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Research article

Studies on intra-ocular vaccination of adult cattle with reduced dose *Brucella abortus* strain-19 vaccine

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ABSTRACT

Brucella abortus vaccines play a central role in bovine brucellosis control with tremendous success worldwide for decades. The study was aimed to evaluate the efficacy of reduced dose (5.0 × 10 9 cfu) of S19 vaccine in adult cattle and its shedding in the milk of vaccinated cattle using molecular techniques. The OIE recommended tests (RBPT, SAT, and iELISA) for brucellosis screening in cattle were used. Seronegative cattle (n = 90) of different age groups (young, old heifers & milking cows, n = 30 each) were selected for the vaccine trials. Antibody titers were recorded at 7th, 21st, 30th, 60th, 90th and 120th days post-vaccination (DPV) to monitor the immune responses following vaccination and at 150th, 180th, 210th and 240th DPB following booster-dose to an intraocular group. The humoral immune responses observed by RBPT and ELISA, proved that antibody titers persisted in s/c group compared to the i/o group in all categories. The IFN-γ stimulation (CMI) due to reduced dose vaccination was noticed early as 30th in all groups and declined after 90th DPV, with higher IFN-γ stimulation among the s/c group. The Bcsp31 and IS711 targeted PCR detected the presence of *Brucella* DNA in milk samples (n = 120) from the vaccinated cows (n = 30) and confirmed by qPCR (TaqMan assay) at 30th, 60th, 90th and 120th DPV. A Significant number, 70% (7/10) was detected in s/c by qPCR. BCSP31 sequence was deposited at NCBI GenBank (accession no. MK881173-6). PCR and qPCR techniques could provide a reliable diagnosis of brucellosis from milk. The intraocular route remains the safer route for vaccinating adult cattle than subcutaneous.

1. Introduction

Brucellosis is a complex zoonotic disease with significant epidemiological, economic, and global health impacts, particularly for human and animal populations in developing countries that rely on cooperative farming and agricultural practices [1]. Evidence of changing ecology and reemergence of *Brucella* over recent years has demonstrated the pathogen's ability to seamlessly and rapidly adapt to the modern world, hence necessitating innovative approaches to an epidemiological study [2]. Brucellosis is caused by the *Brucella* organisms (commonly: *Brucella abortus*, *B. melitensis*, and *B. suis*) with the main clinical signs in animals being abortion and infertility.

Brucella organisms can also affect humans through the ingestion of contaminated milk and milk products derived from infected animals. It can be spread through contact with aborted fetuses, vaginal fluids, placental fluids, milk, and vertically [3]. Infection of female animals can result in abortion at advanced pregnancy, the birth of unthrifty calves, and retention of the placenta with reduced milk yield [3, 4]. The nature of the disease is deceptive, and in most situations, the infected animals remain a carrier of the disease throughout their life with the shedding of *B. abortus* in vast amounts through the placenta, fetus, uterine discharges, and in milk [5]. The disease is affecting approximately 268.81 cases per 100,000 people annually around Asia and 34.86 cases around Sub-Saharan Africa [6]. Brucellosis remains endemic in many regions of

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the world, including Latin America, the Middle East, Africa, Asia, and the Mediterranean basin [2]. However, clinical signs are often misinterpreted; as a result, human brucellosis is underreported and remains a major neglected zoonosis of low-income nations [7].

The serological evidence of infection has been reported from various states within the Indian subcontinent, indicating its endemic situation [8, 9, 10], also demonstrated that brucellosis is endemic in many developing countries in Asia and Sub-Saharan African countries, including India and Nigeria respectively, and evidence of infection is available in cattle, buffaloes, sheep, goats, camels, and human beings (veterinarians, nomads, herdsmen, farmers, cattle owners, butchers, and laboratory staff). According to [8], who reported the isolation of 46 *Brucella* strains from cattle, buffalo, goat, horse, and man. Importantly [9], reported the isolation of 15 *B melitensis* biovars from aborted fetuses of sheep in Hisar, indicating the diversity of *Brucella* strains in the study area.

Vaccination has played an enormous role in reducing brucellosis in many countries. Live vaccines have proved to be superior to inactivated ones. They are practical, inexpensive, and immunity is more persistent. The disadvantages of persistent antibodies associated with the subcutaneous full dose can be minimized by reducing the dose to $1/10^{th}$ and changing the route of administration to the conjunctival route. A reduced dose of *Brucella abortus* strain 19 vaccination remains an effective strategy to prevent the spread of bovine brucellosis with few drawbacks [11].

B. abortus strain 19 vaccine is a smooth, live attenuated, and reference vaccine used extensively for bovine brucellosis control, to which other new mutant vaccines are compared, to evaluate their efficacy and relevance in surveillance and control programs, globally. S19 was isolated in 1923 from the milk of a Jersey cow by Dr. John Buck. In the USA, this vaccine was used for more than five decades since 1941 and is still being used in several other countries, including the Indian subcontinent, Mediterranean basins, and Sub-Saharan Africa. *Brucella abortus*-RB51 vaccine strain was developed in 1982 by Prof. Gerhardt Schurig's group and is derived from a virulent smooth *B. abortus* biovar1 strain 2308 [11].

The RB51 is a rough rifampicin-resistant strain, lacking the O-side chains LPS (OPS) and does not interfere with serodiagnosis hence, differentiation of infected from vaccinated animals (DIVA) strategy is possible while using this vaccine. However, the O-side chain LPS is an immunodominant antigen to which the majority of antibodies resulting from S19 vaccination or natural infection are directed [12]. Recent advances in genomics, proteomics, recombinant technology, and reverse vaccinology have evolved other tools for the development of safer vaccines, without limitations [13, 14]. Thus, an intraocular reduced-dose of S19 vaccine to adult bovines and the potentialities of *Brucella* vaccine candidate genes: *omp25, ialB, fliC, virJ, frlB, acvB* remain the forecast as better options for disease control program [15]. Strain 19 and RB51 are the approved *B. abortus* vaccine strains most commonly used to protect cattle against infection and abortion as smooth and rough strains with intact and mutant O-LPS membrane, respectively [12, 13]. Other *B. abortus* derivatives: SR82 and 45/20 strains were used with little success.

The standard doses for the two vaccines are $0.5–1x10^{11}$ CFU and $1–3.4 \times 10^{10}$ CFU given by subcutaneous route respectively or in reduced-dose administered intraocularly as 5×10^9 CFU for *B. abortus* S19 [16]. The study was undertaken to evaluate the efficacy and shedding of *B. abortus* S19 intra-ocular reduced-dose vaccine in comparison with the subcutaneous route in lactating cattle and heifers.

2. Materials and methods

2.1. Study areas

The research was carried out at the "*Shri Kurukshetra Gaushala," Hisar,* Haryana India, Departments of Veterinary Public Health and Epidemiology, Animal Biotechnology, and College Central Laboratories, LUVAS, Hisar (Lat. 299′ 6.6996″ N, Long. 75° 43′ 16.0428″ E). Software data analysis was carried out at the Department of Pathobiology and Population Sciences, Royal Veterinary College, University of London, Hawkshead Campus, Herts AL9 7TA, UK.

Gaushala is a local *Hindi* name referring to a cattle farm. It is an organized farm *per se* where cattle are kept for spiritual and economic purposes as closed populations under the semi-intensive system.

2.2. Ethical approval

The study was approved by the LUVAS *Institutional* A*nimal Ethical Committee (IAEC)* No.VCC/IAEC/265-93; dated 15/02/2018.

2.3. Study design

This research is an experimental epidemiological study involving adult cattle and heifers. The study was carried out within 12 months encompassing the serological screening of the herd to post-vaccination monitoring and booster vaccination. For this purpose, the cattle were divided into three broad groups based on their age as young and old heifers and adult milking cows. Then further subdivided into an intraocular and subcutaneous group based on the route of administration of the S19 vaccine.

2.3.1. Screening of animals for brucellosis

The OIE recommended tests: Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT), and indirect ELISA (iELISA) for brucellosis screening in cattle were used to ascertain the brucellosis status of the animals under study. Only seronegative cattle were used in this study. The standard procedure for performing RBPT was adopted as described by [17].

Succeeding screening of animals, the seronegative cattle were subdivided into three major groups based on their breed as *crossbred, Hariana,* and *Sahiwal* breeds, and further subgrouping was done based on age and route of administration of the *brucella* vaccine as young-heifers, old-heifers and milking cows having intraocularly and subcutaneously vaccinated cows as provided in section 2.3.2 below.

2.3.2. Experimental design and vaccination

The seronegative animals (n = 90) were grouped into three based on their age groups:

Group-1*"young heifers"* consisted of n = 30 animals (<2–3 years age).
Group-2 *"old heifers"* had n = 30 (3–5 years).
Group-3 *"milking cows"* contained n = 30 animals (5 years and above).

