DEVELOPMENT AND EVALUATION OF MODIFIED SEROLOGICAL AND MOLECULAR DIAGNOSTIC TECHNIQUES IN SEROPREVALENCE STUDIES OF BOVINE BRUCELLOSIS

Thesis

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy
(Veterinary Bacteriology)

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Dedicated to...

My Beloved Father

Late Shree. Venkaraddi B. Jagapur

(20/12/1921 - 23/12/2007)

भारतीय पशु चिकित्सा अनुसंधान संस्थान
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Dated: 12-07-2013

Certificate

This is to be certified that the research work embodied in this thesis entitled **"Development and evaluation of modified serological and molecular diagnostic techniques in seroprevalence studies of bovine brucellosis"** *submitted by* **Dr. Ramesh V. Jagapur, Roll No. 1350,** *for the award of* **Doctor of Philosophy** *Degree in* **Veterinary Bacteriology** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

It is further certified that **Dr. Ramesh V. Jagapur, Roll No. 1350,** *has worked for more than 30 months in the Institute and has put in more than 300 days attendance under me from the date of registration for the Doctor of Philosophy Degree in this Deemed University, as required under the relevant ordinance.*

(RAJESH RATHORE)
Chairman
Advisory Committee

Certificate

We the undersigned members of Advisory Committee of **Dr. Ramesh V. Jagapur, Roll No. 1350,** *a candidate for the degree of* **Doctor of Philosophy** *with the major discipline* **Veterinary Bacteriology,** *agree that the thesis entitled* **"Development and evaluation of modified serological and molecular diagnostic techniques in seroprevalence studies of bovine brucellosis"** *may be submitted in partial fulfillment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Doctor of Philosophy** *Degree of this Institute.*

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Doctor of Philosophy** *Degree of the Deemed University, Indian Veterinary Research Institute, Izatnagar.*

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Place: IVRI, Izatnagar

(Ramesh V. Jagapur)

ABBREVIATIONS

%	-	percent
~	-	about
α	-	alpha
β	-	beta
µg	-	microgram
µl	-	microlitre
µM	-	micromolar
@	-	at the rate of
A260	-	absorbance at 260 nm
A280	-	absorbance at 280 nm
Ab	-	antibody
APS	-	Ammonium persulphate
bp	-	base pairs
CBB	-	Coomassie brilliant blue
DNA	-	Deoxyribonucleic acid
DNAse	-	Deoxyribonucleic neuclease
dNTPs	-	Deoxynucleotide triphosphate
2-ME	-	2-mercaptoethanol
EDTA	-	Ethylene diamino tetra acetic acid
ELISA	-	Enzyme Linked Immuno Sorbant Assay
iELISA	-	Indirect Enzyme Linked Immuno Sorbant Assay
cELISA	-	Competitive Enzyme Linked Immuno Sorbant Assay
Fig.	-	Figure
g	-	gram(s)
g	-	acceleration due to gravity
h	-	hour
IgG	-	Immunoglobulin G
IgM	-	Immunoglobulin M
kb	-	kilo base pairs
KCl	-	Potassium chloride
kDa	-	kilo Dalton
L	-	Litre
LPS	-	Lipopolysacharide
M	-	Molar
mA	-	milliampere
MAbs	-	Monoclonal antibodies
mg	-	milligram(s)

$MgCl_2$	-	Magnesium Chloride
min	-	minutes
ml	-	millilitre
mM	-	millimolar
MW	-	Molecular Weight
NFW	-	Nuclease free water
NaCl	-	Sodium Chloride
ng	-	nanogram
nm	-	nanometre
°C	-	degree centigrade
OMP	-	Outer membrane protein
OPS	-	O-polysaccharide
PBS	-	Phosphate buffered saline
PCR	-	Polymerase Chain Reaction
pH	-	negative log of hydrogen Ion concentration
pmoles	-	picomoles
RNA	-	Ribonucleic acid
RNase	-	Ribonuclease
RPLS	-	Rough lipopolysacharide
rpm	-	rotations per minute
SDS-PAGE	-	Sodium dodecyl sulphate-Polyacrylamide gel
Sec	-	seconds
SLPS	-	Smooth lipopolysacharide
TAE	-	Tris acetate EDTA
TEMED	-	N,N,N',N'-Tetra methyl ethylene diamine
UV	-	ultraviolet
V	-	volt(s)
v/v	-	volume/volume
w/v	-	weight/volume

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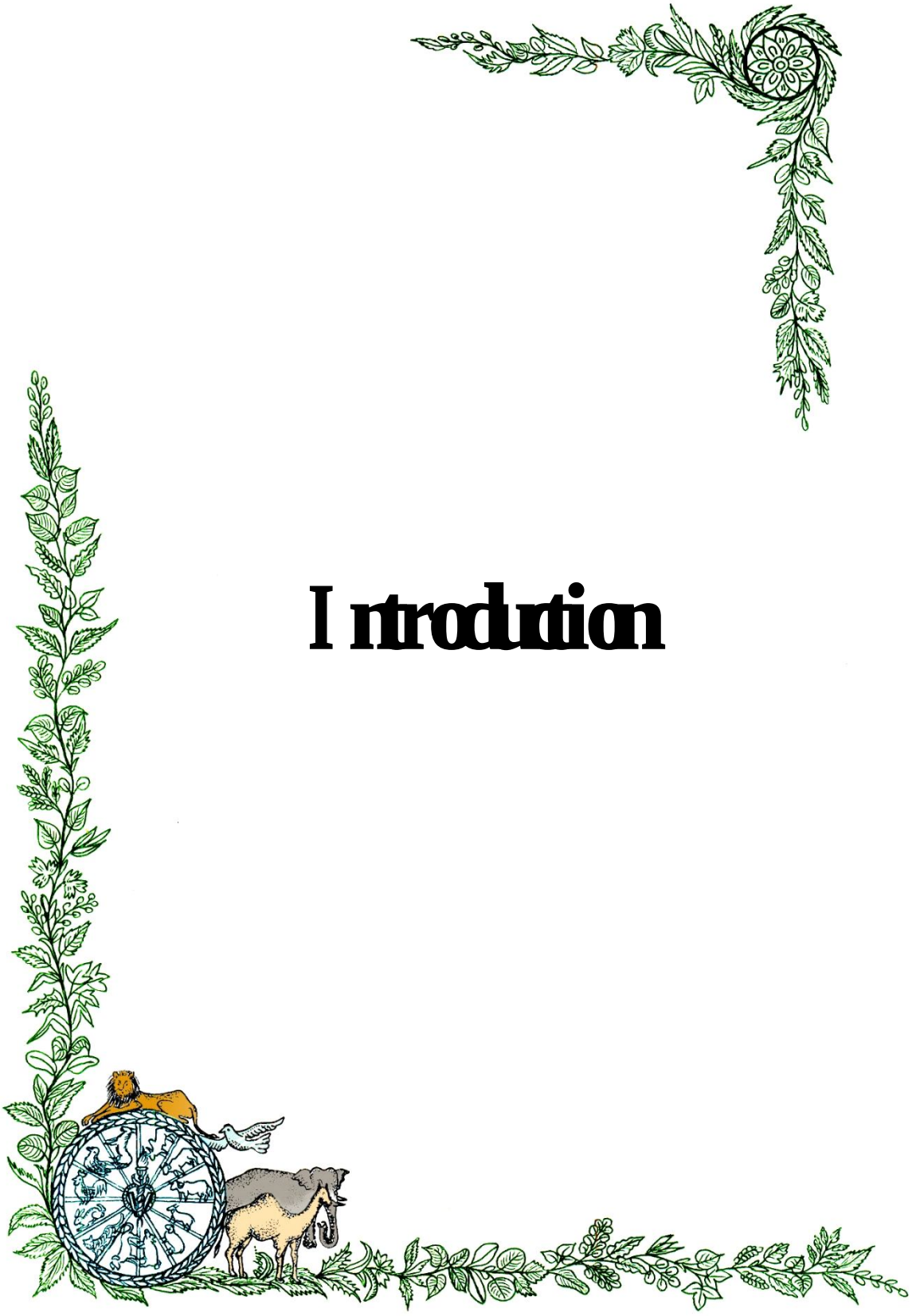


Introduction

1 INTRODUCTION

Bovine brucellosis, also called as “Mediterranean fever” or “Bang’s Disease”or “Contagious Abortion”, is highly zoonotic bacterial infection, mostly transmitted by direct or indirect contact from infected animals or their products. It affects all age groups of both sexes in animals and human beings (Alton *et al.,* 1975). It is found worldwide however, it has been eradicated from many countries and mainly caused by biovars of *Brucella abortus.* In southern Europe and western Asian countries the infection can also be caused by *B. melitensis,* where cattle are kept in close association with sheep or goats. Occasionally, *B. suis* may cause a chronic infection in the mammary gland of cattle (Corbel and Brinley-Morgan, 1984). The disease is clinically characterized by abortion, retained placenta, orchitis, epididymitis, arthritis, excretion of the organisms in uterine discharges and in milk. The disease is usually asymptomatic in males and non-pregnant females but following infection the pregnant adult females develop placentitis, which result in abortion between the fifth and the ninth month of pregnancy.

Comprehensive understanding of humoral immunity and advances in diagnosis of brucellosis (Al Dahouk *et al*., 2006) is necessary to control the disease because i) major zoonosis results in economic hardship due to loss of livestock, ii) highly pathogenic *B. melitensis* and *B. suis* represent emerging pathogen in cattle and iii) as a weapon in biological warfare or bioterrorism (Moreno *et al*., 2002).

