Sero-epidemiology and Comparison of a PCR Assay in Whole Blood, Milk and Serum Specimens for Brucellosis Diagnosis in Bovines with Reproductive Disorders

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OF

MASTER OF VETERINARY SCIENCE

IN

VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE

BY

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(Enrolment No. V-1648/16)

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(2019)

CERTIFICATE

This is to certify that the thesis entitled "**Sero-epidemiology and Comparison of a PCR Assay in Whole Blood, Milk and Serum Specimens for Brucellosis Diagnosis in Bovines with Reproductive Disorders**" submitted by **Dr. Krishna Govind Bohrey**, Enrolment No. V-1648/16 in partial fulfilment of the requirements for the award of the **Master of Veterinary Science** in **Veterinary Epidemiology and Preventive Medicine** of the **U. P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Viswavidyalaya Evam Go-Anusandhan Sansthan (DUVASU), Mathura (UP), India,** is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Barkha Sharma 10.6.19

Dated 10 June 2019

(Barkha Sharma)

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ABBREVIATIONS

%	Per cent
<	Less than
>	More than
°C	Centigrade/ Degree Celsius
bp	base pair
C-ELISA	Competitive Enzyme Linked Immunosorbent Assay
CFT	Complement Fixation Test
D/W	Distilled water
DNA	Deoxyribo Nucleic Acid
gm	gram(s)
I-ELISA	Indirct Enzyme Linked Immunosorbent Assay
mg	Milligram
min.	Minute
MRT	Milk Ring Test
NFW	Nuclease free water
NH	Native Hapten
PBS	Phosphate Buffer saline
PCR	Polymerase chain reaction
RBPT	Rose Bengal Plate Test
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolution per minute
sec.	Second
SSCP	Single Strand Conformational Polymorphism
STAT	Standard Tube Agglutination Test

TAE	Tris acetate EDTA
UV	Ultra violet
μg	Microgram
μl	Microlitre

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Contents

ABSTRACT

In this study, a total of 470 samples from 203 cattle and 59 buffaloes were collected. Including 262 serum samples from 203 cattle and 59 buffaloes which were screened for seroprevalence of bovine brucellosis using RBPT, STAT and I-ELISA. Further, 108 whole blood samples and 100 milk samples were also collected and processed for molecular detection of *Brucella*. This study also considered the effect of various epidemiological parameters like place or geographical area, species, age, rearing practice and health status with reproductive problems on the occurrence of the disease in the population. RBPT, STAT and I-ELISA tests used for diagnosis of brucellosis were compared for sensitivity and specificity of the diagnostic test.

The overall seroprevalence was found 9.92%, 16.03%, 6.48% by RBPT, STAT and I-ELISA respectively. Species wise seroprevalence of brucellosis found in cattle 11.82%, 16.25%, 8.37% by RBPT, STAT and I-ELISA respectively while in buffaloes seroprevalence were found 3.38%, 15.25% and 0% by RBPT, STAT and I-ELISA respectively. On the other hand age wise seroprevalence in young animals were 7.76%, 13.04% and 4.34% by RBPT, STAT and I-ELISA respectively while in adult animals 11.17%, 17.64% and 7.64% by RBPT, STAT and I-ELISA respectively. Unorganized rearing of animals were show seroprevalence 8.68%, 5.43% and 6.52% by RBPT, STAT and I-ELISA while the seroprevalence of organized rearing of animals were 10.58%, 21.76%, 6.47% by RBPT, STAT and I-ELISA respectively. RBPT, STAT and I-ELISA were compared against each other by applying Kappa Statistics and concordance percentage. According kappa statistics, moderate agreement (0.5547) was present between RBPT and I-ELISA while STAT and I-ELISA showed fair agreement (0.3061). Similarly concordance between RBPT and I-ELISA was more (91.98%) as compared to STAT and I-ELISA (85.87%).

Molecular detection of Brucellosis by PCR assay from serum (100 samples), whole blood (108 samples) and milk (100 samples) revealed the total positive samples 5 out of 308 (1.62%). None of the sera samples were found positive by PCR whereas 3(2.8%) and 2(2%) from whole blood and milk samples, respectively were found positive and showed amplicons of 223bp confirming *Brucella* genus and amplicon of 498bp confirming *Brucella abortus*. So among samples processed for PCR, whole blood was found to be better than milk and serum.

Hence, a combination of RBPT, STAT and I-ELISA were found to be the most suitable serological tests for the confirmation of Brucellosis and in case where infection is not established or pronounced, confirmation by PCR using whole blood might be most suitable for the diagnosis of bovine brucellosis.

Introduction

CHAPTER-1

INTRODUCTION

Brucellosis is one of the most important contagious and communicable bacterial diseases, with a worldwide distribution and is endemic in most parts of world especially the developing countries (Trujillo et al., 1994). It is considered a re-emerging though neglected zoonosis with high rates of morbidity and lifetime sterility. The incidence of brucellosis cases is increasing over the recent years especially in developing countries due to poor management, limited resources (Khan and Zahoor, 2018) and increased trade and frequent movement of livestock (Renukaradhya et al., 2002). Though it has been eradicated in many developed countries in Europe, Australia, Canada, Israel, Japan and New Zealand (Geering et al., 1995) yet remains as highly endemic in Africa, Mediterranean, Middle East, parts of Asia and Latin America (Refai, 2002).

The economy of India is dependent mainly on agriculture. Livestock production is a vital source of providing dietary protein for the rapidly growing human population and it is therefore, important to define strategies for controlling infectious diseases that are undermining the livestock industry. The presence of brucellosis in India was first established early in the previous century and since then it has been endemic in almost all the states (Sehgal and Bhatia, 1990; Renukaradhya *et al*., 2002).

Taxonomically, *Brucella* are classified as alfa-proteobacteria having 6 classical species (Moreno and Moriyon, 2001) namely *Brucella abortus* (cattle and buffalo), *Brucella melitensis* (goat, sheep and camel), *Brucella ovis* (sheep), *Brucella canis* (dogs), *Brucella suis* (pigs) and *Brucella neotomae* (desert wood rat). Recently new species have been included are *B. pinnipedialis* and *B. cetaceae* (in marine mammals), *B. microti* (in common vole Microtusarvalis), and *B. inopinat* (in a breast implant) (Foster et al., 2007; McDonald et al., 2006). The main pathogenic species in both animals and human are *B.melitensis, B.abortus, B.suis and B.canis* (Araj, 2010; Cloeckaert and Vizcaino, 2004).

Brucellae are gram negative, facultative intracellular, nonmotile, non spore forming coccobacilli, requiring an optimum temperature of at 37^{o} C to produce visible colonies after 24 hrs, and may require supplementary CO_2 for their growth especially on primary isolation (Corbel and Bannai, 2005). Flagella, endospores and capsule are absent although capsule like structure have been reported in preparation treated with antiserum. They are partially acid fast, as are not decolorized by acetic acid in the modified Ziehl-Neelsen (MZN) staining (Alton et al., 1988).

Transmission of brucellae mainly occurs through the skin, conjunctiva or respiratory mucosa by inhalation and most commonly through oro-faecal route in cattle (Crawford et al., 1990). Infection spreads to local lymph nodes where *Brucella* replicates intracellularly in phagocytes (Anderson et al., 1986). Invasion of lymphatic vessels is followed by bacteraemia leading to systemic infection, favouring colonisation of the gravid uterus, male genital organs, and mammary glands (Ko and Splitter, 2003). The mammary gland is another target organ that is important in transmitting the infection through contaminated milk. *B. abortus* induces a multifocal interstitial mastitis with interstitial accumulation of macrophages and intra-acinar infiltration of neutrophils (Emminger and Schalm, 1943).

The clinical picture of brucellosis is so strange and protean that it can be easily confused with other infectious and non infectious diseases, leading to diagnostic delays (Al Dahouk and Nockler, 2011). The disease is clinically characterized by abortion storms in animal herds along with metritis, mastitis, retention of placenta in the last trimester of pregnancy, causing huge financial losses to the dairy and beef farmer alike. Additional economic losses occur due to reduced milk production, infertility, delayed heat, interrupted lactation, loss of calves, wool, meat and milk production, extended inter-calving period and decreased value of breeding stock and sterility in males (Radostits et al., 2000). Susceptibility to brucellosis increases as the animals approach the breeding age (Tolosa et al., 2008). Although, abortion is rare in chronic cases, but infected animal continues to act as a carrier and source of infection to the herd. Calves born to infected females also serve as carriers in the herd. Infected animals generally develop granulomatous inflammatory response often located within lymphoid tissues and organs with a prominent reticuloendothelial component. The localisation and persistence of brucellae within these tissue and organs follow in the wake of widespread distribution of the bacteria during a generalised stage of infection. Invasion of gravid uterus is marked by characteristic necrotic placentitis. Acute and widespread inflammation of placenta leads to early death of foetus followed by abortion or birth of a live, infected, weak calf associated with retention of placenta and congested, necrotic cotyledons covered with yellowish or sticky brownish exudates extending deep in to the crypts.

In males, orchitis and epididymitis are the manifestation of chronic infection (Chand et al., 2002). During orchitis the testicles gradually enlarge enveloping an area of dry necrosis encapsulated by fibrinous tissue which eventually contracts, often leaving the testicle smaller than normal.

There is no completely reliable and satisfactory diagnostic procedure for the disease. Currently three major approaches are being employed including microbiological, serological and molecular techniques (Alves et al., 2010). The absence of a perfect rapid reference diagnostic test makes evaluation of serological tests difficult. Although isolation is considered as gold standard but in the case of brucellosis, it is seldom successful (Jama'yayah et al., 2011). Bacterial cultures are often negative for infected animals because of the intra-cellular and fastidious nature of the pathogen (Romero et al., 1995), and the test is relatively difficult to use in the field in rural areas as it requires biosafety level 3 (BSL 3) laboratory set up (Bricker, 2002). It also requires long incubation periods and may also cross-reacst with some other gram-negative bacteria, including *Escherichia coli* O157, *Francisella tularensis*, *Salmonella enterica* group N, *Stenotrophomonas maltophilia*, *Yersinia enterocolitica* O: 9 and *Vibrio cholerae*.

For assessment of brucella antibody there are various serological tests like Rose Bengal Plate agglutination Test (Alton et al., 1975), serum agglutination Test (Alton *et al*., 1975), Enzyme Linked Immunosorbent Assays (Nielsen and Wright, 1984), complement fixation using whole cell preparations (Magee, 1980; De Klerk and Anderson, 1985), cell sonic extracts or lipopolysaccharide (LPS) fractions (Lindberg et al., 1982), Native Hapten (NH) gel precipitation tests, fluorescence polarization assay (FPA) etc. The main serological test used for screening of brucellosis is the Rose Bengal Plate Test (RBPT), which has very high sensitivity but low specificity (Barroso et al., 2002). I-ELISA is the most frequently used serological test for confirmatory diagnosis as it yields higher sensitivity and specificity in comparison with the Serum Agglutination Test and Rose Bengal Plate Test (Almuneef and Memish, 2004). Thus I-ELISA has become the standard method for the diagnosis of the brucellosis.

Apart from serological tests, molecular approaches have been explored to overcome these problems. Conventional and multiplex PCR typing are capable of identifying *Brucella* upto species level. A multiplex PCR assay, AMOS PCR (AMOS is acronym for abortus, melitensis, ovis, suis) identifies *B.abortus, B.melitensis, B.ovis,* and *B.suis* simultaneously including biovar level using a combination of different primers. This PCR assay has successfully identified *B.abortus* biovar 5, 6, and 9 and some field strains of biovar 3 *B.abortus* (Sasa et al., 2005).

Brucellosis is an important re-emerging disease with a worldwide distribution (Geering *et al.,* 1995) and continues to be endemic in most parts of the world especially the developing countries like India. *Brucella abortus* strain -19 vaccine is recommended as most effective vaccine for cattle and buffaloes, however application of full dose is restricted in adults due to persistence of antibodies response that interfere with serological diagnosis. Various studies have been conducted in India to establish the prevalence of the disease in bovines but to assess the change in the seroepidemological situation of the disease after the initiation of bovine brucellosis control programme, more studies are needed. Therefore present study was designed to assess the epidemiology of the disease in bovines suffering with various reproductive disorders in and around Mathura region, contribution of *Brucella* to those reproductive disorders, comparative efficacy and diagnostic performance of existing serological and molecular tests with the following objectives.

OBJECTIVE

1) To detect the prevalence of brucellosis in bovines suffering with reproductive disorders using various serological and molecular tests.
2) To study the relationship between brucellosis and associated risk factors in dairy animals with reproductive disorders.
3) To compare the diagnostic performance of different serological and molecular tests employed for diagnosis of brucellosis in bovines.
4) To compare the efficacy of using whole blood, milk and serum as specimens for PCR assay for diagnosis of Brucellosis.

Review of Literature

CHAPTER- 2

REVIEW OF LITERATURE

Brucellosis is a chronic infectious disease caused by small, non-motile, non-sporing, gram-negative, facultative intracellular coccobaccilli of the genus *Brucella*. This disease may affect a range of different mammals including cattle, buffalo, sheep, goat, swine, rodents, man and marine mammals (Manish et al., 2013). It is a major bacterial zoonosis of global public health importance. In most of the animal species, the disease primarily affects the reproductive system with concomitant loss in productivity. Both humoral and cellular immune responses are involved in *Brucella* infection. Bovine brucellosis by *Brucella abortus*, is widely prevalent in India causing economic losses to the tune of US $ 58.8 million (Kollannur et al., 2007, Shome et al., 2014).

2.1 Seroprevalence of Brucellosis around the World

Brucellosis is the most common zoonosis in the world accounting for the annual occurrence of more than 500,000 cases (Pappas et al., 2006). Though, it has been eradicated in many developed countries in Europe, Australia, Canada, Israel, Japan and New Zealand (OIE, 2002). It is still a major problem in the developing world like Mediterranean countries of Europe, Northern and Eastern Africa, Near East countries, India, Central Asia, Mexico and Central and South America. Brucellosis is a significant veterinary and public health problem in India where 80% of the population lives in approximately 575000 villages and thousands of small towns and have close contact with domestic / wild animal population owing to their occupation. Hence, human population stands at greater risk of acquiring zoonotic diseases including brucellosis.

Awareness of risk groups is needed to take appropriate preventive measures and to accept control measures (Smits & Kadri, 2004).

