The Pharma Innovation Journal 2020; 9(4): 300-305

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2020; 9(4): 300-305

www.thepharmajournal.com
Received: 10-02-2020
Accepted: 12-03-2020

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Safety and immunogenicity evaluation of *Brucella abortus* S 19 reduced dose vaccine in comparison with *Brucella abortus* S 19 standard dose vaccine in cattle

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DOI: https://doi.org/10.22271/tpi.2020.v9.i4e.4602

Abstract
Bovine brucellosis, caused by *Brucella abortus*, is a serious zoonotic disease manifested by reproductive disorders in animals and results in huge economic losses to dairy farmers. This study was undertaken to evaluate the safety and immunogenicity of *Brucella abortus* S-19 reduced dose vaccine in comparison with *Brucella abortus* S-19 standard dose vaccine in 4-10 months old female cattle calves by measuring humoral immune responses and cell mediated immune responses. The humoral antibody responses in reduced dose group indicated absence of persistent antibody titres, whereas, those vaccinated with *Brucella abortus* S-19 standard dose vaccine showed persistence in antibody titres till day 240 with i-ELISA and day 150 with c-ELISA post vaccination. The IFN-γ responses in vaccinated groups were found to be diminishing till 3 years post vaccination. It is concluded that *Brucella abortus* S-19 reduced dose vaccine performed similar to that of *Brucella abortus* S-19 standard dose vaccine.

Keywords: Brucellosis, reduced dose vaccine, standard dose vaccine, persistent antibody, IFN – γ responses.

1. Introduction
Bovine brucellosis is an important zoonotic disease that causes major reproductive problems including abortions in advanced pregnancy, retention of placenta, infertility, stillbirths, the birth of weak calf and calf mortalities causing substantial economic losses to the dairy industry. Mantur and Amarnath (2008) observed that brucellosis prevalence varied across different regions and farms in various agro-ecological regions of India. As per Renukaradhya *et al* (2002) [1], long-term serological studies indicated baseline seroprevalence of 5% in cattle and 3% in buffaloes. The Animal Disease Monitoring and Surveillance (ADMAS) [2], Bangalore report on long term survey of bovine brucellosis (1994 - 2002) indicated that the disease is widespread in most parts of the country with a cumulative average of 6.8% in bovines. The overall national average incidence of brucellosis in cattle and buffaloes was 7.2% and 5.25%, respectively. Singh *et al*. (2015) [3], estimated the losses due to brucellosis in livestock to be the US $ 3.4 billion, in addition to the economic and social losses due to brucellosis in humans.

Vaccination has been recognized as the ideal strategy for disease control. Brucellosis Control Program in India as outlined in the 12th Plan envisaged vaccination of female calves aged 4-8 months in all states. Moreno (2002) [4] observed that vaccination of calves of 4-8 months of age with Brucella abortus S-19 vaccine as practiced in India is not very useful and it is a truncated policy for endemic areas. Nicoletti (1990) [5] suggested that the disease can be prevented by the use of attenuated strain 19 vaccines which induces resistance to infection and abortion lasting several years in a significant number of vaccinated animals. Apart from the standard dose, OIE Terrestrial Manual 1996 [6] recommends a reduced dose vaccine with a CFU per dose of 3 x 10^8 to 3 x 10^9 organisms subcutaneously to calves.

The present study was undertaken to evaluate the safety and immunogenicity of Brucella abortus S-19 reduced dose vaccine in comparison with Brucella abortus S-19 standard dose vaccine in 4-10 months old female calves by evaluation of both humoral and cell mediated immune responses.

2. Materials and methods

2.1 Animals

Necessary approvals and informed consent were obtained before starting the experiment. One hundred forty (140) female bovine calves comprising of indigenous and cross bred animals in the age group of 4 to 10 months were randomly allocated to three experimental groups (Table 1), namely, Group 1, Group 2 and Group 3 (Control group). The animals were screened against Brucellosis before initiating the experiments. The details of the vaccines administered with dose, colony forming units (CFU) per dose and route of administration are presented in Table 1.