Diagnostic tests fall into two categories: those that demonstrate the presence of the organisms and those that detect an immune response to its antigens. Isolation is the gold standard, definitive proof for the diagnosis of brucellosis, but it is time consuming, resource-intensive, not all infected animals give a positive culture and requires skilled technical personnel to handle samples. The presumptive diagnosis can be made by assessing specific or serological or cell-mediated responses to *Brucella* antigens. Diagnosis and control of the disease in animals

must be carried out on a herd basis, positive animals give evidence of infection in the herd and negative animals may be with long incubation periods (OIE, 2006).

Virtually at present, all serological tests of *Brucella* utilize the detection of antibody to smooth lipopolysaccharide (SLPS), which is a part of lipopolysaccharide (LPS) or whole cells as the antigen. The immunodominant epitope observed on the surface of the smooth cell is O-polysaccharide (OPS), the outermost portion of LPS. There is substantial similarity in OPS of *Brucella* LPS with other Gram-negative bacteria due to sharing epitopes. The high amount of anti-LPS antibodies persist for more than a year after acute brucellosis and results in low specificity due to false positive reaction (Corbel, 1985). The genetic diversity of some animals respond with low antibody titer to infection, resulting in false negative results. Both scenarios results in a false serological reaction, leading to a major diagnostic problem in some areas, where such microorganisms are endemic.

Many serological tests cannot distinguish these antibody responses, because of the cross-reacting antibody *i.e.*, IgM isotype. The agglutinability limited by use of reducing agents like dithiotreitol, 2-mercaptoethanol and divalent cations (Huber and Nicoletti, 1986) diminishes the number of false positive reactors. Enzyme linked immuno sorbent assays (ELISAs) using *B. abortus* cytoplasmic proteins (CPs) depleted of LPS or the *Brucella* lumazine synthase facilitated the differentiation of active from inactive systemic disease (Goldbaum *et al.*, 1993; and Goldbaum *et al.*, 1999). Therefore, only a well defined selection of *Brucella* proteins will lead to a satisfactory diagnostic test (Rosetti *et al.*, 1996). As alternative strategy, a number of protein antigens have been tried with limited success. For instance, other candidate antigens used are recombinant proteins, monoclonal antibodies and rough LPS (RLPS). Skin testing using a protein antigen derived from *Brucella* (Brucellergene, Brucellin) has certain logistical drawbacks, in combination with serological tests can provide part of a sensitive and specific protocol for detection of infected animals (Bercovich, 2000). To reduce this problem, in cattle vaccination is usually employed in young animals under the age of six months.

ELISAs using crude mixtures of *Brucella* major outer membrane proteins (MOMPs) or CPs lack specificity, if proteins cross-react with heterologous antigens. Most studies on the antigenicity of *Brucella* proteins are either hampered by the limited number of proteins investigated or the complexity of the protein mixtures used. The relative weakness of LPS-based serological tests for brucellosis could be avoided by using specific seroreactive protein

antigen preparations. A well defined single or a mixture of *Brucella* specific, immunoreactive proteins will improve serodiagnostics in all host species (Al Dahouk *et al*., 2006).

There are considerable differences in the accuracy of various serological tests and it is common to use a panel of tests and majority results as an indicator of exposure (OIE, 2008). However, the OIE quoted "perfect diagnostic test has still not been developed and probably never will be". The validation and extensive use of primary binding assays has made diagnosis more manageable (OIE, 2009).

In order to avoid difficulties in isolation and disadvantages of serological tests, alternative methods used to identify nucleic acid of the bacterium using molecular techniques like polymerase chain reaction (PCR). PCR becomes very useful with improved sensitivity and specificity (Yu and Neilson, 2010). The use of PCR to identify *Brucella* DNA at genus, species and even biovar levels has become extended to improve diagnostic test (Poester *et al*., 2010). DNA purification from infectious samples is very risky to persons handling in the laboratory, so purification from serum is safe (Zerva *et al.,* 2001) and which was applied to brucellosis screening (Elfaki *et al*., 2005).

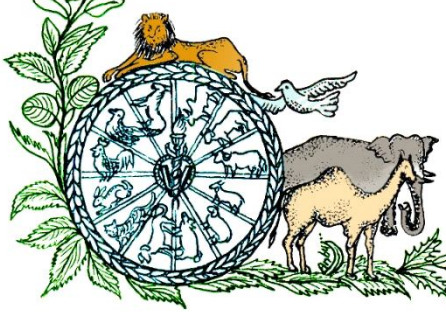
Thus cultural filtrate antigens from *Brucella abortus* S-99 culture, can be the potential candidates for developing improved diagnostic serological assays of brucellosis. The non-infectious serum samples can be the best source for purification of *Brucella* DNA during or after the infections. Thus the detection of *Brucella* DNA from sera can be helpful in seroprevalence studies of bovine brucellosis.

So in purview of the above mentioned information, the present work has been designed with the following objectives.

Objectives:

1. **To develop i-ELISA using cultural filtrate antigen of *Brucella abortus* S-99**
2. **To evaluate the developed i-ELISA by screening the serum samples for bovine brucellosis**
3. **To develop and standardize the modified molecular technique like PCR for detecting *Brucella* DNA in bovine serum samples**

Review of Literature



2 REVIEW OF LITERATURE

Brucella is a facultative intracellular bacterium responsible for causing brucellosis. It is also one of the most frequent bacterial zoonoses in economically backword countries. Brucellosis is responsible for major causes of direct economic losses and an impediment to trade and exportation.

Earlier, brucellosis was discribed by Marston in 1859 (Vassalo, 1992). Brucellosis then called Mediterranean fever, caused debilitating chronic illness with rheumatism from which many Royal Navy seamen used to be affected each year (Wyatt, 1999). An agent called *Micrococcus melitensis* was isolated from human spleen by Captain David Bruce in 1887. Themistocles Zammit, a physician, tested the blood of goats and found that as many as 50% had agglutinins and isolated the bacteria from blood and milk of at least 10% goats. Bernhard Bang, Danish veterinary bacteriologist, described the causative organism of brucellosis in cattle during 1895, named as *Bacillus abortus*. The name of the disease was changed from bovine infectious abortion to Bang's disease in 1930. The strain 19 was introduced as vaccine in the USA during 1941. In the United States, *B. suis* was isolated from an aborted pig fetus in 1953. *B. ovis* was identified as a cause of epididymitis in sheep in Australia and New Zealand and *B. canis* was isolated from aborted canine fetuses in 1965 by Carmichael.

Historically, in the Indian subcontinent, the credit of first investigation of contagious abortion in livestock, associated with brucellosis, goes to the Imperial Veterinary Research Institute (now Indian Veterinary Research Institute), Muketswar, in northern India (Anonymyous, 1918).

2.1 Characteristics of *Brucella*

The *Brucella* organisms belong to family *Brucellaceae.* The genus *Brucella* are small, nonmotile, Gram- negative coccobacilli, facultatively intracellular bacteria and cause disease in a broad range of animal hosts. The size of organisms is 0.5-0.7 µm wide by 0.5-1.5 µm in length and occur as single cells, in pairs or in short chains. In *vivo,* they often occur within the cytoplasm of cells in close-packed clusters. They do not sporulate or have true capsules and are invariably non-motile and aflagellate (Corbel and Brinley-Morgan, 1984).

Brucella organisms are enveloped, which make the study of individual components difficult. Unlike other Gram negative bacteria, most *Brucella* species do not form spheroplasts when treated with Tris, EDTA and lysozyme. Nonionic detergents are less effective, therefore ionic detergents were used in extracting proteins from *Brucella* (Moriyon and Berman, 1982; Winter and Rowe, 1988). Evaluation of immunogenic *Brucella* proteins has been limited to complex protein preparations or a small number of recombinant protein products (Pugh *et al*., 1990; Smith and Ficht, 1990).

They are aerobic; some strains of the species *B. abortus* and *B. ovis* are carboxyphilic and require supplementary CO_2 for growth. *Brucella* has oxidative metabolism, utilize various amino acids, carbohydrate substrates and many strains use i-erythritol as a preferred energy source. Most strains require complex media containing multiple amino acids, thiamine, biotin, nicotinamide and pantothenic acid for growth, especially for primary isolation (Corbel and Brinley-Morgan, 1984). The production of H_2S varies between species and biovars and it has value in differentiating these. Urease activity is consistently high in *B. suis* and *B. canis* but variable in other species, weak or absent in the case of *B. ovis.* Citrate cannot serve as the sole carbon source in *Brucella* metabolism. Acid is not produced from glucose and O-nitrophenol-β-D-galactoside is not usually hydrolyzed. Litmus milk remains unchanged or some times rendered alkaline.

In-vitro sensitivity of organisms to antibiotics, like gentamycin, tetracyclines, chloramphenicol and ansamycins. Differences were observed between the protein components of the species and strains, by high performance liquid chromatography of extracts of live cells.

The DNA composition of all strains is very similar having >94% homology between species (Hoyer and McCullough, 1968). The guanine plus cytosine (G+C) contents range between 55 and 59 mole%. The very similar to that of *Escherichia coli* (50% GC) having *Brucella* as a monospecific genus was described by Verger *et al*., 1987. Even DNA-DNA hybridization is similar between the species of *Brucella*, but electrophoretic analysis of restriction enzyme digests has disclosed species differences (Verger *et al*., 1987).

Brucella-lytic phages have been described in detail, and are used mostly for identification and taxonomy. The *Brucella* has an extremely waxy cell wall, refractory to transformation protocols, using calcium chloride, rubidium chloride and resistant to many of the antibiotics, tetracycline and streptomycin cannot be used, since they are antibiotics of choice to treat the disease (Smith and Ficht, 1990).

