Immunogenicity and protective efficacy of a *Brucella abortus* L7/L12 DNA vaccine

delivered via chitosan modified PLGA nanoparticles in mice

Uddhav Chaudhari [1,2,*], Satyabrata Dandapat [1,*], Sivasankar Panickan [1,3,*], Vimal Kumar [1,4],

Pradeep Mahadev Sawant [3]

[1] Immunology Section, ICAR-Indian Veterinary Research Institute, Izatnagar-243122, India.

[2] ICMR-National Institute for Research in Reproductive and Child Health, Mumbai-400012,

India.

[3] ICMR-National Institute of Virology, Pune-411001, India.

[4] ICMR-National JALMA Institute for Leprosy and other Mycobacterial Diseases, Agra-

282001, India.

[*]**Corresponding author**

drvetuddhav@gmail.com; satyadandapat2008@hotmail.com; sankarsiva905@gmail.com

Abstract

The present study evaluated the immunogenicity and protective efficacy of the chitosan (CS)-

modified poly-lactide-co-glycolic acid (PLGA) nanoparticles (NPs) delivering *Brucella*

abortus L7/L12 DNA in a mouse model. The NPs were prepared by solvent displacement

method and characterized for size, charge, morphology, cellular uptake and cytotoxicity. The

cationic CS-PLGA NPs were spherical with a mean size of ~165 nm with a positive zeta

potential (+20 mV). DNA loading efficiency of 1.2% and DNA adsorption shifted zeta

potential to -45 mV. *In vitro* studies in RAW 264.7 cell line demonstrated efficient uptake of

the DNA loaded cationic NPs and expression of L7/L12 protein. Intramuscular immunization

of the L7/L12 DNA vaccine loaded CS-PLGA NPs elicited both humoral and cell-mediated

immunity with upregulation of Th1 and Th2 cytokines along with induction of IgG antibodies

in mice. IFN-γ, IL-2, and IL-4 levels were significantly (P < 0.001) higher than control

group. The protective efficacy of the DNA loaded NPs against virulent *B. abortus* 544

infection (10^5 CFU) was significantly higher than that of the naked DNA (P<0.001). These

findings suggest that the CS-PLGA NPs were efficiently delivered L7/L12 DNA and

exhibited adjuvant potential, conferring protection against experimental murine brucellosis.

Keywords: *Brucella abortus*, L7/L12 DNA, Cationic PLGA nanoparticles, Chitosan, Mice

1. Introduction

Brucellosis is a global public health threat and an economically important disease caused by

Brucella abortus, an intracellular facultative pathogen that affects domestic animals and

humans. In cattle, brucellosis primarily affects the reproductive system causing late-term

abortion, infertility, delivery of weak calves and significant economic loss to the dairy

industry. Undulant fever, arthritis and endocarditis are the major signs of the disease in

humans. In India, the reported prevalence in humans ranges from 0.8-26.6% and it is mostly

due to *B. abortus*. There is no licensed vaccine available for human brucellosis. However, the

burden of brucellosis in cattle is mainly controlled by vaccination with live attenuated

vaccines, RB51 or strain 19 and in small ruminants with strain Rev 1 (*Brucella melitensis*).

However, these vaccines have certain drawbacks, as they cause abortion in pregnant animals

and also the vaccine virus is pathogenic and prone to the risk of reversion to virulence, which

might spread to humans through contacts. These vaccines cannot be used in humans as these

strains are too virulent and have a low therapeutic index. Further, they interfere with

serological diagnosis and may also be secreted in milk. Being a live vaccine, it is not

recommended for use in brucellosis-free countries. Therefore, safe and efficient vaccines are

required to control the disease in animals and humans. Usually, the *Brucella* organisms

multiply intracellularly, especially in macrophages or reticulo-endothelial system. Protective

immunity against *Brucella* infection seems to be mediated by both humoral and cellular

immunity; though cell mediated immunity (CMI) is expected to play a critical role in

protection, in which IFN-γ and CD8+ lymphocytes are the major effectors (Oliveira and

Splitter, 1995; Skendros and Boura, 2013). IFN-γ activates macrophages which exhibit anti-

Brucella activity (Jones and Winter, 1992). To develop better vaccines, many strategies have

been attempted, but they were not much efficacious than the standard vaccines S-19.

However, DNA vaccines have been used successfully for many diseases (Ulmer et al., 1993;

Boyer et al., 1997) as they can induce better CMI, which might also be effective against

Brucella infection.

