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Intraperitoneal immunization with Urease loaded *N*-trimethyl Chitosan nanoparticles elicits high protection against *Brucella melitensis* and *Brucella abortus* infections

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ABSTRACT

Brucella (B) species are brucellosis causative agents, a worldwide zoonotic illness causing Malta fever in humans and abortion in domestic animals. In this work, we evaluated the vaccine potential of Trimethyl chitosan (TMC) nanoparticles formulation of Urease (TMC/Urease) against brucellosis. TMC/Urease nanoparticles and urease without any adjuvant were separately administered both orally and intraperitoneally. Intraperitoneal (i.p.) administration of urease alone as well as oral administration of both TMC/Urease nanoparticles and urease alone, elicited low titers of specific immunoglobulin G (IgG), while i.p. immunization with TMC/Urease nanoparticles induced high specific IgG production levels. As it was indicated by the cytokine assay and the antibody isotypes, i.p. immunization by urease alone, and TMC/Urease nanoparticles induced a mixed Th1-Th2 immune response, whereas oral administration of both urease alone and TMC/Urease nanoparticles induced a mixed Th1-Th17 immune response. In lymphocyte proliferation assay, spleen cells from i.p.-vaccinated mice with TMC/Urease nanoparticles showed a strong recall proliferative response. Vaccinated animals were challenged with virulent strains of *B. melitensis* and *B. abortus*. I.p. vaccination with TMC/Urease nanoparticles resulted in a high degree of protection. Altogether, our results indicated that TMC nanoparticles are a potent delivery system for i.p.-administered *Brucella* antigens.

1. Introduction

Brucella (B) species are brucellosis causative agents, a worldwide zoonotic illness causing Malta fever in humans and abortion in domestic animals (cattle, sheep, and goats) [1]. Humans are usually infected through mucosal contact, breakage in the skin or inhalation of aerosols contaminated with *Brucella*. Therefore, preventing human brucellosis is principally dependent on the control of the illness in domestic animals. Test and slaughter programs along with immunization are the most important strategies to control the disease. Live-attenuated strains are being used to immunize domestic animals in many regions (*B. melitensis* Rev.1 for goats and sheep and *B. abortus* RB51 and *B. abortus* S19 against bovine brucellosis) [2,3].

In general, application of live-attenuated organisms as a vaccine, possesses some limitations in terms of safety. For instance, through the organism potential to return back to a pathogenic strain or organism shedding into the environment [4]. With these disadvantages, there is an immediate need to improve vaccines to have a combination of safety and efficiency.

Recombinant proteins are promising vaccine candidates, since they can be produced at a high yield and degree of purity, and can be manipulated to improve favorite properties and minimize unfavorable ones. Selecting an antigen for immunization is based on a recombinant protein, is better to be different from what is used in diagnosis tests. This makes it possible to differentiate immunized domestic animals from *Brucella*-infected ones. Additionally, recombinant protein-based vaccines are safer to handle, well defined and not infectious, unlike live-attenuated vaccines [5].

The main disadvantage of recombinant proteins is poor immunogenicity [6]. To enhance their immunogenicity, these type of vaccines required to be co-administered with adjuvants, which indirectly induce the immune response against recombinant proteins. Thus, success of a recombinant vaccine is generally dependent on the application of substances with immunomodulatory properties, which

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direct the selective stimulation of various antigen-specific immune responses [7].

Previous studies indicated that a chitosan derivate, *N*-trimethyl chitosan (TMC), is an efficient vaccine delivery system for systemic and oral vaccination. Additionally, TMC nanoparticles have an intrinsic adjuvant effect on dendritic cells (DCs) [8–10]. Another report suggested that subcutaneous (s.c.) and intraperitoneal (i.p.) vaccination with recombinant urease (rUrease) elicits protection against *B. abortus* and *B. melitensis* infections [11]. To further improve rUrease efficiency, we attempted to investigate the vaccine potential TMC nanoparticles formulation of rUrease *via* oral and i.p. immunization routes.

2. Materials and methods

2.1. Mice and ethics statement

The 4–6 weeks old female specific-pathogen-free Balb/c mice were obtained from Pasteur Institute (Tehran, Iran) and housed in standard polypropylene cages maintained at 20–22 °C, while undergoing 12 h light/dark cycles. All experimental procedures on animals were approved by the local Ethics Committee of Razi Vaccine and Serum Research Institute, Karaj, Iran.

2.2. Bacterial strains

B. melitensis virulent strain 16 M, *B. abortus* virulent strain 544, vaccine strains of *B. melitensis* Rev.1 and *B. abortus* S19 were obtained from Razi Vaccine and Serum Research Institute, Karaj, Iran. *Escherichia coli* BL21 (DE3) and pET32a (Novagen, Madison, WI, USA) were used for recombinant protein expression.

2.3. Antigen production

Methods for gene cloning and expression of rUrease from the synthetic gene (GenBank Accession Number: JQ965699) in *E. coli* BL21, and its purification were previously described [11]. Briefly, a 482 bp long open reading frame of urease alpha middle part subunit gene (Ala_{201} to Leu_{350}) was amplified by specific primers and cloned into the pET32a (Novagen, Madison, WI, USA). The rUrease was successfully expressed in *E. coli* BL21 solubilized with 8 M Urea. The soluble proteins were purified and refolded by affinity chromatography on Ni-agarose beads (Qiagen, Dorking, UK), as previously described [12]. The purity of the recombinant protein was evaluated by SDS-PAGE and then validated by western blotting using anti-His antibody. The concentration of recombinant protein was estimated using Bradford's reagent.