Seroprevalence of bovine brucellosis was reported to be 6.5% in Jordan (Al-Majali et al., 2009), 8.4% in Cameroon (Bayemi et al., 2009) and 32.9 to 39.4% in Turkey (Sahin et al., 2008). The overall seroprevalence of the disease at the individual animal level was 2.9% in three ecological areas of central Oromiya, Ethiopia (Jergefa et al., 2009).

In a cross-sectional study conducted in western Ethiopia, the seroprevalence of bovine brucellosis in 1,152 cattle from 164 herds under traditional extensive husbandry, using RBPT as screening test and CFT as a confirmatory test the apparent seroprevalences were found to be 1% and 4.9% at the animal and herd level, respectively (Adugna et al., 2013).

Egaru et al. (2013) obtained an overall prevalence of 15.4% in cattle using RBPT as screening test in Arapai, Soroti district, Uganda.

Vhoko et al. (2018) estimated the prevalence of brucellosis in Zimbabwe's 156 farms with Rose Bengal Test (RBT), Complement Fixation Test (CFT) and Milk Ring Test (MRT).

Brucellosis has its reservoirs in wild animals and gets transmitted from them to domestic animals in their vicinity (Rhyan et al., 2013).

A study conducted in Dhamar governorate in Yemen with screening of 384 sera samples including 359 females and 25 males collected randomly from unvaccinated cattle using RBT and I-ELISA showed a seroprevelance of cattle brucellosis to be 0.26%. In Ethiopia, an overall seroprevalence of 2.0% and 3.75%, respectively, was reported by Alemu et al. (2014) and Waktole et al. (2018).

An overall prevalence of 2.93% (13/444) of brucellosis in cattle using RBPT as a screening test was found in Kintampo Districts, Brong Ahafo region, Ghana (Foliste et al., 2014) whereas in Morocco, the prevalence of brucellosis in cattle at individual and herd level was 1.9% and 9%, respectively (Azami et al., 2018). In Mbeya region, Southern highlands of Tanzania, the overall animal-level seroprevalence was 9.3% in a cross-sectional study in conducted by (Sagamiko et al., 2018) using the Rose Bengal Plate Test (RBPT) and competitive Enzyme Linked Immunosorbent Assay (c-ELISA) as screening and confirmatory tests, respectively.

2.2 Seroprevalence of Brucellosis in India

Serological evidences are suggestive of high endemicity of brucellosis in India with national average of as high as 5% in cattle, 3% in buffaloes, 7.9% in sheep and 2.2% in goats (Renukaradhyay, 2002). A serological survey of brucellosis in bovine population in 23 states of India using RBPT and STAT showed the prevalence to be 1.9% in cattle and 1.8% in buffaloes (Isloor et al., 1998).

Perusal of the literature indicated prevalence of brucellosis in western region (Gujarat state) ranging from 8.98 to 44.00% (Chauhan et al., 2000), 6.3% in central region (Madhya Pradesh state) (Mehra et al., 2000) and 12.9% (Dhand et al., 2005) and 18.26% by systematic multistage random sampling using milk ELISA in Punjab (Aulakh et al., 2008). In the states of Karnataka, Uttarakhand and Uttar Pradesh, seroprevalence was 31.74% including 27.21 in cattle and 36.34% in buffaloes using i-ELISA (indirect enzyme linked immunosorbent assay) (Jagapur et al., 2013).

A systematic study of 24 organized farms, out of a total of 1359 dairy animals revealed 71 (5.22%), 82 (6.03%), 73 (5.37%) and 54 (3.97%) samples to be positive by RBPT, iELISA, serum and blood PCRs respectively. Combination of iELISA and serum PCR were found most suitable to declare brucellosis status of the animals (Shome et al., 2014).

In a study by (Khajuria et al., 2014), seroprevalence of brucellosis in the state of Jammu was 4.38% and 7.50% by Rose Bengal Plate Test (RBPT) and Standard Tube Agglutination Test (STAT), respectively.

In peri-urban areas of Gujarat, (Patel et al., 2015), found 16.9% (849) bovines to be suffering from various reproductive disorders and of these, 18.30% (155) were found positive for *Brucella abortus* antibodies.

Trangadia et al. (2015) studied six organized cattle herds located in Andhra Pradesh, Gujarat and Odisha and the estimated prevalence of brucellosis in these states was found to be 6, 8.2 and 2.3%, respectively.

Overall prevalence of brucellosis among 559 bovine sera samples collected from organized dairy farms in Southern India was 10.2% by RBPT and 11.63% by iELISA (Krishnamoorthy et al., 2015). In another study involving western UP, the seroprevalence of brucellosis by iELISA, was 12.37% (Kumar et al., 2016).

A total of 570 dairy cattle from 35 herds were screened serologically based on the history of abortion in the farm, 13 (2.28%) were found positive by RBPT while 33 were negative inspite of history of abortion (Geresu et al., 2016).

Study by Kumar et al. (2017) revealed that 4.35% and 5.80% of the milk samples were positive for bovine brucellosis by MRT and m-ELISA respectively from eleven districts of Tamil Nadu. Crossbred cattle, cattle with more than seven years of age and animals having history of abortion recorded more prevalence of brucellosis.

Ul Islam et al. (2018) revealed an overall high sero-prevalence of 15.12% in a cross sectional study in Punjab in 628 Murrah buffaloes using ELISA as screening test.

2.3 Association of Bovine Brucellosis with Epidemiological factors

Both husbandry systems as well as environmental conditions greatly influence the spread of Brucella infection (WHO, 1997). Over half of the cattle are farmed under extensive lowland pastoralist and agro-pastoralist production system.

A higher risk of brucellosis was observed in cattle in intensive livestock systems, in imported and crossed breeds and in animals from larger herds (Azami et al., 2018).

The study by Isloor et al. (1998) showed the prevalence to be more in organized herds (4.1%) then in cattle owned by individual farmers in Karnataka. In a similar study in Debre-Zeit, Central Ethiopia, difference in breed and parity were found to be potential risk factors with disease more prevalent in crossbreds than local dairy cattle and in animals above two years than younger animals. History of abortion and retained fetal membrane were found to be significantly associated with occurrence of bovine brucellosis (Alemu et al., 2014).

The positive reactors to the diseases were significantly more in cows than bulls, and disease acquiring risk seemed to increase with age; more between age 1 to 4 cattle (Foliste et al., 2014).

Sagamiko et al. (2018) found infection to be more prevalent in indigenous cattle than in exotic cattle and maximum in age group of 6–10 years followed by 1–5 years and 11–15years old. The odds of seropositivity in animals which calved on pasture were more than those that calved at home. Similar findings were also cited by Gogoi et al. (2017).

Seroprevalence was zero by both RBPT and STAT in organized dairy farm while in unorganized sector, 5.04 and 8.63% by RBPT and STAT, respectively. Age-wise seroprevalence was higher in 4.5 to 6 years old buffaloes than younger (Khajuria et al., 2014).

No significant statistical variation in the seroprevalence of brucellosis was found in different age and sex groups of the study animals (Degefu et al., 2011; Krishnamoorthy et al., 2015) whereas Egaru et al. (2013) showed that that younger cattle were less infected than adults and prevalence of *Brucellosis* was higher in females compared to males. Similar findings were also cited by Khan et al. (2016) who recorded prevalence in female cattle to be 15.14%) as compared to male (14%). Similarly significantly adult were more susceptible as compare to young ones. Breeding through natural service was at greater risk (94%) as compared to artificial insemination (78%).

In a study by Jagapur et al. (2013), prevalence of brucellosis was found to be more in buffaloes than cattle and prevalence in organized herds was more than unorganized farms or villages.

Higher prevalence of bovine brucellosis was found in cases of abortion than other reproductive disorders (Patel et al., 2015; Krishnamoorthy et al., 2015). Similarly, prevalence was highest in case of aborted cattle (64.24%) followed by retention of placenta (47.13%) in cattle (Gogoi et al., 2017). Higher seroprevalence of brucellosis was found in cases of abortion and retention of placenta, while at lower level from various reproductive disorders indicating endemicity of the infection in villages around Anand city, Gujarat (Ghodasara et al., 2010).

Increased disease prevalence was observed in animals with a history of abortion than in those without such histories Ul Islam et al. (2018).

Maximum numbers of seropositive cases were found in female animals, indigenous breed and in 6 to <9 yrs age group, respectively by all serological tests ELISA, RBPT, STAT and 2-ME . ELISA diagnosed highest number of seropositive cases in different categories *viz.,* females (36.41%), indigenous breed (21.13%) and 6 to <9 yrs (52.15%), respectively. Sex, breed and age of animals were found to influence the antibody titer of animals (Kushwaha et al., 2016).

Kumar et al. (2016) found seropositivity to be higher in organized herds (24.88%) than individual cattle (4.8%). Young calves had significantly higher seropositivity (10.38%) and it further increased in sexually matured adults (12.71%) in comparison to older animals (9.17%).

Another study by Ndazigaruye et al. (2018) in cattle from selected sectors of Nyagatare District, Rwanda showed the prevalence of brucellosis to be more in cows with no history of abortion (38.5%) rather than those with a history of abortion (17.0%) although retention of placenta was a major factor associated with the disease. An increase in calving interval was also noted. The prevalence was more in adult cows (21.4%) than that in heifers (12.8%).

2.4 Diagnosis of Brucellosis

Clinical signs of *Brucella* infection are non-pathognomonic and thus diagnosis of the disease is mainly dependent on laboratory tests. The conventional tests, viz. Rose Bengal plate agglutination test (RBPT), serum tube agglutination test (STAT) and complement fixation test (CFT) are commonly used. Isolation is still the gold standard. Other commonly used tests are enzyme linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), native hapten (NH) gel precipitation tests, brucellin skin test and fluorescence polarization assay (FPA). All reproductive disorders should be monitored using accurate diagnostic tests such as milk-ELISA or I-ELISA for correct and prompt diagnosis as to prevent further spread of infection to apparently healthy animals of the herd and surroundings (Patel et al., 2015).

2.4.1 Molecular Detection of *Brucella abortus*

In the absence of adequate culture facilities the diagnosis of brucellosis either depends on serological or molecular tests. The main drawback with the serological tests is their cross reactivity and low specificity. Alternatively, molecular techniques can be used for diagnosis of bovine brucellosis. The test samples from which DNA can be extracted most commonly for brucellosis diagnosis include tissues from neonates or aborted fetuses, milk, whole blood, serum, semen, body fluids, and foods such as cheese.

Extraction of DNA from body fluids is cumbersome, mainly because of the presence of PCR inhibitors in samples taken from those liquids. Extraction procedures for *Brucella* detection are further complicated because it is an intracellular pathogen.

Leal-Klevezas et al. (1995) described protocols of DNA extraction from blood and milk to overcome these problems, making detection by PCR more applicable for brucellosis.

Rekha et al. (2013), revealed the status of bovine brucellosis in an organized dairy with a past history of *Brucella* abortions. A total of 195 samples including 89 blood samples, 89 serum samples and 17 milk samples were collected and analyzed by isolation and identification by PCR, MRT, RBPT, STAT and ELISA. All the 89 blood and 17 milk samples were negative for culture and PCR. MRT and ELISA tests on all the 17 milk samples and STAT on all the 89 serum samples were also negative. The percent positives for *Brucella* antibodies in serum samples were 4.5 and 6.7 by RBPT and ELISA, respectively.

The number of brucellosis positive samples detected either by Brucella genus specific *bcsp*31-PCR or virulent gene *omp*25- PCR was 297 (94%) and 294 (93%) out of 334 total milk samples tested, respectively (Arasoglu et al., 2013).

In a study by Karthik et al. (2014), out of 370 blood samples collected from three states of Uttarakhand, Uttar Pradesh and Tamil Nadu, the seroprevalence of Brucellosis by RBPT was 16.49%, 15.94% by STAT and 15.13% by whole blood PCR targeting genus specific bcsp31gene and species specific IS711 gene. Sensitivity and specificity of PCR compared with RBPT was 100% and 92.4% while with STAT, these were 100 and 96.16%, respectively.

Hemade et al. (2015) studied detection of brucellosis in animals by isolation and identification of *Brucella* species isolates by conventional method and they recovered 15 *Brucella* spp. isolates with isolation rate of 24.59% which were further confirmed as B*rucella abortus* species isolates 2 different PCR assays using genus specific bcsp31 and species specific IS711 (AB and BM) primers.

Hemade and Gandge (2016) studied PCR-RFLP and PCR-SSCP to understand variabilities in *Brucella* spp. genome to assist in planning epidemiological strategies for control of brucellosis in animal population and there by human transmission. Comparative results of RFLP and SSCP in 15 isolates showed that, PCR-SSCP is more sensitive than RFLP for detection of polymorphism in bcsp31 gene.

A multiplex PCR technique for detection of *Brucella* spp. in samples of bacterial suspension was validated as a complementary tool in the diagnosis of the disease, allowing the characterization of the agent without performing biochemical tests (Orzil et al., 2016).

Daugaliyeva et al. (2016) developed a differential PCR assay for detection of *B.abortus* and *B.melitensis*.

A total of 110 sera sample and whole blood samples were collected separately from unorganized dairy herds of Jersey and Holstein-Friesian (HF) crossbred cattle to evaluate the sero-prevalence of brucellosis by Rose Bengal Plate Test (RBPT), indirect ELISA and its PCR assay (Koushik et al., 2017).

Alamian et al. (2017) described a novel PCR assay for detecting *Brucella abortus* and *Brucella melitensis*.

Lindahl-Rajala et al. (2017) investigated the presence of *Brucella* DNA in bovine milk in the urban and peri-urban area of Dushanbe, Tajikistan. *Brucella* DNA was detected in 10.3% of 564 cow milk samples by IS*711*-based real-time PCR.

