# SAFETY AND IMMUNOGENICITY OF *BRUCELLA ABORTUS* STRAIN-19 REDUCED DOSE VACCINE THROUGH CONJUNCTIVAL ROUTE IN CATTLE - A TRIAL

*By*

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B.V.Sc. & A.H

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# CERTIFICATE

**V. Manoj Kumar** has satisfactorily prosecuted the course of research and that the thesis entitled "**Safety and Immunogenicity of *Brucella abortus* Strain-19 Reduced Dose Vaccine through Conjunctival Route in Cattle - A Trial"** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any University.

**Date: 17 June 2013**
**Place: Hyderabad.**

**(Dr. Y.NARASIMHA REDDY)**
MAJOR ADVISOR

# CERTIFICATE

This is to certify that the thesis entitled **"SAFETY AND IMMUNOGENICITY OF BRUCELLA ABORTUS STRAIN-19 REDUCED DOSE VACCINE THROUGH CONJUNCTIVAL ROUTE IN CATTLE - A TRIAL"** submitted in partial fulfillment of the requirements for the degree of "**MASTER OF VETERINARY SCIENCE"** of the Sri Venkateswara Veterinary University, Tirupati, is a record of bonafide research work carried out by **V.Manoj Kumar** under my guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted for any other degree or diploma. The author of thesis has duly acknowledged all assistance and help received during the course of investigation.

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# DECLARATION

**I, *V. MANOJ KUMAR*** hereby declare that the thesis entitled "***SAFETY AND IMMUNOGENICITY OF BRUCELLA ABORTUS STRAIN-19 REDUCED DOSE VACCINE THROUGH CONJUNCTIVAL ROUTE IN CATTLE - A TRIAL*****"** submitted to Sri Venkateswara Veterinary University, Tirupati for the degree of ***MASTER OF VETERINARY SCIENCE*** is the result of original research work done by me. I also declare that the materials contained in this thesis have not been published earlier.

Date: 17 August 2013 **(V. MANOJ KUMAR)**

Place: Hyderabad.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| % | : percent |
| µL | : Micro litre |
| 2ME | : 2-mercaptoethanol |
| A-B ELISA | : Avidin Biotin Enzyme linked immunosorbent assay |
| ABTS | : 2, 2‟-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) |
| AMOS PCR | : Abortus melitensis ovis suis polymerase chain reaction |
| BCG | : *Bacillus* Calmette Guerin |
| BCSP | : brucella cell surface protein |
| BD | : Beckton Dickinson |

| | |
|---|---|
| Bp | : Base pairs |
| Biovar-1 | : *B. abortus* strain 1 |
| CD4 | : Cluster differentiation factor |
| c-ELISA | : competitive ELISA |
| CFT | : complement fixation test |
| CFU | : colony forming unit |
| CMI | : cell mediated immunity |
| ConA | : Concanavalin A |
| $CO_2$ | : carbon dioxide |
| DNA | : deoxyribonucleic acid |
| ELISA | : Enzyme linked immunosorbent assay |
| FAO | : food and agriculture organization of UN |
| HRPO | : horseradish peroxidase |
| i-ELISA | : indirect enzyme linked immune assay |
| IFN-γ | : Interferon-gamma |
| IRF-1 | : Interferon regulatory factor 1 |
| IgG | : immunoglobulin class G |
| IgM | : immunoglobulin class M |
| IL | : Interleukin |
| IVRI | : Indian Veterinary Research Institute |
| LPS | : lipopolysaccharide |
| Mabs | : mono-clonal antibodies |
| MHC class-II | : major histocompatibility complex class II |
| ml | : millilitre |
| M | : mole |
| MRT | : Milk ring test |
| NK | : Natural Killer cell |
| $^oC$ | : degree centigrade |
| OD | : optical density |
| OIE | : World Organisation for Animal Health |
| OMP | : outer membrane protein |

PD_ADMAS : Project Directorate on Animal Disease Monitoring and Surveillance
PBS : phosphate buffered saline
PBST : phosphate buffered saline-tween 20
PCR : polymerase chain reaction
*Pmm* : phosphomannomutase
PV : post vaccination
RBPT : Rose-bengal plate test
ROP : Retension of Placenta
S19 : *B. abortus* strain 19
S2308 : *B. abortus* strain 2308
SE : standard error
SI : stimulation index
SAT : standard serum agglutination test
RB51 : *B. abortus* strain RB51
T-cells : thymus cells
TH1 : T helper cell-1
v/v : volume/volume
WHO : World Health Organization
WPV : week post vaccination

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# ABSTRACT

The present study is taken up with a view to study the efficacy of brucella vaccine at reduced dose ($5 \times 10^9$ to $8 \times 10^9$ CFU/dose) through conjunctival route and compare it with conventional standard dose ($40 \times 10^9$ to $80 \times 10^9$) vaccine. Female cattle above 8 months age were selected for the study. Thirty animals were inoculated with reduced dose conjunctival vaccine and ten with standard subcutaneous vaccine using *Brucella abortus* S19 antigen. The efficacy of vaccine was studied in terms of both humoral and cell mediated immune responses against brucella for a period of 120 days. Humoral immunity techniques consisted of rose bengal plate test (RBPT), standard tube agglutination test (SAT) and indirect ELISA (i-ELISA). Cell mediated immune response was studied by using interferon gamma (IFN-γ) assay after *in vitro*

stimulation of lymphocytes with both S19 and *B. abortus* 544 as antigens in indirect competitive ELISA (Enzyme Linked Immunosorbent Assay). Of the humoral immunity tests employed for studying the efficacy of vaccines RBPT was least sensitive. It detected 20 to 18.51% positive responders at 21 and 60 days PV in reduced dose conjunctival vaccine group. Similarly it detected 60% and 50% responders on 21 and 60 days PV in standard group.

Standard tube agglutination test (SAT) detected more responders against vaccinates than the RBPT. The positive percentage ranged from 26 to 66% among reduced dose vaccine group and 40 to 80% among standard dose vaccine group. I-ELISA was the most sensitive as it detected up to 78.9% responders among reduced dose vaccination group and up to 100% in standard dose vaccinates group. Humoral immune responses declined after 90 days PV. IFN-γ production as indicator of cell mediated immune response in vaccinates was more consistent. IFN-gamma production was observed during the entire period of the trial (120 days). It is concluded that CMI responses are better indicators of immune response to brucella than the humoral immunity.

# CHAPTER I

# INTRODUCTION

Brucellosis is the second most important zoonotic disease in the world according to OIE (2004). It is also most wide-spread zoonotic infection, in the developed and developing world, transmissible from animals to humans. It is an important neglected zoonotic problem in India accounting for a loss of about 30 million man days per year (Handa *et al*., 1998). Asia is currently considered as one of the high prevalence regions of this disease.

India is an agricultural country and exposure of humans to animals is quite high. Dairy farm workers are constantly exposed to infection due to contamination of hands and arms while milking the animals. The dairy farm workers also spend a considerable amount of time attending to the animals need and attending to them in parturition and often sleeping alongside them. In India, as per ADMAS (Animal Disease Monitoring and Surveillance, Bangalore) report on long term survey of bovine brucellosis (1994-2002) the disease is widespread in most parts, with cumulative average of 6.8% in bovine. The overall national average incidence of brucellosis in cattle and buffalo was 7.2% and 5.25% respectively. The prevalence of brucella antibodies ranged from 2.7% (2/73) in Andaman &Nicobar Island to a maximum of 63.5% (250/394) in West Bengal. As per the study conducted by Mudaliar *et al*. (2003), 12 out of 225 animal handlers (5.33%), 6 out of 133 (4.51%) dairy farm workers and 6 out of 41 (14.63%) veterinary doctors showed the presence of antibodies to *Brucella abortus.* A study of Soman and Kothari (1954), reported 4% of dairy workers positive for Brucellosis. Rana *et al*., (1985) showed a sero positivity of 27.7% in Veterinary workers in Delhi.

Human infections are characterized by large number of somatic illnesses including undulant fever, anorexia, fatigue, malaise, weight loss, depression, endocarditis, pericarditis, arthritis of peripheral joints and spondylitis (Bossi *et al*., 2004). Principal signs of the disease in cattle and buffaloes are reproductive failure such as abortions (in the third trimester of pregnancy) in females and orchitis with sterility in males (Radostits *et al.,* 2004). The organism is sensitive to many antibiotics but due to its intracellular sequestration in lymph-nodes, mammary glands and reproductive organs, treatment is not useful and is too expensive in most of the animal species.

Approaches used to control brucellosis include immunization, testing and removal of infected animals, depopulation and quarantine (Radostits *et al*., 2004). High prevalence rate of brucellosis, socio-economic conditions, veterinary services and methods of livestock farming in the country suggest that the appropriate method for the control of this disease in India is immunization of the susceptible animals. Brucella attenuated strain multiplies within the body of the animal for a shorter period of time than the virulent field strain from which it was derived.

Cattle vaccinated with *B. abortus* RB51 exhibited high level of protection characterized by good cell mediated immunity (Olsen *et al*., 2003). However, efficacy of *B. abortus* SRB51 vaccine in buffaloes is controversial (Fosgate *et al.*, 2003), could not find the SRB 51 vaccine protective against infection in buffaloes on standard dose recommended for cattle. Furthermore, poor cell mediated immune responses in buffaloes, following SRB51 vaccination, were reported by Diptee *et al.* (2005).

Cytokine detection assays have been used extensively for the detection of cell mediated immune responses in cattle and other animal species (Odbileg *et al.,* 2007). Lymphocyte proliferative assays still remain the gold standard in assessing cell mediated responses to brucella antigens.

*Brucella abortus* S-19 strain is stable and has low pathogenicity and high immunogenicity and used as vaccine. It is normally given to female calves aged between 4 and 12 months as a single subcutaneous dose of 5-8 x $10^{10}$ viable organisms. Alternatively, it can be administered to cattle of any age as either one or two doses of 5 x $10^{9}$ viable organisms, given by the conjunctival route. This produces protection without a persistent antibody response and reduces the risks of abortion and excretion in milk when vaccinating adult cattle (OIE, 2004). Hence the present study is taken up to know the efficacy of strain 19 vaccine by conjunctival route and compare it with standard vaccination method with the following objects.

- To study the number of cattle experiencing local and/or systemic reactions after conjunctival vaccination and during follow-up period.
- To determine the humoral immune response of *Brucella abortus* S-19 reduced dose vaccine through conjunctival route at different time intervals using ELISA.
- To study cell mediated immune response in randomly selected statistically significant number of animals from each group at different time intervals using *gamma*-interferon assay.Comparison of ELISA with standard tube agglutination test (SAT) for immunogenicity.

# CHAPTER-II

## REVIEW OF LITERATURE

- ## 2.1 Brucellosis:

- Brucella is named after Dr. David Bruce an English military doctor who identified *Brucella* as disease causing microorganism in the island of Malta in 1887. *Brucella spp*. were later isolated from aborted bovine fetuses by Dr. Bang, a Danish veterinarian, and therefore the test used to identify the disease as well as the disease in cattle are also known as Bang's test and Bang's disease respectively (Nicoletti *et al*., 2002).
- Corbel *et al.* **(**1997) reported that the *Brucella* species possess certain host specificity; *B. melitensis* infects mainly caprine and bovines, *B. abortus* affects bovines and some wild life species such as elk and bisons, *B. ovis* infects sheep, *B. canis* infects dogs, *B. cetacea* or *B. pinnipedae* affect marine mammals.
- Ko *et al*. (2003) reported that animals and humans become infected with *Brucella* when their mucosal membranes or broken skin come in contact with infected secretions (milk, blood, uterine discharge) or aborted fetuses.
- Yingst *et al.* (2003) described the brucellosis symptoms in animals: they may range from no apparent signs of infection, to orchitis and decreased fertility in rams, to abortion in goats, cows, dogs and elk. In humans, usually, it is associated with marked malaise, undulant fever, joint aches, and orchitis. If the disease in humans is not treated appropriately with

antibiotics, it results in chronic brucellosis with relapses of articular problems, neurobrucellosis and in some cases depression.

- ## 2.2 General Characteristics:

- Moreno *et al.* **(**1979) revealed that the structure of the O-chain in smooth *Brucella* consists of repeating units of 4,6-dideoxy-4-formamido−α-D-mannopyranosyl residues known as perosmines (rough organisms lack this chain) linked by an α1-2 linkage. Depending on the LPS linkage variations they were classified into: A and M strains. A strains have homopolymers linked by α 1-2 linkage, while M strains have α 1-3 linkage between every fifth residue.
- Smith and Ficht **(**1990) proved that the lipopolysaccharide (LPS) outer layer of the bacterial cell wall is the major antigenic and toxic component of gram negative bacteria.
- Stevens *et al.* (1996) stated that brucella consists of lipid A (major endotoxic portion of LPS), an oligosaccharide core, KDO (2-keto-3-deoxyoctonic acid), and an O-side chain (O-polysaccharide, O-antigen, O-chain), that is the major antigenic portion of the LPS.
- Ogata *et al*. **(**2000) suggested that the LPS and its O-side chain stimulate the production of antibodies by B cells by initiating a signal transduction cascade upon interaction with specific pattern recognition receptors. LPS of brucella differ from that of other gram negative bacteria in that it is less endotoxic.

- Ficht *et al*. (2002) reported that *brucella* organisms lack the classic virulence determinants such as exotoxins, plasmids, flagella, fimbriae, lysogenic phages, highly endotoxic LPS, cytolysins etc., that are usually associated with pathogenic bacteria.

- **2.3 Sero-prevalence:**

- Mudaliar *et al*. (2003) conducted seroprevalence studies and found that 12 out of 225 animal handlers, 6 out of 133 (4.5%) dairy farm workers and 6 out of 41 (14.63%) veterinary doctors showed the presence of antibodies to Brucella abortus. Out of 225 cases, 12 (5.33%) animal handlers showed presence of antibodies to *Brucella abortus* where as control group of non animal handlers (50) tested negative for Brucella antibodies.
- Jai sunder *et al.* (2005) reported the presence of *B.abortus* agglutinins as 13.83% and 10.4% by RBPT and A-B ELISA respectively whereas the SAT studies showed only 2.29 % in the Andaman and Nicobar Islands.
- Agasthya *et al.* (2007) screened in high risk group individuals for brucellosis and reported the disease prevalence was 41.23% in veterinary inspectors, 30.92% in veterinary assistants, 12.37% in veterinary officers, 6.18% in veterinary supervisors, 6.18% in group D workers, 2.06% in shepherds, 1.03% in butchers in Karnataka state.
- 2.4 Serological Tests Used For the Diagnosis of Brucella

- **2.4.1 Enzyme Linked Immunosorbent Assay (ELISA):**

- Nielsen *et al.* (1995) examined 1446 sera from brucellosis free herds and observed the specificity of a modified C-ELISA as 99.7 percent and it correctly identified 636 sera from *B.abortus* infected cattle as positive accounting for a sensitivity estimate of 100 percent.
- Rojas and Olonso (1995) showed that the indirect enzyme linked immunosorbant assays (I-ELISA) are predominantly used to assess humoral immunity against brucellosis or as serological diagnostic test for the detection of infection in cattle. Smooth LPS are mostly used as antigen in this test. Competitive enzyme linked immunosorbant assay using O-polysaccharides as antigen are employed as differential diagnostic tests in cattle.
- Saravi *et al.* (1995) suggested the use of ELISA in combination with a screening assay such as Brucella Plate Agglutination Test (BPAT) to be cost effective and that it would introduce a high degree of standardization to brucellosis diagnosis in Latin America.
- Uzal *et al.* (1996) recommended the use of I-ELISA monoclonal assay as a useful tool in diagnosis of bovine brucellosis as it is rapid, simple, sensitive and specific for detecting antibodies to *B. abortus* in non-vaccinated, vaccinated and infected animals.
- Renukaradhya *et al.* (2001) developed and validated the A-B ELISA kit at field level using 7040 cattle and 678 buffalo sera samples to reveal 2.7

percent and 10.2 percent positivity respectively. The overall specificity of 98.8 percent and sensitivity of 98.2 percent was observed with the kit.