2.2 Classification of *Brucella*

On the basis of natural host, phage sensitivity, colony morphology, CO_2 requirement, ability to oxidize certain substrates, grow in the presence of dyes, H_2S production and serological properties, the genus has been divided into six species, three of which have been subdivided into biovars. These six species are *B. abortus* (cattle), *B. melitensis* (goats), *B. suis* (hogs), *B. ovis* (sheep), *B. neotomae* (wood rat), and *B. canis* (dogs) (Corbel and Brinley-Morgan, 1984). The *B. melitensis*, *B. suis*, and *B. abortus* are apparently the most virulent and cause the majority of human disease when compared to others. *B. canis* and *B. ovis* have rough LPS and isolates retained the virulence. The organisms of the genus *Brucella* have a close taxonomic relationship and distinguished by rigorous metabolic, biochemical and immunologic analyses (OIE, 2004).

DNA analysis has been used to distinguish between *Brucella* species based on restriction enzyme analysis, and by analysis of restriction polymorphisms within specific genes via Southern blotting and hybridization (Ficht *et al*., 1988).

The species can be differentiated on the basis of oxidative metabolism tests with selected carbohydrate and amino acid substrates. *B. ovis* oxidize only a few amino acids, whereas *B. canis* and *B. suis* oxidize a complete range of substrates, including carbohydrates, amino acids and urea cycle intermediates. Oxidative metabolism patterns show a fairly close correlation

with phage lysis patterns and both procedures are useful for identification of the species (Corbel and Brinley-Morgan, 1984).

2.3 Antigens of *Brucella* in Diagnosis

Several cellular components contribute to the survival and virulence of the *Brucella*, but most of these components have not been elucidated. Unlike other Gram-negative pathogens, the outer surface of the *Brucella* does not have complex structures, such as pili or fimbriae, nor it has capsular material. The outer membrane contains only two components that have been identified as virulence factors: the LPS and outer membrane proteins (OMPs). Besides, several ribosomal and cytosolic proteins have been considered as the main immunogens for development of subunit vaccines and diagnostic reagents (Corbel *et al*., 1984).

During the *in-vitro* growth, *Brucella* organisms may release macromolecular products into the culture medium that have a wide range of biological activities, like antigenicity and antiphagocytic activity (Ellwood *et al*., 1967). The crude *Brucella* culture supernatants have been used as the basis of skin test antigen preparations employed for hyperreactivity purposes (Alton and Jones, 1967).

In most of standard serological tests *i.e.*, serum agglutination, complement fixation and ELISA, whole cell preparations, cell sonic extracts or LPS fractions have been used (Lindberg *et al*., 1982). These antigen preparations are most frequently obtained from smooth *Brucella abortus* strains being rich in SLPS (Al Dahouk *et al*., 2003).

2.3.1 Lipopolysaccharides (LPS)

Virulence is associated with the smooth colony morphotype, designated 'S', which contains LPS. The rough colony morphotype, designated 'R', arises by dissociation from the smooth form and its accumulation is typically observed *in vitro*. The LPS of smooth strains consists of lipid A, a core region containing flucose, mannose, quinovosamine and 2-keto-3-deoxyoctulosonic acid (KDO) and an O chain composed of a homopolymer of about 100 residues of N-formylated perosamine (Nielsen and Duncan, 1990). It consists of two distinct epitopes depending upon the species/biovar of the strain. In *B. abortus* biovar 1, the A epitope is present and probably represents terminal N-formylated perosamine residues which are

linked by the 1 and 2 carbon atoms throughout the chain and in *B. melitensis* biovar 1, the M epitope is present. In this case, the O chain consists of repeating units of four 1,2-linked N-formylated perosamine residues and one 1,3-linked residue. The A, and M epitopes can be detected by cross-absorbed polyclonal antisera (Perry and Bundle, 1990).

Of the four LPS determinants recognized in the sera from *Brucella abortus*-infected and vaccinated bovines, two are present in the OPS (A and C), one in the core oligosaccharide from rough *Brucella* LPS (R), and one in lipid A (LA). LPS preparations from smooth *Brucella* had small amounts of rough-type LPS, while rough *Brucella* did not show smooth-type LPS on immunoblots (Rojas *et al.*, 1994).

LPS provokes a strong antibody response, with major drawbacks (i) The immunodominant epitope of the *Brucella* O-polysaccharide is similar to that of various bacteria, e.g. *Yersinia enterocolitica* O:9, *Vibrio cholerae, Francisella tularensis, Salmonella Urbana* group N, *Escherichia coli* O:157, and *Stenotrophomonas maltophilia* resulting in cross-reactivity (Corbel *et al.*, 1984; Caroff *et al.*, 1984a and 1984b).It lowers the specificity in LPS-based assays, (ii) Anti-LPS antibodies may persist for more than a year after acute brucellosis (Buchanan *et al.*, 1974) and elevate due to repeated exposure the agent without clinical relevance, especially in high risk occupations in endemic areas, *i.e.*, butchers, farmers and veterinarians, (iii) *Brucella* infections caused by rough strains, *i.e., B. canis* and *B. ovis*, cannot be detected by these assays and (iv) In eradication programs, we cannot differentiate vaccinated from infected animals by most of the LPS-based assays (Samartino *et al.*, 1999).

The serological diagnosis of brucellosis with LPS is still a challenge in human and animal disease (Al Dahouk *et al.*, 2003).

2.3.2 Polysaccharide antigens

Various polysaccharides related to *Brucella* LPS O-chain have been described. These include native antigen or native hapten. Smooth O-chain, synthesized and accumulate in the cytoplasm but not assembled into complete LPS nor exported to the cell surface have been observed in *B. melitensis* B115 (Cloeckaert *et al.*, 1992a).

The N-Acylated-D-perosamne also occurs in the O chains of LPS complex of *E. coli* 0157, Salmonells Kauffmann-White group N, *Stenotrophomonas maltophilia* strain 555, *Vibrio cholerae* and *Yersinia enterocolitica*, all of which cross reacts with serologically with smooth *Brucella* (Perrry and Bundle, 1990).

2.3.3 Outer membrane proteins (OMPs)

OMPs are important immunogens of *Brucella*, and sequentially extracted using different enzymatic and detergent treatments. The characterization of these proteins was carried out on the basis of molecular masses (Tibor *et al.*, 1999). The role of various outer-membrane and other cellular proteins has been investigated in cattle and a murine model by immunoblot analysis and humoral as well as cell mediated immune response studies (Onate and Folch, 1995; Vemulapalli *et al.*, 2000). The usefulness of OMPs as differential diagnostic antigens in immunoblotting and ELISAs has been indicated in cattle (Gupta *et al.*, 2007; Mathur *et al.*, 2008).

The *Brucella* outer membrane contains three major proteins with molecular masses ranging from 25 to 27, 31 to 34, and 36 to 38 kDa (Cloeckaert *et al.*, 1990 and 1992b). The largest protein has been identified and characterized as a Porin (Douglas *et al.*, 1984). The genes coding for these proteins have been cloned and sequenced, and the current names for these OMPs are Omp25, Omp31, and Omp2b (Cloeckaert *et al.*, 1996).

The other OMPs identified so far by use of monoclonal antibodies (MAbs) are less abundant (minor) proteins with molecular masses of 10, 16.5, 19, and 89 kDa and cloning predicted the amino acid sequences, and the presence of particular protein motifs (Cloeckaert *et al.*, 1990). The current names for these OMPs are Omp10, Omp16, and Omp19, respectively (Tibor *et al.*, 1999). The Omp16 actually belongs to the peptidoglycan-associated lipoprotein family of proteins found in many gram-negative bacteria. Homologs of Omp10 and Omp19 have not yet been reported for other bacteria. All of these proteins have been found as immunogenic proteins in infected cattle, sheep and goats (Letesson *et al.*, 1997).

ELISAs using crude mixtures of *Bucella* MOMPs or CPs lack specificity as some proteins cross-react with heterologous antigens (Al Dahouk *et al.*, 2006).

The OMPs profile of three strains of *B. abortus i.e.,* S19, RB51 and a local field isolate of biotype-1 indicated, two OMPs of molecular weight 37-38 and 19 kDa were immuno-reactive in all strains in buffaloes. A distinct protein of molecular weight of 151.3 kDa was identified in a field strain but not in both vaccine strains of *B. abortus* and used in diagnostic assay may differentiate between vaccinated and infected animals (Munir *et al.*,2010).

Siadat *et al.* (2011) reported OMPs concentration to be 6.27 mg/ml by the Nanodrop ND-1000 spectrophotometer. SDS-PAGE analysis indicated a band (36-38KDa) which would be classified as *Brucella* porins.

2.3.4 Sonicated lysate antigens

Jerry *et al.* (1970) performed separation and purification of several soluble *Brucella* antigens from *B. suis,* suggesting that these methods can be used to prepare more efficient protective antigens for man and animals.

The sonicated cell lysates from SRB51 (Edmonds *et al*., 1999) has been used for the complement fixation test. The water soluble extract was obtained by sonication of *B. abortus* smooth strains 19 and A/73 and rough strain 45/20. Precipitation tests performed for detection of Component-1 in gel filtration and polyacrylamide gel fractions by double immunodiffusion and single radial diffusion (Robles *et al*., 2009).

2.3.5 Cytoplasmic proteins

Cytoplasmic proteins induced a higher antibody response than outer membrane proteins (Letesson *et al*., 1997). Antibodies directed against proteins may be host specific, like anti-OMP28 antibodies were detected in *Brucella* infected humans and goats, but not in pigs and cattle (Lindler *et al*., 1996).