Table 1: Animal grouping and vaccination details

Group details	Age of the animals	Number of animals	Vaccine for administration	Dose, CFU/dose and route of administration
Group 1	4 to 10 months	65	*Brucella abortus* S-19 reduced dose vaccine	2 ml, 3 to 30 X 10^8, subcutaneous route
Group 2	4 to 10 months	65	*Brucella abortus* S-19 standard dose vaccine	2 ml, 40 to 80 X 10^9, subcutaneous route
Group 3 (Control)	4 to 10 months	10	No vaccination (only diluent administered)	Diluent @ 2 ml per animal by subcutaneous route

2.2 Vaccines and vaccination

Group 1

Sixty-Five female calves in Group 1 were vaccinated with *Brucella abortus* S-19 strain reduced dose vaccine (Brand name "Bruvax Plus" manufactured by Indian Immunologicals Limited) having CFU of 3 to 30 X 10^8 per dose of 2 ml by subcutaneous route after reconstitution with the vaccine diluent provided with the vaccine pack.

Group 2

Sixty-Five female calves in Group 2 were vaccinated with *Brucella abortus* S-19 strain vaccine (Brand name "Bruvax" manufactured by Indian Immunologicals Ltd.) having CFU of 40 to 80 X 10^9 per dose of 2 ml by subcutaneously after reconstitution with the vaccine diluents provided with the vaccine pack.

Group 3

Ten female calves in the same age group served as Control group and did not receive any vaccine and were administered with vaccine diluent only @ 2 ml per animals by subcutaneous route.

2.3 Screening of animals

Initial screening of the animals for the absence of Brucella antibodies was ensured by employing RBPT and in house i-ELISA and only sero negative animals were included in the experimental study.

2.4 Bleeding details

The blood collection details in the experiment are as follows: Day -7, 0, 21, 60, 90, 120, 150, 180, 210 and 240 DPV (days post vaccination), 1 year and 3 years post vaccination. Vacutainers were employed for collection of blood samples from each animal for the separation of serum. For the cell mediated immune response evaluation, whole blood samples in heparin coated vacutainers were collected.

2.5 Serology

Testing for initial screening was carried out by RBPT reagents procured from AHVLA, UK and by i-ELISA, which was an in-house developed method. After the commencement of the experiment, sera samples collected at various time points were analyzed by i-ELISA, c-ELISA (a commercial kit procured from AHVLA, UK) for antibodies. Cell mediated immune responses were evaluated by IFN – γ responses on stimulated peripheral blood cells at various time points of experiment. Commercial kits were used as per the manufacturer's instructions.

2.6 Safety evaluation

All the vaccinated and the control animals were monitored for safety till 14 days of vaccination for adverse events such as pain at the site of inoculation, swelling, rash, skin eruption, redness or induration at the site of injection and systemic signs like rise in body temperature, loss of appetite, diarrhea, restlessness and any other adverse events or allergic reactions.

2.7 Statistical analysis

The OD values at different days were analyzed by applying descriptive statistics and given as mean ± standard error. ANOVA test was performed using summary statistical data among the treatment groups for arriving at statistical significance.

3. Results and discussion

3.1 Safety observations

The observations noticed till 14 days after vaccination did not reveal any safety issues and all the vaccinated animals in both groups 1 and 2 remained healthy and were well tolerant to the vaccine administration. Control animals remained healthy throughout 14 days observation.

3.2 Humoral immune responses

Studies of the responses of cattle calves after *Brucella abortus* vaccination or infection involve the measurement of humoral immune responses and several serological tests are in use such as MRT, Tube agglutination test, RBPT, CFT, Indirect ELISA, c-ELISA, and Fluorescence polarization assay. The percentage of seroconversion analyzed by i-ELISA with both Groups 1 and 2 with mean OD values are presented in Table 2 and Fig 1. With i-ELISA, detectable serum antibody persistence was noticed till day 120 post vaccination in animals vaccinated using a reduced dose vaccine while with standard dose, the antibody persistence was observed till day 210 post vaccination though small percentage of animals were also positive at day 240 post vaccination. From day 150 post vaccination till 3 years post vaccination, no positive reactors were detected with reduced dose vaccine group and in case of the standard dose, antibody persistence was not observed from 1 year post vaccination.