- Sarumathi (2002) conducted a serological survey for the presence of brucella antibodies in cattle in various districts of Andhra Pradesh by using A-B ELISA, and found 12.5% (192 of 1541) samples were positive.

- Charanjeet *et al.* (2004) screened 641 cattle sera and 146 buffalo sera from different regions of Himachal Pradesh and reported a sero-prevalence of 1.56 percent and 1.36 percent respectively using A-B ELISA which was little less compared to RBPT (2.65% and 3.42%) and SAT (2.34% and 2.73%).

- Dhand *et al.* (2004) showed the apparent overall prevalence of brucellosis in Punjab to be 12.09% (true prevalence, 11.23%). The prevalence varied from a low of 0% to a high of 24.3% in various districts. Higher variance (0.08) was noted within villages than between different villages (0.03). The prevalence rates among buffaloes and cattle were 13.4% and 9.9%, respectively by using an avidin-biotin enzyme-linked immunosorbent assay.

- **2.4.2 Standard Tube Agglutination Test (SAT):**

- Kumar *et al.* (1974) reported that a high percent (69%) of breeding bulls in non-endemic areas showed low titer (up to 1:40) and 59-63 percent of bulls and 89 percent of bullocks of endemic areas showed high titer (1:80 and above) after screening with SAT.

- Stemshorn *et al.* (1985) stated that SAT had a higher specificity for detection of brucellosis in non-vaccinated (99.5%) than vaccinated cattle (98.8%).
- Das *et al.* (1990) observed that most of the buffalo sera collected at the acute phase of infection showed high SAT titers (range 80-640) which either remained the same or increased by only one dilution at the convalescent phase. Crossbred cow sera showed low SAT titers (range 10-160) at acute phase of infection, which increased significantly by 1 to 5 dilution steps (80-1280) at the convalescent phase.
- Sharma and Saini (1995) screened cattle and buffaloes in Punjab by using RBPT, SAT and found the prevalence of 8.69 and 14.61 percent respectively. They also found 8 cattle and 10 buffalo herds positive for brucellosis.
- Isloor *et al.* (1998) screened 23,284 cattle and 7,153 buffalo sera for brucella antibodies in 23 States of in India. They initially used the rose bengal plate test: doubtful and positive samples were then titrated in the serum tube agglutination test. The overall prevalence rate of antibodies was 1.9% in cattle and 1.8% in buffalo. In a detailed study of 47 organized farms in the southern State of Karnataka, 207 of 4,995 (4.1%) serum samples from cattle showed titers for brucellosis. This result was in contrast to the low rate of seropositive results reported in cattle owned by individual farmers in Karnataka (0.7% of 2,424 serum samples). In organized farms with a history

of abortion, placenta retention and repeat breeding, the prevalence rate was 17%.

- Mrunalini and Ramasastry (1999) detected the highest serum agglutination titer of 2560 IU in cattle, buffaloes, goats and humans. They found that in cattle more than 50 percent of the seropositive cattle had high titer and less than 50 percent of the seropositive buffaloes had high titers.
- Singh *et al.* (2004) employed SAT to check the authenticity of positive sera detected by RBPT and stated that SAT can be used better for detection of brucellosis in brucellosis eradication campaigns to avoid any unwanted killing or slaughter of animals detected as positive by RBPT.
- Studies of Kaur *et al.* (2006) on aborted cattle and buffaloes revealed that RBPT and SAT were in conformity with each other. However, SAT was more sensitive compared to RBPT.

### 2.4.3 Rose Bengal Plate Test (RBPT):

- In a study conducted by Saravi *et al.* (1995), RBPT, 2ME, CFT and ELISA demonstrated high and comparable specificity estimates in the range 99.7-99.9 percent where as BPAT demonstrated a lower specificity estimate of 90.6 percent. The relative sensitivity of RBPT was 86.3, 81.4 and 79.1 percent compared to CFT, 2ME and ELISA respectively.
- Thakur and Thapliyal (2004) observed that RBPT was more efficient than SAT in detecting brucellosis in all the species (cattle, goats, sheep and humans).

- Ruiz-Mesa *et al*. (2005) conducted trials to analyze the diagnostic yield of the Rose Bengal test. A total 711 Brucellosis patients were divided into three groups: group I, individuals with no regular exposure to or history of brucellosis; group II, individuals exposed repeatedly to Brucella infection; and group III, individuals infected with Brucella who had received appropriate treatment during the previous 12 months. The overall sensitivity of the rose Bengal test was 92.9%. The specificities for groups I, II and III were 94.3%, 91.7% and 76.9%, respectively. They concluded that use of the rose Bengal test as the sole technique for the diagnosis of brucellosis in endemic areas should be considered very carefully in the context of patients who are exposed repeatedly to Brucella or have a history of brucellosis.
- Anuradha and Ganesan (2006) employed RBPT to screen 30 aborted animals, 15 animals with retained fetal membranes and 26 with repeat breeding and arthritis and detected 16.6, 13.35 and 3.8 percent brucella positive animals respectively.
- Akhtar *et al*. (2010) calculated the comparative efficacy of Rose Bengal Plate Test (RBPT) and Milk Ring test (MRT) for the diagnosis of bovine brucellosis in cows (Group A) and buffaloes (Group B). Using bacterial growth as a gold standard RBPT showed high sensitivity values of 100% in both groups with specificity of 96.29% (Group A) and 90.62% (Group B).On the other hand MRT showed low sensitivity (80.0% in Group A; 86.6% in Group B) while its specificity was 100% in all the animals of both groups.

- Cho *et al*. (2010) described a simple way to improve the accuracy and uniformity of reading the RBT reaction by counting the number of agglutinated particles using transparent OHP film with Quantity One, which was originally designed to count the bacterial colony numbers on agar plates. Using this system, the reactivities of three Rose Bengal antigens from different sources against international standard serum (1,000 units, VLA, UK) were numerically measured: the intensity scale ranged from zero to around 1,600.
- Basavaraj *et al.* (2011) screened 380 sera from general people and 45 samples from veterinary staff and found that the seroprevalence was 2.4% in general population and 11.1% in veterinary staff in Davangere (Karnataka). The overall seroprevalence of brucellosis in the study was 3.3%, Prevalence was more among males (3.7%) than in females (1.01%). 20-40 years age group was more affected (3.6%). Shepherds had the highest prevalence rate (5.9%), followed by farmers (5.2%). When compared with STAT, RBPT showed sensitivity of 100% and specificity of 99%. RBPT was highly reliable and had a close relation with STAT in the diagnosis of human brucellosis.
- Mahajan *et al.* (2011) studied a total of 51 specimens, each of milk and serum from clinically suspected subjects in the field. The true prevalence of brucellosis was 13.72% with apparent prevalence of 33.34% and 17.64% by the milk ring test (MRT) and the Rose Bengal plate agglutination test (RBPT), respectively. The overall agreement between RBPT and serum tube

agglutination test (STAT) was higher (96.07%) as compared to MRT and STAT (80.89%). Both MRT and RBPT showed 100% sensitivity. Specificity of RBPT was higher (95.45%) than MRT (77.27%), when compared to STAT.

- **2.4.4 Milk Ring Test (MRT):**

- Wickramasuriya *et al.* (1983) studied the herd incidence of brucellosis using MRT in Polonnaruwa, Batticaloa and Ampara districts and recorded 7.7 percent, 12 percent and 14.4 percent respectively. MRT positives were subjected to serological tests where the incidence of seropositive cows was 1.3 percent of the total number tested with MRT.
- Kerkhofs *et al.* (1990) observed the sensitivity and specificity of MRT as 58 percent and 99.5 percent respectively. They further observed that the specificity of MRT was on par with that of milk ELISA (99.3%).
- Durand *et al.* (1992) compared the results of milk ring tests on bulk milk and serological tests and found that only 10.5% of the ring test positive herds had serologically positive animals whereas 99.5% of ring test negative herds were serologically negative. They also found these false positives more commonly among small herds.
- Sadasivam *et al.* (1996) tested 1761 individual milk samples for brucellosis in non-endemic areas of Tamilnadu, using MRT and found that 13 (0.73%) samples were positive.

- Eze *et al*. (1997) showed that the diagnosis of brucellosis in a dairy herd by means of the MRT on bulk milk, as carried out in temperate climates, was not suitable to the African environment where milk was soured before sale.
- Gonzalez and Samartino (1997) concluded that the milk ring test was useful in monitoring brucellosis but its efficiency was limited as it depends on the secretion of antibodies in the milk, antibody titer in milk, quantity of milk produced by infected cows, size of dairy and frequency of testing.
- Silva Junior *et al. (*2004) performed ring test on 69 individual milk samples and 68 bulk milk samples processed according to the technique after 24 hours of refrigeration and the same samples were processed 6-8 hours after collection during which they were maintained under cooling and obtained the same results with both tests in all the samples tested.

## • 2.5. Diagnosis by Genome Based Techniques

### • 2.5.1 Polymerase Chain Reaction (PCR):

- Ewalt *et al*. (2000) evaluated Brucella AMOS PCR test to determine its accuracy in differentiating *Brucella abortus* into three categories: field strains, vaccine strain 19 (S19), and vaccine strain RB51/parent strain 2308 (S2308). Two hundred thirty-one isolates were identified and tested by the conventional biochemical tests and Brucella AMOS PCR. This included 120 isolates identified as *B. abortus* S19, 9 identified as *B. abortus* strain RB51, 57 identified as *B. abortus* biovar 1, 15 identified as *B. abortus* bv. 2, 1 identified as *B. abortus* bv. 2 (M antigen dominant), 7 identified as *B. abortus* bv. 4, and 22 identified as *B. abortus* S2308 and isolated from

experimentally infected cattle. The Brucella AMOS PCR correctly identified each isolate as RB51/S2308, S19, or a field strain of Brucella.

- Hussein *et al*. (2006) tested peripheral blood samples from 23 males and 17 females by Rose Bengal test (RBT), Standard tube agglutination test (SAT) and nested PCR using four primers to amplify 677 bp fragments. 23 patients (57.5%) were positive by (RBT), 25 patients (62.5%) were positive by (SAT). 28 patients (70%) were positive by nested PCR assay. They concluded that the PCR assay was more sensitive than conventional serological methods and this coupled with its speed and reduction in risk to laboratory workers.
- Miyashiro *et al*. **(**2007) analyzed a total of 192 samples of illegal cheese from different regions of the states of Brazil, for the isolation and detection of *Brucella* spp. DNA by means of microbiological culture and polymerase chain reaction (PCR), respectively. Samples that yielded positive results were submitted to the analysis of the occurrence of *Brucella abortus* (biovars 1, 2 e 4), as well as to the differentiation of DNA in B19 vaccinal strain or *Brucella abortus* field strain using PCR. Although the microorganism was not isolated from any sample, PCR detected 37 positive samples (19.27%) using genus-specific primers (100%) for *Brucella abortus*. Differentiation of the strain showed that 30/37 samples (81.08%) had vaccinal strain B19 and 7 (18.92%) had Brucella *abortus* field strains. It was concluded that diagnostic sensitivity of PCR was greater than the microbiological culture.

- Mukherjee *et al.* (2007) standardized three independent diagnostic PCR assays on six reference strains of Brucella using Brucella-specific nucleotide sequences encoding the BCSP 31 kDa protein, Omp2 and the 16S r-RNA in. They employed16S r-RNA gene as the diagnostic target in the PCR and found only 14 out of these 19 isolates and 2 out of 7 bovine milk isolates as the genus Brucella. They compared antibody-detecting ELISA results of field samples separately with omp2 and bcsp PCRs of blood from a serologically positive herd and found that the bcsp PCR was the most sensitive, the degree of association of ELISA with omp2 blood PCR was similar to that with the bcsp blood PCR. They concluded that the use of more than one marker-based PCR gives increased sensitivity and higher specificity and a more reliable molecular diagnostic approach for screening of field animals.
- Hafez *et al*. (2011) employed single step polymerase chain reaction (PCR) to diagnose brucellosis in sheep using whole blood and compared its sensitivity and specificity with some of the most commonly used serological techniques and modified ones. 300 sera were tested against Rose Bengal test (RBT), Serum Agglutination test (SAT), ELISA using both the whole Brucella antigen (W-ELISA) and the periplasmic protein antigen (P-ELISA). Results showed that 39% of the blood samples were positive to the PCR test, 29.3, 27.0, 28.7 and 28.3% were positive to the serological tests respectively. Finally they recommended the use of this blood PCR assay for

accurate diagnosis of ovine brucellosis especially in the early stage of infection, which is difficult to achieve by the applied serological tests.

- Weiner *et al.* (2012) compared the AMOS, Multiple Locus Variable-Number Tandem Repeat analysis and Bruce-Ladder for typing of Brucella spp. Out of 138 Brucella clinical isolates examined, AMOS PCR could correctly identify only *B. abortus* bv1, bv2, and bv4, *B. suis* bv1, three biovars of *B. melitensis* (bv1, bv2, and bv3) and *B. ovis*. But the Bruce-ladder PCR assay could identify and differentiate all of the Brucella species and the vaccine strains in the same test. They concluded that Bruce-ladder PCR can be a useful tool for the rapid species identification of Brucella strains.

## 2.6 Cell Mediated Immune Tests for Diagnosis

### 2.6.1 IFN-γ Assay:

- Pottumarthy *et al.* (1990) evaluated an *in vitro* test of cell-mediated immunity, the tuberculin gamma interferon assay, QuantiFERON-TB (QIFN), in 455 individuals from three groups: group I, 237 immigrants from high-risk countries; group II, 127 health care workers undergoing Mantoux testing; group III, 91 patients being investigated for possible active tuberculosis (79 patients) or *Mycobacterium avium-Mycobacterium intracellulare* complex infection (12 patients). The QIFN results were compared either to those of the Mantoux test or to microbiological and clinical diagnosis, as appropriate. For group I, the agreement between QIFN

and Mantoux results was 89% for Mantoux-negative and 64% for Mantoux-positive individuals. For group II, when >/=10-mm-diameter induration was taken as positive, the agreement was 81% for Mantoux-negative and 67% for Mantoux-positive individuals. For group III, agreement was 81% for Mantoux-negative and 86% for Mantoux-positive patients. They suggested the tuberculin gamma interferon assay (QIFN) as a potential replacement for the Mantoux test.

- Wood *et al.* (1991) conducted extensive field studies to compare gamma interferon assay and the single intradermal tuberculin test for the diagnosis of bovine tuberculosis in Australia. The specificity of the IFN-gamma assay determined by testing more than 6000 cattle from tuberculosis-free herds, varied from 96.2% to 98.1%, depending on the cut-off point chosen to define a positive reactor. The sensitivity of the IFN-gamma assay was shown to be significantly higher than the single intradermal tuberculin test and varied from 76.8% to 93.6%. They obtained a maximum overall sensitivity of 95.2% by testing with the IFN-gamma and the tuberculin test in parallel.

- Billman-Jacob *et al*. (1992) screened 6 cattle herds for Johne's disease using two interferon gamma assays, the IFN-gamma enzyme immunoassay (EIA), the IFN-gamma bioassay and an absorbed ELISA. Each herd had a history of Johne's disease but the majority of infected animals did not show clinical signs. The sensitivities of the IFN-gamma assays and the absorbed ELISA were determined using test results from infected animals. The sensitivity of

the IFN-gamma EIA in detecting subclinical (71.8 to 93.3%) and clinical animals (100%) was not significantly different. They concluded that the IFN-gamma bioassay and the absorbed ELISA were more sensitive in detecting cattle with advanced infections (80%) than those that were sub-clinically affected (16.7 to 33.3%).