2.4. TMC/Urease nanoparticles

TMC was provided by Dr. Sahebghadam Lotfi (Department of Clinical Biochemistry, Faculty of Medicine, Tarbiat Modares University, Tehran, Iran). TMC/Urease nanoparticles were obtained through ionic complexation with pentasodium tripolyphosphate (TPP) and urease, as described before [12]. Briefly, urease was added to a 0.2% w/v TMC solution in 5 mM Hepes (Sigma-Aldrich) buffer (pH 7.4). Under continuous stirring, TPP was added to a weight ratio TPP: urease: TMC of 2: 1: 10. Particles were washed and harvested by centrifugation on a glycerol bed for 15 min at 14,000*g* and re-suspended in 5 mM Hepes buffer (pH 7.4) [8].

2.5. Nanoparticle characterization

Particles' size was measured by dynamic light scattering (Zetasizer Nano, Malvern Instruments, UK). The morphology and size of the particles were assessed using FE-SEM (JEOL 7500F). The samples were coated with gold before analysis by FE-SEM.

Triplicate samples were examined after encapsulation and the loading efficiency in TMC-loaded nanoparticles was computed from the calibration curve. The loading efficiency of the urease nanoparticles was calculated according to the following formula:

$$\mathrm{LE}(\%) = \frac{\text{Total amount of Urease} - \text{Free Urease}}{\text{Total amount of Urease}} \times 100$$

2.6. Protein integrity

SDS-polyacrylamide gel electrophoresis was performed to determine the effect of preparation process on protein integrity. Urease-loaded TMC nanoparticles were destabilized by adding 10% (w/v) NaCl to the nanoparticles suspension to produce a solution with a protein concentration of 0.37 mg/ml. The urease was electrophoresed at 120 V under reducing conditions in a 10% SDS-Polyacrylamide gel.

2.7. In vitro release study

Urease loaded TMC nanoparticles were separated by centrifugation at 14,000*g* and 4 °C for 15 min. The supernatant was decanted and the nanoparticles were re-suspended in 10 ml of 0.1 M PBS buffer (pH 7.4), then kept at 37 °C under magnetic stirring (150 rpm). At various time intervals, 0.5 ml of the suspension was removed and centrifuged (16,000 × g, 15 min). The Urease concentration in the supernatant was determined by Bradford method. The same amount of fresh PBS was added to the release medium to reach the primary volume. A sample consisting of only non-loaded N-TMC nanoparticles was re-suspended in PBS to be used as a negative control.

2.8. Vaccination

Mice were immunized *via* i.p. and oral routes. Groups of mice either received the vaccine or as the negative control groups are listed in Table 1. The positive control groups were immunized intraperitoneally on the 15th day with 1×10^5 CFU of *B. melitensis* Rev.1 and *B. abortus* S19.

2.9. Antibody responses

Sera for detecting total IgG, IgG1 and IgG2a responses were

Table 1
The groups of immunized mice.

Groups (n = 15)	Administration type	Administration route	Antigen dose	Days of immunization	Goal of administration
PBS	PBS	i.p.	–	0, 15	Negative Control
Urease i.p.	Urease without Adjuvant	i.p.	30 mg	0, 15	Immunogenicity of Urease without adjuvant
Urease oral	Urease without Adjuvant	oral	75 mg	0, 7, 14	Immunogenicity of Urease without adjuvant
NPs i.p.	PBS containing nanoparticles	i.p.	–	0, 15	Negative Control
TMC/Urease s i.p.	Nanoparticles containing Urease	i.p.	30 mg	0	Immunogenicity of TMC/Urease (single-dose)
TMC/Urease m i.p.	Nanoparticles containing Urease	i.p.	30 mg	0, 15	Immunogenicity of TMC/Urease (multi-dose)
TMC/Urease s oral	Nanoparticles containing Urease	oral	75 mg	0	Immunogenicity of TMC/Urease (single-dose)
TMC/Urease m oral	Nanoparticles containing Urease	oral	75 mg	0, 7, 14	Immunogenicity of TMC/Urease (multi-dose)

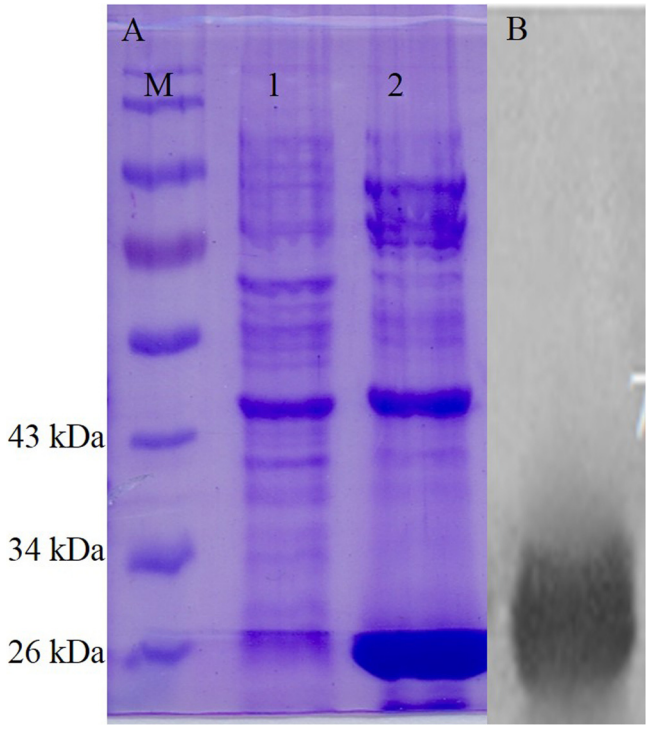

Fig. 1. Expression analysis of recombinant *E.coli*. After induction with IPTG, the rUrease protein produced by recombinant cells was analyzed by SDS-PAGE (A) Lanes 1 and 2 show the uninduced and induced cell lysates of rUrease expressing *E. coli* cells, respectively. Western blot analysis of purified urease with anti-His tag monoclonal antibody (B).

