EVALUATION OF NOVEL MODIFICATIONS TO THE ROSE BENGAL PLATE TEST FOR DIAGNOSIS OF BOVINE BRUCELLOSIS

Thesis

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MASTER OF VETERINARY SCIENCE

in

VETERINARY MICROBIOLOGY
(Minor Subject: Veterinary Pathology)

By

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CERTIFICATE – I

This is to certify that the thesis entitled, **"Evaluation of Novel Modifications to the Rose Bengal Plate Test for Diagnosis of Bovine Brucellosis"** submitted for the degree of **M. V. Sc.** in the subject of **Veterinary Microbiology** (Minor Subject: Veterinary Pathology) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Shubhada Krishna Chothe (L-2010-V-26-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE - II

This is to certify that the thesis entitled, **"Evaluation of Novel Modifications to the Rose Bengal Plate Test for Diagnosis of Bovine Brucellosis"** submitted by **Shubhada Krishna Chothe (L-2010-V-26-M)**, to the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in partial fulfillment of the requirements for the degree of M. V. Sc. in the subject of **Veterinary Microbiology** (Minor Subject: Veterinary Pathology) has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an external examiner.

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ABSTRACT

Brucellosis remains a major emerging zoonosis. In order to control and eradicate brucellosis from humans and livestock it is essential to establish an accurate serological method for diagnosis of the disease. A modified Rose Bengal Plate Test (mRBPT) has recently been developed in the Department of Veterinary Microbiology, GADVASU to reduce the false negative and false positive results of the RBPT. The innovative modifications are aimed at differentiation of non – specific aggregates of antigen particles from true agglutinates and for the enhancement of the clump size. In the present study, evaluation of novel modifications to the conventional RBPT (cRBPT) was carried out to compare its efficacy with other diagnostic tests like the conventional RBPT, STAT, ELISA, CFT and PCR. The sensitivity of mRBPT was found to be the highest of all the tests conducted i.e. 95.88%. It showed a specificity of 89.32% which was higher than that of cRBPT and STAT but less than that of ELISA and CFT. Positive predictive value (PPV) of this test was calculated as 89.42% which was higher than that of cRBPT and STAT but less than iELISA and CFT. The negative predictive value (NPV) of mRBPT was highest of all the tests conducted i.e. 95.83%. The mRBPT enabled an improved visual identification of agglutination reaction by clearly differentiating the clumps into two components, blue, representing the antibody and pink, representing the antigen. False negative results were less in mRBPT when compared to other tests. mRBPT showed highest agreement with cRBPT with a kappa value of 0.850. Agreement between mRBPT and other tests was found to be substantial. The advantages that mRBPT provides, such as higher sensitivity and NPV, better visualization and ease of carrying of the test suggest that this test can serve as a better pen side diagnostic test for Brucellosis when compared with cRBPT and STAT.

Keywords: Brucellosis, RBPT, mRBPT, Sensitivity, Specificity

Signature of Major Advisor **Signature of the Student**

LIST OF ABBREVIATIONS

%	:	Percent
≤	:	Less-than or equal to
°C	:	Degree Celsius
µg	:	Microgram(s)
µl	:	Microlitre(s)
bp	:	Base Pair
CFT	:	Complement Fixation Test
CO_2	:	Carbon Dioxide
cRBPT	:	Conventional Rose Bengal Plate Test
DNA	:	Deoxyribonucleic acid
DW	:	Distilled Water
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme-linked immunosorbent assay
et al	:	Et alibi (and others)
g	:	Gram(s)
GADVASU	:	Guru Angad Dev Veterinary and Animal Sciences University
Fig.	:	Figure
h	:	Hour(s)
HCl	:	Hydrochloric Acid
IgG	:	Immunoglobulin G
i.m.	:	Intra muscular
in vitro	:	Outside the living body and in an artificial environment

in vivo	:	Inside the living body of an animal
KCl	:	Potassium Chloride
Kg	:	Kilogram(s)
L	:	Litre(s)
M	:	Molar
mg	:	Milligram(s)
$MgCl_2$	:	Magnesium Chloride
min	:	Minute(s)
ml	:	Milliliter(s)
mRBPT	:	Modified Rose Bengal Plate Test
nm	:	Nano meter
No.	:	Number
NPV	:	Negative Predictive Value
OIE	:	Office International des Epizootes
PCR	:	Polymerase Chain Reaction
PMN's	:	Polymorphonuclear cells
PPV	:	Positive Predictive Value
RBPT	:	Rose Bengal Plate Test
sec	:	Second(s)
spp	:	Species
STAT	:	Standard Tube Agglutination Test
TBE	:	Tris Borate EDTA
UV	:	Ultra Violet

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CHAPTER-I

INTRODUCTION

Brucellosis is an important zoonotic disease that causes huge economic losses to the dairy farmer and is of public health significance. It is a chronic infectious disease of livestock, rodents, marine animals and human beings caused by the small, non-motile, non-spore forming, gram negative facultative intracellular coccobacilli of genus *Brucella.* It is an important cause of reproductive losses in animals. Abortions, placentitis, epididymitis and orchitis are the most common consequences of the infection.

The genus *Brucella* is currently known to contain nine species classified on the basis of their host specificity; seven of them that affect the terrestrial animals are: *B. abortus* (cattle), *B. melitensis* (goats), *B. suis* (hogs), *B. ovis* (sheep), *B. canis* (dogs), *B. neotomae* (wood rat) (Verger *et al* 1987) *and B. microti* (voles) (Scholz *et al* 2008) and two that affect marine mammals are: *B. ceti* (cetaceans) *and B. pinnipedialis* (seals) (Cloeckaert *et al* 2001 and Foster *et al* 2007). *B. melitensis, B. suis,* and *B. abortus* are apparently the most virulent and cause human disease in the majority of cases. *B. canis* has occasionally caused disease in humans. *B. ceti and B. pinnipedialis* are also known to cause infection in human beings (Ewalt *et al* 1994).

Brucellosis is worldwide in distribution and is more common in countries with poor animal and public health programs (Capasso, 2002). Though it has been eradicated from many developed countries like Europe, Australia, Canada, Israel, Japan and New Zealand (Geering *et al* 1995), it remains an uncontrolled problem in regions of high

endemicity such as Africa, the Mediterranean, Middle East, parts of Asia and Latin America (Refai 2002). It is endemic in India and is prevalent in all states of the country including Punjab (Aulakh *et al* 2008).

Brucellosis is an important disease of public health significance. Humans are often infected due to direct animal contact or ingestion of contaminated dairy products, causing acute febrile illness – undulant fever – which may progress to a more chronic form and can produce serious complications affecting the musculo-skeletal, cardiovascular and central nervous systems (OIE, 2009). Human brucellosis is usually manifested as an acute or subacute febrile illness which may persist and progress to a chronic form (Mantur *et al* 2007). It is a well characterized occupational disease in shepherds, abattoir workers, veterinarians, dairy industry professionals and laboratory personnel (Agasthya *et al* 2007).

Outbreaks of bovine brucellosis are associated with abortion during the last trimester of gestation, and produce weak newborn calves and infertility in cows and bulls (Enright *et al* 1984). The outcome of infection in cattle is dependent on age, reproductive and immunological status, natural resistance, route of infection, infectious challenge and virulence of the infective strain (Nicoletti, 1980). Death may occur as a result of acute metritis followed by retained fetal membranes (Radostits *et al* 2000).

The clinical diagnosis is complicated by variable incubation period and absence of apparent clinical signs, except abortion. While isolation and culture of *Brucella* organisms is regarded as the gold standard test for laboratory diagnosis of brucellosis, its

sensitivity is low because the *Brucellae* are fastidious micro-organisms that can easily be overgrown by other contaminating bacteria. More importantly, the procedure is associated with high risk of infection to laboratory personnel (Alton *et al* 1988). Therefore, serological tests are often relied upon for the diagnosis of brucellosis.

The conventional Rose Bengal Plate Test (cRBPT) is often used as a rapid screening test (Ruiz-Mesa *et al,* 2005) and considered as a reliable test in the diagnosis of brucellosis (Oomen and Waghela, 1974). The sensitivity of RBPT is reported to be very high (>99%) but the specificity can be disappointingly low (Barroso-Garcia *et al* 2002 and Kiel *et al* 1987). As a result, the positive predictive value of the test is low and a positive test result thus requires confirmation by a more specific test (Smits and Kadri, 2005).

The RBPT is a very sensitive test. However, it could sometimes give a false positive result because of S19 vaccination or of false positive serological reactions (OIE, 2009). Also, gram negative bacteria such as *Yersinia enterocolitica*, *Vibrio cholera, Campylobacter fetus, Bordetella bronchiseptica* and *Salmonella* spp. may cross react with smooth *Brucella* spp. (Corbel and Brinley Morgan, 1984). Suitable modifications of the conventional RBPT are, therefore, required to get results with a better sensitivity and a higher specificity. A novel modified RBPT has been developed for the first time in the Department of Veterinary Microbiology, GADVASU to enhance the sensitivity of RBPT and minimize false positive and false negative results.

The present study was undertaken to evaluate the novel modifications in the conventional RBPT, keeping in view the following objectives:

1. To compare the efficacy of the modified RBPT with conventional serodiagnostic tests like RBPT, STAT and ELISA.

2. To compare the efficacy of the modified RBPT with the non-conventional assays like CFT, PCR etc.

3. Evaluation of the modified RBPT on false positive and false negative serum samples and determination of its sensitivity and specificity.

CHAPTER-II

REVIEW OF LITERATURE

2.1 The genus *Brucella*

A British Army Physician, Martson, first described the disease as the "Mediterranean Gastric Fever". The causative agent was isolated in 1887 by Sir Bruce from the spleen of fatally infected soldiers in Malta and placed it in the genus *Micrococcus.* After ten years, M L Hughes coined the term "undulant fever" for the disease. During the same time B Bang, a veterinarian, identified the organism from the fetuses and placenta of aborted cows and called it the "Bacillus of abortion". In the year 1917, A C Evans recognized that Bang's organism was identical to that described by Bruce as an etiological agent of human brucellosis.

2.1.1 General properties of the genus *Brucella*

The genus *Brucella* consists of small, nonmotile, Gram-negative and facultatively intracellular bacteria that cause disease in a broad range of animal hosts. The organisms are coccobacilli, 0.5-0.7 mm wide and 0.5-1.5 mm in length. They can occur as single cells, in pairs or in short chains. *In vivo* they often occur within the cytoplasm of cells in close-packed clusters (Corbel and Brinley-Morgan 1984). Flagella, endospore and capsule are absent although capsule like structure has been reported in preparations treated with antiserum. They are aerobic and do not grow under strictly anaerobic conditions but many strains, especially of the species *B. abortus* and *B. ovis*, are carboxyphilic and require supplementary CO_2 for growth.

Metabolism is oxidative and energy is produced by utilization of various amino acids and carbohydrate substrates. For many strains *i*-erythritol is a preferred energy source. Most strains require complex media containing multiple amino acids, thiamine, biotin, nicotinamide and pantothenic acid for growth, especially on primary isolation (Corbel and Brinley-Morgan, 1984). Citrate cannot serve as the sole carbon source. Acid is not produced from glucose. Litmus milk either remains unchanged or is rendered alkaline.

Brucellae are urease positive, capable of reducing nitrate to nitrite (except *B. ovis* and some *B canis*). Optimum temperature is 37°C. Optimum pH is 6.6-7.4. Catalase positive and usually oxidase positive, but negative strains occur. Chemoorganotropic, most strains require complex media, containing several amino acids, thiamin, nicotinamide, and magnesium ions; some strains may be induced to grow on minimal media containing an ammonium salt as the sole nitrogen source. Growth is improved by serum or blood, but hemin (X-factor) and nicotinamide adenine dinucleotide (NAD: V factor) are not essential. Acid production does not occur from carbohydrates in conventional media, except for *B. neotamae*. It is methyl red negative, does not produce indole, and does not liquefy gelatin or inspissated serum. *Brucella* species do not lyse erythrocytes and do not produce acetyl methyl carbinol (Voges-Proskauer test) (Corbel and Brinley-Morgan 1984).

Growth occurs in the temperature range of 20-40°C, but the optimum temperature is 37°C. All the strains lose viability at 56°C, however temperatures over 85°C may be required to ensure complete killing of *Brucella* organisms. The optimum

pH for growth is between pH 6.6 and 7.4; growth usually results in alkalization of the medium. *Brucella* strains are fairly resistant to drying and can survive in biological material for long periods, especially at low temperature. They are sensitive to a wide variety of disinfectants including formaldehyde, hypochlorite, iodophors and phenols provided that excess organic matter is not present. The organisms are killed by heat under pasteurization conditions.

Brucellae are partially acid fast as they are not decolourized by 0.5% acetic acid in the modified Ziehl-Nelson (MZN) staining (Alton *et al* 1988). The production of H_2S from sulphur containing amino acids varies between species and biovars and is of value in differentiating them. *Brucella abortus* is capable of producing H_2S, except *Brucella abortus* biovar 5 and 6 (European commission report 2001). Proteolytic activity is slight; urease activity is consistently high in *B. suis* and *B. canis* but variable in other species, being weak or absent in the case of *B. ovis*. They are oxidase and catalase positive and capable of reducing nitrate to nitrite, except *B. ovis* and some *B. canis*.

