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ORIGINAL PAPER

Immunological responses to recombinant cysteine synthase A of *Brucella abortus* in BALB/c mice

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Abstract Brucellosis is a worldwide zoonotic disease. No *Brucella* vaccine is available for use in humans, and existing animal vaccines have limitations. There is a need to develop a safe and effective vaccine against human and animal brucellosis. In the present study, we generated recombinant cysteine synthase A (rCysK) of *Brucella abortus* in *Escherichia coli* and purified it up to homogeneity by metal affinity chromatography. The immunogenicity and protective efficacy of purified rCysK were evaluated in BALB/c mice with Freund's adjuvant, aluminium hydroxide gel or without any adjuvant. High titres of anti-rCysK IgG antibody predominated by IgG1 were observed in all immunized mice. After stimulation with rCysK, the spleen lymphocytes of mice immunized with CysK formulated with aluminium hydroxide gel produced significant levels of IFN-γ. Protection against challenge with virulent *B. abortus* strain 544 was determined in BALB/c mice, and significant protection was observed in all CysK immunized groups when compared with PBS control. Among all the CysK vaccine groups, comparatively better protection was observed in mice immunized with aluminium hydroxide gel (1.064 units of protection). Overall, the results of the study suggest that rCysK induces primarily Th2 type of immune response and provides partial protection against *B. abortus* challenge.

Keywords Cysteine synthase A · Brucella · Subunit vaccine · IFN-gamma

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Introduction

Brucellae are Gram negative, facultative, intracellular bacteria that cause an important zoonotic disease called brucellosis (Young 1994). The genus contains six-well recognized (*Brucella abortus, B. melitensis, B. suis, B. canis, B. ovis,* and *B. neotomae*) and four recently added (*B. ceti, B. pennipedialis, B. microti,* and *B. inopinata*) species (Corbel 1997a; Franco et al. 2007). 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). Brucellosis is one of the commonest zoonotic diseases, with more than 500,000 new cases annually (Pappas et al. 2006b). In humans, the major cause of brucellosis is *B. melitensis* (Corbel 1997a), although several cases have also been attributed to *B. abortus* (Young 1994; Ashford et al. 2004). Human brucellosis manifests itself as a chronic infection with undulant fever and general malaise; other clinical signs vary depending on the affected organ systems (Franco et al. 2007). It is a weakening disease that requires prolonged antibiotic treatment, lasting at least 6 weeks in moderate cases and may extend for years in complicated cases (Young 1994; Pappas et al. 2005). 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). *Brucella abortus* is considered potential bioterror agent and has been classified as NIAID Category B priority pathogens because of the ease of aerosolization, diverse symptoms, and chronic persistence (Pappas et al. 2006a). Brucellosis is also one of the most common laboratory acquired infections (Noviello et al. 2004).

Attenuated live *Brucella* strains, such as *B. abortus* RB51 and S19, and *B. melitensis* Rev1, are being used as vaccines to control brucellosis in domestic animals

(Schurig et al. 2002). However, these vaccines are not suitable for humans since they can cause disease even in healthy individuals (Blasco and Díaz 1993; Perkins et al. 2010). The live attenuated *B. abortus* S19 and RB51 veterinary vaccines have limitations, as they may induce abortions when administered to pregnant animals (Schurig et al. 1991; Corbel 1997b) and show cross-reaction with natural infection during sero-diagnosis (Stevens et al. 1994, 1995). Therefore, there is an urgent need to develop efficacious vaccines to combat human form of infection and also to improve the veterinary vaccine on the same time.

In general, the use of live attenuated vaccines is a time tested approach, yet it possess some problems in terms of safety e.g. risk of reversion to the original pathogenic form and shedding in the environment with danger to immunocompromised individuals (Liljeqvist and Stahl 1999; Hansson et al. 2000). Hence, many laboratories, including ours, are working on the development of subunit vaccines. Many immunogenic *Brucella* proteins i.e. p39, Cu–Zn SOD, L7/L12, BLS, Omp31, GroEL, YacJ, DnaK, SurA, Mdh have been tried as recombinant subunit vaccines, but only a few have conferred significant protection (Xiang et al. 2008). In this study, we targeted cysteine synthase A (CysK) of *B. abortus* which was shown to be immunogenic protein in a proteomic analysis of *B. abortus* cell envelope (Connolly et al. 2006). Cysteine synthase A and cysteine synthase B are the key enzymes of cysteine biosynthesis in bacteria and they form a bienzyme complex, called cysteine synthase (Tanous et al. 2008). CysK is involved in various stress conditions as it was shown that its expression increased during bile stress in *Lactobacillus casei* (Wu et al. 2010) and during chromate stress in *E. coli* (Ackerley et al. 2006). In the line with the crucial role of cysteine synthesis in response to environmental changes, we studied immunogenicity and protective potential of cysteine synthase A.