Serum based bcsp 31 genus specific and IS711 species specific PCR assay on 821 bovine sera samples revealed a percent positivity of 821 bovine sera samples whereas by indirect Enzyme Linked Immunosorbent Assay (i-ELISA), the per cent positivity was 5.72. In comparison between serology and molecular test, 44 samples were positive for both assays and 11 and 3 samples were positive for serology and molecular assays individually (Kumar et al., 2018)

2.6 Comparative Performance of the Serological Tests for Brucellosis

There are several methods for diagnosis of *Brucella* spp infection but the gold standard test still remains the culture isolation of the organism. (Alton et al., 1988; Lulu *et al*., 1988). Some ELISAs have similar or better diagnostic performance as compared to complement fixation test (CFT) because they are simple, easy to perform, sensitive and preferred to use (OIE, 2009). All these conventional tests have some limitations, as brucellosis is caused by different *Brucella* species sharing common epitopes and thus might not give accurate diagnosis thus it is imperative to use both direct and indirect methods for accurate and reliable diagnosis of brucellosis (Carmichael and Greene, 1990; Wanke, 2004).

Many laboratories across the world are involved in developing sensitive and specific assays based on the molecular markers of *Brucella* spp in order to eradicate menace of brucellosis. Different serological tests have been developed by keeping various goals in mind but the validation of all these tests is still an issue, the combination of different serological tests with appreciable specificity and sensitivity values can be utilized to know the status of animals (Ariza et al., 1992; Weynants et al., 1996).

The contaminated vaginal discharges, organs of aborted fetuses such as lymph nodes, stomach content, milk, secretions of infected animals have been proved to be important source of bacterial isolation. Phage typing has been a very handy tool for species and biovar characterization along with biochemical tests (Godfroid et al., 2002; Singh et al., 2014).

Gall and Nielsen (2004) found the performance index (PI) of buffered antigen plate agglutination test (BPAT) rated highest (PI = 193.1) among the conventional tests including the Rose Bengal test (PI = 167.6) and the complement fixation test (PI = 172.5). The indirect enzyme linked immune sorbent assay (PI = 189.8) and the competitive enzyme-linked immune sorbent assay (PI = 188.2), were found to be more accurate than the conventional tests, except for the BPAT.

In a serological survey at the abattoir of Dschang (West Cameroon) to determine the prevalence of bovine brucellosis, the seroprevalence of brucellosis was 9.64 and 4.88% by I-ELISA and RBT, respectively. The best estimation of the actual prevalence of brucellosis was based on the I-ELISA results and was close to 10% (Njila et al., 2005).

On comparing three serological tests (RBPT, STAT and i-ELISA), seropositivity of brucellosis was found highest by i-ELISA (25%), followed by STAT (14.45%) and RBPT (10.56%). The results shows higher prevalence of brucellosis in cases of abortion and retention of placenta, while at lower level from various reproductive disorders as detected serologically indicating endemicity of the infection in villages around Anand city, Gujarat (Ghodasara et al., 2010).

Manishimwe et al. (2015) compared RBPT and C-ELISA test in detection of bovine brucellosis in a study conducted in Kigali city, the capital of Rwanda. The study revealed that even if the observed significant difference between prevalence given by RBPT and C-ELISA and the agreement between the two test was perfect (k= 0.92).

Trangadia et al. (2015) evaluated 5 commercially available indirect ELISA kits and one competitive ELISA kit in naturally infected cattle population and found I-ELISA to be better than C-ELISA. They found that all I-ELISA had more than 96% specificity whereas much lower sensitivity, suggesting that the test is optimized for confirmation of negative status of infection.

In a comparative study on bovine serum samples from ten different sources by serological tests (RBPT, STAT and I-ELISA) and molecular technique (serum PCR), the seroprevalence observed was 11.60%, 9.34% and 14.46% by RBPT, STAT and I-ELISA, respectively. Out of 664 sera samples, 148 comprising 98 serologically positive and 50 randomly selected negative samples were subjected to serum PCR and 42.57% were found positive. Maximum relative sensitivity and specificity was found in RBPT than STAT and serum PCR (Sunder et al., 2015).

Kushwaha et al. (2016) conducted four serological methods *viz.*, ELISA, RBPT, STAT and 2-ME tests were used to determine the seroprevalence of brucellosis in an organized dairy farm. Overall, 33.85%, 32.61% and 30.90% animals were diagnosed serologically positive, respectively by ELISA, RBPT and STAT. However, only 13.66% animals were diagnosed positive by 2-ME. Therefore, ELISA could be recommended as a screening test for cattle.

Comparative efficacies of Rose Bengal Test (RBT), Standard Agglutination Test (STAT) and indirect Enzyme Linked Immnosorbant Assay (i-ELISA) were analysed in bovine brucellosis serodiagnosis (n=821) from various districts of Tamil Nadu. Using i-ELISA as a gold standard test, RBT and STAT showed sensitivity of 54.54 & 61.81 and specificity of 99.60 & 99.73 percent respectively. On kappa statistics with comparison to i-ELISA, RBT and STAT showed moderate (0.66) and substantial (0.73) agreement (Kumar et al., 2018).

Kala et al. (2018) also evaluated the comparative efficacy of RBPT and STAT to i-ELISA and determined their sensitivity and specificity in the diagnosis of bovine brucellosis. I-ELISA showed higher efficacy followed by STAT and RBPT. The sensitivity of RBPT and STAT was found to be of 34.68% and 55.86%, respectively, considering i-ELISA as a gold standard test while specificity was found to be of 98.48% and 98.99%, respectively. Thus, STAT was found to be more sensitive and specific than that of RBPT.

Materials and Methods

CHAPTER -3

MATERIALS AND METHODS

3.1 Study area

The present epidemiological study was conducted from October 2017 to July 2018. Samples of milk, blood and serum were collected from different districts viz., Aligarh, Firozabad, Hathras, and Mathura of western part of Uttar Pradesh of India. These districts were selected due to high number of smallholder dairy farmers with animal husbandry practices. The climate of this area is humid subtropical with dry winter. Agriculture with animal rearing is the predominant economic activity. In these area the predominant cattle breeds are Zebu, Sahiwal, and crossbreds of Holstein Friesian and Jersey, while the main buffalo breed is Murrah. The animals in the present study belonged to either organized farms or unorganized. In organized farms, animals were reared under semi-intensive system of management and were frequently in contact with other animals during feeding, watering and housing. In unorganized category, animals were individually reared by marginal or landless farmers relying on grazing in pasture with no or few supplementary feeding. Sample were collected by random sampling with no history of vaccination against brucellosis.

3.2 Place of work

The study was carried out in the Department of Veterinary Epidemiology and Preventive Medicine and Department of Veterinary Public Health, College of Veterinary Science and Animal Husbandry, Uttar Pradesh Pandit Deen Dayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sanathan, (DUVASU), Mathura, India.

3.3 Materials Required

3.3.1 Glass wares, plastic wares and reagents

In the present study glass wares, disposable and autoclavable plastic wares, were procured from Borosil Glass Works Pvt. Ltd. (Mumbai, India), Tarson Product Pvt. Ltd. (Kolkata), Eppendorf (Germany) and Genie Pvt. Ltd. (Bangalore, India) etc.

3.3.2 Reagents for RBPT, STAT and ELISA

For RBPT and STAT, *Brucella abortus* coloured antigen and *Brucella abortus* plain antigen (S-99) were procured from Division of Biological Product Indian Veterinary Research Institute, (IVRI), Izatnagar, India.

For I-ELISA, Protein-G based indirect ELISA kit for Bovine Brucellosis was procured from National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), (Formerly PD_ADMAS), Hebbal, Bengaluru, Karnataka, India.

3.3.3 Equipments

Major equipments used in the study were Autoclave (Sonar, India), Biosafety Cabinet (Sonar, India), Refrigerated Centrifuge CPR-30 (Remi Equipment, India), RM12C Microcentrifuge (Remi Equipment, India), Weighing Balance (Kern, Germany), Thermocycler (Bio–Rad, USA), UV - transilluminator (Genie Pvt. Ltd., Bangalore, India), Micropipette (Eppendorf, Germany) and Gel Electrophoresis (Genie Pvt. Ltd. Bangalore, India).

3.4 Methods

3.4.1 Data collection

The required epidemiological data was collected using the structured questionnaire (annexure-I). The questionnaire sought information about epidemiological determinants influencing the occurrence of brucellosis in animals like species, sex, age, geographical location, rearing practice and disease condition etc.

3.4.2 Selection of animals

Stratified convenient sampling was used to draw sample from the population of animals. In the first stratum, districts were selected followed by dairy units and animals. The total sample size to be drawn was calculated as described by Thrushfield (2008).

3.4.3 Collection of samples

During the present study, a total of 470 samples (serum, whole blood and milk) were aseptically collected from the selected animals (cattle and buffaloes) from different districts of western U.P. (Table 1). Total 262 serum samples were collected for serological diagnosis and PCR assay. The samples were collected according to age, sex, species, disease condition and rearing practice of animals (Table 2, 3, 4, 5, 6). About 5 - 10 ml of blood was collected by puncturing the jugular vein in sterile disposable syringes (Dispovan) or plain vaccutainers (BD, USA) for serum collection. Care was taken to avoid shaking the samples during transportation to prevent the destruction of the RBCs or hemolysis. Serum was collected from the clotted blood by centrifugation at 4000 rpm for 10 min. After the serum was separated, it aliquoted and stored at - 20 º C till further use. To prevent any damage to the protein structure of the serum repeated freezing and thawing was avoided.

A total of 108 whole blood samples were collected for PCR assay in EDTA vaccutainers (BD, USA). 100 milk samples were collected in sterile polypropelene tubes for PCR assay. The samples were transported immediately to the laboratory on ice pack. The individual animals in the present study were identified by their respective identification numbers or names. None of the animals were vaccinated against brucellosis.

Table 1: District wise Collection of Sample (Serum, Whole blood and Milk)

S. No	Name of district	Number of serum sample	Number of whole blood sample	Number of milk sample	Total
1	Aligarh	32	32	36	100
2	Firozabad	48	35	17	100
3	Hathras	43	31	26	100
4	Mathura	139	10	21	170
5	Total	262	108	100	470

Table 2: Collection of Samples (Serum, Whole blood and Milk) According to Age of Animals

S. No	Age	Serum	Blood	Milk
1	Young animals (< 3 years of age)	92	21	7
2	Adult animals (> 3 years of age)	170	87	93
3	**Total**	262	108	100

Table 3: Collection of Samples (Serum, Whole blood and Milk) According to Sex of Animals

S. No.	Sex	Serum	Blood	Milk
1	Male	4	4	00
2	Female	258	104	100
3	**Total**	262	108	100

Table 4: Collection of Samples (Serum, Whole blood and Milk) According to Species

S. No.	Species	Serum	Blood	Milk
1	Cattle	203	61	58
2	Buffaloes	59	47	42
3	**Total**	262	108	100

Table 5: Collection of Samples (Serum, Whole blood and Milk) According to Disease Condition of Animals

S. No.	Disease condition	Serum	Blood	Milk
1	Abortion	18	12	14
2	Pyometra	12	6	6
3	Metritis	8	4	3
4	Stillbirth	10	10	10
5	Retention of placenta	9	9	4
6	Repeat breeding	28	27	25
7	Anoestrus	177	40	38
8	**Total**	262	108	100

Table 6: Collection of Samples (Serum, Whole blood and Milk) According to Rearing Practice of Animals

S. No.	Rearing practice	Serum	Blood	Milk
1	**Unorganized**	92	32	40
2	**Organized**	170	76	60
3	**Total**	262	108	100

3.5 Serological Assays

In the present study, three serological techniques viz. Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT), and Indirect Enzyme Linked Immunosorbent assay (I-ELISA) were compared with each other for their respective sensitivities and specificities. I-ELISA was performed using commercial kit as per the manufacturer's instructions.

3.5.1 Rose Bengal Plate Agglutination Test (RBPT):

RBPT was performed as per the protocol of Alton et al. (1988) and OIE (2008). 30 µl each of sample serum and antigen adjusted to room temperature (18-25 ºC), were mixed by the disposable stirring stick on a clean grease free slide. The slide was rotated manually for 4 minutes and the result was immediately analyzed for the presence or absence of any degree of agglutination against indirect source of light. Any degree of agglutination within 4 minutes was taken as positive.

Interpretation of result:

The result of RBPT was read as strong positive (immediate visible agglutination), weak positive (visible agglutination after some time) or negative (no agglutination).

Limitations of RBPT:

1) Low sensitivity particularly in long evolution (chronic) cases, and relatively low specificity in endemic areas.
2) Moreover, some authors consider that prozones make strongly positive sera appear as negative in RBPT.

3.5.2 Standard Tube Agglutination Test (STAT):

STAT were performed as per Alton et al (1988), OIE (2008) and Nasir et al. (2004). For STAT, plain heat killed phenolised suspension of *Brucella abortus* strain 99 which show 50% agglutination at 1/500 final dilution of serum with Indian standard was used.

3.5.2.1 Procedure:

a) Serological tubes were arranged in a rack from 1 to 11.

b) 0.8ml carbol saline (0.5%) was taken in the first tube and 0.5 ml in the rest of tubes with the help of pipette.

c) Add 0.2ml of list serum in the first tube mix gently than transfer 0.5ml to the next tubes continue the process up to 9th tube.

d) Transfer 0.5 ml of *Brucella abortus* plain antigen to each tubes to make final dilution to 1:10, 1:20, 1:40, 1:80 and so on. The 10th tube is remaining as control and 11th as discard tube.

e) Shake gently the tubes and incubate at 37^0 C for 48 hours and the result was recorded.

Interpretation:

For bovine if the dilution is 1:40 or 80 IU and above than it is positive.

Limitation of STAT:

This test does not yield result during incubation stage of the disease.

It does not give result just after abortion during chronic stage of disease.

3.5.3 Indirect-Enzyme Linked Immunosorbent Assay (I-ELISA)

The test kit was purchased from National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI) (Formerly PD_ADMAS) Hebbal Bengaluru, Karnataka, India and the contents were stored at 4-8 º C until use. The test was performed as per the manufacturer’s protocol. All the reagents of the kit and serum samples were brought to room temperature. Working dilutions of the reagents were prepared as instructed in information booklet provided with the kit.

Step 1: Coating the Microtitre Plate

Antigen from stock (stored at -20^0 C) is added @40microliter/12ml of coating buffer (10ml is sufficient to coat 1 plate). It is mixed properly and then dispensed @100microliter in to each well of microtitre plate. Tap the side of the plate to ensure that the antigen is evenly distributed over the bottom of each well. The microtitre plate are covered with aluminum foil and kept for incubation overnight at 4^{o}C in refrigerator. After incubation, plates are washed 3 times using 100 µl wash buffer and tap on tissue paper to remove residual wash buffer. Avoid drying of the plate between washes and prior to addition of the next reagent. Blocking each well with100microliter of blocking buffer for 1 hour improves the test efficiency.