The DNA vaccine is an attractive alternative to the existing live attenuated vaccines, as they

provoke both humoral and cellular immune responses with many advantages like convenient

to design, good safety profile and low cost of production. The ribosomal protein L7/L12 is a

major antigenic component with a protective role, and the gene is conserved among *Brucella*

species, which is immunodominant that induces both Th1 and Th2 mediated immune

responses (Bachrach et al., 1994; Golshani et al., 2015). Therefore, immunization with such

DNA vaccine along with efficient delivery system might be beneficial for induction of better

immune response against *Brucella*. In this context, the use of poly-lactide-co-glycolic acid

(PLGA) and chitosan nanoparticles (NPs) or microparticles for delivery of vaccine candidates

has advantages because of their property of slow and pulsatile release of antigen and these are

potent inducers of humoral and CMI (Silva et al., 2016; Dmour and Islam, 2022). Better

immune response with caprolactone microparticles coated with *Brucella* outer membrane

complex has been reported against *Brucella ovis* infection in rams (Muñoz et al., 2022). The

PLGA NPs are well recognized as ideal drug delivery carriers for their biocompatibility and

biodegradability and are approved by U.S Food and Drug Administration (FDA) (Blasi,

2019). A recent study reported better immunogenicity and protective efficacy of inactivated

Newcastle disease vaccine with PLGA NPs in chickens (Ananda Kumar et al., 2023). Further,

PLGA coated tuberculosis DNA vaccine has also shown better efficacy than the naked DNA

vaccine (Mollenkopf et al., 2004). In the earlier studies, the recombinant L7/L12 protein with

PLGA NPs has been shown to induce strong CMI and conferred protection against *Brucella*

infection in mice (Singh et al., 2015), although the protective efficacy was shown to be lower

than the live attenuated *B. abortus* S19 vaccine. The main disadvantage with PLGA NPs is

the burst release at initial stage, which can be overcome by coating with chitosan that can

prolong the drug delivery (Wang et al., 2013). Chitosan (CS) is a polysaccharide and

copolymer of glucosamine and N-acetyl glucosamine, derived from chitin which is found in

shells of crustacean (Darwesh et al., 2018). It is also biodegradable, highly biocompatible,

non-toxic with immunomodulatory properties (Abourehab et al., 2022) and its use has been

approved by FDA (Kantak and Bharate, 2022). The CS has been used as a successful

adjuvant with influenza (Ghendon et al., 2008), hepatitis A (AbdelAllah et al., 2020), rift

valley fever (El-Sissi et al., 2020), Newcastle disease (Yang et al., 2020). It induces both Th1

and Th2 mediated immune response (Shim et al., 2020). Recently, our chitosan coated PLGA

nanoparticle with infectious bursal disease virus provided better protection than commercial

vaccine (Kumar et al., 2025). With this background, the present study has been focused

towards preparation and *in vitro* characterization of CS-modified PLGA NPs and evaluation

of immunogenicity and protective efficacy of *Brucella* L7/L12 DNA vaccine formulation

with CS-PLGA NPs in mice model for exploring an alternative strategy for vaccination

against brucellosis.

2. Materials and Methods

2.1 Ethical statement

All experimental procedures in mice were approved by Institutional Animal Ethics

Committee of ICAR-Indian Veterinary Research Institute and conducted in accordance with

guidelines of the Committee for the Purpose of Control and Supervision of Experiments on

Animals (CPCSEA). This work was reviewed and approved by Joint Director Research and

Academic section of ICAR-Indian Veterinary Research Institute (IVRI).

2.2 Experimental mice and the bacterial strains

Apparently healthy 6–8-week-old Swiss albino mice were obtained from the laboratory

animal resource section of Indian Veterinary Research Institute (IVRI), Izatnagar, India. The

mice were maintained in cages and were provided with sterile feed and water *ad libitum*.

Brucella abortus 544 virulent strain and *Brucella abortus* S-19 live vaccine strain were

obtained from the *Brucella* laboratory, Division of Veterinary Public Health, IVRI, Izatnagar.

2.3 Preparation of chitosan coated PLGA NPs

PLGA NPs were prepared by the solvent displacement method (Messai et al., 2005). Briefly,

0.5% (w/v) PLGA was prepared in 10 mL of acetone and added drop-wise to 10 mL of sterile

filtered distilled water containing 1% polyvinyl alcohol (PVA) as emulsion stabilizer with

continuous stirring. The acetone was allowed to evaporate overnight. The NPs were pelleted

by high-speed centrifugation at 24,000 rpm for 30 min and resuspended in limited amount of

filtered pure distilled water and stored at 4°C. The PLGA NPs were negatively charged and to

make them cationic, chitosan was coated on preformed PLGA NPs as per previous method

(Messai et al., 2005). The final coating procedure was done by incubating the preformed NPs

with different concentrations of chitosan (125 µg, 250 µg, and 500 µg) per 5 mg of NPs in 1

mL acetate buffer (pH 5.5) with continuous shaking for 1 h at room temperature (25°C). The

CS-PLGA NPs were pelleted by centrifugation at 20000 rpm for 10 min. The coating of the

chitosan was confirmed by analyzing zeta potential of the NPs and by transmission electron

microscopy (TEM). For cellular uptake study, PLGA NPs encapsulating the fluorescent dye

(coumarin-6) were prepared by solvent evaporation method (Vila et al., 2002).

