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Prophylactic and Therapeutic Efficacy of a Glycoconjugate Vaccine Against Bovine Brucellosis

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Abstract

Bovine brucellosis is a zoonotic disease. It impacts dairy industry since it is a major cause of abortion. The disease is caused by bacteria belonging to the genus *Brucella*. The pathogen is excreted in milk, semen and aborted materials. Bovine brucellosis is currently controlled by calf-hood vaccination of females using live attenuated *B. abortus* strains (S19/RB51), however it does not provide 100% protection, and its use is restricted by age and gender. The vaccine strains are infectious to humans. Review of literature indicated that use of glyco-conjugate (GC) vaccines could address the above problems. Therefore, the objective of study was to assess the protective efficacy and therapeutic potential of GC vaccine prepared from *Brucella abortus* S19 (S19GC) in cattle. Immunization of *Brucella* free animals with two doses of 50µg each at an interval of 90 days by sub-cutaneous route resulted in pronounced Th1 mediated interferon gamma (IFN-γ) response (P=0.0061) as observed in ELISPOT assay compared to unvaccinated controls till 90 days post vaccination (DPV). The ability of S19GC to arrest shedding of *Brucella* was studied in brucellosis positive animals, vaccination of infected animals each with 50µg of S19GC by subcutaneous route resulted in arresting of shedding as evidenced by negative culture results, reduction (2 log folds) in genome copy number as observed by real-time PCR (qPCR). The prime-boost strategy of immunization (50 µg dose/ cattle) of a group of cattle infected with brucellosis (> 53%) was only able to arrest bacterial shedding transiently (7-60 DPV) in 66.67% immunized cattle, and after booster bacterial shedding was not recorded by culture in 83.34% of the animal till 250 PI. Also, *Brucella* genome was not detected transiently (7-150 DPV) in samples; but was detected thereafter till 250 DPV. The most promising effect of immunization with S19GC was observed in a group of cattle with a mixed population of brucellosis negative and positive animals of the above farm. Immunization of this group of animals with 100 µg of S19GC resulted in complete stoppage of shedding as indicated by culture and qPCR. The post immunization anti-LPS and anti-OMP antibody IgG1 and IgG2 response differed (P<0.01) at 30 DPV compared to pre immunization, suggesting the involvement of both Th2 and Th1 cells in the immunity conferred by S19GC vaccine.

Key words: *Brucella abortus*, cattle, glycoconjugate vaccine, lipo-polysaccharide, outer membrane protein.

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Introduction

The genus *Brucella* is a gram-negative, non-motile and facultative intracellular pathogen; and is comprised of ten recognized species (1, 2, 3, 4). *B.abortus* is the most widespread (5) and affects cattle as well as other mammalian species (6). World Health Organization (WHO) ranks brucellosis among top seven "neglected zoonosis", a threat to human health and the cause of poverty (7, 8). The disease is a major cause of concern for dairy industry as it causes reduction in milk yield, abortion and infertility in female cattle, poor quality of semen and orchitis in males. There are two major type of vaccines used for control of brucellosis - live attenuated *B.abortus* smooth S19 strain and live attenuated *B.abortus* rough RB51 strain. The major drawback of these conventional vaccines is their ability to shed the live organism and cause disease in associated cattle (9) and humans (10). Live attenuated vaccines have been used in many countries for a long time for control of brucellosis. However, these vaccines are indicated for female calves of 4 months to 11 of age which is a serious limitation for effective control of brucellosis in many endemic countries where slaughter of infected animals cannot be advocated. The S19 vaccine also induces O-lipo-polysaccharide specific antibodies which interfere with serodiagnostic tests (6). Because of these drawbacks, efforts are currently directed towards the development of improved vaccines, which includes formulations of subunit (11) and recombinant sub-cellular vaccines against brucellosis (12, 13).

Subunit vaccines are promising vaccine candidates since they are non-virulent, non-infectious, and therefore do not pose any biohazard, and are well defined antigens that can be adopted for immunization. The use of GC vaccines for prophylactic immunization in humans has been cited earlier. They have been used against human pathogens *Haemophilus influenzae* (HiB), *Streptococcus pneumoniae* and *Niesseria meningitis* (14, 15, 16). In case of brucellosis earlier reports had indicated the use of lipopolysaccharide (LPS) from *B.melitensis* covalently conjugated to BSA (3) and *B.melitensis* LPS non-covalently conjugated to *Neisseria meningitides* OMP (17). More recently, the immune response of a GC vaccine formulation prepared from LPS and OMP extracted from *B.abortus* S19 strain was studied in mice and cattle calves (18, 19). It was shown that BALB/C mice immunized with S19GC vaccine by sub-cutaneous route were protected upon challenge with *B. abortus* 544 virulent strain (19), and also the GC formulation was able to elicit appropriate cell mediated immune response in mice and cattle calves (18, 19). In the present study the prophylactic and therapeutic potential of S19GC vaccine in adult cattle comprising of brucellosis negative and naturally infected animals raised as closed free mixing herd was explored.

Material and Methods

Preparation of Brucella S19GC Glycoconjugate vaccine: The extraction and purification of lipopolysacharide (LPS) and outer membrane protein (OMP) from *B.abortus* S19 strain and further conjugation of LPS and OMP for the preparation of *B. abortus* glycoconjugate vaccine was done in house as described by Mythili *et al.,* 2010 (18). The *B. abortus* S19 vaccine USDA strain used for the preparation of glycoconjugate vaccine was obtained from Animal Disease Research Laboratory (ADRL), National Dairy Development Board, Anand, India.

Pre-vaccine screening of animals: The prevaccination status of brucellosis in animals of three different farms (Farm I, Farm II and Farm III) was determined by cultural isolation, serology, and qPCR. Cultural isolation was done by using modified *Brucella* selective media (MBS) (20) and serology by indirect ELISA using a commercial kit BRUCELISA (VLA, UK). The qPCR was done as per the method developed in house (unpublished data).

