Evaluation of Different Serological Tests to Study Seroprevalence of Brucellosis in Buffaloes.

M.V.Sc. Thesis

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ABSTRACT

The present study was undertaken to detect the Brucella antibodies in serum as well as in milk of buffaloes of Kota region of Rajasthan. The AB ELISA was used for detection of Brucella antibodies in conjunction with RBPT, STAT, HIT, MET, AGID and MRT to detect their efficacy as compared to AB ELISA.

The overall seroprevalence of was found as; AB ELISA (37.84%), RBPT (35.09%), STAT (29.59%), HIT (27.52%), MET (26.38%) and AGID (13.99%) in total 436 buffalo serum samples and MRT (31.75%) in 63 milk samples.

Highest seroprevalence was found in Chhabra region in all tests whereas lowest seroprevalence was found in Dag region by all the tests except HIT and MET which showed lowest prevalence in district Bundi.
Higher number of females (38.46%) showed seropositivity for brucellosis as compared to males (30.30%) in AB ELISA.

In the present investigation, the relative sensitivity value was highest with RBPT followed by MRT, STAT, HIT, MET and lowest with AGID. The relative specificity values were highest with AGID followed by MET, HIT, STAT, MRT and lowest with RBPT. The relative concordance values were highest with MET followed by HIT, STAT, RBPT, MRT and lowest with AGID.

This study reveals prevalence of *Brucella* infection in buffaloes of Kota division and necessitates early detection and proper monitoring of brucellosis at State level to eradicate the brucellosis.
बैलसो में ब्रुसेलोसिस के सीर्मीय प्रायोगिक के अध्ययन हेतु विभिन्न सीर्मीय परीक्षणों का मूल्यांकन

इस अध्ययन के अन्तर्गत कोटा समाग की बैलसो के सीर्म व दुःख में ब्रुसेला प्रतिपिण्डो की खोज की गई। जिसमें एन्जाइम लिंकूड इम्यूनो सोरबेन्ट ऐसे परीक्षण व अन्य परीक्षण जैसे आर.बी.पी.टी., एस.टी.ए.टी., एच.आई.टी., ए.जी.आई.टी. व एम.आर.टी. परीक्षणों से ब्रुसेला प्रतिपिण्डो की खोज की गई और सभी परीक्षणों की ए.बी. इ.एल.आई.एस.ए. की तुलना में कार्यक्रमता की खोज भी की गई।

सभी सीर्मीय प्रायोगिक निम्न क्रम में पाया गया। ए.बी. इ.एल.आई.एस.ए. (37.84%), आर.बी.पी.टी. (35.09%), एस.टी.ए.टी. (29.59%), एच.आई.टी. (27.52%), एम.इ.टी. (26.38%) एवं ए.जी.आई.टी. (13.99%) कुल 436 सीर्मीय नमूनों में व एम.आर.टी. (31.75%) 63 दुःख नमूनों में पाया गया।
I consider myself fortunate to have worked under the supervision and guidance of Dr. B. N. Shringi, Assistant Professor, Department of Veterinary Microbiology and Biotechnology, R.U.V.A.S., Bikaner, who gave his invaluable suggestions and scholarly guidance throughout my study and research work.

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My reverend parents, my brother and sister, and all my near and dear ones deserve not just thanks but much more than what I can weight in words.

And I would not be what I am, if I do not thank that one heavenly soul, the Almighty for His untold blessings.

Place: Bikaner
Date: Mamta Soni
### ACRONYMS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>μl</td>
<td>Microliter</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>AB-ELISA</td>
<td>Avidin-Biotin Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ABR Antigen</td>
<td>Abortus Bang Ring Antigen</td>
</tr>
<tr>
<td>AGID</td>
<td>Agar Gel Immuno Diffusion</td>
</tr>
<tr>
<td>AI</td>
<td>Artificial Insemination</td>
</tr>
<tr>
<td>BPAT</td>
<td>Buffered Plate Antigen Test</td>
</tr>
<tr>
<td>C- ELISA</td>
<td>Competitive Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>CFT</td>
<td>Complement Fixation Test</td>
</tr>
<tr>
<td>dot- ELISA</td>
<td>dot Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme Immuno Assay</td>
</tr>
<tr>
<td>HIT</td>
<td>Heat Inactivation Test</td>
</tr>
<tr>
<td>I-ELISA</td>
<td>Indirect Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>I.U.</td>
<td>International Unit</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>MET</td>
<td>2-Mercaptoethanol Agglutination Test</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>ml</td>
<td>Mili Liter</td>
</tr>
<tr>
<td>mm</td>
<td>Mili Meter</td>
</tr>
<tr>
<td>MRT</td>
<td>Milk Ring Test</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NSS</td>
<td>Normal Saline Solution</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>RBPT</td>
<td>Rose Bengal Plate Test</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotation per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Rivanol Test</td>
</tr>
<tr>
<td>S LPS</td>
<td>Smooth Lipo Polysaccharide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>STAT</td>
<td>Standard Tube Agglutination Test</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>W</td>
<td>Watt</td>
</tr>
</tbody>
</table>
**APPENDIX**

**Kit for Bovine Brucellosis Avidin-Biotin ELISA (A-B-ELISA)**

The kit components were as follows:

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Items</th>
<th>Quantity</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Immunochemicals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Antigen (vial)</td>
<td>1 ml</td>
<td>-20°C</td>
</tr>
<tr>
<td>2.</td>
<td>Biotin – anti IgG conjugates</td>
<td>100µl</td>
<td>-20°C</td>
</tr>
<tr>
<td>3.</td>
<td>Avidin - HRP</td>
<td>100µl</td>
<td>-20°C</td>
</tr>
<tr>
<td>4.</td>
<td>Control (vial)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C++</td>
<td>0.2 ml</td>
<td>4°C</td>
</tr>
<tr>
<td></td>
<td>C+</td>
<td>0.2 ml</td>
<td>4°C</td>
</tr>
<tr>
<td></td>
<td>C-</td>
<td>0.2 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>Reagents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------</td>
<td>------------</td>
<td>----------</td>
</tr>
<tr>
<td>5. Sodium Carbonate</td>
<td>1 bottle</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>6. Sodium Bicarbonate</td>
<td>1 bottle</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>7. Bovine Gelatin</td>
<td>1 bottle</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>8. Phosphate buffer saline</td>
<td>5 sachets</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>9. Tween 20</td>
<td>1 bottle</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>10. Chromogen OPD</td>
<td>10 tablets of 10 mg</td>
<td>2-8°C</td>
<td></td>
</tr>
<tr>
<td>11. Stopping Solution 1M H₂SO₄</td>
<td>1 bottle</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>12. ELISA microtitre plates</td>
<td>15 plates</td>
<td>RT</td>
<td></td>
</tr>
</tbody>
</table>

**PHENOL SALINE**

NaCl 8.55 g
Dist. water 1 liter

Mix well than add 0.5 ml phenol.
PHOSPHATE BUFFER SALINE SOLUTION (pH 7.4)

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 & \quad 1.09 \text{ g} \\
\text{NaH}_2\text{PO}_4 & \quad 0.353 \text{ g} \\
\text{NaCl} & \quad 7 \text{ g} \\
\text{KCl} & \quad 0.2 \text{ g} \\
\text{Dist. Water} & \quad 100 \text{ ml}
\end{align*}
\]

COATING BUFFER (stock solution)

Solution A

\[
\begin{align*}
\text{Sodium Carbonate} & \quad 1.06 \text{ gm} \\
\text{Dist. Water} & \quad 50 \text{ ml}
\end{align*}
\]

Solution B

\[
\begin{align*}
\text{Sodium Bi-Carbonate} & \quad 0.84 \text{ gm} \\
\text{Dist. Water} & \quad 50 \text{ ml}
\end{align*}
\]

ADDED AS:

\[
\begin{align*}
\text{Sol. A} & \quad 7 \text{ ml} \\
\text{Sol. B} & \quad 17 \text{ ml} \\
\text{Dist. Water} & \quad 76 \text{ ml}
\end{align*}
\]
WASHING BUFFER
PBS 100 ml
Dist. Water 400 ml
Tween20 100ul
Prepared freshly before use.

BLOKING BUFFER
Bovine Gelatin 1.2 gm
1 x PBS 10 ml

STOPPING SOLUTION (1M H$_2$SO$_4$)
Concentrated H$_2$SO$_4$ 5.5 ml
Dist. Water 94.5 ml

1. INTRODUCTION

Brucellosis is one of the most common zoonoses in the world accounting for the annual occurrence of more than 500,000 cases in human beings (Pappas et al., 2006). Although the disease has been controlled in most developed countries, it remains a major problem in the Mediterranean region, Western Asia, Africa, and Latin America (Pappas et al., 2006). The incidence varies considerably between herds, areas and countries (Blood and Hinderson, 1983). In India, brucellosis was first recognized in 1942 (Polding, 1942) and now is endemic throughout
the country (Aulakh et al., 2008; Bandyopadhyay et al., 2009; Brahmbhatt et al., 2009). The disease has also been detected in cattle and buffaloes of Western Rajasthan (Wadhwa, 2007).

The importance of the infection lies in the fact that brucellosis is caused by *Brucella* organism which is a gram negative facultative intracellular bacterium, pathogenic for a wide variety of animals and human beings, thus making it difficult to treat using usual antibiotics. Secondly, the prevalence of this infection depends upon several factors like food habits, social customs, husbandry practices, climatic conditions, socioeconomic status, and environmental hygiene. Environmental sanitation is particularly important in the context of airborne transmission. The disease is almost invariably transmitted to man from infected domestic animals.

The ease of its transmission and difficulty in treating this infection makes it one of the most important organisms as far as bioterrorism is concerned. In addition to this, the disease itself is important because it can cause economic losses in terms of abortions, decreased milk production, sterility, and veterinary care and treatment costs. (Corbel, 1997).

Brucellosis in bovines is usually caused by *Brucella abortus* but it can be caused less frequently by *B. melitensis* (Jimenez et al., 1991) and rarely by *B. suis* (Ewalt et al., 1997).
Brucellosis in cattle and buffaloes appears to be on the increase in recent times, perhaps due to increased trade and rapid movement of livestock (Renukaradhya et al., 2002). The incidence is higher in animals kept at organized farms rather than in small holdings (Mehra et al., 2000).

This is a disease of sexually mature animals and manifested by reproductive failure, abortion between the fifth to ninth month of pregnancy, birth of unthrifty calves and retained placenta. (McMahan, 1944; Corbel, 1988). Lesions in *Brucella* infected male are largely confined to the genital organs including testicles, seminal vesicles and epididymes (Morgan and MacKinnon, 1979).

Bulls can occasionally pass the *Brucella* organisms with semen in uterus during the natural service but the greater danger of transmission is via artificial insemination. (Bendixen and Blom 1947; Manthei et al., 1950; Rankin, 1965).

Early detection, control and elimination of reactors are important considerations for the control of brucellosis. Diagnosis depends on the isolation of *Brucella* from aborted material, udder secretions or from tissues removed at post-mortem. Presumptive diagnosis can be made by assessing specific cell-mediated or serological responses to *Brucella* antigens. Out of all these diagnostic techniques serological tests are used most widely as they are easier to perform and give rapid results.
The various serological tests used for the diagnosis of brucellosis are agglutination, precipitation, complement fixation, enzyme-linked immunosorbent assays etc. All these tests have different sensitivities and specificities.

Rose Bengal plate test, standard tube agglutination test and milk ring tests are conventional tests used for the diagnosis of brucellosis but all these tests have limitations (Nielsen, 2002). Therefore supplemental blood and milk test procedures are used to speed up the eradication of brucellosis.

Heat inactivation test and 2-mercaptoethanol agglutination tests are supplemental blood tests developed to differentiate specific and non specific reactions found in bovine serum because nonspecific agglutinins of bovine origin, associated with suspect *Brucella* titers, can be inactivated when heated (Hess, 1953; Amerault *et al.*, 1961) in case of heat inactivation test and reduced by 2-mercaptoethanol (Anderson *et al.*, 1964; Reddin *et al.*, 1965; Elberg, 1973; Spink, 1977; McMahon, 1983) in 2-mercaptoethanol agglutination test.

Immune precipitation methods like agar gel immunodiffusion test are used to demonstrate antibody in bovine sera which reacts with *Brucella* antigens other than the smooth lipopolysaccharide. (Bruce and Jones, 1958; Hinsdill and Berman, 1967; Corbel, 1973) to improve the diagnosis of this disease.

Recently, indirect enzyme linked immunosorbent assay, competitive enzyme-linked immunosorbent assay and complement fixation test have been shown to be more accurate than the conventional tests (Gall & Nielsen
The diagnostic performance characteristics of enzyme-linked immunosorbent assays (ELISAs) are better than that of the CFT, as these are technically simpler to perform and more robust, so their use is preferred. (Wright et al., 1993; Nielsen et al., 1996). According to Rojas and Alonso 1995, application of the ELISA technique is considered a better test in early detection of infection than complement fixation test.

No single serological test and antigen combination show 100 percent sensitivity and specificity simultaneously. Immunoprecipitation tests are highly specific but less sensitive than the Rose Bengal test, complement fixation, and indirect ELISA. Although no serological test and antigen combination fully resolve the diagnosis of bovine and bubaline brucellosis in the presence of false positive serological reactions, but these are simple and practical alternatives to the Brucella culture (Munoz et al., 2005).

India ranks first in the world in terms of total milk production. The total milk production in India is 94.0 million tons, out of which more than 55% is produced by buffaloes. According to livestock census 2003, the buffalo population in Rajasthan is 10.4 million. So buffaloes are valuable component of rural households having socioeconomic importance and play important role in national economy as these are high milk producing animals, but reproductive disease like brucellosis can cause severe losses to the farmer in form of reproductive failure, abortion between the fifth to ninth month of pregnancy and birth of unthrifty calves. Moreover, the animal is a seasonal breeder where even one abortion in a buffalo can leave the poor farmer helpless till the second breeding season. So brucellosis in buffaloes is an important socioeconomic disease but a limited work has been done on brucellosis in this costly animal and a seasonal breeder.
Keeping in view the above facts, the present study was undertaken to assess the status of brucellosis in buffaloes, the major milch animal species, by examining serum and milk with following objectives:

1. To evaluate the reactivity of bovine serum showing signs of brucellosis against *Brucella abortus* antigen in various serological tests.