In the present work, gene encoding cysteine synthase A (*cysK*) was cloned, expressed in *E. coli* and the recombinant protein was purified up to homogeneity by metal affinity chromatography. Immunogenicity and protective ability of the recombinant protein in formulation with two different adjuvants were evaluated in BALB/c mice. The rCysK was found to generate specific antibody response and release IFN-γ in spleen lymphocytes of immunized mice. Further, rCysK also conferred partial protection against challenge with virulent strain of *B. abortus*.

Materials and methods

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 Institutional Animal Ethics Committee of DRDE, Gwalior. The challenged mice were housed in biocontainment safety level -3 (BSL-3) facility.

Bacterial strains and plasmids

Escherichia coli host strains BL21 (DE3) and DH5α, and pET28a$^+$ expression vector (Novagen, USA) were used for cloning and expression of gene. All *E. coli* strains were routinely grown at 37 °C in Luria–Bertani broth or agar with appropriate concentration of kanamycin whenever needed. *B. abortus* strain NCTC 10093 (544) was obtained from NCTC, UK. Pathogenic *B. abortus* strain S99 and vaccine strain S19 were obtained from our own culture collection. 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 plate count method. All live *B. abortus* manipulations were performed in BSL -3 facility.

Cloning and expression of *cysK* gene and purification of rCysK

Full length open reading frame of *cysK* gene was amplified by PCR from the genomic DNA of *B. abortus* S99. The primers with *NdeI* and *XhoI* restriction sites at the 5′ ends were designed in accordance to the sequence information available in GenBank (Gene ID: 3341091). The primers used were: CysKF- 5′ TATGCACATATGTTCAATT CGGTACTCGA3′ and CysKR- 5′ TAGCAGCTCGA GTTACCCCTCGAACGGTATGT3′. Plasmid pET28a$^+$ and purified PCR product (purified using GenElute PCR Clean-up kit, Sigma, USA) were digested with *NdeI* and *XhoI* followed by setting up of ligation reaction. Ligated product was transformed in *E. coli* DH5α competent cells and the positive clones were selected according to standard protocol (Sambrook and Russell 2001). The integrity of cloned gene was confirmed by restriction digestion analysis and DNA sequencing. The plasmid of the clone containing the insert was used to transform competent cells of *E. coli* BL21 (DE3). The recombinant protein was expressed by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in LB medium containing kanamycin. The rCysK was found to express in soluble fraction. The recombinant protein was purified by Ni$^+$-NTA resin (Qiagen, Germany) using imidazole as the elution reagent

according to manufacturer's instructions. The lysate of IPTG induced cells and the purified protein were identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Confirmation of expressed rCysK was done by Western blotting using monoclonal anti-polyhistidine HRP conjugate (Sigma, USA). The purified rCysK was dialysed against buffer containing 50 mM NaH_2PO_4 (pH 8.0) with reduced concentration of imidazole (150, 100 and 50 mM) and NaCl (200, 100 and 50 mM) for 12 h; and finally with buffer lacking both. The endotoxin level in purified protein was determined by a chromogenic LAL (*limulus* amebocyte lysate) endotoxin assay kit (Lonza, Switzerland).

Immunization

Groups of BALB/c mice (n = 12) were injected with a total three doses (day 0, 14 and 21) of rCysK without any adjuvant (rCysK group), rCysK formulated with Freund's complete/incomplete adjuvant (rCysK-FA group) and rCysK with aluminium hydroxide gel (rCysK-Al group) by subcutaneous route. Each animal was injected with 25 μg (100 μl) of recombinant protein in every dose. First dose in FA group was with Freund's complete adjuvant and boosters were administered with Freund's incomplete adjuvant. A group of mice was injected PBS (0.01 M, pH 7.2) subcutaneously and was included as negative control (PBS Control). Sera from all groups were collected on day 0 and 28 to determine the antibody response.