obtained at 15, 30 and 45 days after the first vaccination. Serum reaction against purified rUrease was analyzed by indirect ELISA, as described previously [10].

Anti-urease IgA was determined in fecal extracts by indirect ELISA using a goat anti-mouse IgA-specific horseradish peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA). Fecal extracts were obtained by suspending 5 fecal pellets in 0.5 ml of extraction buffer (100 mg/ml soybean trypsin inhibitor (Sigma), 10 mg/ml bovine serum albumin (Sigma) and 30 mM disodium EDTA in PBS, pH 7.6). After homogenization and centrifugation at 4 °C, the supernatants of the fecal extracts were analyzed for the presence of IgA in feces. All antibody assays were performed in triplicate.

2.10. Cytokine responses

One month after the final immunization, spleen cells from immunized and control mice (five mice/group) were removed aseptically, and individually cultured at 4×10^6/mL in duplicate wells with 10 mg of purified rUrease or 2.5 mg of concanavalin A (ConA) (Sigma). RPMI 1640 medium (NUNC Thermo Fisher Scientific Inc., Roskilde, Denmark), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% heat-inactivated FBS was used to culture the splenocytes. Cultures were incubated at 37 °C and 5% CO_2 for 48 h. At the end of the incubation period, supernatants were aliquoted and stored at −70 °C. Cytokine responses were evaluated using mouse ELISA kits according to the manufacturer instructions: IFN-γ, IL-4, IL-17 and IL-12 (R & D Systems, Minneapolis, MN, USA). All assays were performed in triplicate.

2.11. Protection experiments

One month after the last vaccination, five mice from each group were challenged with 2×10^7 CFU of virulent *Brucella* spp. *via* the i.p. route of the immunization. One month after the challenge, mice were sacrificed and their spleens were separated in aseptic conditions. Each spleen was homogenized in 1 ml 0.9% NaCl containing 0.1% Triton X-100, serially diluted, and plated on Brucella agar in triplicates and incubated at 37 °C with 10% CO_2 for 4 days. Log_{10} units of protection were computed by subtracting the mean log_{10} CFU for the vaccinated group from the mean log_{10} CFU of the corresponding control group [12].

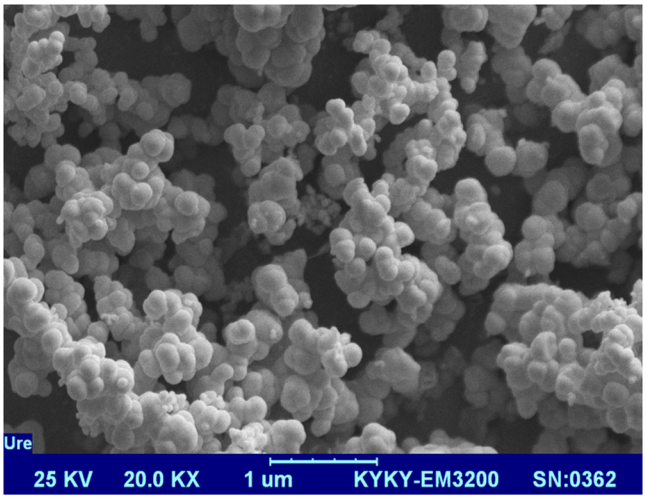

Fig. 2. Appearance and size of the nanoparticles were characterized by scanning electron micrograph of TMC/Urease nanoparticles. Scale bar represents 0.2 mm.

2.12. Lymphocyte proliferation assay

One month after the final immunization, five mice from each group were euthanized and their spleens removed in aseptic conditions. Splenocytes from all five mice from each group were first pooled and then seeded in 96-well microtitre plates with 2×10^5 cells per well in complete DMEM, followed by stimulation with rUrease (0.1 mg/ml). The plates were then incubated at 37 °C, 5% CO_2, 95% humidity for 72 h. Lymphocyte proliferation was determined by MTT assay. Total of 20 ml MTT dye (5 mg/ml in PBS, Sigma–Aldrich) was added to each well and the plates were incubated for 2 h in the dark at 37 °C, 5% CO_2 and 95% humidity. After carefully pipetting out the media from each well, the formazane crystals were solubilized using 90% acidified isopropanol (0.5%, w/v, SDS and 25 mM HCl in 90% isopropanol). The absorbance (OD) was measured at 540 nm.

2.13. Statistical analysis

ANOVA was employed to analyze the data and to evaluate the levels of lymphocyte proliferation, antibodies, and cytokine responses. Mean comparisons were carried out by Dunnett's test in SPSS statistical software. P values ≤ 0.01 were considered to be statistically significant. The CFU data were normalized by log transformation and evaluated by analysis of variance, followed by Dunnett's post hoc test.