Vaccination: Based on the pre-vaccine screening results a total of nine experimental groups were formed (Table 1). The prophylactic

and therapeutic efficacy of GC vaccine was studied post vaccination by the assessment of humoral and cell mediated immune response and shedding pattern of *Brucella* in these groups. Experiments in three groups of brucellosis negative cattle (Group 1, 2 and 3) approved by the Institutional Ethical Committee (IAEC) and the Committee for the Purpose of Control Experiment of Animals (CPCSEA), Ministry of Environment, Forest and Climate Change, Government of India (Approval number: IIL-R&D-LA-53/2010 dated 08.09.2010) were conducted according to the standard operating procedures (SOP) and guidelines of IAEC/ CPCSEA. Immunization studies in rest of the 6 groups of animals naturally infected with brucellosis were conducted upon receipt of request by the proprietors of the farms and after obtaining consent from the farm authorities.

Experimental groups in Farm I: The purpose of the study in Farm I was to measure the cell mediated immune response in immunized cattle by assessing the secretion of interferon gamma (IFN-γ). Animals were housed in separate sheds and were not allowed to mix with each other. A total of three groups (Group 1, 2 and 3) of 6 cattle per group were maintained in the Holding Farm, Indian Immunologicals Limited (IIL), Hyderabad. Cattle of Group 1 and 2 were administered with $2x10^{10}$ cfu/dose of live *B. abortus* S19 vaccine (Bruvax, IIL) and 50 µg/Ml/dose of S19GC formulations, respectively, by sub-cutaneous route. Animals in Group 2 were given a booster dose of 50 µg/Ml/dose of S19GC each at day 90 post primary immunization. Animals in the Group 3 were inoculated sub-cutaneous with 1ml of phosphate buffered saline (PBS) served as placebo controls. Heparinized whole blood samples were collected at 0, 7, 14, 21, 30, 60, 90, 105 and 120 DPV (Table 1). Peripheral blood lymphocytes harvested from blood samples were used to study the cell mediated immune (CMI) response by ELISPOT (Table 1).

Experimental groups in Farm II: The purpose of the study was to assess the prophylactic and therapeutic efficacy of the S19GC vaccine in an infected farm. The farm housed approximately 1000 animals. The animals were maintained by a trust under animal charity. The animals were left free for grazing together during the daytime and were tied in different sheds at night as routine. Milking animals however, were housed in separate sheds and were stall fed. There was a separate shed for the calves born of these milking animals. Separate sheds were maintained for male and female animals. Hence the farm housed a mixed population of animals that were either negative or positive for brucellosis and were in contact with each other. A total of four groups of adult female cattle were formed namely Group 4, 5, 6 and 7. In Group 4 (n=8) and 7 (n=8) all the animals were negative by serology, isolation and qPCR. In Group 5 (n=8) all the animals were positive by serology but negative by isolation and qPCR. In Group 6 (n=7) all the animals were positive by serology and qPCR with one of the animal positive by culture. All the animals from these three groups were vaccinated with 50µg/ml/dose of the vaccine except group 7 which was kept as unvaccinated negative control group and were inoculated with 1ml of PBS. Nasal swabs, vaginal swabs, milk, serum and heparinized blood samples were collected on day 0, 7, 14, 21, 30 and 60 after vaccination. The samples were used for isolation by culture, antibody isotype subtypes ELISA and qPCR (Table 1).

Experimental groups in Farm III Phase 1: All cattle constituting the Group 8 (n=6) in Farm III were positive for brucellosis by serology and qPCR, of which two were also positive by culture. The purpose of study was to evaluate the effects of booster and the nature of protection offered by S19GC in infected animals over an extended period of observation (250 days). The farm housed 70 animals that mixed freely while grazing. Hence all animals were always in contact with each other. Animals were primed with 50µg/ml/dose of S19GC by sub-cutaneous route and boosted with same dose 90 days after primary immunization. Samples were collected on 0, 7,

14, 21, 30, 60, 90, 105, 120, 150, 210 and 250 days after primary immunization (Table 1). Samples were subjected to isolation by culture, antibody isotype subtypes ELISA, qPCR, whole blood IFN-γ ELISA (IGRA) and IFN-γ ELISPOT assay (Table 1).

Experimental groups in Farm III Phase 2: Animals in Group 9 (n=9) constituted of 7 cattle that were negative for brucellosis by isolation, serology and qPCR, and 2 were positive only by qPCR. Animals in Group 9 were vaccinated sub-cutaneously with 100µg/ml/dose of S19GC

Table 1: Experimental design to study the prophylactic and therapeutic efficacy of *Brucella* S19GC vaccine.

Farm ID	Group ID / No. of animals	Brucellosis status of the animal	Type, route date and dose of vaccination	Booster dose	Sample collection days	Tests done
Farm I	Group 1, n=6	Isolation -ve / Serology -ve / qPCR -ve	S19 live sub-cutaneously 10.12.10 2 x 10^{10}cfu	No booster	0,7,14,21,30,60, 90,105,120 days	IFN-γ estimation by ELISPOT
Farm I	Group 2, n=6	Isolation -ve / Serology -ve / qPCR -ve	S19GC sub-cutaneously 10.12.10 50 µg/ml	50 µg/ml at day 90	0,7,14,21,30,60, 90,105,120 days	IFN-γ estimation by ELISPOT
Farm I	Group 3, n=6	Isolation -ve / Serology -ve / qPCR -ve	PBS sub-cutaneously 10.12.10 1ml	No Booster	0,7,14,21,30,60, 90,105,120 days	IFN-γ estimation by ELISPOT
Farm II	Group 4, n=8	Isolation -ve / Serology -ve / qPCR -ve	S19GC sub-cutaneously 16.11.11 50 µg/ml	No Booster	0,7,14,21,30,60 days	Isolation, antibody Isotype ELISA, qPCR
Farm II	Group 5, n=8	Isolation -ve / Serology +ve / qPCR +ve	S19GC sub-cutaneously 16.11.11 50 µg/ml	No Booster	0,7,14,21,30,60 days	Isolation, antibody Isotype ELISA, qPCR
Farm II	Group 6, n=7	Isolation +ve or Isolation -ve / Serology +ve / qPCR +ve	S19GC sub-cutaneously 16.11.11 50 µg/ml	No Booster	0,7,14,21,30,60 days	Isolation, antibody Isotype ELISA, qPCR
Farm II	Group 7, n=8	Isolation -ve / Serology -ve / qPCR -ve	PBS sub-cutaneously 16.11.11 1ml	No Booster	0,7,14,21,30,60 days	Isolation, antibody Isotype ELISA, qPCR
Farm III	Group 8, n=6	Isolation +ve or Isolation -ve / Serology +ve / qPCR +ve	S19GC sub-cutaneously 05.09.12 50 µg/ml	50 µg/ml at day 90	0,7,14,21,30,60, 90,105,120,150, 210,250 days	Isolation, antibody Isotype ELISA, qPCR, IFN-γ estimation by ELISA and ELISPOT
Farm III	Group 9, n=9	Isolation -ve / Serology -ve / qPCR -ve or qPCR +ve	S19GC sub-cutaneously 04.06.13 100 µg/ml	No Booster	0,14,30,60, 90,120 days	Isolation, antibody Isotype ELISA, qPCR, IFN-γ estimation by ELISA and ELISPOT

