of IFN-γ in spleen cells from mice of APLG-pep, EPLG-pep and Pep-Ad groups in comparison to APLG-NP, EPLG-NP and PBS groups was observed.

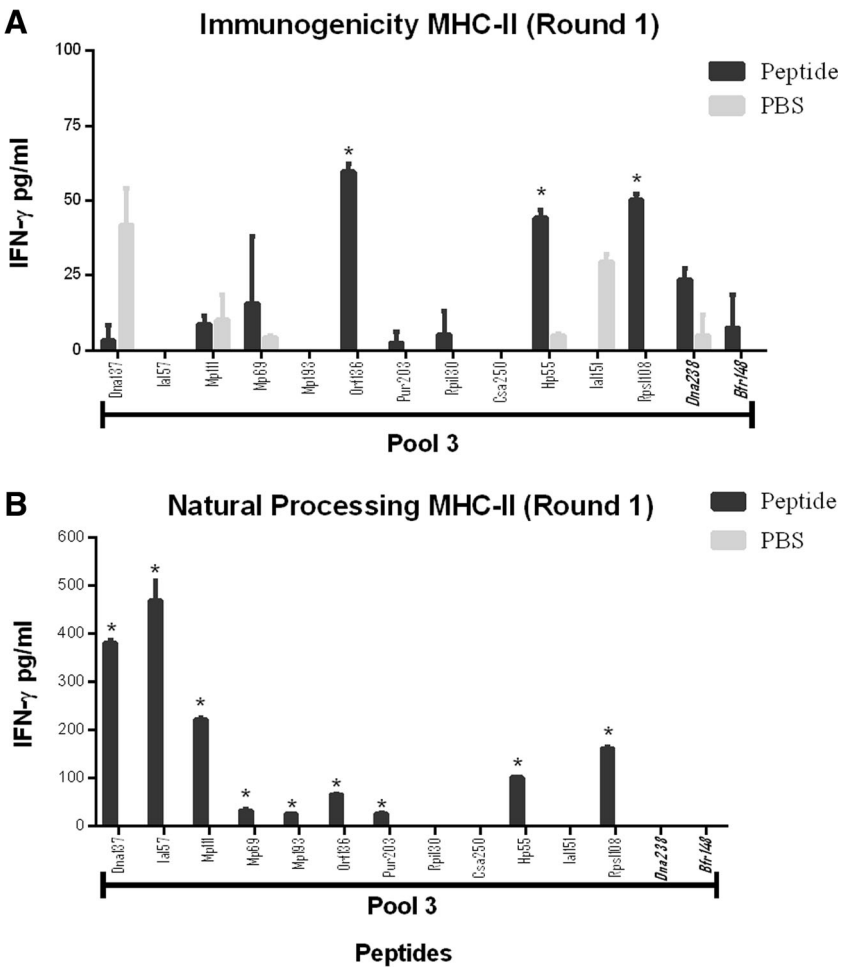

Fig. 2 In vivo peptide screening (round 1) for MHC-II-restricted T cell peptides by peptide immunogenicity (**a**) and natural processing (**b**). Mice were injected with pool of peptides as shown. Release of IFN-γ in culture supernatant of splenocytes was determined after stimulation with individual peptide of the pool. Significant differences of comparison with PBS group were determined by Student's *t* test and are indicated by *asterisk* ($p<0.05$). Results shown at stimulating concentration of 50 μg ml^{-1} of peptide. Pool 4 peptides did not release IFN-γ and, hence, have not been shown