Indirect enzyme linked immunosorbent assay (ELISA)

Levels of anti-rCysK antibodies in the sera of the immunized mice were assayed by ELISA. Briefly, the polystyrene plates (Nunc-Immuno Plate MaxiSorp surface, Denmark) were coated with rCysK (10 μg/ml, 100 μl/well) in carbonate-bicarbonate buffer (0.05 M, pH 9.6) and were incubated overnight at 4 °C. Next morning the plates were washed twice with PBST (PBS, 0.01 M, pH 7.2 containing 0.05 % vol/vol Tween 20). Blocking was done with 1 % (wt/vol) BSA in PBS (200 μl/well) and incubated for 2 h at 37 °C. After three washings with PBST, the plates were incubated with 100 μl of test sera dilution (two fold) in PBS containing 0.1 % BSA for 1 h at 37 °C. The wells were washed five times with PBST. Goat anti- mouse (IgG) antibodies and isotypes (IgG1 and IgG2a) conjugated to horseradish peroxidase obtained from Sigma and Pharmingen, respectively, were diluted as recommended by manufacturers and were added to the wells. After incubation for 1 h at 37 °C and subsequent washes, plates were developed with ortho-phenylenediamine (0.4 mg/ml) and H_2O_2 (6 %, 0.4 μl/ml) in citrate phosphate buffer (pH 4.5). The absorbance was measured at 492 nm in an ELISA reader (BioTek, USA) and values are presented as end point titres. The reciprocal of highest dilution of test sera having OD more than the mean OD (+2 SD) of preimmune serum (1:100 dilution) in the same group was considered as the end point titre.

Lymphoproliferation and cytokine assays

For lymphoproliferation assay, four mice from each group were sacrificed on day 49 (28 days after last immunization) and their spleens were removed aseptically. Spleen cell proliferation was carried out by an earlier described method (Kumar et al. 2009). Briefly, single cell suspension of splenocytes was prepared by maceration of spleens and by lysing erythrocytes with ammonium chloride solution. The splenocytes were suspended in 96-well tissue culture plate at ca. 1×10^6 cells/ml (100 μl/well) along with one volume of antigen (1, 5 or 10 μg/ml) in RPMI1640 medium. Appropriate positive (Con A, 5 μg/ml) and negative controls (without antigen) were also included. After 48 h of incubation, 0.1 volume (20 μl) of alamar blue 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 results are expressed as mean specific absorbance, which was calculated by subtracting the delta absorbance of cells without antigen from the delta absorbance of cells with antigen. Delta absorbance is obtained by subtracting readings of cells at 600 nm from that at 570 nm.

Supernatants from parallel cultures stimulated with 10 μg/ml of recombinant protein were harvested after 72 h and stored at −70 °C until assayed for cytokine interferon-γ (IFN-γ). Appropriate positive (Con A, 5 μg/ml) and negative controls (without antigen) were also included. The levels of IFN-γ were determined in culture supernatants using sandwich ELISA (OptEIA, Pharmingen, USA) according to manufacturer's instructions. The levels of cytokine were determined with the help of standard curves that were plotted using recombinant cytokine (Pharmingen) and are expressed as pg/ml.

Protection assay

A group (n = 6) of mice (vaccine group) was inoculated intraperitoneally with *B. abortus* S19 (5×10^4 CFU/mouse) on day 21 and included as positive control for protection studies. On day 51 (30 days after last injection), six immunized mice from vaccine group and the groups described above were challenged with 2×10^5 CFU/mouse of *B. abortus* strain 544 by intra-peritoneal injection. The challenged animals were sacrificed on day 81 (30 days after challenge) by cervical dislocation and their spleens were removed aseptically. Each spleen was

homogenized, serially diluted in PBS and plated on Brucella agar. The plates were incubated at 37 °C with 5 % CO_2. The numbers of colonies were counted after 48 h and the results were expressed as the mean log CFU/spleen ± SD per group.