Step 2: Addition of Test and Control Sera

Control and test sera should be diluted in blocking buffer separately in perplex plate for which 5 µl of test and control sera are diluted in 500 µl blocking buffer. Serum and blocking buffer is mixed thoroughly 10 times to ensure homogeneity before loading the microtitre plate. The diluted 100 µl test sera samples in duplicate wells (in 2- wells) and to control sera (positive and negative control sera) along with the conjugate samples in quadruplicate wells (in 4- wells) and transferred from the perplex plate to the microtitre plate as per layout and incubate at 37^{o}C for an hour.

Step 3: Addition of the Conjugate

After incubation the plates are taken out and washed three times with washing buffer. The working dilution of the conjugate (Protein – G HRP conjugate) is made by adding 1.5 µl of conjugate to 12ml blocking buffer (1:8000 dilution). Then 100 µl of the working of conjugate is added to each well and incubate at 37^{o}C for an hour.

Step 4: Addition of Substrate/Chromogen

After incubation, the plates are again washed three times with washing buffer. Substrate/chromogen solution is prepared by adding 1 OPD tablet (5 mg) to 12ml distilled water followed by the addition of 50 µl hydrogen peroxide (3%). Chromogen solution @100 µl is added to all the wells of the microtitre plate, incubate at room temperature for 7 minutes or wait until a visible yellow colour develops in the strong positive wells by covering with aluminium foil (In dark).

Step 5: Stopping Solution

After the colour develops, immediately stop the reaction by adding 50 µl of stopping solution to all wells. The plates are read in the ELISA reader at 492 nm immediately. The OD values of the test control in each ELISA test performed should fall within defined upper control limit (UCL) and lower control limit (LCL) for acceptance or rejection of the test. The OD value of UCL for the positive and negative controls should be between 1.2 and 0.2, respectively. Similarly, OD values of the LCL should be of 0.6 and 0.09 for positive and negative controls, respectively.

Step 6: Interpretation of the Results

Percent positivity (PP) values which are used for the diagnostic interpretation are calculated as follows:

$$PP = \frac{\text{Average OD value of test serum}}{\text{Median OD value of positive control sera}} \times 100$$

Any sample that gives PP value between 55% to 65% is considered Moderate Positive and more than 65% is considered Strong Positive. If sample shows a PP value of below 55% is taken as – Negative

3.6 Molecular Detection of *Brucella* by Polymerase Chain Reaction (PCR) Assay

Polymerase chain reaction is rapid, *in vitro* method of enzymatically synthesizing relatively large numbers of copies of DNA molecules from minute quantities of source DNA by using repeated thermal cycle of template denaturation, primer annealing and polymerase extension for amplify a specific sequence of genomic DNA from a minute amount of sample determined by oligonucleotide primers in the presence of a heat stable DNA polymerase such as Taq DNA polymerase that catalyzes the elongation of the primers.

The samples collected for the purpose of molecular diagnosis of Brucellosis using PCR included milk, serum and whole blood samples. A total of 100 milk samples and 108 whole blood and 262 serum samples collected from cattle and buffaloes showing symptoms of reproductive disorders like abortion, repeat breeding, pyometra, metritis, retention of placenta, anoestrous etc were subjected to PCR assay.

3.6.1 Extraction of Genomic DNA from Whole Blood for PCR Assay by Phenol Chloroform Method (Sambrook and Russel, 2001).

Blood lymphocytes are the source of genomic DNA.

3.6.1.1 Reagents

R.B.Cs lysis buffer (1X), DNA extraction buffer, 10% SDS, Proteinase K, Equilibrated phenol (Tris saturated phenol pHmore than 7.8), phenol: chloroform : isoamyl alcohol (25:24:1), chloroform : isoamyl alcohol (24:1), 3M Sodium Acetate (ph 5.2), isopropanol, 70% ethanol, 1X TE Buffer (ph 8.0).

3.6.1.2 Procedure

1) Take out the blood samples stored at 4^{o}C and thaw to make a uniform solution.
2) Filled the tubes with chilled RBCs lysis buffer (1X), mix end to end, and centrifuge at 4000 rpm for 10 minutes at room temperature.
3) Discard the reddish tinged supernatant containing plasma and lysed RBCs by simple inversion of the tubes or by pipetting.
4) Add two volumes of chilled RBCs lysis buffer, tap the tubes ends to disperse the pellet of WBCs or RBCs (if left) and centrifuge at 4000 rpm for 10 minutes.
5) Discard the black tarry coloured supernatant containing lysed RBCs by simple inversion of the tubes or by pipetting.
6) Repeat steps 4-5 till the WBCs pellet appear nearly white in colour.
7) Add 3ml of DNA extraction buffer (@3ml/10ml blood) to the tubes, tap to disperse the WBCs pellet in the extraction buffer and keep in the incubator for half an hour because the buffy coat is completely suspended so that the cells are accessible to SDS and Proteinase K.
8) Add 200 µl of 10% SDS and gently mixed by inverting. The content of the tubes will appear viscous indicating lysis of WBCs. Handle the samples gently from now on to avoid shearing of DNA.
9) Add about 25µl of Proteinase K for 10ml of blood in the tubes and mix gently. Incubate the tubes at 50^{o}C in the water bath overnight.

10) Next morning, transfer the content in to clean, autoclaved polypropylene tubes, add equal amount of Tris Saturated Phenol (pH7.8), mix by inverting gently for 10-15 minutes till a light coffee coloured uniform solution without any balls of phenol forms and centrifuge at 4000 rpm for 10-15 minutes.

11) Transfer the upper aqueous phase containing DNA in to fresh clean, autoclaved polypropylene tubes by mean of a1ml tip whose tip has been cut to widen the bore. Upper aqueous phase is very viscous and care must be taken during transfer of aqueous phase and white interface.

12) Perform similar extraction (as in steps 10-11) once with equal volume of phenol: chloroform: isoamyl alcohol (25 : 24 : 1) and with chloroform : isoamyl alcohol (24 : 1).

13) Add 3M sodium acetate (pH-5.2) (@100 µl / 1ml of aqueous phase) to the final aqueous phase and mix gently.

14) Add two volumes of chilled ethanol / isopropanol (at room temp) in the tubes and mix gently by inversion. Keep it at room temperature to allow precipitation of DNA.

15) Transfer DNA along with 500 µl of chilled ethanol in to fresh eppendorfs by mean of wide bore tips and centrifuge @10,000 rpm for 10 minutes at 4^{o} C.

16) Discard the supernatant by gentle inversion (where DNA pellet is intact), otherwise aspirate (when DNA pellet is loose).

17) Wash the DNA pellet twice in 500 µl of 70% ethanol and centrifuge the eppendorfs@10,000 rpm for 10 minutes at 4^{0} C.

18) Finally air dry the DNA pellet after inverting on blotting paper so that last traces of ethanol are lost.

19) Add approximately 200µl of TE buffer or autoclaved double distilled water and put in water bath at 60^{0} C for 2 hours to inactivate DNAase or other enzymes.

20) Store the eppendorfs at 4^{0} C for a week so that DNA dissolves and subsequently at - 20^{0} C indefinitely.

3.6.2 Genomic DNA Extraction from Serum for PCR Assay by Phenol Chloroform Methods (Sambrook and Russel., 2001).

1) For PCR analysis, briefly 500 µl serum sample take in a 1.8ml eppendorf tube and centrifuge for 15 minutes at 15,000 rpm.
2) The pellet was resuspended in 200µl of nuclease free water and subjected by phenol chloroform method.
3) To remove inhibitory substance, 200µl of sample was treated with SDS and proteinase K with a final concentration of 1 % and 250µg/ml and kept at 56^0 C for 30 minutes in water bath.
4) After taking from water bath, 200µl Tris Saturated Phenol: Chloroform: Isoamyl Alcohol (25:24:1) was add to 200µl of sample in an eppendorf tubes and mix gently.
5) Again centrifuge at 10,000 rpm for 5 min at 4^0 C and supernatant take in other eppendorf tube.
6) Then 1/10 volume of 3M sodium acetate (ph 5.2) and 1ml of chilled ethanol added, mix and kept at -20^0 C for overnight.
7) Next morning the tubes are centrifuge at 12,000 rpm for 15minuntes at 4^0 C.
8) The supernatant was discarded and pellet was washed with 500µl of 70% ethanol, followed by centrifugation at 12,000 rpm for 2 min.
9) The ethanol was discarded and pellet was dried at 37^0 C and resuspended in 20µl of nuclease free water.

3.6.3 Genomic DNA Extraction from Milk for PCR Assay (Pokorska et al., 2016).

Procedures

1. Centrifuge 10 ml of raw milk at 7000g for 10 min (4^0C), remove the milk fat and most of supernatant from above somatic cells and milk protein pellet, and transfer the pellet with the remainder of the supernatant to a 2 ml sterile tube. Centrifuge the mixture at 5000 g for 3 minutes (4^0C) and remove the supernatant.
2. Wash the pellet with 1 ml of buffer (15 mMTris-HCL (pH 7.4-7.6), 25 mMNaCl, 5mMMgCl_2, 15mMNa_2HPO_4, 2.5 EDTA, 1% Sucrose). Centrifuge

this mixture at 5000 g for 3 minutes (4^0C), remove the supernatant, and repeat this step until clear supernatant is obtained.

3. Add 1ml of lysis buffer (ph 8.8; 6% SDS, 3mm $MgCl_2$, 15mm Tris- HCl, 0.5% DMSO, 6% Acetone) to the pellet and incubate this mixture at 65^0C for approximately 20-30 minutes. After this time, the string of DNA clumps will be visible in the liquid.
4. Attach the string of DNA clumps to the wall of new sterile tube by pipette. Then, discard leftover supernatant that has dropped to the bottom and wash DNA twice with 100µl of 70% ethanol. Centrifuge the mixture at 10000 g for 1 minute at room temperature and discard the supernatant.
5. Dissolve the DNA pellet in 50-100 µl of deionized water or TE buffer.

3.6.4. Molecular Identification of *Brucella* spp. By PCR

The DNA extracted from whole blood, milk and serum were subjected to PCR amplification using *Brucella* genus specific bcsp31 primers (table7, 8 and 9) and *B. abortus* species specific PCR (IS711 AB). The PCR was set in a final volume of 25 µl, spinned briefly and set in to thermal cycler (Bio-Rad, USA).

The isolated genomic DNA samples were subjected to agarose gel electrophoresis in ethedium bromide stained agarose gel for assessing their integrity.

Table 7: The Cycling Conditions for *Brucella* genus specific PCR and *B.abortus* species specific PCR

S. No.	Cycling Condition	Temperature	Time
1.	Initial Denaturation	95^0C	3 Minutes
2.	5 cycle of denaturation	95^0C	30 Sec
3.	Anneling	63^0C	45 Sec
4.	Extention	72^0C	45 Sec
5.	Final extension	72^0C	10 Minutes

3.6.4.1. *Brucella* genus specific BCSP31 PCR assay:

The amplification of 223bp region of BCSP 31 genetic element of Brucella was carried out using oligonucleotide primer sequence (Table 8), as per Baily et al. (1992).

Table 8: Oligonucleotide primers for *Brucella* genus-specific PCR (BCSP 31)

Primer	Oligonucleotide sequence	Reference
B4 (Forward)	5’-TGG-CTC-GGT-TGC-CAA-TAT-CAA-3’	Baily et al. (1992)
B5 (Reverse)	5’-CGC-GCT-TGC-CTT-TCA-GGT-CTG-3’	

3.6.4.2. *Brucella* species specific IS711 PCR assay:

The amplification of 498 bp region of IS711 genetic element of *Brucella abortus* was carried out using published oligonucleotide primer sequence (table 9) as per Bricker and Halling (1994) using cycling conditions as mentioned in (Table 7).

Table 9: oligonucleotide primers for *B.abortus* species specific PCR (IS711 AB)

Primers	**Oligonucleotide sequence**	**Reference**
IS711AB (Forward)	5’-TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT-3’	Bricker and Halling (1994)
IS711AB (Reverse)	5’-GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC-3’	

3.6.4.3. Agarose Gel Electrophoresis for PCR Assay

The size of amplicon obtained was confirmed by allowing them to electrophoretically migrate in 1% agarose gel (Agarose, Genei Pvt. Ltd., Banglore) containing ethidium bromide dye.

1. 50 ml of 1% agarose was prepared in 1X TAE buffer and heated it in microwave for 1 minutes on full power.
2. It was allowed to cool for about 45^0 C and 1µl ethidium bromide (stock conc. 5mg/µl) per 10 ml was added giving a final concentration of 0.5µg/ml.
3. Gel was poured in to electrophoresis tank after inserting the comb and was allowed to solidify to form flat surface for 15 minutes.
4. Then electrophoresis tank was filled with 1X TAE buffer containing 0.5 µg/ml ethidium bromide, i.e. 1 µl of 5mg/ml stock to every 10 ml of buffer and comb was removed.
5. The isolated DNA samples (PCR product) were loaded in parallel with molecular weight markers (100 bp DNA ladder, Fermentas, USA).

6. The electrophoresis run was allowed for 45 minutes at 100 volts and gel was viewed under UV- Transilluminator (Genei Pvt. Ltd., Banglore) and the PCR products were confirmed.

3.7. Analysis of data

Analysis of data was done as per standard procedure using methods described by Thrusfield (2008).