It grows best on tripticase soy-based media or other enriched media with a typical doubling time of two hours in liquid culture. Although bacteremia can be detected within one week by using automated culture systems (Yagupsky, 2004), cultures should be maintained for at least 4 weeks with weekly subculture for diagnostic purposes. On serum-dextrose agar (SDA), smooth colonies appear translucent, raised, convex, with an entire edge, and have a smooth shiny surface (Corbel and Brinley-Morgan, 1984). On primary isolation using SDA, *Brucella* colonies are rarely seen before 48 hours. At 48 hours, colonies are approximately 0.5-1.0 mm in diameter. Under transmitted light,

colonies appear pale honey coloured. Colony variants can be classified under four morphological categories: smooth, rough, smooth-rough intermediate, and mucoid. This classification is based on characteristics of the bacterium's lipopolysaccharide (LPS). At the molecular level, smooth organisms have LPS molecules containing a polysaccharide O-side chain made from a homopolymer of perosamine on their surface, while rough organisms lack this chain on their LPS (Caroff *et al*, 1984). Smooth and intermediate colonies are indistinguishable macroscopically. Rough colonies are usually less translucent than smooth variants. They have a dull, granular surface and appear matte white, yellowish white/buff, or brown in colour. Mucoid colonies are similer to rough colonies except that they have a sticky glutinous texture.

In terms of antibiotic susceptibility, nearly all strains of *Brucella* are susceptible *in vitro* to gentamicin, tetracycline (and derivatives), and rifampicin. Additionally, many strains are also susceptible to ampicillin, chloramphenicol, erythromycin, kanamycin, novobiocin, spectinomycin, streptomycin and trimethoprim. Susceptibility to antibiotics can differ among species, biovars and even strains. These differences can aid in the identification of specific *Brucella* strains (Vemulapalli, 2000).

2.2 Brucellosis

Brucellosis is a contagious disease of animals and characterized by abortion in females, orchitis and infection of accessory sex glands in males and infertility in both. It is a disease of the sexually mature animals with predilection for placenta, fetal fluids and testes of male animals. It has zoonotic importance in terms of its transmissibility to

human beings. Manifestation of the disease is different for different hosts. In animals it causes abortion usually in the last trimester of pregnancy (Corbel 1997). Characteristically, all *Brucella* species establish persistent infection in the reticuloendothelial system of the natural host species. In ungulates, the organism shows a marked tropism for the placenta of pregnant animals probably due to the presence of the compound erythritol (Smith *et al* 1961, Williams *et al* 1962 and Keppie *et al* 1965). Erythritol enhances the growth of the bacteria and in many cases, fetuses abort because of endotoxic shock and/or fetal death caused by increased numbers of bacteria and increased concentrations of endotoxin. Most infected cows abort only once although the placenta will be heavily infected at subsequent apparently normal calvings (Morgan 1969). Other clinical signs due to localization of organism in different tissues are hygroma, arthritis, metritis, sub-clinical mastitis etc. The brucellae localize in the supra-mammary lymph nodes and mammary glands of 80% of the infected animals and thus continue to secrete the pathogen in milk throughout their lives (Hamdy and Amin 2002).

In humans the most frequent symptoms are fever, chills or shaking rigors, malaise, generalized aches and pains all over the body, joint and lower back pain, headaches, anorexia, tiredness and general weakness (Corbel 2006). A very few cases of spontaneous abortions from infection with *Brucella* have been described. If untreated, it can cause serious sequel such as arthritis and neurological disorders. Man almost always receives the infection from infected animals; transmission from man to man rarely occurs (Corbel 2006). Therefore most of the research on Brucellosis is focused at the control and eradication of the disease in the animal host.

2.3 Mode of transmission

The most common route of infection in cattle is the gastrointestinal tract (Crawford *et al* 1990) from where infection spreads to local lymph nodes where *Brucella* replicates intracellularly in phagocytes (Anderson *et al,* 1986). Invasion of lymphatic vessels is followed by bacteremia leading to systemic infection. Transmission also occurs through contaminated milk as *B. abortus* induces multifocal interstitial mastitis with interstitial accumulation of macrophages and intra-acinar infiltration of neutrophils (Emminger and Schalm 1943), associated with predominantly intracellular organisms. The most important sources of infection are aborted fetuses, fetal membranes and uterine discharges eliminated after abortion (Samartino and Enright, 1993). Vertical transmission is also possible (Ray *et al,* 1988). Artificial insemination with contaminated semen is a potential source of infection (Rankin, 1965).

2.4 Pathogenesis

Brucella is an unusual organism in several ways i.e. the classical virulence factors in a bacterium like exotoxin, pili, plasmids, flagella etc are found to be lacking in this organism. The *Brucellae* are facultative intracellular parasites. They usually enter the body through cuts and abrasions in the oral mucosa, nasopharynx, conjunctivae, or genitalia and even unbroken skin. After gaining entry to the body, the organisms have been found to survive within the cells of the reticuloendothelial system, particularly within macrophages/monocytes. They can survive within cells derived from either

ectodermal or mesodermal origin, but do not invade tissue of endodermal origin. They can evade the bactericidal activity of phagocytic cells and replicate within them (Pomales-Leborn and Stinebring 1957). They are transported to the lymph nodes. If bacteria do not become localized and are not killed in regional lymph nodes, then they spread to other organs via lymph and blood. Brucellae gain access to the uterus via hematogenous route and the bacteria initially localize within erythrophagocytic trophoblasts of the placentome. Adjacent chorioallantoic trophoblasts become infected and support massive growth of bacteria. These cells eventually rupture and ulcerate the chorioallantoic membrane. Bacteria and inflammatory cells both are present within the lumen of the uterus. Bacteria then spread via haematogenous route to the fetus and to the placentome. Diffuse submucosal inflammatory reaction is present. Endometrium is not infected with brucellae and remains largely intact.

The presence of elevated amounts of erythritol in uterine tissue of cattle plays an important role as the bacteria can utilize this. Moreover, extracts of fetal fluids, placenta, and chorion stimulate the growth of *B. abortus* (Keppie *et al* 1965). Fetuses that become infected during late gestation may be aborted. Extensive fetal inflammatory disease involving multiple organs leads to fetal stress, which is a reason for premature parturition. Mammary gland infection is clinically inapparent. In animals where the acute infection is not controlled, the bacteria become disseminated and eventually localize in the spleen and liver (Cheers 1984).

The organisms have been found within phagocytic cells, they survive both in phagocytic cells and nonphagocytic cells, such as epithelial cells. Survival within

phagocytic cells involves inhibition of phagosome fusion with primary and secondary granules (Kreutzer *et al* 1979 and Riley and Robertson 1984) while survival within cells lacking phagocytic capability requires a bacterial invasion mechanism.

The major clinical sign of the disease in animals is abortion. Animals usually do not abort in subsequent pregnancies but they act as carriers, thus contaminating the environment. Abortion in pregnant animals doesn't occur if the pregnant animals are infected at the end of the pregnancy (European Commission, 2001).

2.5 Virulence

Within a given Brucella spp., strains with smooth colonial morphology (bacteria with surfaces composed of lipopolysaccharides, LPS) are more virulent than those with rough colonial morphology (bacteria lacking a major LPS component in their outer membrane) (Roop *et al,* 1991). The basis for this association of virulence with smooth LPS has been investigated in a variety of *in vitro* culture systems with both phagocytic and nonphagocytic cells from a number of hosts. The studies demonstrate that both smooth (virulent) and rough (non virulent) brucellae are able to enter the host cells. In the studies utilizing phagocytic cells (both neutrophils and macrophages), the smooth brucellae survive and even multiply while the rough bacteria are eventually eliminated. Survival of smooth bacteria has been related to their ability to prevent lysosomal-phagosomal fusion, and their ability to resist the destructive effects of lysosomal enzymes after fusion has occurred (Kreutzer *et al,* 1979; Detilleux *et al,* 1990).

An important property of Brucella spp. associated with virulence is the ability to survive and multiply intracellularly in host phagocytes (Cheville 1994). The survivability of brucellae within phagocytic cells is associated with the composition of the cell wall of the bacteria.

Virulent brucellae may also escape the killing mechanism of leukocytes by resisting the effects of both oxygen dependent and independent bactericidal systems of these cells. Smooth pathogenic strains are more resistant to killing by myeloperoxidase system than were rough strains. The basis for this differential susceptibility is related to LPS component on the surface of the smooth bacteria. (Riley and Robertson, 1984). Superoxide dismutase plays a role in the survival and pathogenicity of the organism (Tatum *et al*, 1992).

2.6 Host Response

The alimentary tract is the major route in the transmission of *B. abortus* in cattle. Licking aborted fetuses and placental membranes or ingesting contaminated milk by calves introduces brucellae to the oral mucosa, tonsils and gastrointestinal mucosa. Passage of *B. abortus* through epithelial barriers results in acute regional lymphadenitis and bacteremia.

Epithelium covering domes of ileal Peyer's patches is an important site of entry. *B. abortus* has several fractions which can generate chemotactic factors (Bertram *et al*, 1986). Neutrophils are considered an important line of defense against Brucellosis. A component of *B. abortus* is capable of inhibiting release of myeloperoxidase by dose-

dependent preferential inhibition of primary granule release from bovine neutrophilic leukocytes (Bertram *et al*, 1986). Ingestion of the organism by PMNs fails to stimulate

the hexose monophosphate shunt; therefore, *Brucella* spp. survive, as certain surface properties fail to generate a suitable stimulus to activate killing mechanisms during interaction with plasma membranes.

The chronic persistence of *B. abortus* infection is due to intracellular localization of the brucellae in macrophages whose bactericidal mechanisms are resistant or refractory to activation. The protective effects of submucosal immune responses and inflammatory reactions against invading organisms alter the ability of *Brucella* spp. to colonize the lymph nodes. Some pregnancy associated factors that suppress immune responses may alter the effectiveness of vaccines.

2.7 Epidemiology and zoonosis

Brucellosis is widely distributed all over the world and it is one of the world's major zoonotic problems. The disease occurs worldwide, except in those countries where bovine brucellosis (*B. abortus*) has been eradicated. These countries include Australia, Canada, Cyprus, Denmark, Finland, The Netherlands, New Zealand, Norway, Sweden and the United Kingdom. Though it has been eradicated in these countries, it remains an uncontrolled problem in regions of high endemicity such as the Africa, Mediterranean, Middle East, parts of Asia and Latin America (Refai, 2002).

Serological evidences are suggestive of high endemicity of brucellosis in India. From India, it was first reported in 1942. *B. abortus* biotype-1 is the predominant

infective biotypes in cattle and buffaloes while *B. melitensis* biotype-1 is in sheep, goats and man. In India, 5% cattle, 3% buffaloes, 7.9% sheep and 2.2% of goats are infected with brucellosis (Renukaradhya *et al* 2002). In Punjab, the prevalence of this disease in cattle and buffaloes has been reported as 20.67% and 16.41%, respectively and the overall prevalence of brucellosis has been found to be 18.26% (Aulakh *et al* 2008).

2.8 Diagnosis

The diagnosis of the cause of abortion in a single animal or in a group of cattle is difficult because of the multiplicity of the causes which may be involved. Even in aborting animals or animals having epididymitis, the clinical diagnosis of brucellosis is difficult. The isolation of the organism is the only way to make a positive diagnosis. The factors determining the diagnosis of bovine brucellosis are: absence of clinical signs other than abortion, the incubation period, the high proportion of inapparent infections, the degree of resistance, either natural or resulting from vaccination, and the presence of natural or non specific agglutinins (Morgan, 1982). The diagnosis should be based upon the disease history of the herd, epidemiological observations, serum antibody tests, cell mediated immunity, and the demonstration of the causal organism.

Because of the costs, difficulty of performance, and lack of sensitivity of culture procedures, there is an indirect method of diagnosis by the way of serological tests. There are many serological tests for demonstrating *Brucella* antibodies in serum, milk, whey, vaginal mucus, semen, and muscle juice. The commonly used tests are the milk ring test (MRT), serum agglutination test (SAT), Rose Bengal Plate Test (RBPT), anti-globulin

(Coombs) test, 2 – mercaptoethanol, rivanol, and the enzyme- linked immunosorbent assay (ELISA) (Morgan, 1982).

2.9 Comparison between various serodiagnostic tests of Brucellosis:

Chachra *et al* (2009) carried out a study to compare the efficacy of RBPT, STAT and Dot ELISA in immunological detection of antibodies to *Brucella abortus* in sera. The study revealed that Dot ELISA was the most sensitive of the three tests used. The authors suggested that in order to get a fool proof diagnosis of *Brucella* infection, a combination of RBPT and Dot ELISA should be used, especially for the samples which are found negative by RBPT or STAT used alone or in combination.

A study was carried out on 180 serum samples by Ghodasara *et al* (2010) with an aim of comparing the RBPT, STAT and i-ELISA for detection of *Brucella* antibodies in cows and buffaloes. The seropositivity was found highest by i-ELISA (25%), followed by STAT (14.45%) and RBPT (10.56%).

Cernyseva *et al* (1977) carried out a study of the plate agglutination test with Rose Bengal antigen for the diagnosis of brucellosis. They opined that the RBPT holds a great promise for animal screening. The purpose of the study was to compare the RBPT with other serological tests. They found that the RBPT has high specificity and sensitivity.