- Kittelberger *et al*. (1997) investigated the specificity of the *in vitro* antigen-specific gamma interferon test for bovine brucellosis diagnosis in experimentally *Yersinia enterocolitica* 0:9-infected cattle using the conventional brucellosis tests: complement fixation test (CFT), serum agglutination test (SAT) and brucella card test (BCT) and Brucella antigen-specific gamma interferon test. Initially, the animals were exposed orally to $10^{10}$ colony-forming units (CFU) of *Y. enterocolitica* 0:9. Four weeks later, they were inoculated intravenously with $10^{8}$ CFU of *Y. enterocolitica* 0:9 cells. After oral inoculation, the response in the conventional brucellosis tests was minimal but the Brucellergen-stimulated release of IFN-gamma peaked at values above the cut-off stimulation index of 2.5 in 80% of the heifers. After intravenous inoculation, stimulation indices above 2.5 were present in only 10% of the animals. Two *B. abortus* infected control cattle showed stimulation indices of 3.1 and 3.4, and a negative control animal exhibited a stimulation index of 1.0. Their findings showed that the Brucellergen-specific IFN-gamma assay can be used as a specific and discriminatory test for *B. abortus* infections.

- Gonzalez-Llamazares *et al*. (1999) compared the interferon gamma assay and the single intradermal cervical tuberculin test (SICT) and found that the sensitivity of IFN-γ assay was shown to be higher (84%) than that of the SICT Test (80.2%), but the combination of both tests offered the highest sensitivity (92.9%).
- Adone *et al*. (2000) evaluated the concordance, specificity and sensitivity of the IFN- γ test with respect to complement fixation (CF) test. They selected 672 cattle from brucellosis-infected and brucellosis-free herds and 4 cattle experimentally-vaccinated with strain RB51 and tested for the production of IFN in response to allergens from *B. melitensis* B115 and *B. abortus*, respectively. IFN was detected in specific allergen-stimulated blood cultures using a commercial enzyme immunoassay and the CF test was performed on sera from all examined cattle, according to the official procedures for bovine brucellosis diagnosis. They suggested that the IFN-γ test used in conjunction with the CF test could improve the diagnosis of brucellosis in cattle. In cattle excreting *Yersinia enterocolitica* 0:9, the IFN-γ test gave negative results, indicating that this test does not give false positives due to the cross-reaction of *Brucella* with this microorganism. They concluded that cattle vaccinated with *B. abortus* RB51, the γ-IFN test performed with the homologous strain RB51 as stimulus, was able to detect treated subjects despite the absence of significant antibody titers, as measured with the official serological methods.

- Cresswell **(**2000) reported that IFN-γ induces the up-regulation of cell surface MHC-I molecules which results in efficient induction of cell mediated immunity and recognition of foreign peptides by cytotoxic lymphocytes. IFN-γ also up-regulates chaperones involved in the assembly of the MHC I.
- Eze *et al.* (2000) established the importance of IFN-γ is during the very early stages of infection and stated that its importance is somewhat diminishing during the latency phase and in chronic stages.
- He *et al.* **(**2001) showed that vaccine strain RB51 induces cytotoxic lymphocytes that secrete IFN-γ and are able to kill infected macrophages. IFN-γ is a type II IFN secreted by Th1 CD4+ T cells, NK cells, CD8+ cytotoxic cells, B cells and other antigen presenting cells ( Schroder *et al.,* 2004).
- Murphy *et al.* **(**2001) stated that the CD8+ cytotoxic cells play a fundamental role in controlling the infection not only because of the direct killing effect on infected cells but also by secretion of IFN-γ which further increases the anti-*Brucella* response.
- Ko *et al.* **(**2002) suggested that during intracellular infections, IFN-γ and TNF-α induce Interferon regulatory factor 1 (IRF-1), which activates macrophages. This was demonstrated, using IRF-1-deficient (IRF-1-/-) mice that are more susceptible to BCG infection than wild type mice, and die when infected with virulent *B. abortus* but not when infected with attenuated strains.

- Odbileg *et al.* (2007) estimated the levels of cytokines produced by camel (*Camelus bactrianus*) peripheral blood mononuclear cells (PBMCs) in response to live attenuated *Brucella abortus* S19 vaccine. Cytokine responses to *B. abortus* S19 were examined at 6 hr, 48 hr and 1, 2 and 3 weeks post-vaccination. Their findings showed that B. *abortus* stimulates cell-mediated immunity by directly activating camel Th1 cells to secrete IFN-γ. This is the first report of *in vivo* camel cytokine quantification after vaccination.
- Segura *et al.* (2007) evaluated the activity of gamma interferon (IFN-γ) when it was either adsorbed onto or loaded into albumin nanoparticles. *Brucella abortus*-infected macrophages and infected BALB/c mice were used as the models for testing of the therapeutic potentials of cytokine delivery systems and concluded that albumin nanoparticles would be suitable as carriers that target IFN-γ to macrophages and, thus, potentiate their therapeutic activity.
- Tittarelli *et al.* (2009) employed an enzyme linked immunosorbent assay (ELISA) for the detection of γ- interferon production in cattle vaccinated with *Brucella abortus* strain RB51. The test was evaluated for 300 days in 10 brucellosis free heifer calves vaccinated at calf-hood with 10 $x10^9$ colony forming units of RB51 and in five control heifers. Vaccinated animals started to give positive results from day 17 post vaccination (PV) until day 239 PV. All vaccinated animals gave a positive reaction at least once (with a stimulation index exceeding 2.5). A stimulation index over 2.5 was also

recorded in three control animals. They suggested that the γ interferon test is not suitable for the detection of cattle vaccinated with RB51, either at the individual or at the herd level.

- Antognoli *et al*. (2011) conducted studies to assess the diagnostic accuracy of the gamma interferon assay for detection of bovine tuberculosis in U.S. They estimated the test sensitivity using a population of 87 confirmed infected cattle from 14 herds and the specificity was estimated using a population of 4123 cattle representing 3000 premises in 3 states. The results showed that the sensitivity of the G-IFN for a cut-off value ≥0.1 was 83.9%. The specificity of the G-IFN was 90.7%, 97% and 98.6% for cut off values of 0.1, 0.3, and 0.5, respectively. Basing on the findings they indicated that the G-IFN performs with high accuracy in the field, yielding sensitivity and specificity.

## • 2.7 Comparison of Serological Tests:

- Heck *et al.* (1981) studied the serological profile of a cow that was experimentally infected with *Brucella abortus* and compared the conventional serologic tests like Card, Rivanol, SAT and CFT with ELISA. They observed that ELISA was capable of identifying *B.abortus* infected cows when conventional serologic methods were negative.

- Mathias Pinto *et al.* (1983) conducted comparative studies of sera from 212 unvaccinated buffaloes against brucellosis and found that CFT was able to reveal significant titers in sera. From 109 sera which did not show agglutination titers in the PAT, four showed complement fixing titers greater than 1 in 200. All the positive sera to the RBPT gave complement fixing titer equal to or greater than 1 in 20. In 131 sera that showed negative result to the RBPT the CFT was able to reveal titers greater than 1 in 60.
- Mahajan *et al.* (1991) made comparative study of the standard tube agglutination test (SAT), Rose Bengal plate agglutination test and counter immuno-electrophoresis (CIEP) on 647 sera from naturally aborting ewes, orchitic, in-contact and apparently healthy sheep with no history of vaccination against brucellosis. They detected more reactors during the early stage of infection using SAT while CIEP performed better in later stages of infection.
- Shrivastava *et al.* (1991) compared dot-ELISA with conventional tests for serodiagnosis of brucellosis and observed that dot-ELISA agreed 87.85 percent with RBPT and SAT. The SAT and dot-ELISA titers were positively correlated ($r=0.3406$) and highly significant ($P<0.01$).
- Suresh *et al.* (1993) screened 743 sera for bovine brucellosis and found 47 positives with RPAT and 57 positives with RBPT indicating a higher sensitivity of RBPT over RPAT. They subjected 58 other suspected samples (by RPAT and RBPT) to SAT and found 17 of them positive.

- Hussain *et al.* (1994) observed 6.3 percent and 4.6 percent incidence of brucellosis in buffaloes using RBPT and SAT respectively with an agreement of 73.35 percent in positive results, while among cows 10.7 percent and 8 percent was noted in both tests with an agreement of 75 percent.
- Barbuddhe *et al.* (1994) compared RBPT and SAT for detection of brucellosis in cattle and found that 39 samples were positive in both the tests but one sample was positive in RBPT but negative in SAT.
- Jai sunder *et al*. (2005) studied the prevalence of bovine brucellosis in 311 cattle using RBPT, SAT and A-B ELISA. They suggested that RBPT and A-B ELISA were equally sensitive since the same percentage of positive sera was detected in both tests.
- Gomez *et al*. (2008) used Rose Bengal test, micro agglutination test, microtiter-adapted Coombs test, and immunocapture-agglutination test (Brucellacapt) for diagnosis of human brucellosis and found that all sera from patients with acute brucellosis were positive but the immunoglobulin G (IgG), IgM, and IgA commercial enzyme immunoassays (ELISAs) failed to show specific antibodies in 3 patients, 10 patients, and 1 patient, respectively. The sensitivity of ELISA was not higher than that of conventional tests.
- Sahin *et al.* (2008) examined 626 sera of cattle from 27 herds with history of abortion in Turkey using RBPT, SAT and ELISA and detected 221 (35.30%), 206 (32.92%) and 247 (39.45%) brucella positives respectively.

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- Shahaza *et al*. (2009) prepared and compared The Rose Bengal Plate Test (RBPT) antigens from *Brucella melitensis* local isolates (in-house RBPT) with RBPT antigen for Brucellosis in sheep and goats prepared by Veterinary Laboratory Agency, UK. Out of 856 sera analyzed by in-house RBPT, commercial RBPT and CFT, 30.84%, 26.40% and 31.65% were found to be *Brucella* positive, respectively. They concluded that in-house RBPT antigens could be prepared and used for epidemiological surveillance of *Caprine brucellosis* in Malaysia.
- Ghodasara *et al.* (2010) tested 180 serum samples (107 cows, 73 buffaloes); 106 from abortion ,R.O.P. and 74 from various reproductive disorders and found overall prevalence of brucellosis by RBPT, STAT and i-ELISA as 34 (32.07) 12 (11.32) 17 (16.04) in abortion ,R.O.P and 11 (14.86) 7 (9.46) 9 (12.16) 9 in various reproductive disorders cases. They found higher prevalence of brucellosis in cases of abortion and R.O.P while at lower level from various reproductive disorders\
- Hadi and Ali (2010) screened 182 sera from suspected patients having brucellosis by different serological tests: Rose Bengal agglutination test, *B.abortus* antigen agglutination test, *B.melitensis* antigen agglutination test as well as ELISA IgM, IgG and found that the Rose Bengal test was the most useful and more sensitive than other two tests: *B.abortus* antigen test and *B.melitensis* antigen test

- Sareyyüpoğlu *et al*. (2010) screened 100 serum samples by using Rose Bengal plate test, serum agglutination test, Micro-agglutination test and 2-mercaptoethanol-micro-agglutination tests and found that 3 (3%) 5 (5%) 5 (5%) 5 (5%) were positive with RBPT and SAT, MAT, 2-ME-MAT.they concluded that MAT was determined to be a fast, reliable and an economic test, which can be easily used in routine diagnostic laboratories for serological diagnosis.
- Mythili *et al*. (2011) developed a competitive ELISA and compared it with Rose Bengal Test which is 94.87% and 92.12% sensitive and specific respectively. The competitive ELISA described was a simple method for the routine screening of animal sera for detecting Brucella-specific antibodies.

## • 2.8 Brucella Vaccines:

- Brucellosis control and eradication requires serological tests and vaccines. Effective classical vaccines (S19 in cattle and Rev 1 in small ruminants), induce antibodies to the O-polysaccharide of the lipopolysaccharide which may be difficult to distinguish from those resulting from infection and may thus complicate diagnosis (Schurig *et al*.2002). Strain 45/20 is reportedly unstable and it is not presently used (Chukwu 1985). RB51 is increasingly used instead of S19 in some countries but it is rifampicin resistant and its effectiveness is controversial. Most studies in cattle reported that RB51 provides protection against moderate challenge (Martin *et al*., 2009), but less effective than S19 against severe challenges (Moriyon *et al*., 2004). RB51 is not useful in sheep and evidence in goats is preliminary and

contradictory (El Idrissi *et al.,* 2001). Strain-19 vaccine can be administered to cattle of any age as either one or two doses of $5x10^9$ viable organisms , given by conjunctival route instead of subcutaneous dose of $5\text{-}8x10^{10}$ viable organisms. This produces protection without a persistent antibody response and reduces the risk of abortion and excretion in milk when vaccinating adult animals (OIE, 2009).

- Ko and Splitter (2003) reported that immunization with live vaccines permits the organism to replicate within the host which provoke TH-1 type of responses and is more Th-1 effective.

## 2.8.1 Existing and Tested Brucellosis Vaccines:

### 2.8.1.1 Strain 19

- Alton (1978) isolated *B. abortus* Strain 19, one of the first vaccine strains from jersey cow in 1923 and was found virulent. The strain after maintaining at room temperature for one year or more was found attenuated in mice.
- Fensterbank and Plommet (1979) selected 22 Friesian heifers of 6.5 to 9 months old and vaccinated subcutaneously with $11.5 \times 10^{10}$ colony forming units (CFU) of *Brucella abortus* strain 19 and 22 others with $6.1 \times 10^9$ CFU by the conjunctival route. All of them were revaccinated six months later with $5.7 \times 10^9$ CFU by conjunctival route. During their first pregnancy challenged with $14.8 \times 10^6$ CFU *B. abortus* strain 544 by the conjunctival route. They found that best protection was afforded by the S + C (subcutaneous+ conjunctival) method.

- Schuurman (1983) conducted vaccination trials to know the feasibility of brucella S19 reduced dose vaccine in Zambia. A total of 250 sero-negative adult cows and 13 (young) heifers were vaccinated with a reduced dose (3 x $10^9$ CFU in S-phase) Strain 19 (S-I9) vaccine. Sera were collected at 1, 4, 6, 9, and 12 months after vaccination and all animals found positive in the RBPT were subjected to SAT to check the serological titer. Twelve months after vaccination all cattle were negative to the serological tests and he supported the use of reduced dose S19 under the local conditions found in Zambia.
- Davis *et al*. (1991) suggested that S19 vaccine was not suitable for pregnant bison (*Bison bison*) due to presence of Persistent antibody titers, vaccine induced abortions, and chronic S19 infections. 48 pregnant bisons were manually inoculated with strain 19 vaccine, 44 were ballistically inoculated with an absorbable hollow pellet containing lyophilized S19 and 46 were manually injected with buffered saline as non-vaccinated control. They observed 58% abortions in bison after 60 days PV. One cow aborted during her second post-vaccinal pregnancy and S19 was identified from the dam and fetus indicating that chronic S19 infection can occur in bison. Challenge tests were conducted during second trimester of pregnancy with 1 x $10^7$ cfu of *B.abortus* strain 2308 via bilateral conjunctival inoculation. Protection against abortion was 67% ($p \leq 0.0001$) for vaccinated bison compared to 4% non-vaccinated control. Protection against *B.abortus*

infection was determined to be 39% ($p \geq 0.001$) for vaccinates and 0% for non-vaccinated control.

- Stevens *et al.* (1995) conducted studies on immune responses and resistance to infection with *Brucella abortus* 2308 (S2308) in mice following vaccination with *B. abortus* 19 (S19) or the lipopolysaccharide (LPS) O-antigen-deficient mutant, strain RB51 (SRB51). Live bacteria persisted for 8 weeks in spleens of mice vaccinated with $5 \times 10^6$ or $5 \times 10^8$ CFU of SRB51 and showed increased resistance to infection with S2308 at 12, 16, and 20 weeks after vaccination, whereas bacteria persisted for 12 weeks in mice vaccinated with $5 \times 10^6$ CFU of S19 and showed less resistance. Mice vaccinated with S19 showed antibody to S2308 bacteria and S2308 smooth LPS at 4, 8, and 12 weeks after vaccination. Only mice vaccinated with the highest dose of SRB51 ($5 \times 10^8$ CFU) had lower and less persistent antibody responses to S2308 bacteria. They found that SRB51vaccinated mice had similar cell-mediated immune responses to S2308 but lower resistance to infection with S2308 compared with S19-vaccinated mice.
- Olsen *et al*. (1996) evaluated the serologic responses of cattle vaccinated as adults with strain RB51 that also had been vaccinated as calves with strain 19 ($n = 40$). They found significantly greater antibody titers ($P < 0.05$) against strain RB51 at 4 and 8 weeks post-vaccination in the dot blot assay than did animals ($n = 10$) not vaccinated with strain RB51. They suggested that use of strain RB51 in cattle vaccinated with strain 19 as calves will not

cause positive responses on confirmatory tests and will not impair brucellosis serologic surveillance efforts.