Table 2: Percentage of sero-conversion analyzed by i-ELISA at different post-vaccination time points with Mean OD values.

Group details	0 day	21 DPV	60 DPV	90 DPV	120 DPV	150 DPV	180 DPV	210 DPV	240 DPV	1 year PV	3 years PV
Group 1	0 (0.122 ±0.012)	100 (1.051 ±0.035)	18.5 (0.306 ±0.048)	12.00 (0.390 ±0.034)	9.00 (0.171 ±0.028)	0 (0.098 ±0.007)	0 (0.1724 ±0.019)	0 (0.2421 ±0.024)	0 (0.1069 ±0.012)	0 (0.1075 ±0.007)	0 (0.3727 ±0.017)
Group 2	0 (0.0978 ±0.006)	100 (1.0782 ±0.031)	33.80(0.4467 ±0.057)	21.50 (0.4171 ±0.040)	18.00 (0.2305 ±0.034)	16.50(0.2117 ±0.030)	11.00(0.2147 ±0.027)	8.00 (0.2451 ±0.024)	2.00 (0.2536 ±0.021)	0 (0.2760 ±0.020)	0 (0.1090 ±0.016)
Group 3 (Control)	0 (0.0722 ±0.019)	0 (0.4105 ±0.048)	0 (0.2502 ±0.057)	0 (0.1697 ±0.041)	0 (0.0995 ±0.027)	0(0.0732 ±0.010)	0 (0.1131 ±0.018)	0 (0.1085 ±0.029)	0(0.1014 ±0.031)	0 (0.1343 ±0.048	10.00 (0.1412 ±0.072)