Statistical analysis

CFU data was logarithmically transformed and statistically analysed by analysis of variance using multiple comparisons versus control group by Dunnett's method. A non parametric Mann–Whitney rank-sum test was used to compare the cellular response (Sigma Stat, Jandel Scientific, USA).

Results

Production of purified rCysK

To obtain the recombinant CysK, *E. coli* BL21 (DE3) cells harbouring pETCysK were induced with 0.5 mM IPTG. SDS-PAGE analysis of the cell lysate showed band of ~39 kDa rCysK (Fig. 1a). Expressed rCysK was confirmed by Western blotting using monoclonal anti-polyhistidine antibody (Fig. 1b). The rCysK protein was purified by affinity chromatography with Ni^{+}-NTA column. Purity of the recombinant protein (>95 %) was estimated by staining 12 % (wt/vol) SDS-PAGE with Coomassie blue stain. Figure 1c shows the SDS-PAGE profile of purified rCysK. Expression level of rCysK was found to be 18.6 mg/l of medium. The protein preparation contained <0.01 EU/μg of protein.

Antibody response elicited by rCysK immunization

Immune response to rCysK was studied in BALB/c mice after subcutaneous inoculation. The generation of antigen-specific antibodies was measured in the sera of the immunized mice 1 week after the last dose by indirect ELISA. Maximum anti-rCysK IgG antibody titres were observed in rCysK-FA group followed by rCysK-Al, rCysK and PBS groups (Fig. 2a). Highest IgG titre of 32,000 was obtained in rCysK-FA group mice. The IgG titre in serum samples of rCysK-Al group mice was 16,000 (Fig. 2b). The sera were also analysed for presence of different IgG isotypes. IgG1 titres predominated over IgG2a in all immunized mice, and maximum IgG titres were obtained in rCysK-FA group (Fig. 2c).

Lymphocyte proliferation and IFN-γ response

The proliferative response of splenocytes harvested 28 days after the last dose from immunized mice was investigated. No significant proliferation was observed in spleen cells of rCysK (with or without adjuvants) immunized mice when compared to PBS group. The ConA mitogen was able to induce spleen cell proliferation in all cultures non-specifically. Splenocytes of only rCysK-Al group induced the significant ($p < 0.05$) production of IFN-γ (Fig. 3) as compared to PBS group. ConA was able to produce IFN-γ in various groups and ranged from 1,000 to 1,500 pg/ml (data not shown).

rCysK partially protects BALB/c mice against *B. abortus* infection

The potential of rCysK to impart protection was evaluated on the basis of their ability to reduce the bacterial load of