2.3.3. Reduced dose vaccine formulation for intraocular and subcutaneous administration

Brucella abortus S19 live-vaccine used in the present study was procured from *Hester* Ltd (Gujarat, India). The vaccine vial usually comes in 5-doses of 2ml each, after reconstitution with a 10ml diluent. Following a 10ml dilution, the standard full dose vaccine contains 40×10^9 CFU viable organisms per 2ml (usually given as s/c). However, a new dilution with a 4ml diluent was adopted to prepare a reduced-dose, instead. Following the 4ml dilution (20×10^{10} CFU) method, each dose contains 5×10^{10} CFU viable organisms per ml. The reduced-dose vaccine ($1/10^{th}$ of the full dose of the S19) contains 5×10^9 CFU viable organisms per 0.1ml (100μl). The reduced-dose vaccine was then administered through conjunctival and subcutaneous routes for both groups respectively. The vaccine was instilled on the conjunctiva in one eye of the animal in the conjunctival group, whereas in the subcutaneous group, it was administered on the mid-neck site, as prescribed in the OIE manual [18].

2.3.3.1. Safety evaluation of Brucella abortus S19 vaccine. There is no single serious adverse event recorded in this study. However, a

temperature rise was recorded in rare cases. Safety evaluation was done by monitoring the adverse events during the first 1-h post-vaccination. The animals were closely observed for local reaction, pain, swelling, rashes, skin outburst, redness at the site of administration, fever, sclerosis, loss of appetite, restlessness, unwanted systemic events, etc.

2.3.4. Vaccination of cattle and booster dose

All the three subgroups received a reduced-dose vaccine (5×10^9 CFU/100μl) using a tuberculin syringe (1ml), which was administered through conjunctival routes (n = 10 animals from each group, irrespective of breed and lactation stage in milking cows). Then the second category received the same dose of vaccine via the subcutaneous route (n = 10 animals from each group), and the control group received only normal saline (placebo) through the conjunctival route.

The booster dose was administered on the 121st day to the intraocularly vaccinated cattle only (n = 30, 10 animals from each group).

2.3.5. Blood collection at different time interval during post-vaccination days (DPV)

During post-vaccination monitoring, blood samples (serum) and milk were collected in labeled sample collection vials, packed on ice, and brought to the laboratory for further investigation. All groups of animals

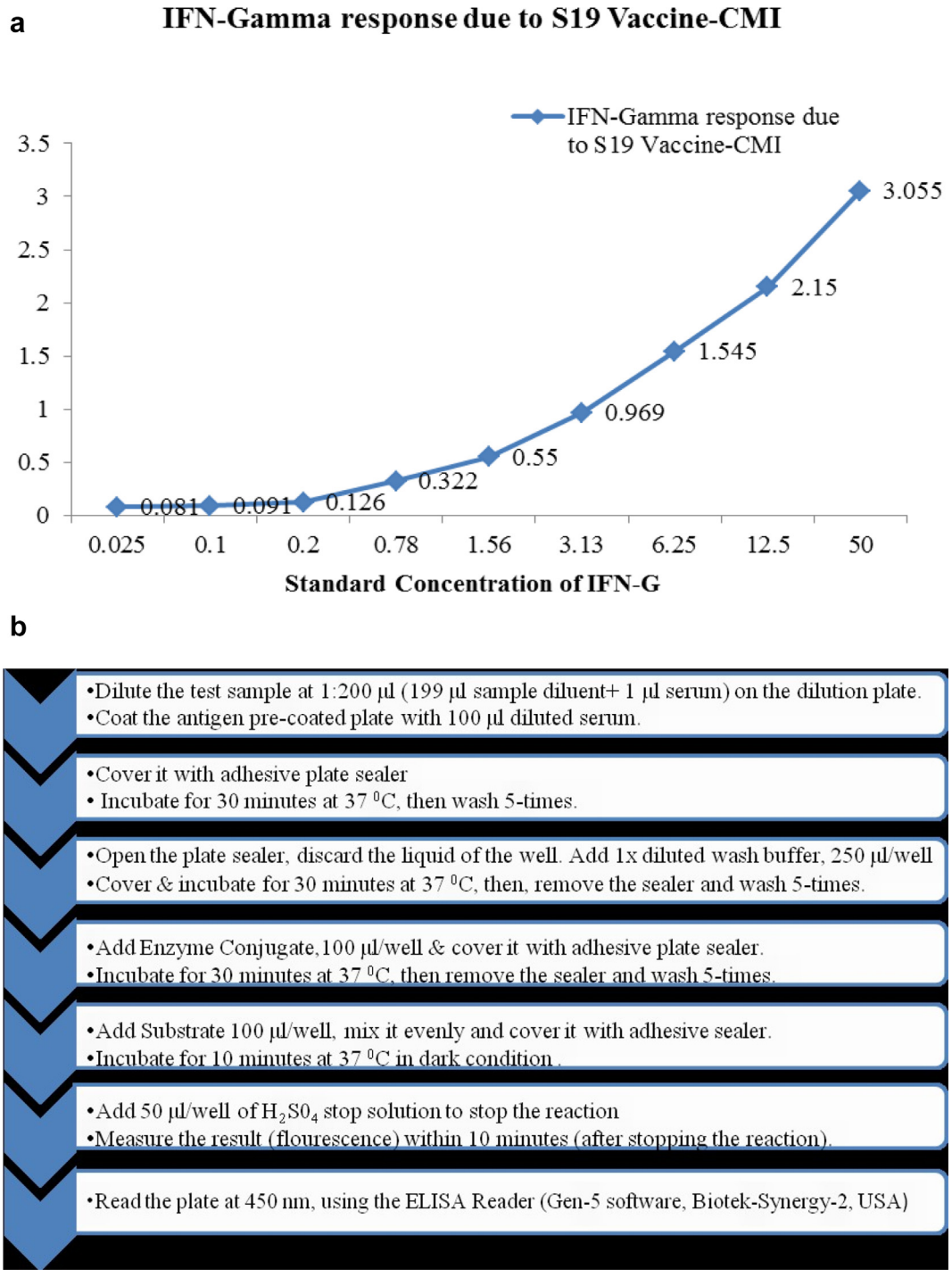

Figure 1. (a) IFN-Gamma Responses due to *Brucella abortus* S19 Vaccine - A Cell-Mediated Immunity. (b) A step-wise flow chart of Indirect Enzyme-Linked Immunosorbent Assay (iELISA).

were monitored for antibody responses to the S19 live vaccine by RBPT, iELISA, and IFN-γ at specific dpv on monthly intervals for 8-months. The animals were screened on days 7th, 21st, 30th, 60th, 90th, and 120th dpv and subsequently for the intraocular booster group at 150th, 180th, 210th, and 240th days post-booster (dpb) for both humoral and cell-mediated immune (CMI) responses respectively.

2.3.6. Serological evaluation of immune responses at DPV

All the serum samples collected during post-vaccination sampling were screened for *Brucella abortus* S19 antibodies, as described in the pre-vaccination subsection (2.3.1). Rose Bengal Plate Test (RBPT), iELISA (Cat. No. LT808001BZ5), and Bovine IFN-γ- Assay were used to evaluate the serological responses due to vaccination (Figure 1b). The percent positivity (%P) was calculated as follows:

$$\%P = \frac{\text{OD value of test sample}}{\text{Average OD value of positive control}} \times 100$$

The iELISA Assay kit and RBPT determined the humoral immune responses (HIR) induced by *B. abortus* S19 vaccine. In iELISA any S/P value above or equal to 40 percent of the positive control *OD-value* was considered positive and below that was considered as negative (cut off).

2.3.7. Bovine interferon-gamma specific ELISA assay (IFN-γ)

A commercially available IFN-γ ELISA kit (Cat. No. mca5638kzz) was used for the detection of IFN-γ in all the serum samples from the vaccinated groups to check for CMI from lymphocyte stimulation due to the S19-vaccine challenge. In principle, this kit is a sandwich enzyme immunoassay designed for the quantitative determination of bovine IFN-γ in serum using two different mouse anti-bovine IFN-γ monoclonal antibodies and recombinant bovine IFN-γ as a standard (BioRad, UK).

2.4. Molecular studies: post-vaccination screening of milk for Brucella abortus S19

Following vaccination, milk samples from vaccinated milking cows were collected at dpv (30th, 60th, 90th, and 120th) and screened for *B. abortus* S19 DNA, using molecular methods. Subsequently, a similar screening was conducted on the booster group at dpb (150th, 180th & 210th). These samples were transported aseptically inside ice packs to the "*Disease Investigation*" laboratory (DI-Lab), Department of Veterinary Public Health and Epidemiology for storage and further processing. The techniques of DNA extraction, PCR, qPCR, and sequencing were carried out in the Department of Animal Biotechnology, LUVAS, Hisar.

2.4.1. DNA extraction from milk

DNA extraction from milk samples was done as per the "blood and body fluid protocol" of Qiagen Blood mini kit (Cat. No. 51306, Germany, and Zymo Research, USA), with slight modifications, which includes: keeping milk samples at 4 °C overnight, to remove the excess fat contents on top of the 50ml sterile plastic tubes (ETO-Sterilized, HiMedia, India). Then an equal volume of PBS (pH 7.4) was added to the tube and centrifuged at 3000rpm for 30 min. The supernatant was discarded, and the pellet was used for DNA extraction. Treatment with lysis buffer (AL-buffer) and proteinase-K @ 200μl and 20μl respectively, for 1 h at 56 °C, extended therapy with chilled ethanol (98–100%) for 20 min was done and final DNA elution in 50–75μl with AE buffer. The eluted total DNA was spectro-quantified and stored at -20 °C until further use for PCR, qPCR, and sequencing.