Table 2. Status of brucellosis by isolation of *Brucella* by culture: Farm-II, Group 4, 5, 6 and 7.

Farm II	Animal ID Group 4	Status of animal before vaccination			Type of vaccine	Date of vaccination	*Brucella* isolation status post vaccination (days)					
		Isolation	Serology	qPCR			0	7	14	21	30	60
1	4a	N	N	N	S19GC 50µg	16.11.2011	N	N	N	N	N	N
2	4b	N	N	N			N	N	N	N	N	N
3	4c	N	N	N			N	N	N	N	N	N
4	4d	N	N	N			N	N	N	N	N	N
5	4e	N	N	N			N	N	N	N	N	N
6	4f	N	N	N			N	N	N	N	N	N
7	4g	N	N	N			N	N	N	N	N	N
8	4h	N	N	N			N	N	N	N	N	N
Farm II	**Group 5**	**Isolation**	**Serology**	**qPCR**	**Type of vaccine**	**Date of vaccination**	**0**	**7**	**14**	**21**	**30**	**60**
1	5a	N	P	N	S19GC 50 µg	16.11.2011	N	N	N	N	N	N
2	5b	N	P	N			N	N	N	N	N	N
3	5c	N	P	N			N	N	N	N	N	N
4	5d	N	P	N			N	N	N	N	N	N
5	5e	N	P	N			N	N	N	N	N	N
6	5f	N	P	N			N	N	N	N	N	N
7	5g	N	P	N			N	N	N	N	N	N
8	5h	N	P	N			N	N	N	N	N	N
Farm II	**Group 6**	**Isolation**	**Serology**	**qPCR**	**Type of vaccine**	**Date of vaccination**	**0**	**7**	**14**	**21**	**30**	**60**
1	6a	N	P	P	S19GC 50 µg	16.11.2011	N	N	N	N	N	N
2	6b	N	P	P			N	N	N	N	N	N
3	6c	N	P	P			N	N	N	N	N	N
4	6d	P	P	P			P	N	N	N	N	N
5	6e	N	P	P			N	N	N	N	N	N
6	6f	N	P	P			N	N	N	N	N	N
7	6g	N	P	P			N	N	N	N	N	N
Farm II	**Group 7**	**Isolation**	**Serology**	**qPCR**	**Type of vaccine**	**Date of vaccination**	**0**	**7**	**14**	**21**	**30**	**60**
1	7a	N	N	N	Phosphate buffer saline 1ml	16.11.2011	N	N	N	N	N	N
2	7b	N	N	N			N	N	N	N	N	N
3	7c	N	N	N			N	N	N	N	N	N
4	7d	N	N	N			N	N	N	N	N	N
5	7e	N	N	N			N	P	P	P	P	P
6	7f	N	N	N			N	N	N	N	N	N
7	7g	N	N	N			N	P	P	P	P	P
8	7h	N	N	N			N	N	N	N	N	N

without booster. Animals of Group 9 were housed in the same cattle shed occupied by cattle in Group 8. The purpose of this study was to assess the effect of increased dose of S19GC on cattle. Samples collected on 0, 7, 14, 21, 30, 60, 90, 105, 120 days post-immunization were subjected to same assays conducted on samples obtained from animals in Group 8 as described above (Table 1).

Assessment of post vaccine response of cattle:

Cultural Isolation: Modified *Brucella* selective medium (MBS) with 1X concentration of antibiotics was prepared as described by Her *et al*., 2009 (20). Two hundred microliters of samples (milk, nasal/vaginal swabs) in tryptic soya broth with 1X concentration of *Brucella* selective supplement (Hi Media, India) consisting of a cocktail of antibiotics comprising of Polymyxin B sulphate, Bacitracin, Nystatin, Cycloheximide, Nalidixic acid, Vancomycin (transport medium) were plated on MBS media plates in a biosafety level 3 (BSL-3) cabinet, and incubated at 37°C with 5%CO_2 for 4 to 5 days. Small pink shiny smooth colonies representative of *Brucella* species was further confirmed by staining with modified Ziehl-Nelson staining and qPCR.

Antibody Isotype IgG1 and IgG2 specific ELISA: The antibody isotype IgG1 and IgG2 titers against LPS and OMP were determined using an indirect ELISA (17). The checker board titration was done using purified LPS and OMP, purified in house as described by Mythili et al., 2010 (18); and known positive and negative cattle serum. The results were used to arrive for optimal concentration of antigen and serum dilution. The optimal concentration of the antigen fixed was 100ng/well and the dilution of serum was from 1:25 till the endpoint of titration. The log2 reciprocal of end point dilution showing optical density value close to or above the cut-off value was taken as the serum antibody titer for IgG1/IgG2. The cut-off value was determined by frequency distribution of the mean value of day zero titers of all negative group of animals and calculated as Mean±3 S.D. The cut off value for IgG1 against LPS and OMP was determined as 0.142 and 0.217 respectively. The cut off value for IgG2 against LPS and OMP was 0.275 and 0.285 respectively.