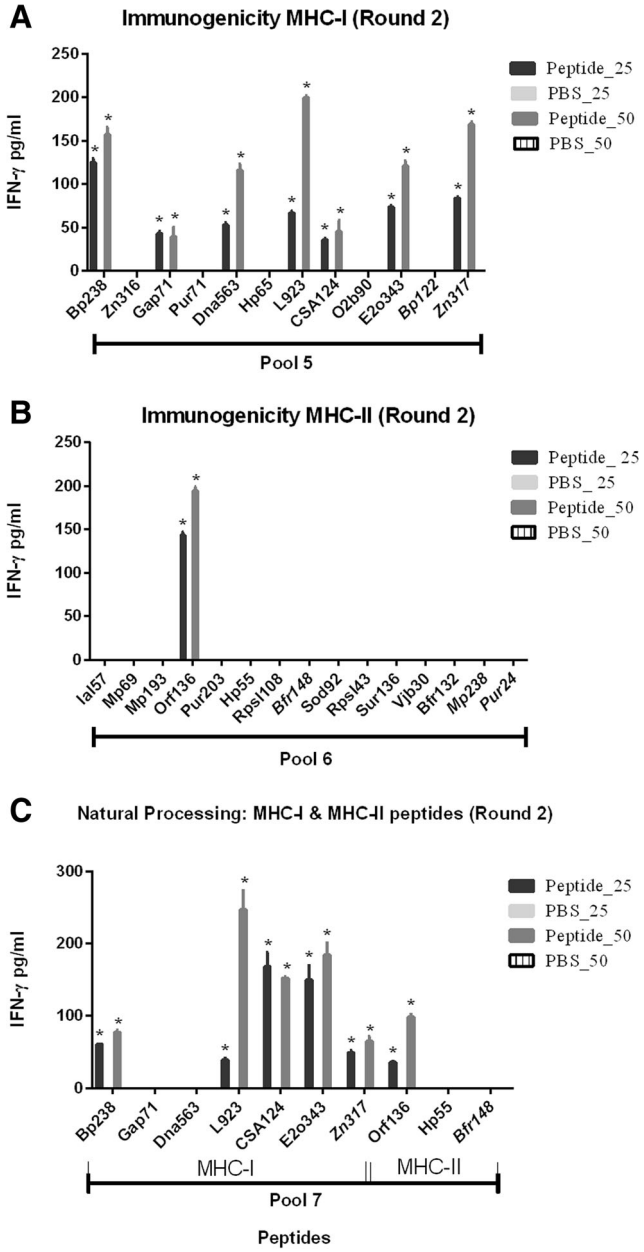

Fig. 3 In vivo peptide screening (round 2) by peptide immunogenicity for MHC-I (**a**)- and MHC-II-restricted T cell peptides (**b**). The shortlisted peptides were tested by natural processing (**c**). Mice were injected with pool of peptides as shown. Release of IFN-γ in culture supernatant of splenocytes was determined after stimulation with individual peptide of the pool. Significant differences of comparison with PBS group were determined by Student's *t* test and are indicated by *asterisk* ($p<0.05$). The results are shown at two stimulating concentrations of 25 and 50 μg ml^{-1}

Splenocytes of mice from groups APLG-NP, EPLG-NP and PBS did not produce IFN-γ (Fig. 4b). Except for peptide L923, stimulation by all other peptides induced higher levels of IFN-γ in EPLG-Pep or APLG-Pep groups than Pep-Ad group ($p<0.05$ to $p<0.0001$). Between EPLG-Pep and APLG-Pep groups, peptides Bp238, Zn317 and L923 produced higher IFN-γ in the former, whereas E2o343 and Orf136 produced higher IFN-γ in the latter ($p<0.001$).

Protection studies were carried out by challenging APLG-Pep, EPLG-Pep, Pep-Ad, APLG-NP, EPLG-NP, PBS and *B. abortus* strain S19 group mice with *B. abortus* strain 544. Mice of EPLG-Pep, APLG-Pep and Pep-Ad groups exhibited a significant degree (1.69, 1.49 and 1.42 log units of protection, respectively) of protection compared to control receiving PBS ($p<0.001$; Table 5). The mice immunized with S19 vaccine conferred 2.23 log units of protection. Results indicate that EPLG-Pep, APLG-Pep and Pep-Ad provided protection against *B. abortus* infection.

Evaluation of epitope-based pDNA vaccine

Synthetic gene of six selected epitopes was custom synthesized, was subcloned in pVax1 vector and was injected in the anterior tibialis of mice by in vivo electroporation. The mice were challenged 30 days after the last dose. Mice injected with pVaxPep exhibited a significant degree (1.66 log units of protection) of protection compared to control receiving PBS ($p<0.001$; Table 6). The mice immunized with S19 vaccine induced 2.05 log units of protection.