Figures in brackets indicate mean OD values ±SE

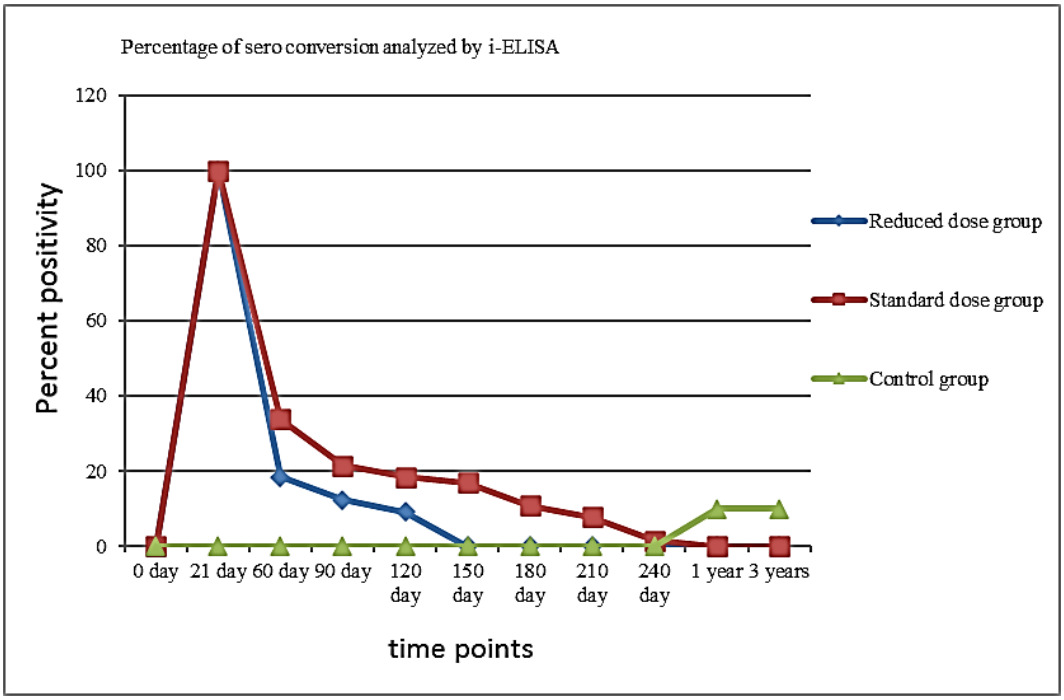

Fig 1: Percentage of seroconversion analyzed by i-ELISA

With reduced dose vaccine, the persistence of antibodies is observed till day 60 post vaccination and by 90 days post vaccination, no antibodies could be detected using c-ELISA and the animals remained sero negative till 3 years of study period. With standard dose vaccine, the persistence in serology was noticed till day 150 post vaccination. The percentage of seroconversion analyzed by c-ELISA with both Groups 1 and 2 with mean OD values are presented in Table 3 and Fig 2.

Similar responses were observed by Carrasco *et al.* (1998) [7] after vaccination with Brucella abortus S-19 vaccine in heifers by c–ELISA and i-ELISA. These authors also explained how different vaccines, dose and age at which an animal is immunized and use of different reagents result in varying results. In their study, a diminished percentage of positive animals towards 253 DPV and 316 DPV were observed after vaccination by c-ELISA and i-ELISA respectively. Poester *et al*. (2000) [8] made similar observations using reduced dose of *Brucella abortus* S-19 vaccine in the evaluation with i-ELISA and could notice that no adult female cattle being positive for the test at day 360 post vaccination.

A few animals in the control groups showed positive titres at 3 years post vaccination, indicating possible exposure to brucella infection in the field. Munir (2009) [9], in a study involving *Brucella abortus* S-19 vaccine in adults, heifers and calves (buffaloes) demonstrated that ELISA tires were maximum at 30 DPV with a gradual decline and by 180 DPV in adults, the titers were negative. Similarly in heifers and calves the gradual decline followed complete absence of positive animals by 120 DPV and 90 DPV. Generally, persistence of antibody titers increases with the age at which animal vaccinated, and to overcome this, vaccination is undertaken on female calves of 3-8 months age with Brucella abortus S 19 vaccines using either the standard dose or the reduced dose. Nicolette P (1990) [10] observed that a reduced dose vaccine in calves greatly helps in minimizing the residual antibody titers and to prevent persistence.

Table 3: Percentage of seroconversion analyzed by c-ELISA at different post-vaccination time points with Mean OD values.

Group details	0 day	21 DPV	60 DPV**	90 DPV	120 DPV	150 DPV	180 DPV	210 DPV	240 DPV	1 year PV	3 years PV
Group 1	0 (0.5479 ±0.008)	100 (0.2266 ±0.011)	12.3(0.5636 ±0.015)	0 (0.653 ±0.011)	0 (0.613 ±0.010)	0 (0.625 ±0.006)	0 (0.618 ±0.008)	0(0.618 ±0.008)	0 (0.595 ±0.007)	0(0.549 ±0.009)	0(0.5715 ±0.007)
Group 2	0 (0.5333	100 (0.2709	33.84 (0.4914	21.53 (0.542	9.23(0.560 ±0.010)	4.60 (0.736	0(0.618 ±0.008)	0 (0.630	0(0.562 ±0.005)	0(0.5396 ±0.008)	0(0.5396 ±0.010)

	±0.007)	±0.010)	±0.019)	±0.014)		±0.12)		±0.010)			
Group 3 (Control)	0 (0.566 ±0.014)	0 (0.5844 ±0.014)	0(0.5859 ±0.018)	0(0.588 ±0.017)	0(0.559 ±0.011)	0 (0.537 ±0.006)	0(0.593 ±0.011)	0(0.571 ±0.011)	0(0.532 ±0.010)	0(0.5520 ±0.024)	10.00 (0.5396 ±0.010)