2. To compare the efficacy of sero-reactivity of various sero-diagnostic tests performed for the diagnosis of brucellosis.

3. Statistical interpretation of anti-*Brucella abortus* antibodies titre determined in various serological tests to decide their sensitivity and specificity.

**2. REVIEW OF LITERATURE**

Brucellosis, an important zoonosis and a significant cause of reproductive losses in animals, found worldwide but well controlled in most developed countries. It is still an uncontrolled serious public health problem in many developing countries including India (Mantur and Amarnath, 2008). In India, brucellosis is yet a very common but often neglected disease in animals especially in buffaloes.

The main impact of the disease is economic; abortions, placentitis, epididymitis and orchitis are the most common consequences. The disease can be diagnosed by cultural or serodiagnostic tests. Serological detection of
antibodies is usually the method of choice for control and eradication of bovine brucellosis. Several conventional tests have been used singly or in combination for the diagnosis of this disease.

The objective of the present investigation included seroepidemiology of brucellosis, study of various serological tests and comparison of these tests; hence the literature has been reviewed under three broad headings viz. seroprevalence of brucellosis, different serodiagnostic tests and comparison of different serological tests.

**SEROPREVALENCE OF BRUCELLOSIS**

Development of the first agglutination test was reported by Wright and Smith (1897) over 100 years ago for the diagnosis of Malta fever in British soldiers, which helped to distinguish it from typhoid fever.

Kataria and Verma (1969) conducted serological survey of bovine brucellosis in Madhya Pradesh and from 6438 sera samples examined, 4.44 percent were found positive.

Nag *et al.* (1977) studied the prevalence of *Brucella* infection among cattle and buffaloes of West Bengal state. Out of 50 serum samples taken, 24 percent showed positive reaction.
Kapoor *et al.* (1985) examined the milk sample of 58 goats by MRT in Bikaner region of Rajasthan. The prevalence was found to be 11.42 percent and 1.92 percent in milch goats and slaughtered goats, respectively.

Chatterjee *et al.* (1986) detected *Brucella* antibodies in cattle and buffaloes in West Bengal and recorded overall seroprevalence of 20.5 and 19.6 percent, respectively by standard tube agglutination test.

Savalgi *et al.* (1987) screened a total number of 464 buffaloes, 82 cows and 20 working staff serologically for brucellosis in Karnataka and the seropositivity was found in 10 buffaloes, 10 cows and 4 working staff.

Sharma *et al.* (1990) tested breeding bulls in a murrah buffalo herd in Vietnam having history of abortion in buffaloes at 6-9 months of pregnancy and suspected for brucellosis. Out of 8 breeding bulls, only 1 positive reactor was found on repeated testing by serological test.

During the study on brucellosis Shrivastava *et al.* (1991) in Anand screened 571 serum samples from cows and buffaloes and reported overall seroprevalence of 38.87 percent.

Chandramohan *et al.* (1992) tested 138 sera samples from zebu cattle and buffaloes by ELISA in which 26 (18.84 percent) were found positive for *Brucella* antibodies.
Sera of 967 camels of both sexes were tested by Gameel et al. (1993) for antibodies to Brucella in Libya using the Rose Bengal plate test, serum agglutination test and the complement fixation test. The prevalence of positive sera was 4.1 per cent.

Ahmad et al. (1994) revealed a much higher prevalence in different species of livestock maintained at organized farms (7.0 percent), compared to those belonging to rural domestic animal holdings (3.5 percent). Out of 2000 buffaloes they studied, 500 from Government farms, 500 from private farms and 1000 from rural domestic animal holdings, the prevalence of brucellosis was 7.00 percent, 6.20 percent and 3.5 percent, respectively.

Lodhi et al. (1995) tested 208 individually owned adult buffalo cows in and around Faisalabad for brucellosis and found seroprevalence 12.98 percent and 2.40 percent through RBPT and SAT, respectively.

Sharma and Saini (1995) in Punjab found 8.69 and 14.91 percent cattle and buffaloes, respectively positive in STAT.

In a seroprevalence study of brucellosis by Rahman et al. (1997) in in Bangladesh the buffaloes with retained placenta showed highest prevalence (7.1%) whereas the repeat breeder buffaloes showed lowest prevalence (1.2%).
Mathias et al. (1998) estimated prevalence of brucellosis among 462 buffaloes from 16 herds from the Ribeira Valley region, Brazil, and observed that 10.39% of the animals were reactors to the complement fixation test.

Nasir et al. (1999) recorded 3.84 percent seroprevalence of brucellosis in 1377 cattle of various government livestock farms in Punjab in Pakistan by using RBPT.

Prahlad et al. (1999) carried out seroprevalence study of brucellosis in buffaloes. Seroprevalence in Punjab was found higher (21.39 percent) than in Uttar Pradesh (11.32 percent). RBPT showed 33.33 percent relative sensitivity using CFT as an indicator test.

Rao et al. (1999) studied the prevalence of brucellosis among Murrah buffaloes and cross breed cows. The incidence was observed to be 16.25 percent and 31.25 percent by dot-ELISA, 11.5 percent and 16.25 percent in RBPT and 8.75 percent and 15.0 percent in STAT, respectively.

Chakraborty et al. (2000) screened 141 sera samples of cattle in Assam for brucellosis antibodies using ELISA, SAT and RBPT and found very high seroprevalance i.e. 56.02 percent, 50.33 percent and 33.33 percent, respectively.
Chauhan et al. (2000) tested 59 sera samples (50 from aborted buffaloes and 9 from pregnant buffaloes) for bovine brucellosis in North Gujarat and found 28 (17.46 percent) and 23 (38.98 percent) samples positive by RBPT and STAT, respectively.

In a seroprevalence study carried out by Kubuafor et al. (2000) in 183 cattle of Ghana using the Rose Bengal plate test, a mean seroprevalence of 6.6 percent was detected.

Silva et al. (2000) in Sri Lanka, found an overall seroprevalence of brucellosis 4.7% in cattle and 4.2% in buffaloes using I ELISA.

Sandhu et al. (2001) studied 666 cows and 750 buffaloes to determine the seroprevalence of brucellosis in Punjab, India and found 10.06 percent cows and 9.33 percent buffaloes positive for brucellosis.

Renukaradhya et al. (2002) studied brucellosis serologically for a long term and indicated that 5 percent of cattle and 3 percent of buffaloes are infected with brucellosis in India.

Chahota et al. (2003) reported an outbreak of brucellosis in an organized dairy farm in Himachal Pradesh employing rose bengal plate test (RBPT) and serum agglutination test (SAT) and revealed involvement of both B. abortus and B. melitensis in all affected cows.
Miranda et al. (2003) estimated the prevalence of bovine brucellosis in Brazil. A random sample of 18 municipalities was selected. These were divided in quadrants and one herd was selected at random from each quadrant. Antibodies to *Brucella abortus* were observed in 8 out of 18 municipalities (44.44%), in 8 out of 72 herds (11.11%) and in 8 animals out of 2343 samples collected (0.34%).

Prevalence of brucellosis in organized farms with abortion storms in Goa region was investigated by Barbuddhe et al. (2004). Out of 107 serum samples tested for brucellosis, 40 (37.38%), 39 (36.45%) and 43 (40.18%) were found positive for antibodies against *Brucella*, by RBPT, STAT and AB-ELISA, respectively.

Chand and Sharma (2004) screened out the cattle serum samples for brucellosis in Haryana, Uttar Pradesh and Madhya Pradesh. Overall prevalence rate of brucellosis was found to be 26.50 percent by ELISA, 20.47 percent by RBPT and 18.89 percent by STAT.

Charanjeet et al. (2004) found only 2.65 percent cattle, 3.42 percent buffaloes, 15.60 percent sheep and 5.4 percent goats positive for brucellosis in Himachal Pradesh.

Das et al. (2004) recorded seroprevalence of brucellosis in buffalo bulls and she-buffaloes at farms as 1.6 percent and 10 percent by RBPT; 1.06 percent and 5.9 percent by STAT and 0.53 percent and 8.18 percent by
DOT-ELISA respectively. Similar data at the abattoir were recorded as 6.5 percent and 22 percent by RBPT; 4.5 percent and 4.5 percent by STAT and 4.5 percent and 15.5 percent by DOT-ELISA respectively.

Mahato et al. (2004) screened serum samples of 40 bulls by STAT and I-ELISA and found 2 bulls positive by both the tests.

Nasir et al. (2004) carried out seroprevalence studies in Punjab and recorded the seroprevalence as 14.70% in cattle and 15.38% in buffaloes at Government and 18.53% in cattle and 35.40% in buffaloes at various private livestock farms using RBPT. Out of these RBPT positive animals, 7.19% cattle and 2.91% buffaloes at Government whereas 9.00% cattle and 23.70% buffaloes at private livestock farms were found seropositive with SAT.

Singh et al. (2004b) found the overall prevalence of brucellosis in different Gaushalas of Mathura as 15.15 percent in cattle and 4.5 percent in man.


In a seroprevalence study carried out by Dhand et al. (2005) in Punjab, the prevalence rates among buffaloes and cattle were reported as 13.4 percent and 9.9 percent, respectively. The seroprevalence was found to be higher in animals with a history of abortion (33.87 percent) than in those without such a history (11.63 percent).
Mishra et al. (2005) in Uttar Pradesh found 1.55 percent of cows and 1.97 percent of buffaloes seropositive for brucellosis by STAT, while 3.11 percent of cows and 4.18 percent of buffaloes by I-ELISA out of the total 579 serum samples of cows and 407 of buffaloes.

Mittal et al. (2005) studied seroprevalence of brucellosis in animals in Uttaranchal. Maximum seroprevalence was observed in goats (39.21 percent) and minimum in buffaloes (29.85 percent). Female animals (38.24 percent) were found more seropositive than male animals (22.50 percent).

Rajkhowa et al. (2005) in India recorded seroprevalence of brucellosis in 98 mithuns using avidin–biotin enzyme-linked-immunosorbent assay, standard tube-agglutination test and Rose-Bengal plate test as 34 percent, 20 percent and 11 percent, respectively.

Chivandi (2006) reported 4.11% prevalence rate of bovine brucellosis in the Gokwe Smallholder Dairy Project Herd of Zimbabwe.

From year 2004 to 2006 a seroepidemiological study was conducted by Omer et al. (2007) in Eastern Sudan using RBPT. An average of 10.9% cattles was found positive for brucellosis.
Otlu et al. (2008) in Turkey serologically investigated the prevalence of brucellosis in cattle, farmers and veterinarians using RBPT, SAT and ELISA. Of the cattle sera analyzed, 32.92 percent and 34.64 percent were determined as positive by RBPT and SAT, respectively.

Upadhyay et al. (2007) carried out serosurveillance of brucellosis in cattle and buffalo in Uttar Pradesh state and found overall 7.25 percent prevalence of bovine brucellosis. Seroprevalence was found to be higher in female animals (16.73 percent in cattle and 4.18 percent in buffaloes) than in males (12.94 percent in cattle and 1.41 percent in buffaloes).

Zafer et al. (2007) investigated the seroprevalence of Brucellosis in human, sheep, and cattle populations in rural regions of Turkey. Among cattle sera, 2.67 percent showed seropositivity with RPBT and 1 percent with the complement fixation test (CFT).

Sahin et al. (2008) studied seroprevalence on total 626 serum samples of cattle with a history of abortions in Turkey. Of the cattle sera analyzed, 35.30 percent and 32.92 percent and 39.45 percent were found to be positive by RBPT, SAT and ELISA, respectively.

Al-Majali et al. (2009) investigated the seroprevalence and risk factors for Brucella seropositivity in Jordan. The prevalence in individual cows and cattle herds was found to be 6.5% and 23%, respectively using a Rose
Bengal plate test and indirect ELISA. The seroprevalence of brucellosis in cows older than 4 years of age was significantly higher than that in the younger cows.

Brahmabhatt et al. (2009) revealed 19.12 percent seroprevalence of brucellosis in buffaloes in the central Gujarat region of India.

Magona et al. (2009) found herd-level seroprevalence and animal-level seroprevalence of brucellosis in cattle as 100 percent and 34.0 percent in the pastoral system and 5.5 percent and 3.3 percent in the zero grazing system, respectively in Uganda.

Samaha et al. (2009) in Egypt employed found no significant difference in brucellosis sero-prevalence between cattle of Friesian and Charolais breeds, or between male and female animals, but brucellosis prevalence was significantly higher in more-than-1-year-old cattle than its prevalence in less-than-1-year-old cattle.

**MRT (MILK RING TEST)**

Jaartsveld and Mathiissen (1976) suggested that performing the milk ring test in herd samples from herds of approximately twenty dairy cows is an acceptable method in checking herds for brucellosis but the larger herds in the threatened areas should be examined by performing the milk ring test in individual samples at regular intervals.
Blythman and Forman (1977) performed *Brucella* milk ring tests (BMRT's) on fresh and pooled samples, preserved with potassium dichromate and observed that preserved milk gave results that were similar to an average result for individual fresh daily samples. When fresh samples gave variable results and the pooled preserved sample gave a negative result, it was considered that the latter result was more reliable, possibly due to factors causing false positive reactions were diluted.

Rahman *et al.* (1978) reported prevalence of Brucellosis using milk ring test (MRT) as 11.44% from Savar, 16.66% from Tangail and 4.19% from Bangladesh Agricultural University (BAU) dairy farm. Higher incidence of disease was observed among cows of organized farms.

Pharo *et al.* (1981) recorded the 62.5 percent prevalence of bovine brucellosis by using Milk Ring test. The scientist reported that 30.7% of MRT positive cows were also positive to RBPT.

Rahman and Rahman (1981) studied the incidence of *Brucella* infection in subclinical mastitic udder using MRT and revealed 5.5, 11.44 and 0.00% of brucellosis in cattle on BAU dairy farm, Central Breeding and Dairy farm, Savar and rural areas, respectively.
Sutra et al. (1986) extracted milk immunoglobulins from the stained cream layer of positive milk ring tests and observed that IgA was always found, associated with IgM in most cases (15/17) and with IgG in a smaller number of cases (11/17).

Vanzini et al. (2001) compared indirect enzyme-linked immunosorbent assay (ELISA) with milk ring test (MRT) for the detection of *Brucella abortus* antibodies in bovine bulk milk samples. The sensitivity of the ELISA (98.1%) was found higher than the BRT (72.2%) but the specificity of BRT (90.5%) was not statistically different from the ELISA (88.1%).