2.3.6 Antigens for T-Cell

T-cell antigens play major role in protection against brucellosis. Induction of a Th1 type of immune response with production of IFN-γ and generation of cytotoxic CD8+ cells are pivotal for protection against *B. abortus* infection (Oliveira and Splitter *et al*., 1994 and 1995). They identified the *B. abortus* L7/L12 ribosomal protein as an immunodominant molecule. L7/L12 from *B. melitensis* (Bachrach *et al*., 1994) provoked strong delayed type hypersensitivity in primed guinea pigs.

Cespedes (2000) reported the occurrence of the 22.9 kDa protein which induced protection in Balb/c mice against *B. abortus.* Al-Mariri *et al.* (2001) have demonstrated that *B. abortus* P39 together with CpG as adjuvant protected mice against experimental challenge. Humoral and cellular immune responses to recombinant glyceraldehyde 3-phosphate dehydrogenase were characterized (Rosinha *et al.*, 2002).

Chaudhari (2008), reported shown efficient cellular uptake of the DNA adsorbed nanoparticles as well as expression of *B. abortus* L7/L12 DNA constructs and induction of moderate humoral and cellular immune responses in mice.

2.4 Immunity against *Brucella*

Both humoral and cellular immune responses are required to get immunity against brucellosis (Araya *et al.*, 1989).

2.4.1 Humoral immune responses

The LPS component of *B. abortus* induces a strong humoral response (Moreno *et al.*, 1981). Many of the constituents of *Brucella* cells are capable of inducing specific antibodies in their hosts (Verstreate and Winter, 1984).

Multiple precipitating antibodies have been reported to be usually detectable within 3-4 months of severe or prolonged active *Brucella* infection (Schurig *et al.*, 1978). These are mostly IgG globulin fraction and they are not as long lasting as the agglutinins (Glenchur *et al.*, 1962).

Infected cattle with *B. abortus* generally produce an early IgM isotype antibody response, amplitude appears between 5 to 15 days post exposure (Allan *et al.*, 1976). Which was followed by very short period production of IgG1 Isotype of antibody and subsequently of IgG2 and IgA (Neilson *et al.*, 1984). Since the IgM response commences early, theoretically it would be most suitable to measure this isotype as an indicator of exposure. But there are number of other microorganisms containing antigens with epitopes similar to those of OPS and so the main antibody response to these cross-reacting antigens is IgM (Corbel, 1985. Measurement of IgM antibody may result in a false positive reaction in serological tests with limited specificity, which would consequence in an early control programme resulting in

unnecessary slaughter in the last stages of an eradication programme. Production of IgG2 and IgA isotypes occurs later in infection and, as a result, measurement of these antibodies would generally lower assay sensitivity (OIE, 2004). Based on these observations, the most useful antibody for serological testing for brucellosis is an IgG1 (Allan *et al.*, 1976; Nielsen *et al.*, 1984). Antibody produced in response to smooth vaccines may also result in positive serological reactions, which may lead to misdiagnosis in *B. abortus* S19 vaccine by the time animals reach sexual maturity (Nicoletti, 1990). Most of these problems have been overcome by the development of improved serological tests, for example, the competitive enzyme immunoassay and fluorescence polarization assay (Nielsen, 2002) and the development of a live vaccine devoid of OPS like *B. abortus* RB51 (Schurig *et al.*, 1991).

2.4.2 Cell-Mediated immune responses

Certain protein components induce the T-cell-mediated response responsible for the elimination of the pathogen from the infected host (Oliveira *et al.*, 1993). Responsible for protection against *Brucella* are the interferon gamma (IFN-γ) and up-regulation of macrophages, the main host cellular reservoir for the bacterium (Baldwin *et al.*, 1993).

The DNA vaccine (L7/L12) with adsorbed cationic nanoparticles on immunization resulted in lymphocyte proliferation observed after the 4th week of last booster. The IFN-γ cytokine production was higher and protective immune response was elicited than naked DNA vaccine (Chaudhari, 2008).

2.5 Diagnosis of Bovine Brucellosis

2.5.1 Isolation and identification of the agent

The gold standard in the diagnosis of brucellosis is the isolation of *Brucella* bacteria. The isolation rate of the bacteria from blood cultures ranges from 47.1% to 94.1%, depending on the methods used and the period of incubation (Ozturk *et al.*, 2002).

However, this direct diagnostic test is not possible when testing large numbers of samples. Further it has several drawbacks: i) It is time consuming to culture *Brucella* from biological samples, ii) The process is complicated and needs trained personnel and iii) Handling the live *Brucella* is hazardous due to the zoonotic nature of the disease (Bricker *et al.*, 2002a).

2.5.2 Detection of *Brucella* antibodies in serum

Serological diagnosis is presumptive evidence of infection or indicator of exposure. There are considerable differences in the accuracy of the various serological tests and it is common to use a battery of tests. The serological tests depend on a reaction between *Brucella* antigen and antibodies produced in response to the infection, vary in sensitivity, specificity and complexity (OIE, 2004 and 2008).

2.5.2.1 Rose Bengal Plate Test (RBPT)

The RBPT is considered to be suitable for screening of individual animals, however, some cross-reacting antibodies have been detected by this test and false negative reaction may occur mostly due to prozoning. Rose and Roepke (1957) introduced a modification of slide plate agglutination test in which the antigen was buffered at pH 4.0 immediately before use. The low pH prevents some agglutination by IgM and encourages agglutination by IgG1 thereby reducing non-specific interactions. RBPT activity was associated with IgG1 and IgM was detected more efficiently than IgG1 or IgG2 in RBPT (Allan *et al.,* 1976). Later on, Stryszak (1986) concluded that both IgM and IgG were active in RBPT.

2.5.2.2 Standard Tube Agglutination Test (STAT)

The STAT has been recognized as the principal serological test used for the diagnosis of brucellosis. IgM isotypes of antibody are the most active agglutinin at neutral pH (Nielsen *et al.,* 1984).

The STAT is susceptible to a false positive reaction by cross-reacting antibodies (Corbel, 1988), so it should not be used as a diagnostic test and its discontinuation is recommended by the OIE. False negative reaction in high titer sera have been reported of prozone phenomenon caused by the presence of high concentration of non-agglutinating IgG1 that competes for binding by the agglutinating IgG2 antibodies (OIE Manual, 2000).

Other limitations of STAT are that agglutination titers may persist after vaccination and infection with field strain, antibodies detected by Complement fixation test and RBPT test ride at the same time or earlier than those detected by STAT. Often, the reaction does not reach significant levels until after the abortion has occurred (MacMillan *et al.*,1990). Modifications

that are done to destroy or inactivate IgM agglutinin includes acidified antigen, rivanol precipitation and use of 2-mercaptoethanol, heating of serum, additions of antiglobulins or chelating agents (Nielsen, 2002).

2.5.2.3 2-Mercaptoethanol Test (MET)

2-mercaptoethonol is a reducing agent that reduces disulphide bridges, reduces IgM to monomeric units thereby reducing its ability to agglutinate. This treatment increases the specificity to some extent but may cause false-negative reactions as IgG molecules containing disulphide bridges may also be reduced to an extent where they are unable to agglutinate. This test is used mainly as confirmatory test, reported 97% of culturally positive cases (Nicoletti, 1969). Ongor (2001) compared ELISA with other serological tests including MET in the diagnosis of ovine brucellosis and found that there is a concordance of 83% between ELISA and MET.

2.5.2.4 Rivanol Test

Rivanol is added to serum causing high molecular weight glycoproteins to precipitate. A rapid agglutination test is performed by using serum diluted 1:25, 1:50, 1:100 and 1:200 after the removal of precipitate by centrifugation (Nicoletti, 1969; Huber and Nicoletti, 1986).

2.5.2.5 Milk Ring Test (MRT)

This test provided a good tool for diagnosing brucellosis both in individuals and herds because samples can be pooled, but the test had not been found effective in milk of sheep and goats. MRT with a sensitivity of about 89% for detection of *Brucella* antibodies in fresh milk was reported. MRT is used to detect the presence of *Brucella* antibodies in the milk. It is an adaptation of the agglutination test using hematoxylin stained whole cell antigen added to milk (Hunter and Allan, 1972; Huber and Nicoletti, 1986).

Bercovich and Taaijke (1990) have compared the ELISA test with MRT, ELISA has been found to be a good substitute for MRT-1 to detect antibodies against *Brucella* in milk from individual cows and in MRT-8. This test has low sensitivity due to positive reactions from samples taken shortly after parturition, near the end of lactation period, or from mastitis quarters (MacMillan *et al.*, 1990).

2.5.2.6 Complement Fixation Test (CFT)

CFT has been considered to be the most sensitive and specific method for serological diagnosis of brucellosis in cattle, sheep and goat (Nicoletti, 1969; MacMillan *et al.*, 1990) and distinguished between live vaccination and natural infection by the results of serological reactions (Alton *et al.*, 1975).

IgG and IgM antibodies are the predominately complement fixing antibodies in infected animals (Morgon *et al.*, 1969). Allan *et al.* (1976) confirmed that IgG2 do not fix complement. CFT is technically challenging in that a large number of different reagents are necessary, a large number of various controls are required, a number of reagent titrations are needed each time the assay is set up and the results are subjective. In spite of these shortcomings, CFT has been a valuable asset in control/ eradication programs as a confirmatory test and it is recommended by the OIE as a test prescribed for international trade (OIE, 2004).

2.5.2.7 Radio Immunoassay (RIA)

The RIA is used to measure the specific antibody of IgG 1 and IgG2 subclass but it is insensitive to IgM, this makes more suitable than the CFT or STAT for distinguishing infected animals from vaccinated with *B. aborts* S-19 (Chappel *et al.*, 1982). Due to biohazard problems involved in handling of radioisotopes, these assays have not been used widely as diagnostic tools.