- Afzal *et al*. (2000) studied the immune response of buffalo calves and heifers to a full or half dose of *Brucella abortus* strain 19 vaccine. Buffalo calves developed high serum agglutination test (SAT) titers following full dose vaccination and the titers declined more rapidly in calves of six months of age than in calves of eleven to twelve months of age. Specific immunoglobulin G titers, as measured by 2-mercaptoethanol-treated serum agglutination, declined much earlier than SAT titers. Buffalo heifers vaccinated with a full or half dose developed high SAT titers and the rate of decline of titers in heifers was much slower than in calves. They suggested that vaccination with a half dose did not appear to offer any advantage in terms of disappearance of SAT titers.
- Geong and Robertson (2000) observed the protective role of S19 vaccine in Bali cattle and noticed that the vaccinated animals were less likely to have abortions and still births.
- Teresita and Cespedes (2000) reported that the smooth strains of Brucella, due to the presence of perosmines in the LPS structure, produce antibodies which interfere in routine serologic surveillance tests carried out for the diagnosis of brucellosis. On the other hand, the titer did not persist for more than 10 months when prophylactic immunization of cattle and buffaloes was carried out between 4 to 7 months of age.

- Cook *et al*. (2002) reported no significant difference in protection with reduced vaccination dose while the titer disappears earlier than full dose. 45 serologically negative female elk calves were selected and 16 were hand-vaccinated with 1 x $10^9$ colony forming units (CFU) of RB51, 16 were vaccinated with 1 x $10^8$ CFU RB51 by biobullet and 13 were given a saline placebo. The elk were bred and they were challenged with 1 x $10^7$ CFU of B. abortus strain 2308 by intra-conjunctival inoculation. Thirteen (100%) control elk aborted, 14 (88%) hand-vaccinated elk aborted, and 12 (75%) biobullet vaccinated elk aborted or produced nonviable calves.
- Bhattacharjee *et al.* (2006) compared protective efficacy of subcutaneous versus intranasal immunization of mice with a *Brucella melitensis* lipopolysaccharide subunit vaccine. Groups of mice were immunized either subcutaneously or intranasally with purified *Brucella melitensis* lipopolysaccharide (LPS) or with LPS as a noncovalent complex with *Neisseria meningitidis* group B outer membrane protein (LPS-GBOMP). Mice were challenged intranasally with virulent *B. melitensis* strain 16M 4 weeks after the second dose of vaccine. They observed no significant difference in clearance of bacteria from the lungs of immunized mice and control mice and revealed that intranasal immunization with either vaccine provided significant protection against disseminated infection of the spleen and liver only but subcutaneous immunization of mice with the vaccines conferred significant protection against infection of the spleen, liver, and lungs also.

- Fiorentino *et al.* (2008) conducted immune-efficacy trials by using M1-luc and Strain I2. M1-luc is a mutant strain derived from S19 vaccine strain in which most of bp26 sequence has been replaced by the luciferase coding gene. Strain I2 is a double mutant derived from M1-luc in which most of omp19 has been deleted without introduction of any genetic markers. They selected four groups of 15 heifers, 6-month old and vaccinated with S19, M1-luc or I2. One group served as control. After conception, heifers were challenged at six months gestation with virulent *B. abortus* S2308. Protection levels against abortion after challenge were 78.6% for S19, 81.8% for M1-luc and 45.5% for I2, compared to the 25% in the non-vaccinated group. They stated that in bovines BP26 had no influence in protective capacity of S19, correlating with the results obtained in mice.
- Aldomy *et al*. (2009) studied the effect of route of administration in 3 groups of Shami goats using Brucevac (Rev. 1). Group 1 was vaccinated subcutaneously with a full dose ($1.54 \times 10^9$ organisms). Group 2 was vaccinated conjunctively with one eye drop ($5.2 \times 10^8$ organisms), while Group 3 was injected subcutaneously with a reduced dose ($7.1 \times 10^5$ organisms) of vaccine. Blood samples were collected before vaccination, 2, 4, 8, 15 and 24 weeks post vaccination. All samples were tested with CFT, ELISA, SAT and Rose Bengal plate test. They found that the conjunctival route of vaccination significantly reduced the intensity and duration of the post vaccination serological response and made the use of this vaccine

compatible with brucellosis eradication programmes, even when these are based on a test-and–slaughter policy.

- Arenas-Gamboa *et al.* (2009) evaluated Strain 19 vaccine efficacy in a sustained release vehicle consisting of alginate microspheres containing live vaccine with a delivery system suitable for prevention and control of the brucellosis in wildlife. Red deer were orally or subcutaneously immunized with 1.5 x $10^{10}$ colony-forming units (CFUs) using microencapsulated S19. The vaccinated red deer and non vaccinated controls were challenged 1 yr post immunization conjunctivally with 1 x $10^{9}$ CFUs of *B. abortus* strain 2308. Red deer vaccinated with oral microencapsulated S19 had a statistically significant lower bacterial tissue load compared with controls. They suggested that protection against Brucella-challenge can be achieved by combining a commonly used vaccine with a novel oral delivery system.
- Cardina *et al.* (2009) performed clinical assay using two groups of 100 animals each to establish the efficacy of the *Brucella abortus* strain 19. One herd was identified as infected with four animal reactors to Card Test (CT) and confirmed by Rivanol Test, isolation and identification of *Brucella abortus* colonies. In 18 months, the number of infected animals increased to eight females, seven within non vaccinated group and one in the vaccinated group for serum reaction rate in the non-vaccinated group of 5.8% and the vaccinated one of 0.8%. They found that strain 19 vaccine is efficacious in the control of brucellosis in herds with a 3% prevalence of the disease; yet

before its use, interference of diagnosis problem that are produced should be assessed to properly evaluate economics and vaccination efficacy.

- Arenas-Gamboa *et al.* (2011) evaluated the immunogenicity, safety, and protective efficacy of live S19 Δ*vjbR*::Kan vaccine encapsulated in alginate microspheres containing a non immunogenic eggshell precursor protein of the parasite *Fasciola hepatica* (vitelline protein B). BALB/c mice were immunized intraperitoneally with either encapsulated or non encapsulated S19 Δ*vjbR*::Kan at a dose of $1x10^5$ CFU per animal. They observed that a single dose with the encapsulated mutant conferred higher levels of protection compared to the non encapsulated vaccine and suggested that S19 Δ*vjbR*::Kan is safer than S19, induces protection in mice, and should be considered as a vaccine candidate when administered in a sustained-release manner.
- El-Razik *et al.* (2011) evaluated the immunological response to RB51 using brucella periplasmic proteins as ELISA antigen. The samples collected were subjected to standard tube agglutination test (SAT) and ELISA. They injected S19 vaccine, RB51vaccine and sterile saline for the three groups respectively through subcutaneous route. Concerning the S19 vaccinated group, significant SAT titers were seen 1 week post vaccination (WPV) till the maximum at the 2nd WPV. After that it gradually decreased till the 7 WPV, then sharply before it completely disappeared at the 13 WPV. The LPS-ELISA showed an antibody titer as early as one WPV and reached its peak at 2 WPV and persisted steadily till the 6th WPV and decreased slowly

when it reached minimal level at the 16 WPV till the end of the experiment (20WPV). But the antibody titer began and reach the maximum as early as the first WPV, still steady till 2 WPV, fluctuating till the 6th WPV, then dropped sharply when it disappeared at 11WPV till the end of the experiment (20WPV) in RB51 vaccinated buffalo calves.

- Lafta (2011) used Anthrax crude protective antigen (PA) as an adjuvant with *Brucella abortus* S19 vaccine in a ratio of 1:1 to immunize mice at dose of 0.1 ml s/c in order to decrease side effects of S19 vaccine and also to enhance its immunogenicity. Three groups of mice were immunized with the mixture composed of anthrax PA + S19, S19 alone, and sterile PBS solution as a control. They observed that the group treated with the mixture was the best regarding skin test, the difference of thickness was 1.51± 0.103 mm, 1.14± 0.103 mm, 0.89± 0.100 mm after 24, 48 and 72 h and also revealed the highest antibody titer.

- **2.8.1.2 H38 Vaccine:**

- This is a formalin killed vaccine used for vaccination of goats and sheep. Because it is made of killed smooth *B. melitensis*, vaccinated animals develop O-chain antibodies that interfere with diagnosis. It confers variable protection and induces local reactions at the inoculation site (Schurig *et al.*, 2002).

- **2.8.1.3 B. *suis* strain 2 Vaccine:**

- This is an attenuated smooth strain derived from biovar 1of *B. suis* originally developed in China. It has been used with varying degrees of successes to vaccinate sheep and swine (Blasco *et al.,* 1993).
- Mustafa and Abusowa (1993) reported that although this vaccine induce the development of O chain antibodies that interfere with the serological diagnosis of the disease, these antibodies seem to decline and disappear by one year post vaccination.
- **2.8.1.4 Strain 45/20 Vaccine:**
- This R vaccine was obtained after twenty passages in guinea pigs of a field isolate of *B. abortus* strain 45 in 1938 and it has also been used in brucellosis control programs (Alton, 1978).
- Schurig *et al.* **(**2002) noticed that the vaccine has the disadvantage of serological interference and more importantly, when administered as a live vaccine it reverts to virulence *in-vivo.* Therefore it needs to be used as a bacterin, which is less effective.
- **2.8.1.5 Rev1 Vaccine:**
- This vaccine is a spontaneous mutant derived from *B. melitensis* (Schurig *et al.*, 2002). It confers protection against *B. melitensis* infection in goats and is widely used in the caprine industry in endemic areas (Al-Khalaf *et al.,* 1992).
- Blasco *et al.* **(**1987) stated that Rev1 induces protection in lambs against *B. ovis* challenge, but the vaccine is still virulent in this species as cases of post vaccinal epididymitis have been observed.

- Adone *et al.* (2005) assessed the protective properties as well as humoral and cellular immune responses induced by *B. melitensis* Rev1 and *B. abortus* RB51 vaccines. Basing on the results they stated that the rough mutants were able to induce a very good level of protection against *B. melitensis* infection, similar to that provided by Rev1 and superior to that of RB51, without inducing antibodies to O antigen. Also found that all *B. melitensis* mutants were able to stimulate good production of gamma interferon.

- **2.8.1.6 Vaccine strain RB51:**

- Strain RB51 is a spontaneous R mutant selected after repeated in vitro passages of *B. abortus* strain 2308 on the media containing sub-inhibitory concentrations of rifampicin (Schurig *et al.*, 1991).

- Vemulapalli *et al.* (2002) stated that RB51 strain possesses at least two mutations in its LPS biosynthetic pathway. One of the mutations they observed is the presence of an IS711 element in the *wboA* gene responsible for a glycosyltransferase involved in the synthesis of the O-chain.

- Stoffregen *et al*. (2007) suggested that feral swine may serve as a reservoir of infection for *Brucella abortus* as well as *Brucella suis* for domestic livestock. They collected samples from feral swine and Brucella was cultured from 62 (77.5%) animals. Males were significantly more likely to be culture positive than females (92.9% vs. 60.6%). Thirty-nine animals (48.8%) were seropositive. Males also had a significantly higher seropositivity rate than females (61.9% vs. 34.2%).

- Tittarelli *et al.* (2008) studied immune response to *Brucella abortus* Strain RB51 after calfhood vaccination in Italian cattle. The antibody levels increased after day 6 of post-vaccination in the 10 vaccinated animals, remained constant for two months, and then progressively decreased. All vaccinated animals remained negative from day 162 PV to the end of the study (day 300 PV). Only at days 13 and 14 PV the RB51-CFT showed 100% sensitivity. These results indicated that the possibility to use RB51-CFT for the identification of cattle vaccinated at calfhood with RB51 is limited in time.
- Arenas *et al.* (2010) studied the maturation process of Brucella- containing phagosomes in phagocytes, by comparatively monitoring the intracellular transport of a virulent strain (2308) with two vaccine strains (S19 and RB51) in J 774 macrophages. They also compared the behavior of all three strains studied through transmission electron microscopy. They suggested that the virulent strain not only occupies two different kinds of compartments but also alters the endocytic pathway of the cell it parasitizes, unlike what has been reported for non-professional phagocytes, like HeLa cell.
- Rashmi *et al.* (2012) evaluated safety and immunogenicity of *Brucella abortus* RB51 vaccine by iELISA using acetone killed strain RB51 antigen. The percentage responders at day 35, 60 and 90 post vaccination were 100%, 95% and 20% respectively. The post-vaccination time point analysis indicated that the cumulative IFN-γ response of whole blood from

vaccinates stimulated with heat killed RB51 antigen was elicited in 80% of calves at 60 days post vaccination. Absence of strain RB51 in the secretions and excretions and lack of local or systemic reaction indicated the safety of the vaccine.

- ## 2.8.2 Experimental Vaccines

- **2.8.2.1 Killed and subunit vaccines:**

- Vaccination attempts using heat killed or otherwise dead *Brucella* vaccines without strong adjuvants have been unsuccessful. Similarly, subunit vaccines have been unable to induce high levels of antigen expression and induce protection against virulent *Brucella* challenge (Schurig *et al.,* 2002).

- **2.8.2.2 Viral vector vaccines:**

- Vaccinia virus and the insect baculovirus have been used as vectors to express *Brucella* antigens (Bae *et al.,* 2002). Although proteins were expressed in these systems, protection against virulent challenge was not obtained.

- **2.8.2.3 *Ochrobactrum anthropi* vector vaccine:**

- This gram negative soil bacterium is the closest genetic relative of *Brucella* (Velasco *et al.,* 2000). It has been used to express *B. abortus* protective antigens. Vaccination of mice with *O. anthropi* expressing *Brucella* Cu/ZnSOD induces immune responses specific to *Brucella* SOD of a mixed Th1/Th2 profile, with high IFN-γ but also high IL-4 levels.

- He *et al.* **(**2002) showed that these vaccines were non-protective unless they were co-administered with the genetic adjuvant CpG which switched the cytokine profile to a Th1 type without IL-4 production.

- **2.8.2.4 DNA vaccines:**

- Velikovsky *et al.* (2002) demonstrated protection in a DNA vaccine system expressing *B. abortus* lumazine synthase gene. The L7/L12 protein gene has also been expressed as a DNA vaccine inducing protection against challenge (Kurar *et al*., 1997).

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- Onate *et al.* (2003) induced protection against challenge with virulent *Brucella* by vaccinating with a DNA vaccine expressing Cu/ZnSOD.

- **2.8.2.5 Recombinant strain RB51 vaccines:**

- Vemulapalli *et al.* **(**2000) compared strain RB51 and recombinant strain RB51 over expressing homologous *B. abortus* Cu/ZnSOD (approximately 10 times the normal level). Recombinant strain vaccine induced significantly increased protection against challenge with a virulent *B. abortus* strain in BALB/c mice.