3. Results

3.1. Antigen production

E. coli BL21 were transformed with pET32a-urease and induced with 1 mM isopropyl b-D-thiogalactoside (IPTG). After induction by IPTG, rUrease was successfully expressed in *E. coli* cells (Fig. 1a). Based on the N-terminal His tag, the recombinant protein was purified by Ni-NTA affinity chromatography. Western blotting results indicated that anti-

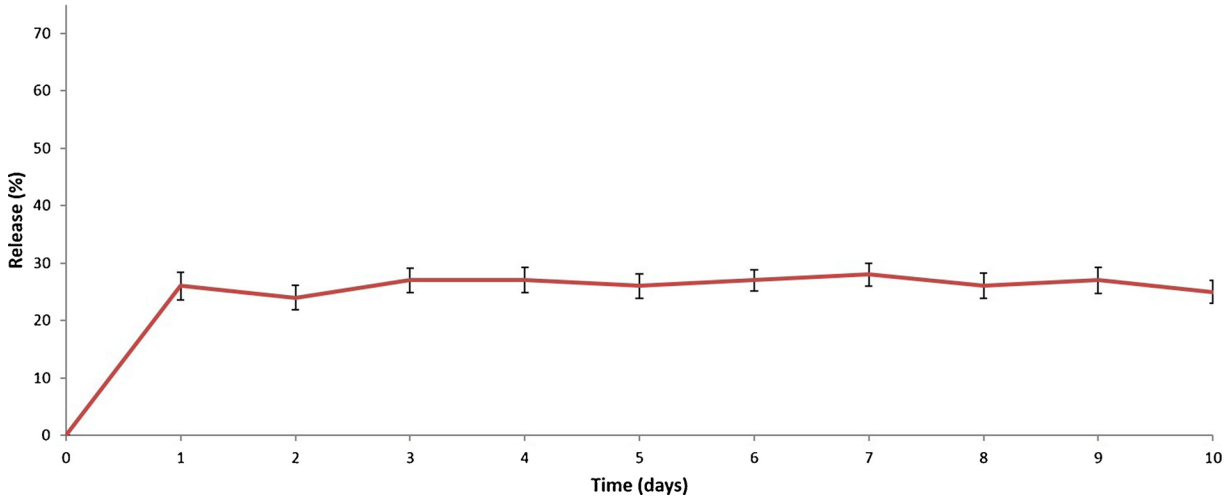

Fig. 3. Release profile of TMC/Urease nanoparticles at pH 7.4 for 10 days.