2.4 Particle size and zeta potential analysis

The size distribution of PLGA NPs was determined by quasi-elastic light scattering at $25^{\circ}C$,

at an angle of 90°, using a zeta sizer 3000 HS (Malvern Instruments, UK). Highly diluted

colloidal dispersions of NPs in pure distilled water were used for size and zeta potential

measurement. Further, the morphology and size of NPs were determined by TEM (FEI 12 BT

120 kv). The diluted NPs sample was loaded on copper grids stained with 2% uranyl acetate

and observed under transmission electron microscope.

2.5 Cytotoxicity study of cationic PLGA NPs

Vero cells were seeded in 96-well tissue culture plate (100 µL/well) at the concentration of

10^{6} cells/mL in DMEM complete medium, supplemented with 10% fetal bovine serum (FBS)

and incubated at $37^{\circ}C$ with 5% CO_2. After attaining 80% confluency, the cells were further

incubated with 100 µL of different concentrations (10, 50, and 500 µg/mL) of PLGA NPs for

24 h at $37^{\circ}C$. The negative control with 100 µL/well of medium alone and positive control

with 0.2% Triton-X 100 (for cytotoxicity) were also included. After incubation the medium

was removed and cells were washed twice with PBS. Then, 85 µL of fresh culture medium

and 15 µL of 3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyl tetrazolium bromide (MTT) dye

solution (5 mg/mL in PBS) was added to the wells and further incubated for 4 h. Then, 100

µL of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan

precipitates formed and finally, optical density (OD) was measured by the microplate reader

at 550 nm. The percentage of relative growth was calculated by using the formula: (OD of

nanoparticle treated sample/OD of negative control) x100.

2.6 Cellular uptake of PLGA NPs

Murine macrophage cell line (RAW 264.7) and Vero cells were used for cellular uptake assay

of NPs as per previous method (Davda and Labhasetwar, 2002). Both cell lines were

maintained in DMEM (Gibco), supplemented with 10% FBS, penicillin (100 IU/mL),

streptomycin (50 µg/mL) and 25 mM HEPES. The cells were seeded at 2×10^4 cells/well on

the cover-slips placed in a 6-well tissue culture plate. After 80% confluency, the culture

medium was replaced with fresh medium without serum, containing 100 µg/mL of coumarin-

6 (dye)-encapsulated PLGA NPs and incubated at 37°C. At different time points (30 min, 1 h,

2 h and 4 h) the cells were harvested, cover-slips were washed thrice with sterile PBS and

fixed with 1:1 methanol and acetone for 15 min. The cover-slips were subsequently mounted

with 90% glycerol in PBS and observed under Confocal Microscope.

2.7 Plasmid DNA adsorption onto CS-PLGA NPs

Plasmid DNA (L7/L12) was adsorbed onto the surface of cationic CS-PLGA NPs as per

previous method (Messai et al., 2005). Briefly, 1 mg aliquots of nanoparticles (NPs) coated

with different concentrations of chitosan (section 2.2) were separately dispersed in 1 mL of

acetate buffer in Eppendorf tubes. These were then mixed with varying concentrations of

plasmid DNA (3–18 µg/mL) and incubated on a magnetic stirrer at 4°C for 6 hours by

placing the tubes in a water-filled beaker. DNA binding to NPs was checked by agarose gel

electrophoresis (1% agarose, 0.5 µg /mL ethidium bromide in Tris-acetate EDTA buffer)

based on mobility of the DNA-NPs complex and the free form of DNA, visualized under UV.

The amount of plasmid DNA adsorbed onto NPs was derived by determining the amount of

free plasmid concentration in the supernatant recovered after centrifugation (20000 rpm, 15

min), by UV spectrophotometry.

2.8 *In vitro* release of plasmid DNA from PLGA NPs

10 mg of plasmid DNA loaded PLGA NPs were suspended in 1 mL of PBS and incubated in

shaking incubator at 37°C. At different time intervals (24 h, 48 h, 7 d, 14 d, 21 d and 30 d),

the PBS was collected after centrifugation at 12000 rpm for 10 min and each time NPs were

resuspended with 1 mL of fresh PBS. The amount of DNA release was determined

spectrophotometrically by taking absorbance at 260 nm and purity of the released DNA was

checked by 1% agarose gel electrophoresis.