RBPT, CFT and i-ELISA were compared in a study on the herd of unvaccinated cattle. It was found that the ELISA titers (≤20) accurately predicted all the negative sera in herds that were also negative by the CFT, the number of seropositive animals was higher by ELISA in herds that had positive animals. It was also suggested that the serum

samples which give higher degrees of agglutination with the RBPT need not be re-tested with CFT. Diagnostic sensitivity of i-ELISA and CFT was found to be 100% and 83%, respectively when 4803 cattle sera were tested against them, whereas their specificities were 99.8% and 100% respectively (Paweska *et al* 2002).

In another study, 141 bovine sera were screened for brucellosis using RBPT and STAT. The relative sensitivity and specificity of STAT and RBPT was found to be 88.61% and 98.59%, respectively for STAT and 56.96% and 96.77% for RBPT, respectively, classification being based on ELISA. (Chakraborty *et al* 2000).

Chand and Sharma (2004) advocated the use of ELISA in comparison to RBPT and STAT for assessing the situations of brucellosis in cattle, to have better results because chances of non detection of an infected animal in ELISA is minimum. ELISA can be used to eliminate false positive results amongst RBPT positive sera. (Erdenebaatar *et al* 2004).

ELISA has been claimed to be more sensitive followed by RBPT and STAT when applied to cattle sera, whereas RBPT was found to be more sensitive followed by STAT and ELISA when applied to buffalo sera (Mittal *et al* 2005). Comparison of dot-ELISA and ELISA for diagnosis of bovine brucellosis proved dot-ELISA to be more sensitive (Ganesan and Anuradha 2006).

Singh *et al* (2010) conducted a study in which an attempt was made to use PCR in diagnosis of sheep brucellosis using serum as sample and the results were compared with

those of RBPT. Out of 36 samples tested 19 were found positive for brucellosis by RBPT. PCR detected 13 samples as positive.

Vivekananda *et al* (2012) carried out a study on a sheep flock with a history of reproductive disorders. Among RBPT, STAT and iELISA, iELISA detected highest positive samples (14.8%) followed by RBPT (5.88%) and STAT (4.17%). They found that vaginal samples were most suitable for isolation and PCR, followed by serum and blood.

Keid *et al* (2010) carried out a comparison of a PCR assay in whole blood and serum specimens for canine brucellosis diagnosis on 72 serum samples. He reported that the sensitivities of blood PCR and serum PCR were, 97.14% and 25.71% respectively. The specificities of both were found to be 100%. The authors concluded that the serum PCR showed little value for the direct diagnosis of canine brucellosis.

Mariri *et al* (2011) carried out a study on a total of 2580 unvaccinated Syrian female sheep serum samples. Presence of *B. melitensis* antibodies was tested using four serological methods: RBPT, SAT, CFT and iELISA. Positivity by RBPT, SAT and iELISA was 66%, 64% and 60%, respectively. Whereas, the CFT revealed the smallest number of positive samples.

2.10 Prevention and Control

Efforts to control brucellosis rely on the use of vaccine prepared from attenuated *B. abortus* strain 19, screening of dairy cattle herds for antibodies using the brucella ring test, detection of infected beef cattle herds by the serological testing of slaughter cows

and bulls and tracing reacting animals to the herd of origin, and identification and removal of *B. abortus*- infected cattle from a herd by use of serological tests and/or

bacteriologic examinations on appropriate tissues and milk. The standard plate and tube agglutination tests have been commonly used for detecting infected animals. Vaccination of calves 4-8 months of age with *B. abortus* strain 19 provides protection in 65-85% of mature animals challenged by natural exposure. Killed *B. abortus* strain 45/20 suspended in adjuvant has been used for vaccinating cattle of all ages. Advantage of this killed vaccine is that it does not include levels of agglutinating antibodies that interfere with interpretation of agglutination tests, but it does stimulate the formation of antibodies detectable by CFT.

The major cycle of transmission of *B. abortus* is from an aborted fetus to mature females. The control programme should ideally constitute components like the test and reduction of reservoir of infection by sending positive animals for slaughter, which will remove infected cows from the herd and it will reduce exposure and transmission within herd; quarantine, which will prevent inter-herd transmission by infected cattle. Quarantine period for brucellosis ranges from 120 days to 1 year or until all breeding animals have completed a gestation without laboratory evidence of infection. Another component is vaccination with strain- 19 vaccine as properly vaccinated cattle are less likely to be infected and, therefore, are not a source of field strains of the organism. In India, due to religious taboos against slaughtering cattle, vaccination programme must be given due importance.

The treatment of brucellosis in the cow has been unsuccessful because of the intracellular sequestration of the organisms in lymph nodes, the mammary gland, and reproductive system. Treatment failures are due to inability of the drug to penetrate the cell membrane barrier (Guerra and Nicoletti, 1986).

CHAPTER III

MATERIALS AND METHODS

3.1 Source and collection of samples

Blood samples from cattle and buffaloes were collected from the veterinary clinics, dairy farms and gaushalas, in and around Ludhiana, Punjab. All the animals were of age more than two years. About 10ml of blood was collected aseptically from the jugular vein of the animal. Serum was collected by centrifuging the clotted blood at 3000 rpm for 15minutes.

Common serological tests i.e. Rose Bengal Plate Test (RBPT) [or conventional RBPT (cRBPT)], STAT, iELISA and CFT along with the modified RBPT (mRBPT) and PCR were applied on all the serum samples.

3.2 Conventional Rose Bengal Plate Test

Equal volumes (10 µl of each) of RBPT coloured antigen (Punjab Veterinary Vaccine Institute, Ludhiana) and test serum were mixed on a clean glass slide (Morgan *et al*., 1978) with the help of a clean sterilized toothpick. The slide was observed for 4 mins for the formation of clumps. The formation of clear clumps was considered a positive test while the absence of clear clumps was considered a negative reaction.

3.3 Modified Rose Bengal Plate Test

Equal volumes (2.5 µl each) of RBPT coloured antigen, test serum stained with 0.1% Coomassie Blue dye, biotinylated anti-bovine IgG and streptavidin were mixed on a

clean glass slide thoroughly in the above mentioned sequence. The slide was observed for 4 mins for the formation of clumps. Ordinary hand lens was used occasionally for better visibility. The slides were viewed under low power (10X) objective lens under an inverted microscope to confirm the clumping in case of doubt. Formation of clear clumps, within which the blue colour (due to the Coomassie Blue dye staining the serum antibodies) and the pink colour (due to the Rose Bengal dye stained RBPT coloured antigen) could be differentiated on magnification, were considered as positive, while absence of clear clumps was considered as negative.

3.4 Standard Tube Agglutination Test (STAT)

The standard method recommended by OIE was followed (Table 1). Twelve agglutination tubes were placed in a rack. 0.8ml of 0.5% carbol saline was added to the first tube of the series. 0.5ml of carbol saline was added to all the other tubes except in tube no. 10, 11 and 12 which contained 1.25, 1.50 and 1.75ml of carbol saline, respectively.

0.2ml Serum was added to the first tube and the contents were mixed. 0.5ml of this mixture was transferred to the second tube. This process was repeated till the 9^{th} tube, whereafter 0.5ml of the content was discarded. 0.5ml of *Brucella abortus* plain antigen (Punjab Veterinary Vaccine Institute, Ludhiana) was then added to tube numbers 1 to 9, giving a final dilution of 1:10, 1:20, 1:40 and so on.

To the tubes 10, 11 and 12, which were kept as controls, 0.75, 0.50 and 0.25ml respectively of *Brucella abortus* plain antigen was added. These tubes were incubated at

Table No. 1: STAT PROTOCOL

Tube No.	1	2	3	4	5	6	7	8	9	10	11	12
Carbol Saline in ml	0.8	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.25	1.5	1.75
Test Serum in ml	0.2	Serial dilution was performed after thorough mixing. 0.5 ml of the contents was transferred from tube no 1 to the next tube up to tube no. 9. Finally 0.5ml of the contents was discarded from tube no. 9.										
B. abortus plain antigen in ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.75	0.5	0.25
Final Dilution	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560			

Controls 1: Tube No. 10 – 25% agglutination

2: Tube No. 11 – 50% agglutination

3: Tube No. 12 – 75% agglutination

37°C for 24 hours and the results were read. The results were compared with the antigen control tube showing 50% agglutination. The highest serum dilution showing 50% agglutination was taken as the end point for the titre of serum. A titre of 1:40 or above was considered positive.

3.5 Indirect ELISA (iELISA)

To perform this test, a commercial kit was procured from Immuno-Biological Laboratories, America (IBL-America). The IBL- America Brucella IgG Antibody ELISA Test Kit has been designed for the detection of IgG class antibodies against Brucella in serum and plasma. The test was performed according to the instructions provided in the kit manual. The test serum samples had to be diluted 1:101 with ready-to-use sample diluent.

Microtiter wells were prepared for standards, controls and samples in duplicate as well as for a substrate blank. 100µl each of the diluted serum samples were pipetted into the wells. Ready-to-use standards and controls were pipetted in the respective wells. One well was left empty as a substrate blank.

The plate was covered and was incubated at room temperature for 60 mins. After the incubation, the wells were emptied by dumping the contents. Washing was carried out by pipetting 300 µl of diluted washing solution into each well followed by discarding the contents. This procedure of washing was repeated three times, after which the rest of the washing buffer was removed by gently tapping the plate over a tissue paper.

100 µl of ready-to-use conjugate was then pipetted into each well except the substrate blank. The plate was then covered and incubated at room temperature for 30 mins. After the incubation, the wells were emptied by discarding the contents. Washing was carried out three times, after which the rest of the washing buffer was removed by gently tapping the plate over a tissue paper.

100 µl of ready-to-use substrate was pipetted into each wells. This time the substrate blank was also pipetted. The plate was covered and was incubated at room temperature for 20 mins. To terminate the substrate reaction, 100 µl of ready-to-use stop solution was pipetted into each well. The substrate blank was also pipetted. After thorough mixing and wiping the bottom of the plate, reading of the absorption was recorded with a spectrophotometer at 450 nm within one hour of the completion of the test. Calculation of the results was carried out as per the instructions on the manual supplied with the kit.

3.6 Complement Fixation Test

The Complement Fixation Test (CFT) is widely used and accepted as a confirmatory test. Barbital (Veronal) Buffered Saline was used as the standard diluent for the CFT. Following is the composition of Veronal Buffer (VB; pH 7.4):

Sodium 5, 5, Diethyl Barbiturate: 3.75g

5, 5, Diethyl-Barbituric Acid : 5.75g

Magnesium Chloride : 1.68g

Calcium Chloride : 0.28g

Sodium Chloride : 85g

All the contents were dissolved in 500ml of hot distilled water (80-90°C) for 10-15 mins. The final volume was adjusted to 3000 ml. This stock buffer was stored in a refrigerator. For making the working dilution of the buffer, one part of the stock buffer was mixed with four parts of cold distilled water and the final pH was adjusted to 7.4.

Blood from a healthy sheep negative for Brucella antibodies was collected under aseptic conditions into equal volume of Alsever's solution. It was mixed thoroughly and centrifuged at 12000 rpm for 10 mins. Thereafter, the supernatant was discarded along with the thin layer of white cells and the erythrocytes were washed twice, first with the PBS and then with the Veronal buffer by centrifugation at 5000 rpm for 10 mins. To make 1% suspension of sheep RBC, 100 µl of erythrocytes were suspended in 9.9 ml of Veronal buffer. Following was the composition of the Alsever's solution used:

Sodium Chloride : 4.2g

Dextrose : 20.5g

Citric Acid : 0.55g

Sodium Citrate : 8.09g

DW : upto 1000ml

The Alsever's solution was autoclaved at 110°C and stored at 4°C. Haemolysin (Rabbit anti-sheep RBC's) was prepared by i/v inoculation of 1% sheep RBC's in rabbits. The immunization for haemolysin was done for duration of 20 days as per the method described by Darter (1953). Rabbits were bled by cardiac puncture and serum was separated. The high titre serum was then stored at 4°C in a refrigerator.

Serum from guinea pig used as the source of good quality complement was obtained from IVRI, Izatnagar. The site of prick near the medial sternum was disinfected

and blood was drawn through cardiac puncture. Serum was separated by centrifuging the clot at 2000 rpm at 4°C for 5 min. The guinea pig serum was stored at 4°C in a refrigerator.

3.6.1 Haemolysin Titration

To obtain a dilution of haemolysin for use in the test proper, a serial dilution of haemolysin was prepared in VB ranging from 1:10 to 1:5120. An equal volume of 1% sheep erythrocyte suspension was added to each dilution and incubated for 30 min at room temperature (Table 2).

An erythrocyte control was set. The highest dilution of haemolysin that produced 100% haemolysis was taken as one unit of the haemolysin. Two units of haemolysin were used in the test proper.

3.6.2 Complement Titration

For the titration of the complement, a dilution of haemolysin containing 2 haemolytic units was prepared. The haemolysin was inactivated by heating at 56°C for 30 min. Serial dilutions of complement were made in VB ranging from 1:10 to 1:5120 in a 'U' bottom 96 well plate. Equal amounts of haemolysin and 1% sheep RBC suspension were added to each dilution of the complement. The plate was then incubated for 2 hours at room temperature (Table 3). The highest dilution giving complete haemolysis was considered as the titre of the complement. In the test proper, two units of complement were used.

