PREVALENCE AND ZOONOTIC IMPORTANCE OF BRUCELLOSIS IN CATTLE IN AND AROUND BIDAR

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CERTIFICATE

This is to certify that the thesis entitled **"PREVALENCE AND ZOONOTIC IMPORTANCE OF BRUCELLOSIS IN CATTLE IN AND AROUND BIDAR"** submitted by **Mr. SHANTVEER GOPA, I.D. No. MVNK-1911** in partial fulfilment of the requirements for the award of **MASTER OF VETERINARY SCIENCE** in **VETERINARY GYNEACOLOGY AND OBSTETRICS** of Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar is a record of bonafide research work carried out by him during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, association ship, fellowship or other similar titles.

BIDAR
FEBRUARY, 2022

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Affectionately
Dedicated to
My beloved late
Parents

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Bidar

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LIST OF ABBREVIATIONS

%	Per cent
et al.	Co-workers
@	At the rate of
^{0}C	Degree Celsius
>	More than
<	Less than
=	Equal
±	Plus or minus
AB-ELISA	Avidin Biotin Enzyme Linked Immunosorobant Assay
Ab	Antibody
Ag	Antigen
C-ELISA	Competative Enzyme Linked Immunosorobant Assay
CFT	Compliment fixation test
Fig.	Figure
H_2O_2	Hydrogen peroxide
HRP	Horse Raddish peroxidise
H_2SO_4	Sulphuric acid
I-ELISA	Indirect-Enzyme Linked Immunosorobant Assay
IgG	Immunoglobulin-G
IgM	Immunoglobulin-M
I.U	International unit
IQR	Inter Quartal Range
LFA	Lateral Flow Assay
M	Molar

MRT	Milk Ring Test
MET	Mercapto Ethanol Test
Min	Minute
mL	Millilitre
η	Nano meter
No.	Number
OD	Optical densitity
OIE	Office International des Epizooties
OPD	O-Phenyldiamine
p	Level of Significance
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PP	Percentage positivity
RBPT	Rose Bengal Plate Test
rpm	Revolutions Per Minute
RT	Room Temperature
STAT	Standard Tube Agglutination Test
Sl	Serial
S-LPS	Smooth Lipopolysaccharide
V/V	Volume/Volume
Viz.	Which are/Namely/That is to say
WHO	World Health Organization
hrs	Hours
®	Trade name
etc	Et cetera

Introduction

I. INTRODUCTION

India is the world's second-largest cattle producer with a population of 8.5 millions cattle in Karnataka and 0.173 millions in Bidar district (20th livestock census by DAHD GOI, 2019) producing 22 percent of the world's milk with an annual output of 10936.438 tonns in Karnataka, 211.943 tonnes in Bidar (The economic survey by GOI, 2019). About 80.83 million households are depends on livestock for their income in India. Bovine brucellosis is widespread in India due to increase trade and rapid movement of livestock (Isloor *et al.*,1998 and Renukaradhya *et al.*, 2002) and prove disastrous wherever they go unchecked. International Trade Rules restrict import and export of animal due to threat of brucellosis from endemic countries. Aggregated data at national or regional levels do not capture these complexities of disease dynamics and high quality research is essential for an accurate assessment of disease (Dean *et al*., 2012).

Brucellosis is the world's second most prevalent zoonosis next to Rabies (FAO, 2005).however, it is also one of the world's seven most neglected zoonotic diseases and it is more common in nations with poor animal health and public health programme (Capasso, 2002). It has been eradicated in many countries such as Europe, Australia, Canada, Japan, Israel and New Zealand although cases still occurs in people returning from endemic countries (Geering *et al.*, 1995). Nonetheless, it continues to be an uncontrolled concern to Africa, Mediterranean, Middle East and parts of Asia (Refai, 2002).

Brucellosis is a highly contagious disease caused by a gram-nagative, aerobic, facultative intracellular bacteria of the genus *Brucella* that are pathogenic for a wide variety of animals and human beings. Bang identified *B. abortus* was casual agent for brucellosis in cattle and humans in Copenhagen in 1897 and Devid Bruce found *Brucella melitensis (B.melitensis)* causes Malta fever in 1886. In June 1905, Maltese scientist Zammit identified unpasteurized goat milk as the key etiologic factor of undulant fever, despite the fact that no one understood at the time that this brucellosis is a one of the zoonotic disease (Berhanu and Pal., 2020).

Brucellosis was originally identified in India in 1942 and it is currently endemic in the country. In India and in Karnataka seroprevalence in cattle was 8.3 percent and 7.37 per cent (Shome *et al*., 2019). The zoonotic significance of brucellosis is related to the prevalence of brucellosis in the local animal population; In North Karnataka, the overall seropositivity of brucellosis in suspected cases was 5.1 per cent (Patil *et al*., 2016) The World Health Organization estimates globally 832,633 cases of human brucellosis per year and annual economic loss in India around US$ 340 billion per year (Sing *et al*., 2015). Prevention of abortions can add 2.63 million new female calves and increase milk production by 5% per annum.

Transmission of *brucella* occurs via infected placenta, aborted fetuses, fetal fluids, vaginal discharges, urine and semen of infected animals. In humans, transmission depends on food habits, milk products, unhygienic husbandry practices and human-to-human transmission of the infection may be through breastfeeding, trans-placental transmission and blood transfusion (Berhanu and Pal, 2020). The common symptoms in

humans are undulant fever, fatigue, malaise, etc. The main symptoms in ruminants are joint ill, metritis, placentitis, abortion (premature or full-term birth of dead or weak calves) which frequently occurs in the second trimester pregnancy, with retention of the placenta, usually asymptomatic chronic carriers after their first abortion and continue to shed *Brucella* in uterine discharges and milk during subsequent pregnancies. Eighty percent of infected animals have *Brucella* in mammary tissues and they continue to release the pathogen in milk throughout their lives (Hamdy and Amin, 2002). As a result, brucellosis screening in herds is critical for effective disease control.

A lot of progress has been made during the last two decades in control of the bovine brucellosis in many countries including India by improving the methods of diagnosis. Several conventional serological tests are available for the rapid diagnosis of brucellosis with some limitations; thus the definitive diagnosis usually requires more than one test (OIE, 2009). The most widely used serological tests for diagnosis of brucellosis are Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT), 2-Merquaptoethanole Test (2-MET) and ELISA (Maurice *et al*, 2013). The diagnostic value may be questionable on individual basis due to high homology among *Brucella* species, neither a single serological test nor combination of tests detects all infected animals. However, for screening of herd these tests remain ideal. Other than serum, *Brucella* antibodies are also excreted in milk which is used for screening of herd by Milk Ring Test (MRT) to know the prevalence of *Brucella* infection. It may give false-positive results shortly after parturition, near the end of lactation and when mastitis is present (Alton *et al.*, 1988). The improvements in diagnostic methods could benefit the effective control and eradication of bovine brucellosis. Regular screening of herd is utmost

important in control disease to understand pattern of disease occurrence This study was designed with the goal of studying the prevalence of brucellosis in humans and cattle in and around Bidar and comparing simple and sensitive methods for its diagnosis. In light of the aforementioned facts, the research was conducted with following objectives;

1. To study prevalence of Brucellosis by RBPT, MRT and Indirect-ELISA in Cattle.
2. Comparative evaluation of RBPT,MRT and I-ELISA in diagnosis of Brucellosis.
3. To study the zoonotic importance of Brucellosis.

Review of Literature

II. REVIEW OF LITERATURE

Brucellosis is an important re-emerging zoonotic disease with a worldwide distribution. It is still an uncontrolled serious public health problem in many developing countries including India (Mantur, 1998).The review of literature is mentioned under three sub headings as below.

2.1 Prevalence of brucellosis in cattle

In India, the existence of the disease has been over a long time. Very first a systematic survey was made by Polding (1942) and reported that it is a common cause of contagious abortion in India.

Several studies and serological evidences suggested that brucellosis is highly endemic in most part of the India (Renukaradhya *et al*., 2002; Pathak *et al*., 2014; Chand *et al.,* 2015; Islam *et al.,* 2018; Shome *et al*.,2019). Highest percentage of true prevalence (23.51%) was reported in Panjab (Shome *et al*., 2019). In Bihar and Assam were 0.3% and 15.9 % respectively by Dekha *et al.,* (2021). The prevalence of disease was reported in both organised and private farms.

Isolation remains a gold-standard for diagnosis brucellosis. However culturing has several drawbacks such as low sensitivity and impractical as screening test. Currently, diagnosis of brucellosis is almost based on serological test (Godfroid, 2002). The ineffective state of the host indirectly determined by RBPT, STAT, 2-MET and ELISA in humans and animals and by MRT in lactating animals (Reddy *et al*., 2014).

2.1.1 Milk ring test (MRT)

For many years MRT has been used for the diagnosis of dairy cows infected with *B.abortus,* by detecting antibodies in milk samples (Alton *et al.*, 1975). It is particularly useful on bulk milk samples and is effective for screening and monitoring small dairy herds for brucellosis. In large herds (>100 lactating animals) and in early infection, the MRT does not show proper sensitivity and milk from recent parturition, near the end of lactation and during the mastitis may give false positive results (Alton *et al.*,1988). Though MRT is a known herd based test, in the present study, the individual milk samples were tested to draw correlation between milk and seropositivity.

Trangadia *et al.*, (2010) found more number of samples becoming positive by ELISA (22.18%) than the MRT (12.82%) where as Muttanagouda *et al*. (2014) found more positive MRT (19.56%) than the I-ELISA(5.76%) when the test is applied to screen organised dairy farm. Shome *et al*. (2015) found 2.55%, (1657) positive by MRT out of 64,818 milk samples.

Kumar *et al.* (2016) did cross-sectional study on brucellosis, a total of 483 milk samples were collected from lactating cows from selected districts of Tamil Nadu. Overall, 4.35% of milk samples were positive by MRT. The lactating cows were divided into 1st, 2^{nd} to 4^{th} and $\geq 5^{th}$ lactation and the prevalence of *Brucella* was 2.94, 3.06 and 6.48 % respectively.

Sing and Reddy *et al.* (2016) studied prevalence of brucellosis in total 240 cows and found 11 (4.58%) milk samples positive by MRT in Vindhya region of Madhya Pradesh.

Al-Bakri *et al*. (2016) reported a total of 09 (10.34%) of 87 cow's milk samples were positive by MRT for brucellosis.

Dalal *et al*. (2017) screened a total of 299 milk samples of cows around Jaipur and overall 65 (21.7%) positive milk samples was found by MRT for brucellosis.

Raghava and Gogoi (2017) studied brucellosis prevalence and reported 3.4% and 10.53% less number of seropositive by MRT than 4.6% and 12.69% positive by RBPT respectively.

Jyothi *et al.* (2018) reported highest 20 (71.4%) positive by MRT out of 28 cows around Hyderabad.

Al-Mashhadany (2019) screened a total of 130 milk samples from cows randomly and prevalence of brucellosis was 9.2% (12/130) by MRT and also reported that MRT was more sensitive and specific than the standard milk culture method.

Naveen Kumar *et al*. (2019) found more number of positive samples by MRT (19.2%) than RBPT (4.34%); Poonati *et al*. (2020) also found more number of positive samples by MRT (17.8%) than RBPT (3.10%) for diagnosis of brucellosis.

Khan *et al.* (2020) screened a total of 176 milk and 402 serum samples from cattle and buffaloes in three districts of upper Punjab, Pakistan. Anti-*brucella* antibodies were detected highest MRT (37/21.02%) positive samples than RBPT (66/16.4%) and i-ELISA (71/17.7%).

2.1.2 Rose Bengal Plate Test (RBPT)

A modified plate agglutination test, which employs suspension of *B. abortus* cell stained with Rose Bengal dye buffered at pH 3.65, was introduced by United States Department of Agriculture and the test was found to be more accurate than SAT as an indicator of infection (Nicoletti, 1967).

Corbel (1972) reported that RBPT activity was associated with $1G_1$. This was disproved by Alton *et al*. (1975) who proved that IgM was detected more efficiently than IG_1 or $1G_2$ in RBPT. However, Stryszak (1986) opined that both IgG and IgM were active in RBPT.

Morgan and Mackinnon et al, (1969) reported RBPT to be a useful and highly sensitive test in screening animals for brucellosis but with a low specificity. WHO expert committee on brucellosis (1986) recommended RBPT as a screening test with positive sera correlated by CFT for confirmation.

Isloor *et al.* (1998) in a serological survey of brucellosis in cattle in 23 states of India, a total of 30,437 bovine samples, comprising 23,284 cattle were screened by RBPT. The overall percentage of antibodies was 1.9 per cent in cattle.

Chachra *et al*. (2009) reported RBPT for the diagnosis of brucellosis to be an economical, rapid, dependable and frequently used test for the prevalence studies. Ghodasara *et al.* (2010) reported seroprevalence of 11.21 per cent out of 108 bovine serum samples tested by RBPT in Anand district of Gujarat.

Trangadia *et al.* (2010) reported overall seroprevalence of bovine brucellosis as 13.78% (82/ 595) and 22.18% (132/595) by RBPT and ELISA respectively.

Sanogo *et al*. (2013) reported sensitivity and specificity was 54.9% per cent and 97.7 per cent by RBPT for diagnosis of brucellosis in 995 serum samples collected from cattle.

Salman *et al.* (2012) revealed that, the positive reactors on serum samples to both RBPT and ELISA were 22.6% for brucellosis. The sensitivity and specificity of RBPT, ELISA on serum was 92 and 94% respectively.

Senthil *et al.* (2013) found comparatively more number of positive samples for brucellosis by ELISA 11.4% (24/210) and 5.23% (11/210) by RBPT. Priyadarshini *et al.* (2013) carried out a study on serosurvey in Odisha, India. A total of 258 serum samples from cattle and brucellosis seroprevalence rate by I-ELISA, RBPT were found to be 8.14%, 4.26% positive respectively.

Banurekha *et al.* (2013) reported percent positives for *Brucella* antibodies in serum samples as 4.5 and 6.7 by RBPT and ELISA respectively.

Sharma *et al.* (2015) screened 303 serum samples from Gaushala and 125 (41.25%) were positive for brucellosis by RBPT and 142 (46.86%) positive by ELISA.

Krishnamoorthy *et al*. (2015) screened 559 sera samples from 6 organized dairy farms in southern India. The diagnostic tests RBPT and indirect ELISA revealed overall seroprevalence for *Brucella* as 10.20% and 11.63% respectively.

Chand *et al*. (2015) screened 1082 adult bovines by RBPT, ELISA and results shown 171 (15.8%) samples seropositive for brucellosis.

Shakunthala *et al*. (2016) screened a total of 1505 serum samples from cattle for brucellosis and the seroprevalence rate was 11.29% by I-ELISA and 5.91% by RBPT.

Raghava *et al.* (2017) screened a total of 500 serum samples and 4.6% prevalence of brucellosis was found in Karnataka by RBPT.

Kavya *et al.* (2017) evaluated serum samples in south Karnataka by RBPT for diagnosis of brucellosis and found 7.84% positive in the cattle.

Jyothi *et al.* (2018) screened a total of 105 cattle serum samples for the brucellosis and 28, 25 samples positive by RBPT and I-ELISA test respectively.

Basak *et al.* (2019) screened a total of 463 serum samples of which 16 (3.46%) and 28 (6.05%) were found positive with RBPT and i-ELISA respectively.

Leishangthem *et al*. (2019) reported an overall prevalence of 15 (50%) animals (4 cattle and 11 buffaloes) out of 30 animals for brucellosis by RBPT and ELISA test.

Poonati *et al*. (2020) subjected a total of 4,998 individual milk and serum samples and found more number of samples positive for brucellosis by RBPT 17.8 % (894) than I-ELISA. However, MRT was 19.2 % (958) shown highest positive samples for brucellosis.

2.1.3 Indirect-Elisa (i-ELISA)

Engvall and Ljungstorm (1975) found Enzyme Immuno Assay to be useful for diagnosis of brucellosis. The I-ELISA was first developed by Carlsson *et al.* (1976) for the diagnosis of human brucellosis. Since then, Magee (1980): Diaz -Aparicio *et al.* (1994) and Renukaradhya *et al.* (2002) employed i-ELISA for the diagnosis of brucellosis in man and animals respectively.

Many versions of the i-ELISA have been described employing different antigen preparations. conjugates and substrate chromogen. Indirect ELISA is considered to be more of a screening test rather than a confirmatory test for testing of herds affected by false positive serological reactions (OIE, 2004).

Jagapur *et al.* (2013) screened 417 (79 cattle and 338 buffalo) serum samples which were collected from Karnataka by i-ELISA and recorded an overall prevalence of (191) 45.80 %.