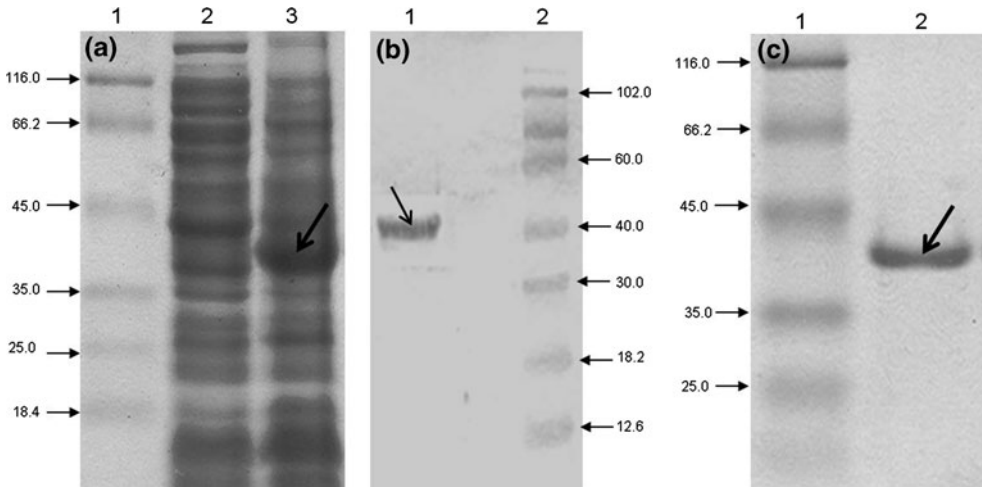

Fig. 1 Expression and purification of rCysK. **a** A 12 % SDS-PAGE profile showing the expression of rCysK in *E. coli* BL21 (DE3) cells. *Lane 1* Protein molecular mass marker (in kilodaltons), *Lane 2* Un-induced *E. coli* BL21 (DE3) cell pellet, *Lane 3* Induced *E. coli* BL21 (DE3) cell pellet. **b** Western blots showing the specific expression of rCysK using monoclonal anti-polyhistidine conjugate. *Lane 1* Induced *E. coli* BL21 (DE3) cell pellet, *Lane 2* Protein molecular mass marker. **c** 12 % SDS-PAGE analysis showing the affinity purified rCysK. *Lane 1* Protein molecular mass marker, *Lane 2* Purified protein by immobilized metal affinity chromatography

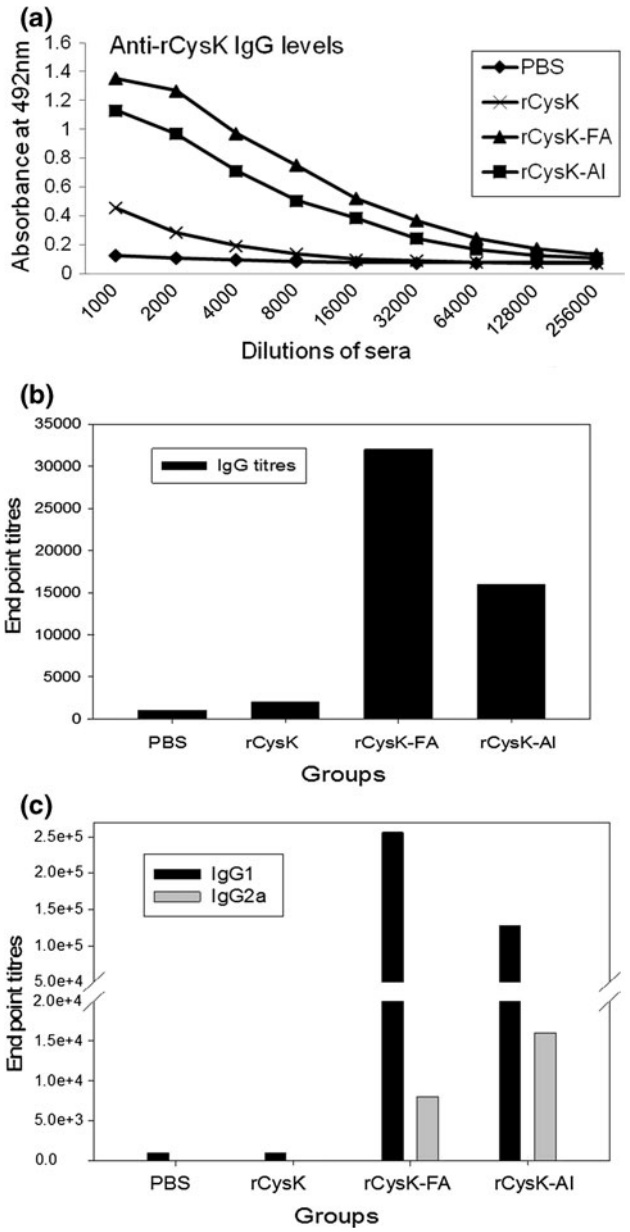

Fig. 2 Anti- rCysK IgG (**a** and **b**) and IgG isotypes (**c**) titres in the sera of rCysK immunized mice and PBS control ($n = 12$). The mice were bled on day 0 and 28 retero-orbitally and antibody titres were evaluated by ELISA. Data are the representative of three separate experiments. Each *bar* represents the mean end point titre ± SD

virulent *B. abortus* strain in spleens of immunized mice. On day 51, mice were challenged with virulent *B. abortus* strain 544 and sacrificed after 30 days to determine the number of CFU in their spleens. Mice inoculated with rCysK with either of the adjuvants or without any adjuvant conferred protection against *B. abortus* infection. To compare the extent to which mice could be protected, a group of mice inoculated with S19 vaccine was included and 2.155 units of protection were obtained in this group. Among rCysK groups, maximum protection was observed