2.5.2.8 Fluorescence Polarization Assay (FPA)

The FPA for detection of antibody to *Brucella* spp. has been validated for use as serological diagnosis of brucellosis in cattle (Samartino *et al.*, 1999) and pigs (Nielsen *et al.*, 1996). FPA has the capability to distinguish vaccine antibody and antibody resulting from exposure to cross-reacting organism such as *Yersinia enterocolitica* O: 9 from antibody to *Brucella* spp. in most of the cases (Gall *et al.*, 2001).

2.5.2.9 Competitive Enzyme Immunoassay (c-ELISA)

c-ELISA appears to be promising, quite easy to perform, less time consuming, repeatable and from a practical point of view, can differentiate infected from vaccinated animals (Rojas and Alonso, 1995). It measures vaccine induced antibody of lower affinity and shorter

exposure to antigen due to immune elimination compared to field infection in which antigen persists, resulting in increased antibody affinity (Nielsen *et al.*, 1989; MacMillan *et al.*, 1990).

ELISA inhibition has been performed using MAbs with inhibitor concentrations ranging from 250 to 0.04 µg/ml and incubated at 22°C (Rojas *et al.*, 1994). MAbs selected were specific for a common epitope of the LPS molecule, allowing use for *B. abortus, B. melitensis* and *B. suis* serology and reported that c-ELISA using conjugate BM-40 showed greater sensitivity than the CFT but lower than i-ELISA. c-ELISA using conjugate BM-38 showed greater sensitivity than both CFT and i-ELISA (Nielsen *et al.*, 1995).

Weynants *et al.* (1996) examined the MAb cross-reactions with smooth LPS of *Brucella* species. The specificity of c-ELISA was greater than the specificities of CFT and RBPT.

Nielsen *et al.* (2001) used c-ELISA and FPA techniques and identified 7 of the 10 farms as brucellosis-free, whereas the conventional techniques identified only 3.

2.5.2.10 Dot Enzyme Immunosorbent Assay (Dot-ELISA)

Chand *et al.* (1988) compared dot-ELISA with STAT and CFT for detection of *Brucella*-specific protein A reactive antibodies in buffaloes and found the least number of positive cases in comparison with other tests when dot-ELISA was used.

The dot-immunoblotting assay is a simple, sensitive and rapid assay in comparison to CFT for the detection of *Brucella* antibodies in sheep sera. Since dot-immunoblotting assay does not require heat inactivation of serum samples, this is an additional advantage. Subsequently, a number of reports described the usefulness of dot-ELISA in the diagnosis of brucellosis (Chand *et al.*, 1989).

Batra *et al.* (1989) evaluated dot-ELISA for detection of *Brucella* antibodies in cow milk and they concluded that results from the dot-ELISA from milk correlated well with serological results obtained using the i-ELISA and CFT.

2.5.2.11 Indirect ELISA (i-ELISA)

i-ELISA, under laboratory conditions was sensitive as RIA and can also be adopted as simple field screening procedures (Voller *et al.,* 1976) and widely applied in diagnosis of animals (Nielsen *et al.,* 2005).

Dohoo *et al.* (1986) recommended that either the buffered plate antigen test or i-ELISA be used as a screening test. Either the CFT or the i-ELISA is appropriate for use as a confirmatory test in situations requiring a high specificity.

The whole cells to purified components, antigen mixtures of soluble antigen (BASA) consist of SLPS, which was probably the major antigen component both in terms of antigenicity (immunodominance) and its ability to absorb to polystyrene. The poly B, a polysaccharide shown to distinguish S-19 vaccinated animals from *B. abortus* field infected animals by gel diffusion (Nielson *et al.,* 1989).

Rojas and Alonso (1995) obtained results of i-ELISA using an FAO/IAEA defined cutoff of PP, 35% being considered as positive. Uzal *et al.* (1996) evaluated an i-ELISA using a monoclonal anti-IgG1 conjugate for diagnosis of bovine brucellosis and this technique seems to be particularly useful in regions where little epidemiological information is available about brucellosis.

Samartino *et al.* (1999) evaluated the performance of i-ELISA and c-ELISA for diagnosis of ovine brucellosis in comparison to conventional tests routinely used in Argentina. They demonstrated that sensitivity of i-ELISA was marginally better than the cELISA but the specificity was lower than that of cELISA for cattle vaccinated with *B. abortus* strain 19.

Abalos *et al.* (2000) evaluated three *Brucella* soluble antigens: An OPS, a hot-water extracted polysaccharide (PS) and a native hapten (NH) in an i-ELISA to discriminate S19 vaccinated animal from infected one. Paweska *et al.* (2002) suggested that ELISA could replace not only the currently used confirmatory CFT, but also another two routine screening test, namely the RBPT and STAT.

ELISA has been reported to be a more sensitive test compared to other conventional tests (Al Dahouk *et al.,* 2003). Neilsen *et al.* (2004) recorded highest false positive result in RBPT and highest false negative result in ELISA.

The SLPS optimization and standardization of concentration, dilution and detection system of antigen made by checkerboard titration. Using *Brucella abortus* strain 99 and a weak positive control serum prepared by mixing positive serum in negative serum at ratio 1:12 and showed the ODs above the cutoff value. The LCL and UCL of C^{++}, C^{+}, C^{-} and Cc were obtained yielded OD of 0.807- 1.199, 0.458-0.77, 0.092-0.22, and 0.045-0.109, and PP at 81-120%, 46-77%, 9-22% and 4-11%, respectively. The positive results were accepted as the cutoff values of e"40 percent positivity on screening bovine serum (Ekgatat *et al*., 2008).

Siadat *et al.* (2011) confirmed the potency of porins and the porins combination with CFA and LPS to promote humoral specific response on using ELISA. A combination of porins + LPS or porins + CFA has been the most potent immunogenic compound to induce higher titer of antibody against *B. abortus* S99 in the animal model.

2.5.3 DNA detection molecular techniques

2.5.3.1 Polymerase Chain Reaction (PCR)

The first published PCR-based diagnostic assay was reported by Fekete *et al.*, (1990) based on the amplification of a 635-bp sequence of a gene encoding a 43 kDa outer membrane protein of *B. abortus* strain S19. Herman and DeRidder (1992) explored *Brucella* genetic with targeting 16S rRNA gene. In 1992, a new PCR assay was published by Baily *et al.* (1992) based on the gene encoding *BCSP31*.

Zerva *et al.* (2001) developed a PCR assay using 31-kDa *Brucella abortus* antigenic protein gene sequence and applied to whole-blood and serum samples. The assay sensitivity was higher with serum samples (94%) than with whole-blood samples (61%) and used as a diagnostic tool for brucellosis (Bricker, 2002b).

Elfaki *et al.* (2005) subjected sera for PCR to detect *Brucella* DNA, a 223 bp amplicon, and obtained it in 96% of tested sera using primers derived from the nucleotide sequence of a gene encoding the 31-kDa *Brucella abortus* antigen. Similarly amplicon of 731 bp was obtained in 60% using *Brucella melitensis*-specific primers.

Kaushik *et al.* (2006) standardized a PCR assay using specific primers from a gene encoding 31 kDa protein for *Brucella* species and reported that it could detect *Brucella* organism up to 40 cfu per ml in cattle semen.

Mukherjee *et al*. (2007) compared ELISA and omp2 based PCR and found similarily between *bcsp*-PCR and ELISA. Kanani (2007) compared three pairs of primers amplifying three different fragments (i) a gene encoding a 31 kDa *B. abortus* antigen (primer B4/B5), (ii) a sequence 16S rRNA of *B. abortus* (primer F4/R2) and (iii) a gene encoding an omp2 (primer JPF/JPR) by testing 101 semen samples from breeding bulls of AI Centers of Gujarat. He found that B4/B5 primer was more sensitive followed by F4/R2 primer and JPF/JPR primer.

PCR has the potential to meet the need for better diagnostic tools and robust test can differentiate in a single step for all of the classical *Brucella* species, including those found in marine mammals and S19, RB51, and Rev.1 vaccine strains (Lopez-Goni *et al.*, 2008).

PCR amplification was done for *IS711* and *omp2a* genes of *B. aborts* and obtained 498 and 966 bp product using BAF/BAR and Omp2aF/Omp2aR primers respectively (Tarekegn, 2010).

2.5.3.2 Real time PCR

Quantitative PCR using serum samples seems to be highly reproducible, rapid and show sensitivity of 93.5% and a specificity of 98.4% (Queipo-Ortuno *et al.*, 2008). It is therefore a useful method for both the initial diagnosis and the differentiation between past and active brucellosis. Marianelli *et al.* (2008) screened milk samples from 53 *Brucella* seropositive buffaloes and found 35 samples positive by real-time PCR.

2.6 Seroprevalence of Bovine Brucellosis In India

History of brucellosis in India dates back to 1897 when Wright and Smith reported serological evidence of the disease in invalidated soldiers from India (Theodorides, 1996). Brucellosis was first recognized in India in 1942 (Renukaradhya *et al.*, 2002). Serological evidence suggested that brucellosis is highly endemic in most parts of the India. Perusal of the literature indicated a prevalence of brucellosis was 17% in the southern region (Isloor *et al.*, 1998) and 6.3% in the central region (Madhya Pradesh state) (Mehra *et al.*, 2000).

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

7.09%, 2.70%, 11.14% and 8.10% by RBPT, STAT, CFT and dot-ELISA, respectively in buffaloes of Delhi (Prahlad *et al.*, 1999). Chakraborty *et al.* (2000) reported the seroprevalance in cattle as 56.02%, 50.35% and 33.33% by employing RBPT, STAT and ELISA respectively.