- Mediavilla *et al.* (2003) suggested the epitope mapping of the *Brucella melitensis* BP26 immunogenic protein for diagnosis of sheep Brucellosis. Sequencing of immunogenic periplasmic protein bp26 gene was performed in the reference strains of *Brucella abortus*, *B. suis*, and *B. ovis*. The epitope mapping of BP26, performed by using a panel of monoclonal antibodies and recombinant DNA techniques, allowed the identification of

an immunodominant region of the protein for the diagnosis. They observed that the use of recombinant fusion protein instead of the entire recombinant BP26 improved the specific serological diagnosis of *B. melitensis* or *B. ovis* infection in sheep**.**

- Monreal *et al.* (2003) obtained *Brucella abortus* rough lipopolysaccharide (LPS) mutants by transposon insertion into two *wbk* genes (*wbkA* [putative glycosyltransferase; formerly *rfbU*] and *per* [perosamine synthetase]), into *manB* (*pmm* [phosphomannomutase; formerly *rfbK*]), and into an unassigned gene. The rough LPS of *manB* mutant lacked the outer core epitope and the gene was designated *manBcore* to distinguish it from the *wbk manBO-Ag*. The mutants *per* and *wbkA* induced protection but less than the standard smooth vaccine S19, suggesting that anti O-polysaccharide antibodies accounted largely for the difference. The core-defective mutant was no effective against *B. ovis*, S19 and RB51. They concluded that rough *Brucella* vaccines should carry a complete core for maximal effectiveness.
- Vemulapalli *et al.* **(**2004) stated that increased protection may be attributed to the higher IFN-γ levels found in splenocytes of mice vaccinated. Complementation of strain RB51 with a functional copy of the *wboA* gene fails to revert the strain to a smooth phenotype. The O-chain remains intracytoplasmic and is not transported to the outer membrane. Mice vaccinated with this construct developed O-chain antibodies and completely protected against infection with virulent strain 2308.

- Yang *et al.* (2006) hypothesized that the *znuA* gene present in *Brucella melitensis* 16 M would be an important factor for survival, normal growth and virulence of *Brucella abortus* under low Zn2+ concentrations. A *znuA* knockout mutation in *B. abortus* 2308 (Δ*znuA*) was constructed and found to be lethal in low-Zn2+ medium. When infected the macrophages, Δ*znuA B. abortus* showed minimal growth and also its virulence in BALB/c mice was attenuated. They confirmed the Δ*znuA* mutant as a potential live vaccine, since protection against wild-type *B. abortus* 2308 challenge was as effective.
- Cyntia *et al*. (2010) reported that the *Brucella abortus* Phosphoglycerate Kinase mutant is highly attenuated and induces protection superior to that of vaccine strain 19 in immunocompromised and immunocompetent mice. The *B. abortus* Δ*pgk* mutant strain exhibited extreme attenuation in bone marrow-derived macrophages and *in vivo* in BALB/c, C57BL/6, 129/Sv, and interferon regulatory factor-1 knockout (IRF-1 KO) mice. At 24 h post infection the Δ*pgk* mutant was not found within the same endoplasmic reticulum-derived compartment as the wild-type bacteria. Challenge experiments revealed that the Δ*pgk* mutant strain induced protective immunity in 129/Sv or IRF-1 KO mice that was superior to the protection conferred by commercial strain 19 or RB51. They concluded that *Brucella* PGK is critical for full bacterial virulence and that a Δ*pgk* mutant may serve as a potential vaccine candidate in future studies.

- Clapp *et al.* (2011) determined the protection levels of oral administration of Δ*znuA B. melitensis* against nasal *B. melitensis* challenge. A single oral vaccination with Δ*znuA B. melitensis* rapidly cleared from mice within 2 weeks and effectively protected mice upon nasal challenge with wild-type *B. melitensis* 16M. They suggested that oral immunization with the live, attenuated Δ*znuA B. melitensis* vaccine provides an attractive strategy to protect against inhalational infection with virulent *B. melitensis*.

# CHAPTER III

# MATERIALS AND METHODS

## 3.1. Study Design

This study was designed to assess the prophylactic efficacy of *Brucella abortus* S19 reduced dose vaccine through conjunctival route manufactured and supplied by Indian Immunologicals Ltd. Study animals were monitored and evaluated for 120 days after vaccination. The blood was collected from jugular vein for the estimation of *Brucella* antibodies. The study animals were also observed for any adverse reactions during the study. The serum obtained was analyzed for immune response using specific tests.

### 3.1.1 Number of Animals and Treatments:

A total of fifty seronegative female adult Holstein Friesian cows above 8 months of age irrespective of stage of lactation were selected enrolled for the trial. The grouping and treatments were as follows.

- **Group-1** consisted of 30 animals. A vaccination dose of 5 x $10^9$ to 8 x 1 $0^9$ CFU strain 19/ animal given by conjunctival route through dropper

- **Group-2** consisted of 10 animals. A vaccine dose of 40 x $10^9$ to 80 x $10^9$ CFU of strain 19/animal was given by S/C route.
- **Group-3** consisted of 10 animals. This group served as control/ placebo group and received only normal saline.

**3.2 Study Procedure:**

At base line (-10days, before enrollment) consent was taken from the farm owner. A venous blood sample 5. 0 ml was collected from 80 cows in plain vaccutainer tubes (Cat.No.367820, BD). Sera samples from the animals were screened for Brucellosis using Commercial ELISA KIT supplied by Veterinary Laboratory Agency

## CHAPTER-IV

# RESULTS

This study was designed to assess the prophylactic efficacy of *Brucella abortus* S19 reduced dose vaccine (Manufactured and supplied by Indian Immunologicals Ltd) through conjunctival route. This was compared with standard subcutaneous S19 vaccine. The animals enrolled belong to Deccan Agro Farms, Choutkur, Medak District. Fifty seronegative female adult Holstein Friesian cows aged above 8 months were selected and enrolled for the trial. Thirty animals were given a vaccine dose of 5 x $10^9$ to 8 x $10^9$ CFU/ dose by conjunctival route, 10 animals were given standard vaccine dose of 40 x $10^9$ to 80 x $10^9$ CFU/ dose by S/C route and remaining 10 animals served as control/ placebo group and received only normal saline. Blood samples were collected from all animals on 0, 21, 60, 90 and 120 days post-vaccination.

## 4.1 Safety

Local reactions due to vaccination such as pain, swelling, redness, and allergic reaction at injection/inoculation site and systemic reactions like anorexia, dullness,

depression, fever were not observed in the animals after vaccination during the entire period of trial.

## 4.2 Immunogenicity

The humoral immune response was determined by using In-House indirect ELISA, rose Bengal test and standard tube agglutination test. Cell mediated immunity was studied by interferon-*gamma* assay with indirect competitive ELISA at different time intervals.

## 4.2.1 Humoral Immunity

### 4.2.1.1 Rose Bengal Plate Test (RBPT):

RBPT is based on the agglutination of serum antibodies with stained whole cell preparation of killed *Brucella*. In the present study animals responded to RBPT only at 21 and 60 days PV. At 21$^{st}$ day of post vaccination only 6/30 (20%) animals responded to the test and on 60 day only 5/27 (18.5%) responded in reduced dose conjunctival vaccination group animals as shown in the table 2.The percentage of responders is 60% (6/10) and 50% (5/10) for the standard subcutaneous vaccine at 21 and 60 days of post vaccination respectively. No single animal responded twice for the reduced dose conjunctival vaccine but in the standard dose vaccine group four out of six (66.6%) responded twice.

The maximum number of vaccinated adult cattle developed antibodies detectable by RBPT at 21 day post vaccination. Thereafter most of the animals were negative and by day 90, antibodies detectable by RBPT declined below the analytical threshold of RBPT. None of the animals were positive, in any group, by RBPT at 90 and 120 days of

post vaccination. The control animals remained negative to RBPT throughout the study period.

**4.2.1.2. Standard Tube Agglutination Test (SAT)**:

Sera from all standard dose, reduced conjunctival dose vaccinated and non-vaccinated control cattle were collected periodically and subjected to SAT. Results are shown in the table 3. The standard tube agglutination test showed the percentage of responders as 26, 66, 57 and 32 in reduced conjunctival dose vaccine at 21, 60, 90 and 120 day PV respectively. The percentage of the positive reac

dose on 60$^{th}$ day. There was a drastic increase in the number of responders from day 21 to day 60 and it gradually decreased on the subsequent days.

The percentage of responders was 80, 80, 40 and 0 for standard dose vaccine at 21, 60, 90 and 120$^{th}$ day PV respectively. The percentage of the positive reactors was higher for standard dose on 21$^{st}$ and 60$^{th}$ days. On day 90 the responders decreased to 40% compared to 80% on day 60. However none of the animals was positive on day 120 PV in the standard dose vaccine.

One control animal seroconverted from day 60 PV because different groups were not segregated.

**4.2.1.3. i-ELISA**

The serum antibody response to *Brucella abortus* S19 reduced dose conjunctival vaccination and standard subcutaneous vaccination was determined by i-ELISA as described by Colby *et al*. (2002). Mean OD values obtained which are = or > mean ± 3SD of negative control sera (day zero/pre-vaccination sera samples from saline

inoculated calves) were considered as positive responders. The titers above the cut-off 0.4241 were considered positive and responsive to immunization. Results are presented in table 4 & 5.

For the reduced conjunctival vaccine only two animals responded at 21$^{st}$ day post vaccination. By 60$^{th}$ day, seroconversion was recorded in 10 of 27 (37%) animals. The highest seroconversion was recorded in 15 of 19 (78%) vaccinated animals at 90$^{th}$ day of post vaccination. The percentage of positives drastically decreased after 90$^{th}$ day PV and only one animal of 28 (3.6%) was positive on day 120 PV as shown in table 6.

For the standard subcutaneous vaccine the response of animals was higher than the reduced dose conjunctival vaccine. At 21$^{st}$ day of post vaccination seven of 10 (70%) animals were positive. Thereafter a moderate increase (80%) was observed by day 60 of post vaccination. Maximum seroconversion was recorded in 10 of 10 (100%) animals at day 90 and the percentage of positives was slightly decreased (80%) after 90$^{th}$ day.

None of the controls animals was positive.

The OD values of i-ELISA for humoral immune response were 0.146927, 0.305915, 0.476975 and 0.239909 on 21, 60, 90 and 120 days respectively in reduced dose compared to 0.69324, 0.677255, 0.96535 and 0.580695 standard dose. The OD values gradually increased by day 90 PV and decreased at day 120 PV in reduced conjunctival dose vaccine. The mean OD values were higher than cut off (0.4241) for reduced dose only at day 90 PV.

There was a great increase in the mean OD value from day 0 to day 21 PV in standard S/C dose vaccine. The OD value decreased at day 60 PV compared to day 21

PV. Again there was a great increase at day 90 PV and the subsequent mean OD values at day 120 were decreased. The mean OD values were higher than the cut off value on 21, 60, 90 and 120 days for standard dose.

### 4.2.2. Cell Mediated Immunity:

The cell mediated immunity was determined by Interferon-gamma assay with Indirect Competitive ELISA (ic-ELISA fig 1 & 2); a useful method to reveal the presence of immune response following lymphocyte stimulation with heat killed *B. abortus* S19 and 544. The interferon-gamma assay results were expressed as stimulation indices (SI), calculated as the ratio between the mean OD value of IFN-γ from blood cells stimulated

with specific antigen at 450nm and the mean optical densities value of IFN-γ from blood cells stimulated with PBS control as described by Weynants *et al*. (1995). The cut-off S.I value was derived from the mean S.I of plasma of saline inoculated controls at 21 and $60^{th}$ day ± 3 SD of this mean value. Two cut-off values 6.442 and 4.2834 were obtained when the blood samples were stimulated against *B. abortus* S19 and 544 respectively.

The IFN-γ response induced by S19 reduced dose conjunctival vaccine as determined by ic-ELISA titer indicated that by day 21 PV 50% of cattle responded to the strain 19 heat killed antigen whereas 66% of animals responded to the *B. abortus* 544 antigens used for stimulation of lymphocytes as shown in table 7.

The percentage of responders at day 21, 60, 90 and 120 days post vaccination were 50, 88, 63 and 53% respectively when whole blood samples were stimulated with S19 heat killed antigen whereas the percentage of responders were 66.6, 96, 66.6 and

53% respectively for the same time intervals when whole blood samples were stimulated with *B. abortus* 544 antigen. B. abortus 544 antigen may be a potent immune stimulator being wild, hence the higher levels observed.

There were 20 and 30% of positive responders found on day 0 for the standard dose subcutaneous vaccine when blood samples were stimulated with S19 heat killed antigen and *B. abortus* 544 respectively. At day 21 PV there was a slight increase in the percentage of responders for S19 antigen, but no change observed for the *B. abortus* 544 as shown in fig 3.

There was a sudden increase in the percentage of responders by the day 60 PV and highest percentage 84.6 and 96 was observed for both antigens. Thereafter there was a gradual decrease in the percentage of responders for the subsequent

no difference was found in the number of responders at days 90 and 120 PV between the two antigens (63% and 53%) as shown in fig 4.

There found a great surge for both the antigens at day 60 PV and highest percentage of responders 90% and 100% were recorded for S19 and *B. abortus* 544 respectively in standard dose group as shown in fig 5. Thereafter slight decrease at day 90 PV and gradually decreased by the day 120 PV for the standard dose vaccine.

However almost all the animals responded for the reduced dose conjunctival vaccine. There were 21.43% of animals that responded once, 42.86% of animals responded twice, 28.57% of animals responded thrice and 7.14% of animals responded four times during the whole trial study for four collection intervals.

The control cattle remained unresponsive throughout the trial period for both the antigens.

# CHAPTER V

# DISCUSSIONS

Brucellosis is an infectious and contagious bacterial disease of animals and humans caused by *Brucella abortus* (OIE 2004). It is very wide-spread infection, in the developed and developing world, transmissible from animals to humans. In India it accounts for a loss of about 30 million man days per year (Handa *et al*., 1998). The organism is sensitive to many antibiotics but due to its intracellular sequestration in lymph-nodes, mammary glands and reproductive organs, treatment is not useful and is too expensive in most of the animal species.

Control and eradication of brucellosis includes measures like immunization of female cattle, surveillance testing and removal of infected animals, depopulation and quarantine (Radostits *et al*., 2004). High prevalence rates of brucellosis, socio-economic conditions, inadequate veterinary services and methods of livestock farming in India suggest that the appropriate method for the control of this disease is immunization of the susceptible animals.

Two live, attenuated vaccines are used in some countries for the control of *B. abortus* infection worldwide in cattle: *B. abortus* strain 19 and *B. abortus* strain RB51. *B. abortus* S19 when given to adult cattle often causes persistent antibody titers leads to confusion in differentiating infected from vaccinated cattle. Use of S19 in pregnant cattle also results in abortions. *B. abortus* strain RB51 is the official vaccine strain in the United States. It does not produce any clinical signs post-vaccination and vaccinates will be seronegative on all subsequent serological tests. It is rapidly cleared from the bloodstream. It is not shed in the nasal secretions, saliva, or urine. But it is rifampicin

resistant and its effectiveness is controversial (Fosgate *et al.,* 2003). Controlled experiments in calves, have shown reduced doses of RB51 to be ineffective, full doses only partially effective. RB51 was also found to be less effective than S19 against severe challenges by some workers (Diptee *et al.,* (2005). Killed vaccines **(***B. abortus* strain 45/20), *Brucella* R mutant vaccines ***(****wboA* mutants other than RB51, mutants in the *wbk* region, *B. abortus* B2211 *pgm*) subunit vaccines and DNA vaccines were also tried but not been commercialized so far.

Brucella S19 vaccine can be administered to cattle of any age as either one or two doses of 5-8 x $10^9$ viable organisms, given by the conjunctival route. This produces protection without a persistent antibody response and reduces the risks of abortion and excretion in milk (OIE, 2004). Hence the present study was taken up to know the efficacy of reduced dose strain 19 vaccine by conjunctival route and compare it with standard subcutaneous vaccination method.

Various serological and molecular tests were used to diagnose the disease and also for assessment of the efficacy of different vaccines. Corbel *et al*. (1983) employed isolation and identification method for diagnosis of the disease and Davis *et al*. (1991) employed it for immunological tests. Hafez *et al*. (2011) employed molecular techniques like single step polymerase chain reaction (PCR) to diagnose brucellosis in sheep using whole blood.

Basavaraj *et al.* (2011) screened sera with Rose Bengal test where as Chapel *et al*. (1982) used the test for vaccine efficacy. Angus and Barton (1984) utilized buffered plate agglutination test for diagnosis of brucella and Mustafa *et al*. (2012) used it for

immune response studies. Other tests were also used. (Alton *et al*. 1988); Aldomy *et al*. 2009 and Nielsen *et al*., 1995).