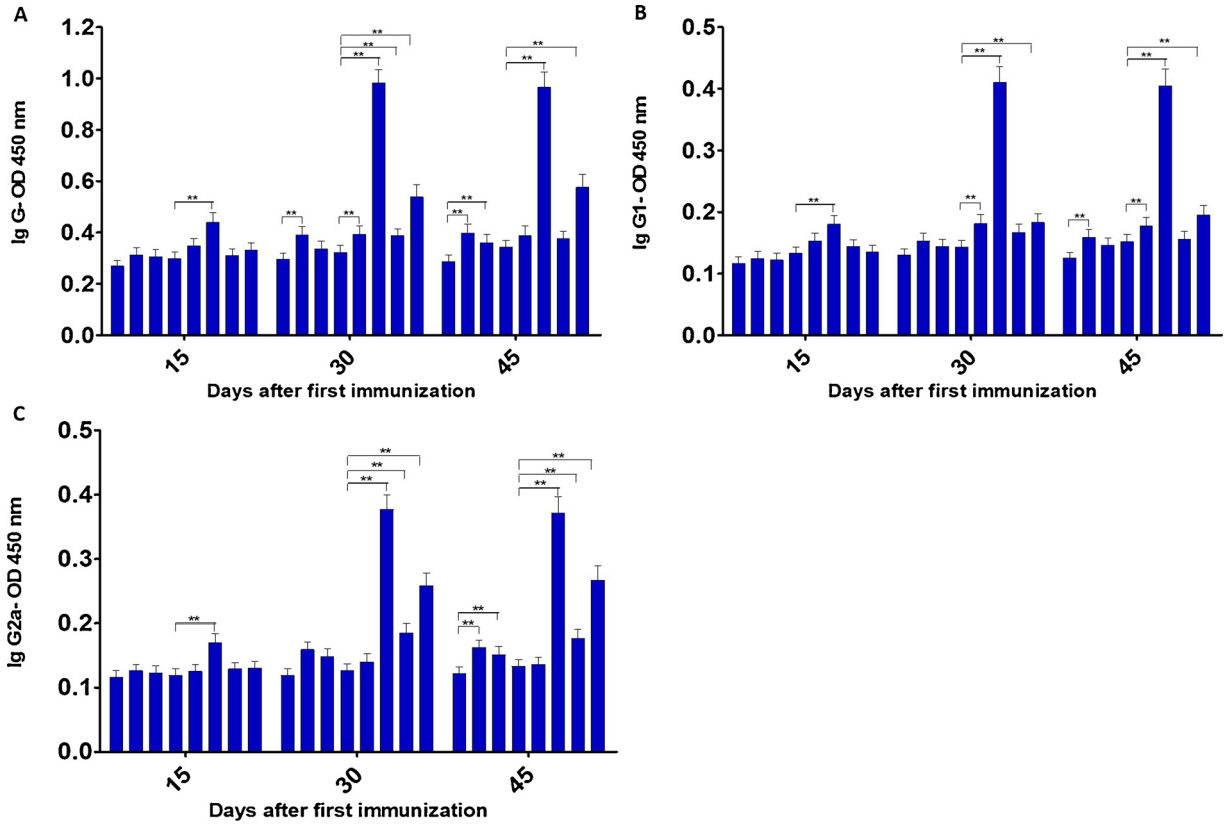

Fig. 4. Anti-Urease antibody levels: The sera were analyzed in triplicates for Urease specific IgG antibodies by ELISA with comparison to the control group. Sera obtained from mice belonging to different experimental groups were collected at regular intervals up to day 45 post-primary immunization, dilution 1:250. (A). Antibody level of intraperitoneally and orally immunized mice (B & C). Antibody isotyping: The isotype profile of Urease specific antibodies in serum of orally and intraperitoneally immunized mice were analyzed by ELISA using HRP conjugated anti-mouse IgG1 and IgG2a (dilution 1:5000) antibodies ($p \leq 0.01$). Immunization groups are based on Table 1. Immunization groups from left to right include PBS, Urease i.p., Urease oral, NPs, TMC/Urease s i.p., TMC/Urease m i.p., TMC/Urease s oral and TMC/Urease m oral, respectively. Dunnett's test was used to evaluate the anti-Urease antibody levels.

His tag antibody detected recombinant Urease protein (Fig. 1b).

3.2. Nanoparticle characterization

DLS showed that most of TMC/Urease nanoparticles had a mean size distribution of 300–400 nm (data are not shown). Due to dehydration of the sample, SEM images depicted the size of the particles as smaller than measured with DLS (between 200 and 300 nm). Typical TMC/Urease nanoparticles showed spherical nature and smooth surface as revealed by SEM (Fig. 2). The loading efficiency of urease was 83.2 ± 6.6%.

3.3. Protein integrity

The SDS-PAGE showed an expected band for the entrapped urease. There were no additional bands to indicate the presence of fragments. Therefore, antigen integrity was not affected by the preparation procedure (data are not shown).

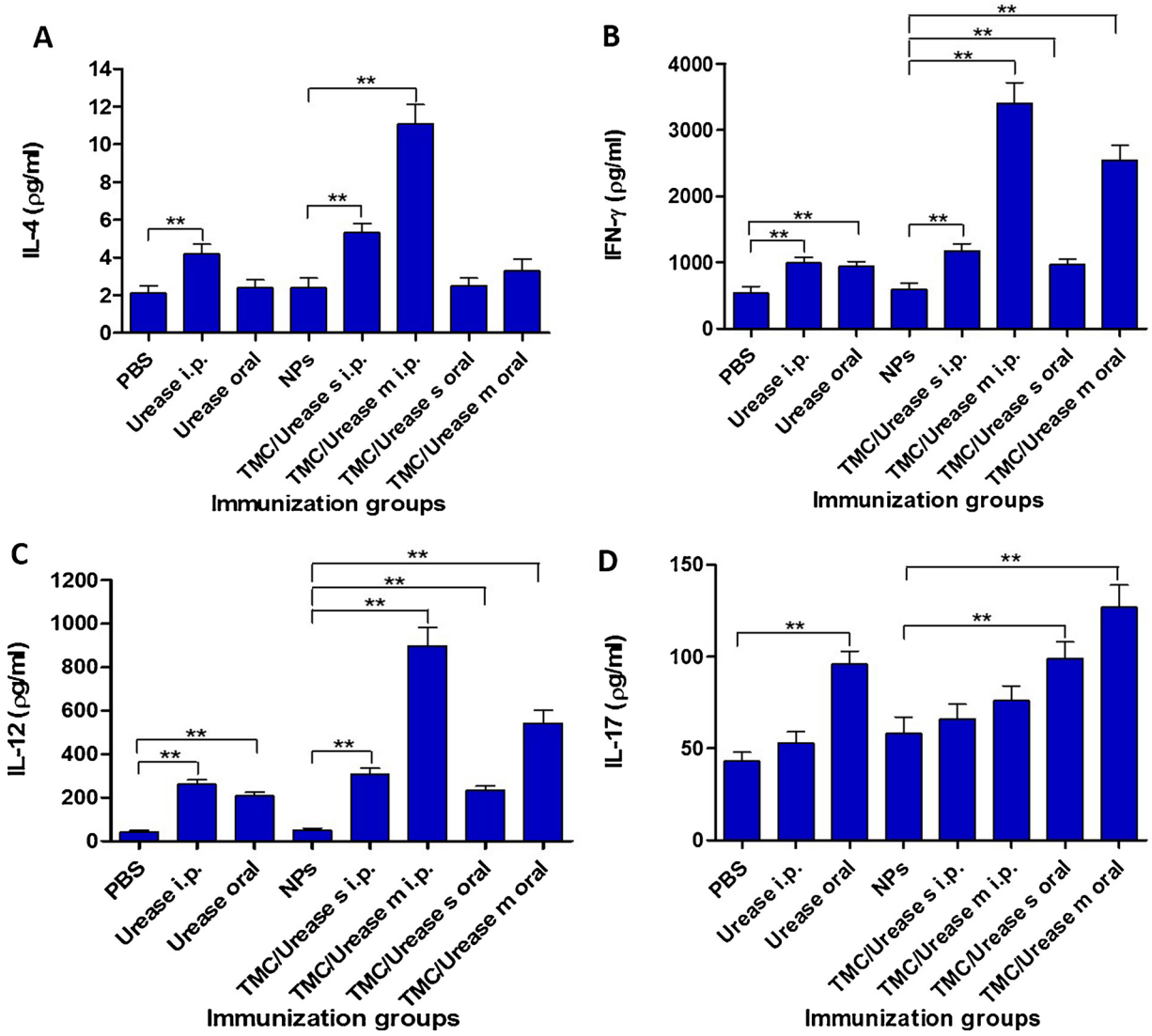

Fig. 5. IL-4 (A), IFN-γ (B), IL-12 (C) and IL-17 (D) levels in cell supernatants were determined by ELISA. Spleen Cells ($4 \times 10^6\,ml^{-1}$ in duplicate wells) were stimulated with rUrease for 48 h ($p \leq 0.01$). Immunization groups based on Table 1. Dunnett's test was used to evaluate cytokine responses.