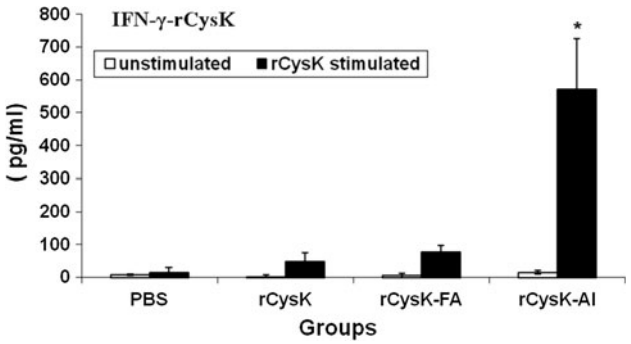

Fig. 3 Release of IFN-γ in the spleen cells of rCysK immunized mice and PBS control. IFN-γ in culture supernatants were measured by sandwich ELISA. Results are expressed in pg/ml and represent the mean (±SE) of the 4 animals in each group. Data are the representative of three separate experiments. Significant differences of comparison with PBS group are determined by *t* test and are indicated by asterisk ($p < 0.05$)

with aluminium hydroxide gel formulation (rCysK-Al, 1.064 units) followed by Freund's adjuvant formulation (rCysK-FA, 0.879 units) (Table 1).

Discussion

Brucellosis is the world's most common zoonosis, with no effective vaccine available against it. Several strategies are being sought to prevent this disease while avoiding the disadvantages of the currently used live vaccines. One of the attractive vaccine development approaches is the development of subunit vaccines. In this study, we investigated the immunogenicity and protective efficacy of recombinant cysteine synthase A. The gene encoding the protein was cloned in prokaryotic expression vector $pET28a^{+}$ and the expressed protein was purified to near homogeneity by affinity chromatography.

The immunogenic and protective potential of rCysK formulated in Freund's complete/incomplete and aluminium hydroxide adjuvant was studied in BALB/c mice. Freund's adjuvant is the most commonly used adjuvant in research setting, whereas aluminium hydroxide is the only adjuvant licensed for human vaccines. The antibody response to rCysK was determined 1 week after the last dose. The results of this work show that rCysK is able to elicit significantly high levels of IgG antibodies in BALB/c mice when administered with FA and aluminium hydroxide adjuvants. Serum samples collected from immunized and control groups were analysed for the presence of different IgG subtypes. Titres of both IgG1 and IgG2a increased in adjuvant formulated immunized groups in comparison to control group, and IgG1 titres predominated over IgG2a. In *Brucella*, like many other intracellular pathogens, role of antibodies in protection is considered

Table 1 Protection against *B. abortus* strain 544 provided to BALB/c mice by vaccination with rCysK protein with different adjuvant formulations

Vaccine ($n = 6$)	Adjuvant	Log_{10} CFU of *B. abortus*/spleen[a]	Units of protection[b]
PBS	None	5.20 ± 0.014	–
rCysK	None	4.49 ± 0.011	0.713[c]
rCysK	CFA/IFA	4.32 ± 0.32	0.879[c]
rCysK	Aluminium hydroxide	4.14 ± 0.47	1.064[c]
B. abortus S19	None	3.05 ± 0.20	2.155[c]

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

[c] significantly different ($p < 0.05$) compared with value of control (PBS group)

dispensable. Though, literature is available suggesting protective effects of antibodies in brucellosis (Araya and Winter 1990; Jaques et al. 1992). However, now it is becoming clearer that a humoral response may not be protective but it is indispensable in host defenses as opsonisation is required for successful uptake of *Brucella* by macrophages (Eze et al. 2000; Baldwin and Goenka 2006) and opsonization may result in increased bacterial uptake by macrophages (Eze et al. 2000).

IFN-γ is the characteristic cytokine of Th1 immune response. The Th1 immune response characterized by IFN-γ is associated with protection against brucellosis (Murphy et al. 2001; Paranavitana et al. 2005; Rafiei et al. 2006). 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 (Boehm et al. 1997). During a CMI response, there is gradual change in the predominant immunoglobulin class of the specific antibody produced. This isotype switch is controlled by T cells and their cytokines. In mice, IFN-γ generally switches activated B cells to the IgG2a and IgG3 isotypes (Roitt et al. 2001). Here, in this study, rCysK induced spleen cells to produce significant levels of IFN-γ in rCysK-Al immunized group, which may have influenced the B cells to secrete IgG2a.