**indicates significant ($p<0.01$) difference at that time point
Figures in brackets indicate Mean OD values ± SE

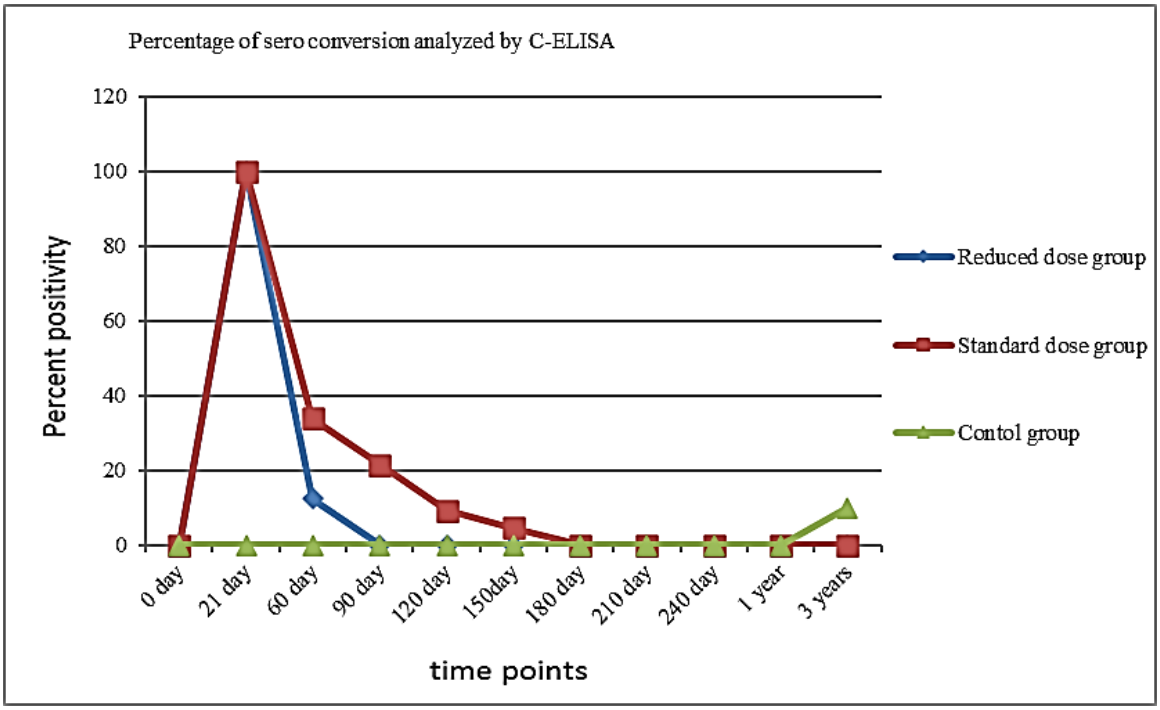

Fig 2: Percentage of seroconversion analyzed by C-ELISA

3.3 Cell mediated immune responses

The percentage of seroconversion in various groups as analyzed by IFN - gamma assay at different post-vaccination time points against *Brucella abortus* S-19 and 544 antigens are presented in Fig 3 and Fig 4 respectively.