3.4. In vitro release study

TMC nanoparticles showed about 26% release within the first day, followed by no release over the next nine days (Fig. 3).

3.5. Antibody responses

I.p. administration of urease as well as the oral administration of TMC/Urease nanoparticles and urease alone elicited low titers of specific IgG, while i.p. immunization with TMC/Urease nanoparticles induced high specific IgG production (Fig. 4A). The main subtype produced after oral immunization was IgG2a, while the titers of IgG1 and IgG2a were both significantly enhanced following i.p. immunization (Fig. 4B and C). Furthermore, oral and i.p. immunization paths of urease did not elicit a detectable specific IgA immune response (data are not shown).

3.6. Cytokine responses

According to the cytokine profile, supernatants of splenocyte cultures from i.p. vaccinated mice contained higher levels of IFN-γ, IL-12 and IL-4 compared to the negative control groups. In the case of oral vaccination, splenocytes from immunized mice secreted IFN-γ, IL-12 and IL-17 (Fig. 5).

3.7. Lymphocyte proliferation assay

To examine the ability of diverse vaccine formulations to stimulate rUrease specific cell-mediated immune responses, an *in vitro* cell proliferation assay was performed one month after the final vaccination and the results are presented as stimulation indices (S.I.). As shown in Fig. 6, a significantly higher cell proliferation rate was observed in i.p. vaccination with TMC/Urease nanoparticles (S.I. = 1.59), or by oral vaccination with TMC/Urease nanoparticles (S.I. = 1.14), as compared to the results obtained when mice were vaccinated with the free form of rUrease. The S.I. values obtained from splenocytes collected from the animals immunized with PBS or nanoparticles only did not show a detectable proliferative response when stimulation was induced *in vitro* by the rUrease protein. The significant proliferation index clearly points to the cell stimulatory activity of TMC/Urease nanoparticles as a reason behind the potent immune response.

3.8. Protection experiments

In the intraperitoneally vaccinated mice group, TMC/Urease nanoparticles stimulated high levels of protection in comparison with the

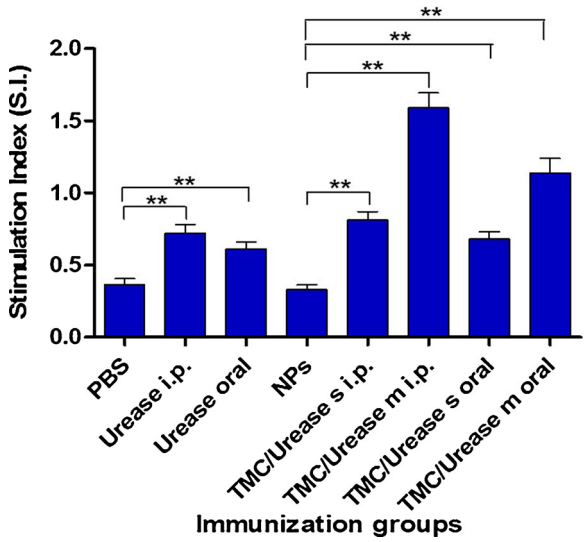

Fig. 6. Lymphocyte proliferation assay of splenocytes from mice vaccinated with rUrease. Mice immunized with PBS and nanoparticles were used as negative controls. Splenocytes from vaccinated mice (2×10^5 cells/well) were stimulated with rUrease (0.1 mg/well) for 72 h and the proliferative response was determined by *in vitro*-MTT assay. The stimulation index corresponds to the count per minute of stimulated spleen cells divided by the count per minute of unstimulated spleen cells. The data are the mean S.I. ± SD of five individual mice from each group with three repeats ($p \leq 0.01$). Immunization groups based on Table 1. Dunnett's test was used to evaluate the levels of lymphocyte proliferation.

negative control groups as it generated 2.11 and 1.83 log units of protection against *B. abortus* and *B. melitensis*, respectively (p value ≤ 0.01). However, orally administered mice with TMC/Urease nanoparticles gave 1.77 and 1.55 log units of protection against *B. abortus* and *B. melitensis*, respectively (p value ≤ 0.01). The protection units obtained indicates that TMC/Urease nanoparticles injected intraperitoneally provides more protection than TMC/Urease nanoparticles administered orally (Table 3). As expected, the vaccine strains *B. abortus* S19 and *B. melitensis* Rev.1 elicited 2.17 and 1.91 log units of protection, respectively (Table 2).

4. Discussion

Repeated studies in brucellosis control have shown that the stealthy spread of the illness in animals can only be prevented or slowed by the application of vaccines. Different components have been designed and examined as protective antigens against *Brucella* spp. [13–17]. Among these immunogens, *Brucella* urease as a recombinant protein has been known to elicit protection against *B. abortus* and *B. melitensis* in BALB/c mice [11].