Gumber et al. (2004) analyzed 970 bulk milk samples by AB milk-ELISA and MRT to know the status of bovine brucellosis in Punjab. The MRT showed lower sensitivity (68.8%) but had comparable specificity (98.9%) than that of milk-ELISA.

Mahato et al. (2004) used MRT to detect *Brucella* antibody in individual milk samples of 67 cows and found 24 (35.82%) positive cases.

Abbas and Aldeewan (2009) collected 420 samples of raw cow and buffalo milk from the Basrah province of Iraq and tested in the milk ring test to detect *Brucella* antibodies. Positive results were obtained in 25.8 percent of samples of cow milk and 35 percent samples of buffalo milk.
RBPT (ROSE BENGAL PLATE TEST)

Nicoletti (1967) devised a rapid card test to detect brucellosis which is now named as Rose Bengal Plate Test (RBPT). The test is a simple spot agglutination test using antigens stained with rose bengal and buffered to a low pH, usually $3.65 \pm 0.05$. The low pH prevents some agglutination by IgM and encourages agglutination by IgG1 thereby reducing non-specific interactions (Corbel, 1972; Allan et al., 1976). The test is considered to be suitable for screening of individual animals, however, some cross-reacting antibodies have been detected by this test (OIE, 2004).

Morgan (1969) and Nicoletti (1969) found RBPT as the most valuable screening test for the diagnosis and control of brucellosis especially in infected herds where the test seems to identify infected material at an early stage than the STAT.

Davies (1971) stated that the RBPT gives few false positive reactions and detects the infection earlier than the plate agglutination test.

While working on brucellosis Gallagher (1973) screened 1,879 sera from vaccinated cattle with RBPT, and all reactors were subjected to the SAT and CFT. The author observed that out of 409 sera reacted to the RBPT,
279 were confirmed positive by the CFT. The correlation both positive of the RBPT with the CFT was thus 68.2 percent.

Beh (1974) concluded that cross reactivity with vaccinal IgM might be accounted for the false positive reaction in RBPT.

Strohl (1974) mentioned that Rose Bengal test was a modification of Plate agglutination test used earlier, replacing the dye Gentian Violet with Rose Bengal.

Diaz et al. (1978) employed the Rose Bengal test to detect antibodies against different structural antigens in spinal fluid of human *Brucella* meningitis. The Rose Bengal test was positive in five out of five patients studied.

Kulshrestha et al. (1978) reported during surveys of brucellosis, that Rose Bengal Plate Test was a reliable diagnostic test for brucellosis in cattle and buffaloes.

Timbs et al. (1978) affirm that RBPT is seldom used for a definitive diagnosis of brucellosis due to its low specificity, although it is used as a screening method.

Stryszak (1986) determined the value of RBPT in diagnosing brucellosis in cattle by making 2 variants of RBPT and concluded that sensitivity of the test can be increased by halving the amount of RBPT antigen used.
Alton et al. (1988) showed that RBPT detected IgM antibodies more efficiently than IgG₁ and IgG₂ antibodies.

Marín et al. (1999) found RBPT as the most sensitive test when they compared the competitive and standard enzyme-linked immunosorbent assays (ELISAs), RBPT, CFT, and agar gel immunoprecipitation with native hapten (AGID-NH) tests in sheep.

While studying on brucellosis in buffaloes Prahlad et al. (1999) showed that RBPT has the highest relative sensitivity (33.33 percent) using CFT as an indicator test.

Chakraborty et al. (2000) estimated relative sensitivity and specificity of RBPT for bovine brucellosis on basis of ELISA 56.96 percent and 96.77 percent, respectively.

In a seroprevalence study of brucellosis carried out by Nasir et al. (2004) in cattle and buffaloes RBPT recorded the 14.70 percent seroprevalence in cattle and 15.38 percent in buffaloes at Government and 18.53 percent in cattle and 35.40 percent in buffaloes at various private livestock farms.

Singh et al. (2004a) reported that relative sensitivity and specificity of RBPT during study in 6 organized dairy farms in Punjab, were 88.46 percent and 97.75 percent using AB-ELISA as the gold standard test.
Rahman and Baek (2005) stated that RBPT, using \textit{B. abortus} strain S1119-3 whole cell antigen, appears to be a potential candidate, as a useful diagnostic method, for brucellosis detection in rats.

In a serological survey of brucellosis conducted by Cadmus \textit{et al.} (2006) in livestock animals and workers, prevalence in cattle was recorded as 5.82 percent by RBPT.

Chakravarty \textit{et al.} (2007) examined 344 heads of cattle belonging to organized and unorganized farms for \textit{Brucella} antibodies using RBPT and 29.07 percent were found positive.

Bandyopadhyay \textit{et al.} (2009) estimated relative sensitivity and specificity for RBPT as 88.76 and 100%, respectively in comparison to I-ELISA while studying the seroprevalence of brucellosis in yaks of North-Eastern hilly yak tracts of Arunachal Pradesh, India.

**AGID (AGAR GEL IMMUNODIFFUSION TEST)**

While working on \textit{Brucellosis} in human sera McMahon \textit{et al.} (1979) found that agreement between the agar gel immunodiffusion (AGID) test and the STAT was 97% when the titer was 1:160 or higher.
Diaz et al. (1968) demonstrated that most antigens present in water soluble extracts of sonicated Brucella sp. are common to smooth and rough members of the genus but are distinct from the soluble antigens of several other Gram-negative bacteria from outside the genus Brucella.

Corbel (1972) demonstrated that serum of a cow naturally infected with B. abortus precipitated five antigens present in an ultrasonically disrupted extract of smooth B. abortus.

During study on brucellosis in buffaloes Chand et al. (1988) used sonicated antigen for precipitation reactions and observed that AGPT is less sensitive than RBPT, STAT, CFT and CIE test.

**STAT (STANDARD TUBE AGGLUTINATION TEST)**

The standard serum agglutination test (SAT) is still widely used for the diagnosis of brucellosis but the test is liable to give false positive results because it fails to differentiate between the antibody response to natural infection and that in response to vaccination with Brucella abortus strain 19 (Beh, 1974; Nielson et al., 1989). But specificity of the test can be significantly improved with the addition of EDTA (Rose and Roepke, 1964; Macmillan and Cockrem, 1985).

Artemov (1963) conducted series of experiments on 3044 animals which were free from brucellosis and reported that agglutination occurred at 1:10 dilution and in few cases in 1:20 or even at 1:40 dilution.
Allan et al. (1976) showed that the STAT measured IgM class of immunoglobulins more efficiently than IgG1 class of antibodies in brucellosis.

Nag et al. (1977) recorded 24 percent seroprevalence of *Brucella* infection among cattle and buffaloes of West Bengal state using tube agglutination test.

Chappel et al. (1978) reported that serum agglutination test detected 35.0 percent apparent false negative reactors and 5.0 percent apparent false positive.

Kalimuddin et al. (1990) examined sera samples from cows of two organized farms for brucellosis and found 18.81 percent as positive by STAT.

Lodhi et al. (1995) found seroprevalence of brucellosis 2.40 percent through SAT in individually owned adult buffaloes in and around Faisalabad.

During serodiagnosis study of brucellosis Ghani et al. (1996) found 11 serum samples positive out of 500 buffaloes using STAT.

Jiwa et al. (1996) screened a total of 13078 cattle in Tanzania for brucellosis using the serum agglutination test (SAT) and revealed an overall prevalence rate of 10.8 percent. The lowest prevalence rate (4.3 percent) was
found in the Local management system, with Dairy intermediate (6.3 percent) and Ranch highest prevalence rate (15.8 percent).

Relative sensitivity and specificity of STAT for bovine brucellosis on basis of ELISA results were found as 88.61% and 98.59%, respectively as recorded by Chakraborty et al. (2000).

Barbuddhe et al. (2004) tested serum samples from organized farms with abortion storms for brucellosis and found 36.45 percent samples positive for antibodies against *Brucella* by STAT.

Nasir et al. (2004) carried out seroprevalence studies of brucellosis in cattle and buffaloes from various Government and private livestock farms and found 2.91 percent buffaloes at Government whereas and 23.70 percent buffaloes at private livestock farms seropositive in SAT.

Nasir et al. (2005) subjected RBPT positive samples to routine serum agglutination (SAT) and modified SAT (EDTA added) tests and concluded that modified SAT can be used as an alternate to routine SAT in the diagnosis of brucellosis.

Chakravarty et al. (2007) examined 344 heads of cattle belonging to organized and unorganized farms for *Brucella* antibodies using STAT and 33.72 percent were found positive.
Otlu et al. (2008) serologically investigated the prevalence of brucellosis in cattle, farmers and veterinarians using RBPT, SAT and ELISA. Of the cattle sera analyzed, 34.64 percent were determined as positive by SAT.

Sharma et al. (2007) screened 2988 animals in 62 dairy farms and gaushalas of Punjab for brucellosis and 540 (18.07%) were found positive by STAT.

Upadhyay et al. (2007) investigated seroprevalence of brucellosis in cattle and buffalo in selected districts of Uttar Pradesh state of India. The authors observed overall prevalence of bovine brucellosis 4.73 percent by STAT.

Bandyopadhyay et al. (2009) recorded the relative sensitivity and specificity for STAT as 79.77 and 100%, respectively in comparison to I-ELISA during seroprevalence study of brucellosis in yaks of North-Eastern hilly yak tracts of Arunachal Pradesh, India.

During a study on bovine brucellosis conducted by Sahin et al. (2008) in Turkey, serum samples of cattle with a history of abortions were examined for Brucella antibodies and 32.92 percent were found positive by SAT.

**HIT (HEAT INACTIVATION TEST)**
Jones (1927) reported that the antibody destruction progressed as temperature increased, and that heating at 65°C for 20 minutes appreciably affected the antibody activity.

Hess (1953) observed that the non specific agglutinins of bovine origin, associated with suspect *Brucella* titres, were inactivated when heated at 70°C for 10 minutes.

Morse *et al.* (1955) concluded that incubation of the tubes in tube agglutination test for bovine brucellosis at a temperature of 56°C, resulted in a decrease in titres of sera from many suspected and low reactor cattle. Reduction in titre was more pronounced with sera from vaccinated animals than from unvaccinated animals.

Rose (1955) found that two non specific *Brucella* agglutinins were present in the bovine serum, one agglutinin was heat stable and the other was heat labile at 56°C.

Amerault *et al.* (1961) reported that nonspecific reactions were inhibited on inactivation of serum at 65°C for 15 min whereas the specific reactivity was remained as such. The scientists showed that *Brucella* could be isolated from 96% of cattle that had serum positive to the HIT.

Kulshrestha *et al.* (1973) reported that out of 251 cattle and 602 buffaloes serum tested with STAT, 31 (12.3 percent) cattle, 234 (5.9 percent) buffaloes were found positive and with HIT 5 (1.9 percent) cattle and 19 (3.1 percent) buffaloes were found positive.
Kalimuddin et al. (1990) found 16.83 percent sera samples positive for Brucella agglutinins by heat inactivation test during their study on brucellosis in dairy cattle.

Sandhu and Joshi (1993) applied mercaptoethanol test and heat inactivation test to confirm the result of screening tests and to differentiate vaccinal titre from that of active infection in bovines.

Shringi (1999) screened 265 cattle sera samples by STAT, HIT and 2-MET. The relative sensitivity of STAT, HIT, 2-MET were 89.62, 90.0, and 92.22 percent respectively.

**2-MET (2- MERCAPTOETHANOL TEST)**

Nicoletti and Muraschi (1966) presented data on comparing several supplemental blood serum and milk tests with the standard plate and tube agglutination tests. The scientists concluded that the use of supplemental procedures would hasten the eradication of brucellosis.

While studying on cattle of infected herds Nicoletti (1969) indicated that 2MET classified 97 percent of the infected cattle as positive for brucellosis.

Buchanan et al. (1974) concluded that the complement fixation test and 2- mercaptoethanol tests were the most accurate indicators of active disease.
McMahon et al. (1979) compared AGID test and 2-MET on 148 human sera that demonstrated a STAT titer of 1:20 or higher for brucellosis. All sera with a 2-ME-agglutination titer of 1:40 or higher were positive with the AGID test.

While studying on brucellosis in human patients Buchanan and Faber (1980) suggested that the 2ME test is superior to the standard tube test in determining the adequacy of antibiotic therapy, and a negative 2ME test is strong evidence against a diagnosis of chronic brucellosis.

Concordance between the ELISA and 2- mercaptoethanol test (2-MET), was found 75.7 percent by Ruppanner et al. (1980) while studying on brucellosis using sera of experimentally infected heifers.

In a research on patients with symptoms of brucellosis Klein and Behan (1981) stated that the IgG antibody titer which is significant for differentiating active from inactive disease can be determined by treating the serum with 2-mercaptoethanol that inactivates the IgM Brucella antibodies while leaving the IgG Brucella antibodies intact.

In a comparison of the 2-Mercaptoethanol test with Dithiothreitol test McMahon (1983) showed that the dithiothreitol test is not a reliable substitute for the 2-mercaptoethanol test to detect immunoglobulin G agglutinating antibody in bovine serum.
Stemshorn et al. (1985) revealed 99.8 percent specificity of 2-mercaptoethanol test (2-MET) based on a study on brucellosis in 1051 bovine sera.

Out of 101 serum samples of cows of two organized farms tested for brucellosis by Kalimuddin et al. (1990), 13 (12.87 percent) were found positive by 2-ME test.

Hadad and Jamalludeen (1992) tested cattle sera for antibodies against *Brucella* by RBPT, TAT, 2-MET and CFT. Out of total 2006 serum samples 6.24 percent gave positive result with 2-MET.

In a comparison of ELISA and 2-ME test Saravi et al. (1995) found that sensitivity of ELISA and 2MET were comparable.

Shringi (1999) tested 265 cattle sera samples by 2-MET and found 92.22 percent relative sensitivity of 2-MET.

Elfaki et al. (2005) examined twenty patients with symptoms of brucellosis before and after antibiotic treatment for the diagnosis of brucellosis. The authors reported that 2-mercaptoethanol test was positive in 60% of the enrolled patients and negative in all patients after the antibiotic treatment.