Protective ability of rCysK formulations was studied in BALB/c mice. BALB/c mouse is a proven animal model to study the protection of *Brucella* vaccine candidates. Immunization with rCysK could confer significant ($p < 0.05$) protective immunity against *B. abortus* infection as compared to PBS group. Vaccination with rCysK-Al provided comparatively better protection (1.064 units) than rCysK-FA (0.879 units) or rCysK (0.713 units), though there was no statistical significant difference in protection provided by the different formulations. The protection offered by CysK, however, was lower than the *B. abortus* S19 vaccine (2.155 units). It is possible that the newer adjuvants which skew immune response to the Th1 type may provide better protection using CysK protein. Nonetheless, the results of this study show that rCysK is able to confer partial protection in mouse model. It is likely that CysK protective efficacy and immunogenicity may further be increased by using other delivery methods like liposome-mediated delivery, escheriosome-mediated delivery and co-stimulation with interleukins. Taken together, we believe that CysK can be a valuable adjunct with other candidates.

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References

Ackerley DF, Barak Y, Lynch SV, Curtin J, Matin A (2006) Effect of chromate stress on *Escherichia coli* K-12. J Bacteriol 188:3371–3381

Araya LN, Winter AJ (1990) Comparative protection of mice against virulent and attenuated strains of *Brucella abortus* by passive transfer of immune T cells or serum. Infect Immun 58:254–256

Ashford DA, di Pietra J, Lingappa J, Woods C, Noll H, Neville B, Weyant R, Bragg SL, Spiegel RA, Tappero J, Perkins BA (2004) Adverse events in humans associated with accidental exposure to livestock brucellosis vaccine RB51. Vaccine 22:3435–3439

Baldwin CL, Goenka R (2006) Host immune responses to the intracellular bacteria *Brucella*: does the bacteria instruct the host to facilitate chronic infection? Crit Rev Immunol 26:407–442

Blasco JM, Díaz R (1993) *Brucella melitensis* Rev-1 vaccine as a cause of human brucellosis. Lancet 342:805

Boehm U, Klamp T, Groot M, Howard JC (1997) Cellular responses to interferon-gamma. Annu Rev Immunol 15:749–795

Connolly JP, Comerci D, Alefantis TG, Walz A, Quan M, Chafin R, Grewal P, Mujer CV, Ugalde RA, DelVecchio VG (2006) Proteomic analysis of *Brucella abortus* cell envelope and identification of immunogenic candidate proteins for vaccine development. Proteomics 6:3767–3780

Corbel MJ (1997a) Brucellosis: an overview. Emerg Infect Dis 3:213–221

Corbel MJ (1997b) Vaccines against bacterial zoonoses. J Med Microbiol 46:267–269

Eze MO, Yuan L, Crawford RM, Paranavitana CM, Hadfield TL, Bhattacharjee AK, Warren RL, Hoover DL (2000) Effects of opsonization and gamma interferon on growth of *Brucella melitensis* 16 M in mouse peritoneal macrophages in vitro. Infect Immun 68:257–263

Franco MP, Mulder M, Gilman RH, Smits HL (2007) Human brucellosis. Lancet Infect Dis 7:775–786

Hansson M, Nygren P, Stahl S (2000) Design and production of recombinant subunit vaccines. Biotechnol Appl Biochem 32:95–107

Jaques I, Cloeckaert A, Linet JN, Dubray G (1992) Protection conferred on mice by combinations of monoclonal antibodies directed against outer-membrane proteins or smooth lipopolysaccharide of *Brucella*. J Med Microbiol 37:100–103

Kumar S, Balakrishna K, Agarwal GS, Merwyn S, Rai GP, Batra HV, Sardesai AA, Gowrishankar J (2009) Th1-type immune response to infection by pYV-cured phoP-phoQ null mutant of *Yersinia pseudotuberculosis* is defective in mouse model. Antonie Van Leeuwenhoek 95:91–100