Discussion

The focus of the present work was to identify the T cell epitopes of *B. abortus* that can be relevant for enhancing the *Brucella*-specific cellular immunity of BALB/c mice. In order to increase the likelihood of getting large numbers of high-affinity binding epitopes as well as to keep the generated epitope design data to manageable levels, instead of using the full *Brucella* proteome, we chose 23 earlier reported protective proteins or virulence determinants to design the T cell epitopes. MHC-I- and MHC-II-restricted T cell epitopes were designed by tools available online, and the peptides that were predicted to be high binders were chosen. The prediction of MHC-II peptides appeared to be less accurate, as large numbers of high-scoring peptides were predicted by the two used softwares.

Since IFN-γ is a key cytokine for providing protection against *Brucella* infection (Jiang and Baldwin 1993; Paranavitana et al. 2005), therefore, the release of IFN-γ in splenocyte culture supernatant of mice was taken as a criterion for in vivo screening of T cell peptides. Further, screening of the peptides in the present study was decided not only by their ability to be immunogenic but also by their ability to be presented by MHC-I or MHC-II after host infection with intact *B. abortus*. Therefore, those peptides that released IFN-γ in peptide immunogenicity as well as in natural processing were selected. Similar approach was earlier used in MHC-I-restricted CD8 *Brucella* T cell peptide screening (Durward

Table 4 Peptides selected for immunogenicity and protection studies

Target Protein	Name peptide	Peptide sequence	Allele	Peptide immunogenicity	Natural processing
BP26	Bp238	SYNVSVNVV	H-2K^d	+	+
ZnuA	Zn317	YPQLIRNLA	H-2L^d	+	+
L9	L923	DGYARNFLL	H-2D^d	+	+
CSA	CSA124	NYVRLSGRL	H-2K^d	+	+
E2o	E2o343	APQSGILGM	H-2K^d	+	+
Orf	Orf136	KGVEAAHAA	H-2A^d	+	+

et al. 2010). In this study, a total of 37 high-affinity MHC-I- and 32 MHC-II-restricted T cell peptides were tested in mouse model, and after multiple rounds of screening, five MHC-I peptides, i.e. Bp238, Zn317, L923, CSA124 and E2o343, and one MHC-II peptide, i.e. Orf136, were finally selected for evaluation as epitope-based vaccine. Of the five MHC-I peptides, three were predicted by four softwares and one was predicted by two softwares, suggesting that prediction by MHC-I prediction tools is good and comparable. One peptide Zn317, however, was initially taken as NC yet showed release of IFN-γ in all experiments and was one of the finally selected peptides. This indicates that immunoinformatics tools for MHC-I prediction though may be good, they are yet to mature. Similar results were shown earlier by Durward et al. (2010) who also found that one of the NC peptides released significant amount of IFN-γ in mouse model. We did not find the prediction for MHC-II T cell epitopes to be that good. Although a large number of high-scoring epitopes were predicted, yet of the 32 synthesized peptides, only one could finally be selected.