**ELISA (ENCEMYE LINKED IMMUNOSORBENT ASSAY)**
Ruppanner et al. (1980) compared ELISA with standard tube agglutination (STAT), 2-mercaptoethanol agglutination (2-MET), microtitration complement-fixation (MCFT), and automated complement-fixation (ACFT) tests. The scientists observed that concordance between the ELISA and other tests were 100% (STAT), 75.7% (2-MET), 97.8% (MCF), and 95.2% (ACF).

Enzyme-linked immunosorbent assay (ELISA) was used by Van Aert et al. (1984) for the detection of antibodies to *Brucella abortus* in bovine sera. Among 785 animals from 23 brucellosis-infected herds, 336 were positive in ELISA.

Chandramohan et al. (1992) used indirect ELISA to screen 138 serum samples from zebu cattle and buffaloes in which 26 animals (18.84 percent) were found positive for *Brucella* antibodies.

Saravi et al. (1995) compared Enzyme-Linked Immunosorbent Assay (ELISA) with the Rose Bengal Plate Test (RBPT) and the buffered plate antigen (BPAT), the 2-mercaptoethanol (2-MET) and the complement fixation (CF) tests and showed that in the testing of vaccinated cattle from *Brucella*-free dairy herds, the diagnostic specificity of the ELISA (99.7%) was comparable to the RBPT (99.7%), 2-MET (99.8%) and CFT (99.9%) and greater than the BPAT (90.6%).
Uzal et al. (1995) compared I-ELISA with rose Bengal plate test (RBPT), the buffered plate antigen test (BPAT), 2-mercaptoethanol test (2-MET) and CFT. The specificity of the I-ELISA (98.9%) was similar to that of the BPAT, RBPT, 2MET and CF tests for negative animals and its sensitivity (98.7%) was higher than the BPA test (96.1%) and the CF test (95.2%) in case of positive animals.

Kerby et al. (1997) evaluated an I-ELISA for detection of brucellosis in comparison to the CFT and concluded that, against sera from the positive population, the ELISA gave many more positive reactions than that of CFT.

Chakraborty et al. (2000) screened 141 serum samples collected from 111 cows and 30 heifers of various organized and unorganized farms by rose Bengal plate test (RBPT), serum agglutination test (SAT) and indirect ELISA and considered ELISA as more sensitive test than the other two tests.

Dangolla and Kulachelvy (2000) used the indirect enzyme-linked immunosorbent assay (I-ELISA) test to screen 3916 cattle and buffaloes for brucellosis and the overall seroprevalence in cattle and buffaloes were 4.7% and 4.2%, respectively.
Omer et al. (2001) compared Rose-Bengal Plate test (RBPT), the complement-fixation test (CFT) and an indirect enzyme-linked immunosorbent assay (I-ELISA) for the detection of *Brucella*-infected animals in unvaccinated cattle herds and reported that ELISA titers accurately predicted all the negative sera in herds.

Renukaradhya et al. (2001) developed and calibrated AB ELISA kit in India using the indirect brucellosis ELISA kit of the International Atomic Energy Agency (IAEA) as a reference and reported the overall specificity of A-B ELISA was 98.8% and overall sensitivity was 98.2%.

Sarumathi et al. (2003) compared the efficacy of avidin-biotin ELISA (A-B ELISA), RBPT and STAT in detecting antibodies to *Brucella* in serum samples from cattle. The authors noted that AB-ELISA had a sensitivity of 100% and specificity of 88.22% and 90.59% when compared with RBPT and STAT respectively.

Chand (2006) compared monoclonal antibody based competitive ELISA (CELISA) for detecting brucellosis in cattle with Standard Tube Agglutination Test (SAT) and indirect ELISA (I ELISA). Out of 411 animals 104 were positive in CELISA, 107 in I ELISA and 93 in SAT. The results of CELISA were comparable with I ELISA.

Upadhyay et al. (2007) investigated seroprevalence of brucellosis in cattle and buffaloes in selected districts of Uttar Pradesh state of India. Overall prevalence was observed 7.25 percent by I-ELISA out of 1034 serum samples screened.
During his work with 280 human patients Heydari et al. (2008) compared the sensitivity of STAT and ELISA and demonstrated that ELISA was more sensitive than STAT In a diagnostic and follow up study of brucellosis.

Sahin et al. (2008) conducted study on bovine brucellosis in Turkey. Out of total of 626 serum samples of cattle with a history of abortions examined 247 (39.45%) were found positive by ELISA.

Osoba et al. (2001) evaluated the diagnostic value of Brucella ELISA IgG and IgM in 83 patients with brucellosis. Blood culture of 30/83 (36.1%) patients was positive. Among the 30 bacteremic patients, 24 (80%) had an increased IgM titer. Of the 53 non-bacteremic patients, 41 had IgM titer, while 22 had IgG titer. The ELISA IgM and IgG tests achieved a specificity and sensitivity of 100% and 96% respectively. The scientists concluded that Brucella ELISA is a reliable and sensitive test in the diagnosis of brucellosis.

**COMPARISON OF DIFFERENT SEROLOGICAL TESTS**

Ruppanner et al. (1980) compared ELISA with STAT, 2- mercaptoethanol test (2-MET), microtitration complement-fixation (MCF), and automated complement-fixation (ACF) tests for brucellosis using sera of experimentally infected heifers. Concordance between the ELISA and other tests were found 100 percent (STA), 75.7 percent (2-MET), 97.8 percent (MCF), and 95.2 percent (ACF).
Mathias and Pinto (1983) compared complement fixation (CFT), plate agglutination (PAT), tube agglutination (TAT) and Rose Bengal plate tests (RBPT) for serodiagnosis of brucellosis in 180 Indian buffaloes. The results showed that the CFT is useful for diagnosis of bovine brucellosis.

Stemshorn et al. (1985) compared six agglutination and two complement fixation tests with respect to specificity, sensitivity and relative sensitivity based on 1051 bovine sera. The scientists revealed 98.9 percent specificity of buffered plate antigen test (BPAT), 99.2 percent and 99.3 percent for standard tube and plate agglutination tests (STAT and SPAT) respectively, and 99.8 percent for the 2-mercaptoethanol test (2-MET).

Rahman et al. (1990) conducted serodiagnostic tests on 13 serum samples from buffaloes suspected for brucellosis, the higher percentage of positive cases were obtained with the single radial haemolysis test, followed by the rose bengal test.

Hadad and Jamalludeen (1992) tested cattle sera for antibodies against *Brucella* by RBPT, TAT, 2-MET and CFT. Out of total 2006 serum samples 5.8 percent gave positive reaction to RBPT and CFT, 7.78 percent gave positive reaction to TAT and 6.24 percent gave positive result with 2-MET.
Sandhu and Joshi (1993) screened serum samples from 1134 vaccinated and non vaccinated bovines using RBPT, PAT and STAT. All the positive sera samples were further tested by 2-MET and heat inactivation test to confirm the result of screening tests and to differentiate vaccinal titre from that of active infection.

Saravi et al. (1995) compared ELISA with RBPT, BPAT, 2-MET and CF tests. The ELISA demonstrated the higher sensitivity as compared to CF tests and comparable to 2-MET.

Ghani et al. (1996) evaluated relative efficacies of SPAT, STAT, RT and 2-MET in serodiagnosis of brucellosis in 500 buffaloes from Peshawar city, Pakistan. Positive reaction against *B. abortus* antigen was detected in 13, 11, 9 and 6 blood samples using SPAT, STAT, RT and 2-ME tests, respectively.

Kerby et al. (1997) evaluated an I-ELISA for detection of brucellosis in comparison to the CFT on sera from a non-vaccinated negative population, a vaccinated negative population and a brucellosis positive population of unknown vaccination status. The CFT and ELISA showed specificities of 100% and 98%, respectively against the negative non-vaccinated group. The specificity of CFT was 98% against the S19-vaccinated negative group, but ELISA specificity fell to 94% using a cut-off of 40% of positive control and against sera from the positive population, the ELISA gave many more positive reactions than that of CFT.
Omer et al. (2001) compared RBPT, CFT and I-ELISA for the detection of *Brucella*-infected animals in unvaccinated cattle herds in Eritrea. ELISA was found equal sensitive to CFT but its specificity was higher than CFT.

Fosgate et al. (2002) evaluated different serological tests for the diagnosis of *Brucella*-specific antibodies in cattle and water buffalo by use of SPAT, card test (CT), BPAT, and STAT. They reported that BPAT had the highest sensitivity for screening cattle and water buffalo.

Shringi (1999) screened 265 cattle sera samples by RPAT, STAT, HIT, 2-MET. The relative sensitivity of STAT, HIT, 2-MET were 89.62, 90.0, and 92.22 percent, respectively.

Sarumathi et al. (2003) compared the efficacy of avidin-biotin ELISA (A-B ELISA), RBPT and STAT in detecting antibodies to *Brucella* in serum samples from cattle. A-B ELISA gave sensitivity of 100 percent in comparison to RBPT and STAT.

Gen et al. (2005) detected the antibodies against *B. abortus* in the serum samples of aborted dairy cows as 68.1 percent, 65.6 percent, 58.9 percent and 55.2 percent by the Competitive ELISA, CFT, RBPT and SAT, respectively.
Agrawal et al. (2007) compared three serological tests namely RBPT, STAT and ELISA by applying to the 142 cattle and 61 buffalo sera of the state Uttaran chal. They found that ELISA was more sensitive followed by RBPT and STAT.

Jain et al. (2007) evaluated the accuracy of various immunodiagnostic techniques and determine the brucellosis seroprevalence in animals in Uttaran chal, India. The authors revealed that ELISA proved more sensitive in sheep and goats, while RBPT detected more seropositive cattle (7.53%), and equal numbers of seropositive buffaloes (6.67%) were detected by both test.

Muma et al. (2007) evaluated diagnostic performance of three serological tests for brucellosis, Rose Bengal Plate Test (RBPT), competitive ELISA (C-ELISA) and Fluorescence Polarization Assay (FPA) in naturally infected cattle in Zambia. They revealed the highest sensitivity was achieved by the C-ELISA and the highest specificity by the FPA.

Munir et al. (2008) developed an Indirect ELISA (I-ELISA) using lipopolysaccharide (LPS) as antigen and compared with the commercial kit using 100 negative and positive sera each from buffaloes. The agreement for the positive result between the developed and commercial I-ELISA was 78 percent and for the negative it was 100 percent.
Heydari et al. (2008) compared the sensitivity and specificity of the STAT and ELISA in diagnosis and follow up of the brucellosis. A total of 280 patients were studied and ELISA was demonstrated to be more sensitive to STAT.

Chachra et al. (2009) evaluated serological tests using a total of 28 serum samples which included 18 samples from brucellosis suspected and 10 from normal healthy cattle. Out of 18 sera from suspected cases, 5.55 percent were found positive by STAT and 50 percent were found positive by RBPT, whereas Dot ELISA could detect antibodies in 100 percent samples.

3. MATERIALS AND METHODS

The present work on seroprevalence studies and antibodies detection for Brucella infection was carried out in buffaloes and buffalo bulls. Blood and milk samples were collected from Sangod, Mandana, Bundi, Talera, Chhabra, Jhalrapatan and Dag regions of Kota division and subjected to various diagnostic serological tests.
The study was conducted at Department of Veterinary Microbiology and Biotechnology, College of Veterinary and Animal Sciences, Bikaner. During the present study a total of 436 serum samples and 63 milk samples were collected for anti *Brucella* antibodies detection.

Details of samples collected from buffaloes of Kota division:

**Table 3.1: Area wise collection of samples**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Area</th>
<th>Serum samples</th>
<th>Milk samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Buffaloes</td>
</tr>
<tr>
<td>1.</td>
<td>Sangod (Kota)</td>
<td>52</td>
<td>47</td>
</tr>
<tr>
<td>2.</td>
<td>Mandana (Kota)</td>
<td>47</td>
<td>43</td>
</tr>
<tr>
<td>3.</td>
<td>Bundi</td>
<td>67</td>
<td>61</td>
</tr>
<tr>
<td>4.</td>
<td>Talera (Bundi)</td>
<td>91</td>
<td>85</td>
</tr>
<tr>
<td>5.</td>
<td>Chhabra</td>
<td>85</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>(Baran)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Jhalrapatan</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>(Jhalawar)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.1 GENERAL MATERIALS

Glass wares and Plastic wares

During the course of this study, properly cleaned, neutral and standard glass wares and plastic wares compatible with molecular biology work were used.

Chemicals, Buffers, Reagents etc.

The details of chemicals, buffers and reagents used during the study were as per the Appendix.

3.2 COLLECTION OF SAMPLES

3.2.1 Serum

About 10 ml of blood was collected aseptically from the jugular vein of individual animal in a sterile test tube. These test tubes were kept in upright position at room temperature for about 2 hrs, and then clot was detached from the walls with glass rod and the tubes were stored in refrigerator for 12 to 24 h to permit clot contraction. Then serum was decanted into clean tubes and centrifuged at 2500 rpm for 30 min and serum was carefully removed by decanting. The separated serum was collected in a screw capped plastic vials in duplicates and transported to the laboratory. serum sample were preserved by adding 0.20% sodium azide to each sample vial and sera were stored at -20°C till further use. These collected serum samples were subjected to different serological tests.
3.2.2 Milk

The udder was thoroughly washed and cleaned with potassium permanganate solution (1:1000) and dried with clean cloth. Teat openings were disinfected with 70% of ethyl alcohol. After discarding few drops of milk, approximately 10 ml of milk from each quarter was collected in two sets of sterile screw capped plastic vials and transported on the ice to the laboratory and stored at -20°C for future use. The milk samples were used for Milk Ring Test (MRT).

3.3 MILK RING TEST

The test was performed according to the method of Huber and Nicoletti (1986).

Procedure

The Abortus Bang Ring antigen (ABR antigen) obtained from the I.V.R.I., Izatnagar was used for the test.

MRT was performed on individual milk samples. Antigen and milk samples were brought to the room temperature prior to performing the test. About 30-50 μl of antigen was added to the 2 ml of milk in a narrow test tube and mixed thoroughly. The tubes then were incubated at 37°C for 1 h together with positive and negative working standards.
Observation of Result

A strongly positive reaction was indicated by formation of dark pink cream layer above a white milk column. The test was considered to be negative if the pink color of the underlying milk exceeds that of the cream layer.