Varasada (2003) recorded seroprevalence of brucellosis in animals (cattle and buffaloes) as 22.11% while species wise prevalence was found to be 24.12% and 19.12% in cattle and buffaloes respectively in Gujarat. Sarumathi *et al.* (2003a) reported higher prevalence of brucellosis among crossbred female cattle (11.1%) than indigenous animals (6.6%).

Singh *et al.* (2004) conducted a study in organized farms from Punjab and found seroprevalence to be significantly lower in well managed farms (5.2%) when compared with poorly managed farms (14.81%).

As per OIE (2004), i-ELISA should be considered more as a screening test rather than a confirmatory test for testing of vaccinated cattle or herds. Chand and Sharma (2004) recorded the highest prevalence of 26.50% by ELISA in cattle and buffaloes. Similarly higher efficacy of i-ELISA was reported by Chakraborty *et al.* (2000) and Sarumathi *et al.* (2003b) in cattle and buffaloes.

Mishra *et al*. (2005) studied the seroprevalence in cattle and buffalo in Gorakhpur district of Uttar Pradesh, using STAT and reported that 1.55% of cows and 1.97% of buffaloes were *Brucella* positive. In contrast, on i-ELISA, 3.11 and 4.18% of cows and buffaloes were found to be positive for *Brucella* antibodies.

Bandyopadhyay *et al.(* 2009) tested 374 yak animals and found 23.79%, 21.11% and 18.98% positive for brucellosis using AB-ELISA, RBPT and STAT respectively in the North-Eastern hilly yak tracts of Arunachal Pradesh, India. The alarming prevalence as recorded was highest among the yak cows (31.42%) followed by heifers (23.85%) and bulls (8.88%).

Trangadia *et al.* (2010) used ELISA and RBPT for screening brucellosis in organized dairy farms with a history of abortion. The test results indicated that 22.18% and 13.78% animals were sero-positive by ELISA and RBPT respectively.

Ghodasara *et al*. (2010) recorded the overall seropositivity for *Brucella* antibodies by i-ELISA as 25% (45), 24.30% (26) in cows and 26.03% (19) in buffaloes. They concluded

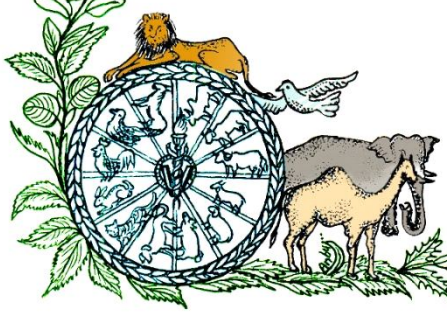
that i-ELISA is a better serological test compared to RBPT and STAT and it could be advocated for screening of Brucellosis.

Sukumar *et al.* (2012) recorded 23.52% seroprevalence in cattle in Tamilnadu using RBPT. Trangadia *et al.* (2012) reported 7.57% seroprevalence of bovine brucellosis, in Gujarat and 12.27% in Andhra Pradesh. Sharma and Bist (2012) reported 28.57% seroprevalence in cattle by RBPT, STAT and dot-ELISA in Mathura district of Uttar Pradesh.

Jagapur *et al.* (2013) reported seroprevalence in Karnataka as 46.83% and 45.56% in cattle and buffaloes respectively on organized farms and 27.36% and 16.87% in cattle and buffaloes respectively at unorganized farms. Seroprevalence of 22.39% and 8.57% was recorded on organized cattle farms in Uttar Pradesh and Uttarakhand respectively.

Sachan and Nautiyal (2013) reported seroprevalence of 11.6% in cattle and 10.9% in buffaloes in Uttar Pradesh.

Materials and Methods



3 MATERIALS AND METHODS

The present work deals with development of modified i-ELISA for antibody detection, screening of serum samples using many serological tests and development of modified molecular detection tests like PCR to identify *Brucella* infection in bovines (cattle and buffaloes) by using serum samples.

3.1 General Materials

3.1.1 Glasswares and Plasticwares

During the course of this study, properly cleaned, neutral and standard glass wares and plastic wares compatible with molecular biology work were used. Glasswares used in the study were procured from Borosil (India) and Schott Duran (Germany). Similarly, plasticwares were procured from Tarsons (India) and Axygen (USA). Vacutainers for collection of blood and serum were from BD Franklin lakes (USA).

3.1.2 Media, Stains, Chemicals, Buffers, Reagents etc.

The details of media, stains, chemicals, buffers and molecular biological reagents used during the study were procured as per the Appendix.

3.2 Chemicals and Reagents

All the chemicals and reagents used were of analytical grade and were procured from the SD fine Chemicals Limited, GENEI, SIGMA, Merck India Limited, Glaxo, SRL, IBI, Thermo scientific , MBI Fermentas, Biosafe Oligos, IDEXX, Zymo Research and HiMedia Limited.

Table 3.1 : Sources of bovine serum samples tested for brucellosis

Sl. No	Source of serum samples	Cattle		Buffalo		Total	
		Male	Female	Male	Female	Male	Female
Karnataka							
1.	Organized farm – A	-	-	2	76	2	76
2	Organized farm – B	-	22	-	33	-	55
3	Organized farm – C	-	-	-	35	-	35
4	Organized farm – D	-	-	2	198	2	198
5	Unorganized farm – A	-	19	-	14	-	33
6	Unorganized farm – B	-	84	-	89	-	173
7	Unorganized farm – C	-	47	-	38	-	85
8	Unorganized farm – D	-	26	-	19	-	45
9	Unorganized farm – E	-	30	-	-	-	30
	Total	**-**	**228**	**4**	**502**	**4**	**730**
Uttar Pradesh							
10	Organized farm – A	41	-	-	-	41	-
11	Organized farm – B	1	2	1	-	2	2
12	Organized farm – C	-	164	-	-	-	164
13	Organized farm – D	40	20	30	30	70	50
14	Organized farm – E	-	14	-	1	-	15
	Total	**82**	**200**	**31**	**31**	**113**	**231**
Uttarakhand							
15	Organized farm – D	-	35	-	-	-	35
	Grand Total	**82**	**463**	**35**	**533**	**117**	**996**
	Grand Total (C+B)	**(545)**		**(568)**		**(1113)**	

Table 3.2: Distribution of serum samples of bovine according to breed and sex

Sl.No	Breed	Sex		Total
		Male	Female	
A.	**CATTLE**			
1	Jersey	-	22	22
2	Khillar	-	98	98
3	Sahiwal	16	-	16
4	HF	53	20	73
5	Haryana	8	-	8
6	Vrindavani	1	-	1
7	Crossbred	4	215	219
8	ND (Non-descript)	0	103	103
B.	**BUFFALO**			
9	Murrah	35	340	375
10	Surthi	-	33	33
11	ND (Non-descript)	0	165	165
TOTAL		**117**	**996**	**1113**

3.3 Equipments

In the present study, equipments used were- ELISA reader (Bio-Rad Model 680), Electronic balance (Sartorius), Micro centrifuge (REMI), Deep freezer -20°C and -80°C, Millipore water purification system (Millipore, USA), Semidry western blotting apparatus (Atto, Japan), SDS PAGE assembly (Atto, Japan), Power pack (Bangalore genei), shaker incubator (Gallen Kamp), Gel documentation system (UVP), Vortex (Spinix), Horizontal gel electrophoresis apparatus (Bangalore genei, India), UV trans-illuminator (Genei), Vertical Autoclave (York Scientific), Vertical Laminar flow (Base Biosafe), Nanodrop® (Thermoscientific), Incubator (SD scientific Co), Micropipette of variable volume (Eppendorf) and thermo cycler (GeneAmp 9700, Applied biosystems).

3.4 Collection of Serum Samples

The research work was carried out at the CADRAD, IVRI, Izatnagar, UP. A total of 1113 bovine sera samples were collected for the present study, from Karnataka (734), Uttar Pradesh (344) and Uttarakhand (35). Out of 1113 samples, 545 were from cattle (82 male + 463 females) and 568 from buffaloes (35 male + 533 females (Table 3.1). The samples were also recorded breed wise. Among total sera samples collected 117 were of males and 996 of females (Table 3.2).

Bovine serum samples were collected from nine farms of Karnataka, five farms of Uttar Pradesh and one farm of Uttarakhand. About 5 ml of blood was collected in a sterile syringe and transferred to a sterile gel clot activator blood collection tube (VAC-T). After collection, the blood samples were kept overnight at 4ºC in refrigerator and centrifuged at 3,000 rpm (R8C Centrifuge, REMI) for 15 minutes. Serum was separated, collected in 2 ml sterile micro centrifuge tubes than 0.01% sodium azide was added. The serum samples were heat inactivated at 56ºC for 30 min and stored at – 20ºC. The collected bovine serum samples were subjected to different serological tests and DNA isolation for PCR.

3.5 Reference Bacterial Strains

The *Brucella abortus* S99 and *Brucella abortus* S19 were obtained from Biological Standardization, IVRI, Izatnagar and used as reference bacterial strains for preparation of antigen, cultural and molecular work.

3.6 Characterization of *Brucella abortus* S99

For characterization and identification of *Brucella abortus* S99, serological, biochemical and molecular tests were performed.

The procured culture was subjected to Gram staining and Modified Ziehl-Neelsen (MZN) staining for checking the purity of cultures and morphological characters.

3.6.1 Identification

The procured culture was subjected to agglutination and biochemical tests as described below.

3.6.1.1 Rapid slide agglutination test: One drop (0.03 ml) of *Brucella* positive field serum was taken on a glass slide by using micropipette. Loopful of culture from single colony was mixed thoroughly with the spreader and then the slide was rotated for four min. The result was read immediately. The clumping/agglutination was obtained considered as positive reaction.

3.6.1.2 Acriflavine test: One drop of acriflavine solution (1:1000 diluted in distilled water) was placed on a glass slide. Loopful of culture was mixed thoroughly and observed for agglutination. Since smooth colonies, remained in suspension no agglutination was formed.