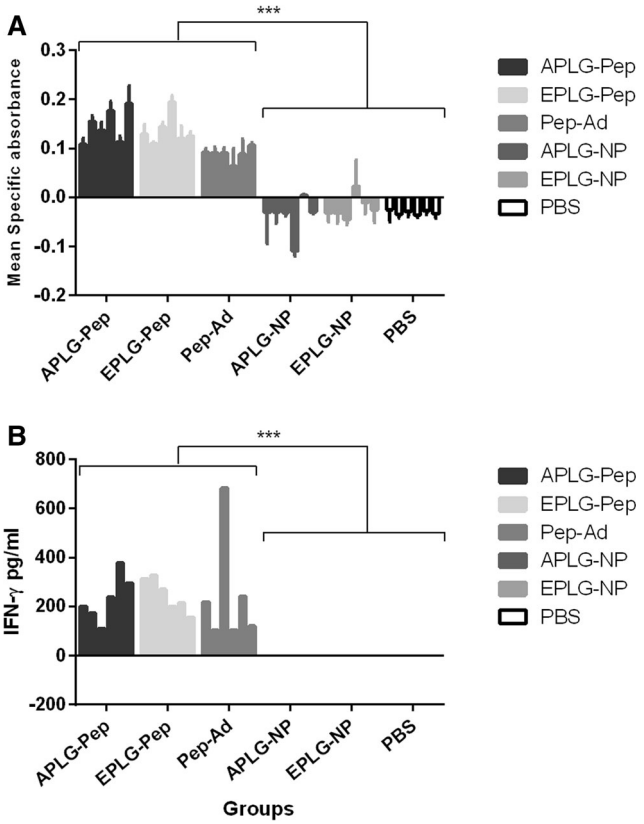

Fig. 4 Immunogenicity determination of peptide PLG formulations by splenocyte proliferation response (**a**) or IFN-γ production in the spleen cells (**b**). Splenocyte proliferation in response to peptides Bp238, Zn317, L923, CSA124, E2o343 and Orf136 is shown. APLG-NP and EPLG-NP group was stimulated with irrelevant peptides. Significant differences of comparison among groups were determined by one-way ANOVA and are indicated by ***$p<0.0001$

The selected peptides were evaluated for immunogenicity and protective ability in mouse model. The peptides were formulated with PLG microparticles for administration. Biodegradable lactide polymers, such as PLG microparticles, have been used extensively in research pertaining to delivery of novel vaccine and drug candidates. The encapsulation of antigen in a polymer matrix limits access of the biological fluid into the antigen until the time of degradation. The adjuvant effect achieved through encapsulation and adsorption of antigens to PLG microparticles was demonstrated by several groups (O'Hagan et al. 2004). The microparticles used in this study were of size smaller than 1.5 μm. Particle size was shown to be an important parameter affecting the immunogenicity of microparticles, and smaller particles (<10 μm) were found to be significantly more immunogenic than the larger ones (O'Hagan et al. 2004). Immunogenicity of peptides with PLG microparticles was evaluated in BALB/c mice. After stimulation with individual peptide, a significant proliferation of splenocytes and release of IFN-γ were observed in mice of APLG-Pep and EPLG-Pep groups when compared with equivalent NC (APLG-NP, EPLG-NP) or PBS groups. A similar response but of lower intensity was observed in Pep-Ad group. Similar to our work, more potent responses of PLG formulations than the established aluminium-based adjuvant have been shown earlier (Singh et al. 2004). Comparable immunogenicity between Freund's adjuvant formulated and microparticle-entrapped staphylococcal B enterotoxoid has also been shown (Eldridge et al. 1991). Delivery of hepatitis B virus peptides in PLG microparticles has earlier been shown by Moynihan and Howard (2001) to elicit T cell response. For determination of protective ability of any *Brucella* vaccine in mouse model, the reduction in the number of bacteria in

Table 5 Protection against *B. abortus* strain 544 in BALB/c mice by vaccination with peptides in formulation with PLG microparticles

Vaccine (n=6)	Adjuvant	[a]Log_{10} CFU of *B. abortus*/spleen	[b]Units of protection
EPLG-Pep	None	4.06±0.54	1.69*,***,†††
APLG-Pep	None	4.26±0.31	1.49*,***,††
Pep-Ad	Freunds' adjuvant	4.33±0.11	1.42*,**,†
EPLG-NP	None	5.13±0.64	0.62
APLG-NP	None	5.22±0.24	0.53
PBS	None	5.75±0.49	–
B. abortus S19	None	3.52±0.11	2.23*,***,†††

Representative data from two separate experiments

[a] The number of bacteria in spleens (CFU/spleen) is represented as the mean log CFU±SD per group

[b] Units of protection were obtained by subtracting the mean log CFU/spleen of the vaccinated group from the mean log CFU/spleen of the control (PBS) immunized group

Significantly different compared with value of PBS control (*p<0.001), EPLG-NP (**p<0.05), EPLG-NP (***p<0.01), APLG-NP (†p<0.05), APLG-NP (††p<0.01) and APLG-NP (†††p<0.001)

spleen is observed. Expectedly, APLG-Pep, EPLG-Pep and Pep-Ad immunization conferred protection against *B. abortus* infection (1.49, 1.69 and 1.42 units of protection). EPLG-Pep conferred best protection in terms of protection units, which suggests that peptides entrapped in PLG microparticles can be most suitable as vaccine delivery method. However, the protection conferred by S19 vaccine strain was superior to PLG-entrapped peptides.