2.4.2. PCR amplification

PCR was carried out in a total volume of 25μl, using a 2X-Top Taq^{R} and Hotspot^{R} master mix (Qiagen, Germany), nuclease-free water, forward and reverse primers, DMSO, and DNA template. The reactions were performed using a thermocycler (Applied Biosystems, UK). Primer sequences of *BCP-31 and IS711 genes* and PCR assay conditions are presented in Table 1. PCR products were analyzed on 2.5% (w/v) agarose gel in 1x TBE buffer (pH 8.0).

2.4.3. Screening of milk samples using Brucella abortus specific TaqMan probe-based qPCR

All samples were further analyzed using an optimized, highly sensitive, and specific TaqMan assay. A TaqMan probe-based qPCR reaction containing preformulated PCR master-mix with throughput amplification efficiency over a range of biological samples was used. The reaction was performed in StepOne-Plus V2.2.2 (Real-Time PCR System), using "JOE" and "NFQ" as reporter and quencher, respectively. Primers and probe sequences of *the TaqMan* assay conditions are presented in Table 2.

2.5. Data analyses

Data generated during this study were presented in the form of Tables and charts using the Microsoft Words and Excel version 2019 for Windows 10. Statistical analyses: like the Kappa test, a chi-squared test of association and 95%CI were determined with cross tabulations of all data, using a Statistical Package for Social Sciences (SPSS, V23.0).

3. Results

This study provides explanations for the safety and efficacy of the i/o reduced-dose vaccination of adult cattle over the subcutaneous route. The risk of persistent antibody titers associated with the conventional full dose of interfering with a screening of cattle herd for brucellosis was circumvented by using the reduced-dose S19 vaccine via conjunctival route. The serological monitoring following vaccination was carried out using Rose Bengal Plate Test (RBPT) and indirect Enzyme-Linked Immunosorbent Assay (iELISA) and Interferon-gamma assay (IFN-γ) similarly as in pre-vaccination screening as detailed below.

3.1. Humoral immune responses by RBPT and iELISA assay

The immune response among the young heifers in the i/o group; started as early as the 7th Day Post-Vaccination (dpv) with a higher titer through the 30th until at 90th dpv when both tests subsided. But the HIR titers in the subcutaneous group were relatively higher throughout the dpv than the conjunctival group by both RBPT and iELISA. As expected, none of the animals in the control group responded among the three age groups (Table 3). In contrast, the older heifer's category had a higher response than the young heifers and this persisted until 90th and 120th dpv in the conjunctival and subcutaneous groups, respectively.

Furthermore, by Rose Bengal Plate Test (RBPT), the number of animal responses in the subcutaneous (S/C) route is consistently higher than the intraocular (I/O) route except on the declining phase (@120dpv) where animals responded in both. Peak titer by S/C administration was achieved on day 7 with a consistent decline up today 120. Titer was the same by I/O administration except for day 21 where the highest response was recorded and days 90 and 120 with no response. Whereas, by iELISA, the same S/C route of administration produced more titer compared to the I/O route. Peak titer for S/C was obtained on days 21 and 30 and declined thereafter. The I/O titer was the same except for day 21 with higher responses when compared to the remaining days. Titer to IFN-G was on day 30–90, with the peak on day 60, and none on day 120. While, in old-heifers, there was not much difference in titer response between S/C and I/O route by RBT. Whereas, in iELISA, the S/C route of administration among old heifers produced a higher response when compared to the I/O route. However, I/O appears to be more stable (Table 3).

Regarding the HIR of the milking cows, more profound responses were found by both iELISA and RBPT compared to the heifers' category (Table 3). However, iELISA was more sensitive than the RBPT. The HIR due to reduced-dose vaccination was noticed as early as 7th dpv throughout the 120th dpv in conjunctival and subcutaneous groups by

Table 1. *Brucella Genus-Specific* (B4F and B5R) for *bcsp-31gene and B. abortus*-Specific Primer Sequences (BAF and BAR) for *IS711- gene.*

Parameters	Oligonucleotide sequences	Amplicon size	Reference
Forward primer (F)	5′-GGCTCGGTTGCCAATATCAA-3′	223 bp	[50] *bcsp31*gene
Reverse primer (R)	5′-CGCGCTTGCCTTTCAGGTCTG-3′		
Forward primer (F)	5′TGCCGATCACTTAAGGGCCTTCAT-3′	498 bp	[51] *IS711* gene
Reverse primer (R)	5′GACGAACGGAATTTTTCCAATCCC-3′		
Protocol:			
Initial Denaturation	95 °C for 5 min	**30 cycles**	
Denaturation	94 °C for 1 min		
Annealing	65 °C (IS711)/60 °C (bcsp-31) for 1 min		
Extension	72 °C for 1 min		
Final extension	72 °C for 10 min		

Table 2. *Brucella abortus* probed-Based TaqMan assay used in this study.

Parameters	Oligonucleotide sequences (5′- 3′)	Amplicon Size	Reference
Forward primer (F)	GCACACTCACCTTCCACAACAA	81 bp	[45]
Reverse primer (R)	CCCCGTTCTGCACCAGACT		
TaqMan® probe (P)	FAM-TGGAACGACCTTTGCAGGCGA G ATC-BHQ-1		
Amplification cycle:			
Initial denaturation	95 °C for 10 min	**40-cycles**	
Denaturation	95 °C for 15 s		
Annealing	60 °C for 1 min		

Table 3. Overall immune responses among old-heifers, young-heifers and milking cows during days post-vaccination.

Route of Vaccination	Days post Vaccination (DPV)						
	Serological Tests	7st No. (%)	21st No. (%)	30th No. (%)	60th No. (%)	90th No. (%)	120th No. (%)
A) Young Heifers (N = 30, < 1-< 2yrs)							
Intraocular (n = 10)	RBPT	1 (10.0)	2 (20.0)	1 (10.0)	1 (10.0)	0 (0)	0 (0)
	iELISA	1 (10.0)	2 (20.0)	1 (10.0)	1 (10.0)	0 (0)	0 (0)
	IFN-G	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Subcutaneous (n = 10)	RBPT	4 (40.0)	3 (30.0)	2 (20.0)	2 (20.0)	1 (10.0)	0 (0)
	iELISA	2 (20.0)	5 (50.0)	5 (50.0)	2 (20.0)	1 (10.0)	0 (0)
	IFN-G	0 (0)	0 (0)	1 (10.0)	2 (20.0)	1 (10.0)	0 (0)
Control (n = 10)	RBPT	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	iELISA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	IFN-G	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
B) Old-Heifers (N=30, 2-4 yrs)							
Intraocular (n = 10)	RBPT	2 (20.0)	1 (10.0)	1 (10.0)	1 (10.0)	1 (10.0)	0 (0)
	iELISA	1 (10.0)	2 (20.0)	2 (20.0)	2 (20.0)	1 (10.0)	0 (0)
	IFN-G	0 (0)	0 (0)	1 (10.0)	1 (10.0)	2 (20.0)	0 (0)
Subcutaneous (n = 10)	RBPT	2 (20.0)	2 (20.0)	1 (10.0)	1 (10.0)	1 (10.0)	1 (10)
	iELISA	0 (0)	4 (40.0)	5 (50.0)	3 (30.0)	2 (20.0)	1 (10)
	IFN-G	0 (0)	1 (10.0)	1 (10.0)	3 (30.0)	2 (20.0)	0 (0)
Control (n = 10)	RBPT	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	iELISA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	IFN-G	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
C) Milching Cow(N=30, < 4yrs)							
Intraocular (n = 10)	RBPT	1 (10)	1 (10)	2 (20)	1 (10)	1 (10)	1 (10)
	iELISA	3 (30)	3 (30)	4 (40)	4 (40)	3 (30)	3 (30)
	IFN-G	0 (0)	0 (0)	1 (10)	0 (0)	1 (10)	0 (0)
Subcutaneous (n = 10)	RBPT	8 (80)	8 (80)	6 (60)	6 (60)	3 (30)	1 (10)
	ELISA	3 (30)	3 (30)	5 (50)	8 (80)	8 (80)	4 (40)
	IFN-G	0 (0)	0 (0)	0 (0)	2 (20)	3 (30)	1 (10)
Control (n = 10)	RBPT	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	ELISA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	IFN-G	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

RBPT = Rose Bengal Plate Test, **iELISA** = indirect enzyme-linked immunosorbent assay, **IFN-G** = interferon gamma, **7TH**, **21ST**, **30TH**, **60TH**, **90TH**, **120TH** = Days Post-Vaccination (**DPV**), * **IFN-**specificity ranges 0.025–50.0 ng/ml (Detection limit).

Table 4. Age-wise immune responses among intraocular booster groups during DPB (N = 30).