In the present study female adult cattle above 8 months were divided into three groups, viz., Group-I, Group-II and Group-III which were given reduced dose vaccine through conjunctival instillation, standard dose vaccine through subcutaneous route and normal saline through subcutaneous route (control group) respectively. No local/systemic untoward reactions were observed throughout the study period. The humoral and cell mediated immune (CMI) responses were also studied by using i-ELISA and gamma interferon assay respectively. The i-ELISA results were compared with the Rose Bengal Plate Test and Standard Tube Agglutination Test.

**5.1 Humoral immune Responses in the Vaccinates:**

RBPT is based on the agglutination of serum antibodies with stained whole cell preparation of killed *Brucella*. In the present study, animals of reduced dose group responded only at 21 (20%) and 60 days (18.51%) post vaccination for conjunctival reduced dose vaccine. There was no response in Rose Bengal Plate Agglutination test in the later periods. Similar results were reported by Plommet and Plommet (1976) and Fensterbank and Plommet (1979). The low percentage of reactors reported by RBPT to the conjunctival vaccination, have been attributed to weak response as detected by serological tests (Fensterbank *et al.,* 1982). Secondly, the conjunctival route of vaccination significantly reduced the intensity and duration of post vaccination serological response and found compatible with brucellosis eradication programmes (Jimenez de Bagues *et al*., 1989; Diaz-Aparicio *et al*., 1994; Marin *et al*., 1999; Aldomy *et al*., 2009).

Sera from all standard dose vaccinated, reduced conjunctival dose vaccinated and non-vaccinated control cattle were collected in different time intervals and subjected to SAT. The standard tube agglutination test showed the percentage of responders as 26, 66, 57 and 32, in reduced conjunctival dose vaccine group at 21, 60, 90 and 120$^{th}$ day PV respectively while the percentages for standard dose group was 80, 80, 40 and 0 respectively. The percentage of the positive reactors high for reduced dose on 60$^{th}$ day compare to other days and later declined gradually. These results agreed with the vaccination results of Poester *et al.* (2006). The test also showed high titers and these titers were present throughout the experiment. These results agree with vaccination responders in a study Afzal *et al*. (2000).

For the reduced conjunctival vaccine group the percentage of responders by iELISA was 6.6, 37, 78.9 and 3.6 at 21, 60, 90 and 120 days PV respectively. These results exhibited higher antibody titers at day 60 and 90 post-vaccination and revealed downward trend at 120 day PV. Similar reports were reported by Weynants *et al*. (1995). Standard dose vaccine showed 70, 80, 100 and 80% of positive responders on 21, 60, 90 and 120 days PV and then revealed lower trend at 120 days PV. However Poester *et al*. (2010) reported opposite results. The different findings of Poester *et al*. (2006) may be because of the dose of vaccine and strain of bacteria used in the vaccine.

Overall RBPT was found to be the least effective test for studying humoral immune response, maximum responders of 20% and found to be least sensitive among the humoral immunity tests, since the test could detect responders only up to 60 day PV. SAT was found to better than RBPT as higher percentage of responders was detected and also for longer period. However the reduced dose group showed the persistent

antibody response, since it detected antibodies even on the day 120 PV, while there was no response on 120 day PV in standard dose group. I-ELISA was found to be more sensitive as it detected 100% of responders among standard dose group even on 120 days PV. It can be seen that humoral immune response tended to decrease after 90 days of vaccination.

**5.2 Cell Mediated Immunity:**

The cell mediated immunity was determined by Interferon-gamma (IFN-γ) assay with Indirect Competitive ELISA (IC-ELISA). The IFN-γ response induced by S19 reduced dose conjunctival vaccine as determined by ic-ELISA titer indicated that at days 21, 60, 90 and 120 PV percentage responders were 50, 88, 63 and 53 respectively with the strain 19 heat killed antigen. The highest number of responders was observed at day 60 pv (88%). These results are in agreement with the results reported by Rashmi *et al*. (2012). If the criterion for a positive CMI response is multiple, consecutive positive test results as reported previously (Weynants *et al*., 1995), then the results of our observation that 21% vaccinates tested positive at least once and that 43% vaccinates tested positive at least twice 29% vaccinates tested positive at least thrice from four sampling intervals indicates of CMI response to S19 exposure. IFN-γ response tended to be more stable with IFN-γ presence up to the end of the trial on 120 day PV.

**5.3 Humoral VS CMI Responses in brucella vaccinates:**

Humoral immune response responses tended to become weak after 60-90 days depending on the test employed. However CMI responses as revealed by IFN- γ assay was made consistent and present till the end of the trial. IFN-γ assay was previously

reported to be a more reliable test for studying immune responses to brucella (Weynants *et al*., 1995).

**Conclusions:**

Reduced dose conjunctival vaccine elicited lower immune response compared to standard dose vaccine. iELISA was found to be the most sensitive test to study humoral immune response. Humoral immune responses declined after 90 days PV. CMI responses as studied by IFN-γ assay were present throughout the study period. Reduced dose conjunctival proved to be less effective than standard dose vaccine. INF-γ assay may be a better test for studying immune response to brucella as previously suggested by Weynants *et al*. (1995).

# CHAPTER VI

# SUMMARY

The present study was taken up with a view to study the comparative effects of brucella reduced dose vaccine with standard dose vaccine. Female cattle above eight months age irrespective of the breed, stage of lactation and pregnancy were selected. These animals were made into 3 groups; group-1 consisted of 30 animals, group-2 consisted of 10 animals and group-3 (control) consisted of 10 animals. Further group-1 was given a reduced conjunctival vaccine dose of $5x10^9$ to $8x10^9$ CFU/ animal, group-2 was given standard subcutaneous dose of 40 $x10^9$ to $80x10^9$ CFU/ animal and saline for control group.

They were observed for local and/or systemic reactions after vaccinations and during follow-up period. Blood samples were collected on $0^{th}$, $21^{st}$, $60^{th}$, $90^{th}$ and $120^{th}$ days post-vaccination (PV). The humoral immune response, cell mediated immune responses to *Brucella abortus* S-19 reduced dose vaccine and standard dose vaccines were studied at different time intervals using i-ELISA and *gamma*-interferon assay (ic-ELISA) respectively. The i-ELISA was compared with Rose Bengal Plate Test (RBPT) and Standard Tube Agglutination Test (SAT).

Humoral immune response to RBPT was very low. Only 20% and 18.51% of animals for reduced dose vaccine, 60% and 50% of animals for standard dose vaccine responded at 21 and 60 days of PV respectively. None of the animals were positive in both the groups by RBPT at 90 and 120 days of PV. The standard tube agglutination test (SAT) showed high percentage of responders: 66 and 80 at 60 day PV for conjunctival dose vaccine and standard dose vaccine respectively. SAT was found to better than RBPT as

higher percentage of responders was detected and also for longer period. However the reduced dose group showed the persistent antibody response, since
it detected antibodies even on the day 120 PV. While there was no response on 120 day PV in standard dose group.

For the reduced conjunctival vaccine only two animals responded at 21$^{st}$ day post vaccination, highest seroconversion was recorded 78% of vaccinated animals at day 90 PV. For the standard subcutaneous vaccine the response of animals was higher than the reduced dose conjunctival vaccine. Maximum seroconversion was recorded in 10 of 10 (100%) animals at day 90 PV. i-ELISA was found to be more sensitive as it detected 80% of responders among standard dose group even on 120 days PV.

Cell mediated immunity (CMI) is an essential component of host defense and particularly important for intracellular pathogens like Brucella. The CMI responses are mediated by various sub populations of T lymphocytes. Lymphocyte proliferation assays are the most widely correlates of CMI. CMI was determined by Interferon-gamma assay with Indirect Competitive ELISA. The highest number of responders was observed in reduced dose conjunctival vaccine group at day 60 PV (88% against S19 antigen and 96% against *B. abortus* 544). A total of 21% vaccinates tested positive at least once and 43% vaccinates tested positive twice and 29% vaccinates tested positive at least thrice from four sampling intervals indicating sufficient stimulation of CMI.

There was a sudden increase in the percentage of responders by the day 60 PV and highest percentage 88 and 96 was observed for both antigens. Thereafter there was a gradual decrease in the percentage of responders for the subsequent periods. However

no difference was found in the number of responders at days 90 and 120 PV between the two antigens (63% and 53%) for reduced conjunctival dose vaccine.

It can be concluded that reduced dose conjunctival vaccine was less effective but studies on larger group of animals and for longer period may be necessary before drawing definite conclusions. Among the humoral tests i-ELISA was found to be most sensitive and do not suffer from subjective interpretations. CMI responses measured by IFN-γ assay were more consistent and may be better indicator of immune response to brucella vaccines/infection.

# LITERATURE CITED


Adone R, Ciuchini F, Pistoia C, Piccininno G 2000 Use of the γ-interferon test to assess cell-mediated immunity in *Brucella* infected cattle. Selezione Veterinaria 2000 supplement pp.225-232.

Adone R, Ciuchini F, Marianelli C, Tarantino M, Pistoia C, Marcon G, Petrucci P, Francia M, Riccardi G, and Pasquali P 2005 Protective Properties of Rifampin-Resistant Rough Mutants of *Brucella melitensis.* Infection and Immunity 73 (7): 4198–4204.

Afzal M, Mirza M A and Jahangir M 2000 Immune response of buffaloes to vaccination with *Brucella abortus* strain 19. Revue Scientifique et Technique. Office International des Epizooties 19(3): 867-870.

Agasthya A S, Isloor S and Prabhudas K 2007 Brucellosis in high risk group individuals. Indian Journal of Medical Microbiology 25 (1): 28-31.

Akhtar Raheela, Zafar Iqbal Chaudhry, Shakoori A R, Mansoor ud Din Ahmad and Asim Aslam 2010 Comparative efficacy of conventional diagnostic methods and evaluation of polymerase chain reaction for the diagnosis of bovine brucellosis. Veterinary World 3(2): 53-56.

Aldomy F, Alkhawaldeh M and Younis B 2009 Immune Responses of Goats (Shami breed) to Vaccination with a full, Reduced and Conjunctival Dose of Brucevac (*Brucella melitensis* rev.1) Vaccine. Pakistan Veterinary Journal 29(4):149-153.

Al-Khalaf S A, Mohamad B T, and Nicoletti P 1992 Control of brucellosis in Kuwait by vaccination of cattle, sheep and goats with *Brucella abortus* strain 19 or *Brucella melitensis* strain Rev. 1. Tropical Animal and Health Production 24 (1): 45-9.

Alton G G 1978 Recent developments in vaccination against bovine brucellosis. Australian Veterinary Journal 54 (12): 551-557.

Alton G G, Corner L A, Plackett P 1980 Vaccination of pregnant cows with low doses of Brucella *abortus* strain 19 vaccine. Australian Veterinary Journal 56 (8): 369-72.

Alton G, Jones L M, Angus R D and Verger J M 1988 Techniques for the Brucellosis Laboratory INRA, Paris.

Angus R D and Barton C E 1984 The production and evaluation of a buffered plate antigen for use in a presumptive test for brucellosis. Dev Biol Stand 56: 349-356.

Antognoli M C, Remmenga M D, Bengtson S D, Clark H J, Orloski K A, Gustafson L L and Scott A E 2011 Analysis of the diagnostic accuracy of the gamma interferon assay for detection of bovine tuberculosis in U.S. herds. Preventive Veterinary Medicine 101(1-2): 35-41

Anuradha P and Ganesan P I 2006 Use of RBT and C-ELISA in the diagnosis of brucellosis in cattle. Indian Veterinary Journal 83:999-1000.

Arenas N Graciela, Diego J Grilli, Luis E Samartino, Javier Magadán and Luis S Mayorga 2010 *Brucella* alters endocytic pathway in J774 macrophages. Virulence 1 (5): 376-385.

Arenas-Gamboa A M, Rice-Ficht M, Kahl-McDonagh M and Ficht T A 2011 Protective Efficacy and Safety of *Brucella melitensis* 16MΔmucR against Intraperitoneal and Aerosol Challenge in BALB/c Mice. Infection and Immunity 79 (9): 3653–3658.

Arenas-Gamboa Angela M, Thomas A Ficht, Donald S Davis, Philip H Elzer, Melissa Kahl- McDonagh, Alfredo Wong-Gonzalez and Allison C Rice-Ficht 2009 Oral Vaccination with Microencapsuled Strain 19 Vaccine Confers Enhanced Protection against *Brucella abortus* Strain 2308 Challenge in Red Deer (*Cervus elaphus elaphus*). Journal of Wildlife Diseases 45(4): 1021–1029.

Bae J E, Schurig G G, Toth T E 2002 Mice immune responses to *Brucella abortus* heat shock proteins: Use of baculovirus recombinant-expressing whole insect cells, purified *Brucella abortus* recombinant proteins and a vaccinia virus recombinant as immunogens. Veterinary Microbiology 88 (2): 189-202.

Barbuddhe S B, Yadava V K and Singh D K 1994 Comparison of Dot-ELISA with conventional serological tests for diagnosing ovine brucellosis. Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases 15 (1/2): 1-5.

Basavaraj Metri C, Baragundi Mahesh C, Jyothi P, Lava R, Basavarajappa, Hanumanthappa A R, Chandrappa N R 2011 Seroprevalence Of Brucellosis In Davangere Karnataka. Journal of Clinical and Diagnostic Research 5(1): 41-44

Bhattacharjee K Apurba, Mina J Izadjoo, Wendell D Zollinger, Mikeljon P Nikolich and David L Hoover 2006 Comparison of Protective Efficacy of Subcutaneous versus Intranasal Immunization of Mice with a *Brucella melitensis* Lipopolysaccharide Subunit Vaccine. Infection and Immunity 74 (10): 5820–5825.

Billman-Jacobe H, Carrigan M, Cockram F, Corner L A, Gill I J, Hill J F, Jessep T, Milner A R, Wood P R 1992 A comparison of the interferon gamma assay with the absorbed ELISA for the diagnosis of Johne's disease in cattle. Australian Veterinary Journal 69 (2): 25-8.

Blasco J M, Marin C M, Barberan M, Moriyon I and Diaz R 1987 Immunization with *Brucella melitensis* Rev 1 against *Brucella ovis* infection of rams. Veterinary Microbiology 14 (4): 381-92.

Blasco J M, Marin C, Jimenez de Bagues M P and Barberan M 1993 Efficacy of *Brucella suis* strain 2 vaccine against Brucella ovis in rams. Vaccine 11 (13): 1291-1294.

Bossi P, Tegnell A, Baka A, Van F Loock, Hendriks J, Werner A, Maidhof H, Gouvras G 2004 Bichat Guidelines for The Clinical Management of Brucellosis and Bioterrorism related Brucellosis. Eurosurveillance 9(12): (1-5).

Cardina Peniche.A, Martinez Herrera D, Franco Zamora J L, Barradas Pina F, Molina Sanchez B, Gutierrez Ruiz E J, Williams J J, Morales Alvarez F, and Flores Castro R 2009 Evaluation of Vaccination with *Brucella abortus* S19 Vaccine in Cattle Naturally Infected with Brucellosis in Productive Systems Found in the Mexican Tropic. International Journal of Dairy Science 4 (4): 142-151.

Chappel R J, Hayes J, Rogerson B A and Shenfield J 1982 The serological response of cattle to vaccines against brucellosis, as measured by the brucellosis radioimmunoassay and other tests. Journal of Hygiene Cambridge 88: 11-19

Charanjeet, Mandeep Sharma, Katoch R C, Dhar P and Kumar R 2004 Application of RBPT, SAT and Avidin-Biotin serum ELISA for detecting brucellosis among livestock in Himachal Pradesh. Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases 25(1): 15-18.

Cho Donghee, Hyangmi Nam, Jongwan Kim, Eunjeong Heo, Yunsang Cho, Inyeong Hwang, Jaemyung Kim, Jongman Kim, Sukchan Jung, Simon More 2010 Quantitative Rose Bengal Test for diagnosis of bovine brucellosis. Journal of Immunoassay and Immunochemistry 31 (2) 120-130 (2010).