Polymeric nanoparticles formulated from biodegradable polymers are being investigated as carriers for controlled delivery of agents including peptides, plasmid DNA (pDNA), proteins, and low molecular weight compounds [18,19]. Therapeutic applications of chitosan are principally limited by its low solubility and permeability above pH 5.6. TMC is more soluble in neutral and alkaline pH; hence it is more efficient than chitosan for drug delivery. Therefore, TMC is preferred for a range of biomedical uses, such as controlled drug delivery system for vaccines, therapeutics, and biomolecules. Moreover, it was indicated that co-administration of TMC with an antigen gives rise to an increased immune response and protection in comparison with the administration of antigen alone [8,10,20,21]. In this study, we examined the immunogenicity and protective responses of urease alone, and in combination with TMC nanoparticles in different administration routes.

The Urease release profile from the TMC nanoparticles represented a primary burst release (Fig. 3). After the primary surge equilibrium was reached, it displayed no further release during the next nine days. Our results are in line with results obtained by Bal *et al.,* and Amidi *et al.,* whom attributed the burst release to an antigen, which was weakly bounded to the nanoparticle surface [20,22]. This means that most of the rUrease are encapsulated in the TMC nanoparticles. The protein release mechanism from nanoparticles depends on the protein location in the carrier and nanoparticles matrix properties. Protein encapsulation in the TMC nanoparticles changes the nanoparticles surface morphology to a significant extent, where some of the protein were generally adsorbed on the nanoparticle surface. The protein adsorbed on the surface resulted from the primary burst release of protein. This phenomenon is referred to as primary protein burst.

As the *Brucella* bacterium frequently enters the body *via* contaminated food and water, mucosal immunity can act as a primary barrier against the infection before bacteria reaches the bloodstream [23]. Our data showed that oral administration of TMC/Urease nanoparticles does not elicit a detectable specific IgA immune response. However, i.p. administration of TMC/Urease nanoparticles is able to stimulate a strong IgG response in comparison to negative control groups, and orally immunized mice. However, our results differ from the observations by Chen *et al.,* who reported that oral administration of *Helicobacter pylori* urease-loaded TMC nanoparticles elicits titers of both IgA and IgG antibodies [24].

Since the isotypes of IgG are detected by the pattern of cytokines secreted by CD4+ helper T cells, we assessed the titers of both urease-specific IgG1 and IgG2a antibodies raised against urease alone and TMC/Urease nanoparticles. High amounts of IgG1 and IgG2a were detected in the sera of mice that were intraperitoneally vaccinated with TMC/Urease nanoparticles, while the main subtype produced after oral immunization was IgG2a. IgG2a isotype plays a critical role in anti-

Table 2
Protection against *B. abortus* 544 and *B. melitensis* 16 M in BALB/c mice immunized with rUrease compared with the vaccine strains S19 and Rev.1.

Vaccine (n = 5)	Adjuvant	$\log_{10}$ CFU of B. abortus 544 in spleen[1]	Protection units[2]	$\log_{10}$ CFU of B. melitensis 16 M in spleen	Protection units[2]	Significant different value
PBS	–	6.3 ± 0.23[a]	0	6.14 ± 0.21[a]	0	$p \leq 0.01$
Urease i.p.	–	5.69 ± 0.16[b]	0.61	5.57 ± 0.14[b]	0.57	$p \leq 0.01$
Urease oral	–	5.81 ± 0.12[b]	0.49	5.66 ± 0.16[b]	0.48	$p \leq 0.01$
B. abortus S19	–	4.13 ± 0.18[c]	2.17	–	–	$p \leq 0.01$
B. melitensis Rev.1	–	–	–	4.23 ± 0.23[c]	1.91	$p \leq 0.01$

The difference between groups was assessed by the ANOVA and comparisons were considered significant at $p \leq 0.01$. Different letters (a, b and c) represent significant difference between groups. The CFU data were normalized by log transformation and evaluated by analysis of variance, followed by Dunnett's post hoc test.

[a] $\log_{10}$ CFU of Brucella in negative control group ($p \leq 0.01$ estimated by Dunnett's post hoc test).

[bandc] Significantly different from negative control group ($p \leq 0.01$ estimated by Dunnett's post hoc test).

[1] The content of bacteria in spleens is represented as the mean log CFU ± SD per group.

[2] Units of protection were determined by deducting the mean log CFU of the vaccinated groups from the mean log CFU of negative control groups.

Table 3
Protection against *B. abortus* 544 and *B. melitensis* 16 M in BALB/c mice immunized with TMC/Urease nanoparticles.

Vaccine (n = 5)	Adjuvant	log_{10} CFU of B. abortus 544 in spleen[1]	Protection units[2]	log_{10} CFU of B. melitensis 16 M in spleen	Protection units[2]	Significant different value
NPs	TMC	6.12 ± 0.28[a]	0	5.91 ± 0.29[a]	0	$p \leq 0.01$
TMC/Urease s i.p.	TMC	5.45 ± 0.14[b]	0.67	5.28 ± 0.15[b]	0.63	$p \leq 0.01$
TMC/Urease m i.p.	TMC	4.01 ± 0.16[d]	2.11	4.08 ± 0.14[d]	1.83	$p \leq 0.01$
TMC/Urease s Oral	TMC	5.61 ± 0.13[b]	0.51	5.41 ± 0.16[b]	0.5	$p \leq 0.01$
TMC/Urease m Oral	TMC	4.35 ± 0.12[c]	1.77	4.36 ± 0.13[c]	1.55	$p \leq 0.01$

The difference between groups was assessed by the ANOVA and comparisons were considered significant at $p \leq 0.01$. Different letters (a, b, c and d) represent significant difference between groups. The CFU data were normalized by log transformation and evaluated by analysis of variance, followed by Dunnett's post hoc test.