3.4 ROSE BENGAL PLATE TEST

The test was carried out following the method described by Davies (1971).

Procedure

The RBPT antigen obtained from the Indian Veterinary Research Institute (I.V.R.I.), Izatnagar, Uttar Pradesh was used for the test.

Serum samples and RBPT antigen were brought to the room temperature and then one drop (0.03 ml) of serum was taken on a clean, dry and non greasy glass slide by micropipette. The antigen bottle was shaken well to ensure homogenous suspension and then one drop (0.03 ml) of the antigen was added. The antigen and serum were mixed thoroughly with the spreader and then the slide was rotated for four min. Negative control was prepared by adding antigen to known brucellosis negative serum and positive control was prepared by adding antigen to known brucellosis positive serum. The result was noted after four min.
RBPT antigen and normal saline solution were mixed thoroughly on a separate glass slide in order to detect autoagglutination.

Observation of Result

Definite clumping/agglutination was considered as positive reaction, whereas no clumping/agglutination was considered as negative.

3.5 AGAROSE GEL IMMUNODIFFUSION TEST

The test was carried out following the method described by Stemshorn and Nielsen (1981).

Procedure

Antigen for agarose gel immunodiffusion test consisted of 40µl of a dilute sonicate of the *Brucella abortus* plain antigen. One liter of this 8.0% (v/v) suspension of heat killed cells in 0.5% phenolized 0.85% NaCl solution was sonicated in an ice bath for six 5 minute periods at optimum output using an Ultrasonic Disintegrator equipped with a 2 cm diameter probe. Cell debris and intact cells were removed by centrifugation at 10,000 x g at 4°C for 15 minutes. The supernate was recovered and stored at -20°C.

The agarose gel immunodiffusion test (AGID) was conducted using 1.0 % agarose in NSS. Glass slides (75 x 50 mm) were precoated with 1 ml of 1% agarose in distilled water and dried. These were then coated with 7.5 ml of AGID gel. Six serum test wells of 5.0 mm diameter were punched in a hexagonal pattern around a 5.0 mm
central antigen well. Serum wells were 3.0 mm apart and 3.0 mm from the antigen well. Antigen well and serum wells were filled with 40 µl antigen and serum samples, respectively. Gels were incubated at room temperature for 4 to 5 days.

**Observation of Result**

Slides were then washed for at least 48 hours in three changes of NSS and then slides were observed for the presence of precipitation lines between antigen well and serum wells. Presence of precipitation lines was considered as positive reaction, whereas no precipitation was considered as negative.

### 3.6 STANDARD TUBE AGGLUTINATION TEST

The technique followed for tube agglutination test was as per recommendation of WHO (1971).

**Procedure**

The *Brucella* abortus plain antigen obtained from the I.V.R.I., Izatnagar was used for the test.

All serum samples were tested up to minimum five dilutions. For high titre sera, more dilutions were prepared in order to achieve the end point titre.

Five agglutination tubes were placed in a rack. In the first tube 0.8 ml of 0.5 % phenol saline was taken and in rest of the tubes 0.5 ml phenol saline was taken. Than in first tube 0.2 ml of serum was added, mixed well and 0.5 ml of diluted serum was transferred to the second tube. The process was continued up to the fifth tube and 0.5
ml was discarded from the last tube after mixing. Than in each tube 0.5 ml of plain antigen was added and mixed thoroughly. Hence, final dilutions in the tubes were 1:10, 1:20, 1:40, 1:80 and 1:160 and so on.

**Preparation of control tubes**

Five control tubes were prepared by taking plain antigen, 2ml (I), 0.75 ml (II), 0.50 ml (III), 0.25 ml (IV) and 0.00 ml (V). Phenol saline was added, 0.00ml (I), 1.25 ml (II), 1.50 ml (III), 1.75 ml (IV) and 2.00 ml (V). So the final volume in each tube was 2.00 ml.

The test serum sample tubes and control tubes were incubated at 37ºC for 20 h in water bath before the results were recorded.

**Observation of Result**

The degree of agglutination was judged by comparing the turbidity of the supernatant of test serum samples with that of control tubes. The highest serum dilution showing 50 per cent agglutination (50 % clearing) was considered as the end titre of the serum. Twice the reciprocal of the serum titre showing 50% agglutination was expressed as International Unit (I.U.) of antibodies.

**Interpretation of Result**

Serum samples showing 80 I.U. or above were considered as positive, 40 I.U. as doubtful, 20 I.U. or less were considered as negative for brucellosis in case of buffaloes.
In case of buffalo bulls serum samples showing 20 I. U. was considered as doubtful for brucellosis.

3.7 HEAT INACTIVATION TEST

The technique was followed as per recommendation by Amerault et al. (1961).

Procedure

*Brucella abortus* plain antigen was used for heat inactivation test.

*Brucella abortus* plain antigen and test serum were kept at room temperature before proceeding the experiment. In HIT, dilution of the serum and antigen were identical to serum tube agglutination test. Both, the serum antigen mixtures and antigen control were immediately incubated in a water bath at 65°C for 15 min, incubation was timed after initial 3 minute warming period. After incubation, the test samples were removed and placed immediately in cold water bath at 18°C for 3 minutes to terminate the incubation. Test samples were centrifuged at 2000 rpm for 5 minutes. After the supernatant fluid was decanted and replaced with 2 ml of NSS solution, each tube was shaken gently and degree of agglutination was read. Agglutination titres were recorded by comparing the turbidity of test serum dilutions with 50% antigen control.

Interpretation of Result
Agglutination titres were interpreted in international units as IU/ml of serum samples as that of STAT.

3.8 2 MERCAPTO ETHANOL TUBE AGGLUTINATION TEST

The test was performed as per recommendation by Nicoletti (1969).

Procedure

In 2-MET the antigen was identical to that of STAT except that 2-mercaptoethanol was added 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. The dilution of the serum and quantity of antigen were identical to serum tube agglutination test. Both, the serum antigen mixtures and antigen control were incubated at 37°C for 20 h in water bath before result was recorded. Agglutination titres were recorded by comparing the turbidity of test serum dilutions with 50% antigen control.

Interpretation of Result

Agglutination titres were interpreted in international units as IU/ml of serum samples as that of STAT.
3.9 INDIRECT-ENZYME LINKED IMMUNOSORBANT ASSAY

The smooth lipopolysaccharide (S-LPS) based avidin biotin ELISA (AB ELISA) kits supplied by the All India Coordinated Research Project (AICRP) on Animal Disease Monitoring and Surveillance (ADMAS), Bangalore, was used in the present study. The test was followed as per the manufacturer’s instruction.

**Bovine Brucellosis Avidin-Biotin ELISA (A-B-ELISA)**

Reagents were prepared as per the manufacturer’s instruction

a) Warming up of reagents: Samples, reagents (Appendix) and plates were brought to room temperature prior to starting the test.

b) Preparation of serum samples: Serum samples were diluted 1:100 in blocking buffer (Appendix).

c) Preparation of controls: Positive and negative controls were diluted 1:100 in blocking buffer before loading to microplates.

d) Preparation of antigen: Antigen from stock was added at the rate of 5µl/ml of coating buffer (Appendix) to prepare working solution.
e) Preparation of conjugates: The stock solution of antibovine IgG conjugated with biotin was prepared by adding 10µl of neat conjugate from the supplied vial with 235µl of sterile distilled water and then stored at –20°C. Working dilution was prepared by adding 1.25µl of stock solution to per ml of blocking buffer.

f) The stock solution of Avidin-HRP was prepared by adding 10µl/ml of neat conjugate from the supplied vial with 235µl of sterile distilled water and then stored at -20°C. Working dilution was prepared by adding 2.5µl of stock solution to per ml of blocking buffer.

g) One 10mg OPD (O-Phenylene Diamine) tablet was dissolved into 25 ml of distilled water and 10 ml aliquots were made and stored at -20°C until it was used. A working dilution of substrate/chromogen solution (Appendix) was prepared by adding 48 µl of 3% hydrogen peroxide to 12.5 ml of chromogen solution.

h) Preparation of stopping solution (Appendix): 5.5 ml of concentrated H₂SO₄ are dissolved in 94.5 ml of distilled water.

Test Procedure
(i) **Coating of the micro plate:** Using a pipette set at 100µl working solution of antigen was transferred to plain plate. The sides of plate were tapped to ensure that the S- LPS dilution was evenly distributed over the bottom of each well. The plate was covered and kept for incubation on a shaker for one hour at 37°C.

(ii) **Washing of wells:** After incubation, the plate was washed three times with washing buffer (Appendix) using micro plate washer. After washing the inverted plates were struck 2 to 3 times sharply on a clean paper towel.

(iii) **Addition of the test and control sera:** After drying the plate, 100µl test serum and control serum (diluted 1:100 in blocking buffer) were dispensed in respective antigen coated wells. Test serums were added in duplicates whereas controls were added in quadruplets in the wells and then plate was kept on the shaker at 37°C for one hour.

(iv) **Addition of the conjugate:** The plate was removed out of shaker and washed three times with washing buffer. Then 100µl of the working dilution of conjugate was added to all the wells of the plate and plate is returned to incubator on the shaker at 37°C for an hour.

(v) **Addition of avidin-HRP conjugate:** The plate was removed out of shaker and washed three times with washing buffer. Then 100µl of the working dilution of Avidin-HRP conjugate was added to all the wells and plate was returned back to the shaker incubator at 37°C for 20 minutes.
(vi) **Addition of substrate/ chromogen and stopping solution**: Plate was taken out of shaker and washed three times with washing solution. Add 100µl of working dilution of chromogen solution to all the wells. Plate was kept at room temperature for 5-10 min until a visible color developed in the strong positive wells. Then the reaction was stopped by adding 100µl of stopping solution to all 96 wells.

(vii) **Reading and recording of test result**: Immediately after adding the stop solution, the results were read on ELISA reader at wavelength of 492nm.

**Interpretation of result**

The median absorbance of the four strong positive control wells was calculated.

Percent Positivity (PP) of test serum and control was calculated as under

\[ PP = \left( \frac{\text{OD of test wells or Control wells}}{\text{Median OD of C++ well}} \right) \times 100 \]

PP value greater than 40 per cent in test wells was considered as positive.
Fig. 1: Results shown in Avidin Biotin ELISA test.
3.10 STATISTICAL ANALYSIS OF DIFFERENT SEROLOGICAL TESTS

Relative sensitivity (Rse): It is the capacity of a test to detect diseased animals, when compared with the gold standard test.

Relative specificity (Rsp): It is the capacity of a test to detect non-diseased animals, when compared with the gold standard test.

Concordance (c): It is the proportional similarity of the results of both the tests
To compare the relative sensitivity, relative specificity and concordance between various tests, the statistical formula given by Samad et al. (1994) was used as described below.

<table>
<thead>
<tr>
<th>Gold standard test</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>a</td>
</tr>
<tr>
<td>Negative</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>a+c</td>
</tr>
</tbody>
</table>

The notations used above are defined as under.

- **a** = Number of samples positive to both conventional and the gold standard tests
- **b** = Number of samples positive to conventional but negative to the gold standard test
- **c** = Number of samples negative to conventional but positive to the gold standard test
d = Number of samples negative to both conventional and the gold standard tests

\[ a + b + c + d = \text{Total number of samples (N)} \]

Formulae of the indices used for comparing the different assays are described follows.

Relative sensitivity (Rse) = \( \frac{a}{a+c} \times 100 \)

Relative specificity (Rsp) = \( \frac{d}{b+d} \times 100 \)

Concordance = \( \frac{a+d}{N} \times 100 \)
4. RESULTS

Brucellosis is still one of the most important zoonotic diseases creating problems in different cities of India and especially in Rajasthan. To prevent the transmission of this highly infectious disease to other in contact animals as well as to humans, it is utmost essential that diseased animals must be diagnosed at an early stage and separated from healthy animals. For rapid diagnosis of brucellosis various serological tests are employed with varying degree of sensitivity and specificity.

The present study was carried out for determining seroprevalence of brucellosis in buffaloes and buffalo bulls of private farms in Kota division by employing Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT), Heat Inactivation Test (HIT), 2-Mercaptoethanol Test (2-MET), Agar Gel Immunodiffusion Test (AGID) and Avidin Biotin Enzyme Linked Immunosorobent Assay (AB ELISA) to detect *Brucella* antibodies in bubaline serum while Milk Ring Test (MRT) was performed to detect antibodies in bubaline milk. The efficacy of these tests to detect *Brucella* antibodies in blood and milk of buffaloes were compared to ELISA in order to find out single test that is most suitable in the epidemiological conditions of Rajasthan.
4.1 SEROPREVALENCE

4.1.1 Overall Seroprevalence

In all 436 blood samples were collected from different parts of Kota division to detect *Brucella* antibodies. Overall seroprevalence of brucellosis in the buffaloes of Kota division was recorded as 37.84% by AB ELISA, 35.09% by RBPT, 29.59% by STAT, 26.15% in HIT, 25.23% in 2-MET and 13.99% in AGID as presented in Table 4.1.1.

Milk Ring Test was applied on a total of 63 milk samples collected from buffaloes of different farms and it showed overall prevalence of 31.75%.

Overall Seroprevalence in females was 38.46% whereas in males was 30.30%.
Fig. 2: Map of Kota division in Rajasthan
Table 4.1.1: Overall Seroprevalence of Brucellosis by Different Diagnostic Tests

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Total Number of Animals Tested</th>
<th>Name of Test</th>
<th>Number of Seropositive animals</th>
<th>Percent seroprevalence recorded</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>436</td>
<td>RBPT</td>
<td>153</td>
<td>35.09%</td>
</tr>
<tr>
<td>2</td>
<td>436</td>
<td>STAT</td>
<td>129</td>
<td>29.59%</td>
</tr>
<tr>
<td>3</td>
<td>436</td>
<td>HIT</td>
<td>114</td>
<td>26.15%</td>
</tr>
<tr>
<td>4</td>
<td>436</td>
<td>2-MET</td>
<td>110</td>
<td>25.22%</td>
</tr>
<tr>
<td>5</td>
<td>436</td>
<td>AGID</td>
<td>61</td>
<td>13.99%</td>
</tr>
<tr>
<td>6</td>
<td>63</td>
<td>MRT</td>
<td>20</td>
<td>31.75%</td>
</tr>
<tr>
<td>7</td>
<td>436</td>
<td>AB ELISA</td>
<td>165</td>
<td>37.84%</td>
</tr>
</tbody>
</table>
4.1.2 Area wise Seroprevalence of brucellosis

A total of 436 blood samples were collected from buffaloes of Sangod (52), Mandana (47), Bundi (67), Talera (91), Chhabra (85), Jhalrapatan (25) and Dag (69) regions of Kota division. Out of these areas 21(40.38%),
20(42.55%), 23(34.32%), 38(41.75%), 37(43.53%), 8(32%) and 18(26.09%) were found positive by AB ELISA, respectively as presented in Table 4.1.2.