Category	Tests	Days Post-Booster (DPB)			
Booster group	Serological Tests	150^{th} No. (%)	180^{th} No. (%)	210^{th} No. (%)	240^{th} No. (%)
Young Heifer (n = 10)	RBPT	0 (0)	1 (10)	1 (10)	1 (10)
	ELISA	0 (0)	0 (0)	1 (10)	1 (10)
	IFN-G	0 (0)	0 (0)	0 (0)	0 (0)
Old-Heifers (n = 10)	RBPT	0 (0)	1 (10)	1 (10)	1 (10)
	ELISA	1 (10)	2 (20)	2 (20)	2 (20)
	IFN-G	0 (0)	1 (10)	1 (10)	0 (0)
Milking Cows (n = 10)	RBPT	2 (20)	2 (20)	2 (20)	2 (20)
	ELISA	2 (20)	4 (40)	3 (30)	2 (20)
	IFN-G	0 (0)	1 (10)	2 (20)	0 (0)

RBPT = Rose Bengal Plate Test, **iELISA** = indirect enzyme-linked immunosorbent assay, **IFN-G** = interferon gamma, **150^{TH}**, **180^{TH}**, **210^{TH}**, **240^{TH}** = Days Post-Booster Vaccination (**DPB**), I/O = intraocular route.

both tests (Table 3). The same trend was noticed following a booster dose in the intraocularly vaccinated group (Tables 4, 5).

Furthermore, by RBT test, the s/c route of administration among the milking cows showed a higher response up to about 8-fold when compared to the i/o route of administration. Milking cows demonstrated a better ability to develop antibodies when administered by the s/c route in comparison with young and old heifers. While there was a consistent decrease in response titers in the s/c route of administration as days go until day 120, the response remains the same all through for the i/o route, except day 30. While, by iELISA, the same titer was produced, but beginning from day 30 there was a higher response by s/c administration compared to i/o. Increase in titer by s/c route until day 90 where the peak was achieved before subsiding during day 120. The antibody-response in the i/o route was constant all through except for days 30 and 60 when the peak was achieved.

3.2. Cell-mediated immune responses by IFN-gamma assay

In this study, we followed a standard cut-off OD-values (0.081–3.055) for considering an animal as positive or said to have produced IFN-γ due to reduced-dose vaccination, an indication of cell-mediated immunity (CMI) due to intracellular pathogens (Bio-Rad, UK). The standard IFN-γ titer sensitivity was "0.025–50.0 ng/ml" with *OD-values* of "0.081–3.055," respectively (Figure 1). Among the 3-categories, the percentage of i/o responders ranged between 10-20% at 30th through 90th dpv. However, it was a contrary outcome in the subcutaneously vaccinated groups. Among the old heifers (n = 10), few animals produced IFN-γ at 21st, 30th, 60th, and 90th dpv with little higher responses (Table 3). Similarly, it was an intermittent production of IFN-γ that was noticed among the subcutaneously vaccinated young heifers with a relatively lower proportion. However, a higher IFN-γ production was noticed among the subcutaneously vaccinated milking cows' category (n = 10) viz: 2(20%), 3(30%), and 2 (20%) at 30th, 60th, and 90th DPV respectively (Table 3). Furthermore, an irregular production of IFN-γ was noticed among the intraocularly vaccinated milking category at 21st and 60th DPV. Generally, the IFN-γ stimulation (CMI) due to reduced-dose vaccination was noticed at later days, 30th dpv (first month) in most groups. It subsided after the 90th dpv (3-months later), except among the old heifers, which started as early as the third week (21st) dpv (Table 3). The overall humoral and CMI responses are presented in Tables 3, 4 and 5.

3.3. Humoral immune response during booster dose in intraocularly vaccinated cattle

The same trend of the HIR during dpv happened during days post-booster (dpb). However, the latter started at 121^{st} onward during days post-booster (dpb).

Table 5. Humoral immune responses among young-heifers, old-heifers, and milching cows during days post-vaccination (DPV) and post-booster dose (DPB).

Groups (Young-Heifers)	Tests	Days Post-vaccination							Days Post-Booster			
		0	7^{th}	21^{st}	30^{th}	60^{th}	90^{th}	120^{th}	150^{th}	180^{th}	210^{th}	240^{th}
Conjunctival [No. (%)]	ELISA	0	1 (10)	2 (20)	1 (10)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	1 (10)
	RBPT	0	1 (10)	2 (20)	1 (10)	1 (10)	0 (0)	0 (0)	0 (0)	1 (10)	1 (10)	1 (10)
Subcutaneous [No. (%)]	ELISA	0	2 (20)	2 (20)	3 (30)	2 (20)	1 (10)	0 (0)	**NA**	**NA**	**NA**	**NA**
	RBPT	0	4 (40)	2 (20)	2 (20)	2 (20)	1 (10)	0 (0)	**NA**	**NA**	**NA**	**NA**
Control		0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Group (Old-Heifers)												
Conjunctival [No. (%)]	ELISA	0	1 (10)	2 (20)	2 (20)	2 (20)	1 (10)	0 (0)	1 (10)	2 (20)	2 (20)	2 (20)
	RBPT	0	2 (20)	1 (10)	1 (10)	1 (10)	1 (10)	0 (0)	0 (0)	1 (10)	1 (10)	1 (10)
Subcutaneous [No. (%)]	ELISA	0	1 (10)	4 (40)	5 (50)	3 (30)	2 (20)	1 (10)	**NA**	**NA**	**NA**	**NA**
	RBPT	0	2 (20)	2 (20)	1 (10)	1 (10)	1 (10)	1 (10)	**NA**	**NA**	**NA**	**NA**
Control		0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Group (Milking Cows)												
Conjunctival [No. (%)]	ELISA	0	3 (30)	3 (30)	4 (40)	3 (30)	3 (30)	2 (20)	2 (20)	4 (40)	3 (30)	2 (20)
	RBPT	0	1 (10)	2 (20)	2 (20)	1 (10)	1 (10)	0	2 (20)	2 (20)	2 (20)	2 (20)
Subcutaneous [No. (%)]	ELISA	0	3 (30)	5 (50)	8 (80)	7 (70)	4 (40)	2 (20)	**NA**	**NA**	**NA**	**NA**
	RBPT	0	8 (80)	6 (60)	6 (60)	3 (30)	1 (10)	0	**NA**	**NA**	**NA**	**NA**
Control		0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

NA = Not applicable.

3.3.1. Milking cows

Following a booster dose of *B. abortus* S19 (intraocular group only) at **121st** dpv (a declining phase), the immune responses rose again throughout the dpb in an ascending manner as it was at 90 and 120 dpv, before booster dose was given. Though, in RBPT, the immune responses/responders were uniform 2 (20%) throughout until at 240th dpb. Whereas in iELISA, titers/responders were more pronounced at 180th due to its high sensitivity, but its consistency remains the same throughout dpb as presented in Tables 4 and 5. This shows that the immunity has been sustained following booster without much persistence antibodies in intraocularly vaccinated cows compared to the subcutaneous category. However, this differs from that of the heifer's category, the response was less and started to respond later at 210th and 240th dpb.

3.3.2. Heifers

The humoral response of both heifer's groups was less compared to the milking group. By RBPT, the immune responses were noticed a bit later at 180th, 210th, and 240th, with the same proportion of 1 (10%) in all. Whereas, in the ELISA assay, the *old-heifers* had higher and persisted immune responses throughout DPB as compared to the young heifers (Tables 4 and 5). The interval between the booster inoculation and beginning of immune response is the same one month in all the groups. However, as stated above under 3.3.1, the milking group had much more responders than the vaccinated heifers and began to respond as early as 150th and persisted until 240th dpb.

3.4. Cell-mediated immune responses of booster groups

As per the CMI, the IFN-γ was elicited only at 180th and 210th dpb as 1 (10%) and 2 (20%), respectively, in the milking group. In the *heifer's* category, the *old-heifers* had CMI response due to booster dose at 180th and 210th dpb. There was no IFN-γ response among the young heifers following a booster dose (Table 4 and 5). However, the control cattle remained unresponsive throughout the study period (Tables 3, 4, 5, and 6).

There was a significant agreement among the serological screening tests (RBPT, SAT and IFN-γ) employed in this study for screening and post-vaccination monitoring, as indicated by the Kappa statistic. The details of the strong agreements between the tests are presented in Table 7 below.

Table 7. Kappa statistic among the serological tests.

Agreement	Kappa 95% CI	P-value
RBPT and iELISA	0.87 (0.857–0.882)	0.001*
SAT and iELISA	0.70 (0.684–0.718)	0.001*
RBPT and SAT	0.82 (0.809–0.834)	0.05*

* Significant at 5% level.