The LPS and OMP components of *Brucella* of concentration 100ng/well in carbonate bicarbonate buffer (coating buffer, pH 9.0) were coated on 96 well plates (Nunc Maxisorp™, The Netherlands) and incubated overnight at 4°C. Plates were washed with wash buffer, 0.05% Tween-20 in phosphate buffer saline (PBST, pH-7). Further the plates were blocked with 200µl/well of blocking buffer (3% skimmed milk in PBST). The plates were incubated at 37°C for 1 hour. After incubation the plates were washed and the test sera diluted 1:25 in blocking buffer was added and diluted serially and incubated at 37°C for 1 hour. After the incubation the plates were washed and antibovine IgG1/IgG2 (AbD Serotec, Germany) diluted 1:500 was added 100µl/well and incubated at 37°C for 1 hour. After incubation the plates were washed and recombinant protein A/G conjugated with horse radish peroxidase diluted 1:20000 in blocking buffer was added 100µl/well to all the plates and incubated at 37°C for 1 hour. After incubation the plates were washed and 100µl of chromogen or substrate solution (Tetra methyl benzidine, TMB with hydrogen peroxide, Sigma, USA) was added to the plates. The plates were incubated in dark for 10 minutes at room temperature. The reaction was stopped by adding 1.25M sulphuric acid (Merck, Germany) 100µl/well. Absorbance was measured at 450 nm in the ELISA reader (Multiscan®Titertek™, Finland).

Real time PCR: Quantitative real time PCR (qPCR) targeting *Brucella bcsp31* gene encoding for cell salt extractable outer membrane 31kDa protein, using TaqMan chemistry was developed and standardized (unpublished data). The assay had an analytical sensitivity of 30 fg and detected up to one copy number of the positive control plasmid construct, and $1x10^4$ *Brucella* cells/reaction from spiked bovine tissue matrices like blood, milk, nasal and vaginal swabs. The

diagnostic sensitivity and specificity of the qPCR was 100% and 86.55%. The qPCR was reliable, reproducible and could be completed in 72 minutes. This assay was used in the present study to monitor *Brucella* shedding in vaccinated animals. Briefly, the details of the assay (*viz.,* primers, probe sequence, thermal profile) are furnished below:

The sequence of the primers and probe is as follows:
bcsp31 forward primer :
5' CTCGGTTGCCAATATCAATG 3';
bcsp31 reverse primer :
5' ATATGGATCGTTTCCGGGTA 3';
bcsp31 probe :
FAM 5'CCGGTGCCGTTATAGGCCCA 3' TAMRA.

The selected primers were expected to generate an amplicon of 165 bp in the qPCR. The reaction was performed in 0.2 ml PCR strip-tubes (Qiagen, Germany) with a total reaction volume of 25µl which contained 12.5µl of master mix (Qiagen, Germany) and 10 pico-moles of each primer, 10 pico-moles of probe and 5µl of the template. Reaction conditions were set as follows: Hold at 95°C for 5 minutes, cycling at 95°C for 5 seconds and 60°C for 30 seconds consisting of 40 cycles. Reaction was carried out in Rotor Gene Q qPCR cycler (Qiagen, Germany). Plasmid constructs (pCR™2.1-TOPO®-Bru-bcsp31) harboring the 165bp fragment of *bcsp31* was developed in house and used as internal amplification positive control standard. The plasmid was serially diluted from 10^{10} to 1 copy number and real time reaction was performed for each dilution of the standard, positive, negative controls and the test samples (blood, nasal/vaginal swabs and milk). Reaction threshold (C_q) values of the standards were plotted on a graph against the initial copy numbers of the plasmid. The sample C_q values were placed on the standard graph to know their copy numbers. C_q value of 38 was set as the cut off for positive and negative samples. Samples showing a C_q value below 38 were decalred as positive and the samples showing C_q value above 38 were declared as negative for brucellosis.

For conducting the qPCR the DNA from the test samples (blood, milk, nasal and vaginal) of vaccinated animals was extracted by 'blood and body fluid protocol' of Qiagen Blood mini kit, Germany with slight modifications. Prior to DNA extraction all the clinical samples were spiked with known amount of unrelated linearized plasmid DNA containing E6 gene of human papilloma virus (HPV). This exercise was done to normalize the values of *Brucella* copy number obtained. Normalization was necessary to minimize the variations in qPCR results due to sampling, handling, and other technical errors. After the DNA extraction the samples were assayed by both the qPCRs targeting *bcsp31gene* and HPV E6 gene , using the same thermal profile and PCR parameters as described above, to determine the respective copy numbers of them in each sample. The sequence of primers and probe of HPV qPCR used is as follows:

HPV forward: 5'-
TGGAGACCATCCGATAACAC-3';
HPV reverse: 5'-
GGATGTCTTGTTTGTTTCCG-3';
HPV probe: 5'-FAM/TCT GTG TTC ACC ACC CGG GC/36-TAMS/-3'.

The normalized copy number of *Brucella* genome was calculated as bcsp31: HPVE6 copy numbers for a particular sample. When the copy numbers of the bcsp31 gene were compared without normalization, the variation in copy numbers was up to 10^3 folds (though the samples were spiked with equal amount of *Brucella* organisms). However, the variations were reduced to less than 10 folds, when the copy numbers were normalized with the copy numbers of exogenous control. This indicated that normalization with extraneous DNA was essential.