Chhabra region of Baran district showed the highest seroprevalence whereas Dag area showed lowest seroprevalence of brucellosis.

### 4.1.3 Sexwise Seroprevalence

In the present investigations, out of 403 buffaloes tested 155 (38.46%) were found positive while out of 33 buffalo bulls tested 10 (30.30%) were found positive by AB ELISA. Highest prevalence was found among buffaloes and buffalo bulls of Chhabra region 43.75% and 40.00%, respectively. Lowest prevalence was found among buffaloes of Jhalrapatan region (26.56%) whereas buffalo bulls of Dag and Sangod region showed lowest prevalence (20.00%). Details of the results of AB ELISA are presented in Table 4.1.3.

### 4.2 DETECTION OF BRUCELLA AGGLUTININS BY MILK RING TEST

MRT was performed on 63 individual milk samples taken from buffaloes of four different farms of Kota division. Twenty (31.75%) milk samples showed positive reaction i.e. pink colored ring formation. Chhabra region of Baran district showed the highest prevalence (37.50%) whereas Dag area showed lowest prevalence (18.18%) of brucellosis. The area wise prevalence of brucellosis by MRT is presented in Table 4.2.
Table 4.1.2: Area wise Seroprevalence of Brucellosis in Buffaloes.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Region</th>
<th>Number of Serum Samples Tested</th>
<th>Number of Serum Samples Showing Positive Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sangod</td>
<td>52</td>
<td>21 (40.38%)</td>
</tr>
<tr>
<td>2</td>
<td>Mandana</td>
<td>47</td>
<td>20 (42.55%)</td>
</tr>
<tr>
<td>3</td>
<td>Bundi</td>
<td>67</td>
<td>23 (34.33%)</td>
</tr>
<tr>
<td>4</td>
<td>Talera</td>
<td>91</td>
<td>38 (41.76%)</td>
</tr>
<tr>
<td>5</td>
<td>Chhabra</td>
<td>85</td>
<td>37 (43.53%)</td>
</tr>
<tr>
<td>6</td>
<td>Jhalrapatan</td>
<td>25</td>
<td>8 (32%)</td>
</tr>
<tr>
<td>7</td>
<td>Dag</td>
<td>69</td>
<td>18 (26.09%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>436</td>
<td>165 (37.84%)</td>
</tr>
</tbody>
</table>
Fig. 4: Overall Area wise Seroprevalence of Brucellosis

Table 4.1.3: Sex wise Seroprevalence of Brucellosis in Buffaloes.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Region</th>
<th>Total Serum samples</th>
<th>Buffaloes tested</th>
<th>Positive Serum Samples</th>
<th>Buffalo bulls tested</th>
<th>Positive Serum Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sangod</td>
<td>52</td>
<td>47</td>
<td>20(42.55%)</td>
<td>5</td>
<td>1(20.00%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>----</td>
<td>----</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Mandana</td>
<td>47</td>
<td>44</td>
<td>19(43.18%)</td>
<td>3</td>
<td>1(33.33%)</td>
</tr>
<tr>
<td>3.</td>
<td>Bundi</td>
<td>67</td>
<td>61</td>
<td>21(34.43%)</td>
<td>6</td>
<td>2(33.33%)</td>
</tr>
<tr>
<td>4.</td>
<td>Talera</td>
<td>91</td>
<td>85</td>
<td>36(42.35%)</td>
<td>6</td>
<td>2(33.33%)</td>
</tr>
<tr>
<td>5.</td>
<td>Chhabra</td>
<td>85</td>
<td>80</td>
<td>35(43.75%)</td>
<td>5</td>
<td>2(40.00%)</td>
</tr>
<tr>
<td>6.</td>
<td>Jhalrapatan</td>
<td>25</td>
<td>22</td>
<td>7(31.82%)</td>
<td>3</td>
<td>1(33.33%)</td>
</tr>
<tr>
<td>7.</td>
<td>Dag</td>
<td>69</td>
<td>64</td>
<td>17(26.56%)</td>
<td>5</td>
<td>1(20.00%)</td>
</tr>
<tr>
<td>Total</td>
<td>436</td>
<td>403</td>
<td>155</td>
<td>33</td>
<td>10(30.30%)</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 5: Overall Sex wise Seroprevalence of Brucellosis

Table 4.2: Area wise Prevalence of Brucellosis in Buffaloes in MRT

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Area</th>
<th>Milk samples</th>
<th>Positive Milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-----------</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Sangod (Kota)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Mandana (Kota)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Bundi</td>
<td>15</td>
<td>5 (33.33%)</td>
</tr>
<tr>
<td>4.</td>
<td>Talera (Bundi)</td>
<td>21</td>
<td>7 (33.33%)</td>
</tr>
<tr>
<td>5.</td>
<td>Chhabra (Baran)</td>
<td>16</td>
<td>6 (37.50%)</td>
</tr>
<tr>
<td>6.</td>
<td>Jhalrapatan (Jhalawar)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Dag</td>
<td>11</td>
<td>2 (18.18%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>63</td>
<td>20 (31.75%)</td>
</tr>
</tbody>
</table>
4.3 DETECTION OF *BRUCELLA* AGGLUTININS BY VARIOUS SEROLOGICAL TESTS
The results of various serological tests like RBPT, AGID, STAT, HIT, 2-MET and AB ELISA employed to detect prevalence of *Brucella* agglutinins in buffalo serum are as follows:

### 4.3.1 Rose Bengal Plate Test

All 436 serum samples were subjected to Rose Bengal Plate Test using Rose Bengal colored antigen, out of which 153 samples were found positive *i.e.* showing agglutination reaction. Buffaloes of Chhabra region showed highest prevalence (40.00%) whereas buffaloes of Dag region showed lowest prevalence of brucellosis (26.09%). Females and males of Chhabra region showed highest seroprevalence (40.00%) of brucellosis. Lowest seroprevalence was found in females of Dag region (26.56%) and in males of Sangod and Dag region (20.00%). Percent seropositivity for brucellosis was 35.09% in the tested buffalo serum. Overall seroprevalence in females and males were 35.48 percent and 30.30 percent, respectively. The results of the test are presented in Table 4.3.1.
TABLE 4.3.1
Fig. 7: Area wise Seroprevalence of brucellosis by RBPT
Fig. 8: Sex wise Seroprevalence of brucellosis by RBPT

4.3.2 Agar Gel Immunodiffusion
Agar gel immunodiffusion test was employed in order to demonstrate antibody in cattle sera which reacts with *Brucella* antigens other than the smooth lipopolysaccharide. Sera from buffaloes naturally infected with precipitated sonicated antigens extracted from *B. abortus*.

In all 436 serum samples were subjected to AGID, out of which 61 were found positive *i.e.* showing precipitation lines. The percent seropositivity for brucellosis was 13.99% in the tested buffalo serum. Buffaloes of Chhabra region showed highest prevalence (16.47%) whereas buffaloes of Bundi region showed lowest prevalence of brucellosis (10.45%). Females of Sangod region (17.02%) and males of Mandana and Jhalrapatan region (33.33%) showed highest seroprevalence. Lowest seroprevalence was found in females of Bundi region (11.48%) whereas males of Sangod, Bundi, Talera, and Dag region did not show any precipitation in AGID. Overall seroprevalence in females and males were 14.39 percent and 9.09 percent, respectively. Area wise and sex wise details of results are shown in Table 4.3.2
Table 4.3.2
Fig. 9: Area wise Seroprevalence of Brucellosis by AGID

Percentage of Seropositive Animals

- Sangod
- Mandana
- Bundi
- Talera
- Chhabra
- Jhalrapatan
- Dag
Fig. 10: Sexwise Seroprevalence of Brucellosis by AGID
4.3.3 Standard Tube Agglutination Test

Four hundred thirty six buffalo serum samples were subjected to STAT that detected 129 samples as brucellosis positive. Out of which fourteen samples were found doubtful for *Brucella* agglutinins. The percent brucellosis seropositivity was found to be 29.59% in the STAT.

Amongst 129 brucellosis positive samples 21 sera showed the titre 640 IU, 39 sera showed 320 IU, 40 sera showed 160 IU and 15 sera showed titer 80 IU. Fourteen animals showed titre of 40 IU. Rest of the 307 *bubaline* sera failed to show agglutination reaction in STAT.

Buffaloes of Chhabra region showed highest prevalence (34.12%) whereas buffaloes of Dag region showed lowest prevalence of brucellosis (23.19%). Overall seroprevalence in females and males were 30.52 percent and 18.18 percent, respectively. Females and males of Chhabra region showed highest seroprevalence of brucellosis (33.75% and 40.00%, respectively). Lowest seroprevalence was found in females of Dag region (25.00%) whereas males of Bundi and Dag regions did not show any titre in STAT. Area wise and sex wise details of results are presented in table 4.3.3.1 and 4.3.3.2.
Table 4.3.3.1
Fig. 11: Area wise Seroprevalence of Brucellosis by STAT

Percentage of Seropositive Animals

- Sangod
- Mandana
- Bundi
- Talera
- Chhabra
- Jhalrapatan
- Dg
Table 4.3.3.2: Sexwise Seroprevalence of Brucellosis in Buffaloes in STAT

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Region</th>
<th>Total Number of Females Tested</th>
<th>Females Positive by STAT</th>
<th>Total Number of Males Tested</th>
<th>Males Positive by STAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sangod</td>
<td>47</td>
<td>15(31.91%)</td>
<td>5</td>
<td>1(20.00%)</td>
</tr>
<tr>
<td>2</td>
<td>Mandana</td>
<td>44</td>
<td>14(31.82%)</td>
<td>3</td>
<td>1(33.33%)</td>
</tr>
<tr>
<td>3</td>
<td>Bundi</td>
<td>61</td>
<td>20(32.79%)</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Talera</td>
<td>85</td>
<td>25(29.41%)</td>
<td>6</td>
<td>1(16.66%)</td>
</tr>
<tr>
<td>5</td>
<td>Chhabra</td>
<td>80</td>
<td>27(33.75%)</td>
<td>5</td>
<td>2(40.00%)</td>
</tr>
<tr>
<td>6</td>
<td>Jhalrapatan</td>
<td>22</td>
<td>6(27.27%)</td>
<td>3</td>
<td>1(33.33%)</td>
</tr>
<tr>
<td>7</td>
<td>Dag</td>
<td>64</td>
<td>16(25.00%)</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>403</td>
<td>123(30.52%)</td>
<td>33</td>
<td>6(18.18%)</td>
</tr>
</tbody>
</table>
4.3.4 Heat Inactivation Test

Heat inactivation test was applied to inactivate nonspecific agglutinins in bubaline sera.
Four hundred thirty six buffalo serum when subjected to HIT revealed 114 samples as brucellosis positive. The HIT recorded 26.15 percent seroreactivity in tested animals. Buffaloes of Chhabra region showed highest prevalence (31.76%) whereas buffaloes of Bundi region showed lowest prevalence of brucellosis (14.92%).

Amongst 114 brucellosis positive samples 17 sera showed the titre 640 IU, 30 sera showed 320 IU, 28 sera showed 160 IU and 21 sera showed titer 80 IU. Eight sera showed the titre of 40 IU. Females and males of Chhabra region showed highest seroprevalence i.e. 31.25 percent and 40.00 percent, respectively. Lowest seroprevalence was found in females of Bundi region (16.39%) whereas males of Talera, Bundi and Dag regions did not show any titre in HIT. Overall seroprevalence in females and males were 27.05 percent and 15.15 percent, respectively.

Area wise and sex wise details of results are presented in table 4.3.4.1 and 4.3.4.2.
Table 4.3.4.1
Fig. 13: Area wise Seroprevalence of Brucellosis by HIT

- Sangod
- Mandana
- Bundi
- Talera
- Chhabra
- Jhargapan
- Dag

Percentage of Seropositive Animals
### Table 4.3.4.2: Sexwise Seroprevalence of Brucellosis in Buffaloes in HIT

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Region</th>
<th>Total Number of Females Tested</th>
<th>Females Positive by HIT</th>
<th>Total Number of Males Tested</th>
<th>Males Positive by HIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sangod</td>
<td>47</td>
<td>14(29.79%)</td>
<td>5</td>
<td>1(20.00%)</td>
</tr>
<tr>
<td>2</td>
<td>Mandana</td>
<td>44</td>
<td>13(29.54%)</td>
<td>3</td>
<td>1(33.33%)</td>
</tr>
<tr>
<td>3</td>
<td>Bundi</td>
<td>61</td>
<td>10(16.39%)</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Talera</td>
<td>85</td>
<td>25(29.41%)</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Chhabra</td>
<td>80</td>
<td>25(31.25%)</td>
<td>5</td>
<td>2(40.00%)</td>
</tr>
<tr>
<td>6</td>
<td>Jhalrapatan</td>
<td>22</td>
<td>6(27.27%)</td>
<td>3</td>
<td>1(33.33%)</td>
</tr>
<tr>
<td>7</td>
<td>Dag</td>
<td>64</td>
<td>16(25.00%)</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>403</td>
<td>109(27.05%)</td>
<td>33</td>
<td>5(15.15%)</td>
</tr>
</tbody>
</table>
Fig. 14: Sexwise Seroprevalence of Brucellosis by HIT

4.3.5 2-Mercaptoethanol Agglutination Test

2-Mercaptoethanol agglutination test was carried out as serum tube agglutination test under the same protocol except that phenol in saline was replaced with 0.05% 2 Mercaptoethanol: a disulfide bond reducing agent.
The test was applied on 436 buffalo serum samples to differentiate *Brucella* antibodies appeared in response to *Brucella* infection. Out of 436 serum samples tested, 110 were found positive. The seropositivity for brucellosis was 25.22 percent in the tested buffalo serum. Amongst 110 brucellosis positive samples 18 sera showed the titre 640 IU, 24 sera showed 320 IU, 38 sera showed 160 IU and 21 sera showed titer 80 IU. Nine samples showed titre below 80 IU.