Pandian *et al.* (2015) screened randomly 430 cattle from nine districts of Bihar and found seroprevalence of *B. abortus* as 12.2% by ELISA test kit.

Chettri *et al.* (2015) screened a total of 118 serum samples in HF and Jersey cows and seroprevalence rate was 17.5% by I-ELISA. Prevalence was higher in HF than Jersey cows. The incidence of positive samples with history of abortion, repeat breeding, retention of placenta, mastitis and in pregnant was 50%, 21.21%, 16.16%, 11.11% and 7.69% respectively.

Kushwaha *et al.* (2016) conducted a serological survey for brucellosis in 644 cattle in an unorganized dairy farm and reported 210 (32.61%) and 218 (33.85%) positive by RBPT and I-ELISA respectively. Crossbreed and 6-9 years age group cows found highest positive.

Katoch *et al.* (2017) screened serum samples of 132 crossbred cattle for brucellosis from districts of Himachal Pradesh and prevalence rate was found as 19.7% by ELISA test.

Ahasan *et al*. (2017) reported true prevalence of brucellosis in cattle was 9.70% by I-ELISA in Bangladesh.

Shome *et al.* (2019) reported prevalence of brucellosis in cattle and buffaloes at national level as 8.3% and 3.6% respectively. The highest prevalence in the state of Punjab in both cattle and buffaloes as 23.51% and 10.2% respectively by I-ELISA.

Yuguda *et al.* (2019) reported prevalence of brucellosis as 9.15 % (27/295) and 11.19 % (33/295) positive by RBPT and ELISA respectively in cattle. Also stated that ELISA is the standard test for detecting *Brucella* antibodies having more sensitivity and specificity.

Poonati *et al*. (2020) subjected a total of 4,998 individual serum samples and found prevalence of 389 (7.78%) samples positive for brucellosis by I-ELISA.

Lindahl *et al.* (2020) did a cross-sectional study on cattle brucellosis in peri-urban cities with seroprevalence rate at Bangalore 2.9%, Bhubaneswar 2.0%, Guwahati 73.5%, Ludhiana 4.9%, Udaipur 5.9% and overall rate was 17.8% by ELISA test.

Shakya *et al*. (2020) reported the overall prevalence of brucellosis in cattle and buffaloes as 12.13% and 20% respectively in Chhattisgarh state of India.

Deka *et al.* (2021) conducted seroprevalence study in 740 dairy animals in Assam and Bihar. Seropositivity rate was 15.9% (58) in Assam and 0.3% (01) in Bihar by I-ELISA for *Brucella* infection.

2.2 Comparative evaluation of serological tests

2.2.1 Rose Bengal Plate Test, Milk Ring Test and Enzyme Linked Immunosorbent Assay

Saz *et al.* (1987) performed ELISA for the detection of antibodies to *Brucella* species, in comparison with RBPT and concluded that ELISA was the most sensitive test (97.1%) showing greater specificity (96%) and good predictive positive and negative values (98% and 94 %) respectively.

Varasada (2003) tested 344 cattle and 251 buffaloes sera samples for brucellosis in Gujarat and reported an overall seroprevalence of 16.80 and 22.01 per cent by RBPT and I-ELISA respectively.

Chand and Sharma (2004) carried out seroprevalence study of brucellosis in cattle by RBPT and ELISA and reported an overall prevalence rate of 20.47 per cent by RBPT

and 26.50 per cent by ELISA. They opined ELISA to be better than RBPT; because of chances of non-detection of *Brucella* infected animal is rare in ELISA.

Mittal *et al.* (2005) compared RBPT and ELISA by testing 217 cattle and 67 buffalo sera samples. They found ELISA to be more sensitive followed by RBPT for cattle samples, whereas RBPT to be more sensitive followed by ELISA for buffalo serum samples.

Office International des Epizooties (OIE) (2009) reported that, it is imperative to diagnose the brucellosis by a combination of tests, especially in developing countries, where the disease is usually diagnosed by field based simple tests like RBPT, STAT and MRT. These tests are widely used for preliminary screening followed by confirmation by ELISA or molecular detection of *Brucella* DNA by PCR or by isolation of the organism.

Moussa *et al.* (2012) assessed the performance of the RBPT and an ELISA in diagnosing brucellosis in 995 serum samples collected from cattle and reported that the specificity of RBPT and ELISA were 96.1 and 54.9 per cent whereas the sensitivity for both the tests was found to be 97.7 and 95.0 per cent respectively by highlighting the importance of using these two tests in combination as part of any brucellosis control programme.

Salman *et al*. (2012) stated that, the prevalence of *Brucella* antibodies was higher in milk compared with serum samples. Prevalence rate by Milk Ring Test was 32.5%, while using the serum was 27% with the RBPT and 24.4% when using serum-ELISA. The sensitivity and specificity of MRT were 85 and 95% and for RBPT were 92 and 94%

respectively. Milk based tests can provide significant contribution to detect brucellosis compared to other serological tests.

Sengupta *et al*. (2014) tested milk and serum samples from 298 cattle for the diagnosis of *Brucella* antibodies and found more number of positive by serum ELISA30 (10.06%) followed by MRT 22 (7.38%) than RBPT 21 (7.04%).

Gogoi *et al.* (2017) reported prevalence of brucellosis as 10.53%, 12.69% and 13.84% in bovine by MRT, RBPT and I-ELISA respectively. Where as, Kylla *et al*. (2017) found specificity of (2.8%) RBPT was higher when compared to (2.24%) I-ELISA test in cattle.

Kala *et al.* (2018) reported 12.46% and 35.92% of serum samples were found to be positive by RBPT and i-ELISA respectively and showing higher efficacy of i-ELISA followed RBPT. The sensitivity and specificity of RBPT was found to be of 34.68% and 98.48% respectively, considering i-ELISA as a gold standard test.

Chandrashekar *et al.* (2018) recorded seroprevalence of brucellosis by MRT, RBPT, and indirect ELISA were found that 11.08%, 20.40% and 38.77% respectively. However, Jyothi *et al.* (2018) found (26.6%) RBPT were higher specificity than (23.8%) I-ELISA test and (71.4%) MRT found even more positive samples than RBPT and ELISA. Therefore, MRT, RBPT and indirect ELISA in combination can be used for diagnosis of brucellosis.

Naveenkumar *et al*. (2019) screened a total of 483 sera and milk samples from 483 lactating cattle from various places of Tamil Nadu state. The highest prevalence rate was found as 6.62%, 4.34% and 3.10% by i-ELISA, MRT and RBPT respectively.

Basak *et al.* (2019) recorded prevalence rate was 3.46% (16/463) positive by RBPT and6.05% (28/463) by i-ELISA. Similarly, Yuguda *et al.* (2019) reported 9.15 % (27/295) and 11.19 % (33/295) were found positive by RBPT and ELISA respectively. Also stated that ELISA is the standard test for detecting *Brucella* antibodies having more sensitivity and specificity.

Khan *et al.* (2020) stated that, out of 176 milk samples, 37 (21.02%) were positive by MRT. 66(16.4%) and 71(17.7%) bovine sera were found positive for RBPT and i-ELISA respectively. The PPVs were highest for i-ELISA (50%) than RBPT (44%).

Poonati *et al*. (2020) screeneda total of 4,998 individual milk and serum samples and prevalence rate as 19.16%, 17.88% and 7.78% samples were found positive by MRT, RBPT and I-ELISA respectively in cattle.

2.3 ZOONOTIC IMPORTANCE OF BRUCELLOSIS

Human brucellosis is a significant public health issue in India and an accurate prevalence is unknown. Inadequate clinico-epidemiological data, low physician awareness, poor availability of diagnostic facilities contributes towards the continued existence of zoonosis in India. (Handa *et al*., 1998). Screening of family members by tracing index cases of brucellosis in an endemic area will help to collect further unrecognized cases. (Mantur *et al*., 2007). Human brucellosis can be caused by

B.abortus, B.melitensis, B.suis and marine *brucella* species (The centre for Food Security and Public Health, 2009).

2.3.1 Prevalence in humans

Husbandry workers have shown pyrexia of unknown origin (PUO) cases for evidence of brucellosis. Kadri *et al*. (2000) identified 28(0.8%) seropositive cases in a group of 3,532 patients and Sen *et al*. (2002) identified 28(6.8%) seropositive cases in a group of 414 patients with PUO.

Desai et al. (1995) screened 102 human serum samples from Bidar area of Karnataka by RBPT and the prevalence of brucellosis recorded was 5.9 per cent in humans.

Kalla *et al*. (2001) screened atotal 48 patients with polyarthritis and out of which 44 (91.6%) persons presented were positive for brucellosis by RBPT in Kanvari village of Churu district in Rajasthan.

Priyadarshini *et al.* (2013) reported a total 88 serum samples from human beings and were 5.68% and 3.41% positive in RBPT and STAT respectively.

Mantur *et al*. (2006) screened a total of 26,948 blood samples (from adult humans) for serological evidence of brucellosis over a period of 16 years. The RBPT and 2-MET gave positive results in 517 patients and of which 509 had detectable titres by the STAT.

Kochar *et al.* (2007) reported 175 cases of brucellosis from Bikaner (124 males and 51 females) and prevalence rate was 81.14% in occupational contact with animals, 62.28% in handling of infected materials and 16% in household contact by STAT.

Kollannur *et al.* (2007) screened a total 119 sera of in contact persons working in cattle farms were tested and of these 34.4% was found positive for Brucellosis

Reddy *et al.* (2014) studied a total of 201 serum samples and positivity rate of RBPT was 30 (14.92%), STAT 33 (16.41%), 8 (3.98%) to 2-MET and to I-ELISA 39 (19.40%). The prevalence of Brucellosis was found to be highest among individuals in the age group of >50 yrs and lowest in the age group of ≤ 20 years.

Mantur *et al.* (2014) reportedserological diagnostic test's sensitivities were 88.9% (standard RBST), 92.6% (SAT), and 57.4% (2ME). The specificities were found to be 87.7% (standard RBST), 86.2% (SAT), and 95.7% (2ME) by 200 serum samples from BRIM hospital, Belgaum, Karnataka.

Pathak *et al.* (2014) screened 282 serum samples for brucellosis from occupationally exposed humans and prevalence was found 4.25%, 3.54%, 6.02% and 4.96% positive by RBPT, SAT, indirect ELISA and IgG ELISA respectively. None of the sample was positive for IgM ELISA.

Sumankumar *et al.* (2015) stated that the overall prevalence around Junagadh district of Gujarat state was 7.9% and 7% in cattle and 9.3% and 5.3% in human samples by RBPT and STAT respectively.

Kavi *et al.* (2015) screened a total of 190 humans sera samples of which 42.2% were shown positive for brucellosis by RBPT and 28.8% by SAT. About 15.4% of them showed positive result with 2-ME test.

Mangalgi *et al.* (2015) screened a total of 1,733 humans serum samples andprevalence rate of brucellosis were found 10.50% (182), 7.32% (127) and 5.88% (102) by RBPT, SAT and 2-MET respectively.

Patil *et al.* (2016) conducted a study from 2006 to 2015 at tertiary human hospitals in North Karnataka. A total of 3610 serum samples were collected and overall seropositivity of brucellosis in suspected cases was 5.1% by RBPT and STAT test.

Shome *et al.* (2017) conducted survey on a total of 1050 sera samples of humans and prevalence rate for brucellosis found as6.76%, 6.38%, 3.90%, and 2.67% positive by IgG ELISA, RBPT, SAT and IgM ELISA respectively. An overall prevalence recorded was 7.04% in personnel engaged in veterinary health care in Karnataka, India.

Jyothi *et al.* (2018) reported that,7 out of 19 people working in the farm were found to be seropositive for breucellosis by STAT.

Proch *et al.* (2018) screened a total 279 humans serum samples and seroprevalence of brucellosis found as 61 (21.9%), 67 (24.0%), 55 (19.7%) and 150 (53.8%) positive in RBPT, STAT, IgM and IgG ELISA, respectively.

Lakew *et al.* (2019) screened 211 humans serum samples and 5 (2.4%) were positive for *Brucella* infection using RBPT.

Mangtani *et al.* (2020) investigated a total of 1801 humans sera samples and seropositivity for brucellosis was 11 by RBT, 23 by ELISA and 7 by both tests and the overall prevalence was 2.24% (41).

Holt *et al*. (2021) reported seroprevalence rate of brucellosis was 15.1% in bovines and 9.7% in humans by ELISA test in Ludhiana, Punjab.

Niaz *et al*. (2021) screened 302 humans serum samples for brucellosis from Malakand district Pakistan and prevalence recorded as 32.25% in females with age group 21–30 by ELISA ($P < 0.05$).

Ghugey *et al.* (2021) reported 5 (1.3%) and 7 (1.83%) positive for brucellosis in 382 human sera samples by RBPT and IgG ELISA test respectively in Nagpur, Maharashtra.

Global scenario of brucellosis

Brucellosis remains a major debilitating illness. It is more prevalent in western parts of Asia, India, Middle Eastern, Southern European and Latin American countries. Human brucellosis is found to have significant presence in rural/ nomadic communities where people live in close association with animals. Additionally, it is recognized as the world's most common laboratory-acquired infection. True incidence is estimated to be 5,000,000 to 12,500,000 cases annually (WHO). For example, Egypt, the Islamic Republic of Iran, Jordan, Oman, Saudi Arabia and Syrian Arab Republic reported a combined annual total of more than 90,000 cases of human brucellosis in 1990 (Awad 1998). The low incidence reported in known brucellosis-endemic areas may reflect the

absence or the low levels of surveillance and reporting (McDermott and Arimi, 2002). The true incidence of human brucellosis however, is unknown for most countries including India. Human brucellosis is not considered a contagious disease. Hence, clustering could result from common-source outbreaks or time-space clustering of factors that increase the risk of infection (Chomel *et al.*, 1994; Fosgate *et al.*, 2002). The species that may infect man are *B. melitensis, B. suis, B.abortus, and B. canis. B. melitensis* colonizes ovine stock and is the frequent cause of brucellosis, globally in humans. Recent re-emergence in Malta and Oman indicates the difficulty of eradicating this infection (Amato-Gauci, 1995). Sheep and goats and their products are the main sources of infection by *B. melitensis* in humans, but *B. melitensis* infection in cattle is emerging as a potential problem in some southern European countries, Israel, Kuwait, and Saudi Arabia. *B.melitensis* infection is particularly problematic because *B. abortus* vaccines do not protect effectively against *B. melitensis* infection; the *B. melitensisRev1* vaccine has not been fully evaluated for use in cattle. In some South American countries, particularly Brazil and Colombia *B. suis biovar 1* has become established in cattle leading to human infections. The importance of screening of household members of acute brucellosis cases in endemic areas has recently been emphasized (Almuneef *et al.,* 2004; Mantur *et al.,* 2006). This is an important epidemiological step. This must be taken into account by the family clinicians, so that timely diagnosis and provision of therapy occur, resulting in lower morbidity. The recent isolation of distinctive strains from marine mammals and humans has extended the ecologic range of the genus and potentially its scope as a zoonosis. Since new strains may emerge and existing types adapt to changing social and agricultural practices, the picture remains incomplete. Males are affected more commonly

than females which may be due to risk of occupational exposure. Although human brucellosis affects all age groups, it is said to be rare in childhood. However, in areas, where *B. melitensis* is endemic, pediatric cases are seen (Caksen *et al.,* 2002; Mantur *et al.*, 2004a).