Prevalence (%)

$$= \frac{\text{No. of individuals having disease at aparticular point of time}}{\text{No. of individuals in the population at risk at that point of time}} \times 100$$

Age specific prevalence (%)

$$= \frac{\text{No. of individuals having disease in a specified age group at particular time}}{\text{No. of individuals in the specified age group at that point of time}} \times 100$$

Sex specific prevalence (%)

$$= \frac{\text{No.of individuals having disease in a particular sex at a particular point of time}}{\text{No.of individuals in a specified sex groupat that point of time}} \times 100$$

Place specific prevalence (%)

$$= \frac{\text{No.of individuals having disease atspecified place at aparticalar point of time}}{\text{No.of individuals at the specified placeat that point of time}} \times 100$$

Rearing practice Specific Prevalence (%)

$$= \frac{\text{No.disease individuals in a specific rearing practice at particular point of time}}{\text{No.of individuals in the specified rearing practice group at that point of time}} \times 100$$

Health status Specific Prevalence (%)

$$= \frac{\text{No.of diseased individuals in a specific health status at a particular point of time}}{\text{No.of individuals in the specified health status group at that point of time}} \times 100$$

Sensitivity and specificity were calculated by as per the standard method (Thrusfield, 2008)

		Standard Test		**Total**
		Positive	**Negative**	
The test to be compared	**Positive**	**a**	**b**	**a+b**
	Negative	**c**	**d**	**c+d**
	Total	**a+c**	**b+d**	**a+b+c+d = N**

The notation used above are defined as under:

a = Number of samples positive to the diagnostic test as well as the standard test (True positives)

b = Number of samples positive to conventional but negative to the standard test (false positives)

c = Number of samples negative to conventional but positive to standard test (False negatives)

d = Number of samples negative to both conventional and standard test (true negatives)

a + b + c + d = Total number of samples (N)

Definition and formulae of the indices used for comparing the different assays are described as following:

Diagnostic Sensitivity (true positive rate): proportion that a test results will be positive when a disease is present.

$$Sensitivity\ (\%) = \frac{a}{a+c} \times 100$$

Diagnostic Specificity (true negative rate): proportion that a test result will be negative when the disease is not present.

$$Specificity\ (\%) = \frac{d}{b+d} \times 100$$

3.7 Statistical Analysis

3.7.1. Agreement between the test: Kappa statistic

The agreement between the tests was evaluated by applying kappa statistic as explained by Thrusfield (2008). Kappa value ranges from 0 – 1. Arbitrary benchmarks for evaluation observed Kappa values are:

>0.81	:	Almost perfect agreement
0.61 – 0.80	:	Substantial agreement
0.41 – 0.60	:	Moderate agreement
0.21 – 0.40	:	Fair agreement
0 – 0.20	:	Slight agreement
0	:	Poor agreement

3.7.2. Concordance percentage

Concordance percentage was calculating using the formula (Perrin and Sureau, 1987)

$$= \frac{\text{No.of serum positive in two test} + \text{No.of serum negative in two test}}{\text{Total No.of serum sample tested}} \times 100$$

Results

CHAPTER- 4

RESULTS

During present study, out of 470 samples (262 serum, 108 whole blood and 100 milk) were collected from dairy animals (cattle and buffaloes) during the period from October, 2017 to July, 2018. These sera samples were examined by Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT) and Indirect Enzyme Linked Immunosorbent Assay (I-ELISA) to know the presence of antibodies against the *Brucella* antigen. The predisposing factors for the *Brucella* antigens were also visulised from the informations collected through performa. This study was conducted to know the prevalence of brucellosis infection in cattle and buffaloes from the Aligarh, Firozabad, Hathras, Mathura.

4.1 District wise Seroprevalence of Bovine Brucellosis

Out of 262 sera samples tested, 26 (9.92%), 42 (16.03%), 17 (6.48%) samples was found to be positive by RBPT, STAT and i-ELISA, respectively (Table 10). On the basis of I-ELISA district wise seroprevalence was higher in Firozabad (10.41%), followed by Mathura (6.47%), Hathras (4.65%) and Aligarh (3.12%). The results showed that antibodies against *Brucella* were widely present in animals of different areas of U.P.

Table 10: District wise Seroprevalence of Bovine Brucellosis

Name of district	Number of animals tested (serum samples)	RBPT	STAT	I-ELISA
Aligarh	32	1(3.125)	9 (28.125)	1 (3.125)
Firozabad	48	9 (18.75)	7 (14.58)	5 (10.41)
Hathras	43	4 (9.30)	8 (18.60)	2 (4.65)
Mathura	139	12 (8.63)	18 (12.94)	9 (6.47)
Total	262	26 (9.92)	42 (16.03)	17 (6.48)

Value in parenthesis is in percentage

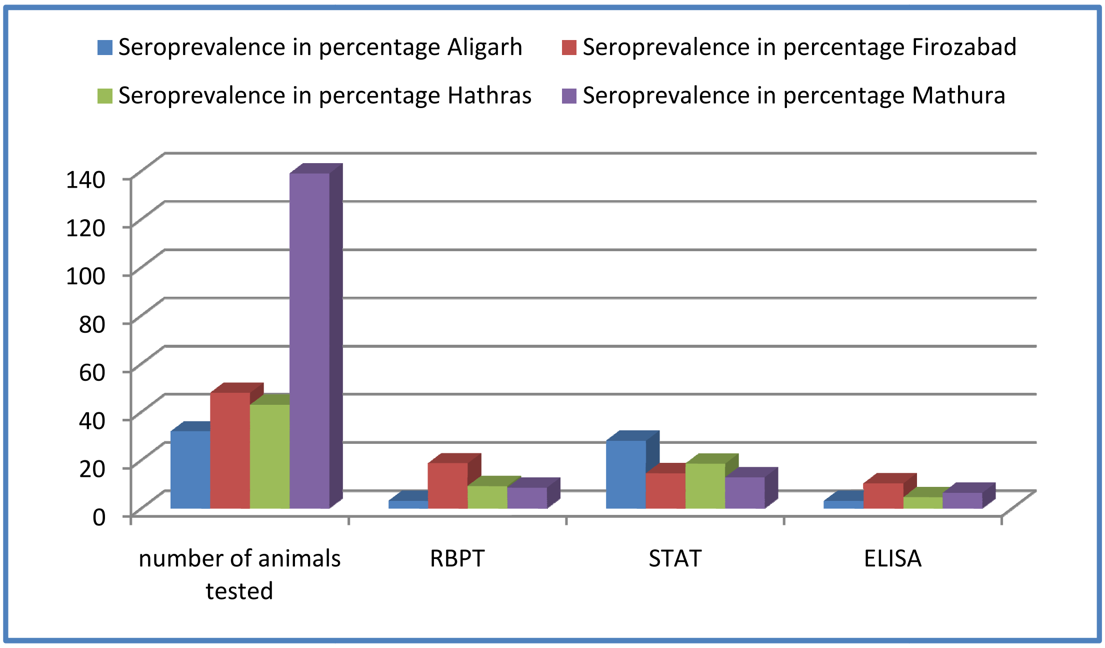


Figure 1: District wise Seroprevalence of Bovine Brucellosis

4.2 Species wise Seroprevalence of Bovine Brucellosis

Species wise seroprevalence analysis showed that distribution of antibodies against *Brucella* antigen varied significantly between cattle and buffaloes (Table 11). The seroprevalence of brucellosis in 203 cattle serum sample tested was found to be 11.82%, 16.25% and 8.37% by RBPT, STAT and I- ELISA, respectively. On the other hand, in 59 buffalo serum samples, the seroprevalence was 3.38%, 15.25% and 0% by RBPT, STAT and I- ELISA, respectively.

Table 11: Species wise Seroprevalence of Bovine Brucellosis

S. No.	Species	Number of animals tested	Number of positive animals by RBPT	Number of positive animals by STAT	Number of positive animals by I-ELISA
1	Cattle	203	24 (11.82)	33 (16.25)	17 (8.37)
2	Buffaloes	59	2 (3.38)	9 (15.25)	00 (0)
3	Total	262	26 (9.92)	42 (16.03)	17 (6.48)

Value in parenthesis is in percentage

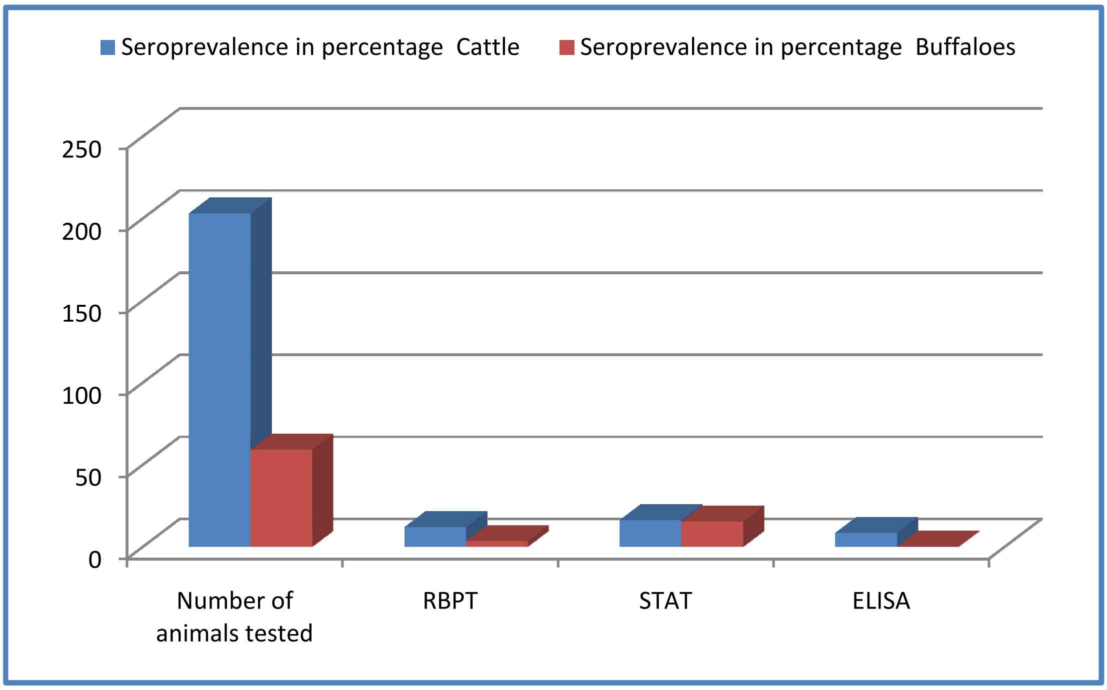


Figure 2: Species wise Seroprevalence of Bovine Brucellosis

4.3 Age wise Seroprevalence of Bovine Brucellosis

Analysis of age wise seroprevalence of bovine brucellosis was done by dividing all 262 total animals in two groups viz. young animals less than 3 years of age (92 animals) and adult animals more than 3 years of age (170 animals). The seroprevalence of brucellosis in young animals was 7.6%, 13.04% and 4.34% by RBPT, STAT and I- ELISA, respectively, while in adult animals the seroprevalence was 11.17%, 17.64% and 7.64% by RBPT, STAT and I-ELISA, respectively (Table 12). Thus the seroprevalence of bovine brucellosis was significantly higher in adult animals as compared to young animals.

Table 12: Age wise Seroprevalence of Bovine Brucellosis

S. No.	Age of animals	Number of animals tested	Number of positive animals by RBPT	Number of positive animals by STAT	Number of positive animals by I-ELISA
1.	Young animals (< 3 years of age)	92	7 (7.6)	12 (13.04)	4 (4.34)
2.	Adult animals (> 3 years of age	170	19 (11.17)	30 (17.64)	13 (7.64)
3.	Total	262	26 (9.92)	42 (16.03)	17 (6.48)

Value in parenthesis is in percentage

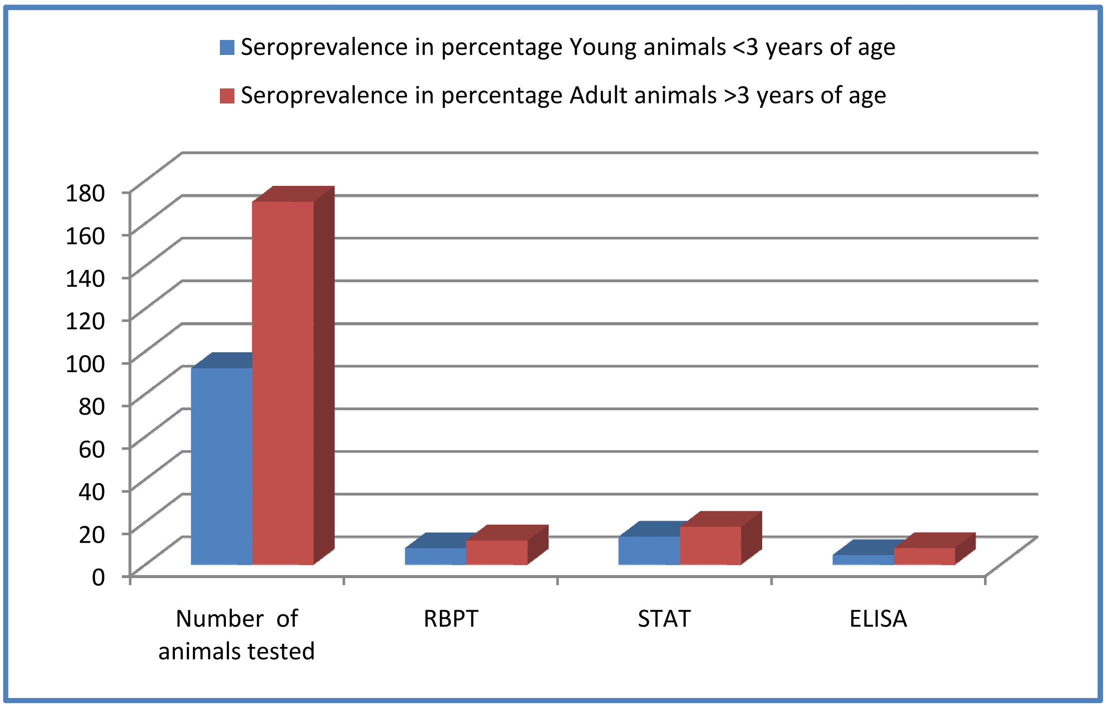


Figure 3: Age wise Seroprevalence of Bovine Brucellosis

4.4 Sex wise Seroprevalence of Bovine Brucellosis

Only 4 serum samples could be obtained from males and none came positive samples thus in females, the disease was considerably higher. Still analysis yielded insignificant results due to small male sample size.

Table 13: Sex wise Seroprevalence of Bovine Brucellosis

S. No.	Sex of animals	Number of animals tested	Number of positive animals by RBPT	Number of positive animals by STAT	Number of positive animals by I-ELISA
1.	Male	4	00	1	00
2.	Female	258	26	41	17
3.	Total	262	26	42	17

4.5 Disease wise Seroprevalence of Bovine Brucellosis On the basis of disease condition the present study estimate the seroprevalence of bovine brucellosis, on the basis of I-ELISA, out of 18 aborted animals, only 4 were positive and out of 12 animals suffering with pyometra, only 2 were found positive. So maximum seropositivity was found in case of stillbirth followed by cases of abortion then repeat breeding (Table 14).