Buffaloes of Chhabra region showed highest prevalence (31.76%) whereas buffaloes of Bundi region showed lowest prevalence of brucellosis (14.92%). Females and males of Chhabra region showed highest seroprevalence *i.e.* 31.25 percent and 40.00 percent, respectively. Lowest seroprevalence was found in females of Bundi region (16.39%) whereas males of Mandana, Bundi and Dag regions did not show any titre in HIT. Overall seroprevalence in females and males were 26.05 percent and 15.15 percent, respectively.

Area wise and sex wise details of results are presented in table 4.3.5.1 and 4.3.5.2.

Table 4.3.5.1
Fig. 15: Area wise Seroprevalence of Brucellosis by MET

- Percentage of Seropositive Animals

- Areas: Sangod, Mandana, Bundi, Talera, Chhabra, Jhalrapatan, Dog.
## Table 4.3.5.2: Sexwise Seroprevalence of Brucellosis in Buffaloes in 2-MET

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Region</th>
<th>Total Number of Females Tested</th>
<th>Females Positive by 2-MET</th>
<th>Total Number of Males Tested</th>
<th>Males Positive by 2-MET</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sangod</td>
<td>47</td>
<td>14 (29.79%)</td>
<td>5</td>
<td>1 (20.00%)</td>
</tr>
<tr>
<td>2</td>
<td>Mandana</td>
<td>44</td>
<td>13 (29.55%)</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Bundi</td>
<td>61</td>
<td>10 (16.39%)</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Talera</td>
<td>85</td>
<td>22 (25.88%)</td>
<td>6</td>
<td>1 (16.66%)</td>
</tr>
<tr>
<td>5</td>
<td>Chhabra</td>
<td>80</td>
<td>25 (31.25%)</td>
<td>5</td>
<td>2 (40.00%)</td>
</tr>
<tr>
<td>6</td>
<td>Jhalrapatan</td>
<td>22</td>
<td>6 (27.27%)</td>
<td>3</td>
<td>1 (33.33%)</td>
</tr>
<tr>
<td>7</td>
<td>Dag</td>
<td>64</td>
<td>15 (23.44%)</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>403</td>
<td>105 (26.05%)</td>
<td>33</td>
<td>5 (15.15%)</td>
</tr>
</tbody>
</table>
4.3.6 Avidin-Biotin Enzyme-Linked Immunosorbent Assay

Avidin biotin ELISA is highly sensitive and highly specific test which revealed 165 serum samples positive for brucellosis out of 436 buffalo serum samples tested. Serum samples showing PP value greater than 40 per cent in test wells were considered as positive.
The percent seropositivity for brucellosis was 37.84% in the tested buffalo serum. Buffaloes of Chhabra region showed highest prevalence (43.53%) whereas buffaloes of Dag region showed lowest prevalence of brucellosis (26.09%). Females and males of Chhabra region showed highest seroprevalence (43.75%) and (40.00%), respectively. Lowest seroprevalence was found in females of Dag region (26.56%) whereas in males of Sangod and Dag regions (20.00%). Overall seroprevalence in females and males were 38.46 percent and 30.30 percent, respectively.

Area wise and sex wise details of results are presented in table 4.3.6.1 and 4.3.6.2.
Table 4.3.6.1: Area wise Seroprevalence of Brucellosis in Buffaloes in AB ELISA

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Area</th>
<th>Total Number of Serum samples</th>
<th>Number of Seropositive Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sangod</td>
<td>52</td>
<td>21 (40.38%)</td>
</tr>
<tr>
<td>2</td>
<td>Mandana</td>
<td>47</td>
<td>20 (42.55%)</td>
</tr>
<tr>
<td>3</td>
<td>Bundi</td>
<td>67</td>
<td>23 (34.33%)</td>
</tr>
<tr>
<td>4</td>
<td>Talera</td>
<td>91</td>
<td>38 (41.76%)</td>
</tr>
<tr>
<td>5</td>
<td>Chhabra</td>
<td>85</td>
<td>37 (43.53%)</td>
</tr>
<tr>
<td>6</td>
<td>Jhalrapatan</td>
<td>25</td>
<td>8 (32%)</td>
</tr>
<tr>
<td>7</td>
<td>Dag</td>
<td>69</td>
<td>18 (26.09%)</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>436</strong></td>
<td><strong>165 (37.84%)</strong></td>
</tr>
</tbody>
</table>
Fig. 17: Area wise Seroprevalence of Brucellosis by AB ELISA
### Table 4.3.6.2: Sex wise Seroprevalence of Brucellosis in Buffaloes in AB ELISA

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Region</th>
<th>Total Serum samples</th>
<th>Buffaloes tested</th>
<th>Positive Serum Samples</th>
<th>Buffalo bulls tested</th>
<th>Positive Serum Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sangod</td>
<td>52</td>
<td>47</td>
<td>20 (42.55%)</td>
<td>5</td>
<td>1 (20.00%)</td>
</tr>
<tr>
<td>2.</td>
<td>Mandana</td>
<td>47</td>
<td>44</td>
<td>19 (43.18%)</td>
<td>3</td>
<td>1 (33.33%)</td>
</tr>
<tr>
<td>3.</td>
<td>Bundi</td>
<td>67</td>
<td>61</td>
<td>21 (34.43%)</td>
<td>6</td>
<td>2 (33.33%)</td>
</tr>
<tr>
<td>4.</td>
<td>Talera</td>
<td>91</td>
<td>85</td>
<td>36 (42.35%)</td>
<td>6</td>
<td>2 (33.33%)</td>
</tr>
<tr>
<td>5.</td>
<td>Chhabra</td>
<td>85</td>
<td>80</td>
<td>35 (43.75%)</td>
<td>5</td>
<td>2 (40.00%)</td>
</tr>
<tr>
<td>6.</td>
<td>Jhalrapatan</td>
<td>25</td>
<td>22</td>
<td>7 (31.82%)</td>
<td>3</td>
<td>1 (33.33%)</td>
</tr>
<tr>
<td>7.</td>
<td>Dag</td>
<td>69</td>
<td>64</td>
<td>17 (26.56%)</td>
<td>5</td>
<td>1 (20.00%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>436</td>
<td>403</td>
<td>155 (38.46%)</td>
<td>33</td>
<td>10 (30.30%)</td>
</tr>
</tbody>
</table>
Fig. 18: Sex wise Seroprevalence of Brucellosis by AB ELISA

Table 4.4
4.5 Comparison of relative sensitivity (Rse), relative specificity (Rsp) and relative concordance of RBPT, STAT, HIT, MET, AGID and MRT with AB ELISA:

The relative sensitivity (Rse), relative specificity (Rsp) and relative concordance of RBPT, STAT, HIT, 2-MET, AGID and MRT compared with AB ELISA are presented in Table 4.4.1.

The percent values of Rse and Rsp in comparison with AB ELISA for RBPT are 48.48 and 73.06; for STAT are 42.42 and 78.23; for HIT are 40.00 and 80.07; for 2-MET are 38.79 and 81.18; for AGID are 18.18 and 88.56 and for MRT are 44.44 and 77.77 respectively.

From these results it could be inferred that the Rse value is highest with RBPT followed by MRT, STAT, HIT, 2-MET and lowest with AGID. It is also evident from the table that the Rsp values are highest with AGID followed by 2-MET, HIT, STAT, MRT and lowest with RBPT.
The results given in table also indicate that the percent concordance of 2-MET with AB ELISA is highest and lowest for AGID.

<table>
<thead>
<tr>
<th></th>
<th>AB ELISA Vs</th>
<th>AB ELISA Vs</th>
<th>AB ELISA Vs HIT</th>
<th>AB ELISA Vs</th>
<th>AB ELISA Vs</th>
<th>AB ELISA Vs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Table 4.5:</strong> Relative Sensitivity (Rse), Relative Specificity (Rsp) and Concordance of various serological tests as compared with AB ELISA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RBPT</td>
<td>STAT</td>
<td>2-MET</td>
<td>AGID</td>
<td>MRT</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>------</td>
<td>------</td>
<td>-------</td>
<td>------</td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>No. of samples tested (n)</td>
<td>436</td>
<td>436</td>
<td>436</td>
<td>436</td>
<td>436</td>
<td>63</td>
</tr>
<tr>
<td>Both test (a) positive</td>
<td>80</td>
<td>70</td>
<td>66</td>
<td>64</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>Both test (d) negative</td>
<td>198</td>
<td>212</td>
<td>217</td>
<td>220</td>
<td>240</td>
<td>28</td>
</tr>
<tr>
<td>Concordance (%) (a+d/n)</td>
<td>63.76</td>
<td>64.68</td>
<td>64.90</td>
<td>65.14</td>
<td>61.92</td>
<td>63.49</td>
</tr>
<tr>
<td>Rse (percent)</td>
<td>48.48</td>
<td>42.42</td>
<td>40.00</td>
<td>38.78</td>
<td>18.18</td>
<td>44.44</td>
</tr>
<tr>
<td>Rsp (percent)</td>
<td>73.06</td>
<td>78.23</td>
<td>80.07</td>
<td>81.18</td>
<td>88.56</td>
<td>77.77</td>
</tr>
</tbody>
</table>
5. DISCUSSION

Brucellosis has been recognized as an important zoonotic disease which persists in the region where not much attention is given for its control. Over the last few decades much progress has been made in the direction to reduce prevalence of brucellosis through standardization of traditional diagnostic methods and application of new procedures. The attention is now diverted towards development of serological test of better sensitivity and specificity with quick and economical diagnosis of brucellosis. But the problem with serological tests is the validation and correct interpretations. The problem is further compounded because of the vaccination of animals which lead to an increase in the antibody titres against the infectious agent. Moreover, the different serological tests have varying degrees of specificity and sensitivity to different Brucella infection.
Serological tests have the advantage of screening large population in short time with cost effectiveness and easy application. In the present investigation efforts were made towards evaluation and comparison of some newer tests with conventional tests on the basis of results of studies with regards to sensitivity and specificity.

Nowadays, I-ELISA is considered as the gold standard test to detect bovine brucellosis. Standard Tube Agglutination Test (STAT), Rose Bengal Plate Test (RBPT) and Milk Ring Test (MRT) are traditional tests for rapid diagnosis of brucellosis in animals. New serological tests like HIT and MET are used along with traditional tests to improve efficacy. Agar Gel Immunodiffusion Test (AGID) with sonicated *Brucella* antigen has been used by many workers in order to demonstrate antibodies which react with *Brucella* antigens other than the smooth lipopolysaccharide displayed on the surface of the bacterial cells.

**SEROPREVALENCE OF BRUCELLOSIS IN BUFFALOES**

The Seroprevalence of brucellosis of animals in India was first reported by Polding (1942) and since then many workers have shown its existence in various parts of the country. This survey is probably the first attempt to assess the seroprevalence among buffaloes in Kota division of Rajasthan.

Four hundred thirty six sera samples of buffaloes were tested for brucellosis, out of which 165 (37.84%) were found positive by AB ELISA. Similar findings were reported by some other workers; 38.87 percent by

Brucellosis in cattle is endemic in many parts of the country including different areas of Rajasthan and thus it is expected to find the presence of antibodies in buffaloes of Kota region somewhat in the same range as found in other areas of the country by other researchers.


The seroprevalence rate of brucellosis in cattle ranged from 0.3% in Himachal Pradesh (Renukaradhya et al., 2002) to 56.2% in Assam (Chakraborty et al., 2000). Higher seroprevalence of brucellosis was also shown by other workers; 47.76 percent by Mahato et al. (2004) in cows in Assam; 55.2 percent by Gen et al. (2005) in
aborted dairy cows in Turkey. Dhand et al. (2005) in Punjab found higher seroprevalence in animals with a history of abortion (33.87 percent) than in those without such history (11.63 percent).

In the present study, the seroprevalence of brucellosis in females and males was detected as 38.46 and 30.30 percent, respectively. The number of male animals (n = 33) studied was much smaller in comparison to females (n = 403). However, the apparently high seroprevalence figure in female animals compared to males in this study agrees with other works; 38.24 percent in females and 22.50 percent in males by Mittal et al. (2005); 4.18 percent in females and 1.41 percent in males by Upadhyay et al. (2007). Lower seropositivity in males may be due to the fact that generally males are not vaccinated for brucellosis. Further, there are reports that male cattle are more resistant than females (Nicoletti, 1980).

AREA-WISE SEROPREVALENCE OF BRUCELLOSIS

Since this type of study has been conducted for the first time in the buffalo population of Kota division of Rajasthan, no references of seroprevalence of brucellosis in this area are available. However, reports from other areas of state have been discussed earlier.
Out of 436 buffalo serum samples collected, 52 were from Sangod, 47 were from Mandana, 67 were from Bundi, 91 were from Talera, 85 were from Chhabra, 25 were from Jhalrapatan and 69 were from Dag regions of Kota division.

Seroprevalence was found highest in Chhabra region in all tests followed by Mandana, Talera, Sangod, Bundi, Jhalrapatan and minimum prevalence was found in Dag region by AB ELISA. All the tests except HIT and 2-MET detected lowest prevalence in Dag region whereas HIT and 2-MET showed lowest prevalence in Bundi region.

MRT was conducted on 63 milk samples taken from buffaloes of Bundi (15), Talera (21), Chhabra (16) and Dag (11) regions and highest prevalence was found in Chhabra region (37.50 %), Talera and Bundi regions showed 33.33 percent prevalence and lowest prevalence was detected in Dag area (18.18 %).

In the present study, proportionately higher number of animals of Chhabra region showed antibody titre by all the tests. It might have been due to earlier history of stream of abortions with retention of placenta as reported by farm owners that could be attributed to brucellosis. However, no confirmatory test had been attempted in such cases.

MILK RING TEST
MRT was performed to detect lacteal anti-Brucella IgM and IgA antibodies bound to milk fat globules present in individual milk samples taken from buffaloes (Sutra et al. 1986). However, this test gives false positive reaction for colostrum, milk at the end of the lactation period, milk from cows suffering from a hormonal disorder or milk from cows with mastitis are tested (Bercovich and Moerman, 1979). Milk that contains low concentrations of lacteal IgM and IgA or which is lacking in the fat-clustering factors tests false-negative (Keer et al., 1959; Tanwani and Pathak, 1971; Patterson and Deyoe, 1977).