3.5. PCR and qPCR analyses

3.5.1. Detection of Brucella abortus S19 in milk during DPV

On account of the vaccination route, among the intraocularly vaccinated cows (n = 10); PCR couldn't detect *Brucella* DNA in all DPV except from only one cow, in which *Brucella organism* (DNA) was detected in milk by both PCR and qPCR at 90th DPV. As expected among (-) control groups, nothing was detected by both tests. However, on the contrary, *Brucella abortus* S19 DNA was detected in milk from the subcutaneously vaccinated cows. Significant numbers were detected at 60th, 90th, and 120th DPV respectively by PCR (Figure 2). Whereas higher numbers 3 (30%), 1 (10%), 7 (70%), and 6 (60%) of S19 DNA were detected by qPCR during dpv (Table 6). Similarly, *B. melitensis* specific Taqman probe-based was used to screen out any possibility of *B. melitensis* natural infection among the vaccinated cattle, but no single amplification was detected by qPCR (Figure 3).

Subsequently, the intraocular booster groups were also screened for *B. abortus* S19 at specific dpb (150th, 180th, and 210th). There was only one animal in which *B. abortus* DNA was detected in its milk following booster dose at 150th and 210th dpb by both PCR and qPCR (Table 6). Significant Ct-values of 32.23 and 32.49 were noticed at 150th and 210th DPB, respectively, the same with the internal positive control (IPC) Ct value of 32.2.

Table 6. Molecular Detection of *Brucella abortus* S19 in vaccinated Milking Cows Based on Route of Vaccination at DPV and Booster dose at DPB by PCR and qPCR.

Group	Test	Days Post-Vaccination (DPV)					Days Post-Booster (DPB)		
		0	30th No. (%)	60th No. (%)	90th No. (%)	120th No. (%)	150th No. (%)	180th No. (%)	210th No. (%)
Conjunctival [No. (%)] n = 10	PCR	0	0 (0)	0 (0)	1 (10)	1 (10)	1 (10)	0 (0)	1 (10)
	qPCR	0	0 (0)	0 (0)	1 (10)	1 (10)	1 (10)	0 (0)	1 (10)
Subcutaneous [No. (%)] n = 10	PCR	0	0 (0)	1 (10)	6 (60)	1 (10)	**NA**	**NA**	**NA**
	qPCR	0	3 (30)	1 (10)	7 (70)	6 (60)	**NA**	**NA**	**NA**
(-) Control		0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Conventional and Real-Time PCR of Positive Milk Samples from Subcutaneous Group (n = 10)

Category	Test	Days Post Vaccination (DPV)			
Milking group (N = 30)	Molecular Tests (DNA in milk)	30th No. (%)	60th No. (%)	90th No. (%)	120th No. (%)
Intraocular (n = 10)	PCR	0 (0)	0 (0)	1 (10)	1 (10)
	qPCR	0 (0)	0 (0)	1 (10)	1 (10)
*Subcutaneous (n = 10)	PCR	0 (0)	1 (10)	6 (60)	1 (10)
	qPCR	3 (30)	1 (10)	7 (70)	6 (60)
(-) Control (n = 10)	PCR	0 (0)	0 (0)	0 (0)	0 (0)
	qPCR	0 (0)	0 (0)	0 (0)	0 (0)
*Subcutaneous Milching Group (DPV)		Conventional PCR % (no)		qPCR % (no)	
30th PV		0% (0/10)		30% (3/10)	
60th PV		10% (1/10)		10% (1/10)	
90th PV		60% (6/10)		70% (7/10)	
120th PV		10% (1/10)		60% (6/10)	

* **PCR** = Polymerase Chain Reaction, **qPCR** = Real-Time PCR. **DPB** = Days Post Booster (**150TH**, **180TH**, **210TH**), **NA** = not applicable.

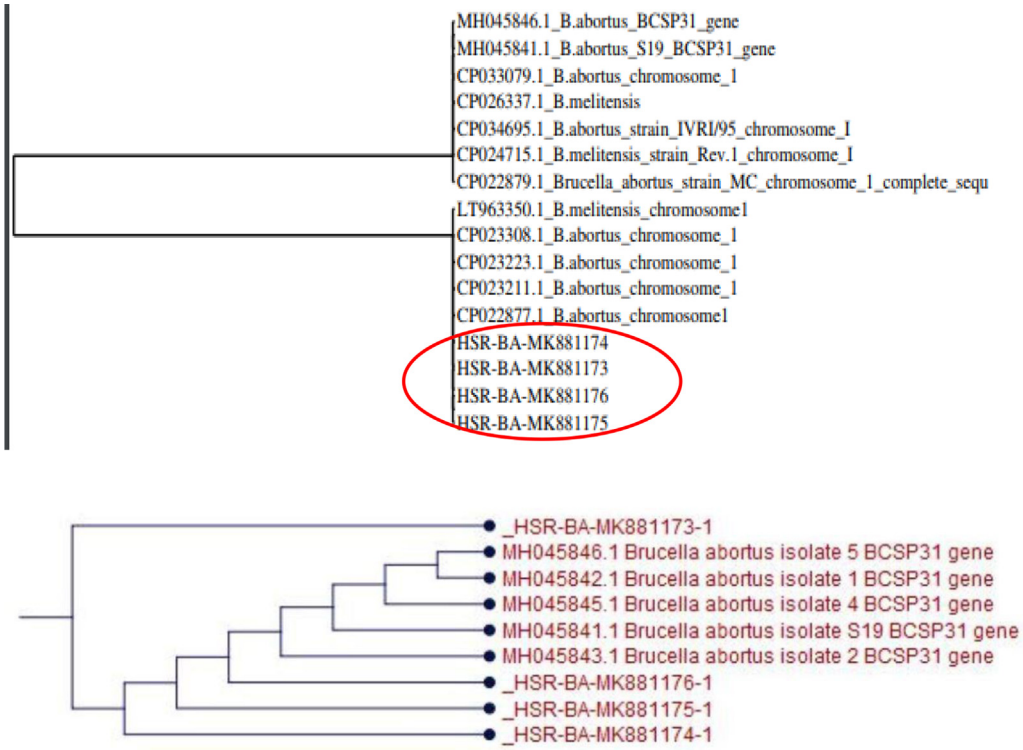

Figure 2. Phylogenetic Tree Analysis of related Brucella abortus Isolates (with 99.9-100% homology).

3.5.2. Nucleotide sequencing of the PCR amplified products and sequence analyses

Purification of the PCR products (*bcsp31*) had revealed *B. abortus* S19 vaccine strain and the sequencing results from different groups (intraocular and subcutaneous isolates) revealed 100% homology with other global isolates from the NCBI Genbank following BLASTn analysis (http://www.ncbi.nlm.nih.gov/). The phylogenetic tree also revealed a 99.9% relationship with other *B. abortus* isolates from within and outside the country. The accession numbers were assigned as MK881173, MK881174, MK881175, and MK881-176 [19]. The details of the sequencing results are presented in Figures 4 and 5.

4. Discussion

The *B. abortus* S19, a live attenuated constituent of the vaccine is of smooth type and possesses non-toxic, unconventional, and immunodominant antigen, lipopolysaccharide (LPS) that stimulates the immune responses in vaccine situations. Generally, immunity due to *Brucella* in vaccinated and infection involves both antibody-based HIR and CMI [11, 20], and both were detected in this study. Furthermore, *Brucella abortus* Strains-19 and RB51 are live vaccines and therefore can elicit immune response due to intracellular stimulation of macrophages and subsequently that of pro-inflammatory interleukins, cytokines, and other cellular mediators due to phagolysosome fusion [21]. Therefore, strong HIR not accompanied by CMI cannot provide total protection against *Brucella* infection. Vaccine candidates that evoke a profound CMI–IFN–γ response confer a better level of immunoprotection in vaccinated adult cattle. In this study reduced-dose, S19 provided a better response than other vaccination strategies as earlier reported in American research [22, 23].

There was significant agreement among the serological screening tests employed in this study, as indicated by the kappa statistic; which is in line with the earlier reports [24]. Similarly, other studies have reported ELISA as an alternate test to both RBPT and SAT for diagnosis of brucellosis in animals with high sensitivity and specificity [25, 26]. The results in this study are also in line with the recommendations set by the OIE [27], that two tests at a time are recommended in screening for brucellosis for import and export of animals and animals' products across borders [18].

Generally, in this study, the proportion of animals responding to the vaccine was less than those that did not respond to the vaccine except for mulching cows. Among young and old heifer categories, the response rate ranged from 10-50% with all types of tests. In comparison, milking cows' administration through S/C resulted in up to 80% of the animals responding, this could be due to age and immunocompetence of the adult cows compared to the heifer's group [25]. But this is contrary to the findings of [21, 29] who reported 100% responders in the s/c group and more than 60% in the i/o category. This might be as a result of the different vaccines used in the two studies, in which India Immunological Limited (IIL, India), and Merck Sharp & Dohme (MSD, France Company) vaccines were used instead of the Hester vaccine, Gujarat, India, used in our study coupled with the breed of animal, geography, and age, as referenced earlier age and breed are palpable risk factors to respond to infection and vaccination in animals. However [35], had reported consistent findings of low responders with no persistent antibodies, following reduced-dose vaccination in adult cattle in Zambia.