2.9 *In vitro* transfection of DNA loaded CS-PLGA NPs

The RAW 264.7 cells were grown on cover-slips in 6-well tissue culture plate (10^6 cells/well)

with complete DMEM incubated at 37°C with 5% CO_2. After attaining 80% confluency the

medium was removed and the cells were incubated with either 10 µg of CS-PLGA+DNA

loaded NPs or commercial transfection reagent lipofectamine (Invitrogen) with 10 µg DNA

or naked plasmid in 0.5 mL of serum-free medium for 4 h at 37°C. Then, 1.5 mL of complete

fresh medium was added and further incubated for 48 h at 37°C. The expression of

transfected DNA was determined by detecting the L7/L12 protein in the cells by indirect

immunofluorescence test (IIFT). Briefly, the cells grown on cover-slip were washed with

PBS and fixed with chilled 1:1 methanol and acetone for 15 min. After fixation cells were

again washed with PBS and blocked with 2% BSA. Then primary antibody (anti-rL7/L12

protein, raised in rabbit) at the dilution of 1:200 was added and incubated at 37°C for 1 h.

Subsequently, cover-slips were washed with PBS and incubated with secondary antibody,

goat anti-rabbit FITC (Sigma) at the dilution of 1:100 for 1 h at 37°C. Finally, the cover-slips

were washed with PBS and removed from the plate and mounted with 90% glycerol in PBS

on clean glass slides and observed under Confocal Laser Scanning Microscope.

2.10 Immunization experiment

Seventy-five healthy Swiss albino mice were randomly divided into five groups (n=15 per

group) and immunized intramuscularly in the left tibialis anterior muscle with various

formulations (Table 1) in 100 µL of phosphate buffered saline (PBS).

2.11 Serum antibody levels

The levels of L7/L12 antigen specific total IgG/IgG1 and IgG2a antibodies in the serum

samples of vaccinated and control groups at two weeks post priming, two weeks post booster

and two weeks post-challenge were measured by indirect ELISA. Briefly, 96 well ELISA

plates (Nunc) were coated with 100 µL of purified L7/L12 antigen (2.5 µg/mL) in carbonate-

bicarbonate buffer (0.05M, pH 9.6) at 4°C by overnight incubation. The plates were washed

thrice with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) and blocked with

1% BSA in PBS-T. After washing again thrice, 100 µL of the serially diluted sera samples

were added and incubated for 90 min at 37°C. Then, after washing the plates were incubated

with 100 µL/well of goat anti-mouse IgG or IgG1or IgG2a HRPO conjugates (Sigma, USA)

at 1:10,000 dilution for 1 h at 37°C. After washing, 100 µL of substrate solution containing

200 µmol of o-phenylenediamine (OPD) and 0.04% H_2O_2 was added to each well and

incubated for 15 min at room temperature. The reaction was stopped by addition of 50

µL/well of 1 M H_2SO_4 and the absorbance was measured at 492 nm. The mean OD of 10

unimmunized control mice sera samples at 1:40 dilutions plus 2 times standard deviation of

its mean value were taken as the cut off. The titer of each sample was calculated as the

reciprocal of the highest dilution that gave OD higher than the cut off value. The experiment

was performed in triplicates and repeated twice.

2.12 Lymphocyte proliferation assay

After 1st, 2nd and 4th week of booster immunization, three mice from each group were

sacrificed and their spleens were collected under aseptic conditions for splenocytes separation

to detect *in vitro* cell mediated immune response by lymphocyte proliferation assay. Several

perfusions were made into spleen with sterile PBS by using 1 mL tuberculin syringe to wash

out splenocytes. About 3 mL of collected splenocytes was layered over 2 mL of histopaque-

1077 (Sigma, USA) and centrifuged at 1600 rpm for 40 min at 25°C (Chaturvedi et al., 2010).

The interface ring rich with mononuclear cells were collected carefully and washed twice

with PBS at 1200 rpm for 10 min. Finally, the cells were suspended in phenol red free RPMI-

1640 complete medium and 100 µL of cell suspension (5 x 10^6 cells /mL) were added to

triplicate wells in 96 well flat-bottom tissue culture plates. The splenocytes were stimulated

with 100 µL/well of either purified rL7/L12 protein (10 µg/mL) or Con A (20 µg/mL) or

medium alone (unstimulated control) by incubating at 37°C with 5% CO_2. After 96 h, 100 µL

of supernatant was carefully removed and 15 µL of MTT solution (5 mg/mL in PBS) was

added to each well and further incubated for 4 h at 37°C. Finally, 150 µL of DMSO was

added to each well and mixed thoroughly in order to dissolve formazan crystals. The intensity

of colour development was measured by taking absorbance at 550 nm in a microplate reader.

Stimulation index (S.I) was calculated as the ratio between mean OD value of stimulated

cells and the mean OD value of the unstimulated control.