3.6.1.3 Oxidase test: Standard oxidase discs (HiMedia Laboratories Ltd., Mumbai) were used to perform the test. Loopful culture from single colony was just touched on the disc. Since, immediately blue color developed, the test was considered as positive.

3.6.1.4 Catalase test: This test was performed by taking 2-3 drops of 3 per cent H_2O_2 on clean grease-free glass slide and single colony from petry plate was mixed with the help of a wire loop. Appearance of gas bubbles indicated positive test.

3.6.1.5 Nitrate reduction: Few drops of 4 days old broth culture were added to peptone water containing 0.1 percent potassium nitrate and then incubated under 5% CO_2 tension at 37°C for 2 days. Presence of nitrate was detected by adding approximately 1.0 ml of sulfanilic acid and 1.0 ml of α-naphthylamine reagent to nitrate broth culture. Development of a distinct red color (which may turn to brown rapidly) was considered as positive test.

3.6.1.6 Urease test: Urea agar slants were inoculated and incubated under 5% CO_2 tension at 37°C and observed up to 7 days. A positive reaction was observed by development of pink color in the slant.

3.6.1.7 Indole test: Few drops of xylene were added in a 4-day-old growth of the isolate in two ml of tryptone water and mixed thoroughly to dissolve indole and about 0.2 ml of Kovac's reagent was added from the side of the test tube. A Pink layer of xylene was considered as positive reaction.

3.6.1.8 Motility and production of H_2S: Motility Sulphide Medium (HiMedia Laboratories Ltd., Mumbai) was used for detection of motility and H_2S production. Loopful culture from single colony was stabbed into the tube and incubated at 37ºC. H_2S production was indicated by blackening of the medium. Since non-motile organisms revealed growth along the stabbed line.

3.7 Confirmation by Polymerase Chain Reaction (PCR)

PCR was used for confirmation of the *Brucella* isolates. The template DNA from the colony was prepared according to Wilson (1987) with minor modifications.

3.7.1 Preparation of material for nucleic acid extraction from the colony

The characteristic colonies picked from Brucella or Blood agar plates and suspended in 2 to 4 ml of PBS (pH 6.4). The suspension in PBS was centrifuged at 10,000 rpm for 10 min at 5°C. The supernatant was discarded and the pellet was used for extraction of nucleic acid.

3.7.2 Solutions used for extraction

i) 10 mM Tris-HCL
ii) Tris-EDTA (pH 8.0)
iii) 1 mM EDTA
iv) Proteinase K solution (20mg/ml, w/v) (MBI Fermentas)
v) SDS (10% w/v)
vi) 5 M Sodium chloride
vii) CTAB (Hexadecyl trimethyl ammonium bromide, 10% solution in 0.7M NaCl)

viii) Tris saturated phenol (pH 8.0)

ix) Chloroform

x) Isoamyl alcohol

xi) 7.5 M Ammonium acetate

xii) 70% Ethanol

xiii) Chilled absolute ethanol

xiv) 0.3X TE (Appendix)

3.7.3 Isolation of genomic DNA (template DNA) by proteinase K-SDS method

1. Cenrifuged the broth culture or suspended the loopful of colony in PBS. Resultant pellet containing bacterial cells was suspended in 2 ml of Tris-EDTA (pH 8.0), 250 µl of SDS (10% w/v) and 10 µl of proteinase K solution (20 mg/ml, w/v) added and incubated for 1 h at 37ºC.
2. Subsequently, 500 µl of 5 M NaCl followed by 100 µl CTAB (10% solution in 0.7 M NaCl) was added and incubated in a water bath for 10 min at 65ºC.
3. The solution was spun at 8,000 rpm for 10 min after mixing with equal volume of chloroform : isoamyl alcohol (24:1). The upper phase was transferred to a clean microfuge tube.
4. Equal volumes of phenol : chloroform : isoamyl alcohol (25:24:1) was added, mixed well by inverting, then spun for 10 min at 10,000 rpm. The upper aqueous phase was transferred to a clean microfuge tube.
5. In the collected supernatant, the DNA was precipitated with equal volume of chilled absolute ethanol in the presence of one-tenth volume of 7.5 M ammonium acetate.
6. The tube was centrifuged for 10 min at 11,000 rpm and the supernatant was discarded.
7. The pellet was washed with 70% ethanol and again spun for 10 min at 11,000 rpm.
8. Supernatant was discarded and the above step was repeated twice.
9. The pellet was dried at room temperature for overnight.
10. DNA was resuspended in 200 µl sterile distilled water or 0.3X TE and kept in water bath at 65ºC for one hour and stored at –20ºC till use.

3.7.4 PCR

For molecular characterization of *B.abortus* S99 culture the PCR technique used, the details of materials and methods mentioned in 3.15.5.5.2 and 3.15.5.5.3.

3.8 Preparation of Antigen from *B.abortus* S99

3.8.1 Growth on potato-infusion agar

For antigen production, the seed culture was used to inoculate a number of potato-infusion agar slopes that were incubated at 37°C for 48 hours. The growth checked for purity and resuspended in sterile PBS, pH 6.4, was used to seed layers of potato-infusion agar in Roux flasks. These were then incubated at 37°C for 5-6 days with the inoculated surface facing down. Each flask was checked for purity by Gram staining or samples of the growth, and the organisms were harvested by adding 20 ml of PBS, pH 7.2 to each flask. The flasks were gently agitated and the suspension was decanted. Then the suspension was centrifuged at 20,000g for 30 min at 4°C to settle the cells. The supernatant was used for the preparation of cultural filtrate antigen and the packed cells were subjected for preparation of killed whole cell antigen.

3.8.2 Growth on Brucella broth

The seed culture were inoculated in to sterile Brucella broth without any supplements. Since the *B.abortus* S99 was lab adopted, it do not require any corbon dioxide or Brucella selective supplements. The broth was incubated at 37°C for 6 days in a shaker incubator. The culture was centrifuged at 20,000g for 30 min at 4°C to settle the cells (Ellwood *et al*., 1967). The resulted supernatant was used for preparation of cultural filtrate antigen.

3.8.3 Preparation of cultural filtrate antigen

The supernatant from above processed potato-infusion agar and Brucella broth were filtered using 0.2µm membrane filters to remove the remaining bacteria. The filtrate was treated with 50-100mg each of ribonuclease and deoxyribonuclease and heat inactivated by keeping at 56°C for 30 min in a water bath. And later filtrate was dialyzed in 10 kDa cutoff dialysis membrane against sterile PBS (0.15 M-NaCl in 0.01 M phosphate buffer, pH 7.2) at 4°C for 2 to 3 days with intermittent change of PBS to remove unwanted low molecular proteins (<10

kDa). The filtrate was further concencentrated with PEG 8000 to reduce the volume 80 to 90% from original. To remove the contents of PEG 8000, it was again dialyzed with sterile PBS (0.15 M-NaCl in 0.01 M phosphate buffer, pH 7.2) at 4°C for a day. The resultant cultural filtrate was made aliquots; protein concentration was assessed and stored at -80°C till further used. Some aliquots were freeze dried in a lyophilizer and stored at -80°C for further use.

3.8.4 Killed whole cell antigen

The packed cells were resulted from 3.8.1 or organisms were directly harvested by adding 50-60 ml of phenol saline (0.5% phenol in 0.85% sodium chloride solution) to each flask. The flasks were gently agitated and suspension was collected in sterile tubes. Later, purity of culture was checked by Gram staining or modified Ziehl-Neelsen (MZN). The organisms were killed by heating at 80ºC for 90 minutes. Following a viability check, protein concentration assessed and the antigen was stored at -20ºC.

3.9 Protein Content of the Antigen

The protein content of prepared cultural filtrate and killed whole cell antigen of *Brucella abortus* S99 was estimated by Lowry (1951).

3.10 Raising of Antisera in Rabbits

The New Zealand White rabbits weighing 2 to 2.5 kg were used for raising antisera. For each antigen group was consisted of two rabbits. Animal models immunized with two different compounds: 1) Cultural filtrate antigen of *B. abortus* S99: 120 µg/ml. 2) Killed whole cell antigen of 120 µg/ml mixed with the same volume of complete Freund's adjuvant (CFA) solution using repeated aspiration with 18 G syringe (Siadat *et al.*, 2011). Prepared the site of injection with alcohol. Prepared antigen was given using $1\frac{1}{2}$" 23 G needle, 0.5 ml was injected into four sites intradermally on the back, 0.3 ml into two sites intramuscularly (thigh) and 0.2 ml into two sites subcutaneously in the neck region of rabbits. Immunization was carried out on 0, 7 and 14 days and hyperimmunized sera was collected on 0 (before the first injection and as the negative control), 7 (before the 2nd injection), 14 (before the 3rd injection) and 21 (one week after the 3rd injection) days. The serum samples were pooled and stored at -20°C.

3.11 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis was carried out as described by Laemmli (1970) in a vertical mini gel electrophoresis apparatus (Atto, Japan) with some modifications.

1. Glass plates were cleaned and set in a gel molding tray of electrophoresis apparatus and 12% resolving acrylamide gel solution for 6.503 ml was poured and allowed to polymerize.
2. After polymerization, 3 ml of stacking gel was poured over resolving gel and a suitable comb was placed over the gel.
3. The polymerized gel was mounted into the electrophoresis chamber and buffer reservoirs were filled with 1x Tris glycine Buffer.
4. Before loading, 30 µl of each sample along with 2x sample buffer were mixed and boiled for 5 min. A standard molecular weight protein marker was also run along with the samples.
5. Electrophoresis was carried out at constant current (110 volt), until the tracking dye reached the bottom of the gel.
6. The gel was taken out from the plates and stained with 0.25% (w/v) Coomassie brilliant blue G-250 and destained with destaining solution (Annexure).