Indian scenario of brucellosis

Brucellosis is a significant and increasing veterinary and public health problem in India. In India 80% of the population live in approximately 6,64,369 villages and thousands of small towns have close contact with domestic/ wild animal population owing to their occupation. Hence, human population stand at a greater risk of acquiring zoonotic diseases including brucellosis. The disease has an added importance in countries like India, where conditions are conducive for wide-spread human infection on account of unhygienic conditions and poverty. Species of main concern in India are *B. melitensis, and B. abortus. B. melitensis* is the most virulent and common strain for man and it causes severe and prolonged disease with a risk of disability. *B. abortus* is the dominant species in cattle. Bovine brucellosis is widespread in India and appears to be on the increase in recent times, perhaps due to increased trade and rapid movement of livestock (Renukaradhya *et al,* 2002). The preponderance of natural bull service in rural India, especially in buffalo, is perhaps yet another important factor in the maintenance and spread of infection.The presence of brucellosis in India was first established early in the previous century and since then has been reported from almost all states. (Sehgal, 1990; Renukaradhya *et al.,* 2002), but the brucellosis situation varies widely between states. Several published reports including recent ones indicate that human brucellosis is quiet common disease in India.The seroprevalence rates have been recorded to be as high as

17-34% in high-risk groups like abattoir workers, veterinarians and animal attendants (Appannanavar *et al*., 2012). In a separate study carried out by Mathur (1968) in Haryana, concluded the goats and sheep as the sources of human infection by isolating *B. melitensis* as a predominant strain from human blood as well as milk samples from goats and sheep. As many as 4.2% aborted women were seropositive for the disease (Randhawa *et al.,* 1974). In Gujarat, 8.5% prevalence of brucellosis was recorded in human cases (Panjarathinam and Jhala, 1986). The study conducted by Thakur and Thapliyal (2002) revealed a prevalence rate of 4.97% in samples obtained from persons exposed to animals. The much higher seroprevalence rate has been also noted in specific risk groups such as abattoir workers (Barbuddhe *et al.,* 2000; Chadda *et al.,* 2004). A prevalence of 3% was observed among patients attending Karnataka Medical College, Hubli (Mantur, 1988). A study by Mantur *et al.,* (2004a) reported 93 children with brucellosis in Bijapur with a prevalence of 1.6% by SAT (≥ 1:160). A publication by Mantur *et al.,* (2006) reported 495 adult patients in Bijapur with the prevalence of 1.8%. Subsequent continuation of the study in Bijapur, additional 111 cases were reported (Mantur *et al.,* 2007a, 2008a,b). A case of meningitis due to *B. melitensis biotype* 1 in a 11-month-old infant transmitted by breast milk (Tikare *et al.,* 2008). In a separate study by Mantur and colleagues identified 63 cases at Belgaum Institute of Medical Sciences, Belgaum. In a study at Vellore, Koshi *et al.,* (1971) reported 10 cases of brucellosis diagnosed by serology or by isolation.However, the epidemiological data on this disease is frequently incomplete. This is partly explained by the absence of proper laboratory facilities, lack of awareness of endemicity, under-reporting as well as poor co-operation and exchange of information between veterinary and health services.

Materials and Methods

III. MATERIALS AND METHODS

3.1 Laboratory practices

During the course of research study, glassware and test tubes from Hi-Media, Sisco Research Laboratories Pvt. Ltd., Mumbai were used. The buffers and other chemical reagents were prepared in autoclaved double distilled water. Plastic-wares including syringes, vacutainers, eppendorf tubes, microplates, milk sample collection bottle, micropipette tips were procured from M/s Tarson products Ltd., Kolkata. The borosilicate test tubes used in this study were prepared by soaking them in a detergent solution over night. Next morning they were washed in running tap water followed by rinsing with distilled water. Then, the packed air dried test tubes were sterilized in hot air oven for one and half hour at 160°C. The study was conducted after the approval of the Institutional Animal Ethics Committee. *Brucella abortus* Milk Ring antigen and Rose Bengal coloured antigen were procured from the Institute of Animal Health and Veterinary Biologicals (IAH & VB), Bangalore.

3.2 Prevalence study

A total of 100 serum and 100 milk samples were collected from lactating cattle with history of abortion and repeat breeding and 25 serum samples were also collected from humans, animal farmers / handlers for the present study.

3.3 Collection of samples

In the present study, cases presented to Department of Veterinary Clinical Complex, Veterinary College, Bidar and five organized dairy farms in and around Bidar were selected. The farms with herd size of more than 20 cows, having a history of abortion, infertility, repeat breeding and retained placenta were selected and were designed as farm A, B, C, D and E. The animals of selected farms reared under semi intensive / intensive system and they were fed with green fodder and concentrate. Animal handlers / attenders involved in animal related activities were also included for the present study.

Table 1: Animal and human samples collected from different area

Sl. No	Farms and VCC cases	Farms	No. of Total animals	No. of Total humans
1	Aravatti farm (Shivnagar)	A	18	03
2	Patil farm (Chidri)	B	20	05
3	Ramkrishna Ashram Goshala	C	28	05
4	Deoni farm (KVAFSU)	D	25	04
5	VCC college bidar.	E	09	09
	TOTAL		100	25

3.3.1 Blood sample collection

Blood samples were collected aseptically by using the sterile needle through jugular venipuncture into the vaccutainers without anticoagulant from the selected animals.Approximately 3-4mL of blood samples were collected and allowed to clot with utmost precaution to avoid hemolysis and transported to laboratory at 4°C.

3.3.3 Blood collection from animal attender

The blood of the in-contact owner / animal attender of the cattle were collected by Medical Lab Technician in the primary health centre aseptically and collected specimens were labelled with the specific participant's identification number for screening of Brucellosis.

3.3.4 Separation of serum

Collected blood inclot activator vaccutainer were kept in slant position for 1-2 hours for clotting and centrifuged at 3000 rpm for 3 minutes. Separated serum samples were transferred into the serum collecting tubes (Eppendorf tubes) and stored with proper labeling at– 20℃ in deep freezer until further processing.

3.4 Collection of milk samples

3.4.a Livestock

The udder was thoroughly washed and cleaned with potassium permanganate solution (1:1000) and dried with clean cloth. After discarding few drops of milk, approximately 10 ml from each quarter was collected into sterile screw capped sample collection bottle from 100 animals and transported in ice pack to the laboratory for *Brucella* screening.

3.5 Serological assays

In present study five different serological techniques viz. Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT), 2-Mercaptoethaline Test (2-MET), Indirect Enzyme Linked Immunosorbent assay (I-ELISA) for humans samples and for

animal serum samples Rose Bengal Plate Test (RBPT), Milk Ring Test (MRT) and Indirect Enzyme Linked Immunosorbent assay (I-ELISA) were compared with each other for their respective sensitivities and specificities.

3.5.1 Screening of milk samples

3.5.1 Milk Ring Test (MRT) / Abortus Bang Ring Test (ART)

The standard method suggested by Alton *et al.*, (1988) was employed in the present study to carry out the MRT for individual fresh raw whole milk samples of cattle.

3.5.1a Material required

a) Abortus Bang Ring antigen (*B abortus* S99)

The antigen contain *Brucella Abortus* strain 99 (Weybridge) that is coloured with haematoxylin with acidic pH 3.3-3.7 and packed with cell volume of eight percent.

b) Mid stream fresh raw whole milk samples pooled from four quarters from an individual animal.

c) Sample collection bottle.

d) Borosilicate test tubes.

e) Pooling containers/ tubes, tips, micropipettes, test tube racks, incubater.

3.5.1b Procedure

1 Milk samples and antigen were brought to room temperature (24℃)

2 Milk samples were mixed thoroughly to dispense the cream evenly and 1 millilitre transferred into a narrow test tube.

3 The test was performed by adding 30 μL of antigen to a one millilitre volume of whole milk.

4 Mixture was allowed to stand for a minute so that antigen thoroughly mixed with the milk.

5 The milk/ antigen mixtures were normally incubated at 37℃ for one hour.

6 The test was read using a uniform light source.

3.5.1c Interpretation

Interpretation of test was done as per the methods described by Corner *et al*, (1987).

Colour of the cream ring	Colour of milk column	MRT reading
Intensively blue	White	++++
Definite blue	Moderate blue	+++
Same colour as milk	Same color as cream milk	+ or -
White or slightly blue	Blue milk	-

3.5.2 Screening of serum samples

3.5.2 Rose Bengal Plate Test (RBPT)

In present study Alton *et al.* (1975) technique was adopted to perform RBPT with serum samples from cattle and humans.

3.5.2a Material required

a) Rose Bengal Antigen: *B. abortus* S99 smooth 11 percent cell suspension buffered to a low pH, usually 3.65 (Morgan, 1969).

b) Test serum sample.

c) Control positive and control negative serum (maintained at The Institute of Animal Health and Veterinary Biologicals (IAH & VB) were used)

d) Slides, tips, micropipettes, magnifying lense, etc.,

3.5.2 b Procedure

i. The test serum samples and antigen were brought to room temperature (22 to 24℃).

ii. Only sufficient antigen for the day's test was removed from the refrigerator.

iii. Then 30 µL of each serum sample was added on a clean microscopic slide.

iv. After shaking the antigen bottle well, an equal volume of antigen was placed near each serum drop.

v. Serum and antigen were mixed thoroughly using a clean glass/plastic rod/ microtip for each test to produce a circular or oval zone approximately two centimetres in diameter.

vi. The mixture was agitated gently for three minutes at ambient temperature on a rocker or three-directional agitator or by tilting the slide three times both in clock wise and anti-clockwise direction.

vii Any degree of agglutination varying from mild (+), moderate (++) and strong (+++) positive was recorded as positive. A control positive and negative serum that gives a

minimum positive should be tested before each day's test has begun to verify the sensitivity of test conditions.

3.5.3 Standard Tube Agglutination Test (STAT)

3.5.3a Material required

a. *Brucella* plain antigen: *B. abortus S*99 smooth cells, one per cent cell buffered suspension to a low pH, usually 6.

b. Phenol saline ($Nacl_2$ 0.85 % [w/v] and phenol at 0.5 % (v/v))

c. Test serum sample.

d. Serological tubes, tips, micropipettes, test tube racks and incubator.

3.5.3b Procedure:

i. To the first tube 0.8 ml of phenol saline and to successive tubes 0.5 ml was added.

ii. Then, 0.2 ml of the test serum was added to the first tube and mix thoroughly.

iii. From the first tube, 0.5 ml of mixture was transferred to the next tube and mixed. From this tube 0.5 ml to the next tube and this process continued up to the last tube from which 0.5 ml was discarded.

iv. The above step results in double dilution of the serum 1:5, 1:10, 1:20, 1:40 and so on.

v. Then, each tube was added with 0.5 ml of antigen and mixed well giving a final serum dilution of 1:10, 1:20, 1:40, 1:80 and so on.

vi. In the control tube 0.75 ml of phenol saline and 0.25 ml of antigen were added.

vii. The tubes were then incubated at 37°C for 20 hrs before the result were read.

3.5.3c Interpretations

The tubes were examined without shaking against the dark background with a source of light coming from above and besides the tubes. The highest dilution of the serum which showed 50 percent agglutination was taken as end titre. When the serum agglutination test was used, complete agglutination at dilutions of 1:80 IU/ml and above was considered positive in humans whereas 40 IU/ml as doubtful and below 401.U as negative for brucellosis in case of STAT.

3.5.4 2-Mercapto Ethaline Test (2-MET)

This test was performed as per the method described by Alton *et al.* (1975). The test remains same as that of STAT except for the use of 2-mercaptoethanol (0.1 mol/litre mercaptoethaline solution in normal saline) was added as diluents to each test tube to a final concentration of 0.05 M, and 0.85% saline was used to dilute the antigen rather than 0.85% saline containing 0.5% phenol. *B. abortus* plain antigen for the test was procured from Project Directorate on Animal Disease Monitoring and Surveillance (PD_ADMAS), National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI) . A titre of 80 I.U. and above was taken as positive in humans samples.

3.5.5 Protein-G Indirect ELISA Kit For Bovine Brucellosis.

3.5.5 a Principle

The protein-G based indirect ELISA kit provides sensitive and specific detection of antibodies in serum of cattle. The enzyme-linked immunosorbent assay (ELISA) detects antibodies to smooth lipopolysacchrides (SLPS) against smooth *Brucella spp.* The

sample antibodies bind to *Brucella* SLPS molecules attached to the polysorp microtitre plate wells. Binding of these antibodies is detected by reaction with Protein-G HRP conjugate labeled affinity purified antibodies. Attached complex (antigen+antibodies + HRP conjugate) are detected by addition of chromogen substrate O-Phenylenediamine dihydrochloride (OPD) with H_2O_2. The degree of color that develops (OD measured at 492nm) is directly proportional to the amount of antibody present in the sample. The diagnostic interpretations are made comparing the OD of the sample with the OD of the two control sera.

3.5.5 b Materials required

1. Protein-G based indirect ELISA kit for bovine brucellosis (Plate)
2. ELISA microplate reader.
3. Wash bottle or manual multichannel washing device.
4. Single and multichannel adjustable volume pipettors and disposable micro tips.
5. Non-antigen-coated transfer plates or perplex plates.
6. De-ionized or distilled water, Paper towels/ Towels.
7. Graduated cylinders for mixing conjugate and wash solution.
8. Stopping solution (1 M H_2SO_4).

8. Carrier surface

The ELISA was carried out in polysorp (Nune, Denmark) flat bottomed 96 well plates as described by Voller *et. al* (1976) with slight modification.

Procedure :

The procedure for i- ELISA to detect antibodies against *brucella* in cattle was carried out using Protein-G based indirect ELISA kit made available by Project Directorate on Animal Disease Monitoring and Surveillance (PD_ADMAS), National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Bangalore and it was carried out in Antigen department of Institute of Animal Health and Veterinary Biologicals, Hebbal, (IAH & VB), Bangalore.

1. Antigen (sLPS) were coated each well of the micro-plates (prepared by PDADMAS, ICAR-NIVEDI).
2. Control and test sera were diluted in blocking buffer separately in perplex plate for which 5µL of test and control sera were diluted in 495µL blocking buffer. Serum and blocking buffer were mixed thoroughly 10 times to ensure homogeneity.
3. The diluted test and control sera 100µl were transferred from the perplex plate to the antigen coated microtitre plate as per the ELISA plate layout.
4. The plates were kept for incubation at 37 °C for one hour on the ELISA plate shaker @300 rpm.
5. The plates were taken out of the shaker and washed 3 times with washing buffer.
6. The working dilution of the conjugate was prepared by adding 1.5µl of the conjugate (Protein-G HRP conjugate) from supplied tube to 12ml of blocking buffer (1:8000 dilution).
7. Then, 100µl of the working dilution of conjugate was added to each wells and incubate at 37 °C for one hour on the shaker @300 rpm. (sufficient for one plate)

8. The plates were washed three times with washing buffer.
9. Then, 100µl of Substrate/Chromogen solution (1 OPD tablet (5 mg) to 12ml distilled water followed by the addition of 50 µl hydrogen peroxide 3%) was added to all the wells, (sufficient for 1 plate).
10. The microtitre plate were incubated at room temperature for 7 minutes or wait until a visible yellow colour develops in the strong positive wells by covering with aluminium foil (In dark).
11. After the colour development, the immediately 50 µl of stopping solution was added to all wells. The plates were read in the ELISA reader at 492 nm immediately.

The OD values were converted to the percentage positivity (PP) values.

Interpretation of the results

Percentage positivity (PP) values which were used for the diagnostic interpretations are calculated as:

$$PP = \frac{\text{Average OD values of test serum}}{\text{Median OD of strong positive}} \times 100$$

Any sample that gives PP value above 55% was considered **Positive** and below 55% was taken **Negative**. If value with 55% was considered suspected.

3.6 Statistical analysis

Chi-square test was used to find the significance of study parameters on categorical scale between two or more groups. The statistical software, SPSS 16.0, Medcalc- version 17.1 were used for the analysis of data.

3.6.1 Diagnostic statistics.

The diagnostic statistics was carried out as per the methods described by Mandrekar and Manderkar (2004).

	Comparator kit				
Test kit	Positive	n	Negative	n	Total
Positive	True Positive(TP)	a	False Positive(FP)	c	a + c
Negative	True Negative(TN)	b	False Negative(FN)	d	b + d
Total	TP + TN	a + b	FP+FN	c + d	

The notation in the above table were described as under.

a=Number of sera positive to both conventional and gold standard tests.

b =Number of sera positive to conventional but negative to gold standard tests.

c=Number of sera negative to conventional but positive to gold standard tests.

d= Number of sera negative to both conventional and gold standard tests.