Table No. 2: Haemolysin Titration Protocol

Reagent	Well No. 1	Well No. 2	Well No. 3	Well No. 4	Well No. 5	Well No. 6	Well No. 7	Well No. 8	Erythrocyte Control
VB in µl	90	50	50	50	50	50	50	50	50
Haemolysin in µl	10	Contents in well no. 1 were mixed thoroughly and 50 µl was transferred to the next well. Serial dilution was performed and 50 µl of the contents were discarded from the last well.							---
1% sheep RBC in µl	50	50	50	50	50	50	50	50	50
	Incubated the plate at 37°C for 30 min. The highest dilution that produced complete haemolysis was taken as one unit of haemolysin.								
Dilution of Haemolysin	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	

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Table No. 3: Complement Titration Protocol

Reagent	Well No. 1	Well No. 2	Well No. 3	Well No. 4	Well No. 5	Well No. 6	Well No. 7	Well No. 8
VB in µl	90	50	50	50	50	50	50	50
Complement in µl	10	Contents in well no. 1 were mixed thoroughly and 50 µl was transferred to the next well. Serial dilution was performed and 50 µl of the contents were discarded from the last well.						
Haemolysin in µl	25	25	25	25	25	25	25	25
1% Sheep RBC in µl	25	25	25	25	25	25	25	25
The plate was incubated at 37°C for 2 hrs. The highest dilution that produced complete haemolysis was taken as one unit of Complement								
Dilution of Complement	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280

Controls: a) Haemolysin (50µl) + 1% Sheep RBC (50µl) b) VB (50µl) + 1% Sheep RBC (50µl)

3.6.3 Antigen Titration

A known positive serum was used for titration of antigen. The serum was inactivated at 56° for 30 min and diluted 1:2 in VB. Further 2 fold serial dilutions of the inactivated serum were made in VB and 25µl was dispensed in the vertical rows of the plate. Two fold serial dilutions of the antigen were made and dispensed in the horizontal rows of the plate. About 25µl of the complement having a final dilution of 1:320 was added to each well. An anti-complementary control for each antigen dilution containing VB, antigen and complement was incubated at 37°C. About 25µl of the sensitized sheep RBC was added to each well and incubated at 37°C for 30 min. The highest dilution of the antigen giving complete haemolysis was selected as the dilution for antigen in the test proper.

3.6.4 Test Procedure

Test serum samples were inactivated at 56°C for 30 min. Each serum sample was serially diluted in VB in horizontal rows in a 'U' bottom-96 well microtitre plate. To prepare the serial dilutions of the test serum, 25µl of VB was added to each well in the horizontal row except in the first well in which 50µl of VB was added. 5µl of the inactivated test serum was added to the first well and serial dilution was carried out by transferring 25µl of the content to the next well and discarding 25µl from the last well. Equal volume (25µl) of *Brucella abortus* plain antigen was added in each well, followed by 25µl of complement in all the wells. The plate was incubated at 37°C for 60 min to allow fixation. Freshly prepared 50µl sensitized RBCs were added to each well and

further incubated at 37°C for 30 min. The plates were checked for the presence of hemolysis. The absence of anti-complementary activity was checked in the controls.

3.6.5 Calculation of Positive Predictive Value (PPV) and Negative Predictive Value (NPV)

The PPV and NPV for each diagnostic test were calculated using following formulae.

$$PPV = \frac{\text{Number of True Positive cases}}{\text{Number of True Positive cases + Number of False Positive cases}}$$

$$NPV = \frac{\text{Number of True Negative cases}}{\text{Number of True Negative cases + Number of false negative cases}}$$

3.7 Polymerase Chain Reaction (PCR)

PCR is a sensitive, specific and rapid test for detecting a variety of pathogens. Using serum as a clinical sample for PCR is a new approach (Zerva *et al*, 2001). In the present study all the serum samples were subjected to PCR analysis.

3.7.1 Preparation of Solutions/Buffers.

3.7.1.1 Stock Solution of Tris EDTA

Tris EDTA was prepared with the following composition:

3.7.1.2 10X TBE – Stock Buffer

Tris-hydroxymethyl amino methane :108.0g

Boric acid : 55.0g

EDTA : 9.38g

DW : upto 1000ml

3.7.1.3 1X TBE – Working Buffer

10X TBE : 10ml

DW : To make volume up to 100ml

3.7.1.3 Lysis Buffer

The Lysis buffer contained 100mM KCl, 20mM Tris HCl (pH8.3), 5mM $MgCl_2$, 0.2mg of gelatin per ml and 0.9% polysorbate 20.

3.7.1.4 One percent Agarose Gel (Molecular Biology Grade)

1g of agarose was dissolved in 100 ml of working buffer (1X TBE) and Ethidium Bromide was added at 0.5 µg/ml of 1X TBE buffer.

3.7.2 Extraction of genomic DNA

For PCR, DNA was extracted from serum samples using the method described by Yamakami *et al* (1996). Briefly, 100 µl of the serum sample was mixed with 100 µl of the lysis buffer. Proteinase K was added to a final concentration of 60µg/ml and the mixture was incubated for 60 mins at 55°C. Proteinase K was inactivated by heating the mixture to 95°C for 10 min followed by centrifugation at 12000 g for 10 min at 4°C. The supernatant was collected in a fresh centrifuge tube to which 0.1 volume of sodium acetate (3M) and 0.6 volume of isopropanol were added. The contents were mixed gently

and kept on ice for 1 hour and then centrifuged at 8000 g for 10 min. The pellet was washed with 70% alcohol twice and dried at 37°C. Finally, the pellet was suspended in 20 µl of Tris EDTA buffer and stored at -20°C till further use.

3.7.3 Primer Sequence

The PCR assay was carried out using *Brucella* genus specific F4/R2 primers (Romero *et al* 1995). These primers are derived from 16S rRNA of *B. abortus*. The details of primers used are given in the Table 4.

Table 4: Sequences of primers used for PCR amplification

Primers	Sequences	Size of amplified product	Reference
F4	5'-TCGAGCGCCCGCAAGGGG-3'	905bp	Romero *et al* (1995)
R2	5'-AACCATAGTGTCTCCACTAA-3'		

The PCR reactions were carried out in a Gradient Thermal Cycler (Sensoquest, Germany) in a 25µl reaction mixture (Table 5) with the cycling conditions described in Table 6.

Table 5: *Brucella* PCR reaction mixture

S No.	PCR components	Amount (µl)
1	PCR Master Mix	12.5
2	Forward Primer	0.75
3	Reverse Primer	0.75
4	DNA Sample	10
5	Distilled Water	1
6	Total Volume	25

Table 6: *Brucella* PCR programme

Stage	Step	Temperature (^{0}C)	Duration	No. of cycles
I	Initial denaturation	94	5 min	1
II	Denaturation	94	45 secs	35
	Annealing	53	45 secs	
	Extension	72	5 min	
III	Final extension	72	5 min	1

3.7.4 Agarose Gel Electrophoresis of PCR products, image visualization and documentation

The PCR products obtained were analysed on 1% agarose gel made in 1X TBE buffer containing 8 µl of eithidium bromide (10mg/ml) per 100ml of the gel. The gel was poured into the electrophoresis tank after inserting the comb. It was allowed to solidify to form a flat surface for 15 mins and the comb was then removed. The electrophoresis tank was filled with 1X TBE. Samples were prepared by mixing 5µl of PCR product and 1µl of loading buffer. Molecular weight marker (100 bp DNA ladder) was loaded alongside the samples. The electrophoresis was allowed for 60 mins at 80 volts and the agarose gel was examined under UV rays using the Gel Documentation System (AlphaInnotech) to visualize the amplified products. The duration of the UV exposure was optimized and photographs were taken. These photographs were normalized with the AlphaImager software for better visibility. The amplicon size and concentrations were determined by comparing with the standard molecular ladder (GeneRuler 100 bp plus, Fermentas) which was run along with the samples.

CHAPTER IV

RESULTS AND DISCUSSION

Brucellosis is an important zoonotic disease causing huge economic losses in cattle industry. Serological tests are found to be practically more useful in diagnosis of brucellosis when compared to direct demonstration of the infectious organism by culture. Conventional serological procedures e.g. Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT) and Complement Fixation Test (CFT) are all based on the detection of anti-lipopolysaccharide (LPS) antibodies, which remain very high even after the recovery from the disease (Lulu *et al* 1988). However no single serological test can be considered confirmatory and therefore a combination of tests along with accurate clinical data is helpful in diagnosis of the disease.

RBPT is a rapid screening test, but at times it shows false positive reactions which arise due to residual antibody activity from vaccination, cross reaction with certain bacteria and laboratory error. False negative reactions in RBPT may arise during early incubation of disease or immediately after incubation. However, the RBPT is still considered to be an excellent test for the large scale screening of sera (Radostits, 2000).

The CFT is useful in differentiating the titers of antibody due to calf hood vaccination from those due to infection; also, CFT reaches diagnosis level sooner than the STAT following natural infection (Radostits, 2000). ELISA has gained wide acceptance for serological diagnosis of bovine brucellosis because of its ability to detect antibodies of all isotypes unlike the conventional tests (Nielsen *et al* 1988).

Serological cross-reactions have been demonstrated between smooth *Brucella* spp. and *E. coli* 0:116 and 0:157, *Francisella tularensis, Salmonella* serotypes of Kauffman-White group N, *Pseudomonas maltophilia, Vibrio cholerae* and *Yersinia enterocolitica* serotype 0:9 and *Stenotrophomonas maltophilia* (Corbel *et al*, 1984). The problem of serological cross-reactions has led to considerable research and investigations to find laboratory tests which will accurately distinguish positive infected animals from non infected animals (Radostits, 2000). In the present study, a modified RBPT based on novel modifications in the RBPT, developed recently in the department, have been evaluated for the diagnosis of bovine brucellosis by comparing its results with those of conventional RBPT, STAT, iELISA, CFT and PCR.

Incidence of Brucellosis in large animals in and around Ludhiana.

A total of 200 bovine serum samples were collected from various farms and gaushalas in and around Ludhiana. Out of these, 181 serum samples were from cattle and 19 serum samples were from buffaloes. All the animals were of age more than two years. These serum samples were tested by various serological tests to evaluate the efficacy of mRBPT, by comparing it with conventional and non-conventional serological tests. The tests performed on all the 200 samples were mRBPT, conventional RBPT, STAT, iELISA, CFT (Table 7 and 8) and PCR. The efficacy of mRBPT was determined by comparing its sensitivity, specificity, positive predictive value and negative predictive value with those of other tests. Table 9 presents the difference between mRBPT and other serological tests regarding positive and negative samples identified.

Table 7: Results of various serological tests

SERIAL No.	SAMPLE NUMBER	STAT TITRE	cRBPT	mRBPT	ELISA	CFT
1.	2218	80	Positive	Positive	Positive	Positive
2.	2308	160	Positive	Positive	Positive	Positive
3.	2362	320	Positive	Positive	Positive	Positive
4.	2417	160	Positive	Positive	Positive	Positive
5.	2426	>1280	Positive	Positive	Positive	Positive
6.	2452	80	Positive	Positive	Positive	Positive
7.	2467	>1280	Positive	Positive	Positive	Positive
8.	2490	640	Positive	Positive	Positive	Positive
9.	2554	>1280	Positive	Positive	Positive	Positive
10.	2567	>1280	Positive	Positive	Positive	Positive
11.	2581	80	Positive	Positive	Positive	Positive
12.	29(15/9/11)	40	Negative	Negative	Negative	Negative
13.	40(15/9/11)	40	Negative	Negative	Negative	Negative
14.	101(15/9/11)	80	Positive	Positive	Positive	Positive
15.	103(15/9/11)	160	Negative	Positive	Negative	Negative
16.	104(15/9/11)	>1280	Positive	Positive	Positive	Positive
17.	13(20/4/11)	80	Negative	Positive	Negative	Negative
18.	15(20/4/11)	40	Negative	Negative	Negative	Negative
19.	17(20/4/11)	40	Negative	Negative	Negative	Negative
20.	19(20/4/11)	>1280	Positive	Positive	Positive	Positive
21.	20(20/4/11)	40	Negative	Negative	Negative	Negative

22.	23(20/4/11)	20	Negative	Negative	Negative	Positive
23.	32(20/4/11)	>1280	Positive	Positive	Positive	Positive
24.	34(20/4/11)	40	Negative	Negative	Negative	Negative
25.	42(20/4/11)	40	Negative	Negative	Negative	Negative
26.	51(20/4/11)	40	Negative	Negative	Negative	Negative
27.	53(20/4/11)	40	Negative	Negative	Negative	Negative
28.	54(20/4/11)	40	Negative	Negative	Negative	Negative
29.	69(20/4/11)	00	Negative	Negative	Negative	Negative
30.	72(20/4/11)	40	Negative	Negative	Negative	Negative
31.	73(20/4/11)	20	Negative	Negative	Negative	Negative
32.	76(20/4/11)	40	Negative	Negative	Negative	Negative
33.	79(20/4/11)	80	Negative	Positive	Negative	Negative
34.	80(20/4/11)	00	Negative	Negative	Negative	Negative
35.	83(20/4/11)	00	Negative	Negative	Negative	Negative
36.	100(20/4/11)	80	Negative	Positive	Negative	Negative
37.	T1	40	Negative	Negative	Negative	Negative
38.	T3	40	Negative	Negative	Negative	Negative
39.	T4	160	Positive	Negative	Negative	Negative
40.	T6	20	Positive	Negative	Negative	Negative
41.	T8	40	Negative	Negative	Negative	Positive
42.	2(1/3/11)	10	Negative	Negative	Negative	Negative
43.	6(1/3/11)	160	Negative	Positive	Negative	Negative
44.	4(1/3/11)	40	Negative	Negative	Negative	Negative
45.	1(1/3/11)	20	Negative	Negative	Negative	Negative