Liljeqvist S, Stahl S (1999) Production of recombinant subunit vaccine: protein immunogens, live delivery systems and nucleic acid vaccines. J Biotechnol 73:1–33

Murphy EA, Sathiyaseelan J, Parent MA, Zou B, Baldwin CL (2001) Interferon-gamma is crucial for surviving a *Brucella abortus* infection in both resistant C57BL/6 and susceptible BALB/c mice. Immunology 103:511–518

Noviello S, Gallo R, Kelly M, Limberger RJ, DeAngelis K, Cain L, Wallace B, Dumas N (2004) Laboratory-acquired brucellosis. Emerg Infect Dis 10:1848–1850

Oliveira SC, Harms JS, Rech EL, Rodarte RS, Bocca AL, Goes AM, Splitter GA (1998) The role of T cell subsets and cytokines in the regulation of an intracellular bacterial infection. Braz J Med Biol Res 32:77–84

Pappas G, Akritidis N, Bosilkovski M, Tsianos E (2005) Brucellosis. N Engl J Med 352:2325–2336

Pappas G, Panagopoulou P, Christou L, Akritidis N (2006a) *Brucella* as a biological weapon. Cell Mol Life Sci 63:2229–2236

Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV (2006b) The new global map of human brucellosis. Lancet Infect Dis 6:91–99

Paranavitana C, Zelazowska E, Izadjoo M, Hoover D (2005) Interferon-γ associated cytokines and chemokines produced by spleen cells from *Brucella*-immune mice. Cytokine 30:86–92

Perkins SD, Smither SJ, Atkins HS (2010) Towards a *Brucella* vaccine for humans. FEMS Microbiol Rev 34:379–394

Rafiei A, Ardestani SK, Kariminia A, Keyhani A, Mohraz M, Amirkhani A (2006) Dominant Th1 cytokine production in early onset of human brucellosis followed by switching towards Th2 along prolongation of disease. J Infect 53:315–324

Roitt J, Brostoff J, Male D (2001) Immunology. Mosby Publications, London

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, Vol. 1, 3rd edn. CSHL press, New York

Schurig GG, Roop RMII, Bagchi T, Boyle S, Buhrman D, Sriranganathan N (1991) Biological properties of RB51: a stable rough strain of *Brucella abortus*. Vet Microbiol 28:171–188

Schurig GG, Sriranganathan N, Corbel MJ (2002) Brucellosis vaccines: past, present and future. Vet Microbiol 90:479–496

Stevens MG, Hennager SG, Olsen SC, Cheville NF (1994) Serologic responses in diagnostic tests for brucellosis in cattle vaccinated with *Brucella abortus* 19 or RB51. J Clin Microbiol 32:1065–1066

Stevens MG, Olsen SC, Cheville NF (1995) Comparative analysis of immune responses in cattle vaccinated with *Brucella abortus* strain 19 or RB51. Vet Immunol Immunopathol 44:223–235

Tanous C, Soutourina O, Raynal B, Hullo MF, Mervelet P, Gilles AM, Noirot P, Danchin A, England P, Martin-Verstraeteal I (2008) The CymR regulator in complex with the enzyme CysK controls cysteine metabolism in *Bacillus subtilis*. J Biol Chem 283:35551–35560

Vrioni G, Pappas G, Priavali E, Gartzonika C, Levidiotou S (2008) An eternal microbe: *Brucella* DNA load persists for years after clinical cure. Clin Infect Dis 46:e131–e136

Wu R, Sun Z, Wu J, Meng H, Zhang H (2010) Effect of bile salts stress on protein synthesis of *Lactobacillus casei* Zhang revealed by 2-dimensional gel electrophoresis. J Dairy Sci 93:3858–3868

Xiang Z, Todd T, Ku KP, Kovacic BL, Larson CB, Chen F, Hodges AP, Tian Y, Olenzek EA, Zhao B, Colby LA, Rush HG, Gilsdorf JR, Jourdian GW, He Y (2008) VIOLIN: vaccine investigation and online information network. Nucleic Acids Res 36:D923–D928

Young EJ (1994) An overview of human brucellosis. Clin Infect Dis 21:283–289