The HIR induced by both intraocular reduced-dose of S19 vaccination, found in this study, was promising and protective enough to protect the adult cattle vaccinated intraocularly (above-calfhood) from *Brucella* infection, and for up to 4 months dpv with no booster. Though the immune response was shown to persist in the subcutaneously vaccinated cattle, there is a tendency of interference with the serological screening of brucellosis by increasing the number of positive reactors in a herd. This may probably make it difficult to differentiate vaccinated from naturally infected animals (DIVA), especially if the DIVA test is not available to judge the argument.

The results in this study were consistently similar to the earlier reports [11, 28, 29], which also reported that animals vaccinated with a reduced-dose intraocularly were found to be free from detectable antibodies after three months post-vaccination. Implying that, there could be

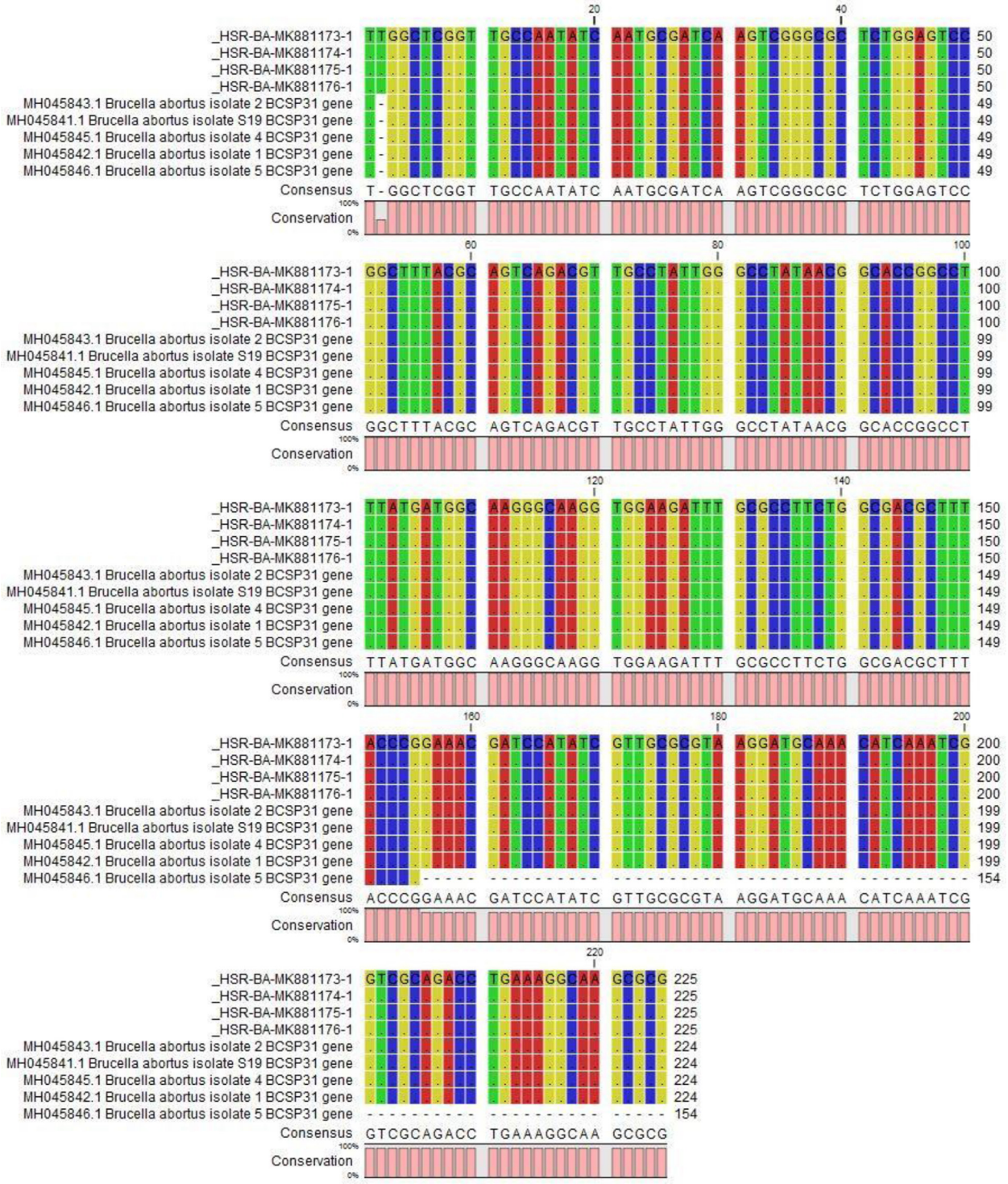

Figure 3. Multiple Alignments of selected nucleotide sequence homologues of the bcsp-31 gene (223bp).

no persistence antibodies with reduced-dose given intraocularly. Similarly, Raghunandan et al. [30] had reported a consistent negative result from milk samples of vaccinated cattle and buffaloes within 30 dpv by both culture and ELISA techniques. The rapid decrease in the antibody suggested that this practice could be adopted as a new strategy in the national control program against Brucellosis, especially for those cattle that missed their calfhood vaccination, to increase the vaccine coverage as the only means of brucellosis disease control in India, aside from the "Test and Slaughter" method. A booster dose was administered to the intraocular group to ensure immunocompetence over a long period [27]. The same scenario following booster-dose in the intraocularly vaccinated cattle was reported by [31], who also reported on the efficacy and safety of reduced-dose over the full dose subcutaneous vaccination. A bit higher increase in responders was noticed among vaccinated milking cows compared to that of the two heifers' category, which was another peculiar finding of this study. Among young and old heifer categories, the response rate ranged from 10-50% with all types of tests. In Comparison, milking cows administration through S/C resulted in up to 80% of the animals responding, for a reason related to the age of the animal. The immune response to most antigens in cattle progresses with the

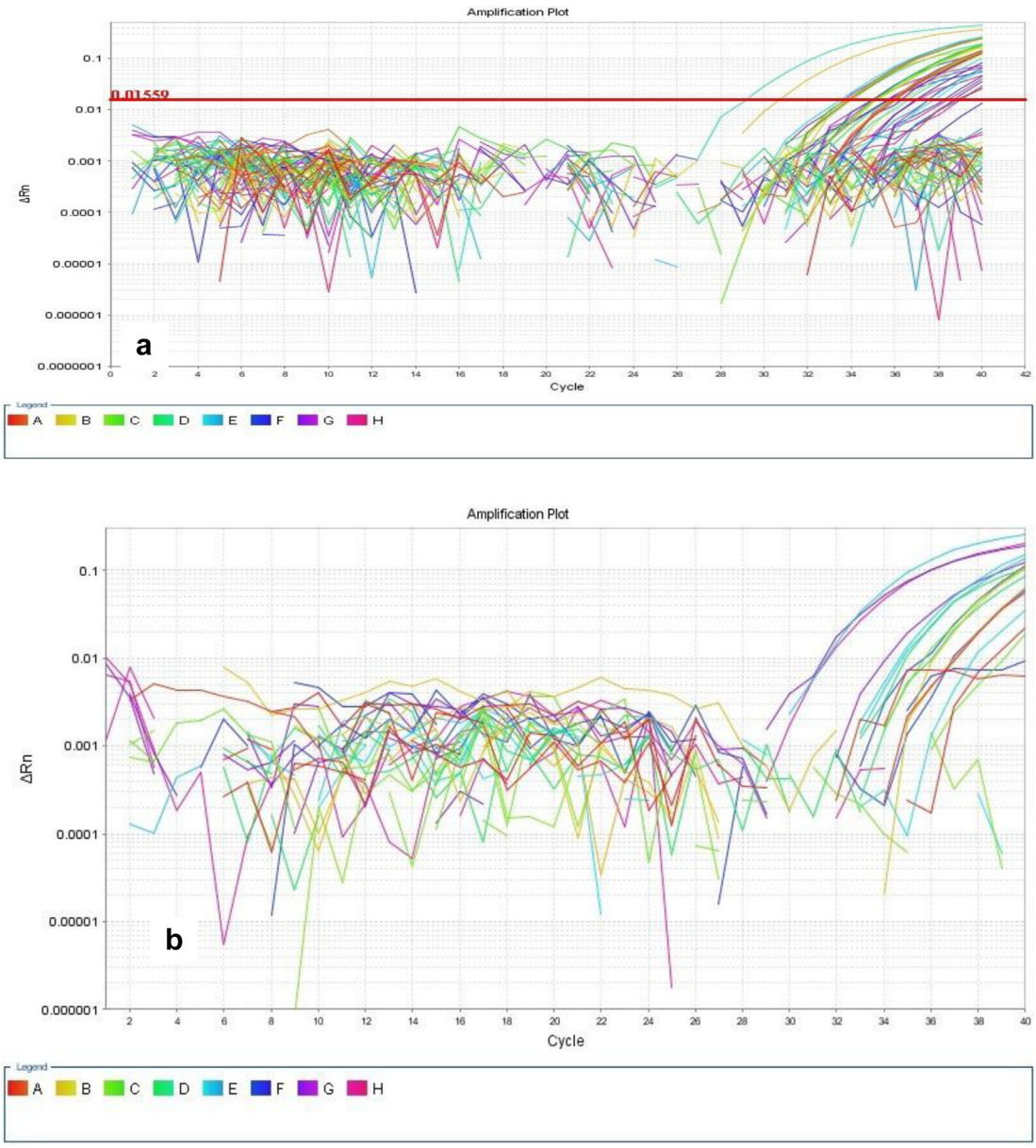

Figure 4. a) Real Time PCR Amplification Plot for B. abortus DNA in milk from S19 vaccinated milking cows (n=30). b) Real Time PCR Amplification Plot for B. abortus S19 DNA in milk from Booster milking cows (n=10).*Note: Real-time PCR amplification pattern using the Brucella abortus probe. Fluorescence ratio is plotted against the number of PCR cycles to monitor amplification in real-time mode. Samples with less Ct values (29.12-35.0) had Brucella DNA and were considered +ve in this assay. While Ct value above 35 was considered doubtful. The baseline value is 0.01559.

increasing age [32]. This is consistent with the findings of [33, 34], who reported that the HIR of the conjunctival vaccine was comparable with that of subcutaneous standard-dose vaccine after booster dose, indicating the great advantage of a booster dose [33, 35], with fewer complications.