46.	11(1/3/11)	80	Negative	Negative	Negative	Negative
47.	13(1/3/11)	10	Negative	Negative	Negative	Negative
48.	19(1/3/11)	80	Negative	Negative	Negative	Negative
49.	20(1/3/11)	20	Negative	Negative	Negative	Negative
50.	23(1/3/11)	10	Negative	Negative	Negative	Negative
51.	21 (T 5/10/11)	20	Negative	Negative	Negative	Negative
52.	76(T 5/10/11)	10	Negative	Negative	Negative	Negative
53.	79(T 5/10/11)	40	Negative	Negative	Negative	Positive
54.	82(T 5/10/11)	80	Negative	Negative	Negative	Negative
55.	86(T 5/10/11)	160	Positive	Negative	Positive	Positive
56.	87(T 5/10/11)	>1280	Positive	Positive	Positive	Positive
57.	89(T 5/10/11)	160	Positive	Positive	Positive	Positive
58.	92(T 5/10/11)	40	Negative	Negative	Negative	Negative
59.	5(13/10/11)	20	Negative	Negative	Negative	Negative
60.	10(13/10/11)	10	Negative	Negative	Negative	Negative
61.	11(13/10/11)	40	Negative	Negative	Negative	Negative
62.	A	00	Negative	Negative	Negative	Negative
63.	B	00	Negative	Negative	Negative	Negative
64.	E	10	Negative	Negative	Negative	Negative
65.	F	00	Negative	Negative	Negative	Negative
66.	G	00	Negative	Negative	Negative	Negative
67.	AT	00	Negative	Negative	Negative	Positive
68.	BA	10	Negative	Negative	Negative	Negative
69.	BB	00	Negative	Negative	Negative	Negative

70.	BF	10	Negative	Negative	Negative	Negative
71.	BH	00	Negative	Negative	Negative	Negative
72.	BR	00	Negative	Negative	Negative	Negative
73.	CD	>1280	Positive	Positive	Positive	Positive
74.	CE	20	Negative	Negative	Positive	Positive
75.	CH	>1280	Positive	Positive	Positive	Positive
76.	CM	160	Positive	Positive	Negative	Positive
77.	CN	20	Positive	Positive	Negative	Negative
78.	CS	>1280	Positive	Positive	Positive	Positive
79.	CX	20	Positive	Positive	Positive	Positive
80.	CY	10	Negative	Negative	Negative	Negative
81.	DD	00	Negative	Negative	Negative	Negative
82.	DL	00	Negative	Negative	Negative	Negative
83.	DN	10	Negative	Negative	Negative	Negative
84.	DQ	40	Positive	Positive	Positive	Negative
85.	DR	10	Negative	Negative	Negative	Negative
86.	DU	80	Positive	Positive	Positive	Negative
87.	T2	640	Positive	Positive	Positive	Positive
88.	12(13/10/11)	80	Positive	Positive	Negative	Negative
89.	T 81 (25/11/11)	40	Negative	Positive	Negative	Positive
90.	T 85 (25/11/11)	40	Negative	Negative	Negative	Positive
91.	T 2062 (25/11/11)	40	Negative	Positive	Positive	Positive
92.	H	40	Positive	Positive	Positive	Positive
93.	I	80	Positive	Positive	Positive	Positive

94.	K	00	Negative	Negative	Negative	Negative
95.	L	00	Negative	Negative	Negative	Negative
96.	M	00	Negative	Negative	Negative	Negative
97.	N	00	Negative	Negative	Negative	Negative
98.	O	40	Negative	Negative	Negative	Positive
99.	P	80	Positive	Positive	Negative	Positive
100.	Q	320	Positive	Positive	Positive	Positive
101.	R	160	Positive	Positive	Negative	Negative
102.	S	00	Negative	Negative	Negative	Negative
103.	T	00	Negative	Positive	Negative	Negative
104.	U	160	Positive	Positive	Positive	Positive
105.	V	00	Negative	Negative	Negative	Negative
106.	W	40	Positive	Positive	Positive	Positive
107.	X	40	Positive	Positive	Positive	Positive
108.	Y	160	Positive	Positive	Positive	Positive
109.	Z	10	Positive	Positive	Positive	Positive
110.	AA	160	Positive	Positive	Negative	Positive
111.	AB	10	Negative	Negative	Negative	Negative
112.	AC	00	Negative	Negative	Negative	Negative
113.	AD	20	Negative	Negative	Negative	Negative
114.	AE	10	Negative	Negative	Negative	Positive
115.	AF	00	Negative	Negative	Negative	Negative
116.	AG	00	Negative	Negative	Negative	Negative
117.	AH	80	Positive	Positive	Positive	Positive

118.	AI	10	Positive	Positive	Negative	Negative
119.	AJ	20	Positive	Positive	Negative	Negative
120.	AK	160	Positive	Positive	Positive	Positive
121.	AL	40	Positive	Positive	Negative	Positive
122.	AM	320	Positive	Positive	Positive	Positive
123.	AN	10	Positive	Positive	Positive	Positive
124.	AO	20	Positive	Positive	Positive	Positive
125.	AP	00	Negative	Negative	Negative	Negative
126.	AQ	40	Positive	Positive	Positive	Negative
127.	AR	320	Positive	Positive	Positive	Positive
128.	AS	40	Positive	Positive	Positive	Positive
129.	AU	320	Positive	Positive	Positive	Positive
130.	AV	20	Negative	Negative	Positive	Positive
131.	AW	160	Positive	Positive	Positive	Positive
132.	AX	640	Positive	Positive	Positive	Positive
133.	AY	40	Positive	Positive	Negative	Positive
134.	AZ	00	Negative	Negative	Negative	Negative
135.	BC	10	Positive	Positive	Negative	Negative
136.	BG	00	Negative	Negative	Negative	Negative
137.	BL	20	Negative	Negative	Negative	Negative
138.	BP	20	Negative	Negative	Negative	Positive
139.	BW	10	Positive	Positive	Positive	Positive
140.	BX	160	Positive	Positive	Positive	Negative
141.	BY	10	Positive	Positive	Negative	Negative

142.	BZ	640	Positive	Positive	Positive	Positive
143.	CB	00	Negative	Negative	Negative	Negative
144.	CI	40	Positive	Positive	Negative	Negative
145.	CK	00	Negative	Negative	Negative	Negative
146.	CR	80	Positive	Positive	Positive	Positive
147.	CT	160	Positive	Positive	Positive	Positive
148.	CV	80	Positive	Positive	Negative	Negative
149.	CZ	160	Positive	Positive	Positive	Negative
150.	DB	160	Positive	Positive	Positive	Positive
151.	DC	160	Positive	Positive	Positive	Positive
152.	DG	80	Positive	Positive	Positive	Positive
153.	DJ	80	Positive	Positive	Positive	Positive
154.	DO	80	Positive	Positive	Positive	Positive
155.	DP	160	Positive	Positive	Positive	Negative
156.	2489	>1280	Positive	Positive	Positive	Positive
157.	2574	640	Positive	Positive	Positive	Negative
158.	2582	640	Positive	Positive	Positive	Negative
159.	16(20/4/11)	80	Positive	Positive	Negative	Negative
160.	25(20/4/11)	640	Positive	Positive	Positive	Positive
161.	66(20/4/11)	20	Negative	Negative	Negative	Negative
162.	68(20/4/11)	00	Negative	Negative	Negative	Negative
163.	70(20/4/11)	00	Negative	Negative	Negative	Negative
164.	71(20/4/11)	00	Negative	Negative	Negative	Negative
165.	74(20/4/11)	10	Negative	Negative	Negative	Negative

166.	77(20/4/11)	00	Negative	Negative	Negative	Negative
167.	78(20/4/11)	00	Negative	Negative	Negative	Negative
168.	81(20/4/11)	00	Negative	Negative	Negative	Negative
169.	82(20/4/11)	320	Negative	Positive	Negative	Positive
170.	T5	160	Positive	Positive	Negative	Positive
171.	3(1/3/11)	20	Negative	Negative	Negative	Negative
172.	9(1/3/11)	160	Negative	Positive	Negative	Negative
173.	88(T 5/10/11)	>1280	Positive	Positive	Positive	Positive
174.	3 (13/10/11)	10	Negative	Negative	Negative	Negative
175.	80(T 5/10/11)	>1280	Positive	Positive	Positive	Positive
176.	2379	40	Positive	Positive	Negative	Positive
177.	2413	40	Positive	Positive	Positive	Negative
178.	102(15/9/11)	20	Negative	Negative	Negative	Positive
179.	7(20/4/11)	20	Negative	Negative	Negative	Positive
180.	T7	20	Negative	Negative	Negative	Negative
181.	6(13/10/11)	20	Negative	Negative	Negative	Negative
182.	7(13/10/11)	20	Negative	Positive	Negative	Negative
183.	T 29 (25/11/11)	>1280	Positive	Positive	Positive	Positive
184.	T 77 (25/11/11)	>1280	Positive	Positive	Positive	Positive
185.	T 78 (25/11/11)	>1280	Positive	Positive	Positive	Positive
186.	T 8 (25/11/11)	>1280	Positive	Positive	Negative	Positive
187.	T 84 (25/11/11)	>1280	Positive	Positive	Positive	Positive
188.	T 86 (25/11/11)	>1280	Positive	Negative	Positive	Positive
189.	T2061(25/11/11)	20	Negative	Negative	Negative	Negative

190.	J	20	Negative	Negative	Negative	Negative
191.	BD	160	Positive	Positive	Negative	Negative
192.	BE	40	Positive	Positive	Positive	Negative
193.	BI	80	Positive	Positive	Positive	Negative
194.	BJ	80	Positive	Positive	Negative	Negative
195.	BK	00	Negative	Negative	Negative	Negative
196.	BM	80	Positive	Positive	Negative	Negative
197.	BO	320	Positive	Positive	Negative	Positive
198.	BQ	320	Positive	Positive	Negative	Positive
199.	BS	320	Positive	Positive	Positive	Positive
200.	BT	40	Positive	Positive	Positive	Positive

Table 8: Number of positive and negative samples in each of the test conducted

Test conducted	Number of samples		
	Positive	Negative	Total
mRBPT	104	96	200
cRBPT	97	103	200
STAT	119	81	200
iELISA	75	125	200
CFT	86	114	200

Table 9: Difference between mRBPT and other serological tests regarding positive and negative samples identified

	Difference with the other tests							
	Positive samples				Negative samples			
	cRBPT	STAT	iELISA	CFT	cRBPT	STAT	iELISA	CFT
mRBPT	+12	+12	+33	+29	-03	-25	-04	-09

+ = More number detected by mRBPT

- = Less number detected by mRBPT

4.1 Comparison of the modified RBPT with conventional serodiagnostic tests - RBPT, STAT and ELISA.

4.1.1 Conventional RBPT

Conventional RBPT was performed on all the 200 serum samples. Out of the 200 samples, 97 were found to be positive by this test. The test showed a sensitivity of 93.33% and a specificity of 88.18%. This test was assessed to have a PPV of 86.6% and NPV of 94.17% (Figure 1). This finding is in accordance with the findings of Akhtar *et al* (2010), who demonstrated a high sensitivity and a low specificity of RBPT, indicating the poor efficacy of the test. They suggested using a combination of the conventional tests and PCR for an accurate diagnosis of brucellosis.

In the RBPT, the antigen is used at a pH of 3.65. The low pH prevents some agglutination by IgM and encourages agglutination by IgG1 thereby reducing non specific interactions (Corbel, 1972 and 1973; Allan *et al*, 1976). RBPT is considered to

be suitable for screening of individual animals, however, some cross reacting antibodies have been detected by this test and false negative reactions may occur mostly due to prozoning (OIE, 2004). Inaccurate serological results leading to incorrect diagnosis is a persistent problem when testing for infectious disease agents in an outbred population of animals. Because of the genetic diversity of populations, some animals will respond with low antibody levels on exposure to *Brucella* species, resulting in a false negative outcome. Other animals will respond with very high levels of antibody which may be due to prozoning in some of the older assays.

4.1.2 mRBPT

All the 200 serum samples were subjected to mRBPT. A total of 104 samples were detected positive by this test. The test showed a sensitivity of 95.88% and a specificity of 89.32%. Positive predictive value (PPV) of this test was calculated as 89.42% and Negative predictive value (NPV) was determined to be 95.83% (Figure 2).

4.1.3 STAT

STAT could detect 119 samples out of the 200 samples as positive. A titre of 1:40 and above was considered as positive. Among the 200 serum samples, 81 samples had titres below 1:40, 36 samples had a titre of 1:40 and 83 samples had titres more than 1:40.