The following statistics were defined:

Sensitivity: Probability that a test result will be positive when the disease is present. (True Positive rate expressed as percentage) = **a /(a+b)**

Specificity: Probability that a test result will be negative when the disease is not present. (True Negative rate expressed as percentage) = **d /(c+d).**

Positive predictive value: Probability that the disease is present when the test is positive

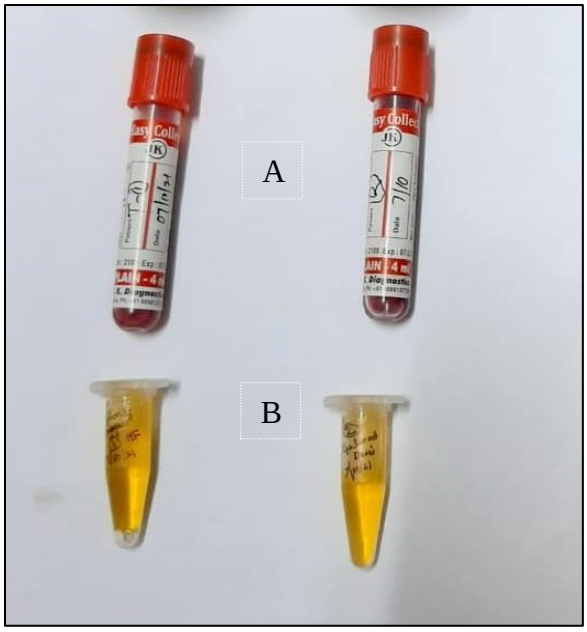
Negative predictive value: Probability that the disease is not present when the test is negative (expressed as percentage) = **d/(b+d).**

Accuracy is the sum of true positive and true negative divided by number of cases. (expressed as percentage) = **a/ (a+c).**

Plate 1: Organised cattle farm selected in present study

Plate 2: Collection of blood sample in cattle

Plate 3: Collection of blood sample from human

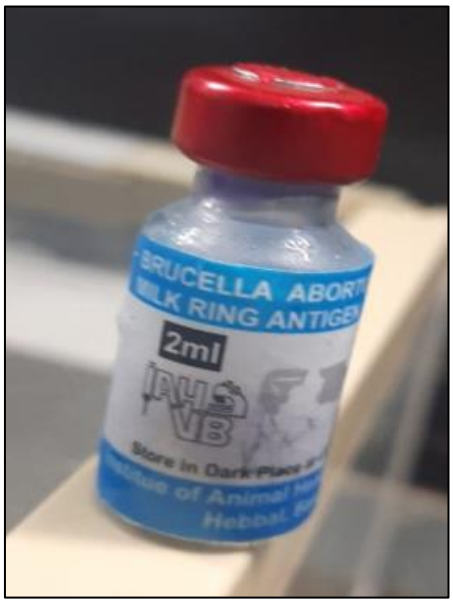


A-Vacutainer clot activator B-Serum in eppendorf tube

Plate 4: Serum samples collected in regular work

Plate 5: Collection of milk sample in cattle

A



B

Plate 6: MRT antigen (A) and Rose Bengal coloured antigen (B) used in present study

Plate 7: Indirect-ELISA kit used in study

Results

IV. RESULTS

4.1 Prevalence of brucellosis by RBPT, MRT and I-ELISA in cattle

Out of 100 cattle sera subjected for RBPT, MRT, I-ELISA, prevalence of 6 per cent in RBPT, 5 per cent in MRT and 8 per cent in I-ELISA was found. The overall prevalence of brucellosis found 8 per cent in cattle by I-ELISA. (Table-2 Fig.1).

Table 2: Prevalence of brucellosis by RBPT, MRT and I-ELISA in cattle

Tests	**RBPT** (n=100)		**MRT**(n=100)		**I-ELISA**(n=100)	
Total no. of positive / Negative	Positive	Negative	Positive	Negative	Positive	Negative
	6	94	5	95	8	92
Prevalence of brucellosis (%)	**6**		**5**		**8**	
Z-test	23.46*, $p<0.05$		34.33*, $p<0.05$		41.98**, $p<0.01$	
CI-95%	1.10% to 9.93%		2.23% to 12.60%		3.52% to 15.16%	

* @5(%) level of significance, ****** @ 1(%) level of significance, CI95%- Confidence of interval.

As per the analysis I-ELISA was found to be statistically significant at a 1% level of significance and positively associated with diagnostic tests of brucellosis as compared to MRT and RBPT.

4.2 Overall prevalence of brucellosis in different farms of cattle.

In the present study, the percent prevalence of brucellosis in selected farms had IQR values of 5 - 16 per cent in case of RBPT, 3.57 - 16 per cent in case of MRT and

5 -22.2 per cent in case of I-ELISA. The highest percentage of seropositive cases was observed in farm A while farm D had no cases. (Table-3, Fig-2. Plate-1,2 & 3).

Table 3: Farm-wise prevalence of brucellosis in cattle by RBPT, MRT and I-ELISA methods

Sl. No.	Farms/VCC Bidar	No. of serum samples tested	No. of milk samples tested	No. of positive			Total
				RBPT (%)	MRT (%)	I-ELISA (%)	
1	**A**	18	18	0 (16.00%)	03 (16.00%)	04 (22.20%)	**7%**
2	**B**	20	20	0	0	01 (5.00%)	**1%**
3	**C**	28	28	02 (7.14%)	01 (3.57%)	02 (7.14%)	**5%**
4	**D**	25	25	0	0	0	**0%**
5	**E**	09	09	01 (11.10%)	01 (11.10%)	01 (11.10%)	**3%**
	Overall prevalance	**100**	**100**	**6 %**	**5 %**	**8 %**	
Chi-square (Yates Correction)				**<0.1**			
P-value				**>0.05**			

The data in the table were tested by Chi-square test (Yates Correction), results showed the statistically significant at a (p>0.05) .

Figure 1: Prevalence of brucellosis in cattle detected in RBPT, MRT and I-ELISA tests

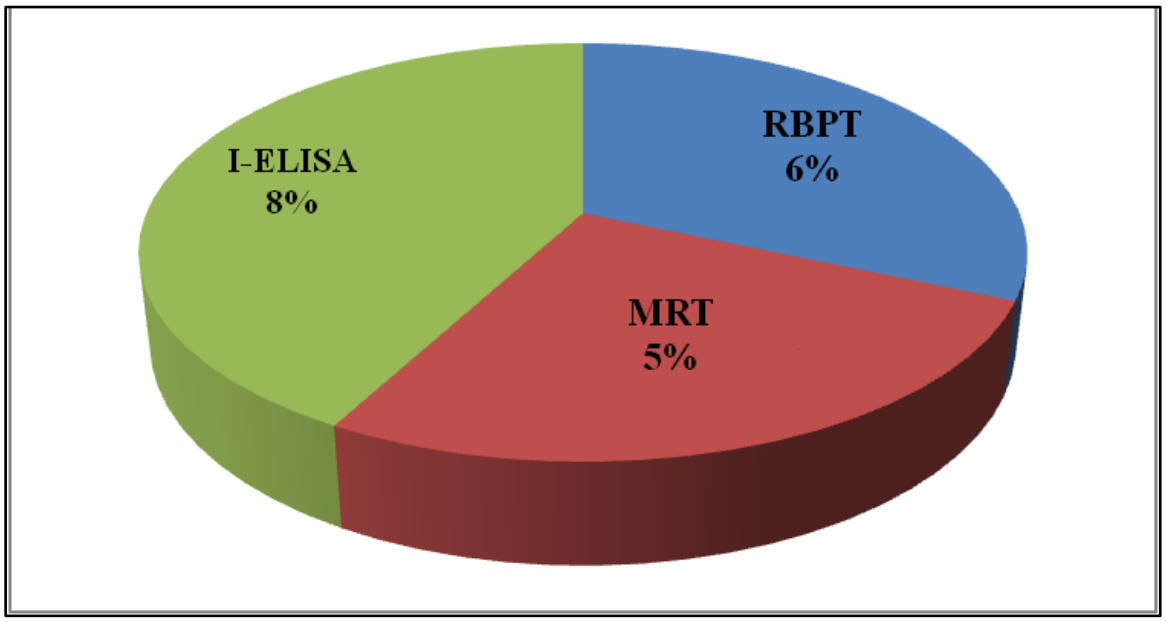
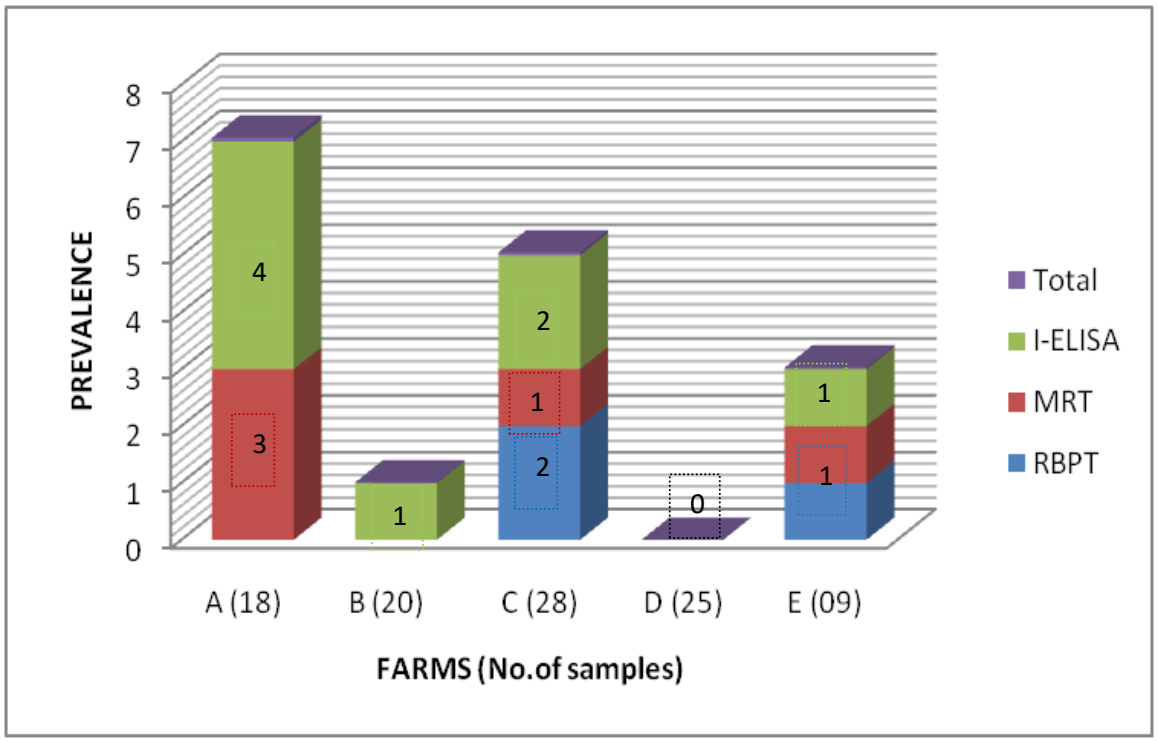


Figure 2: Farm wise prevalence of brucellosis in cattle by RBPT, MRT, I-ELISA tests



4.3 Prevalence of brucellosis in different age groups of cattle

Age-wise distribution and seroprevalence of brucellosis among cattle have been presented in Table-4; Fig.-3. In the present study, the highest seroprevalence was observed in cattle age group > 6 years (9.30 %) followed by the 4-6 years age group (8.57%) and the lowest in age group of 0-4year by RBPT, MRT and I-ELISA, respectively. As age-advanced seropositivity rate was increased comparatively in all diagnostic tests. (Table 4, Fig. 3).

Table 4: Age-wise prevalence of brucellosis with different tests in cattle

Age	Total (n=100)	RBPT	MRT	I-ELISA
0-4 years	22	01 (4.54%)	01 (4.54%)	01 (4.54%)
4-6 years	35	02 (5.71%)	01 (2.85%)	03 (8.57%)
6 and above	43	03 (6.97%)	03 (6.97%)	04 (9.30%)
Chi-square (Yates correction)	<1			
P-value	>0.05			

The hypothesis was tested by Chi-square test (Yates Correction), the results showed the statistically insignificant at a (p>0.05) 5% level of significance.

4.4 Lactation stage-wise prevalence of brucellosis in cattle.

The prevalence rate was recorded more in first and second lactation stages, over the period of time, the results were not significantly correlated (p>0.05), the percent prevalence was reduced. The details of the samples with results are presented in Table 5, Fig. 4.

Table 5: Lactation stage-wise prevalence of brucellosis in cattle

No. Lactation	Samples tested	RBPT	MRT	I-ELISA
1-2	53	03 (5.70%)	03 (6.30%)	**05 (9.40%)**
3 and above	47	03 (6.30%)	02 (3.70%)	**03 (6.30%)**
Total	100	06 (6.00%)	05 (5.00%)	**08 (8.00%)**
Chi-square test		**<1**		
P-value		**>0.05 (p=0.993)**		

There was no significant difference (p>0.05) in occurence of brucellosis between the stages of lactation when tested by three different tests.

Figure 3: Age-wise prevalence of brucellosis in cattle by RBPT, MRT and I-ELISA tests.

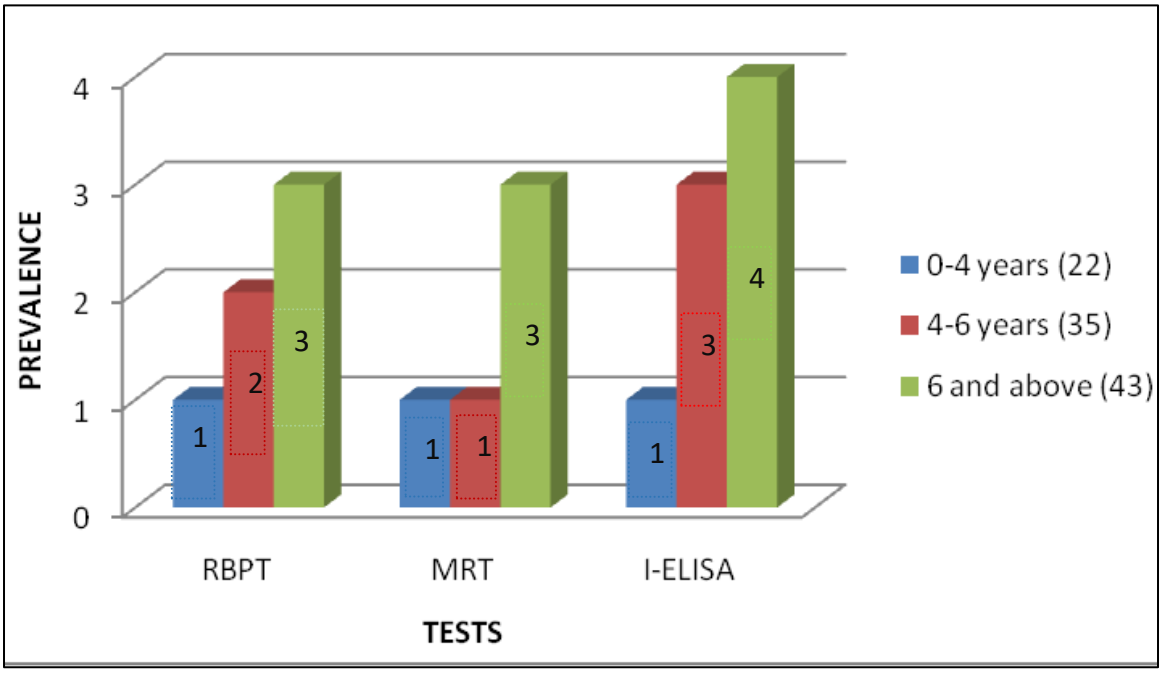
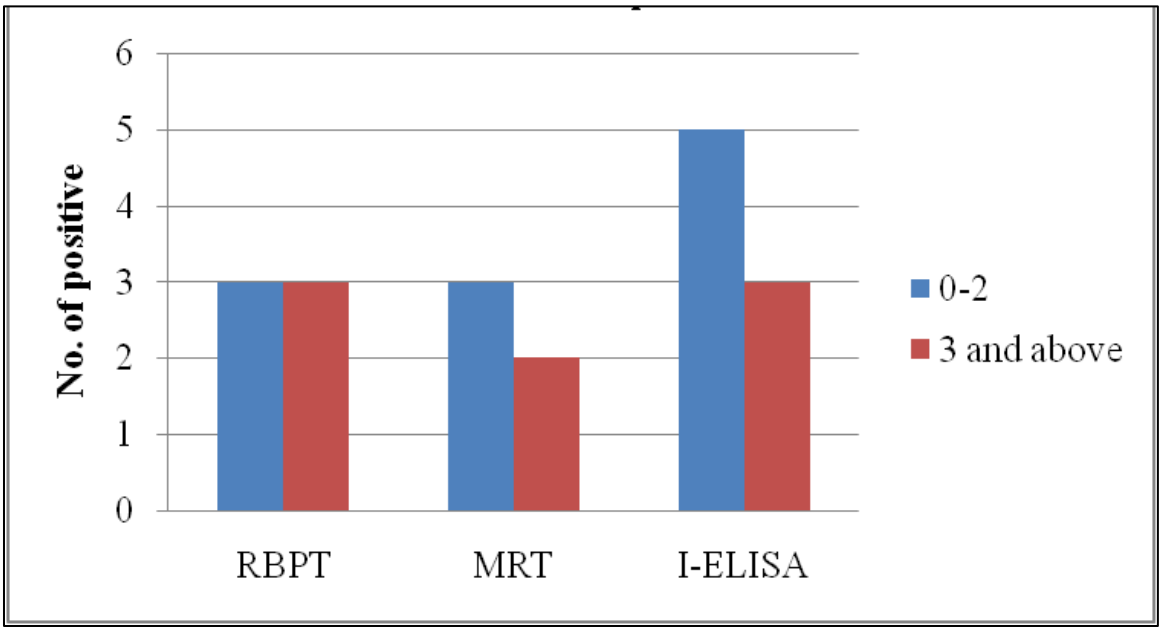