Chukwu CC 1985 The instability of *Brucella abortus* strain 45/20 and a note on significance of using an unstable rough strain in the diagnosis of bovine brucellosis. International Journal of Zoonosis 12 (2): 120-125.

Clapp Beata, Jerod Skyberg A, Xinghong Yang, Theresa Thornburg, Nancy Walters and David Pascual W 2011 Protective Live Oral Brucellosis Vaccines Stimulate Th1 and Th17 Cell Responses. Infection and Immunity 79 (10): 4165–4174.

Colby L A, Schurig G G & Elzer P H 2002 An indirect ELISA to detect the serologic response of Elk (*Cervus Elaphus Nelsoni*) inoculated with *Brucella abortus* strain RB51. Journal of Wildlife Diseases 38: 752.

Cook W E, Williams E S, Thorne E T, Kreeger T J, Stout G, Bardsley K, Edwards H, Schurig G, Colby L A, Enright F and Elzer P H 2002 *Brucella abortus* strain RB51 vaccination in elk. I Efficacy of reduced dosage. Journal of Wildlife Diseases 38(1): 18–26.

Corbel MJ, Cockrem DS and Brewer RA 1983 Differentiation of smooth and rough Brucella strains by lectins. Veterinary Record 113: 261-262

Corbel M J 1997 Brucellosis: an overview. Emerging Infectious Diseases 3:213-21.

Corner L A and Alton G G 1975 Persistence of *Brucella abortus* strain 19 infection in adult cattle vaccinated with reduced doses. Australian Veterinary Journal 51 (2): 57-63.

Cresswell P 2000 Intracellular surveillance: controlling the assembly of MHC class I-peptide complexes. Traffic **1:**301-305.

Cyntia Trant G M C, Thais Lacerda L S, Natalia Carvalho B, Vasco Azevedo, Gracia Rosinha M S , Suzana Salcedo P, Jean-Pierre Gorvel and Sergio Oliveira C 2010 The *Brucella abortus* Phosphoglycerate Kinase Mutant Is Highly Attenuated and Induces Protection Superior to That of Vaccine Strain 19 in Immunocompromised and Immunocompetent Mice. Infection and Immunity 78(5): 2283–2291.

Das V M, Paranjape V L and Corbel M J 1990 Investigation of brucellosis-associated abortion in dairy buffaloes and cows in Bombay. Indian Journal of Animal Sciences 60(10): 1193-1194.

Davis S Donald, Joe Templeton W, Thomas Ficht A, Jan Huber D, Dale Angus R and Garry Adams L 1991 *Brucella abortus* in Bison II Evaluation of Strain 19 vaccination of pregnant cows. Journal of Wildlife Diseases 27(2): 258-264.

Dhand N K, S Gumber, B B Singh, Aradhana, M S Bal, H Kumar, D R Sharma, J Singh & K S Sandhu 2005 A study on the epidemiology of brucellosis in Punjab (India) using Survey Toolbox. Reveu Scientifique *et* Technique. Office International Des Epizooties 24 (3): 879-885.

Diaz-Aparcio E, Marin C, Alanso- Urmeneta B, Aragon V, Perez-Ortiz S, Pardo M, Blasco J M , Diaz R and Moriyon I 1994 Evaluation of serological tests for diagnosis of Brucella melitensis infection of goats. J Clin Microbiol 32(5): 1159-1165.

Diptee M D, Adesiyun A A, Asgarali Z, Canpbell M and Fosgate G T 2005 Evaluation of cell mediated immune response and bacterial clearance in 6-10 months old water buffaloes (*bubalus bubalis*) experimentally vaccinated with four dosages of commercial *Brucella abortus* St. RB-51 vaccine. Veterinary Immunology and Immune Pathology 106: 209-220.

Durand B, Garin B B, Dufour B, Moutou F, Cotton T, Broardelle M and Damour M 1992 National investigation on the ring test used on bulk milk for bovine brucellosis screening (June 1991-November 1991). Epidemiologie et Sante Animale 22; 83-102.

El Idrissi A H, Benkirane A, el Maadoudi M, Bouslikhane M, , Berrada J, Zerouali A 2001 Comparison of the efficacy of *Brucella abortus* strain RB51 and *Brucella melitensis* Rev. 1 live vaccines against experimental infection with *Brucella melitensis* in pregnant ewes. Rev Sci Tech 20(3): 741-7.

El-Razik Abd Khaled A, Salama Abd El-Hafez M, Waleed Shell S and Yasser Ghazi A 2011 The immunological response of RB51 vaccinated buffalo calves using brucella periplasmic proteins as ELISA antigen. African Journal of Biotechnology 10(51): 10562- 10566.

Ewalt Darla R and Betsy Bricker J 2000 Validation of the Abbreviated *Brucella* AMOS PCR as a Rapid Screening Method for Differentiation of *Brucella abortus* Field Strain Isolates and the Vaccine Strains 19 and RB51. Journal of Clinical Microbiology 38(8): 3085– 3086.

Eze E N 1997 The fate of *Brucella abortus* and associated antibodies in traditionally soured milk. Bulletin of Animal Health and Production in Africa 25(1): 5-8.

Eze Michael O, Liang Yuan, Robert Crawford M, Chrysanthi M, Paranavitana, Ted Hadfield L, Apurba Bhattacharjee K, Richard Warren L and David Hoover L 2000 Effects of Opsonization and Gamma Interferon on Growth of *Brucella melitensis* 16M in Mouse Peritoneal Macrophages In Vitro. Infection and Immunity 68 (1): 257–263.

Fensterbank R and Plommet M 1979 Vaccination Against Bovine Brucellosis with a Low Dose of Strain 19 Administered by The Conjunctival Route IV Comparison between Two Methods of Vaccination. Ann Rech Vet 10 (1): 131-139.

Fensterbank R, Pardon P and Marly Jeevamitra 1982 Comparison between subcutaneous and conjunctival route of vaccination with Rev.1 strain against Brucella melitensis infection in ewes. Ann. Rech. Vet. 13:295–301.

Ficht T A 2002 Discovery of Brucella virulence mechanisms using mutational analysis. Veterinary Microbiology 90:311-5.

Fiorentino M A, Campos E, Cravero S, Arese A, Paolicchi F, Campero C, Rossetti O 2008 Protection levels in vaccinated heifers with experimental vaccines *Brucella abortus* M1- luc and INTA 2. Veterinary Microbiology 132: 302–311.

Fosgate G T, Adesiyun A A, Hird D W, Johnson W O, Hietala S K, Schurig G G, Ryan J, Diptee M D 2003 Evaluation of brucellosis RB51 vaccine for domestic water buffalo (*Bubalus bubalis*) in Trinidad. Preventive Veterinary Medicine 58(3-4): 211-225.

Geong M and Robertson I D 2000 Response of Bali Cattle (*Bos Javanicus*) to vaccination with *Brucella abortus* strain 19 in West Timor. Preventive Veterinary Medicine 47(3): 177- 186.

Ghodasara S N, Ashish Roy and Bhanderi B B 2010 Comparison of Rose Bengal Plate Agglutination, Standard tube agglutination and Indirect ELISA tests for detection of *Brucella* antibodies in Cows and Buffaloes. Veterinary World 3(2): 61-64.

Gomez Concepcion M, Jose Nieto A, Carmen Rosa, Paloma Geijo, Angeles Escribano M, Amparo Munoz, and Clara Lopez 2008 Evaluation of Seven Tests for Diagnosis of Human Brucellosis in an Area Where the Disease Is Endemic. Clinical and Vaccine Immunology 15 (6): 1031–1033.

Gonzalez T J and Samartino L E 1997 Efficacy of the milk ring test in the control and eradication of bovine brucellosis. Veterinaria Argentina 14(137): 449-459.

González Llamazares O R, Gutiérrez Martín C B, Alvarez Nistal D, de la Puente Redondo V A, Domínguez Rodríguez L, Rodríguez Ferri E F 1999 Field evaluation of the single intradermal cervical tuberculin test and the interferon-gamma assay for detection and eradication of bovine tuberculosis in Spain. Veterinary Microbiology 70 (1-2): 55-66.

Hadi Mohammad Ali H, Ali Mohammad Ameen H 2010 Evaluation of Different Serologic Laboratory Tests Used For Diagnosis of *Brucella* Antibodies among Patients in Azadi Teaching Hospital in Kirkuk City. Journal of Kirkuk University Scientific Studies 5:53- 60.

Hafez Abdel Salama M, Khaled Abd El-Razik A, Hany Hassan M, and Ibrahim Gad 2011 Comparative Diagnosis of Ovine Brucellosis Using Single Step Blood-PCR with Old and New Serological Tools. African Journal of Microbiology Research 5(23): 3976-3980.

Handa R, Singh S, Singh N, Wali JP 1998 Brucellosis in north India: results of a prospective study. Journal of Communicable Diseases 30(2): 85-87.

He Y, Vemulapalli R and Schurig G G 2002 Recombinant *Ochrobactrum anthropi* expressing *Brucella abortus* Cu Zn superoxide dismutase protects mice against *B. abortus* infection only after switching of immune responses to Th1 type. Infection and Immunity 70(5): 2535-43.

He Y, Vemulapalli R, Zeytun A and Schurig G G 2001 Induction of specific cytotoxic lymphocytes in mice vaccinated with *Brucella abortus* RB51. Infection and Immunity 69**:**5502-8.

Heck F C, Williams J D, Zink D L, Gilmore W C and Adams L G 1981 Serologic profile for a cow experimentally infected with *Brucella abortus*. British Veterinary Journal 137(5): 520-526.

Hussain K A, Saleem A N and Fatoohi F A M 1994 Prevalence of brucellosis in buffaloes, cattle and sheep in Mosul region. Iraqi Journal of Veterinary Sciences 7(3): 233- 239.

Hussein Mohamed Zakaria, Saad-Eldin A Abou-Elnoeman, Aziza Abou–El elanin AlFikky, El– Agamy Ibrahim El-Samadouny, Abeer Adel Mohammed Shaaban 2006 Evaluation of Rose Bengal Test, Standard Tube Agglutination Test and Nested PCR for the diagnosis of Human Brucellosis. Egyptian Journal of Medical Microbiology 15 (2): 249-256.

Isloor S, Renukaradhya G J and Rajasekhar M 1998 A serological survey of bovine brucellosis in India. Revue Scientifique et Technique. Office International des Epizooties 17: 781-785.

Jai Sunder, Rai R B, Kundu A, Chatterjee R N, Senani S and Jeyakumar S 2005 Incidence and prevalence of livestock diseases of Andaman and Nicobar Islands. Indian Journal of Animal Sciences 75(9): 1041-1043.

Jimmanez de Bagues M P, Marin C M, Barberan M and Blasco J M 1989 Response of ewes to B. melitensis Rev1 vaccine administered by subcutaneous or conjunctival routes at different stages of pregnancy. Ann Rech Vet. 20: 205-213.

Kaur P, Sharma N S, Jand S K and Oberoi M S 2006 Isolation and identification of *Brucella abortus* from aborted cattle and buffaloes and evaluation of their antibiogram. Indian Journal of Animal Sciences 76(2): 105-108.

Kerkhofs P, Botton Y, Thiange P, Dekeyser P and Limet J N 1990 Diagnosis of bovine brucellosis by enzyme immunoassay of milk.Veterinary Microbiology 24: 73-80.

Kittelberger R, Reichel M P, Joyce M A, Staak C 1997 Serological cross reactivity between *Brucella abortus* and *Yersinia enterocolitica* 0:9. III. Specificity of the *in vitro* antigen-specific gamma interferon test for bovine brucellosis diagnosis in experimentally *Yersinia enterocolitica* 0:9-infected cattle. Veterinary Microbiology 16:57 (4): 361-71.

Ko J and Splitter G A 2003 Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans. Clinical Microbiology Reviews **16:**65-78.

Ko J, Gendron-Fitzpatrick A, Ficht T A and Splitter G A 2002 Virulence criteria for *Brucella abortus* strains as determined by interferon regulatory factor 1-deficient mice. Infection and Immunity **70:**7004-12.

Kumar R, Quader S A and Arunachalam T N 1974 Incidence of brucellosis in bulls and bullocks in Tamilnadu. Cheiron 3(2): 124-127.

Kurar E and Splitter G A 1997 Nucleic acid vaccination of *Brucella abortus* ribosomal L7/L12 gene elicits immune response. Vaccine 15 (17-18): 1851-57.

Lafta Inam Jasim 2011 Evaluation of Crude Anthrax Protective Antigen as an Adjutant for *Brucella abortus* Vaccine in Mice. Journal of Engineering and Technology 30(3): 500- 511.

Mahajan N K and Kulshreshtha R C 1991 Comparison of serological tests for *Brucella melitensis* infection in sheep. Tropical Animal Health and Production 23(1): 11-16.

Mahajan Sumit, Rajesh Agrawal and Nishi Pande 2011 A comparative evaluation of different diagnostic tests for brucellosis in buffaloes. Buffalo Bulletin 30 (1): 75-78.

Marin C, Moreno E, Moriyon I, Diaz R, Basco JM. 1999 Performance of competitive and indirect ELISAs, gel immunoprecipitation with native hapten polysaccharide and standard serological tests in diagnosis of sheep brucellosis. Clin Diagn Lab Immunol 6: 269–72.

Martin H, Garin-Bastuji B, Lima F, Flora L, Pina Fonseca A, and Boinas F 2009 Eradication of bovine brucellosis in the Azores, Portug-Outcome of a 5-year programme (2002–2007) based on test and slaughter and RB51 vaccination. Preventive Veterinary Medicine 90(1-2): 80-89.

Mathias L A and Pinto A A 1983 Comparative study among Complement Fixation, Serum Agglutination and Rose Bengal Plate Tests in the serodiagnosis of bovine brucellosis. Int J Zoonoses 10(1): 1-6

Mediavilla Patricia Seco, Jean-Michel Verger, Maggy Grayon, Axel Cloeckaert, Clara Marín M, Michel Zygmunt S, Luis Fernandez-Lago and Nieves Vizcaíno 2003 Epitope Mapping of the *Brucella melitensis* BP26 Immunogenic Protein: Usefulness for Diagnosis of Sheep Brucellosis. Clinical and Diagnostic Laboratory Immunology 10 (4): 647–651.

Miyashiro Simone, Eliana Scarcelli, Rosa Piatti M, Fabíola Campos R, Airton Vialta, Lara Borges Keid, Ricardo Augusto Dias, Margareth Genovez E 2007 Detection of *Brucella abortus* DNA in illegal cheese from São Paulo and Minas Gerais and differentiation of B19 vaccinal strain by means of the polymerase chain reaction (PCR). Brazilian Journal of Microbiology 38 (1): 17-22.

Monreal D, Grillo M J, Gonzalez D, C Marín M, De Miguel M J, Lopez-Goni I, Blasco J M, Cloeckaert A and Moriyon I 2003 Characterization of *Brucella abortus* O-Polysaccharide and Core Lipopolysaccharide Mutants and Demonstration that a Complete Core is Required for Rough Vaccines To Be Efficient against *Brucella abortus* and *Brucella ovis* in the Mouse Model. Infection and Immunity 71 (6): 3261–3271.

Moreno E, Pitt M W, Jones L M, Schurig G G and Berman D T 1979 Purification and characterization of smooth and rough lipopolysaccharides from *Brucella abortus*. Journal of Bacteriology 138:361-9.

Moriyón Ignacio, María Jesús Grilló Ignacio, Daniel Monreala, González David, Marín Clara, López-Goñi Ignacio, Raúl C. Mainar-Jaimec, Edgardo Moreno, José María Blasco 2004 Rough vaccines in animal brucellosis: Structural and genetic basis and present status. Veterinary Research 35(1): 1-38.

Mrunalini N and Ramasastry P 1999 Serological survey on the occurrence of brucellosis in domestic animals and man in Andhra Pradesh. Indian Veterinary Journal 76: 483-484.