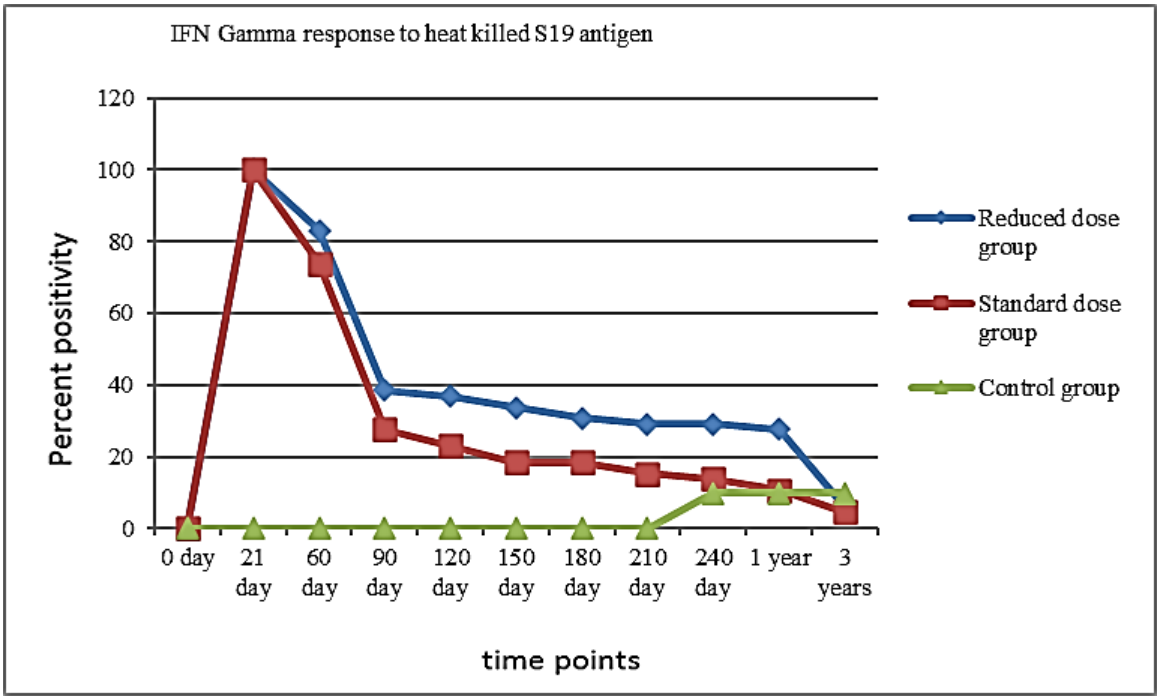

Fig 3: IFN Gamma response to heat killed S19 antigen

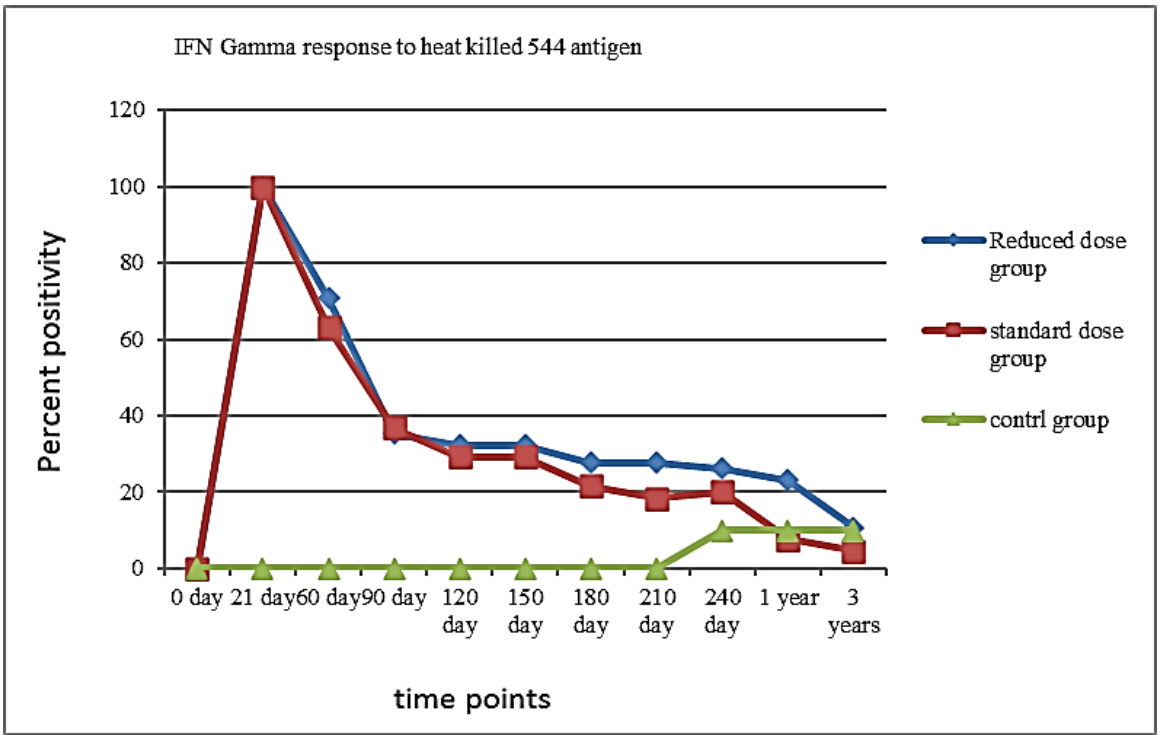

Fig 4: IFN Gamma response to heat killed 544 antigen

Protection against Brucella, as an intracellular pathogen is believed to be associated with the secretion of interleukin 12, IFN – γ by Th1 type helper T cells and the subsequent activation of cytotoxic T cells, NK and macrophages (Winter *et al*, 1998 and Murphy *et al*, 2001) [11, 12]. The stimulation indices of the standard dose group were slightly lesser compared to the reduced dose group till 3 years of study. Davies *et al.* (1980) [13] demonstrated a minimum protective dose with S-19 vaccine in the range of 4.5 X 10^9 viable units and 9 X 10^7 viable units. Their study also revealed that when groups of calves vaccinated with a normal dose (with 9 x 10^{10} viable units), 1/20th of the normal dose (4.5 x 10^9 viable units), and 1/100th of the normal dose (9 x 10^7 viable units) responded to vaccination. McDiarmid (1954) [14] showed that when S 19 vaccine given either in full dose form or at 0.04 dose of the full dose conferred protection in pregnant heifers by subcutaneous route.

Similar observation was presented by Manthei *et al.* (1952) [15] in 12-15 months old heifers given a full dose, 0.04 dose by subcutaneous respectively and 0.04 dose by intradermal route. There was little difference in the immune response of the three groups. Similarly experiments conducted by Campbell and Rodwell (1945) [16] showed reduced dose provoked satisfactory agglutinin response and a minimum local reaction. McDiarmid (1957) [17] demonstrated the immunity of the cattle vaccinated between ages 6-8 months does not decrease from the first through fifth pregnancy. The present study revealed that S-19 strain at a reduced dosage form can be safely administered to the animals. Using both RB 51 and Strain 19 vaccines in doses of 3 X 10^8 to 3 X 10^9 including pregnant cows in Mexico, Barradas Pinna *et al.