STAT showed a sensitivity of 94.25% and a specificity of 68.14. PPV of this test was 69.49% and NPV was 93.90%. The high sensitivity of STAT can be attributed to its ability to detect antibody titre as low as 1:40 which was probably missed by other tests. In serum agglutination tests, cross reactions with various bacteria for example *Yersinia*

Figure 1.The visual readout of the conventional RBPT on a glass slide

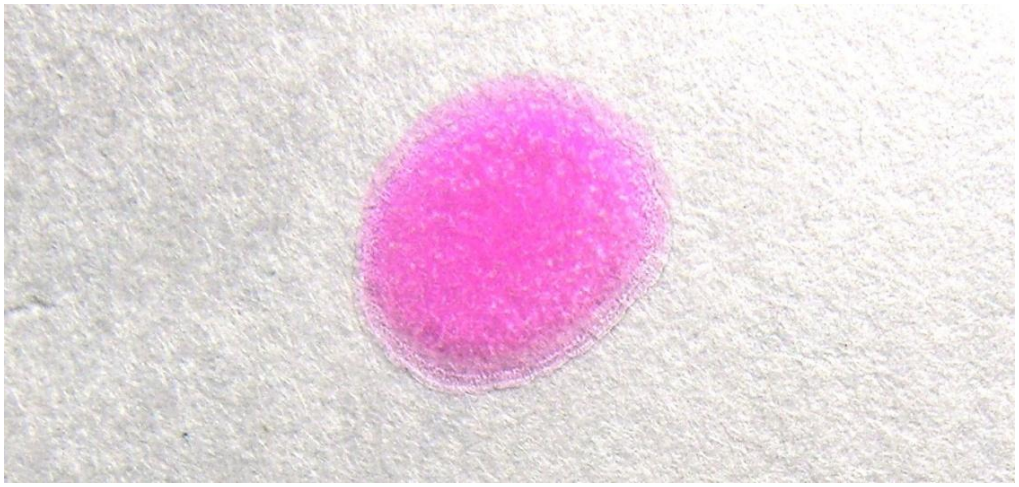
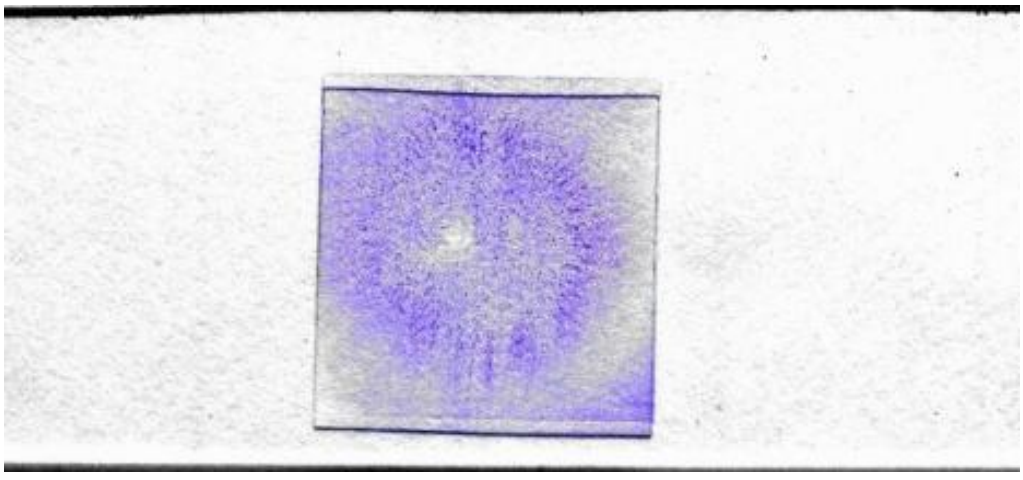


Figure 2.The visual readout of the modified RBPT on a glass slide



enterocolitica O:9, *E. coli* O:157, *Francisella tularensis*, *Salmonella urbana* group N, *Vibrio cholera* and *Stenotrophomonas maltophilia* have been reported (Corbel and Brinley Morgan, 1984). Therefore a confirmatory test is always recommended to avoid false positive results due to the low specificity of agglutination tests.

4.1.4 iELISA

iELISA kit was procured from Immuno-Biological Laboratories, America (IBL-America). All the 200 samples were subjected to this test. The absorbance was measured at 420nm. Out of the 200 samples 75 were detected as positive. Sensitivity of this test was calculated to be 74.47% and the specificity was found to be 95.24%. PPV of this test was 93.33% and NPV was found to be 80.65% (Figure 3). As the symptoms are not unique to the disease and hence difficult to interpret, serological testing is important in the diagnosis of brucellosis. ELISA is considered to be a more specific and sensitive test (Radostits, 2000).

4.1.5 CFT

In the present study, the CFT showed a sensitivity of 82.8% which is in accordance with Gupta *et al* (2010) who demonstrated a sensitivity of 80% while carrying out a comparative evaluation of recombinant BP26 protein for serological diagnosis of *B. melitensis* infection in goats. In spite of the number of reagents required for the complement fixation test and its technical complications, it is widely used as the confirmatory test for brucellosis. The complement fixation test is technically challenging because a large number of reagents must be titrated daily and a large number of controls

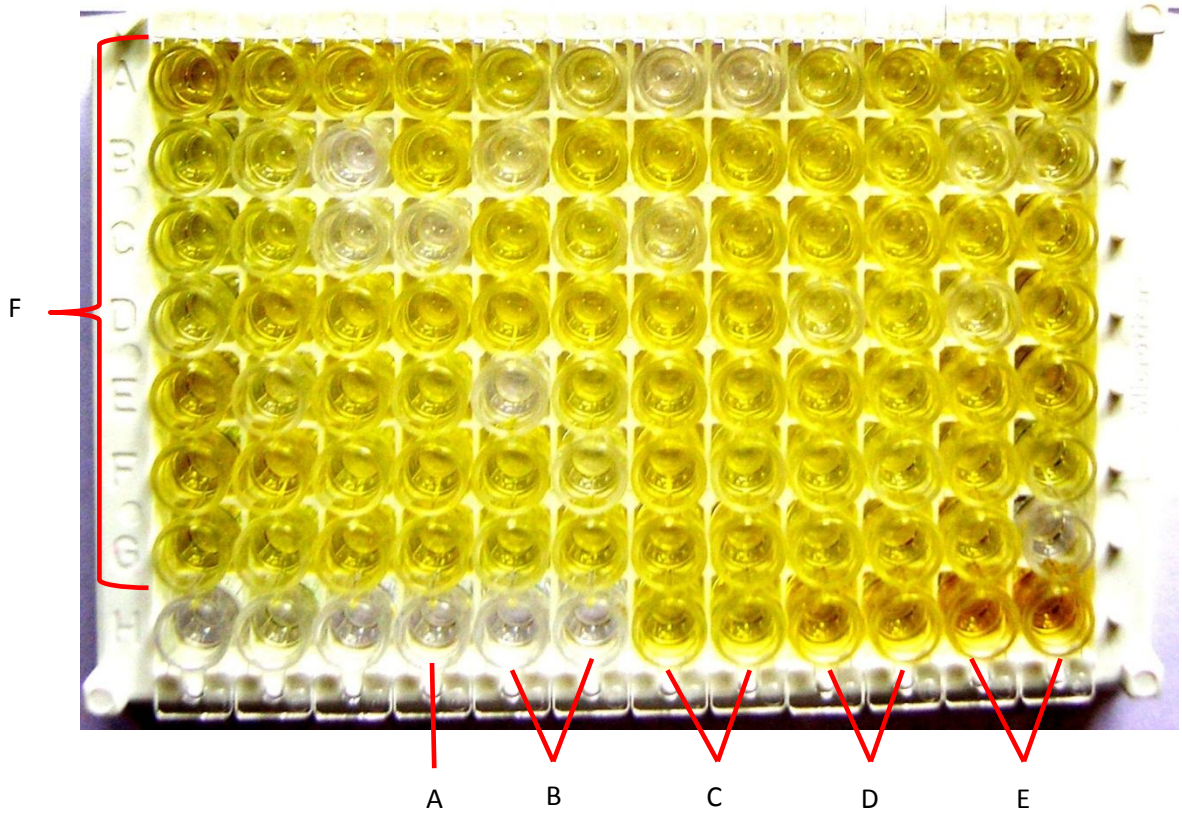


Figure 3. ELISA on serum samples from Brucellosis suspected cattle and buffaloes.

A: Blank; B: Negative control; C: Cut-off standard; D: Weak Positive control; E: Positive control; F: test samples.

of all the reagents is required. It is an expensive test and is labour intensive. However, since only IgG1 isotype of antibody fixes complement well, the test specificity is high (Poester *et al*, 2010).

The present study demonstrated a specificity of 93.46% for the CFT (Figures 4 and 5). Unfortunately, the test does not allow for discrimination of antibody due to infection from *B. abortus* S19 vaccination derived antibody. Other problems include the subjectivity of the interpretation of results, occasional direct activation of complement by serum (anticomplementary activity) and the inability of the test for use with haemolysed serum samples. In spite of the shortcomings, the complement fixation test has been and is a valuable asset as a confirmatory test in control/eradication programs (Poester *et al*, 2010).

The results of our study are also in accordance with those of Stemshorn *et al* (1985) who demonstrated a sensitivity of 79% for CFT while carrying out a comparative study of standard serological tests for the diagnosis of bovine brucellosis in Canada.

4.1.6 Comparison of the serological tests used

Table 10 presents a comparison between the mRBPT, conventional RBPT, STAT, iELISA and CFT based on sensitivity, specificity, PPV and NPV. The graphical representation is presented in the Figure 6.

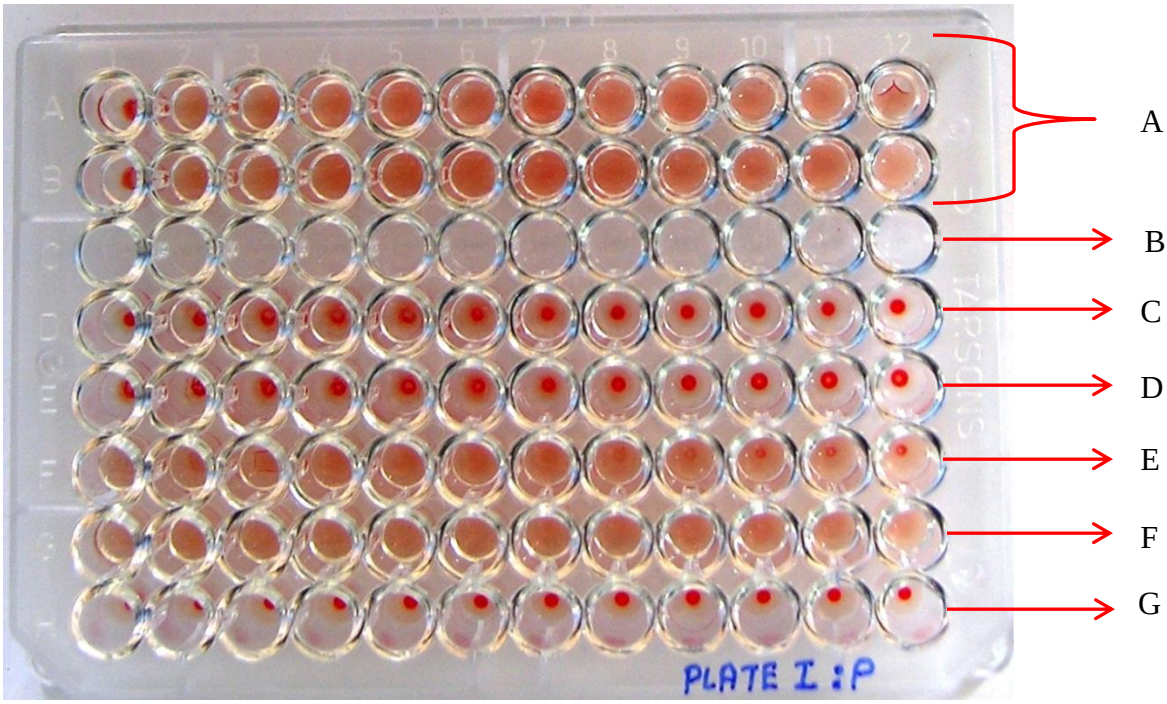


Figure 4. Standardization of Complement Fixation Test

A: CFT on a positive serum sample; B: Antigen + Antibody; C: Antigen + Antibody + Complement + 1% Sheep RBC; D: Antigen + Antibody + 1% Sheep RBC; E: Haemolysin Titration; F: Complement Titration; G: Only 1% Sheep RBC.