The roles of both CD8+ and CD4+ T cells have been described in protection against *Brucella* infection in mice. There are reports that suggest either in vivo depletion of CD8+ T cells results in higher bacterial load of *B. abortus* in infected BALB/c mice or CD8+ T cells kill *Brucella* specifically (He et al. 2001; Murphy et al. 2001). Five MHC-I-restricted peptides used in this study are likely to be responsible for conferring protection in mice. Earlier, two MHC-I restricted CD8+ T cells peptides were not tested for protection (Durward et al. 2010). It is difficult to comment to what extent one MHC-II-restricted CD 4+ T cell peptide contributed for protection in mice in this study. It has been suggested earlier that IFN-γ-producing CD4+ T cells have a major role in clearing the *Brucella* bacteria and CD8+ T cells and humoral response have only modest role to play (Vitry et al. 2012). We do not know whether only one MHC-II-restricted T cell peptide is one of the reasons for lesser protection by PLG-formulated epitope vaccine than S19 vaccine in this study. Nonetheless, our results show that MHC-I- and MHC-II-restricted CD8+ and CD4+ T cell peptides contribute to release of IFN-γ and protection in mouse model.

The pDNA vaccines seem to offer the best approach to activate both cellular components of the immune response (Th1 and CD8+ T cell), owing to the intrinsic feature of DNA vaccine to produce endogenous antigen in professional antigen-presenting cells (Liu et al. 2004). Therefore, to further evaluate the usefulness of selected peptides as vaccine, a pDNA vaccine construct was made and injected to mice using in vivo electroporation. In vivo electroporation-enhanced delivery of pDNA has been used in many animals and has resulted in increased DNA uptake in muscle, leading to robust trans-gene expression levels and enhanced humoral and cellular responses (Estein et al. 2009). It was observed that the protection conferred by pDNA vaccine delivered by in vivo electroporation was significantly higher when compared with PBS control or naked vector (pVax1) control groups.

Table 6 Protection against *B. abortus* strain 544 in BALB/c mice by vaccination with plasmid DNA construct encoding epitope sequences

Vaccine (n=6)	Route	[a]Log_{10} CFU of *B. abortus*/spleen	[b]Units of protection
PBS	Intramuscular	5.58±0.45	–
pVax1	Intramuscular followed by in vivo electroporation	5.21±0.032	0.33
pVaxPep	-Do-	3.92±0.052	1.66[c]
B. abortus S19	Intraperitoneal	3.53±0.086	2.05[c]

[a] The number of bacteria in spleens (CFU/spleen) is represented as the mean log CFU±SD per group

[b] Units of protection were obtained by subtracting the mean log CFU/spleen of the vaccinated group from the mean log CFU/spleen of the control (PBS) immunized group

[c] Significantly different compared with PBS or pVax1 control group (p<0.001)

Protection offered by pDNA vaccine was 1.66 units and was comparable to S19 vaccine conferred protection.

Overall, results of this study prove that screened peptides are immunogenic and protective against *B. abortus* infection in mouse model. It is evident that the protection is mediated through release of IFN-γ cytokine. However, the protection conferred by selected peptides is lesser than the available S19 vaccine. Presence of more antigenic epitopes in S19 or CpG DNA in pDNA vaccine construct may have been the factors for better protection by S19 live or pDNA vaccine than the PLG-formulated epitope vaccine. It is also likely that cytokine other than IFN-γ or any other factors may also have a role in protection. New research on immunology of *Brucella* can give us better insight on this. Nonetheless, the results of this study support the feasibility of epitope-based brucellosis vaccine.

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Ethical statement All animal experiments conducted in this study complied with the relevant federal and institutional guidelines regarding use of laboratory animals.

Conflict of interest Authors declare that we have no conflict of interest.

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