2.13 Cytokine assay by Sandwich ELISA

Splenocytes of three mice from each group were isolated and plated in 96-well flat bottom

tissue culture plates as described (section 2.11). Cells were stimulated with either rL7/L12

protein (10 µg/mL) or Con A (20 µg/mL) or media alone (unstimulated control) for 48 h at

37°C with 5% CO_2. After stimulation, supernatant from all the wells were collected in sterile

eppendorf tubes for cytokine assay. Quantitation of murine IFN-γ, IL-2, and IL-4 was done

by using Sandwich ELISA kit (Cytolab/ Pepro Tech Inc.) as per instructions in the kit.

2.14 Protective efficacy

Four weeks after the booster immunization, all the mice were challenged intraperitoneally

with 10^5 colony forming units (CFU) of *Brucella abortus*, strain 544 in 200 µL of sterile

PBS. Four weeks after challenge, five mice from each group were sacrificed for bacterial

clearance assay in spleen. The spleen was removed aseptically and homogenized in 2 mL of

tryptose phosphate broth (TPB). 20 µL of suspension was placed on TP agar, incubated for 96

h at 37°C and *B. abortus* 544 colonies were counted. The difference of log organism burden

between the PBS control group and each vaccinated group was considered as protection in

terms of bacterial clearance. At the end of experiment all mice were humanely euthanized in a

CO_2 chamber.

2.15 Statistical analysis

Data are presented as mean ± standard error of mean. The appropriate statistical test used are

indicated in the corresponding figure legends. *P* values less than 0.05 were considered as

statistically significant. (* *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001 and **** *P* < 0.0001).

GraphPad Prism version 8.0.2 (San Diego, CA, USA) was used for data analysis.

3. Results

3.1 Characterization of CS-PLGA NPs

On zeta potential analysis, the blank PLGA NPs showed negative charge of -5 mV. The

surface of PLGA NPs was modified by adsorbing chitosan (cationic polymer) to facilitate

binding of negatively charged DNA. The chitosan coated PLGA NPs were spherical in shape

with almost homogeneous size distribution, with mean size of 165 nm as determined by

particle size analyzer and by TEM. The coating of chitosan polymer on PLGA NPs was

indicated by observation of two layers consisting of PLGA core surrounded by a thin layer of

chitosan under TEM. The chitosan coating was further confirmed by the zeta potential of CS-

PLGA NPs, which exhibited positive charge (+20 mV) in distilled water.

262 **3.2 Cytotoxicity study of cationic PLGA NPs**

263 Cytotoxic effect of cationic PLGA NPs (if any) on Vero cells was examined by MTT assay.

264 Different concentrations of cationic PLGA NPs (10 µg, 50 µg and 500 µg/mL), when

265 incubated with cells showed 98%, 97% and 95% relative growth, respectively, indicating that

266 cationic NPs were non-toxic, whereas the positive control with Triton-X (0.2%) showed

267 relative growth below 10%.

268 **3.3 Cellular uptake of PLGA NPs**

269 A time dependent cellular uptake of the coumarin-6 fluorescent dye-encapsulated PLGA NPs

270 was observed in both RAW 264.7 and Vero cells. The confocal microscopic images revealed

271 that after 30 min of incubation NPs were distributed at the periphery of the cytoplasm and as

272 the time of incubation increased from 1 h to 4 h, there was increase in intensity of

273 fluorescence and the number of cells showing fluorescence in all over the cytoplasm and the

274 nucleus of the cells (Fig. 1A, B). The cellular uptake of PLGA NPs was quicker and more

275 efficient in RAW 264.7 cells as compared to non phagocytic Vero cells at various time points.

276 The z-stack image of cells by confocal microscopy showed internal localization of NPs at 4 h

277 of incubation.

278 **3.4 Plasmid DNA adsorption to CS-PLGA NPs**

279 Polyanionic L7/L12 DNA binding with the cationic CS-PLGA NPs was confirmed by

280 immobilization of the DNA-NPs complex in agarose gel. The optimum DNA binding (12 µg

281 of DNA per 1 mg of CS-PLGA NPs) was observed, when PLGA NPs were coated with 125

282 µg/mL of chitosan (section 2.2), with 90-100% binding efficiency and 1.2% loading

283 efficiency. Further, DNA adsorption resulted in a shift of the charge of CS-PLGA NPs to

284 negative zeta potential -45 mV.

285 **3.5 *In vitro* DNA release kinetics**

The *in vitro* release rate of plasmid DNA from the cationic PLGA NPs were initially about

25% within 24 h in PBS at 37°C. Subsequently, the rate of release was slower, but up to 30 d

of incubation about 90% of the DNA was released. The DNA released at different time points

i.e. 24 h, 48 h, 7 d, 14 d, 21 d and 30 d were intact and showed single band on electrophoresis

on 1% agarose gel indicating stability of DNA even after adsorption onto PLGA NPs.