Mudaliar S, Bhore A and Pandit 2003 Detection of antibodies to *Brucella abortus* in animal handlers. Indian Journal of Medical Sciences 57:181-6.

Mukherjee F, Jainendra Jain, Vipul Patel and Mrinalini Nair 2007 Multiple genus-specific markers in PCR assays improve the specificity and sensitivity of diagnosis of brucellosis in field animals. Journal of Medical Microbiology 56: 1309– 1316.

Murphy E A, Parent M, Sathiyaseelan J, Jiang X and Baldwin C L 2001 Immune control of *Brucella abortus* 2308 infections in BALB/c mice. FEMS Immunology and Medical Microbiology **32:**85-8.

Mustafa A A, Abusowa M 1993 Field-oriented trial of the Chinese *Brucella suis* strain 2 vaccine on sheep and goats in Libya. Veterinary Research 24 (5): 422–429.

Mustafa Al Hassan M, Mahmoud Abd Ellah R, Essam El Din M Elbauomy and Ali Sadik H 2012 Comarative Studies of different Serological Tests for Diagnosis of Brucellosis in Vaccinated Sheep and with Special reference to Competitive ELISA. Veterinary Research 5(2): 31-36

Mythili Tadepalli, Lingala Rajendra, Trangadia Bhavesh, Dorairajan Thiagarajan, Villuppanoor Alwar Srinivasan 2011 Development and Comparative Evaluation of a Competitive ELISA with Rose Bengal Test and a Commercial Indirect ELISA for Serological Diagnosis of Brucellosis. Indian Journal of Microbiology 51(4): 528–530.

Nicoletti P 2002 A short history of brucellosis Veterinary Microbiology 90:5-9.

Nielsen K H, Kelly L, Gall D, Nicoletti P and Kelly W 1995 Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis. Veterinary Immunology and Immunopathology 46(3-4): 285-291.

Odbileg Raadan, Byambaa Purevtseren, Dorj Gantsetseg, Bazartseren Boldbaatar, Tumurjav Buyannemekh, Zagd Galmandakh, Janchivdorj Erdenebaatar, Satoru Konnai, Misao Onuma, and Kazuhiko Ohashi 2007 Cytokine Responses in Camels (*Camelus bactrianus*) Vaccinated with *Brucella abortus* strain 19 Vaccine. Journal of Veterinary Medical Science 70 (2): 197–201

Ogata H, Su I, Miyake K, Nagai Y, Akashi S, Mecklenbrauker I, Rajewsky K, Kimoto M and Tarakhovsky A 2000 The toll-like receptor protein RP105 regulates lipopolysaccharide signaling in B cells. Journal of Experimental Medicine 192:23-9.

OIE 2004 Bovine brucellosis In: OIE manual of the diagnostic tests and vaccines for terrestrial animals, mammals, birds and bees. 5th edition Office International des Epizooties Paris 409-438.

OIE 2009 Bovine brucellosis In: OIE manual of the diagnostic tests and vaccines for terrestrial animals 6th editon Office International des Epizooties Paris.

Olsen S C, Evans D, Hennager S G, Cheville N F and Stevens M G 1996 Serologic responses of *Brucella abortus* strain 19 calfhood-vaccinated cattle following adult vaccination with strain RB51. Journal of Veterinary Diagnostic Investigation 8**:**451-4.

Olsen S C, Fach S J, Palmer M V, Sacco R E, Stoffregen W C and Waters W R 2006 Immune responses of elk to initial and booster vaccinations with *Brucella*

*abortus* strain RB51 or 19. Clinical and Vaccine Immunology 13(10): 1098-1103.

Olsen S C, A E Jensen, W C Stoffregen and M V Palmer 2003 Efficacy of calfhood vaccination with *Brucella abortus* strain RB51 in protecting bison against brucellosis. Research in Veterinary Science 74: 17–22.

Onate A A, Cespedes S, Cabrera A, Rivers R, Gonzalez A, Munoz C, Folch H and E Andrews 2003 A DNA vaccine encoding Cu Zn superoxide dismutase of *Brucella abortus* induces protective immunity in BALB/c mice. Infection and Immunity 71 (9): 4857-4861.

Plommet M, Plommet A M 1976 Vaccination against bovine brucellosis with a low dose of strain 19 administered by the conjunctival route. I. Protection demonstrated in guinea pigs. Ann Rech Vet.7 (1): 1-8.

Poester FP, Goncalves VS, Paixao TA, Santos RL, Olsen SC, Schurig GG, Lage AP 2006 Efficacy of Strain RB51 Vaccine in Heifers against Experimental Brucellosis. Vaccine. 24(25): 5327-5334.

Poester Fernando Padilla, Klaus Nielsen, Luis Ernesto Samartino and Wei Ling Yu 2010 Diagnosis of Brucellosis. The Open Veterinary Science Journal 4: 46-60

Pottumarthy S, Morris A J Harrison A C, Wells V C 1990 Evaluation of the tuberculin gamma interferon assay: potential to replace the Mantoux skin test. Journal of Clinical Microbiology 1999 37 (10): 3229-32.

Radostits O M, Gay C C, Blood D C and Hinchdiff K W 2004 Veterinary Medicine, A text book of diseases of cattle, sheep, pigs, goats and horses, 9th edition. W. B. Sanders Company Ltd. Paris, p. 881.

Rana UVS, Sehgal S, Bhardwaj M A 1985 Sero-epidemiological study of brucellosis among workers of veterinary hospitals and slaughter house of Union Territory of Delhi. International Zoonoses 12: 74-79.

Rashmi Singh, Sanjay Singh Basera, Kamal Tewari, Shweta Yadav, Sumith Joshi, Brajesh Singh, and Falguni Mukherjee 2012 Safety and Immunogenicity of *Brucella abortus* strain RB51 vaccine in cross bred cattle calves in India. Indian Journal of Experimental Biology 50: 239-242.

Renukaradhya G J, Isloor S, Crowther J R, Robinson M and Rajasekhar M 2001 Development and field validation of an avidin-biotin enzyme-linked immunosorbant assay kit for bovine brucellosis. Revue Scientifique et Technique. Office International des Epizooties 20 (3): 749-756.

Rojas X and Alonso O 1995 ELISAs for the diagnosis and epidemiology of *Brucella abortus* infection in cattle in Chile. Archivos de Medicina Veterinaria, 27: 45-50.

Ruiz-Mesa J D, Sanchez-Gonzalez J, Reguera J M, Martın L, Lopez-Palmero S and Colmenero J D 2005 Rose Bengal test: diagnostic yield and use for the rapid

diagnosis of human brucellosis in emergency departments in endemic areas. Clinical Microbiology and Infection 11: 221–225.

Sadasivam P R, Chitravel V, Arunachalam T N and Rahman A H 1996 Survey on brucellosis in non endemic areas of Tamil Nadu. Indian Journal of Animal Sciences 66(9): 889-890.

Sahin M, Genc O, Unver A and Otlu S 2008 Investigation of bovine brucellosis in the Northeastern Turkey. Tropical Animal Health and Production. 40(4): 281-286.

Smith L D and T A Ficht 1990 Pathogenesis of Brucella. Critical Reviews in Microbiology 17:209-30.

Saravi M A, Wright P F, Gregoret R J and Gall D E J 1995 Comparative performance of the enzyme-linked immunosorbent assay (ELISA) and conventional assays in the diagnosis of bovine brucellosis in Argentina. Veterinary Immunology and Immunopathology 47(1-2): 93-99.

Sareyyüpoğlu Baris, Zafer Cantekin and Kaan Müştak H 2010 Investigation of *Brucella* antibodies in bovine sera by Rose Bengal PlateTest (RBPT), Serum Agglutination Test (SAT),MicroagglutinationTtest (MAT) and 2-Mercaptoethanol- Microagglutination (2- ME-MAT) test. Ankara Üniversitesi Veteriner Fakultesi Dergisi, 57: 157-160.

Sarumathi C 2002 Epidemiological studies on brucellosis and infectious bovine rhinotrcheitis in various districts of Andhra Pradesh. Thesis submitted to Acharya NG Ranga Agricultural University.

Schurig G G, Sriranganathan N and Corbel M J 2002 Brucellosis vaccine: past, present and future. Veterinary Microbiology 90 (1-4): 479-496.

Schurig G G, Roop R M 2nd Bagchi T, Boyle S, Buhrman D and Sriranganathan N 1991 Biological properties of RB51; a stable rough strain of *Brucella abortus*. Veterinary Microbiology 28 (2): 171-188.

Schuurman H J 1983 The Serological Response of Adult Cattle to Vaccination with Reduced Dose *Brucella abortus* Strain 19 A trial Under Zambian Conditions. The Veterinary Quarterly 5 (2): 94-96.

Segura S, Gamazo C, Irache J M and Espuelas S 2007 Gamma Interferon Loaded onto Albumin Nanoparticles: In Vitro and In Vivo Activities against *Brucella abortus*. Antimicrobial Agents and Chemotherapy 51(4) 1310–1314.

Shahaza O, Khairani-Bejo S, Zunita Z and Bahaman A R 2009 In House Rose Bengal Plate Agglutination Test (RBPT) for a Rapid Diagnosis of Brucellosis in Goats in Malaysia. International Journal of Tropical Medicine 4 (3): 116-118.

Sharma J K and Saini S S 1995 Sero-prevalence of Brucellosis among farm animals of Punjab. Indian Veterinary Journal 72 : 881 – 882 .

Shrivastava P K, Tongaonkar S S, Mukherjee F and Rana S K 1991 A comparision of dot- enzyme-linked immunosorbent assay (dot-ELISA) with other conventional tests for the serodiagnosis of bovine brucellosis. Indian Journal of Animal Sciences 61(2): 123-125.

Silva Junior F F, Nozaki C N and Megid J 2004 Evaluation of the Ring Test in milk without previous cooling. Arquivos do Instituto Biologico (Sao Paulo) 71: suplemento RAIB 588-590.

Singh S V, Gupta V K and Singh N 2000 Control of ovine brucellosis in high performance sheep flock using multiple serological tests. Indian Journal of Animal Sciences 70(3): 231-234.

Singh G, Sharma D R and Dhand N K 2004 Seroprevalence of bovine brucellosis in Punjab Indian Veterinary Journal 81: 620 - 623.

Soman S and Kothari G 1954 Human brucellosis. Journal of Indian Medical Association 24: 44-48.

Stemshorn B W, Forbes L B, Eaglesome M D, Nielsen K H, Robertson F J and Samagh B S 1985 A comparison of standard serological tests for the diagnosis of bovine brucellosis in Canada. Canadian Journal of Comparative Medicine 49: 301-304.

Stevens M G and Olsen S C 1996 Antibody responses to Brucella abortus 2308 in cattle vaccinated with *Brucella abortus* RB51. Infection and Immunity 64:1030-4

Stevens Mark G, Steven Olsen C, George Pugh W, Jr and Dominique Brees 1995 Comparison of Immune Responses and Resistance to Brucellosis in Mice Vaccinated with *Brucella abortus* 19 or RB51. Infection and Immunity 63 (1): 264–270.

Stoffregen William C, Steven Olsen C, Jack Wheeler C, Betsy Bricker J, Mitchell Palmer V, Allen Jensen E, Shirley Halling M, David Alt P 2007 Diagnostic characterization of a feral swine herd enzootically infected with *Brucella*. Journal of Veterinary Diagnostic Investigation 19: 227–237.

Suresh S, Ramakrishna J, Saseendranath M R, Tresamol PV and Bhat MN 1993 Serosurvey of bovine brucellosis in Tamilnadu A recent study. Cheiron 22: 1-7.

Teresita A M and Cespedes N J 2000 Serological response and persistence of titer in vaccinated and revaccinated Argentine Friesian calves, with strain 19 of *Brucella abortus* between the ages of 3 to 10 months. Veterinaria Argentina 17(170): 743-751.

Thakur S D and Thapliyal D C 2004 Seroprevalence of animal and human brucellosis in Kumaon and adjoining parts of Uttar Pradesh with comparison of serological tests. Indian Journal of Animal Sciences 74(9): 932-935.

Tittarelli Manuela, Barbara Bonfini, Fabrizio De Massis, Armando Giovannini, Mauro Di Ventura, Donatella Nannini and Vincenzo Caporale 2008 *Brucella abortus* Strain RB51 Vaccine: Immune Response after Calfhood Vaccination and Field Investigation in Italian Cattle Population. Clinical and Developmental Immunology Volume 2008, Article ID 584624: 1-6.

Tittarelli Manuela, Fabrizio De Massis, Barbara Bonfini, Mauro Di Ventura and Massimo Scacchia 2009 An ELISA for the Evaluation of gamma interferon production in cattle vaccinated with *Brucella abortus* strain RB51. Veterinaria Italiana 45 (2): 355-361.

Uzal F A, Carrasco A E, Nielsen K, Echaidec S and Cabrera R F 1996 An indirect ELISA using a monoclonal anti IgG1 enzyme conjugates for the diagnosis of bovine brucellosis. Veterinary Microbiology 52 (1-2): 175-180.

Velasco J, Bengoechea J A, Brandenburg K, Lindner B, Seydel U, Gonzalez D, Zahringer U , Moreno E and Moriyon I 2000 *Brucella abortus* and its closest phylogenetic relative, Ochrobactrum spp., differ in outer membrane permeability and cationic peptide resistance. Infection and Immunity 68 (6): 3210-3218.

Velikovsky C A, Cassataro J, Giambartolomei G H, Goldbaum F A, Estein S, Bowden R A, Bruno L, Fossati C A and Spitz M 2002 A DNA vaccine encoding lumazine synthase from *Brucella abortus* induces protective immunity in BALB/c mice. Infection and Immunity 70 (5): 2507-2511.

Vemulapalli R, He Y, Sriranganathan N, Boyle S M and Schurig G G 2002 *Brucella abortus* RB51: enhancing vaccine efficacy and developing multivalent vaccines. Veterinary Microbiology 90 (1-4): 521-32.

Vemulapalli R, Contreras A, Sanakkayala N, Sriranganathan N, Boyle S M, and Schurig G G 2004 Enhanced efficacy of recombinant *Brucella abortus* RB51 vaccines against *B. melitensis* infection in mice. Veterinary Microbiology 102: 237-245.

Vemulapalli R, He Y, Buccolo L S, Boyle S M, Sriranganathan N and Schurig G G 2000 Complementation of *Brucella abortus* RB51 with a functional wboA gene results in O- antigen synthesis and enhanced vaccine efficacy but no change in rough phenotype and attenuation. Infection and Immunity 68 (7): 3927-32.

Weiner M, Iwaniak W, Szulowski K (Pulawy PL) 2012 Comparison of AMOS, MLVA and Bruce-Ladder assays for typing of *Brucella* spp. 22nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 31-03-2012.

Weynants Vincent, Jacques Godfroid, Bernard Limbourg, Claude Saegerman, and Jean-Jacques Letesson 1995 Specific Bovine Brucellosis Diagnosis Based on In Vitro Antigen-Specific Gamma Interferon Production. Journal Of Clinical Microbiology 33(3) 706–712

Wickramasuriya U G J S, Peiris G S, Kendaragama K M T and Karunadasa W M 1983 A survey on the incidence of bovine brucellosis in three districts of Sri Lanka. Sri Lanka Veterinary Journal 31(1/2): 27-31.

Wood P R, Corner L A, Rothel J S, Baldock C, Jones S L, Cousins D B, Mc Cormick B S, Francis B R, Creeper J, Tweddle N E 1991 Field comparison of the interferon- gamma assay and the intradermal tuberculin test for the diagnosis of bovine tuberculosis. Australian Veterinary Journal 68 (9): 286-90.

Yang Xinghong, Todd Becker, Nancy Walters, and David W. Pascual 2006 Deletion of *znuA* Virulence Factor Attenuates *Brucella abortus* and Confers Protection against Wild-Type Challenge. Infection and Immunity 74 (7): 3874–3879.

Yingst S and Hoover D L 2003 T cell immunity to brucellosis. Critical Reviews in Microbiology 29:313-31.