Table 14: Disease wise Seroprevalence of Bovine Brucellosis

S. No.	Disease condition	Number of animals tested	Number of positive animals by RBPT	Number of positive animals by STAT	Number of positive animals by I-ELISA
1	Abortion	18	7 (38.88)	9 (50)	4 (22.22)
2	Pyometra	12	5 (41.66)	7 (58.33)	2 (16.66)
3	Metritis	8	00	1 (12.5)	00
4	Stillbirth	10	2 (20)	4 (40)	3 (30)
5	ROP	9	00	1 (11.11)	1 (11.11)
6	Repeat breeding	28	10 (35.71)	13 (46.42)	6 (21.42)
7	Apparently healthy (anoestrus)	177	2 (1.12)	7 (3.59)	1 (0.56)
8	Total	262	26 (9.92)	42 (16.03)	17 (6.48)

Value in parenthesis is in percentage

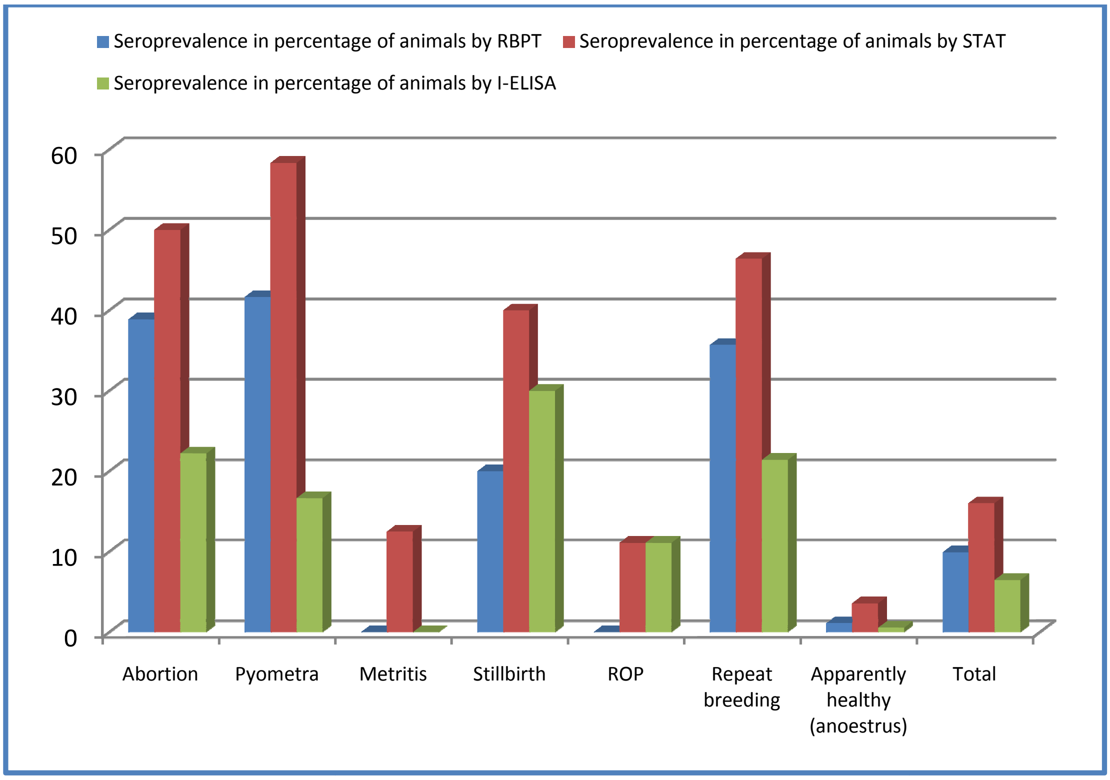


Figure 4: Disease wise Seroprevalence of Bovine Brucellosis

Table 15: Seroprevalence of Bovine Brucellosis According to Animal Rearing Practice

S. No.	Type of animals rearing	Number of animals tested (serum samples)	Number of positive animals by RBPT	Number of positive animals by STAT	Number of positive animals by I-ELISA
1.	Unorganized rearing	92	8 (8.68)	5 (5.43)	6 (6.52)
2.	Organized herd	170	18 (10.58)	37 (21.76)	11 (6.47)
3.	Total	262	26 (9.92)	42 (16.03)	17 (6.48)

Value in parenthesis is in percentage

4.6 Seroprevalence of Bovine Brucellosis According to Animal Rearing Practice

To find out the seroprevalence of bovine brucellosis on the basis of animals rearing practice, total 262 serum samples were classified in two groups, unorganized (92 serum samples) and (170 serum samples) organized herd. The seroprevalence of bovine brucellosis in organized herd was 10.58%, 21.76%, 6.47% on the basis of RBPT, STAT and IELISA, respectively. Whereas in unorganized herd, the seroprevalence was found to be 8.68%, 5.43%, 6.52% by RBPT, STAT and ELISA respectively (Table 15). Thus organized rearing practice showed more prevalence of bovine brucellosis than unorganized rearing practice.

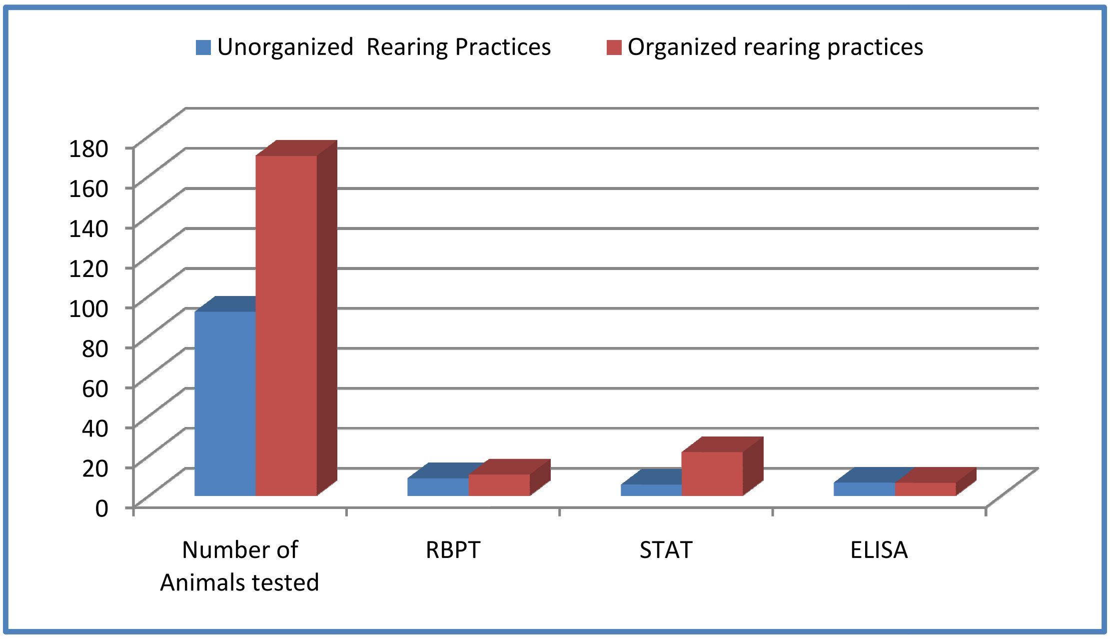


Figure 5: Seroprevalence of bovine brucellosis according to animals rearing practice.

4.7 Molecular detection of *Brucella* spps by PCR

Extraction of DNA was done from three types of samples viz., serum, whole blood and milk, obtained from different animals. Quantification of DNA extracted was done spectrophotometrically at 260 and 280 nm using nanodrop spectro-photometer for determination of sample concentration and purity. The isolated genomic DNA samples were subjected to agarose gel electrophoresis in ethidium bromide stained agarose gel for assessing their integrity (Brown, 2007).

Brucella genus specific BCSP31 PCR Assay: Using genus specific primer pair BCSP31 B4/B5, an amplification product of 223bp was obtained in total 5 samples tested (3 whole blood samples and 2 milk samples) confirming their identity as members of genus *Brucella*. In IS 711 AB PCR, all the 5 isolates positive for genus specific bcsp31 gene exhibited B. abortus specific amplification product of 498bp.

So the percentage positivity of *Brucella abortus* by PCR was found to be 2.8% and 2% from whole blood and milk samples respectively. (Table 16) Thus, among samples processed for PCR, whole blood was found to be better than milk and serum. A combination of RBPT, STAT and I-ELISA is found to be the most suitable combination of serological tests and in cases where disease is not frank or serological cross reactions are confounding the results, molecular confirmation by PCR assay using whole blood might be suitable for diagnosis of bovine brucellosis.

Table 16: Comparison of PCR assay from Serum, Whole Blood and Milk samples

S. No.	Type of samples	Number of samples	Number of positive samples by PCR
1.	Serum	100	0
2.	Whole Blood	108	3 (2.8)
3.	Milk	100	2 (2)
	Total	308	5 (1.62)

Value in parenthesis is in percentage

4.8 Relative sensitivity (Rse) and Relative specificity (Rsp) of RBPT and STAT compared to I-ELISA:

The I-ELISA was taken as a standard test and relative sensitivity and relative specificity of the RBPT and STAT were calculated. The relative sensitivity of RBPT and I-ELISA was higher (75%) as compared to STAT (64.7%). On the other hand the relative specificity of RBPT and I-ELISA was also higher (94.71%) as compared to STAT and I-ELISA (87.34%). (Table 17)

Table 17: Relative sensitivity (Rse) and Relative specificity (Rsp) of RBPT and STAT compared to I-ELISA

S. No.	Test	Relative sensitivity (Rse)	Relative specificity (Rsp)
1.	RBPT and ELISA	75%	94.71%
2.	STAT and ELISA	64.7%	87.34%

4.9 Concordance Percentage between Test applied for Diagnosis of Bovine Brucellosis:

The concordance percentage between of RBPT and STAT were calculated on taking I-ELISA as standard test. The concordance between RBPT and I-ELISA was more (91.98%) as compared to STAT and I-ELISA (85.87%) (Table 18).

Table 18: Concordance percentage between RBPT and STAT on taking I-ELISA as standard test

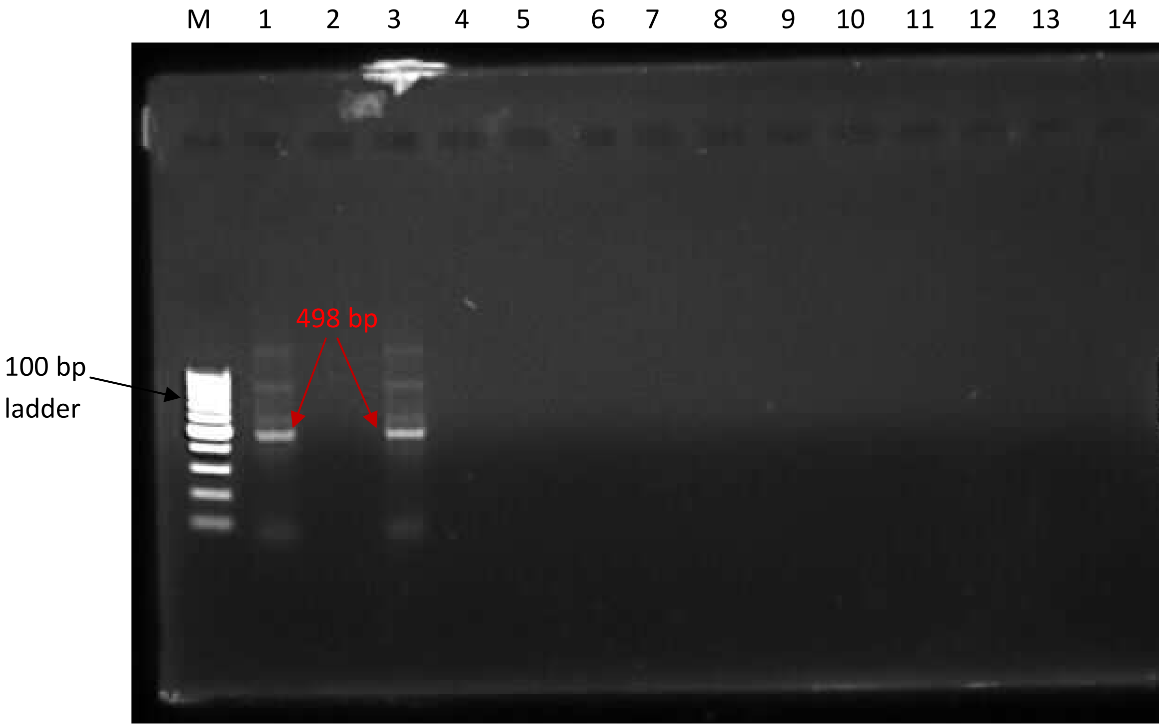
S. No.	Test	Concordance percentage
1.	RBPT and ELISA	91.98%
2.	STAT and ELISA	85.87%

4.10 Kappa values for different combination of tests applied for diagnosis of Bovine Brucellosis:

RBPT, STAT and I-ELISA were compared against each other by applying kappa statistic. According to kappa statistic moderate agreement (0.5547) was found between RBPT and I-ELISA while STAT and I-ELISA show fair agreement (0.3061) (Table 19).