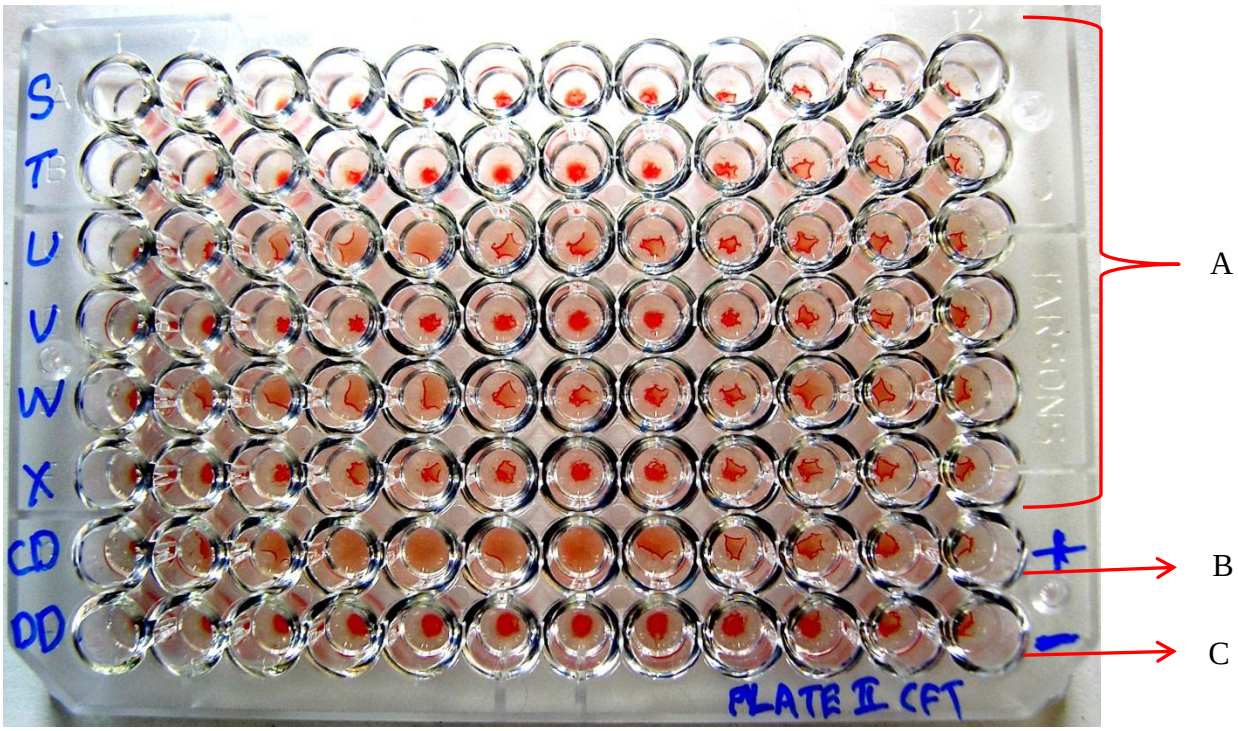


Figure 5. Complement Fixation Test Analysis of Sera

A: CFT on serum samples from Brucellosis suspected animals; Negative samples: S, T, V and X; Positive samples: U and W; B: Positive Control; C: Negative Control.

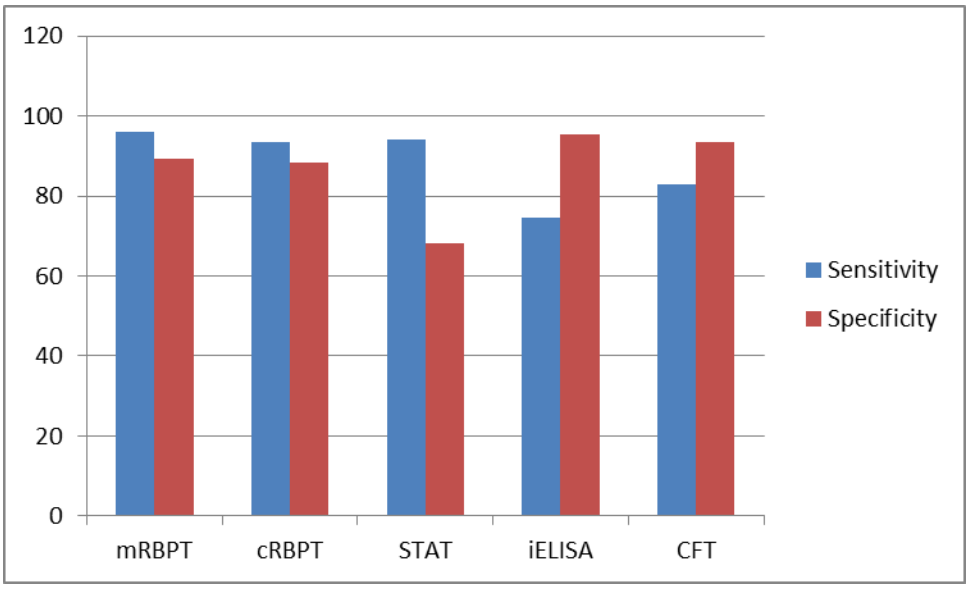


Figure 6. A comparison between the sensitivities and specificities of different serological tests used in the diagnosis of Brucellosis.

Table 10: Sensitivity and Specificity of the mRBPT and other common serodiagnostic tests for Brucellosis.

Test	Sensitivity in per cent	Specificity in per cent	PPV in per cent	NPV in per cent	Disease Prevalence in per cent
mRBPT	95.88	89.32	89.42	95.83	48.50
cRBPT	93.33	88.18	86.60	94.17	45.00
STAT	94.25	68.14	69.49	93.90	43.50
iELISA	74.47	95.24	93.33	80.65	47.24
CFT	82.80	93.46	91.67	86.21	46.5

The modified RBPT showed the highest sensitivity of 95.88% among all the tests which can be attributed to the fact that the number of false negative results with mRBPT was less when compared to the other tests. The anti-bovine IgG plays a role in increasing the sensitivity as, if fewer number of bovine antibodies are present in the serum against the infectious organism, the anti-bovine IgG cross links these antibodies resulting in an increase in the clump size. Streptavidin binds to the biotinylted IgG increasing the clump size further by up to 4 fold due to four binding sites for biotin on each molecule of avidin.

The specificity of the mRBPT, though higher than that of conventional RBPT and STAT, was found to be lower than that of iELISA and CFT. More number of false positive cases may possibly have caused reduction in its specificity. This may be due to the cross reactions of anti bovine IgG with nonspecific antibodies.

In case of mRBPT, when the slide was viewed under a light microscope, the agglutinate could be differentiated into two parts, the antibodies were blue in colour due

to the Coomassie blue dye and the antigen was pink in colour due to the Rose Bengal dye. Each clump had both the blue and the pink colour, which aided in differentiation of the true agglutinates from the non-specific aggregates of the antigen. The antigen and antibodies which did not participate in agglutination reaction could be viewed under the microscope as aggregates of blue or pink particles alone lying separately (Figure 7 and 8). However, the Coomassie blue dye had to be prepared freshly before conducting the test as old solution of the dye tends to concentrate due to evaporation leading to difficulty in reading the test. Ordinary hand lens was generally effective in visualizing the agglutination.

The mRBPT reaction mix had 2.5µl each of the test serum, the RBPT coloured antigen, biotinylated anti-bovine IgG (1:50 dilution), streptavidin and Coomassie blue dye, respectively. The contents were mixed thoroughly. Proper controls were set each time to check for non specific agglutination. Statistical agreement between the mRBPT and other tests was calculated. Observed proportion of agreement (OPA) and agreement beyond chance (kappa values) were determined using winepiscope-2 software package with 95% confidence level.

The kappa values and the observed proportions of agreements are presented in the table 11 and a graphical representation is shown in figure 9.

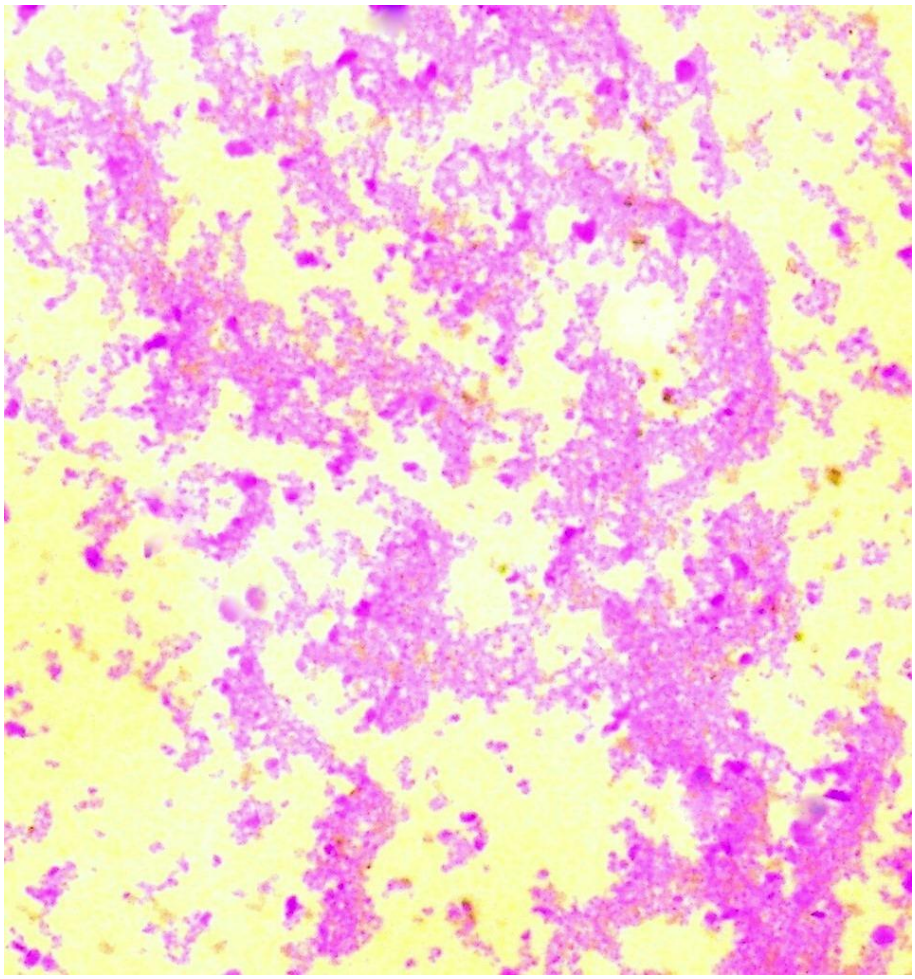


Figure 7: A clump formed by the cRBPT

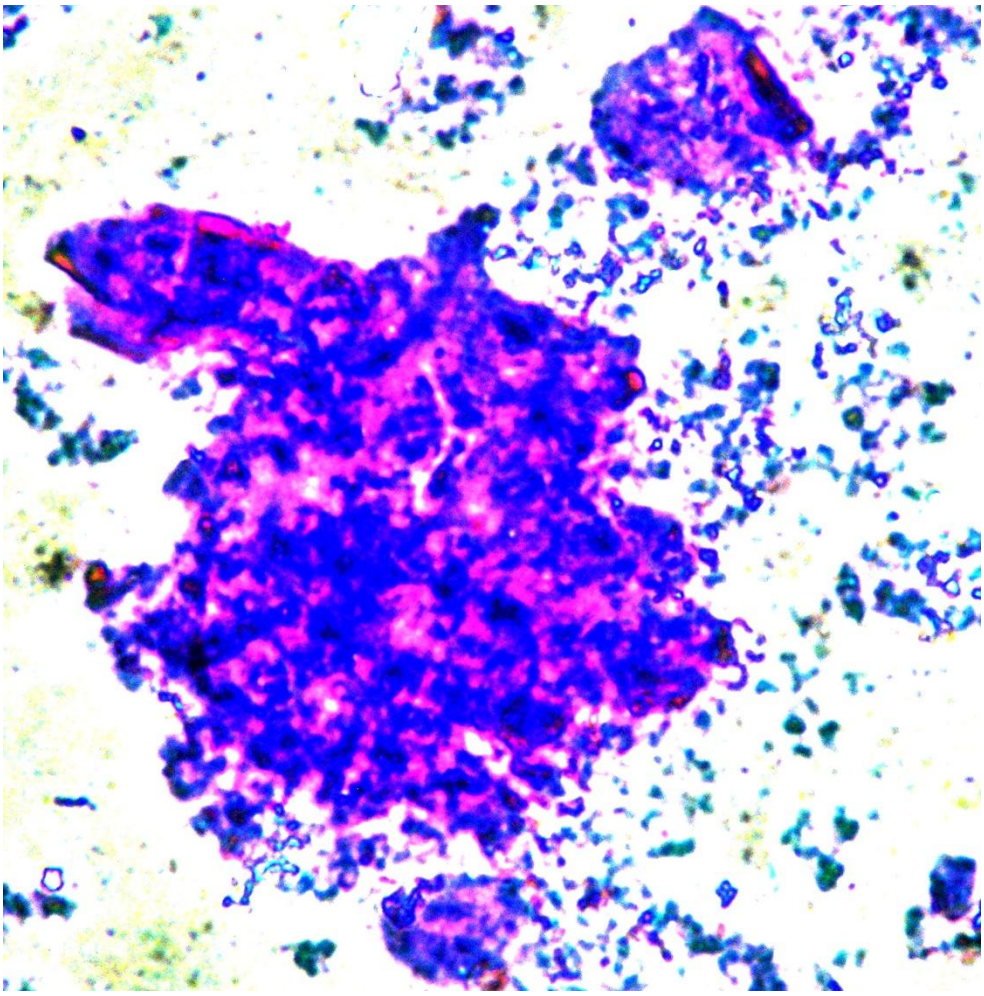


Figure 8. A two colored agglutinate formed in the mRBPT showing blue colored antibodies bound to the pink colored antigen particles.