Whole blood Interferon gamma by ELISA (IGRA): The whole blood IFN-γ stimulation assay was performed as described by Wood and Jones

(21). Firstly the IFN-γ was expressed by stimulating heparinized whole blood of the vaccinated and control group of animals on different days of pre and post vaccination. Secondly a capture ELISA was performed to measure the IFN-γ expression. The IFN-γ was expressed as a Stimulation Index (S.I.) value calculated as (Test sample O.D – Blank) / (PBS O.D – Blank). The cut-off S.I. value was determined by frequency distribution of mean of day zero S.I. values of all negative animals and calculated as Mean±3 S.D. 1ml blood of each animal was stimulated with 25µg of LPS and OMP, concanavalin A (8µg/well) as positive control and PBS as negative control in 24 well tissue culture plates. The plates were incubated in CO_2 incubator at 37°C with 5% CO_2 for 24 hours. After 24 hours the plates were centrifuged at 1000 rpm for 10 minutes. The plasma from each well was collected and stored at -20°C till the testing is done. The capture ELISA was performed using IFN-γ specific antibody obtained from AbD serotec (UK). Suitable standards were also kept for data analysis. Standard curve obtained from the standards was used to estimate the IFN-γ quantity.

Interferon gamma estimation by ELISPOT: Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood collected from the individual animal within 24 hours. PBMCs were isolated by density gradient centrifugation using Lymphoprep™ solution (Axie-Shield PoC AS, RodelØkka, Oslo, Norway). The cell count was taken by trypan blue exclusion technique and the cells were stored in horse serum with 10% DMSO under liquid nitrogen until use. ELISPOT assay was done as per the manufacturer's instructions of the kit ELISpot^{PLUS} for Bovine/Ovine/Equine IFN-γ (MABTECH AB, Sweden) with slight modifications. The assay was standardized and done against *Brucella* OMP component. The OMP concentration was optimized at 25µg/well after testing with various dilutions. The 96 well ELISPOT plate was prewet with 70% ethanol (100µl/well) for exactly two minutes. The plate was then washed with sterile distilled water. The coating antibody (bIFN-γ-I) was added 100µl/well with a concentration 7.5µg/ml to the plate. Next morning the plate was washed with sterile PBS. Later 200µl/well of RPMI with 10% horse serum (cRPMI) was added to the plate and incubated at 37°C for ≥ 30 minutes. While the plate was under incubation the PBMCs were revived and washed two times with RPMI media (Hyclone, U.S.A) +10% Fetal bovine serum (cRPMI) by spinning them at 1500 rpm for 8 minutes. After washing the cells were resuspended in cRPMI and were counted. The cells were added $1x10^6$ per well. OMP antigen and concanavalin A (8µg/ml) was added to the cells in triplicates. Three of the wells were left as blank. Hence total 9 wells were used to test one sample or animal. The plate was incubated at 37°C for 24 hours. After 24 hours the plate was washed with sterile PBS. The detection antibody (PAN-Biotin) of concentration 0.25µg/ml in PBS with 0.5% calf serum (PBS-0.5% FCS) was added 100µl/well to the plate. The plate was incubated for 2 hours at room temperature. After the incubation the plate was washed with sterile PBS and 100µl/well streptavidin-ALP 1:1000 dilution was added. The plate was incubated for one hour at room temperature. After the incubation the plate was washed with PBS and 0.45 micron filtered substrate solution was added 100µl/well and incubated at room temperature for 9 minutes. The reaction was stopped and washed by adding 200µl/well of purified sterile distilled water. The plate was then allowed to dry. The dried ELISPOT plate was scanned to measure the number of spots developed using Immunospot® Series 5 UV Reader (CTL, USA). The results were analysed on the same machine using its automated software features Immunocapture® and Immunospot® for user independent setting of counting parameters (Smartcount®) and the gates (Autogate®).

Statistical Analysis: The anti-*Brucella* specific isotype antibodies were expressed as the reciprocal of log2 end point dilution ± Standard Deviation. The significance of differences between groups and days for both anti-*Brucella*

specific antibodies and T cell population producing IFN-γ were analyzed by employing ANOVA followed by Tukey's HSD. For ANOVA and Tukey's HSD, P value of <0.05 were considered statistically significant (22, 23, 24).

Results

Safety of S19GC vaccine: In all the animals after vaccination no local reaction at the site of inoculation except a pea size swelling was observed that persisted for 72 hours post vaccination. All vaccinated animals were healthy and did not exhibit any unwanted systemic reaction during the observation period of 21 days.

Farm I: In this farm for both the Groups 1 and 2, the mean spot forming units (SFU), wherein each spot indicated a IFN-γ secreting cell against outer membrane protein of *B.abortus* S19 strain (S19OMP) were found to be significantly different when compared with unvaccinated controls (P = 0.0061) (Fig 1A, Fig 1B). The mean SFU values in Group 1 and 2 also differed on different days (P <0.0001) post vaccination (7, 14, 21, 30, 60, 90 DPV) but not at post-booster (PB) (P =0.3715) (Fig 2). Also, the IFN-γ response in placebo controls did not differ statistically between different days of observation.

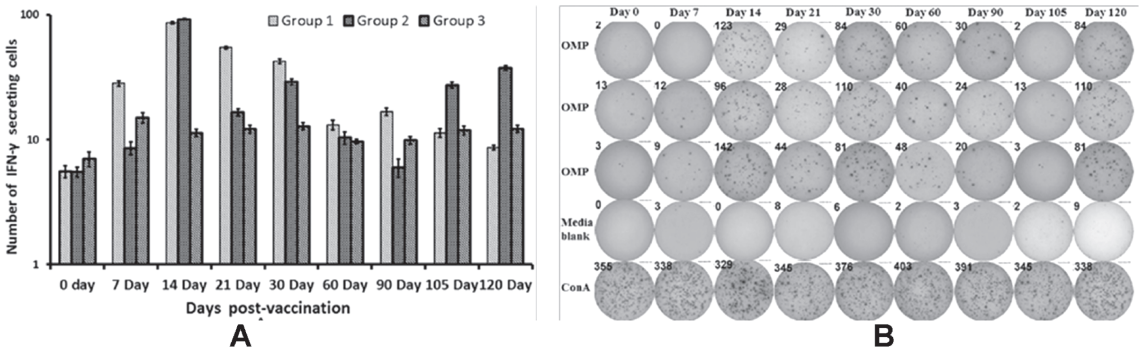

Fig.1. (A) CMI response by ELISPOT: Farm-I, Group 1, 2 and 3 (B) CMI response by ELISPOT-picture: One of the animals of Farm-I, Group 2.