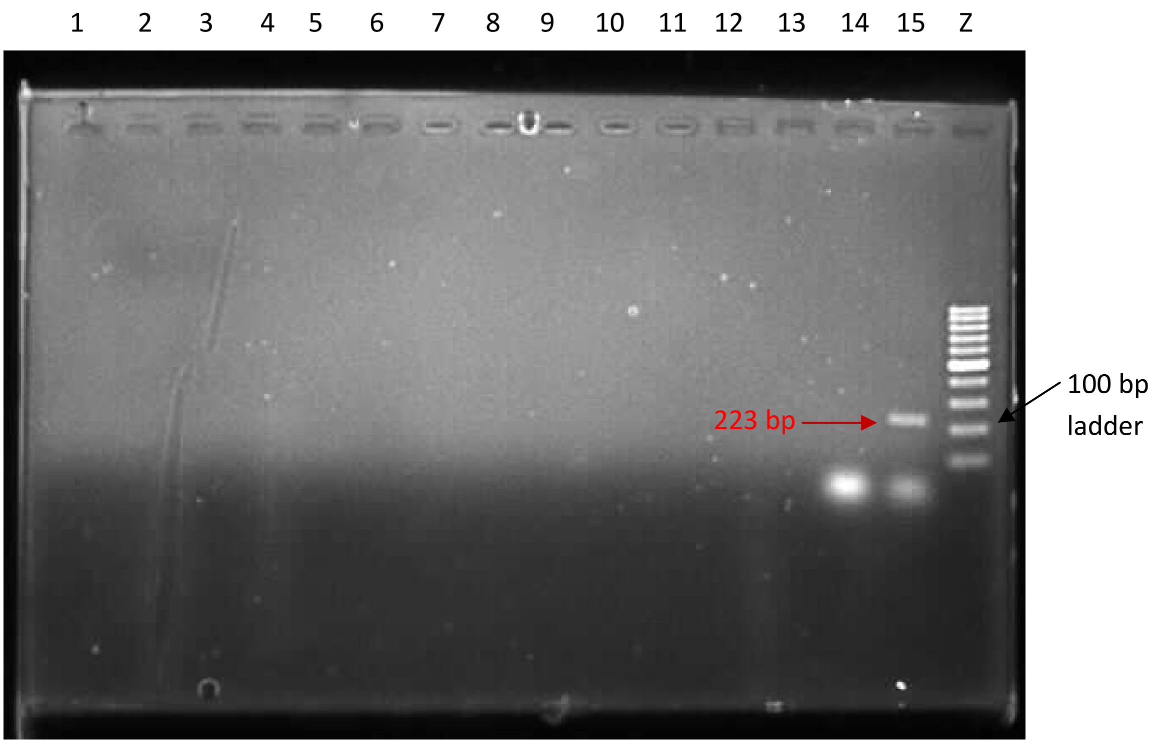
Table 19: Agreement between RBPT, STAT and I-ELISA by applying Kappa statistic

S. No.	Combination of test	Kappa value	Comment
1.	RBPT and ELISA	0.5547	Moderate agreement
2.	STAT and ELISA	0.3061	Fair agreement



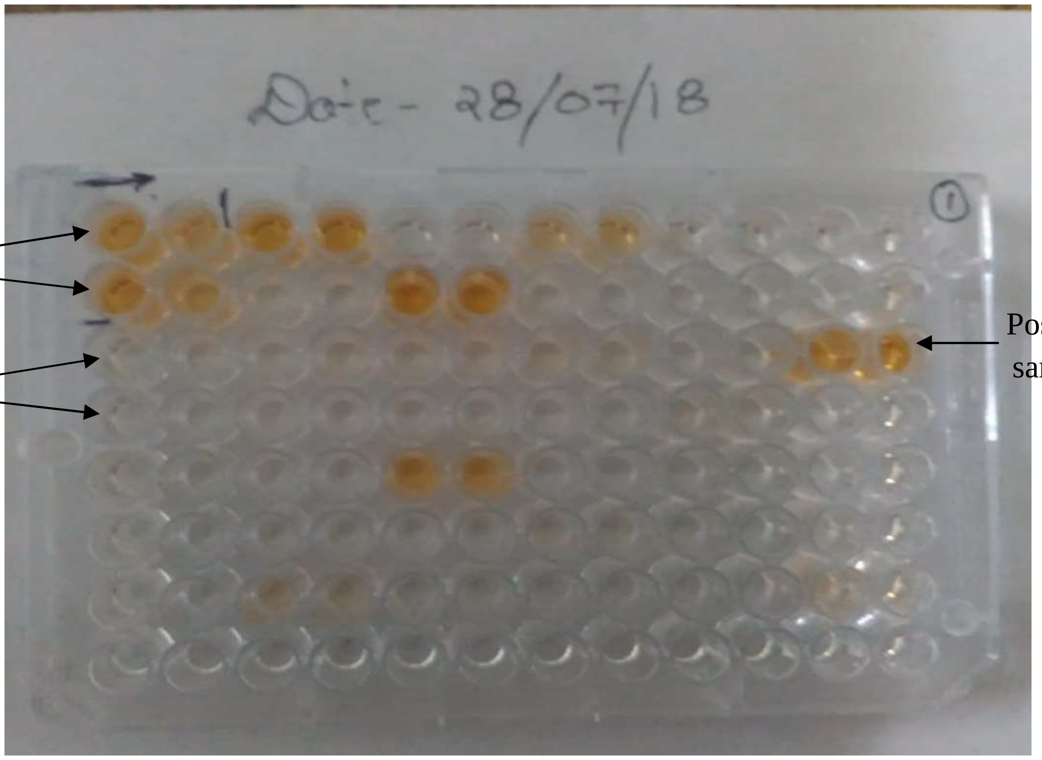
Photograph 1: Species specific detection of *B.abortus* based on IS711/AB PCR Assay (498bp).

Lane M- 100 bp DNA ladder, Lane-1 and 3 positive samples



Photograph 2: *Brucella* genus specific PCR assay BCSP31 (223bp)

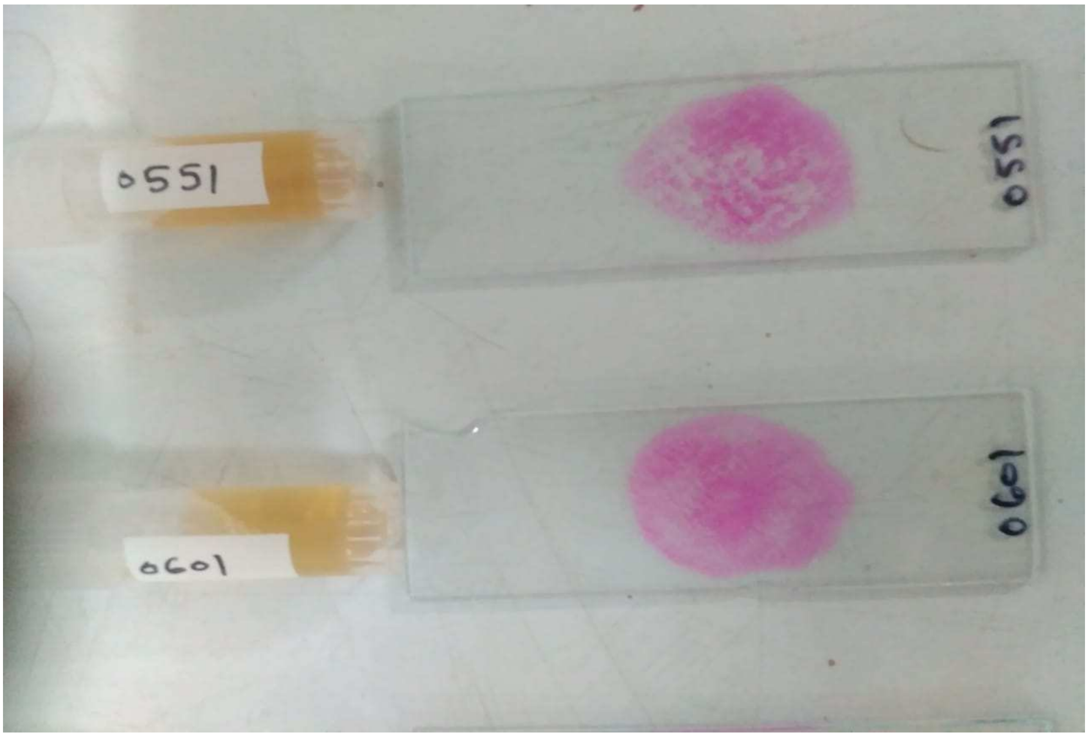
Lane Z- 100 bp DNA ladder, Lane-14 and 15 positive samples



Photograph 3: ELISA plate showing positive and negative controls and positive sample

Photograph 4: Protein-G based indirect ELISA kit for Bovine Brucellosis

Photograph 5: Showing Standard Tube Agglutination Test



Photograph 6: Positive case showing Rose Bengal Plate Test

Discussion

CHAPTER-5

DISCUSSION

Brucellosis is the most common zoonosis reported worldwide and is responsible for considerable economic losses due to abortion and culling of infected animals. About 90 percent of global Brucellosis occurs in resource –limited countries or tropical climatic regions of the world where livestock provides major mean of livelihood. Brucellosis causes huge economic losses to the farmer through birth of weak calves, stillbirths, prolonged inter-calving intervals, infertility in male animals and interference in the breeding programme of cows that aborted (McDermott, Grace & Zinsstag 2013; Singh, Dhand & Gill 2015; Tasaime et al. 2016). Disease is a serious occupational hazard in humans with farm workers, veterinarians, paravets, abattoir workers and alb workers being at maximum risk.

First recorded in India in 1887 (IVRI, 1977), brucellosis has now become endemic throughout the country with prevalence ranging from 6.5% to 16.4% in various livestock species (Aulakh et al., 2008; Kollannur et al., 2007; Lone et al., 2013; Shome et al., 2006; Thoppil, 2000). It is a serious concern to dairy industry of India. The major factors responsible for such unprecedented spread are absence of a control policy, failure to vaccinate young female calves, non implementation of test and slaughter, ban on cow slaughter in many regions of the country, absence of treatment regimen and usual practice of selling positive reactor animals to other farmers.

In the present study, the prevalence of bovine brucellosis in cattle and buffaloes was determined by using Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT), Indirect Enzyme Linked Immunosorbent Assay (I-ELISA) and PCR assay. A total of 262 serum samples, 100 milk samples and 108 whole blood samples from cattle and buffaloes were collected and processed to assess the prevalence of bovine brucellosis in four districts of western UP in India.

In the study, an overall seroprevalence of bovine brucellosis in the western Uttar Pradesh including the Aligarh, Firozabad, Hathras and Mathura district was revealed to be 9.92%, 16.03% and 6.48% using RBPT, STAT, I-ELISA respectively. Among the studies done in India, the results obtained similar to our study were 12.9%

in Punjab (Dhand et al., 2005), 8.98 to 44% in Gujarat state (Chauhan et al., 2000), 12.37% by iELISA in western UP (Kumar et al., 2016) and 18.26% in Gujarat by Patel et al. (2015) although slightly lesser seropositivity of 6.3% was found in M.P. by Mehra et al. (2000). A large study conducted in 23 states of India with 30,437 cattle and buffalo samples found an overall sero-prevalence of 2% (Isloor et al., 1998) which is in accordance with the findings of this study.

Other studies by Krishnamoorthy et al. (2015) and Tragandia et al. (2015) which estimated the prevalence to be 10.20% by RBPT and 11.63% by I-ELISA in southern India and that in Andhra Pradesh, Gujrat and Odisha to be 6, 8.2 and 2.3 % respectively supported the findings of this study.

Previous studies have suggested the seroprevalence of brucellosis to be 6.5% in Jordan (Al-Majali et al., 2009), 8.4% in Cameroon (Bayami et al., 2009) and 32.9% to 39.4% in turkey (Sahin et al., 2008), and 15.4% in Soroti district Uganda (Egaru et al. 2013).

An overall seroprevalence of 30.1% by RBPT in Harare district of Zimbambwe (Vhoko et al., 2018) was quite higher than the results obtained in the present study whereas lesser seroprevalence of 4.9% was estimated by Adugna et al. (2013) in Western Ethiopia using RBPT.

Almost similar seroprevalence was obtained by Jergefa et al. (2009) who got an overall seroprevalence at the individual animal level to be 2.9% in central Oromiya, Ethiopia.

A high prevalence of bovine brucellosis in Punjab state was estimated by Aulakh et al. (2008) at 18.26% while present study estimates the overall seroprevalence to be 6.48% on the basis of I- ELISA. In a study by Aher et al. (2011) in Maharashtra also, overall seroprevalence of 56.75%, 54.05% and 47.29% was found by iELISA, RBPT and STAT which was much higher than the results obtained in this study. Similarly very high seropositivity of 45.8% and 41.55% by iELISA was found by Jagapur et al. (2013) and Pathak et al. (2016), respectively, in their studies.

This study showed higher seroprevalence in cattle than buffaloes. Similar results have been shown by various other authors (Aulakh et al., 2008; Kumar et al., 2016; Patel et al., 2015) whereas results opposite to that of this study were obtained by other workers like (Jagapura et al, 2013; Krishnamoorthy et al., 2015) who found higher seropositivity of brucellosis in buffaloes rather than cattle. Buffalo might show some natural resistance to brucellosis causing lesser seropositivity in them (Fosgate et al., 2011). Another epidemiological study found 5% sero-prevalence in cattle and 3% in buffalo in India (Renukaradhaya et al., 2002)

The prevalence estimated in organized herds of Aligarh, Firozabad, Hathras and Mathura region in the present study was higher as compared to prevalence in the unorganized herds. The disease is supposed to be more prevalent in areas of intensive animal husbandry practices such as organized farms (PD-ADMAS, 2011). It might have been due to break in chain of disease spread among discreet populations. Also organized herds usually adopt artificial insemination over natural service and brucellosis spreads very rapidly through AI if the semen is contaminated. Also exposure to contaminated material and horizontal transmission of disease in organized farms because of poor management practices and overcrowding increases the propensity of spread of disease. Close contact between animals, high animal density, poor hygienic practices like improper disposal of aborted fetuses and fetal membranes and vaginal secretions, which ultimately help in rapid spread of infection from one animal to another in organized farms might be the key reasons for such a finding (Manish et al., 2013). If an infected animal remain for a longer time on a farm then chance of more positive animals on that farms increases with time (Radostits et al., 2000). Various other authors have also reported similar findings (Shome et al., 2014; Jagapur et al., 2013; Kumar et al., 2016; Kachhawaha et al., 2005; Khan et al., 2016). Mufinda et al. (2015) also estimated the seroprevalence of bovine brucellosis at individual level to be 14.96% and at herd level to be 40.16%; Isloor et al. (1998) also showed the prevalence to be more in organised herd (4.1%) than cattle owened by individually. Higher seropositivity was also reported in organized farms from Punjab region of Pakistan (Nasir et al., 2004).