3.6 *In vitro* transfection of DNA loaded CS-PLGA NPs

After *in vitro* transfection, the localization of expressed protein in RAW 264.7 cells were

detected by IIFT using specific antiserum. The cells transfected with L7/L12 DNA loaded

CS-PLGA NPs and commercial reagent lipofectamine with DNA showed fluorescence,

indicating transfection and subsequent expression of specific protein in the cells. However,

commercial transfection reagent (lipofectamine) showed a higher number of transfected cells

as compared to the plasmid DNA loaded CS-PLGA NPs (Fig.2).

3.7 Serum antibody levels in immunized mice

The levels of IgG, IgG1 and IgG2a antibodies at 14 d post priming and 14 d post booster

immunization as well as at two weeks post challenge infection with *B. abortus* are shown in

Fig. 3A, 3B, 3C, respectively. There was anamnestic response observed after booster and post

challenge with the maximum antibody titer recorded after challenge infection. The antibody

titers of IgG in group-I (DNA+NPs) and group-II (naked DNA) after booster immunization

were 750 and 450, respectively. Similarly, the titers of IgG1 were 113 and 75, and IgG2a

were 225 and 163, respectively. The mice, sham-immunized with vector DNA (group III) and

PBS control (group IV) did not show any specific antibody response.

3.8 Lymphocyte proliferation

There was gradual increase in proliferation of splenocytes of the mice immunized with

L7/L12 DNA loaded NPs (group I) and naked DNA (Group II) at different time points post

booster immunization. Splenocytes from group I and II showed significantly higher ($P < 0.01$)

proliferation than that of control group. Mice of group I exhibited highest lymphocyte

proliferation (1.60±0.028) followed by group II (1.42±0.043) at 4th week of booster (Fig. 3D).

3.9 Cytokine levels

The IFN-γ levels in the culture supernatant was maximum at 4th week post-booster

immunization in group I (2500±0.053 pg/mL) and group II (1768±0.034 pg/mL) as compared

to the control group IV (67±0.027 pg/mL) (Fig. 4A). The production of IL-2 and IL-4 upon

stimulation with rL7/L12 was found to be weak as compared to IFN-γ. The group I and II

showed highest amount of IL-4 (255 ±0.063 and 185±0.14 pg/mL, respectively) at 2nd week

post-booster. However, highest production of IL-2 was detected at the 4th week in group I

(419±0.078 pg/mL) and group II (349±0.045pg/mL) (Fig. 4B & 4C).

3.10 Protection against challenge infection

Following challenge infection in the vaccinated mice with virulent *B. abortus* 544, the

difference in log organism burden between the PBS control group (unimmunized mice) and

each of the vaccinated groups was considered as protection in terms of reduction in bacterial

load (Fig. 5). The log protection in group I (DNA+NPs), group II (naked DNA) and group V

(*B. abortus* S19 vaccine) was 1.9, 1.2 and 2.37, respectively (Table 2).   These findings

demonstrate group I (DNA+NPs, *P*<0.0001), group II (naked DNA, *P*=0.0017) and group V

(S-19 vaccine, *P* <0.0001) showed significantly higher protection as compared to the PBS

control group.

4. Discussion

Brucellosis is an important zoonotic disease that mostly affects cattle and many cases in

humans have also been reported. As there is no availability of licensed human vaccine,

controlling the disease in animals might reduce the spread of infection to humans. Although

few vaccines are available to control the disease in animals using *B. abortus* S-19, RB-51 and

Rev-1 strains etc., these live vaccines are pathogenic to humans and induce abortion in

pregnant cattle. These drawbacks highlight the need for improved vaccine strategies. Hence,

some previous studies were focused to deliver safe vaccine that can confront the

disadvantages associated with the existing vaccines, but there was limited success and none

of them was better than the standard live vaccines S-19 or RB-51. However, an alternate

strategic approach using DNA vaccines was able to induce both humoral and cellular immune

responses. Since *Brucella abortus* is an intracellular pathogen, the cell-mediated immunity

plays a crucial role for control of the disease, in which, L7/L12 DNA has been reported to

induce strong T cell response (Mallick et al., 2007). The recombinant L7/L12 protein and

plasmid encoding the L7/L12 gene can elicit strong CMI and protection from *Brucella*

infection in mice; however, the protective efficacy is much lower than the live attenuated

Brucella abortus vaccine S19 (Kurar, 1997; Luo et al., 2006; Yu et al., 2007; Isore et al.,

2014). In earlier studies, *Brucella* genes encoding ribosomal protein L7/L12 (Senevirathne et

al., 2020), divalent L7/L12-Omp 16 (Luo et al., 2006), lumazine synthase DNA (Velikovsky

et al., 2002), bacterioferritin DNA (Al-Mariri et al., 2001b) have also been tested as DNA

vaccines in mice.