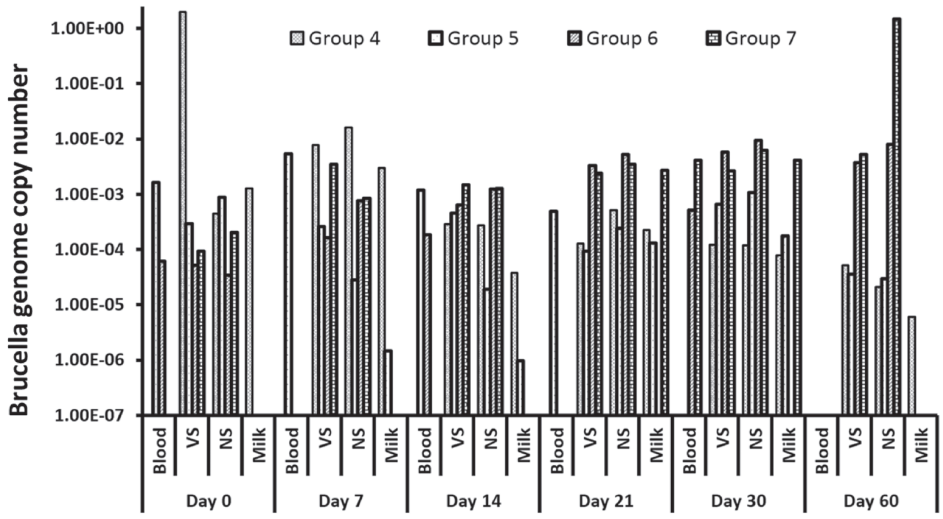

Fig. 2. *Brucella* shedding in blood, nasal, vaginal swabs and milk by bcsp31 qPCR: Farm-II, Group 4, 5, 6 and 7.

Farm II: In this farm one of the animals from Group 6 which was positive by cultural isolation stopped shedding from 7 DPV till 60 DPV. Similarly, in case of qPCR positive and serology positive animals from Group 5 and 6 no bacterial shedding was noticed till 60DPV. Also the vaccinated *Brucella* negative animals in Group 4 remained negative till 60 DPV. Whereas in the unvaccinated control (Group 7) two animals showed shedding from 7 DPV till 60DPV (Table 2). The qPCR results of animals of Group 4, 5 and 6 showed on an average 2 log reduction of *Brucella* genome in case of blood and vaginal secretions on 7 DPV; and also in milk and nasal secretions on 21 DPV. Whereas in unvaccinated animals in Group 7 the *Brucella* genome copy number showed 3 log folds increase in case of nasal secretion and 1 log fold increase in vaginal secretions on 60DPV (Fig 2). The isotype antibody ELISA results of group 4, 5, and 6 indicated that the IgG1 and IgG2 response elicited in terms of log2 end point titers against LPS and OMP components of S19GC vaccine were significantly different from day 0 pre-vaccination when compared with 60 DPV ($P<0.05$) (Figs. 3, 4).

Farm III Phase 1: Two animals in Group 8 positive by culture on day 0, continued to shed *Brucella* till 7DPV. From 14 DPV the shedding was stopped till 30 DPV. On 60 DPV one of the animals out of the two resumed shedding; and in addition, another animal of the group started shedding. The shedding continued till day 90 PI. After the administration of booster dose on day 90 PI, both the animals stopped shedding again, till day 120 PI. From day 150 PI, one animal in the group which was positive by culture from day 0 resumed shedding (Table 3). The *Brucella* genome was not detectable from 7 to 150 DPV. The mean isotype antibody titers in post-vaccinated samples were non-significant when compared to pre-vaccine titers. Similarly, pre-immunization SFU values of IFN-γ secreting cells observed by ELISPOT were not significant when data was compared for all sampling time points after primary immunization and PB. Similarly, the IGRA-ELISA values were non-significant till 90DPV; and analysis of data of samples collected PB showed inconclusive results.

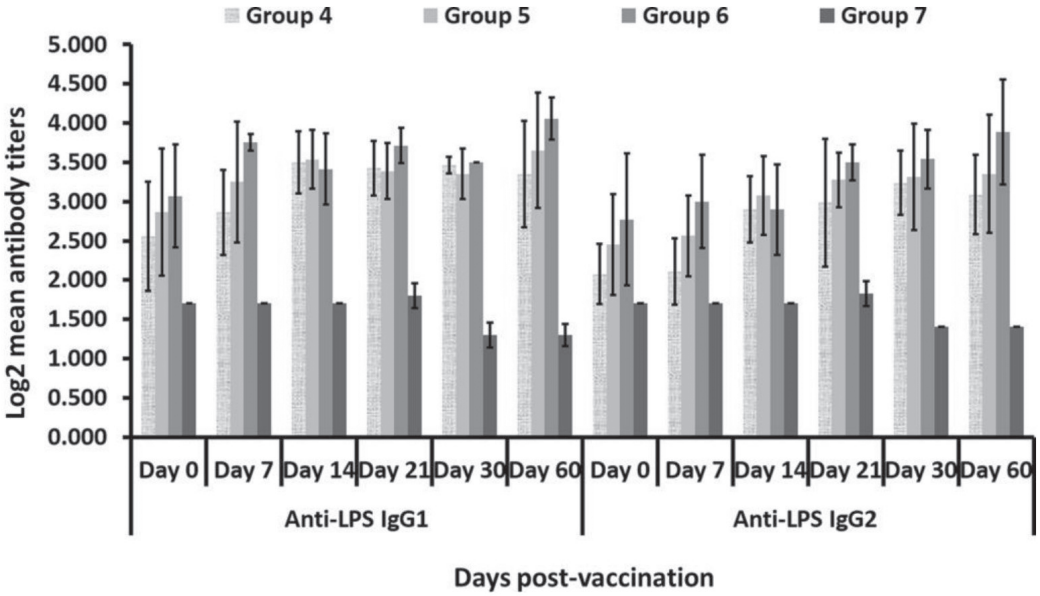

Fig.3. Anti LPS IgG1 and IgG2 response by ELISA: Farm-II, Group 4, 5, 6, and 7.