Moreover, the HIR induced by S19 reduced-dose vaccine in this study was also determined by iELISA. During the dpv, iELISA was found a more useful test in detecting antibody titers than RBPT due to its high sensitivity. The kappa statistic results revealed agreement between the tests (RBPT and ELISA, SAT and ELISA and RBPT and SAT) to be 0.87 (95% CI: 0.857–0.882), 0.70 (95% CI: 0.684–0.718), and 0.82 (95% CI: 0.809–0.834) respectively, which indicated strong agreement between serological tests. Subsequently, the strategy of conjunctival S19 reduced-dose vaccination was adopted in the USA by [34] to control brucellosis in problem cattle herds under an endemic situation. Similarly [35], reported that the use of the S19 reduced-dose vaccination strategy had been proved widely accepted in Zambian settings to control brucellosis. Nevertheless, OIE also prescribed conjunctival vaccination of bovines with a reduced-dose of the S19 vaccine [16, 36], which can be used as a tool for the prevention of brucellosis in animals above-calfhood age under an endemic situation like Hisar, Gaushala.

The CMI-Response by IFN-γ Assay was detected in this study. A reasonable amount of IFN-γ was produced due to reduced-dose vaccination and the standard concentration of IFN-γ titer (0.025–50.0 ng/ml) was detected among the three groups of vaccinated cattle, an indication of CMI due to intracellular pathogens (live-vaccine). The immunoprotection against brucellosis due to challenged and infection involves both HIR and CMI responses, as earlier confirmed by [11]. The signaling of the presence of LPS by cellular defense such as macrophages, dendritic cells, and monocytes has evolved over centuries to provide the host with a rapid recognition and response towards infections due to *Brucella* and other Gram-negative bacteria [37]. This quick innate response against LPS involves the release of a range of pro-inflammatory mediators such as

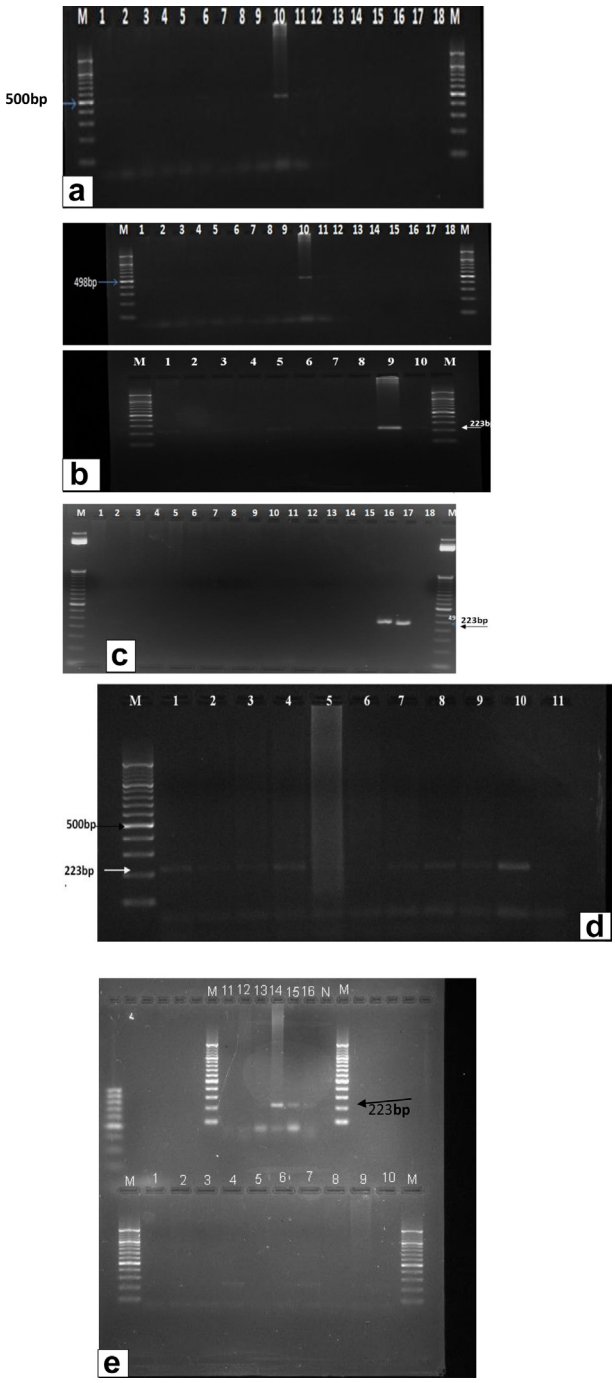

Figure 5. (a) Agarose gel electrophoresis of PCR amplification of IS711 gene from milk samples @ 30 DPV. Lane M 100bp molecular weight marker (Fermentas, USA); lanes1-lane 10 is positive control specific for (B. abortus S19) at 498 bp, lane 18 is a negative control (nuclease free water), and lanes 1–9 & 11–17 are milk DNA samples. (b) Agarose gel electrophoresis of PCR amplification of bcsp-31 gene from milk samples @ 30DPV. Lanes 1–8 are milk DNA samples & lane 9 is a positive (þve) control and lane 10 is a non-template control (NTC). (c) Agarose gel electrophoresis of PCR amplification of bcsp-31 gene from milk samples @ 60DPV. Lane M 50bp molecular weight marker (Fermentas, USA); Lane 17 is a positive control (B. abortus S19 vaccine), lane 18 is negative control (nuclease free water), and lane 16 is a positive sample specific for B. abortus S19 bcsp31 (223bp). Lanes 1–15 are samples from vaccinated cows. (d) Agarose gel electrophoresis of PCR amplification of bcsp31 gene from milk samples @ 90 DPV. Lane M 100bp molecular weight marker (Fermentas, USA); lane 10 is positive control (B. abortus S19 vaccine), lane 11 is a negative control (nuclease free water), lanes 1–4 and 6–9 are positive samples specific for B. abortus S19 (223bp). (e) Agarose gel electrophoresis of PCR amplification of bcsp-31 gene from milk samples @ 120 DPV. Lane M 100bp molecular weight marker (Fermentas, USA); Lane 14 is positive control (B. abortus S19 vaccine), Lane N is negative control (nuclease free water), Lane 11–13 are negative samples and lane 15 is positive sample specific for B. abortus S19 (223bp).

Interleukins (IL-6, IL12, IL1β), cytokines (IFN-γ), and other cellular mediators in enough quantities to mediate the host substantially by promoting inflammation and priming the immune system to eliminate the invading organism and development of memory immune cells against subsequent attacks. *Brucella's* ability to stimulate IL-12 secretions allows it to drive Th0 cells to differentiate into Th1 effector and memory cells that are the central feature of the potential use of the *B. abortus* as a vaccine carrier [38].

Lymphocyte proliferation and cytokine detection assays are the most widely used index of CMI responses. A useful method to reveal the

presence of CMI response against *B. abortus* S19 could be the detection of IFN-γ following lymphocyte stimulation with the specific antigen. In principle, the IFN-γ test (Bio-Rad, UK) utilizes the "*in vitro*" technique, the same mechanism that could be stimulated "*in vivo*" by the antigen. According to the previous studies, on the reduced-dose vaccination conducted by [39], who demonstrated that *Brucella* species can elicit a macrophage response through the production of IFN-γ by stimulated T lymphocytes both in the mice model and in cattle infected with *B. abortus* [39, 40].