The present study revealed the agewise seroprevalence in case of young animals (92/262) to be 7.76%, 13.04% and 4.34% by RBPT, STAT and I-ELISA while adult animals (170/262) showed seroprevalence 11.17%, 17.64% and 7.64%. Thus higher seroprevalence was found in adult animals as compared to young animals. The finding of the present study were in concordance with the studies conducted by previous workers (Egaru et al., 2013; Khan et al., 2016; Kushwah et al., 2016’ Khajuria et al., 2014). Study by Kumar et al., (2016) showed that young claves had significantly higher seropositivity (10.38%) and it further increased in sexually matured adults (12.71%). Gogoi et al. (2017) found the highest prevalence in the age group 3-7 years. Lower seroprevalence of brucellosis in young animals could be attributed to resistance of sexually immature cattle to infection, or to less time of risk of exposure. Increased susceptibility to clinical disease with age, could be more associated with sexual maturity due to the effects of sex hormone and placenta erythritol on the pathogenesis of brucellosis (Asmare et al., 2013).

On the basis of disease condition, in the present study, maximum seropositivity was associated with still birth (30%) followed by abortion (22.22%), repeat breeding (21.42%) and retention of placenta (11.11%). No correlation was found between occurrence of brucellosis and anoestrus in apparently healthy animals. A significant association between *Brucella* infection band risk markers, such as abortion, retention of placenta and repeat breeding is reported by some researchers. Aulakh et al. (2008) found significant association between brucellosis and abortion and retention of placenta, but not between brucellosis and repeat breeding. Mugizi et al. (2015) and Asmare et al. (2013) found no significant association between *Brucella* seropositivity with abortion and retention of placenta. Similar results have also been found by other workers like Ndazigaruye et al. (2018). Gogoi et al. (2017) correlated abortion to be the main risk factor associated with the disease (64.24%) followed by retention of placenta (47.13%). Abortion as a significant risk factor for brucellosis has also been put forth by workers like Patel et al., (2015) who showed the higher prevalence of bovine brucellosis in case of abortion (45.50%) and Krishnamoorthy et al. (2015) who reported a seropositivity of 45-50% in animals with history of abortion. *Brucella's* primary source in dairy animals is uterine fluid placenta or aborted faetus expelled by infected cattle during parturition. Although not all abortion are due to brucellosis, abortion is a major clinical sign of the brucellosis in cattle and buffaloes and therefore disease should be suspected whenever these signs are observed (Godfroid et al., 2010).

For the diagnosis of bovine brucellosis, serological methods like RBPT, STAT and I-ELISA etc are used quite rampantly. RBPT has been used as an individual screening test for bovine brucellosis but has its limitations. There might be chances of false positive reactions which arise due to fibrin clots or due to cross reactivity of antibodies produced in animals due to other microbe that share epitope with *Brucella* species. The acid pH further diminishes agglutination by IgM but encourages agglutination by IgG_1, thereby reducing cross reactions (Corbel, 1972).

Achievement of an infallible diagnosis of brucellosis is a tedious process, because isolation of the etiological agent is influenced by numbers of factors viz. highly fastidious growth requirement, lower number of viable organism in the sample and delay in sample transportation to the laboratory and due to zoonotic nature of disease it causes potential health hazard for laboratory workers. This requires BSL 3 facilities which are not easily available. Thus molecular detection is a tool which can be used very reliably in absence of ability to isolate the organism.

And for comparison of serological and molecular tests, serum (100 samples), whole blood (108 samples) and milk (100 samples) were subjected to PCR assay and amplicons of 223bp of *Brucella* bcsp 31 gene (genus specific) (Baily et al., 1992) and 498bp of *Brucella* species specific IS711 AB gene (Bricker and Halling, 1994) were obtained. Out of 100 serum samples no positive sample was detected by PCR while from 108 whole blood samples, 3 were found positive and from 100 milk samples, 2 were found positive by PCR. The wide variation in the number of samples detected as positive by RBPT (26), STAT (42), ELISA (17) and PCR (5) might be due to many factors. None of the serum samples yielded DNA which implied that *Brucella* organism was not present in the serum of those animals even though the antibody titre was quite high leading to positive results in serology (Singh et al., 2010). It has been reported that PCR could detect 5fg of DNA (Kaushik et al., 2006). Various PCR procedures have been developed for the detection of Brucella (Zamain et al., 2015; Probert et al., 2004; Tanmay, 2007). *B.abortus* remains intracellularly and this poses a problem for selection of a suitable sample (Wattam et al., 2009). Only during acute phase of infection, it circulates in blood, mostly inside the white blood cells and hides itself in mammary organs, genital organs and lymph nodes (Morgan and Mackinnon, 1979). Hence sample should be selected according to the phase of Brucella's life pattern which is not practically possible to find out. In the present study, comparison was made based on the ease of DNA extraction from serum, whole blood and milk and whole blood was found to be the better sample for DNA extraction to perform PCR assay as DNA might have been in very low or negligible quantity in serum and in milk, presence of fat globules and other proteins etc might have inhibited the DNA yield. Karthik et al. (2014) also performed bcsp 31 gene based PCR and species specific IS 711 gene based PCR using whole blood samples to detect 15.13% positivity. Their results indicated that whole blood can be used for studying the molecular epidemiology of *B.abortus* in bovine and particularly detecting the active phase of infection. Further, at the time of equilibrium of host parasite interaction, the Brucellae may persist in circulation for some time before getting localized in their preferred sites. Similar results that DNA can be extracted from whole blood and used as a sample for screening for brucellosis has been reported by Guarino et al. (2000) in buffaloes, Keid et al. (2010) in dogs and Khamesipour et al. (2013) in cattle and sheep. Al Nakkas et al. (2002) and Leat-Klevezas et al. (2000) used buffy coat instead of whole blood for DNA extraction as macrophages take up brucellae but buffy coat needs additional steps (Mitka et al., 2007) and hence the use of whole blood as such was tried in this study and it showed better results. Use of commercially available kits have been said to have improved the quality as well as the quantity of extracted DNA (Queipo-Ortuno et al., 2008; Keid et al., 2010) as compared to conventional DNA extraction.

Alamian et al. (2017) described a noval PCR assay for detecting *Brucella abortus*. Daugaliyeva et al. (2016) developed a differential PCR assay for detection of *Brucella abortus*. Orizil et al. (2016) conducted a multiplex PCR technique for detection of brucella spp. Hemande and Gandge (2016) showed that PCR-SSCP is more sensitive than PCR-RFLP for detection of polymorphism in BCSP31 gene. Arasoglu et al. (2013) detected Brucella genus specific BCSP31 PCR from tested milk samples. In a study by Rekha et al. (2013), none of the samples of blood, milk and serum were positive for brucellosis. Various factors like time of sample collection, infection status of the animal, condition of farm, number of samples collected can influence the results.

The present study adds to the prevalence data available regarding to *B.abortus* infection in bovine population and highlights the usefulness and advantage if using molecular tools as PCR over serological tests. The results of this study provided serological as well as molecular evidence of widespread endemic presence of brucellosis in dairy animals in western UP India. Present study also indicates that species, age, rearing system and disease condition were significant risk factors in the epidemiology of bovine brucellosis in western Uttar Pradesh, India. Presence of brucella antibodies in non vaccinated animals is always suggestive of infection. The seropositivity in organized farms is at alarming level. Brucellosis being a zoonotic disease poses continuous hazard to animal handlers/farmers exposed to the infected animals of acquiring the infection. Thus brucellosis is not only the cause of reproductive and production losses but also may be the potential biohazard in this region. A combination of RBPT and I ELISA can be successfully used for screening brucellosis when prevalence is low. However, molecular approach to identify *Brucella* at species level is advantageous over serological or other conventional approaches like bacteriology which require long duration for isolation of the organism, sophisticated laboratory facilities etc. Serological tests may be plagued with extensive cross reactions and hence false positive reactions leading to over estimation of the spread of the disease. The BCSP31 B4/B5 and IS*711* A/BPCR is very specific, rapid, safe and confirmatory diagnostic tool for detection of brucellosis where whole blood can be used as a sample of choice. Further use of PCR-RFLP will be a valuable tool for epidemiological and evolutionary studies of *Brucella.*

Summary and Conclusions

CHAPTER-5

SUMMARY AND CONCLUSIONS

Brucellosis is one of the most important contagious and communicable bacterial diseases, of cattle and buffaloes caused by *Brucella abortus*. It is worldwide in distribution and is endemic in most parts of world especially the developing countries. It is considered a remerging though neglected zoonosis with high rates of morbidity and lifetime sterility. The incidence of brucellosis cases is increasing over the recent years especially in developing countries due to poor management, limited resources and increased trade and frequent movement of livestock.

Brucellae are gram negative, facultative intracellular, nonmotile, non spore forming coccobacilli, requiring an optimum temperature of at 37^{o} C to produce visible colonies after 24 hrs, and may require supplementary CO_2 for their growth especially on primary isolation. Transmission mainly occurs through the skin, conjunctiva or respiratory mucosa by inhalation and most commonly through oro-faecal route in cattle. The disease is characterized by abortion storms in the animal herds leading to retension of placenta and infertility. The disease is also an important public health threat and causes chronic debilitating sickness in humans if not treated efficiently and in time. Currently three major approaches are being employed including microbiological, serological and molecular techniques. The absence of a perfect rapid reference diagnostic test makes evaluation of serological tests difficult. Although isolation is considered as gold standard but in the case of brucellosis, it is seldom successful. Apart from serological tests, molecular approaches have been explored to overcome these problems. Various studies have been conducted in India to establish the prevalence of the disease in bovines but to assess the change in the seroepidemological situation of the disease after the initiation of bovine brucellosis control programme, more studies are needed. Therefore present study was designed to assess the epidemiology of the disease in bovines suffering with various reproductive disorders in and around Mathura region, contribution of *Brucella* to those reproductive disorders, comparative efficacy and diagnostic performance of existing serological and molecular tests with the following objectives:

- To detect the prevalence of brucellosis in bovines suffering with reproductive disorders using various serological and molecular tools.
- To study the relationship between brucellosis and associated risk factors in dairy animals with reproductive disorders.
- To compare the diagnostic performance of different serological and molecular tests employed for diagnosis of brucellosis in bovines.
- To compare the efficacy of using whole blood, milk and serum as specimens for PCR assay for diagnosis of Brucellosis.

In this cross sectional study a total of 470 samples collected from cattle and buffaloes of different districts viz Aligarh, Firozabad, Hathras and Mathura were screened for prevalence of bovine brucellosis using Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT) and Indirect Enzyme Linked Immunosorbent Assay (I-ELISA) and PCR.

An overall seroprevalence of brucellosis in cattle and buffaloes was calculated to be 9.92%, 16.03% and 6.48% by RBPT, STAT and I-ELISA respectively. Analysis of seroprevalence of brucellosis with respect to district, age, species, rearing practice and diseased condition with reproductive problems of dairy animals was determined and this study estimate that these epidemiological factors significantly affects the seroprevalence of brucellosis.

District wise seroprevalence of brucellosis in cattle and buffaloes on the basis of I-ELISA estimate that brucellosis was widely prevalent in animals of different district viz. Aligarh, Firozabad, Hathras and Mathura and seroprevalence was 3.12%, 10.41%, 4.65% and 6.47% respectively, using I-ELISA.

The seroprevalence of brucellosis was observed to be higher in sexually mature adult animals more than 3 years of age (7.64% using I-ELISA) than in young animals (4.34% using I-ELISA) as sexually mature adult animals are continuously exposed to the infectious.

Species wise seroprevalence was higher in cattle (11.82%, 16.25% and 8.37% using RBPT, STAT and I-ELISA respectively) than buffaloes (3.38%, 15.25% and 0% using RBPT, STAT and I-ELISA respectively).

On the basis of animals rearing practices the seroprevalence was higher in organised herd (10.58% and 21.76% using RBPT and STAT) than unorganised herd (8.86% and 5.43% using RBPT and STAT respectively) but seroprevalence was nearly similar by using I-ELISA (6.47%) in organised herd and (6.52%) in unorganised herd.

The seroprevalence of bovine brucellosis was maximum in cases of stillbirth (30%) followed by abortions (23.52%), repeat breeding (21.42%), pyometra (16.66%), retension of placenta (11.11%) by I-ELISA. There was almost no positive samples among animals suffering with metritis or anoestrus.

All the serological test RBPT, STAT and I-ELISA used for diagnosis of bovine brucellosis were compared for sensitivity and specificity. The relative sensitivity and specificity of RBPT (75% and 94.71%) respectively was much more than STAT (64.70% and 87.34%) respectively by taking I-ELISA as the standard test. RBPT and STAT and I-ELISA were compared against each other by applying Kappa statistics and concordance percentage. According to kappa statistics, moderate agreement (0.5547) was present between RBPT and I-ELISA while STAT and I-ELISA showed fair agreement (0.3061). Similarly concordance between RBPT and I-ELISA was more (91.98%) as compared to STAT and I-ELISA (85.87%).

Among the suitability of different samples viz. whole blood, serum and milk for PCR assay, whole blood was found to be better than the other two. As the percent positivity by whole blood was found to be 2.8% in comparison to that of milk (2%) and none by serum. So combination of RBPT, STAT and I-ELISA and confirmation by PCR assay using whole blood was found to be the most suitable combination for the confirmatory diagnosis of bovine brucellosis in absence of isolation of the organism.

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Annexure -I

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U. P. Pt. Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura-281001

Proforma for collection of specimens

Location (farms/gaushala/others): ....

Animal (cattle/buffalo): ....

Breed (indigenous /exotic/cross breed) : ....

Breed: ....

Age: ....

Sex(male/female) ....

Mating (natural/A.I.):..

Season :..

Healthstatus(vaccinated/nonvaccinated)..

Date of collection: ....

Samples Collected:

1. Blood: ....
2. Milk : ....

2. Other (if any): ....

Rearing type(organised/unorganised): ....

Calving status: ....

CV OF STUDENT

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Degree	University/ Board	Year of Passing	Percentage of marks	Subjects
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10.06.19

Date: **(Signature)**

Place:

UNDERTAKING OF COPY RIGHT

I **Krishna Govind Bohrey,** Enrolment No **V-1648/16** undertake that I give copy right to the DUVASU, Mathura of my thesis entitled **"Sero-epidemiology and Comparison of a PCR Assay in Whole Blood, Milk and Serum specimens for Brucellosis Diagnosis in Bovines with Reproductive Disorders"**

I also undertake that patent, if any, arising out of research work conducted during the programme shall be filed by me only with due permission of the competent authority of DUVASU, Mathura (UP).

10.06.19

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