Farm III Phase 2: Samples from this group of animals were negative by culture (Table 4). The qPCR also did not detect any *Brucella* genome till 120DPV. Comparison of isotype antibody ELISA results between the days showed that the IgG1 and IgG2 titers against LPS and OMP of day 0 pre-vaccination was significantly different from those observed at 30 DPV ($P<0.05$) (Fig 5). However, the IFN-γ response against LPS and OMP as determined by IGRA-ELISA and ELISPOT displayed inconclusive results.

Discussion

Vaccination is a critical component in control and eradication program of bovine brucellosis. In the U.S.A, Australia and in most of the EU countries the disease has been eradicated after large investments and many cycles of culling and vaccinations. As discussed

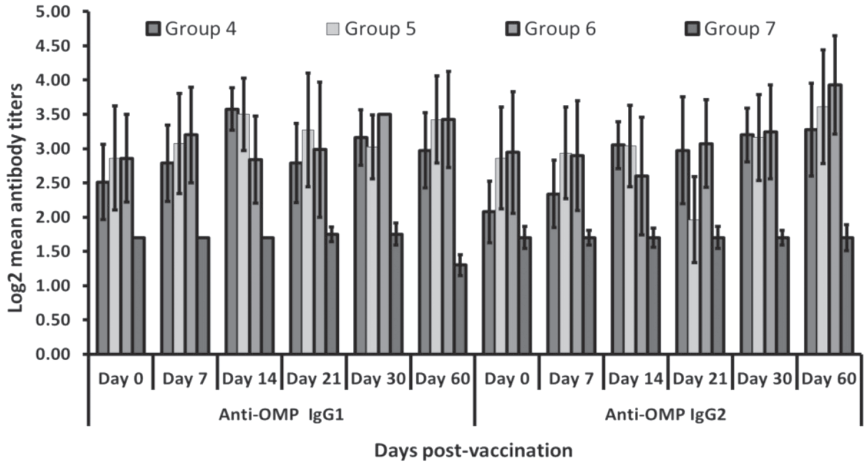

Fig.4. Anti OMP IgG1 and IgG2 response by ELISA: Farm-II, Group 4, 5, 6, and 7.

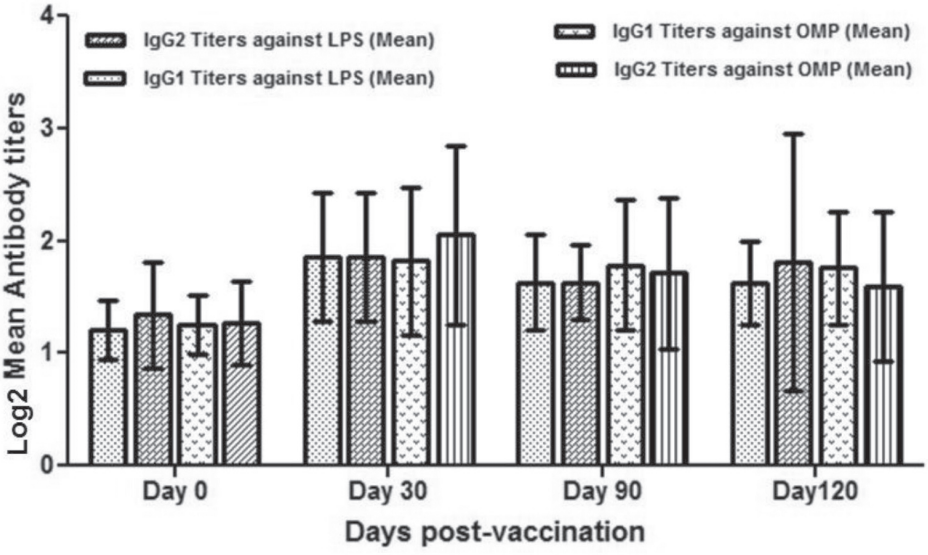

Fig. 5: Anti LPS, anti OMP IgG1 and IgG2 response by ELISA: Farm-III-Phase-2, Group 9.

Table 3. Status of brucellosis by isolation of *Brucella* by culture: Farm-III-Phase-1, Group 8.

Farm III	Animal ID Group 8	Status of animal before vaccination			Type of vaccine	Date of vaccination	*Brucella* isolation status post vaccination (days)					
		Isolation	Serology	qPCR			0	7	14	21	30	60
1	8a	P	P	P	S19GC 50ug	5.09.12	P	P	N	N	N	N
2	8b	P	P	P			P	P	N	N	N	P
3	8c	N	P	P			N	N	N	N	N	N
4	8d	N	P	P			N	N	N	N	N	P
5	8e	N	P	P			N	N	N	N	N	N
6	8f	N	P	P			N	N	N	N	N	N
Farm III	**Group 8**	**Isolation**	**Serology**	**qPCR**	**Type of vaccine**	**Date of vaccination**	**90**	**105**	**120**	**150**	**210**	**250**
1	8a	P	P	P	S19GC 50ug	5.09.12	N	N	N	N	N	N
2	8b	P	P	P			P	N	N	P	P	P
3	8c	N	P	P			N	N	N	N	N	N
4	8d	N	P	P			P	N	N	N	N	N
5	8e	N	P	P			N	N	N	N	N	N
6	8f	N	P	P			N	N	N	N	N	N

Table 4. Status of brucellosis by isolation of *Brucella* by culture: Farm-III-Phase-2, Group 9.