Figure 4: Lactation-wise prevalence of brucellosis in cattle by different tests



4.5 Overall prevalence of brucellosis with different clinical history of cattle.

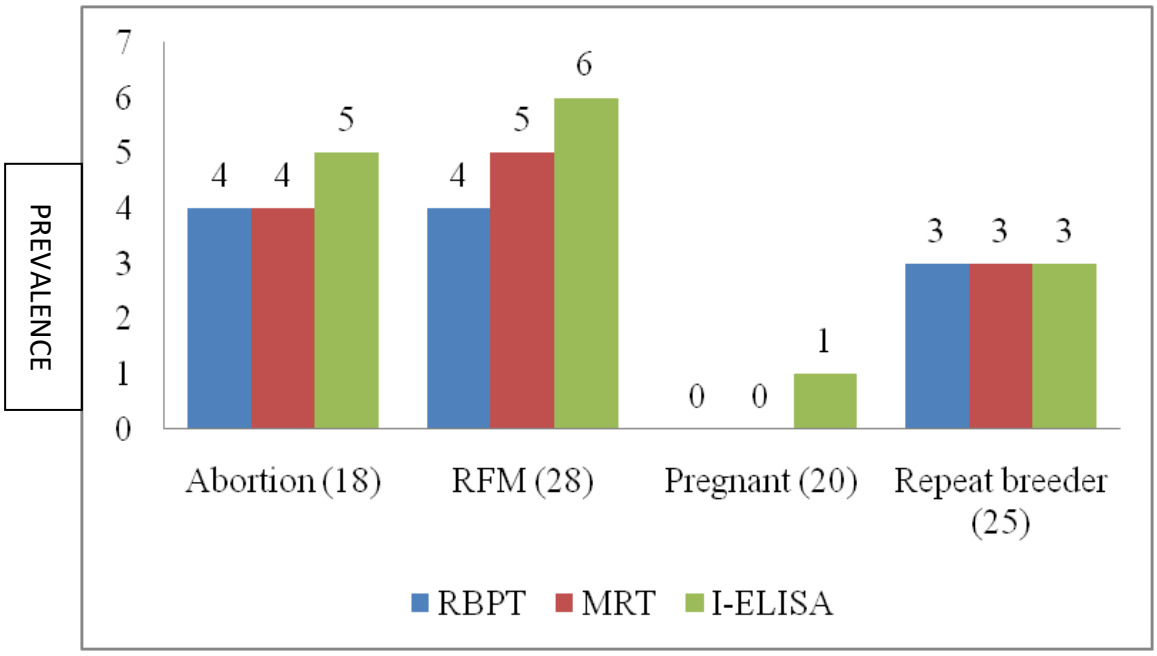
Out of 100 cows, cases found positive for brucellosis 5/18 (27.70%) abortions, 6/28 (21.40%) retention of fetal membrane (RFM), 3/25 (12%) repeat breeder (RB), and 5/20(5%) pregnant cases were found positive for brucellosis. Three abortion cases had a common history of RB and RFM and 2 RFM cases had a common history of abortion while one pregnancy case had the previous history of abortion (Table 6, Fig. 5).

Table 6: Prevalence of brucellosis in cattle with history of abortion, RFM, pregnancy and repeat breeder

History of animal	Samples tested	RBPT	MRT	I-ELISA	P-value
Abortion	**18**	04 (22.20%)	04 (22.20%)	**05 (27.70%)**	**<0.05**
RFM	**28**	04 (14.20%)	05 (17.80%)	**06 (21.40%)**	**<0.05**
Pregnant	**20**	0	0	**01 (5.00%)**	
Repeat breeder	**25**	03 (12.00%)	03 (12.00%)	**03 (12.00%)**	**>0.05**
Chi-square value	**<1**				
P-value	**>0.05**				

Fisher exact and chi-square test was employed to test the hypothetical statement about the different tests and history of the animals. The abortion and RFM history was found to be statistically significant ($p<0.05$) . Within the observations or diagnostic tests were not found to be statistically significant.

Figure 5: Prevalence of brucellosis in cattle with different clinical history



4.6 Comparative (efficacy %) evaluation of RBPT, MRT and I-ELISA.

A battery of three serological tests viz**.** RBPT, MRT and ELISA were evaluated for their sensitivity and specificity. An Indirect- ELISA was considered as the gold standard test to evaluate and to compare other diagnostic tests. The details of the result in respect to sensitivity and specificity of RBPT and MRT evaluated and results were cross tabulated with respect to different diagnostic tests - i-ELISA, considering i- ELISA is the gold standard which is presented in Table-7 and 8; Figures-7 and 8. The sensitivity of RBPT recorded was (85.71%) which is higher than MRT (83.33%) and specificity of RBPT(98.92%) it was found to be higher than MRT (97.87%) and statistically correlated ($p<0.01$).

The positive predictive value (PPV), negative predictive value (NPV) and accuracy for RBPT and MRT were found to be 85.71, 98.92, 98 per cent and 71.42, 98.92, 97 per cent respectively (Table 7; Fig 7).

Table 7: Correlation between RBPT and MRT by keeping I-ELISA as standard test.

	RBPT	**MRT**
True Positive	06	05
False Positive	01	02
False Negative	01	01
True Negative	92	92
Positive Predictive Value (%)	85.71	71.42
Negative Predictive Value (%)	98.92	98.92
Sensitivity (%)	85.71	83.33
Specificity (%)	98.92	97.87

2x2 contingency table chi-square test was employed to explore the TP, FP, FN, and TN. The I-ELISA was recorded more specific and sensitive as compared with RBPT and MRT were statistically differ at (p<0.001).

Table 8: Correlation between PPV, NPV and accuracy by keeping I-ELISA as standard test

Test		**I-ELISA**			**PPV (%)**	**NPV (%)**	**Accuracy (%)**
		Positive	**Negative**	**Total**			
RBPT	**Positive**	06	01	07	85.71	98.92	98
	Negative	01	92	93			
	Total	07	93	100			
MRT	**Positive**	05	02	07	71.42	98.92	97
	Negative	01	92	93			
	Total	06	94	100			

Figure 6: Correlation between RBPT, MRT by keeping I-ELISA as standard test

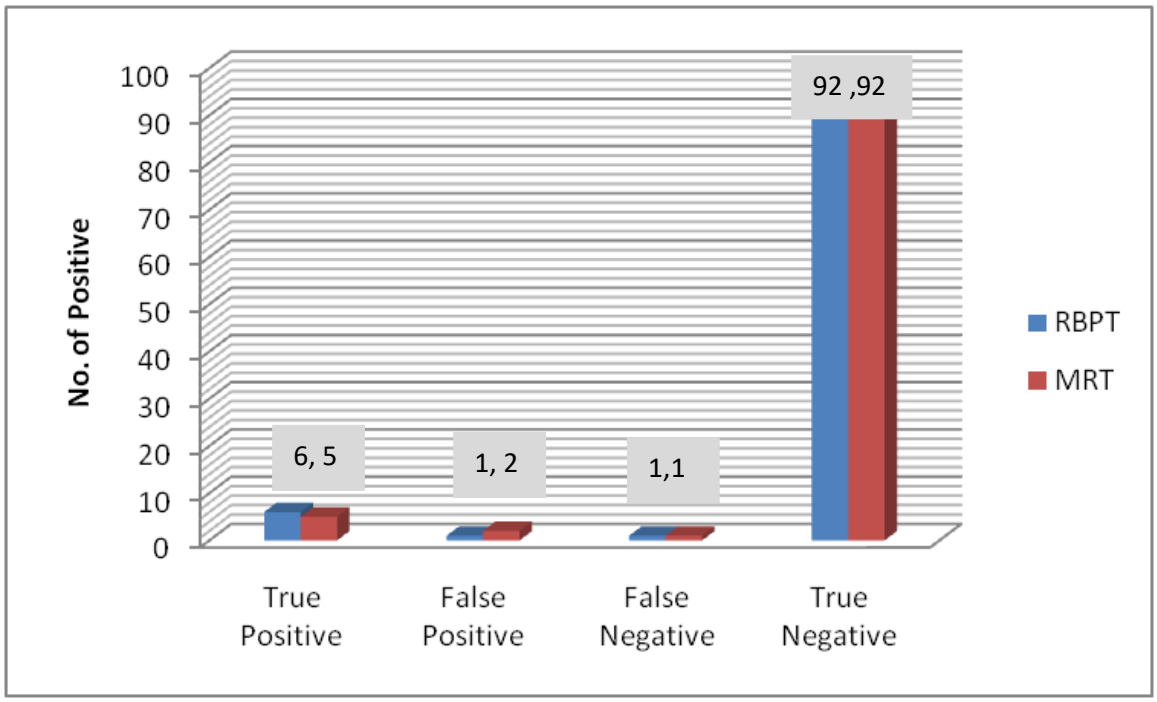


Figure 7: Positive predictive values (PPV%), negative predictive value (NPV%) and accuracy(%) of brucellosis diagnosis

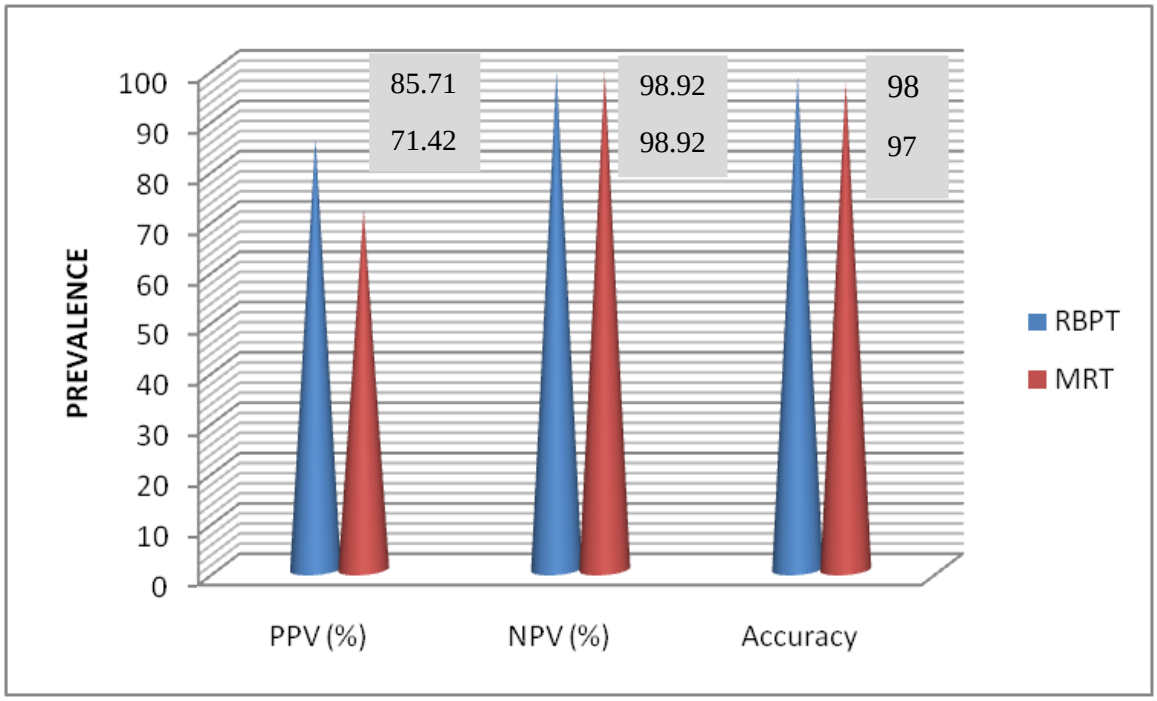
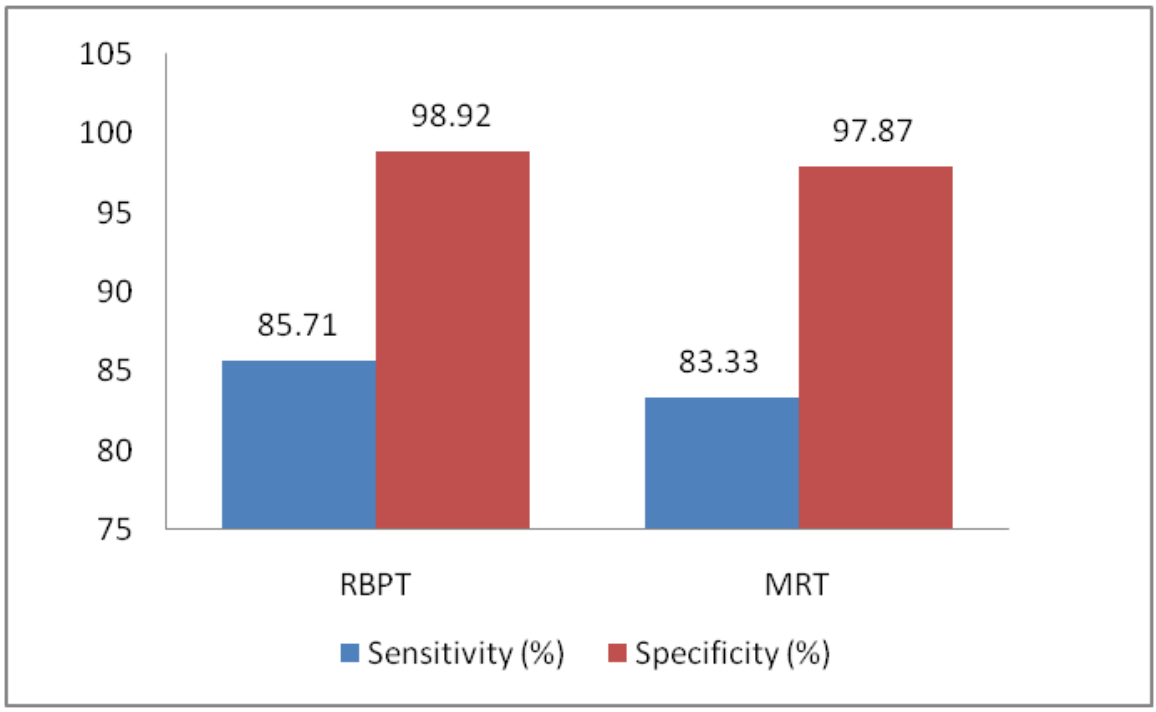


Figure 8: Sensitivity and specificity of RBPT, MRT by comparing with standard test I-ELISA for brucellosis in cattle



4.7 Overall prevalence of brucellosis in humans cases

The overall prevalence of brucellosis was found to be 12 per cent in humans by I-ELISA).

In present study, a total four serodiagnostic tests for humans brucellosis were considered. Out of 25 human sera analysis, 2 (8%) cases were observed positive to RBPT, 1 (4%) to STAT, 2 (8%) to 2-MET, 3 (12%) to IgG-ELISA. It was observed from the table that 03 cases were found to be positive to IgG-ELISA; among these, 2 samples were positive to 2-MET, RBPT and 1 (4 %) sample was positive to STAT. The prevalence of brucellosis was more prevalent in female 1/2(50%) as compared to male 2/23(8.70%) in I-ELISA test. The details of the cases were presented in Table 1, Fig. 1 and different Plates 1, 4, 5 & 6.