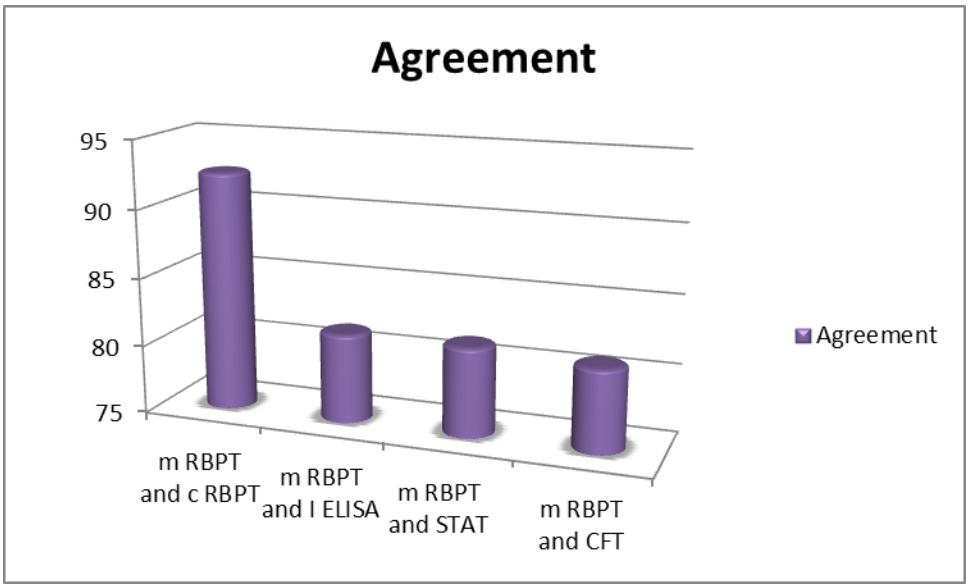


Figure 9. A comparison between agreements of mRBPT and other serological tests.

Table 11: Agreement among the various serological tests.

Tests	OPA in percentage	Kappa value	Degree of agreement
mRBPT and cRBPT	92.5	0.850	Almost perfect
mRBPT and iELISA	81.5	0.634	Substantial
mRBPT and STAT	81.5	0.626	Substantial
mRBPT and CFT	81.0	0.623	Substantial

The agreement between mRBPT and cRBPT was found to be almost perfect, whereas the agreement between mRBPT and other tests was found to be substantial. Considering the fact that cRBPT is widely used as a screening test for brucellosis, this agreement combined with the higher specificity and sensitivity of mRBPT ensure that it can serve as a more efficient screening test than cRBPT.

4.1.7 Serum PCR

PCR was carried out on all the 200 serum samples. Only three samples showed a band of size 905bp, typical of *Brucella* spp (Figure 10). Other samples failed to show positive reaction by PCR. The *Brucellae* are facultative intracellular parasites and they can evade the bactericidal activity of phagocytic cells and replicate within them (Pomales-Leborn and Stinebring 1957). The organisms have been found within phagocytic cells, they survive both in phagocytic cells and nonphagocytic cells (Kreutzer *et al*, 1979 and Riley and Robertson, 1984). As these cells do not appear in serum it is difficult to detect the organism by PCR in the serum.

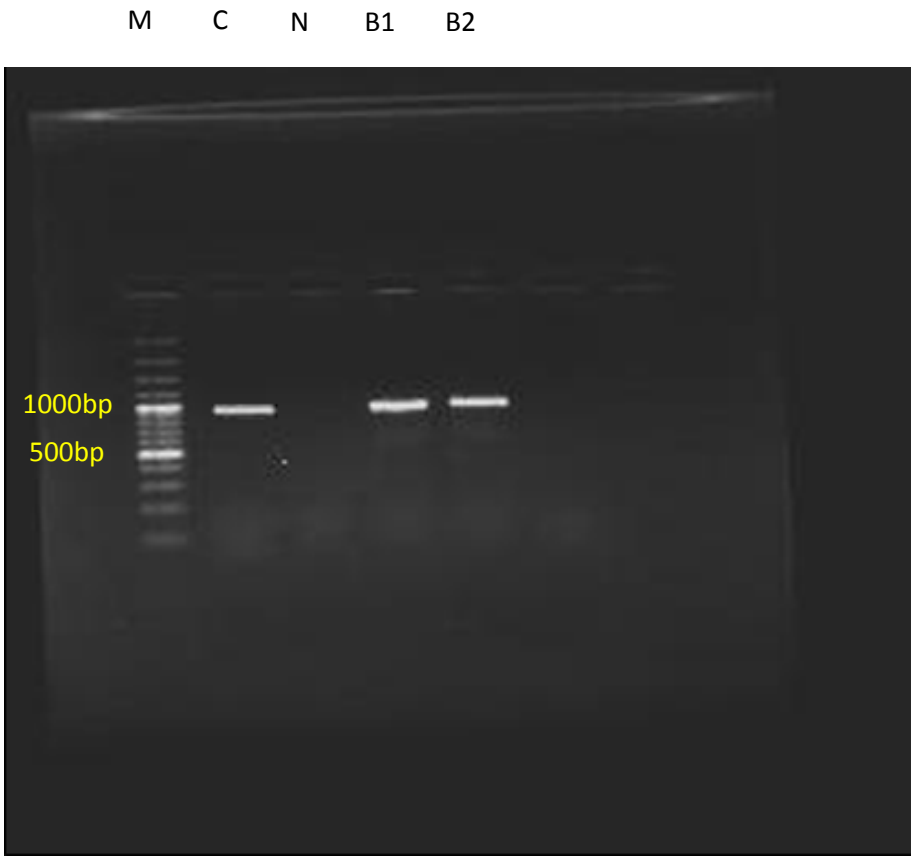


Figure 10. Gel electrophoresis of PCR amplified products of serum samples suspected for brucellosis.

M: 100 bp DNA C: Positive control; N: Negative control; B1 and B2: Test samples;

Keid *et al* (2010) carried out a comparative study between blood PCR assay and serum PCR assay, where sensitivity of blood PCR was 97.14% and that of serum PCR was 25.71%. Serum PCR showed little value for the direct diagnosis of canine brucellosis as the assay had a low diagnostic sensitivity and fewer positive dogs were detected by this test than by blood PCR. In another study carried out by Takele *et al* (2009), a comparison between PCR assay with serum and whole blood samples for detection and differentiation of *Brucella melitensis* was done. They suggested that though the use of serum-PCR may lead to assay simplification and shorten turnaround time, the optimal clinical specimen for this test was not serum but whole blood, which leads to maximum assay sensitivity. Hafez *et al* (2011) recommended the use of the blood PCR assay for accurate diagnosis of ovine brucellosis especially in the early stage of infection, which was difficult to achieve by the applied serological tests.

4.2 Evaluation of the overall efficacy of the modified RBPT

It can be concluded from this study that the mRBPT has a higher sensitivity and a negative predictive value than the other serodiagnostic tests like cRBPT, STAT, ELISA and CFT commonly used for brucellosis. Its specificity and PPV were found to be better than cRBPT and STAT but lower than ELISA and CFT. The test can be used in the penside diagnosis of bovine brucellosis in combination with other tests like STAT with better results than conventional RBPT which is routinely used as a pen side test for brucellosis. ELISA and PCR are not cost effective tests when screening has to be performed at herds with large number of animals. In such situations, mRBPT can offer an advantage of increased sensitivity of screening compared to cRBPT and STAT.

CHAPTER V

SUMMARY AND CONCLUSION

Brucellosis is a major zoonosis caused by the small, non-motile Gram-negative and intracellular coccobacilli belonging to the genus *Brucella*. It causes a great economic loss to the livestock industries through abortion, infertility, delivery of weak or dead offspring, increased calving interval and reduction of milk yield. Brucellosis is mainly a disease of sexually matured animals and commonly transmitted to other animals by direct or indirect contact with infected animals or discharges such as aborted fetuses, placental membranes or fluids. Infection to humans results from direct contact with infected animals and consumption of contaminated milk and milk products. Isolation, serological tests and molecular methods are adopted for the diagnosis of brucellosis in cattle herds with history of reproductive disorders.

The diagnosis of brucellosis is usually carried out by serological testing. The absence of a perfect reference diagnostic test (gold standard) makes evaluation of serological tests difficult. Although isolation of the pathogen is considered as the confirmatory test in bacterial infection, bacterial cultures are often negative in the case of brucellosis because of the intra-cellular and fastidious nature of the pathogen. The test is relatively difficult to use in the field in rural areas. Hence, the use of bacterial culture as gold standard may result in incorrect estimation of specificity, thereby misdiagnosing infected animal as non-infected.

The classical Rose Bengal Plate Test (RBPT) is often used as a rapid screening test and considered as a reliable test in the diagnosis of brucellosis; however it could sometimes give a false positive result because of S19 vaccination or due to false positive serological cross reactions. Also gram negative bacteria such as *Yersinia enterocolitica*, *Vibrio cholera*, *Campylobacter fetus*, *Bordetella bronchiseptica* and *Salmonella* spp. may cross react with smooth *Brucella* spp.

The present study was undertaken to evaluate a modified RBPT, developed recently in the department. Bovine sera samples were analysed to compare the efficacy of the modified RBPT with the conventional serodiagnostic tests like RBPT, STAT and ELISA; and other tests like CFT and PCR.

A total of 200 serum samples from cattle and buffaloes were collected from the veterinary clinics, dairy farms and gaushalas, in and around Ludhiana, Punjab. All the 200 samples were subjected to analysis by modified RBPT (mRBPT), conventional RBPT (cRBPT), STAT, iELISA, CFT and PCR.

In mRBPT, equal volumes (2.5µl each) of RBPT coloured antigen, test serum stained with Coomassie blue dye, biotinylated anti-bovine IgG and streptavidin were mixed thoroughly on a clean glass slide and the slide was observed for 4 mins. Ordinary hand lens was used occasionally for better visibility.

STAT was performed as per the OIE recommended protocol using the plain antigen of *Brucella abortus*. The results were compared with the antigen control tube showing 50% agglutination. A titer of 1:40 or above was considered positive.

iELISA kit was procured from Immuno-Biological Laboratories, America (IBL-America). The test was carried out as per the instructions on the manual supplied with the kit.

In CFT, haemolysin titration, complement titration and antigen titration were carried out as per OIE protocol. Test serum samples were inactivated at 56°C for 30 min. The plates were checked for the presence of hemolysis. The absence of anti-complementary activity was checked in the controls.

For PCR, DNA was extracted from serum samples using the method described by Yamakami *et al* (1996). PCR assay was carried out using *Brucella* genus specific F4/R2 primers (Romero *et al*, 1995).

A total of 104 samples were detected positive by mRBPT. The test was found to have a sensitivity of 95.88% and a specificity of 89.32%. The positive predictive value (PPV) of this test was calculated as 89.42% and the negative predictive value (NPV) was 95.83%.

Out of the 200 samples, 97 were detected as positive by cRBPT. The test showed a sensitivity of 93.33% and a specificity of 88.18%. This test showed a PPV of 86.6% and NPV of 94.17%.

STAT was found to have a sensitivity of 94.25% and a specificity of 68.14%. A titre of 1:40 and above was considered as positive. Among the 200 serum samples, 81 samples had titres below 1:40, 36 samples had a titre of 1:40 and 83 samples had titres more than 1:40. STAT could detect 118 out of the 200 samples as positive.

Out of the 200 samples 75 were detected as positive by iELISA. Sensitivity of this test was calculated as 74.47% and specificity was found to be 95.24%. PPV of this test was 93.33% and NPV was found to be 80.65%.

The CFT showed a sensitivity of 82.8% and a specificity of 93.46%. PCR could detect only three out of 200 serum samples as positive for brucellosis. Agreement between mRBPT and cRBPT was found to be higher than the agreement between mRBPT and other tests. As cRBPT is widely used as a screening test for brucellosis, this agreement combined with the higher specificity and sensitivity of mRBPT ensures that it can serve as a more efficient screening test than cRBPT.

The modified RBPT showed the highest sensitivity of all the tests (95.88%) which can be attributed to the lesser number of false negative results obtained with mRBPT compared to the other tests. The anti-bovine IgG played a role in increasing the sensitivity as, if less number of antibodies against the infectious organism is present in the serum, anti-bovine IgG binds to these antibodies resulting in an increase in the clump size. Streptavidin binds to the biotinylated IgG increasing the clump size further by up to 4 fold due to the four binding sites for biotin present on each molecule of avidin.

The specificity of the mRBPT was found to be lower than iELISA and CFT. More number of false positive cases could have possibly caused reduction in its specificity. This may be due to the cross reactions of anti-bovine IgG with nonspecific antibodies or bacteria.

Agglutinate in case of mRBPT could be differentiated into two parts, the antibodies were blue in colour due to the Coomassie blue dye and the antigen having pink colour due to the Rose Bengal dye. Each clump had both the blue and the pink colour, which aided in identification of the true agglutinates. Antigen and antibodies which did not participate in agglutination reaction could be viewed under the microscope as blue and pink particles lying separately.

The results obtained in this study suggest that mRBPT has a higher sensitivity than other serodiagnostic tests commonly used for brucellosis. The specificity of mRBPT was higher than cRBPT and STAT but lesser than that of ELISA and CFT. The test can be used in the diagnosis of bovine brucellosis in combination with other tests like STAT. As ELISA and PCR are not cost effective tests when screening has to be performed at herds with large number of animals, using mRBPT can have an advantage of increased sensitivity of screening when used in combination with STAT.

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