Farm III	Animal ID Group 9	Status of animal before vaccination			Type of vaccine	Date of vaccination	*Brucella* isolation status post vaccination (days)					
		Isolation	Serology	qPCR			0	14	30	60	90	120
1	9a	N	N	P	S19GC 100µg	4.06.13	N	N	N	N	N	N
2	9b	N	N	N			N	N	N	N	N	N
3	9c	N	N	P			N	N	N	N	N	N
4	9d	N	N	N			N	N	N	N	N	N
5	9e	N	N	N			N	N	N	N	N	N
6	9f	N	N	N			N	N	N	N	N	N
7	9g	N	N	N			N	N	N	N	N	N
8	9h	N	N	N			N	N	N	N	N	N
9	9i	N	N	N			N	N	N	N	N	N

the present vaccines (S19 and RB51) do not provide 100% protection and are virulent to the animals and humans. Also these vaccines cannot be given to all the age groups and genders. Hence development of subunit vaccines is an attractive alternate approach to immunization since these are expected not to have a set of drawbacks as mentioned above.

It has been shown earlier that immunization with LPS from *B. melitensis* non-covalently linked

to *Neisseria meningitidis* OMP was able to offer protection in mice (17). It has been also shown that S19GC vaccine was able to protect mice against the virulent challenge with *B. abortus* strain 544 and cure splenic infection in 91% mice (19). In addition, the S19GC has been also shown to elicit appropriate and specific antibody and cell mediated immune response in cattle calves immunized by subcutaneous route (18). Recently, Cherwonogrodsky *et al*., 2014 (25) has shown that mice immunized sub-cutaneously with a GC formulation using 90% of polysaccharides (PS) and 1-3% of protein from *B.suis* 145 strain are protected upon challenge with virulent strains of *B. abortus*, *B. melitensis* and *B.suis*.

GC vaccine derived from *B. abortus* S19 (S19GC) was tested in cattle for assessing protective efficacy and therapeutic potential. The assessment of S19GC to elicit appropriate immune response compared to a live *B.abortus* S19 vaccine was studied in a brucellosis free farm (Farm-I) by IFN-γ response employing ELISPOT assay. The 50 µg sub-cutaneous dose of S19GC vaccine was found safe for cattle. The ELISPOT assays demonstrated the ability of S19GC to elicit specific IFN-γ response after stimulation with S19 OMP that indicated a Th1 mediated immune response event. The cattle immunized with S19GC differed significantly from controls and at different time points of observation during the pre-booster stage. A similar IFN-γ response has been shown in cattle calves in whole blood stimulation assays (18). The IFN-γ response plays a significant role in killing of *Brucella* by activation of the macrophages (26). Protective immunity to the intracellular pathogen *Brucella* is mostly cell mediated.

The ability of S19GC to stop shedding was assessed in a brucellosis infected farm (Farm-II) maintained as a closed free mixing herd, in 4 groups of animals (Group 4, 5, 6 and 7) at weekly interval till 60 days post immunization. During the pre-vaccine stage 48.3% (15/31) of the animals screened from the four groups were positive for brucellosis. Following immunization shedding of *Brucella* was arrested in cattle from Group 6 beginning 7 DPV till the end of the study period (60 DPV) as animals remained negative by culture; and the qPCR indicated a 2 log fold reduction in the genome copy number. Immunization of animals in Groups 4 and 5 with S19GC prevented them from acquiring fresh infection by natural challenge up to 60 DPV. However 2 out of 7 animals from the brucellosis free unvaccinated controls (Group 7) continued shedding *Brucella* till 60 DPV. The nasal and vaginal secretions collected from these animals on different days during the period of the study (0, 7, 14, 21, 30, 60 DPV) showed progressive increase in the number of bacteria as estimated by qPCR as illustrated in the Figure 2. Overall they showed a 1 to 3 log folds of increase in the genome copy number. The antibody isotype IgG1 and IgG2 titers against LPS and OMP were significantly different when compared to pre-vaccination status in vaccinated group of animals (Group 4, 5 and 6). Similar response has been observed in cattle calves by Mythili *et al*., 2010 (18), and in mice after immunization with subunit vaccines of *B. melitensis* (17). The antibody isotype subtypes (IgG1 and IgG2) response reported in the current study indicated a Th1 as well as Th2 type of response (27) induced by the S19GC vaccine.

The effect of booster and dose of S19GC in cattle was studied in two phases, in another farm (Farm-III) infected with brucellosis, in two groups of cattle (Group 8 and 9). At pre-immunization 53.33% (9/15) animals included in the study had brucellosis as determined by isolation/serology/qPCR. The maintenance of the herd was closed and free-mixing. The shedding in 66.67% (4/6) cows from Group 8, vaccinated sub-cutaneously with 50 µg dose at Day 0 and 90 DPV, was temporarily arrested for 53 days (from 7 to 60 DPV). The effect of booster was clearly observed since only 1/6 animals resumed shedding 60 days post booster, and in the remaining cows (83.34%) shedding was absent till the end of the observation period (250 DPV). Further, the *Brucella* genome was undetectable in nasal/ vaginal secretions from 7 to 150 DPV, but not thereafter, suggesting that the

immunization regimen adopted was successful in arresting the shedding transiently in an endemic situation. Finally, 9 cows in Group 9 from Farm-III, who were in contact with animals of Group 8, from which 2 were positive by qPCR, upon immunization with 100 µg S19GC by subcutaneous route without booster showed complete stoppage of shedding as evidenced by culture and qPCR till the end of study (120 DPV). The antibody isotype ELISA showed a significant difference of IgG1 and IgG2 response against LPS and OMP components of S19GC between pre-immunization and 30DPV.

The overall results indicated that 100µg dose of S19GC vaccine when administered subcutaneously to adult female cattle is able to induce therapeutic as well as prophylactic effect up to 120 DPV. The vaccine was found to be safe and could trigger both humoral and cell mediated immune response. The involvement of Th1 and Th2 cells was marked by pronounced antibody response which in turn was able to stop shedding in case of infected animals and prevented brucellosis free animals from acquiring fresh infection from in contact animals carrying *Brucella* infection.

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