Given the potential of DNA vaccines in inducing both humoral and CMI response, we have

investigated the immunogenicity and protective efficacy of chitosan-modified PLGA NPs

with L7/L12 plasmid in mice against *Brucella* infection. Our results revealed that delivery of

plasmid DNA through the CS-PLGA cationic NPs was successful and further *in vitro*

transfection study established the protein expression in RAW 264.7 cell line (Murine

macrophage). Upon immunization the plasmid was likely expressed in host cells and

stimulated immune response. The cationic NPs were optimum in size to be captured by

antigen presenting cells to elicit immune response as evidenced by our results. The CS-PLGA

NPs release DNA in a controlled manner as demonstrated in our *in vitro* release kinetics,

which favors prolonged immune response. In correlation to our findings, previous studies

reported that PLGA-chitosan NPs provide sustained release of the drug up to 140 h *in vitro*

and could be a good approach to improve bioavailability of the entrapped drug (Bandil et al.,

2023). Recently, chitosan-modified PLGA NPs have also been used for drug delivery to

enhance gastric retention and controlled release (Lestari et al., 2024). Our immunization

experiment revealed that the DNA loaded CS-PLGA NPs have induced significant levels of

antibody as well as CMI response. Further, this experimental vaccine formulation afforded

protection in terms of bacterial clearance in the spleen up to 1.9 $\log_{10}$. Previous studies

reported that vaccine candidates (L7/L12 protein) generated a log protection of 1.69 (Singh et

al., 2015), 1.61 (Mallick et al., 2007) and the L7/L12 DNA vaccine showed 1.26 (Kurar,

1997). In correlation with previous reports, the higher protection level observed in the present

study (1.9 log□□ units) may be attributed to the use of CS-PLGA nanoparticles, which

favors in sustained release and the adjuvant effect helps in potent induction of humoral as

well as CMI response that ultimately conferred efficient protection against virulent *Brucella*

organisms. Earlier workers also have reported that chitosan was able to enhance neutralizing

antibody titers against rift valley fever virus and pertussis toxin (Jabbal-Gill et al., 1998; El-

Sissi et al., 2020). Further, the induction of cytokines is the prime criterion for generating a

potent immune response. In our study, cytokine profiling revealed that the L7/L12 loaded

NPs elicited a significant expression of IFN-γ in the immunized mice (Fig. 4A), which might

have played a crucial role in the activation of macrophages and cytotoxic T-lymphocytes

(CTL) and facilitated the clearance of bacteria. The earlier workers also reported that the

IFN-γ plays an important role in the resolution of brucellosis (Zhan and Cheers, 1993; Eze et

al., 2000). In addition, the CS-PLGA NPs with L7/L12 DNA vaccine has stimulated strong T

cell response than the L7/L12 DNA alone as evidenced by antigen specific lymphocyte

proliferation (Fig.3D). The earlier worker also reported that intramuscular injection of the

L7/L12 gene resulted in specific antibodies and T-cell responses with significant protection

against *B. abortus* infection (Kurar, 1997). Similarly, a bivalent DNA vaccine encoding the

Omp16 and L7/L12 genes induced strong humoral and cellular immune responses with

significant protection against *B. abortus* 544 (Luo et al., 2006).

The IgG2a is essential for protection against intracellular pathogens (Rostamian et al., 2017).

The enhanced titer of IgG2a over IgG1 in the present study indicated the isotype switching

under the influence of IFN-γ (Th1 cytokine) due to a predominant stimulation of cellular

response. In correlation with the present findings, earlier workers also reported an induction

of IgG2a by the *Brucella* DNA vaccine encoding lumazine synthase and bacterioferritin/P39

(Al-Mariri et al., 2001a; Velikovsky et al., 2002). Cationic microparticles adsorbed with

plasmid DNA encoding Japanese encephalitis virus envelope protein also induced IgG2a over

IgG1 (Kaur et al., 2004). Induction of strong IFN-γ along with IgG2a indicates stimulation of

Th1 biased cell mediated immunity. In the present study, production of IL-4 by lymphocytes

of the immunized mice indicating its role in humoral immunity and IL-2 production supports

the CMI response. In our study following challenge, an anamnestic response in the antibody

titer and cytokines levels were observed. The similar finding of post-challenge enhancement

in antibody titer has been reported in avian influenza vaccine experimentation (Panickan et

al., 2022). Overall, the L7/L12 DNA loaded CS-PLGA NPs elicited efficient humoral and

cell-mediated immune response along with better protection against *Brucella* infection.

Further, unlike live vaccines the L7/L12 DNA will be safe as it is a subunit vaccine that lacks

virulence factor. Therefore, vaccines containing recombinant protein or their coding DNA can

be suitable alternatives to the conventional vaccines, because they can reduce unwanted side

effects. To the best of our knowledge, this is the first study on chitosan-modified PLGA NPs

with L7/L12 DNA vaccine in mice against *Brucella* infection.