[a] Log_{10} CFU of Brucella in negative control group ($p \leq 0.01$ estimated by Dunnett's post hoc test).

[b,candd] Significantly different from negative control group ($p \leq 0.01$ estimated by Dunnett's post hoc test).

[1] The content of bacteria in spleens is represented as the mean log CFU ± SD per group.

[2] Units of protection were determined by deducting the mean log CFU of the vaccinated groups from the mean log CFU of negative control groups.

Brucella immunity by facilitating phagocytes. Therefore, i.p. administration of TMC/Urease nanoparticles, a new urease derivative, can stimulate robust IgG2a response.

As with other intracellular pathogens, in *Brucella* infection protection is conferred through cell-mediated immunity. The IFN-γ production by T helper (Th) 1 cell and Cytotoxic T Lymphocyte (CTL) responses has a significant role in protection against *Brucella*, whereas Th2 responses have a minor role in this context [25]. Moreover, it was shown that IL-17 production plays a key role in generating immunity against this pathogen [26]. Our results indicated that IFN-γ and IL-12 production was raised in mice vaccinated orally and intraperitoneally with TMC/Urease nanoparticles (Fig. 5). In addition, IL-17 production increased in orally vaccinated mice. By contrast, intraperitoneally vaccinated mice produced a high level of IL-4 in comparison with orally vaccinated mice. Hence, our data showed that oral administration of TMC/Urease nanoparticles induces a low cellular mixed Th1-Th17 immune response; whereas i.p. administration of TMC/Urease nanoparticles elicits a potent cellular mixed Th1-Th2 immune response. The results are in accordance with a previous study showing that oral administration of *B. abortus* 19 kDa outer membrane protein (Omp19) induces Th1-Th17 cell responses. On the contrary intraperitoneally administered TMC/Omp19 nanoparticles elicited Th2 immune responses; whereas our data indicated that i.p. immunization with TMC/Urease nanoparticles elicits Th1-Th2 immune responses. Since antigen type plays a key role in the type of induced immune response, TMC/Omp19 and TMC/Urease nanoparticles induce different immune responses after i.p. immunization [10]. Stimulation of immune responses is highly dependent upon the type of antigen and the delivery system. Hence, various antigens and delivery systems display different patterns in inducing immune responses.

Although oral vaccination has the advantages easing the administration and a potential for mass and pain-free vaccination, oral immunization with TMC/Urease nanoparticles exhibits a lower degree of protection than i.p. immunization. Higher levels of Th1 and Th2 immune responses in i.p. administration of TMC/Urease nanoparticles can be a reason behind the high degree of protection obtained against *B. melitensis* and *B. abortus* challenges compared to the protection level obtained in oral administration of TMC/Urease nanoparticles.

Vaccination route selection can be an important factor in success or failure of an antigen under development. Although an incorrect vaccination route might render an ineffective antigen or mask its potential efficiency, the antigen within its formulation can be highly efficient *via* another route [27,28]. Based on protection units obtained in this study, i.p. administration of TMC/Urease nanoparticles can generate a better immune response as compared to oral administration of TMC/Urease nanoparticles. In accordance with previous studies, the advantageous effect of TMC nanoparticles as a carrier system was clearly observed in this study. The vaccination studies, showed that although oral administration of urease-loaded TMC nanoparticles was able to induce Th1 and Th17 immune responses, it failed to induce the highest level of protection against virulent strains of *Brucella* spp. By contrast, i.p. administration of urease-loaded TMC nanoparticles elicited high levels of Th1 and Th2 immune responses, indicating that TMC nanoparticles are promising carriers for i.p. vaccination.

In contrast to Goel *et al.,* where a single dose of another *Brucella* antigen (rOmp25) encapsulated in liposomes was sufficient to induce a high protective response, the results of our work showed that single-dose TMC/Urease nanoparticles vaccination was not sufficient for such a response [12]. Furthermore, in another study, single dose vaccination with TMC/Omp19 nanoparticles *via* i.p. and oral routes did not induce good protection against virulent strains of *B. abortus* and *B. melitensis* [10].

Yang *et al.,* indicated that nasal administration of trigger factor plus BP26 with CT induced local immune responses and a low degree of protection against systemic infection. Similar to our report, the authors did not examine protection against mucosal challenge [29].

The cell proliferative response obtained in urease-vaccinated mice points to the activation of cellular immune responses, which is considered to be important for the control of *Brucella* infections. Data obtained from the cell proliferation assay showed that immunization with urease elicits a robust antigen specific cell proliferative response, which could be further increased after i.p. administration of TMC/Urease nanoparticles.

5. Conclusions

In conclusion, i.p. administration of TMC/Urease nanoparticles enhances specific immune responses and improves the protective efficacy. The present study also provides a hint that TMC nanoparticles can be employed as components of future vaccines to control brucellosis *via* i.p. administration. This is an ongoing project and further investigations focusing on increasing the efficacy of urease-based vaccine using various adjuvants or specific delivery systems are underway in our lab.

Conflict of interest

The authors declare no conflict of interest.

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