In our study, twenty (31.75%) out of 63 individual milk samples showed positive reaction. Although this test is usually performed for herd diagnosis on pooled milk sample but can be conducted on individual milk sample in endemic areas in the animals of larger herds at regular intervals (Jaartsveld and Mathiissen, 1976).

Our findings regarding seroprevalence in buffaloes are in agreement with earlier findings on MRT as; 35.82 percent by Mahato et al. (2004); 35 percent by Abbas and Aldeewan (2009). However, lower seroprevalence than the present study was also found by Kang’ethe et al. in 2000 (3.9%); Gumber et al. in 2004 (11.85%), respectively. In present study the slightly higher positivity by MRT in buffalo milk might have been due to false positivity of MRT because of recent parturition, end of lactation and due to sub-clinical mastitis (Alton et al., 1988).

ROSE BENGAL PLATE TEST
In RBPT, antigen is buffered to a lower pH, usually 3.65 ± 0.05 (Morgan et al., 1969). The low pH prevents some agglutination by IgM and encourages agglutination by IgG1 thereby reducing non-specific interactions (Corbel, 1972; Allan et al., 1976) but the temperature of the antigen and the ambient temperature at which the reaction takes place may influence the sensitivity and specificity of the RBPT (MacMillan, 1990).

The test is believed as very sensitive and used as a screening test (Morgan et al., 1969 and Nicoletti, 1969). However, like all other serological tests, it could sometimes give a positive result due to S19 vaccination or due to false-positive serological reactions. False-negative reactions occur rarely, mostly due to prozoning. Nevertheless, RBPT appears to be adequate as a screening test for detecting infected herds as it is more likely to give false positive results than to miss brucellosis (Nakavuma, 1994).

In the current study the test was applied on 436 serum samples and detected 153 (35.09%) samples as positive. Similar results were recorded by Nasir et al. (2004) finding 35.40 percent buffaloes at various private livestock farms positive by RBPT. Lower seroprevalence values than our study was recorded by Chakravarty et al. in 2007 (29.07%); Chand and Sharma in 2004 (20.47%); Cadmus et al. in 2006 (5.82%).

AGAR GEL IMMUNODIFFUSION
Agar gel immunodiffusion test was employed in order to demonstrate antibody in cattle sera which reacts with precipitated sonicated antigens extracted from \textit{B. abortus} as antigens other than those involved in the standard serological tests can be used to improve the diagnosis of this disease.

Corbel (1972) demonstrated that serum of a cow naturally infected with \textit{B. abortus} precipitated five antigens present in an ultrasonically disrupted extract of smooth \textit{B. abortus}.

Out of 436 samples, only 61 (13.99 \%) were found positive i.e. showing precipitation lines. The test was found to be least sensitive in the current study. Chand \textit{et al.} (1988) and Stemshorn and Neilsen (1981) also reported that AGPT is less sensitive than other serological tests and of limited value for general diagnostic use.

\textbf{STANDARD TUBE AGGLUTINATION TEST}

The serum agglutination test (SAT), which historically has been the principal serological test used to detect brucellosis, measures agglutinating antibodies like IgM, IgG1 and IgG2 (Levieux, 1974). It can be used to detect acute infections, as antibodies of the IgM type usually appear first after infection and are more reactive in the SAT than antibodies of the IgG 1 and IgG 2 types (Beh, 1974; Levieux, 1974; Allan \textit{et al.}, 1976). However, the STAT may yield both false negative or false positive results (Corbel \textit{et al.}, 1984)
In the present study STAT detected 129 (29.59 percent) samples out of 436 samples as brucellosis positive out of which fourteen samples were found doubtful for Brucella agglutinins. Nasir et al. (2004) found 23.7 percent and Nag et al. (1977) found 24.00 percent seroprevalence in bovines in West Bengal.

Higher seroprevalence than our finding was recorded in bovines by some workers; 36.45 percent by Barbuddhe et al. (2004); 33.72 percent by Chakravarty et al. (2007); 34.64 percent by Otlu et al. (2008) and 32.92 percent by Sahin et al. (2008) by STAT.

Lower seroprevalence was recorded in bovines by Chand and Sharma (2004), 18.89 percent in Haryana, Uttar Pradesh and Madhya Pradesh; Sharma et al. (2007), 18.07 percent in Punjab and Kalimuddin et al. (1990), 18.81 percent; Lodhi et al. (1995), 2.40 percent; Jiwa et al. (1996) 10.8 percent and Upadhyay et al. (2007) 4.73 percent by STAT.

**HEAT INACTIVATION TEST**

Heat inactivation test was applied as a supplementary test of STAT to inactivate nonspecific agglutinins in bovine sera by heat (Rose, 1955; Amerault et al., 1961). The test can differentiate vaccinal titre from infection titre in bovines (Sandhu and Joshi, 1993).
In the present study HIT detected 114 (26.14%) samples out of 436 samples as brucellosis positive. Higher seroprevalence (33.96%) than our study was recorded by Shringi (1999) in cattles by HIT. Lower seroprevalence than the present study was also recorded in earlier reports. Kalimuddin et al. (1990) and Kulshrestha et al. (1973) found 16.83 percent cattle and 3.1 percent buffaloes respectively, positive by HIT.

**2-MERCAPTOETHANOL AGGLUTINATION TEST**

Nicoletti and Muraschi (1966) concluded that the use of supplemental procedures would hasten the eradication of brucellosis. 2-MET is considered as a highly specific test as it indicates agglutination only due to IgG antibodies (Klein and Behan, 1981). Stemshorn et al. (1985) found 99.8 percent specificity of 2-MET. Nicoletti (1969) indicated that 2-MET classified 97 percent of the infected cattle as positive for brucellosis. Buchanan et al. (1974) concluded that 2- mercaptoethanol tests were the most accurate indicators of active disease.

In the present study, 2-Mercaptoethanol agglutination test was applied on 436 buffalo serum samples out of which 110 (25.22%) were found positive. Shringi (1999) found higher seropositivity than our study in cattles (31.32%) by 2-MET.

Lower seroprevalence was recorded in bovines by Kalimuddin et al. (1990) and Hadad and Jamalludeen (1992), who recorded 12.87 percent and 6.24 percent seropositivity respectively, with 2-MET.
AVIDIN-BIOTIN ENZYME-LINKED IMMUNOSORBENT ASSAY

The test is considered as highly sensitive and specific by many workers (Magee, 1980; Saravi et al., 1995; Pati et al., 2000; Omer et al., 2001; Sarumathi et al., 2003; Mahato et al., 2004; Agrawal et al., 2007; Heydari et al., 2008; Brahambhatt et al. 2009).

In the current study, Avidin biotin ELISA revealed 165 (37.84%) serum samples positive for brucellosis out of 436 buffalo serum samples tested. These findings are comparable to Sahin et al. (2008) who found 39.45 percent seropositivity. Van Aert (1984) detected higher seroprevalence i.e. 42.8% out of total 785 bovines using ELISA.

Slightly lower prevalence was found by Chandramohan et al. (1992); Dangolla and Kulachelvy (2000); Upadhyay et al. (2007) and Chand (2006). They found 18.84 percent; 4.2 percent; 7.25 percent and 26.03 percent seropositivity, respectively.

The test sometimes is not capable of differentiating between antibodies resulting from S 19 vaccination or other false positive serological reactions (FPSR) and that induced by pathogenic Brucella strains (OIE, 2004).

Thus, no single test appears to be free from demerits. This has prompted many workers to carry out comparative studies and to determine the efficacy of different tests. Nielsen (2002) and Gall and Nielsen (2004)
after reviewing various serological tests concluded that no individual test was found perfect, however, error could be minimized using the most reliable test.

**COMPARISON OF RBPT, STAT, HIT, 2-MET, AGID AND MRT WITH AB ELISA**

In the present investigation, the Rse value was highest with RBPT followed by MRT, STAT, HIT, 2-MET and lowest with AGID. The Rsp values were highest with AGID followed by 2-MET, HIT, STAT, MRT and lowest with RBPT.

Relative sensitivity (Rse) value of RBPT was 48.48% whereas relative specificity (Rsp) and concordance values are 73.06% and 63.76%. These results are in conformity with the earlier findings wherein relative sensitivity and specificity of RBPT for bovine brucellosis, classified on the basis of ELISA results, were found to be 56.96% Rse and 96.77% Rsp respectively, Chakraborty et al. (2000); 88.46 percent and 97.75 percent, Singh et al. (2004a); 88.76 percent and 100 percent, Bandyopadhyay et al. (2009).

Relative sensitivity (Rse), relative specificity (Rsp) and concordance values of MRT were detected as 44.44, 77.77 and 63.49 percent in our study. Nazem et al. (1998) found similar sensitivity and specificity of MRT as compared to ELISA (48.15% and 72.22%), respectively but lower value (48.10%) of concordance. Patel (2007)
found higher value (69.81%) of concordance, in his study, the sensitivity and specificity of MRT as compared to ELISA were 30.00% and 93.93 %, respectively.

In our study, the percent values of Rse, Rsp and concordance in comparison with AB ELISA for STAT are 42.42, 78.23 and 64.68; for HIT are 36.36, 80.07 and 63.53; for 2-MET are 35.76, 81.18 and 63.99 and for AGID are 18.18, 88.56 and 61.92 respectively. Higher value of concordance, sensitivity and specificity was found 87.87percent, 61.19 percent and 98.78 percent respectively by Patel (2007) as compared to ELISA; 88.61 percent Rse and 98.59% percent Rsp by Chakraborty et al. (2000); 79.77 percent Rse and 100 percent Rsp by Bandyopadhyay et al. (2009). Ruppanner et al. (1980) found higher (75.7%) concordance between 2-MET and ELISA. AGID was found least sensitive and most specific test in our study. Similar results were obtained by Chand et al. (1988). Shringi (1999) reported that relative sensitivity of STAT, HIT, 2-MET were 89.62, 90.0, and 92.22 percent respectively.

**CONCLUSION**

The analysis of the findings from the present study implies following conclusions.

1. The overall seroprevalence of brucellosis in buffaloes of Kota region was found in different tests as; AB ELISA (37.84%) followed by RBPT (35.09%), MRT (31.75%), STAT (29.59%), HIT (27.52%), 2-MET (26.38%) and AGID (13.99%).
2. Comparatively much higher seroprevalence (38.46%) was observed in females than in males (30.30%) by AB ELISA.

3. Highest seroprevalence was found in Chhabra region in all tests and lowest seroprevalence was found in Dag region in ELISA, RBPT, MRT, STAT and AGID.

4. Buffaloes of Bundi region showed minimum seroprevalence (14.92%) in HIT and 2-MET.

5. Seropositivity in buffaloes of Chhabra region was 43.53 percent in AB ELISA, 40.00 percent in RBPT, 37.5 percent in MRT 34.12 percent in STAT, 31.76 percent in HIT, 31.76 percent in 2-MET and 17.64 percent in AGID.

6. Seropositivity in buffaloes of Dag region was 26.09 percent in AB ELISA, 26.09 percent in RBPT, 18.18 percent in MRT 23.19 percent in STAT, 23.19 percent in HIT, 21.74 percent in 2-MET and 10.14 percent in AGID.

7. Considering ELISA as a gold standard test, the sensitivity of RBPT, STAT, HIT, 2-MET, AGID and MRT were found to be of 48.48%, 42.42%, 36.36%, 35.76%, 18.18% and 44.44%, respectively, while specificity were found to be of 73.06%, 78.23%, 80.07%, 81.18%, 88.56% and 77.77%, respectively.

8. Relative concordance of RBPT, STAT, HIT, 2-MET, AGID and MRT with AB ELISA were 63.76%, 64.68%, 63.53%, 63.99%, 61.92% and 63.49%, respectively.
9. Finally, the study revealed presence of *Brucella* antibody in serum and milk of buffaloes of Kota division. Thus to eradicate the brucellosis from animals as well as for public health point of view, proper measures must be taken at State level. Therefore, all animals must be tested periodically for detection of *Brucella* antibody and effective vaccination must be done.
6. SUMMARY

The present study was undertaken to detect *Brucella* antibodies in serum and milk of buffaloes of Kota division of Rajasthan for effective diagnosis of brucellosis. Buffaloes are valuable components of rural households having socioeconomic importance and play important role in national economy as these are high milk producing animals. But limited work has been done on brucellosis in this seasonal breeder where even one abortion in a buffalo can leave the poor farmer helpless till the second breeding season.

For detection of *Brucella* antibodies in serum, six serological methods viz., AB ELISA, RBPT, STAT, HIT, MET and AGID were employed, whereas in milk, MRT was employed on 436 serum and 63 milk samples respectively.

Among the six serological tests employed for antibody detection, the highest seropositivity was obtained by AB ELISA (37.84%) followed by RBPT (35.09%), MRT (31.75%), STAT (29.59%), HIT (27.52%), MET (26.38%) and AGID (13.99%).

Highest seroprevalence was found in Chhabra region in all of the tests. One of the possible reasons for it may be the previous history of stream of abortions with retention of placenta as reported by farm owners. Lowest
prevalence was found in Dag region by all the tests except HIT and MET which showed lowest prevalence in Bundi region may be the due of reduction of vaccinal titre.

Higher number of females (38.46%) showed seropositivity for brucellosis as compared to males (30.30%) in AB ELISA. This might be due to comparatively high resistance in males for infection and absence of vaccination in male animals.

Considering AB ELISA as a gold standard test, the sensitivity of RBPT, STAT, HIT, MET, AGID and MRT were found to be of 48.48%, 42.42%, 36.36%, 35.76%, 18.18% and 44.44%, respectively, while specificity were found to be of 73.06%, 78.23%, 80.07%, 81.18%, 88.56% and 77.77%, respectively.

Relative concordance values for RBPT, STAT, HIT, MET, AGID and MRT with AB ELISA are 63.76%, 64.68%, 64.90%, 65.14%, 61.92% and 63.49%, respectively.

The study indicated that no single test is free from demerits for detection of *Brucella* infection but the error could be minimized using the most reliable test like ELISA. The study also revealed prevalence of *Brucella* infection in buffaloes of Kota division.
7. LITERATURE CITED