* (2012) [18] demonstrated the protection in vaccinated females against abortions and both vaccines were safe for administration. Subcutaneous vaccination of mature non pregnant cattle with 3 X 10^8 CFU of S19 strain produced immunity as good as that produced by calfhood vaccination by the same route and subcutaneous vaccination of infected dairy herds with 3 x 10^9 CFU of strain 19 has also been shown to reduce effectively the natural infection rate in 6 months (Alton *et al*, 1984) [19]. Kaneene *et al.* (1979) [20] found that cellular responses persisted longer in calves than humoral responses following *Brucella abortus* strain 19 vaccination and that there was an absence of correlation between the responses.

4. Conclusions

In calves, to minimize residual antibody titers and to prevent persistent vaccinal infection, vaccination with *Brucella abortus* S19 reduced dose vaccine can be advocated. The data obtained in the present study show that RBPT with standard reagents can be used as a test for preliminary sero negative status establishment for proceeding with vaccine studies. It is concluded that the safety and immunogenicity of *Brucella abortus* S 19 reduced dose vaccine is comparable with that of the standard dose *Brucella abortus* S 19 vaccine in calves and both vaccine formulations are well tolerated by the animals and can be safely administered.

Acknowledgments

Authors acknowledge the support and help received from Indian Immunologicals Limited.

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