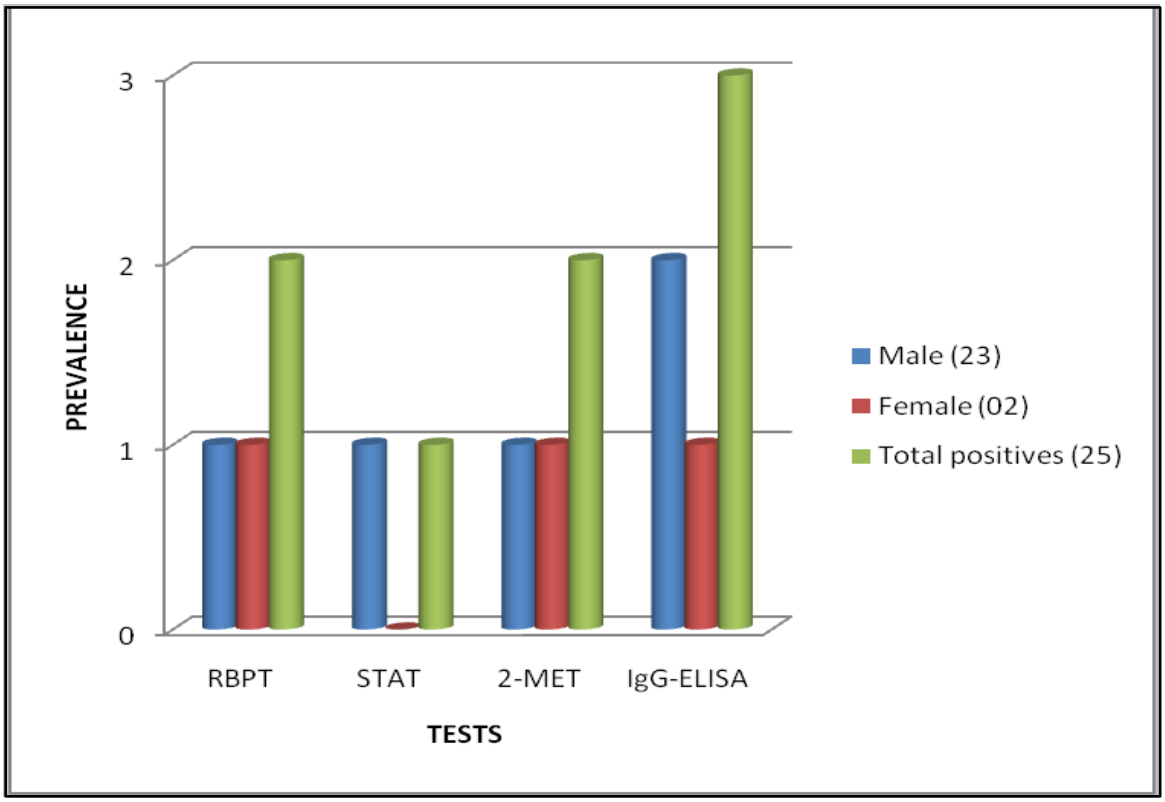
The gender wise correlation with the different diagnostic tests was correlated by the test of proportion and results showed that, the IgG-ELISA was found to be positively associated with a diagnostic test of brucellosis (12%) and it was statistically significant at a 1% level of significance (Z=13.45, p<0.01 CI95% - 0.03% to 18.82%). In case of RBPT (8.74, p<0.05, CI 95% 0.00- 17.21%), STAT (Z= 7.42, p<0.05, CI 95% 0.00- 15.52%) and 2-MET (Z=13.45, p<0.05, CI 95% 0.00-17.21%) was found to be statistically significant at a 5% level of significance (Table-1).

Table 9: Gender wise correlation of different brucella diagnostic tests in humans.

Gender	No. of samples	RBPT	STAT	2-MET	IgG-ELISA
Male	23 (92%)	01	01	01	02 (8.70%)
Female	2(8%)	01	0	01	01 (50%)
Total	25	**02 (8.00%)**	**01 (4.00%)**	**02 (8.00%)**	**03 (12.00%)**
Z-test		8.74* $p \leq 0.01$	7.42* $p \leq 0.01$	8.74* $p \leq 0.01$	13.45** $p \leq 0.01$
CI-95%		0.00% to 17.21%	0.00% to 15.52%	0.00% to 17.21%	0.03% to 18.82%

*** @**5(%) level of significance, ** @ 1(%) level of significance, CI95%- Confidence of interval.

Figure 9: Prevalence of brucellosis in humans as detected in RBPT, STAT, 2-MET and I-ELISA tests



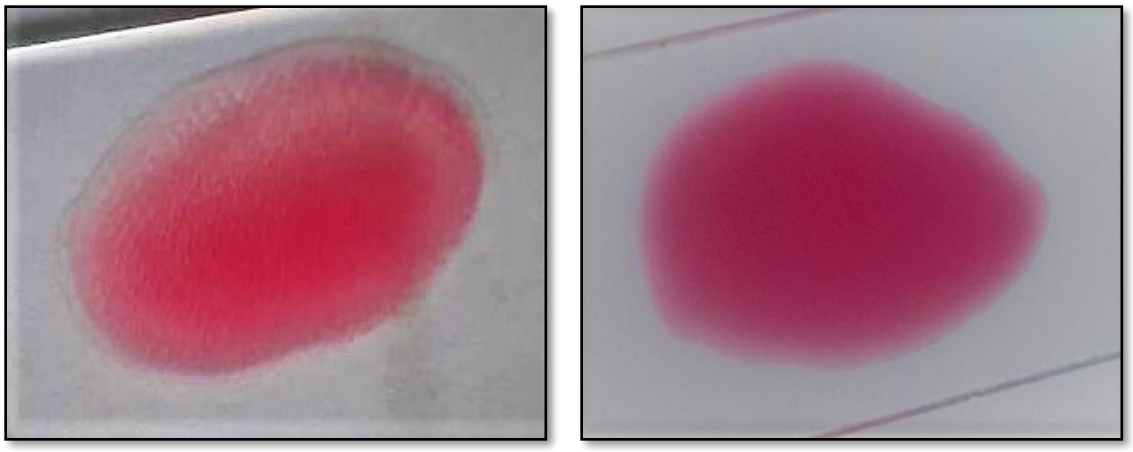


Plate 08. Clumping of Ag-Ab complex **No Clumps of Ag-Ab complex**
Rose Bengal Plate Test Positive **Rose Bengal Plate Test Negative**

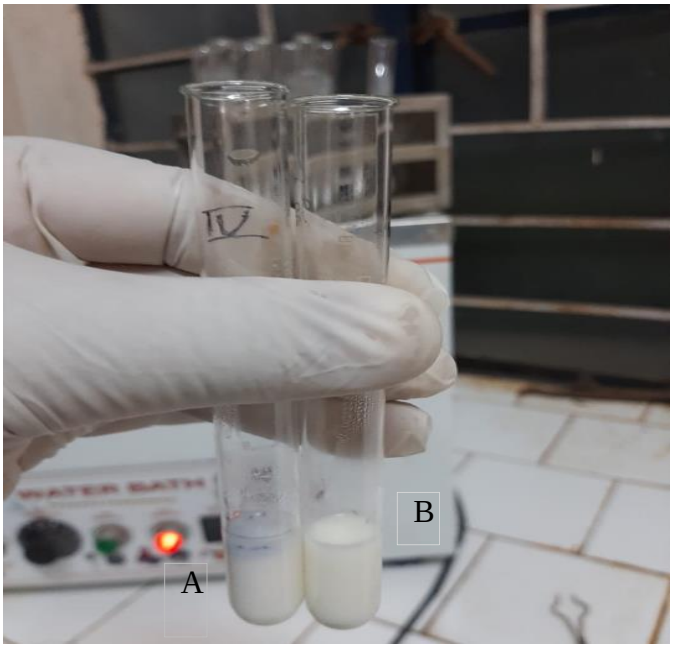


Plate 09. Milk Ring Test. A-Positive sample (blue ring) and B- Nagetive sample

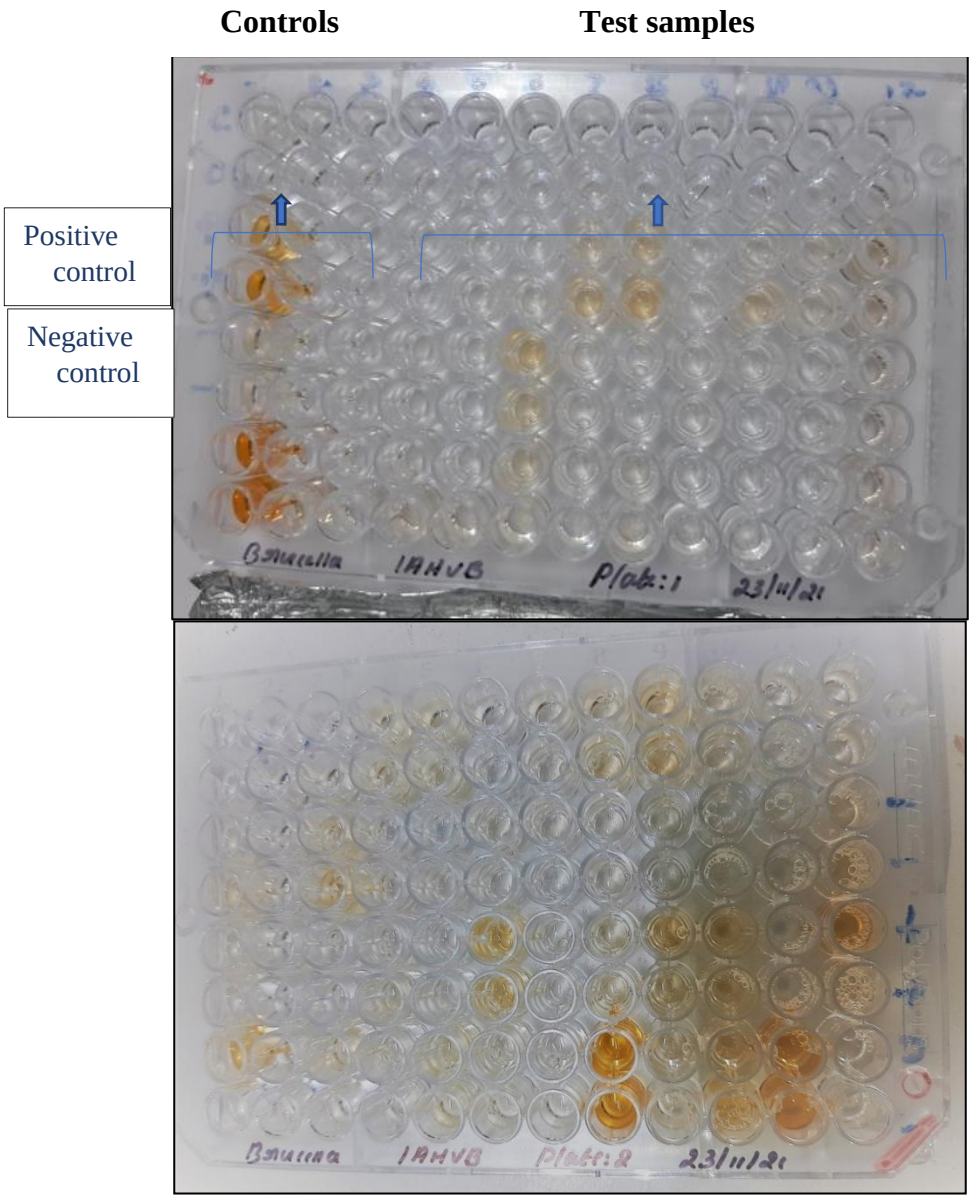
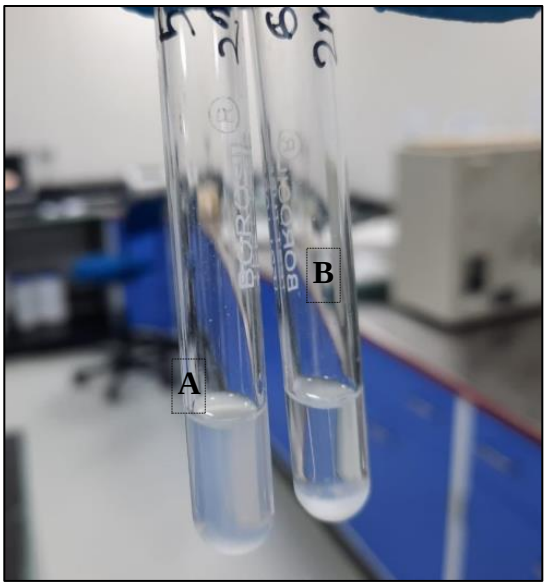


Plate 10: Protein- G based indirect ELISA results for brucella detection in cattle



A-Negative sample B- Positive sample

Plate 11: 2-2-Mercaptoethanol test (2-MET) results for humans brucellosis

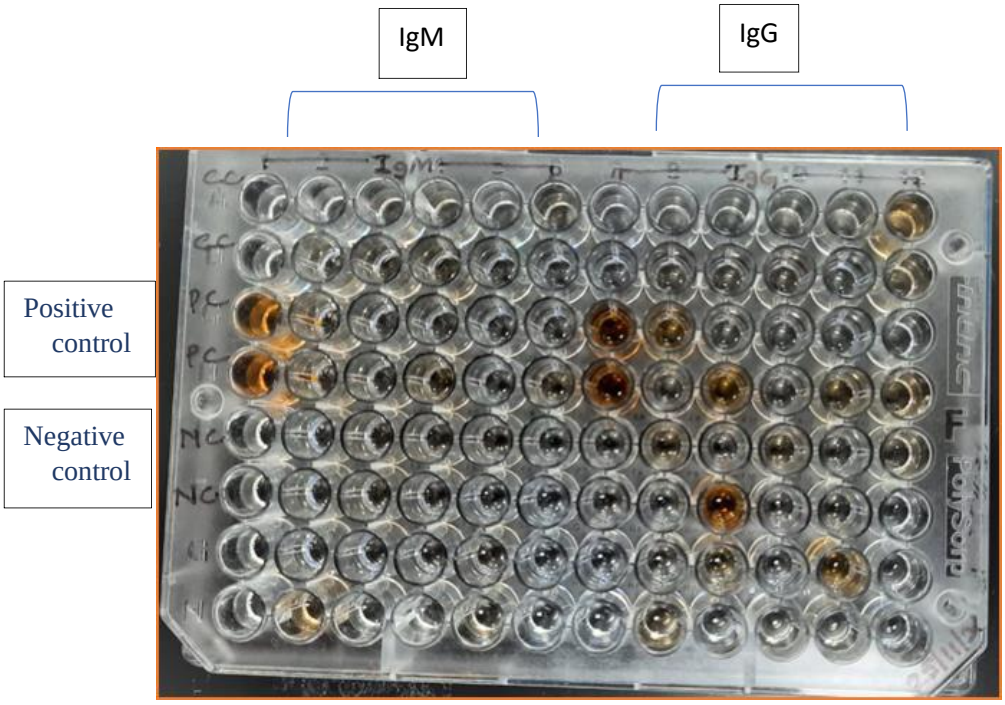


Plate 12: IgM and IgG indirect ELISA results in humans brucellosis

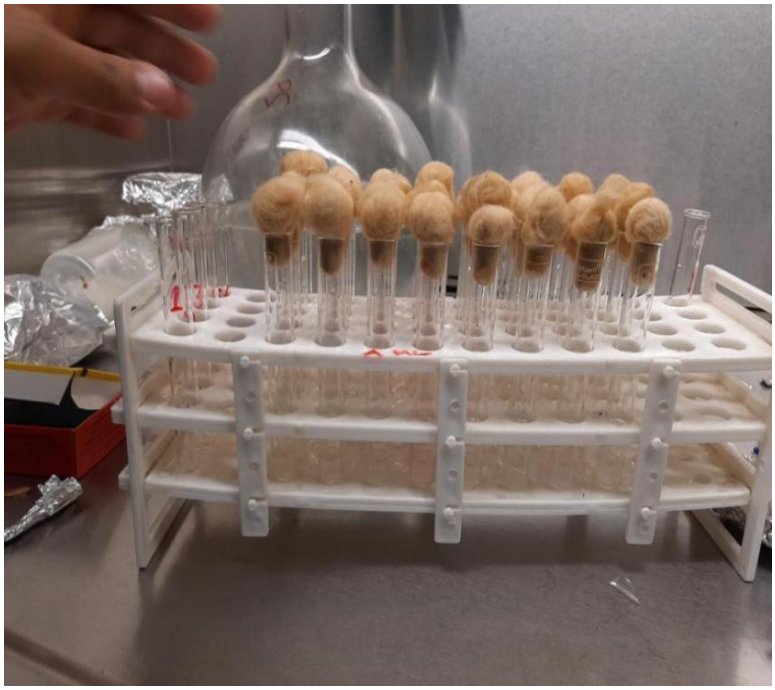
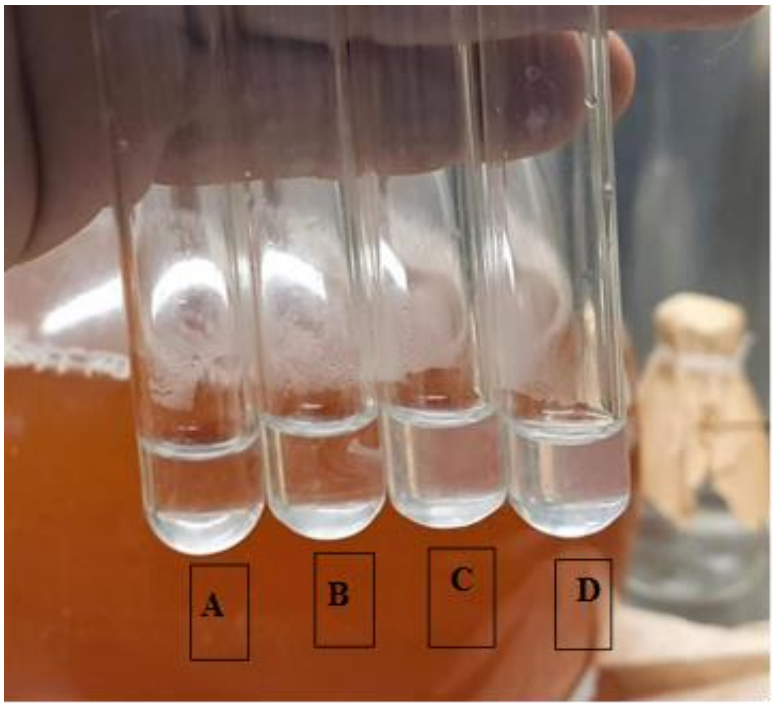


Plate 13a: Pictures showing STAT result without dilution



A-1:120 dilution, B- 1: 80 dilution (positive), C- 1:40 dilution, D- 1:20 dilution

Plate 13b: Pictures showing STAT result with dilutions

Discussion

V. DISCUSSION

Brucellosis is an economically important reproductive disease of livestock and it is prevalent in most of developing countries, including India and also constitutes an important public health problem (Gwida *et al.*, 2011; Ariza, 1999; Corbel, 1997). Brucellosis is a highly contagious zoonotic disease of worldwide importance in cattle. The disease is transmitted to humans through contact with infected livestock. To overcome these problems techniques for diagnosis and prophylaxis have been widely investigated.