The CMI response against the *B. abortus* S19 vaccine studied by IFN-γ assay revealed a promising intervention in the old heifer's group of cattle and the milking cows inoculated with a reduced-dose vaccine. Generally, the IFN-γ stimulation (CMI) due to reduced dose vaccination was noticed lately in all groups, on the 30th (first month). It declined after the 90th-day post-vaccination, except among the old heifers, which started as early as the third week (21st) day post-vaccination. The IFN-γ response was noticed around the 30th-day post-vaccination, and comparatively high on 60 DPV in reduced dose intraocularly vaccinated group in comparison with the cattle in the subcutaneous group but also showing a higher serological titer at the same peak of 60th DPV. This finding was an indication of good CMI response, which is the critical indicator in controlling and eliminating intracellular pathogens like *Brucella* species. However, a contrary view was reported by [41], who observed the increased level of IFN-γ during the first-week post-vaccination. It reduced the level of IFN-γ on the second and third week after vaccination with *B. abortus* S19 reduced-dose subcutaneously route vaccine. However, from 90th to 120th DPV, the percentage of responders drastically declined in all groups, hence the need for the booster dose after 120th DPV among intraocular groups only, as per the recommendation of [16]. It appears there are instances of delayed response to the vaccine or levels undetectable with the test employed in all categories and with all test types and this is not unrelated to the fact that the dose has been reduced coupled with the change of the route of administration and the antibodies stimulation due to vaccine could be delayed especially that of IgM and IFN-γ by stimulated T lymphocytes. However, the immunoprotection was there and the animals were protected.

Similarly, the intraocularly vaccinated cattle have shown good CMI responses in all categories but with a low proportion of animals compared to subcutaneously vaccinated groups. On the contrary, this finding is relatively lower compared with the most recent study by [21]; and previously by [29]. The discrepancies might be related to the different types of animals' breed, age group, vaccine dose, and vaccine type used in the studies. Most importantly, the vaccine vials used in those previous studies contain 80×10^{10} CFU compared to the 40×10^{9} CFU used in this study, this alone may cause an increase in the immune response to be higher than our findings due to relatively low number of viable organisms. Secondly, the immune response is age and breed-dependent. Comparatively, the subcutaneous group, on the other hand, had higher CMI responses in all the groups. The pattern of immune-response found in this study demonstrated that vaccination of adult animals with a reduced-dose of S19 vaccine by conjunctival route did not induce persistent antibody titers, and the level of protection achieved is comparable to that of the full-dose subcutaneous counterpart. This finding was earlier reported by [24], who confirmed the efficacy and safety of reduced-doses due to good immunoprotection with no persistent antibody titers and no abortion, which are the noteworthy limitations in the case of standard subcutaneous full dose vaccination.

In this study, the vaccination by S/C route with standard dose produced almost 80–100 % seroconversion. In the low dose conjunctival vaccine group, there was less seroconversion, and even after booster 100% seroconversion was not noticed. This could be attributed to vaccine strain colonization mostly restricted to the lymph nodes of the head in case of conjunctival vaccination [33], whereas, S/C vaccination systemically extends colonization to other lymphoid organs including the spleen; hence a higher serological response.

Detection of *Brucella abortus* S19 in Milk based on the vaccination route at DPV was also a peculiar finding in this study. It has been speculated that *Brucella organisms* could be secreted intermittently in the milk of infected cattle, depending on the number of infective doses, age, and stage of infection (chronicity of the disease). Most recent reports by [38] and [39] confirmed the detection *of Brucella organisms* from the milk of infected cows. In our study, *Brucella abortus* S19 DNA was detected and characterized from the milk of vaccinated milking cows using the *bcsp-31* and *IS711* targeted PCR and confirmed by TaqMan probe-based qPCR assay, and this is precisely consistent with the approach of [40]. The intraocularly vaccinated cows remain safer in terms of shedding of *Brucella* organisms in milk as compared to the subcutaneously vaccinated cows. Because DNA of *B. abortus* could not be detected by PCR in the intraocular group during DPV except in one cow, in which it was detected in milk (DNA) by both PCR and qPCR at 90th DPV. The reason maybe because of the age and immune status of the animal at the beginning of the experiment. Also, the animal may have become infected from the shedding of subcutaneous counterparts or infected animals at the *Gaushala* due to mixing. Shedding of *Brucella* species in milk from vaccinated and infected cows has a tremendous public health consequence. It might increase the herd-level prevalence over time due to the shedding of the live bacteria in milk as reported by earlier researches [42, 43, 44].

However, among the subcutaneous route, a significant number (>20–50%), *Brucella* DNA was detected in milk at some DPV by PCR. In contrast, a much higher number (60–70%) of *S19* DNA was detected by qPCR in all the DPV. This might not be unrelated to the high sensitivity and specificity of the qPCR over the conventional PCR in the detection of *Brucella* nucleic acid in milk and other biological samples [43]. Hinic et al. [45] reported the usefulness of TaqMan real-time PCR as a rapid, easy, and discriminative method as compared to conventional PCR and bacteriological technique for the diagnosis of brucellosis from aborted fetuses and other biological samples. Subsequently, the intraocular booster group was also screened for *B. abortus* S19 at specific days post-booster and significant Ct-values at 150^{th} and 210^{th} DPB, with a higher positivity compared to the initial load during DPV, and this might be due to booster dose. Hinic and others [45] and [46] also confirmed the use of molecular methods for the detection and identification of *Brucella* species from diverse biological samples including milk.

Similarly [47], had reported the presence of *Brucella* DNA in bovine milk in the urban and peri-urban areas of Dushanbe, Tajikistan, in which 10.3 % was detected from the cow milk samples by *IS711*-based real-time PCR. Ning et al. [48], also identified significant numbers of *Brucella* DNA from the milk samples positive by MRT and SAT after subjecting them to qPCR and IS711 targeted PCR, respectively. However, a contrary view had been reported by [49] that there was not much difference between PCR and bacteriological detection methods. It is concluded that the conventional or real-time PCR will supersede the current diagnostic methods for the detection of *B. abortus* in milk. It could be a reliable detection method with acceptability in the nearest future.

Similarly, *B. melitensis* specific TaqMan probe-based was used to screen for any possibility of *B. melitensis* natural infection among the vaccinated cattle. Nevertheless, no single amplification was detected, an indication of immunoprotection due to S19 reduced-dose vaccination and the absence of *B. melitensis* in the herd. This is consistent with the findings of [49], who also found negative ct-values following S19 vaccination in cattle. Those genes from NCBI with 100% similarity with our isolates were from Maharashtra, India [19].

5. Conclusion

The present study concluded that the conjunctival reduced-dose vaccination of adult cattle with S19 vaccine overcomes the problems of persistent antibodies post-vaccination associated with subcutaneous full-dose, which may interfere with herd screening, which is a downside of full dose subcutaneous inoculation of S19 vaccine. The reduced-dose

strategy could be used on dairy farms endemic for brucellosis. To control brucellosis on a dairy farm in the shortest possible time, then separation of positive animals coupled with immunization of negative cows is needed. The conjunctival vaccination could also be used on farms that are free from brucellosis so that entry through carrier animals could be avoided otherwise huge losses could occur on entry of brucellosis. If a calf misses calfhood S/C vaccination at the age of 4–8 months, an alternate way to immunize animals through conjunctival vaccine could be adopted to achieve maximum vaccination coverage against brucellosis in the country.

The immunogenicity provided by the reduced-dose intraocular vaccination was almost similar to that of the subcutaneous counterpart, the s/c administration produced higher titer when compared to i/o administration and there is a possibility of farm contamination due to shedding of *Brucella* organisms in s/c. Therefore, the intraocular route was far safer compared to the subcutaneous vaccination.

Sequencing results of the *bcsp31 gene* further confirmed the shedding of the *Brucella* vaccine in milk following subcutaneous vaccination with *B. abortus* S19. Also, the BLAST analysis and Phylogenetic tree showed >99% relatedness with other global isolates from the NCBI GenBank. This is the first study of its kind in Hisar, that detected the shedding of the S19 vaccine in milk following subcutaneous and intraocular inoculation and we have contributed to the NCBI database MK881173-6. Therefore, there is a need to enlighten the farmers/public on the risk of shedding *Brucella* organisms in the milk of vaccinated cows and discourage the practices of consumption of raw milk in villages.

5.1. Statement of animal rights

Before our study, a proposal was submitted to the LUVAS Institutional Animal Ethics Committee (IAEC) and was approved vide No. VCC/IAEC/265-93; dated 15/02/2018.

Declarations

Author contribution statement

A. S. Saidu: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Mahavir Singh, Aman Kumar: Performed the experiments.

N.K. Mahajan: Conceived and designed the experiments; Wrote the paper.

Dinesh Mittal, Rajesh Chhabra, Vinay G. Joshi, Usman Sadiq: Contributed reagents, materials, analysis tools or data.

Imadidden I. Musallam: Analyzed and interpreted the data.

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Data availability statement

Data associated with this study has been deposited at National Centre for Biotechnology and Informatics (NCBI GeneBank) under the accession numbers MK881173, MK881174, MK881175, and MK881176 (http://www.ncbi.nlm.nih.gov/).

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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Supporting Statement

The i/o administration of *Brucella abortus* Strain-19 vaccine remains the safer route for vaccinating adult cattle (above-calfhood) than subcutaneous counterparts with no persistent antibodies and good immunoprotection.

The study proved that the i/o reduced-dose vaccination with S19 remains cheaper and a better strategy to be adopted to argue the shedding associated with the conventional subcutaneous route vaccination.

The study also evaluated the immunoprotection and safety of reduce-dose (5.0×10^9 CFU) of *Brucella abortus* S19 vaccine in adult cattle following booster dose at 120DPV without persistent antibodies and shedding of *Brucella* organisms in the milk.

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