5. Conclusion

The plasmid DNA encoding *B. abortus* L7/L12 with the CS-PLGA NPs elicited both cell mediated and humoral immune response in the immunized mice and also afforded protection against the virulent *Brucella abortus* 544 challenge infection. The study indicated that the NPs made up of a combination of PLGA and chitosan is a promising DNA delivery system that is more effective than the traditional adjuvant and also provides an insight for the development of effective vaccines against *Brucella*. The present findings warrant further investigation of the mechanistic activity and the potential benefits of PLGA-chitosan NPs based delivery system cum adjuvant.

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Authors' contributions: S.D and U.K.C conceptualized and designed the study, performed experiments and wrote the manuscript. S.P. performed data analysis and prepared figures. V.K and P.M.S edited and reviewed the manuscript. All the authors read and approved the final manuscript.

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Table captions

Groups (n=15)	Inoculum	Dose	Route
I	Chitosan coated PLGA nanoparticles with L7/L12 DNA	50 µg	I/M
II	L7/L12 naked DNA	50 µg	I/M
III	Vector (control)	50 µg	I/M
IV	PBS (negative control)	100 µl	I/M
V	*Brucella abortus* S19 vaccine	1×10^5	I/P

	(positive control)	CFU/mice	

606 **Table 1.** Experimental design and immunization schedule. All the groups were boosted 3

607 weeks post priming.

Experimental groups	Log$_{10}$ CFU of *B. abortus* in spleen (mean±SE)	Log$_{10}$ units of protection
I. Chitosan coated PLGA nanoparticles with L7/L12 DNA	3.79±0.07^a	1.9
II. Naked DNA	4.49±0.31^b	1.2
III. Vector	5.56±0.054	0.13
IV. PBS	5.69±0.067	0.00
V. *B. abortus*-S19 vaccine	3.32±0.049^a	2.37

Table 2. Bacterial loads in the spleen of different immunized groups of mice after challenge

infection. Four weeks after the booster immunization, all the mice were challenged

intraperitoneally with 10^5 CFU of *Brucella abortus*, strain 544 and then organism burden in

the spleen was determined four weeks post challenge. Units of protection was calculated by

subtracting mean log$_{10}$ CFU in the spleen of vaccinated groups from that of PBS control

group and differences of *Brucella* organism counts were analyzed by student's-t test. Values

marked with a (P <0.0001) and b (P <0.001) significantly differ from PBS control

624 **Figure captions**

Fig. 1 Confocal microscopic images of cellular uptake of coumarin-6 dye encapsulated PLGA NPs in murine macrophage (RAW 264.7) cell line (1A) and in non phagocytic Vero cells (1B). Cells were imaged at 30 min (a), 1 h (b), 2 h (c) and 4 h (d) post adsorption.

Images were obtained by FITC filter showing time dependent increase in the intensity of

fluorescence in cytoplasm and nucleus

Fig. 2 Confocal microscopic images of murine macrophage (RAW 264.7 cells) transfected

with (A) L7/L12 plasmid DNA loaded CS-PLGA NPs, (B) plasmid DNA complexed with

lipofectamine showing *in vitro* protein expression, detected by IIFT and (C) cells transfected

with naked plasmid DNA as control

Fig. 3 *Brucella abortus* rL7/L12 specific antibody titers; IgG (3A), IgG1 (3B) and IgG2a (3C) in the serum samples of mice injected with CS-PLGA+DNA nanoparticles or naked DNA or vector or PBS, at different time intervals, determined by indirect ELISA. Proliferation of splenocytes of mice immunized with CS-PLGA+DNA nanoparticles or naked DNA or vector or PBS, determined by MTT colorimetric assay (3D). At each time point 3 mice were sacrificed to collect splenocytes and the assay was set up in triplicates. Within weeks, each group was compared to PBS group by student's-t test.

Fig. 4 IFN-γ (4A), IL-4 (4B) and IL-2 (4C) induced by the splenocytes of different groups of

immunized mice. At each time points 3 mice were sacrificed to collect splenocytes. After

stimulation of splenocytes, the culture supernatants were collected and quantitation of

cytokines was done by Sandwich ELISA kit. Samples were tested in triplicates. Each bar

represents mean concentration of cytokine in pg/mL and the differences were analyzed within

weeks by one-way ANOVA.

658 **Fig. 5** The mean *Brucella* copies (CFU) in the spleen of immunized and control groups of

659 mice were calculated on 28 d post-challenge with virulent *Brucella abortus* 544. The data

660 represent the mean $\log_{10}$ CFU± standard error. Each group was compared to PBS group by

661 student's-t test.