In the last decade, much progress has been made in this direction to reduce prevalence of brucellosis through standardization of improved traditional diagnostic methods and application of new molecular procedures. The attention is now diverted towards the development of serological tests of better sensitivity and specificity for quick and economical diagnosis for brucellosis in animals and humans. The only constraint with the serological tests is variation in their results. This is mainly due to the ability of different serological tests to detect various isotypes of immunoglobulins and varied sensitivities to different *Brucella* infections, type and purity of antigen and variation in the duration of incubation period in which the test employed remain positive or negative (Chand and sharma, 2004).

Milk ring test and serological tests are mainly used for the diagnosis of disease in cattle. Detection of *B. abortus* antibodies in milk is considered as the principal method for identifying infected herds and for the diagnosis of brucellosis in an individual animal (Noviello, 2004). The results of a single serological test are not always appropriate to

interpret; rather a combination of tests is often required to ensure accurate diagnosis of brucellosis.

Several workers have reported the detection of *Brucella* antibodies in the sera of infected animals as an indirect means of diagnosis by employing several serological tests like RBPT, MRT, ELISA etc.. These tests have an advantage of screening large population in short time with cost effectiveness and are easy to apply. Efforts are being made to evaluate and to compare tests with conventional tests on the basis of sensitivity and specificity.

Prevalence

5.1 Serological tests

Several serological tests are considered to be suitable for screening herds and individual animals. However, no single serological test is appropriate in all the epidemiological situations. Therefore, a battery of tests was employed to identify the prevalence of brucellosis in livestock.

5.1.1 Milk Ring test

Milk ring test is a screening test of great value for locating infected cattle herds, especially in areas of low prevalence and it is also an efficient means of screening dairy herd. Milk can be obtained cheaply and more frequently than blood samples. The principle of milk ring test is, immunoglobulin's present in the milk attached to fat globules via the Fc portion of the molecule. If antibodies to *Brucella* species are present, it come to the top along with the fat globules and combine with antigen to form a ring in

the milk and fat interface. If antibodies are absent, the fat layer will remain a buff colour and the antigen will be distributed throughout the milk. This test may be applied to individual animals (Alton *et al*., 1988) or to pooled milk samples using a larger volume of milk relative to the pool size.

In the present study, MRT was employed for screening 100 individual fresh milk samples, of these 05 were found positive accounting for a prevalence of 5 per cent. Our results are in close agreement with Sing *et al.* (2016) reported prevalence as 4.58 per cent in individual milk samples by MRT in Vindhya region of Madhya Pradesh. Similarly, Naveenkumar *et al.* (2019) recorded prevalence as 4.34 per cent of milk samples were positive by MRT in Tamil Nadu. Raghav *et al.* (2017) recorded the overall prevalence of 3.4 per cent in individual milk samples by MRT which is comparatively less than present study. Shome *et al.* (2015) recorded 6.71 per cent positive samples by MRT in and around Bangalore district and they opined that confirmatory diagnosis should be done by serological tests. On the contrary, very high prevalence was observed by Jyoti *et al.* (2018) where found 20 out of 28 (71.4%) cows were infected owing to the false positive reactions of MRT. Higher positivity by MRT in cows milk could be due to false positivity of MRT on an account of recent parturition, end stage of lactation and may also be due to sub-clinical mastitis (Alton *et al.,* 1988)

Though MRT is a known herd based test, in the present study, the individual milk samples were tested to draw correlation between milk and seropositivity. The MRT positives 05 out of 100 milk samples had a varied ELISA and serological test results. There was less correlation between MRT and other serological tests. Hence, possible

multiple serological tests should be considered for diagnosis of brucellosis where MRT can be used as surveillance test in the control programme instead of restricting to testing of individual animal.

5.1.2 Rose Bengal Plate Test (RBPT)

The RBPT has proven to be a quick, cheap and simple spot agglutination test with high sensitivity. It is reported to be oversensitive particularly in vaccinated animals (Anonimous, 1986). Because of RBPT being oversensitive, it is used as screening test where all positive samples were retested with a confirmatory test. It is used in many screening test prescribed for international trade. The buffered acid Rose Bengal antigen interacts with serum antibody to produce agglutination which is used for the detection of *brucella* specific antibodies. The test detects IgG_1 type than lg M and IgG_2. The STAT is one of the most widely used tests in diagnosis of brucellosis ever since its introduction by Wright and Smith (1897). But, it has many disadvantages such as non-specific reactions, cross reactivity and prozone because of which its use has been restricted by OIE in international trade (OIE Manual, 2009) even though STAT is extensively used in European Union (Ferreira *et al*., 2003).

Rose and Roepke (1957) introduced a modification of the plate-agglutination test in which the antigen was buffered at pH 4.0 immediately before use. They observed that, at this pH, agglutination of *B.abortus* cells by the non-specific agglutinins of bovine serum was inhibited, whereas the activity of specific *Bucella* antibodies was largely unaffected. Subsequent experience showed that the acid plate-agglutination test was of value as a supplemental test in screening field serum samples for *Brucella*-specific

agglutinins. A modification of the acid plate-agglutination test, employing a suspension of *B abortus* cells stained with Rose Bengal dye and buffered at pH 3.65 was introduced by the United States Department of Agriculture, National Animal Diseases Laboratory. In field trials this test was found to be a more accurate indicator of infection.

In the present study seroprevalence of brucellosis in cattle by RBPT is found to be 6 per cent. These results are in agreement with Shakunthala *et al.* (2016) reported 5.91 per cent prevalence by RBPT. Similarly, Muttanagouda *et al.* (2014) reported 5.76 per cent prevalence by RBPT in South Karnataka. Senthil *et al.* (2013) reported 11 (5.23%) were found positive by RBPT around Tamil Nadu. Slightly higher prevalence recorded as compare to present study by Kavya *et al.* (2017) reported 7.84 per cent were found positive by RBPT in and around Bangalore, Karnataka and Sengupta *et al.* (2014) reported 7.04 per cent positive by RBPT out of 298 cattle. Lowest by Priyadarshini *et al.* (2013) reported 4.26 per cent prevalence in Odisha, India. Berhe *et al*. (2007) detected 4.5 per cent prevalence by RBPT in Central Ethiopia. Mohammed *et al.* (2011) who reported a positivity of 4.04 per cent, out of 570 serum samples screened in Jigawa state of Nigeria and concluded that RBPT can be used in brucellosis screening and eradication programme.

Like any other serological test, RBPT could sometimes give a positive result due to vaccination or false positivity due to cross reactions and false negative reactions due to low specificity. Therefore these reactors should be further investigated using suitable diagnostic tests. The sensitivity of RBPT is low, so chances of getting a false positive outcome are more when compared to ELISA. False positive results are a major problem

which makes serological diagnosis of brucellosis difficult in some cases. Virtually all serological tests for antibody to smooth *brucella* species use LPS, part of LPS or whole cells as antigens. The immuno-dominant epitope on the surface of the smooth cell is OPS the outer most portion of LPS. Most but not all of the problems arise from an immune response of the animal to other microorganisms which share epitopes with *brucella* species. However, Sharma *et al.* (2015) recorded a high prevalence rate of 41.25 per cent and have attributed high prevalence in animals to pastoralist husbandry practices that involved cattle sharing grazing areas and drinking from the same point.

5.1.3 Indirect ELISA (i-ELISA)

Enzyme linked Immunosorbent assay was first developed by Carlsson *et al.* (1976) for the diagnosis of human brucellosis. Since then, a large number of alterations have been described. sLPS antigen, passively coated onto a polystyrene matrix is most commonly used in ELISA (Gall and Nielsen, 1994). I-ELISA has been standardized by several researchers to screen brucellosis in livestock (Renukaradhya *et al.*, 2002; Agasthya *et al.*, 2007 and Shome *et al.*, 2006). All of them have used either *B.abortus* 99 or *B. melitensis* 16M strains as a source of sLPS antigen. The present study has shown that the commonly used conventional serodiagnostic tests for brucellosis may not be absolutely reliable. In general, I-ELISA was the most sensitive, specific and reliable test that can be applicable for mass screening of livestock and human brucellosis, which is in concurrence with the published results of others (Chachra *et al*. 2009; Ahasan *et al.* 2017; Shome *et al.* (2019; Shakya *et al*. 2020; Dekha *et al.* 2021).

In the present study seroprevalence of brucellosis in cattle by I-ELISA is found to be 8 per cent and our results are in close agreement with the results reported by Shome *et al.* (2019) recorded in a national survey on seroprevalence of brucellosis in cattle by I-ELISA was 8.30 per cent. Similarly, Priyadarshini *et al.* (2013) recorded prevalence of brucellosis as 8.14 per cent in Orisa, India. and Poonati *et al*. (2020) reported 7.78% prevalence of brucellosis by I-ELISA in Guntur, Andra Pradesh. Similarly. Sharma *et al.* (2015) have reported the apparent seroprevalence of 7.26 per cent in Gujarat by I-ELISA and 6.62 per cent in Tamil Nadu by Naveenkumar *et al.* (2019). Shome *et al.* (2014) reported seroprevalence of 7.50 per cent by I-ELISA in (Goa, Karnataka and Andra Pradesh) India. However, Jagapur *et al*. (2013) have recorded high prevalence of 45.80 per cent in an organized dairy farm with history of abortion in Karnataka; whereas Chakraborty *et al.* (2000) recorded 56.02 per cent in cattle by employing I-ELISA in Gujarat.

As per the analysis I-ELISA was found to be statistically significant($P<0.01$) and positively associated with diagnostic tests of brucellosis as compared with MRT and RBPT. (CI 95% 3.52-15.16%).

5.2 Comparison of serological tests

The application of multiple serological assays currently available for the detection of *Brucella* antibodies in various species of animals indicates that no single test can detect all infected animals and therefore a combination of serological tests with better sensitivity are to be designed to minimize the false negative reactions which otherwise may account for the persistence of *Brucella* infection in the herd. In the present

investigation, RBPT in conjunction with MRT and I-ELISA were employed to compare their efficacy.

5.2.1 Comparing RBPT with MRT and ELISA tests

Out of six RBPT positive samples five were detected as positives by MRT and Similar findings were also reported by Gogoi *et al*. (2017) reported 66(12.69%) samples positive by RBPT of which 16 (10.53%) samples positive by MRT tested in Assam. Further, 72(13.08%) highest positive were detected by I-ELISA. The researchers were of the opinion that results of I-ELISA were observed to be more clear and easy to read than routine MRT and concluded that RBPT may be used as in alternate to routine MRT in the diagnosis of brucellosis. Similarly, Chandrashekhar *et al.* (2018) found 20.08 per cent serum samples positive by RBPT out of 1112 sera samples tested in Hassan dist. Karnataka and out of 442 milk sample, 49 (11.08%) samples were detected as positives by MRT.

5.2.1 Comparing I-ELISA with MRT and RBPT tests

In the present study, keeping I-ELISA as a reference test, the sensitivity of RBPT and MRT was found to be of 85.71, 83.33 and 55.56 per cent respectively, while specificity was 98.92 per cent and 97.87 per cent respectively in cattle. However, variable levels of sensitivity and specificity was recorded by many researchers. Present findings corroborated the reports of Salman *et al.* (2012) who reported higher sensitivity of RBPT (92%) than MRT (85%) where as slightly higher specificity of MRT (95%) than RBPT (94%). Similarly, Naveenkumar *et al*. (2019) reported higher specificity of RBPT (99.11%) than MRT (98.66%) where as higher sensitivity of MRT (46.87%) than RBPT

(34.37%). Kala *et al.* (2018) found sensitivity of RBPT (34.68%) was lower than the specificity (98.48%). Al-Mmashhadany., (2019) reported lower sensitivity of MRT (83%) and higher specificity (98%), with accuracy of 97%. Sharma *et al.* (2015) recorded the sensitivity and specificity of RBPT were calculated as 80.78% and 100% respectively. Shome *et al.* (2015) reported the sensitivity and specificity of RBPT were 62.96% and 97.40%, respectively. Kala *et al*. (2018) reported the sensitivity and specificity of RBPT were 34.68% and 98.48% respectively. Poonati *et al.* (2021) recorded the sensitivity, specificity of RBPT and MRT were 75.51%, 87.07% and 71.97%, 85.38% respectively also PPV, NPV and Accuracy of RBPT were 33.45%, 97.64% and 86.15 and for MRT were 29.75%, 97.25% and 84.31. The overall agreement was RBPT found higher sensitivity and specificity as compare to MRT with ELISA kept as gold standard.

The pH of the buffer in which RBPT antigen suspended is acidic (3.3 to 3.8) enabling both Ig M and Ig G immunoglobulins to induce agglutination, contributing for an increased sensitivity. However the low specificity of the RBPT antigen reported is attributed to cross agglutination with some other gram negative bacteria. On the other hand, ELISA is highly sensitive and specific mainly on account of purified antigens being used. Further, ELISA detects only IgG immunoglobulin and not the IgM could be a probable reason for better sensitivity of RBPT than MRT observed in the present study (Muthanagouda, 2014).

5.2.2 Age-wise prevalence in cattle

In the present study cattle > 6 years age group showed highest seroprevalence (9.30%) followed by 4-6 years age group (8.57%) and 0-4 age group showed (4.54 %) by I-ELISA. These findings are in agreement with the observations of Krishnamurthy *et al.* (2015) who reported higher prevalence (18.46%) of brucellosis in cattle of > 6 year age group. Similarly, Gogoi *et al*. (2017) reported highest percentage of *Brucella* positive reactors among cattle recorded in the age group of 3 to 7 years (16.98%) followed by more than 7 years (12.86%) of age group, whereas lowest prevalence was recorded in the age group of less than 3 years (16.98%) by I-ELISA. On contrary, Kylle *et al*. (2017) observed higher seroprevalece of brucellosis in bovines of 2-4 years of age group (2.85%) followed by 4-6 group (2.61%) and least in > 6 years age group which is not in concurrence with the result of present study. Higher prevalence of brucellosis in animals above 4 years might be due the fact that this is the most suitable age for breeding. It could be also attributed to marked decrease in immune status with the advancement of the age. The results of the present study suggested that animals older than 4 years of age are more likely to become sero-positive to *brucella*. Similar observations were made by other researchers (Amin *et al.*, 2005; Sarumathi *et al.*, 2003; Kazi *et al*., 2005). Lower prevalence of brucellosis in young ones, which become susceptible to disease with age could be attributed to resistance of sexually immature cattle to infection (Paul, 1980), or passive immunization of calves through colostrum of their infected dams. Similar results have been reported by various authors Ahmad and Munir (1995). It appears that the high prevalence of brucellosis among older cows might be related to maturity with the advancing age, where the organism may have propagated either to remain as a latent

infection or it may cause clinical manifestation of the disease (Kazi *et al.,* 2005). Although susceptibility to disease increases with age, it seems to be more commonly associated with sexual maturity than age (Radostits *et al.*, 2000). It is also likely that the animal might have been exposed before reaching maturity, but did not seroconvert at the time of testing and remained in an incubatory stage instead.

5.2.3 Prevalence with clinical history of cattle.

Out of 18 abortion cases 5 (27.70 %) were found positive by I-ELISA. Out of 25 repeat breeding (RB) cases 3 (12.00%), out of 28 retention of fetal membrane (RFM) cases 6 (21.40%) and out of 20 pregnant cases 1(5.00%) were found positive for brucellosis (Table 6, Fig. 5). Similarly, Krishnamurthy *et al.* (2015) who reported higher prevalence as 15/36 (41.67%) in abortion, 4(6.06%) in RB, 0 in RFM and 12(13.33%) in pregnant animals by ELISA. Prevalence of brucellosis in organized farms with abortion storms in Egypt as assessed by Khan *et al*. (2020) was higher (41.02%) when 363 sera samples were screened by ELISA. Similarly, Gogoi *et al.* (2017) recorded the highest percentage of *Brucella* positive reactors among previous history of abortion (64.21%) followed by retention of fetal membrane (47.13%) in case of cattle by ELISA. Khan *et al.* (2020) and Kushwaha *et al.* (2016) reported 39/177(22.03%) and 45/324 (13.88%) positive results respectively in cases of pregnancy. Roberts *et al.* (1971) stated that, multiplication of the *Brucella* organism is enhanced with the increased concentration of erythritol in gravid uterus leads to increase percentage of abortion, placentitis and adhesion usually accompanied with sever endometritis leading to retention of placenta after birth in aborted animals due to brucellosis. Large herd size enhances the exposure potential, especially following abortions, through increased contact and common feeding

and watering points promoting transmission of *brucella* organisms (Hellmann *et al.* 1984). The higher incidence of abortion in third trimester may be due to the fact that the uterine environment becomes conducive for the multiplication of bacteria, which in turn causes fetal death and abortion by invading placental cotyledons and destroying the villi. Similar to the results of the present study, Kylle *et al.* (2017) reported seroprevalence of brucellosis to be 11.22 per cent in animals with a previous history of abortion and RB as compared to only 1.47 per cent without such history. Barman *et al.* (1989) opined that among the aborted animals, non-pregnant infected animals develop repeat breeding condition. It can be inferred from the previous reports and the present study that brucellosis is confirmed to be the major etiological agent of abortion in farm animals worldwide.

5.2.4 Lactation-wise prevalence in cattle

In present study, lactating cows were divided into 1st to 2nd and ≥ 3th lactation and the prevalence was 9.40 and 6.30 per cent respectively. The highest prevalence was recorded in 1^{st} to 2^{nd} lactation groups which were already documented by Mohamand *et al.* (2014) found more prevalence in 2^{nd} to 4^{th} lactation group than $\geq 5^{th}$ lactation groups. Similar results have been reported by various authors. Contradictory to present study, Kumar *et al.* (2016) reported highest prevalence was recorded in ≥ 5^{th} lactation groups than 1^{st} to 4^{th} lactation group and pleuriparous cows showed increased prevalence of brucellosis as age advanced. Aulakh *et al.* (2008) reported that, the disease prevalence was found less common in young animals which were attributed due to resistance of sexually immature cattle to infection, which become susceptible to disease with age or passive immunization of calves through colostrum of their infected dams. These study

variation might be due to sampling size, demography, disease epidemiology and clinical condition of individual animals.

To conclude, MRT should be used for bulk samples or pooled milk or herd samples rather than individual samples to locate infectious herd. RBPT considered as reference test in the absence of isolation as it is widely used in country because of its simplicity in performing and recommended by OIE. Enzyme linked immunosorobent assay, robust test with high sensitivity and specificity serve as quick surveillance test.

5.3 Zoonotic importance of brucellosis

Human brucellosis is a neglected disease of poverty often found in highly agrarian, livestock dependent societies (World Health Organization, 2006). *Brucella* species differ markedly in their capacity to cause invasive human disease. *B. melitensis* is the most pathogenic; *B. abortus* is associated with less frequent infection and a greater proportion of subclinical cases (Alton, 2006). Human infection can occur through consumption of infected raw milk, their products and raw meat. Other means of infection include skin abrasions and inhalation of airborne animal manure particles. In addition, occupational exposure of abattoir workers, veterinarians and laboratory technicians can result in transmission of the disease through contaminated aerosols. Transmission of brucellosis from person to person has also been reported in the literature (Mantur *et al.*, 1988; Wyatt, 1996). The control and eradication of animal brucellosis is closely linked with the elimination of the disease in man, (Morgan, 1969).

Humans in India live in close proximity with the animals thereby stand at a greater risk to zoonotic infections. Mathur, (1964) stated that brucellosis occurred more

frequently in villages than in cities. Moreover, Human brucellosis is reported from most parts of the country and is closely related to animal husbandry activities (Hemashettar and Patil, 1991). Several reports indicate it to be a common disease in India.

5.3.1 Prevalence in humans

In the present study, out of 25 sera of humans, 8, 4, 8, 12 per cent samples were positive to RBPT, STAT, 2-MET and I-ELISA respectively. These findings are in agreement with the observations of Sumankumar *et al.* (2015) recorded prevalence of 9.3% and 5.3% by RBPT and STAT respectively. Similarly, Desai *et al.* (1995) reported 5.9 per cent by RBPT around Bidar area of Karnataka; 3.41 per cent by STAT reported by Priyadarshini *et al.* (2013). Holt *et al.* (2021) found 9.7 per cent by ELISA. The percent prevalence by RBPT in our study was little higher i.e., 8% when compared to Patil *et al.* (2016) who reported a seropositivity of 5.1 per cent by RBPT in Karnataka. An overall prevalence recorded was 7.04% in personnel engaged in veterinary health care in Karnataka, India by Shome *et al.*, (2017) and the study by Thakur and Thapliyal (2002), revealed a prevalence rate of 4.97% in samples obtained from persons exposed to animals. On contrary, Among all, Punjab reports the highest (26.6%) cases of human brucellosis. Reddy *et al.* (2014) reported as 14.92, 16.41, 3.98, 19.40 per cent positivity rate by RBPT, STAT, 2-MET and I-ELISA respectively, 8.5% in Gujarat and Belgaum, 11.51% in Andhra Pradesh, 19.83% in Maharashtra. Patil *et al.* (2016) reported disease from Gadag (21.1%), Haveri (17.4%) and Koppal (18.5%) districts of Karnataka which are slightly higher than present study and lesser prevalence of 0.8% in Kashmir, 0.9% in Delhi, 1.3% in Nagpur, Maharashtra by Ghugey *et al.* (2021), 6.8% in Varanasi. Thus systematic review suggests that the following states have endemicity of the disease.

5.3.2 Prevalence in different risk factors

The study also indicated moderate prevalence of brucellosis (12%) in animal handlers. Another study in Punjab during 2012-13 revealed maximum in dairy workers (16.10%). A 10 year study conducted in Chandigarh on persons with PUO reported 9.94% prevalence on serological basis. However, Fever and upper back pain were also assessed as significant predictors for both acute and chronic forms of brucellosis (Patra *et al.,* 2019). Proch *et al.* 2018 observed that in India, the seroprevalence rates have been recorded to be as high as 17-34 per cent in high-risk groups like abattoir workers, veterinarians and animal attendants (Appannanavar *et al.*, 2012). About 4.2 per cent women with abortion were reported by Randhawa *et al.* (1974) to possess *Brucella* agglutinins.

In our study, regarding sex association, higher percentage of infection in female 1(50%) than male 2(8.70%) by I-ELISA. Similarly, Dutta *et al., (*2018) reported female children (14.3%) was observed higher prevalence as compared to male children (10.9%) unlike Patil *et al*. (2016). On contrary, Reddy *et al.* (2014) reported prevalence of brucellosis was more among males (21.64%) compared to females (14.92%) with I-ELISA detecting highest number of positive cases compared to other four tests. Here was a predominance of females in this study. However, Al Sekait (1999) suggested that gender does not influence the immune response to *Brucella*.

In the present study, most of the positive individuals had a history of livestock workers in Bidar. The history of the positive individuals also revealed other risk factors such as residence in rural area, lack of awareness about zoonosis, contact with parturient

animals, participation in vaccination, occupation- related mishap and eating during working hours were identified as the main risk factors. Al-Fadhli *et al.* (2008) reported that raw milk was the major source of infection. This finding is reasonably in agreement with Ali *et al*. who reported contact with animals (32%); occupation, mainly farmers or butchers (18%) and raising animals in the vicinity of residence (14%) as risk factors for brucellosis. Meky *et al.* (2007) reported that workers in occupations dealing with animals had a 2.4-fold higher risk of brucellosis than those in occupation not dealing with animals. In view of the limited efficacy of the vaccine, maintaining the hygiene especially while being associated with the livestock and consumption of properly processed milk and milk products is the only way to prevent brucellosis among the general human population.

Thus, on the basis of the present study, it can be concluded that human brucellosis is moderately prevalent in the regions of Bidar, Karnataka from where samples are collected. Nicoletti and Tanya (1993) also observed that I-ELISA was the most efficient test in detecting early infection of brucellosis and it has proved to be highly sensitive and specific test for use in diagnosing of brucellosis in cattle and human. However, this needs further evaluation using large number of serum samples preferably from animals of known status with respect to bacteriological isolation which gives most incontrovertible diagnosis of brucellosis.

Brucellosis causes persistent infection by its capacity to escape from innate and adaptive immunity and till date there is no vaccine for human use for prevention of brucellosis (Fitcht T.A , 2003). However, a new candidate vector vaccine against human

brucellosis based on recombinant influenza viral vectors (rIVV) by Bugybayeva *et al.* (2021) under research. There for it is very important to deal with occupation-related disease like brucellosis, knowledge of risk factors is vital in control and prevention programmes. Thus, an extension education campaign, particularly in high-risk areas, such as veterinary practitioners and livestock owners, could aid in decreasing the incidence of brucellosis. In addition, regular surveillance of the disease needs to be integrated into control and prevention programme at a local and national level. An integrated approach to disease surveillance involving both human health and veterinary services would allow a better understanding of disease dynamics at the animal-human interface, as well as a more cost-effective utilization of resources. Further calfhood vaccination should be presented in high risk areas to control infection in 4-8 month old female calves.

Summary

VI. SUMMARY

The present study was conducted to know the prevalence of brucellosis in cattle and humans by screening with RBPT, MRT and I-ELISA serological tests and to evaluate their efficacy and to suggest the test on priority for field application.

In the present study, a total of 100 serum samples of cattle and 25 human sera of animal handlers were collected from the farms/ cases to the VCC college/Gosahalas (A,B,C,D and E) of Bidar district, Karnataka. Out of 100 serum samples screened 6 (6.00%), 5 (5.00%) and 8(8.00%) were detected as positive by RBPT, MRT and I-ELISA tests respectively. In age wise, above > 6 years age group showed the highest prevalence (9.30%) followed by 2-4 years age group (8.57%) and least in 1-2 years age group (4.54%). Prevalence of 27.70 per cent brucellosis was observed in the cattle with previous history of abortion. Further, prevalence of 21.40%, 12%, 5 % was found in RFM, repeat breeder and pregnant cattle, respectively.

The overall prevalence of brucellosis in humans was found to be 2 (8.00%), 1 (4.00%), 8 (8.00%) and 3 (12.00%) by RBPT ,STAT, 2-MET and I-ELISA and also found higher prevalence in female (50%) than male (8.70%) among the animal handlers.

The efficacy of RBPT and MRT to compared to find out the sensitivity, specificity, positive predictive value, negative predictive value and accuracy by keeping I-ELISA as standard test for detection of brucellosis in cattle. Among two tests RBPT showed higher sensitivity (85.71%), positive predictive value (85.71%), negative predictive value (98.92%) and accuracy (98%) than MRT which showed lower sensitivity,

positive predictive value, negative predictive value and accuracy as 83.33%, 97.87 % 71.42 % , 98.92% and 97 %, respectively.

From this study its opined that human and cattle brucellosis is fairly endemic in and around Bidar, Karnataka and it deserves due attention because of the public health significance of the disease.

Conclusions:

1. The overall prevalence of brucellosis in cattle was 8 per cent and in humans was 12 per cent by I-ELISA.
2. Highest prevalence of brucellosis in cattle (12%) and humans (8%) were detected by I-ELISA followed by RBPT and MRT in cattle.
3. Higher prevalence of brucellosis was recorded in cattle of more than 6 years age group, 1st to 2nd stage of lactation and with the history of abortion.
4. The sensitivity, specificity and accuracy of RBPT was higher than MRT in detection of brucellosis in cattle.
5. I-ELISA was superior over the RBPT and MRT in detection of brucellosis in cattle.

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Abstract

VIII. ABSTRACT

PREVALENCE AND ZOONOTIC IMPORTANCE OF BRUCELLOSIS IN CATTLE IN AND AROUND BIDAR

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Brucellosis is one of the major notifiable contagious bacterial disease of zoonotic importance in worldwide including India causing heavy economic loss. A limited work carried out to determine the prevalence of brucellosis in cattle and human in the districts of Bidar, Karnataka. So, the present study was undertaken to determine the sero-prevalence of brucellosis in cattle and animal handlers (human). A total of 100 serum and 100 milk samples were collected farm-wise and subjected to RBPT, MRT and I-ELISA for confirmation of brucellosis in cattle. Animal handlers 25 sera were subjected to RBPT, STAT, 2-MET and I-ELISA. The overall prevalence of brucellosis was found to be 6 per cent, 5.00 per cent and 8 per cent by RBPT, MRT and I-ELISA respectively. Higher prevalence of brucellosis was recorded by I-ELISA followed by RBPT and MRT. I-ELISA revealed that prevalence of brucellosis was observed in humans 12 per cent and in case of cattle 8 per cent. It was also observed that higher prevalence of brucellosis found in cattle with the previous history of abortion (27.70%). On age-wise study, the higher prevalence was noticed in the above > 6 years (9.30%) age groups followed by 2-4 years (8.57%) and least in 1-2 years age group (4.54%). Among the tests performed for the diagnosis of brucellosis, sensitivity and specificity of RBPT was found to be high when compared to MRT, taking I-ELISA as standard test. Thus, Rose bengal plate test is a better screening test in detection of brucellosis in field level when compared to MRT.

Appendices

APPENDICES

1. Protein-G based indirect ELISA kit for bovine brucellosis (Plate) consisting of

Packs	Items	Quantity	Storage
1	ELISA polysorp uncoated microtitre plates	15 plates	RT
2	sLPS Antigen	650µL × 1vial	- 20℃
3	Protein-G HRP conjugate	65 µL x 1vial	- 4℃
4	Positive control serum	110 µL x 1 vial	- 4℃
5	Negative control serum	110 µL x 1vial	- 4℃
6	Sodium carbonate	18gm x 1bottle	RT
7	Sodium bicarbonate	15gm x 1bottle	RT
8	Bovine gelatin	18gm x 1bottle	RT
9	Tween-20	7ml x 1bottle	RT
10	Phosphate Buffered Saline (PBS)	5sachets	RT
11	Chromogen OPD (5 mg)	15 tablets	- 4℃
12	Hydrogen Peroxide (30%)	2ml x 1vial	- 4℃
13	Protocol	1	RT

Preparation of reagents

1. Coating buffer:

Solution - A	Sodium Carbonate	1.06 gm
	Distilled water	50 ml
Solution - B	Sodium Bicarbonate	0.84 gm
	Distilled water	50ml

To prepare 25ml coating buffer (sufficient for coating 2 plates) added:

Solution A	1.75 ml
Solution B	4.25 ml
Distilled water	19.0 ml

Note: The pH of the coating buffer should be 9.6 and it is to be prepared fresh every time.

2. Phosphate Buffer Saline (IX):

NaCl	7.0 gm
KCI	0.2 gm
NaH_2PO_4	0.353 gm
Na_2HPO_4	1.09 gm
Distilled water	1000 ml

Note: 1 sachet of PBS was dissolved provided with the kit in 1000 ml water to make IX PBS and stored at 4°C for reuse.

3. Washing buffer: Every time fresh was prepared

PBS (1X)	500 ml
Tween-20	0.25 ml

4. Blocking buffer: Sufficient for performing 2 plates

Bovine gelatin	2.0 gm
PBS (1X)	100 ml

Note: 1) Kept in water bath at 37°C until bovine gelatin dissolves completely (15-20min).
2) After taking it out of the water bath 50µL of Tween-20 was added.

5. Stopping solution (IM H_2SO_2): Sufficient for performing 2 plates

Conc. H_2SO_4 (Sulfuric acid)	5.5 ml
Distilled water	94.5 ml
Total	100 ml

6. Hydrogen peroxide (3%): Sufficient for performing 2 plates

Hydrogen peroxide (30%)	10 µL
Distilled water	90 µL
Total	100 µL

7. Chromogen - Substrate Solution

OPD	5 mg
H_2O_2 (3%)	50 µl
Distilled water	12 ml

[1]Protein-G based indirect ELISA kit, NIVEDI.