The molecular weight of proteins in cultural filtrate antigen was determined by comparing the relative mobility with that of standard molecular weight markers (43 to 300 kDa, Fermentas life sciences)

3.12 Western Blot Analysis

To assess the immunoreactive proteins in the prepared antigen was done under test, immunoblotting was done as per the method of Towbin *et al.* (1979) with little modifications. After SDS-PAGE of the antigen prepared, the protein was transferred onto a nitrocellulose membrane (SIGMA, USA) from the gel using a semi-dry electroblotting apparatus (Atto, Japan) at a constant current of 20 volts for 90 minutes. Before putting the membrane into the

assembly, it was dipped in 100% methanol for 30 seconds. After blotting, the membrane was air dried briefly and then transferred to a solution of 3% skim milk powder in 0.01 M PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T) overnight at 4°C to block nonspecific sites. The nitrocellulose membrane was then washed three times with PBS-T and incubated for 2 hours at 37°C in 1:100 dilution of *B. abortus* hyperimmune serum. The membrane was then washed thoroughly with PBS-T and incubated with rabbit/anti-bovine HRPO-conjugate (1:200) for 2 hours at 37°C. It was again washed with PBS-T and developed in substrate solution containing 1mg/ml diaminobenzidene tetrahydrochloride (DAB, SIGMA) and 0.25µl/ml of 30% hydrogen peroxide in PBS. The color development was stopped by washing the membrane in distilled water.

3.13 Dot Blot of Antigens

The immunoreactive proteins in prepared antigens were quickly detected under this test. A nitrocellulose membrane (SIGMA, USA) was made into strip of 1-2 cm width and length depending on number of antigens. About 2-3 µl of different anigens was placed at 2-3 cm apart; control of positive and negative (PBS) was included. The drops were allowed to dry at room temperature or in an incubator. The blocking, primary antibody (serum), conjugate treatment and washing with PBST were similar like Western blotting. Finally color developed, was carried with a substrate solution containing 1mg/ml DAB and 0.25µl/ml of 30% hydrogen peroxide in PBS. The color development was further stopped by washing the membrane in distilled water and dried at room temperature before evaluation.

3.14 Development of i-ELISA Using Cultural Filtrate Antigen

Development and standardization of i-ELISA$^{Dev.}$ was done (Ekgatat *et al.*, 2008) with cultural filtrate antigen, which was extracted from *B. abortus* S 99.

3.14.1 Titration of antigen and serum using checker board method

The optimization of the required concentration of antigen and serum dilution in i-ELISA for the prepared cultural filtrate antigen was done with the checker board titration method. The microtitre plate planned for i-ELISA with a half plate for positive *Brucella abortus* and the remaining half with negative for the same disease. The antigen with reducing concentrations were taken in columns and serum with serial dilutions were taken in different rows. The highest

OD (P/N ratio) corresponding antigen concentration and serum dilution were used in developed i-ELISA.

3.14.2 ELISA Test Proper (i-ELISA$^{Dev.}$)

The cultural filtrate antigen of different concentrations was prepared by diluting in coating buffer (carbonate/bicarbonate buffer; pH-9.6), vortexed and 100µl per well was dispensed in flat bottom microtitre plate. The plate was then incubated at 4ºC overnight. Next day the plate was washed thrice using the phosphate buffer saline (PBS; 0.01M; pH-7.4) containing 0.05 percent Tween-20 (PBS-T). For blocking the remaining unbound surface area of the wells of the microtitre plate made with 5% skimmed milk powder was used, 200 µl of which was dispensed per well and incubated 2 h at room temperature. After incubation the plate was washed thrice with PBS-T. Before the final wash, the different serially diluted serum concentrations of both positive and negative was prepared. After the final wash, 100 µl of each diluted serum was dispensed into each well of the microtitre plate and incubated for 1 h at 37 ºC. At the end of incubation, the plate was washed four times with PBS-T. A 100 µl of working dilution anti-bovine conjugate tagged with HRPO (1:10,000) was dispensed in each well and the plate was again incubated at 37 ºC for 1 h. Then the plate was washed four to five times with PBS-T. After the last wash, 100 µl of substrate solution containing 6 mg OPD (Sigma) and 6 µl H_2O_2 (30%) mixed in 10 ml of substrate buffer (Citrate buffer; pH-4.5), was dispensed to each well. The plates were kept in the dark for 15 to 20 min for color development. After 15 min, the plate was checked for color development and in the case of no colour development the plate was left for 20 min and the reaction was stopped by adding 50 µl of 3M H_2SO_2 solution and absorbance was measured at 492 nm in an ELISA reader.

The optimum concentration of antigen and serum dilution for i-ELISA$^{Dev.}$ were determined by P/N ratio, which was calculated by corresponding OD of positive and negative serum samples**.** The cutoff value was calculated comparing gold standard tests like i-ELISA kit. The values above cutoff were recorded as positive and calculated by ROC curve analysis and dot diagram in Medcalc program.

3.14.3 Evaluation of i-ELISA$^{Dev.}$ by screening serum samples for bovine brucellosis

All the serum samples collected were subjected for bovine brucellosis testing using the i-ELISA$^{Dev.}$. Each sample placed in duplicate on microtitre plate and average OD was taken for test and compared with standard test *i.e.,* ELISA kit reading, the cutoff value calculated for the test by placing the values in a ROC curve. Above the cutoff OD values were declared as positive. The i-ELISA$^{Dev.}$ can be compared with other tests including ELISA kit to calculate sensitivity, specificity, concordance and kappa values. Based on these values the developed test is suitable for screening the serum samples to arrive the diagnosis of bovine brucellosis.

3.15 Diagnostic Tests

3.15.1 Rose Bengal Plate Test (RBPT)

The RBPT was performed according to the method described by Alton *et al.* (1975).

3.15.1.1 RBPT antigen

The antigen obtained from the I.V.R.I., Izatnagar, Uttar Pradesh was used for the test.

3.15.1.2 Procedure

The test was performed according to the manufacturer's literature. Serum samples and RBPT antigen were brought to the room temperature and then one drop (0.03 ml) of serum was taken on a clean, dry and non greasy glass slide by micropipette. The antigen bottle was shaken well to ensure homogenous suspension and then one drop (0.03 ml) of the antigen was added. The antigen and serum were mixed thoroughly with the spreader and then the slide was rotated for four min. The result was noted immediately after four min.

3.15.1.3 Observation of result

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

3.15.2 Standard Tube Agglutination Test (STAT)

The test was performed in clean glass tubes (14 mm x 100 mm) according to the method described by Alton *et al.* (1975).

Fig.3.1 : ELISA kit - CHEKIT Brucellose Serum, *Brucella abortus* Antibody Test Kit (IDEXX, Netherland)

Fig. 3.2 : Kit for isolation of DNA from serum - ZR Serum DNA Kit (Zymo Research, USA)

3.15.2.1 Brucella STAT antigen

The antigen obtained from the I.V.R.I., Izatnagar was used for the test.

3.15.2.2 Procedure

The test was performed according to the manufacturer's literature. All serum samples were tested up to a minimum of five dilutions. For high titre sera, more dilutions were prepared in order to achieve end point titer. Five agglutination tubes were placed in a rack. 0.8 ml of 0.5 % phenol saline was taken in a first tube and 0.5 ml in the rest of the tubes. 0.2 ml of serum was added in the first tube, mixed well and transferred 0.5 ml of diluted serum to the second tube. The process was continued up to the fifth tube and 0.5 ml was discarded from the last tube after mixing. 0.5 ml of antigen was added to each tube and mixed thoroughly. This provided final dilutions of 1:10, 1:20, 1:40, 1:80 and 1:160 and so on. Considering the special significance of 50% end point, a control tube was set up to simulate 50% clearing by mixing 0.5 ml of antigen with 0.5 ml of 0.5% phenol saline in an agglutination tube. All the tubes were incubated at 37ºC for 20 h before the result was recorded.

3.15.2.3 Observation of result

The degree of agglutination was judged by the opacity of the supernatant fluid. The highest serum dilution showing 50% or more agglutination (50% clearing) was considered as the titer of the serum. The titer so obtained was expressed in unit system by doubling of the serum titer as an International Unit (I.U.) per ml of serum.

3.15.2.4 Interpretation of result

40 I.U. per ml or above was considered positive for brucellosis in breeding bulls (cattle as well as buffaloes**).**

3.15.3 i-ELISA using IDEXX: CHEKIT Brucellose Serum – *Brucella abortus* Antibody Test Kit (i-ELISA^{Kit})

ELISA kit along with the user manual was procured from IDEXX, CHEKIT Brucellose serum-*Brucella abortus* Antibody Test Kit (Netherland) and the test was performed as per the protocol outlined in the user manual. The contents of the kit were as in Table 3.3 and Fig 3.1.

Table 3.3: Contents of *Brucella* ELISA Kit

1.	CHEKIT-Brucellose microtiter plate coated with *Brucella abortus* Antigen plates	10(2X5)
2.	CHEKIT-Brucellose Anti-Ruminant- IgG-PO conjugate, monoclonal, Antibody labelled with horseradish peroxidase	110 ml
3.	CHEKIT-Brucellose Control-Serum, weak positive	1.5 ml
4.	CHEKIT-Brucellose Control-Serum, negative	1.5 ml
5.	CHEKIT -10X wash concentrate	480 ml
6.	CHEKIT –TMB substrate solution	100 ml
7.	CHEKIT - Stop solution	100 ml

Store all reagents at 2-8º C

3.15.3.1 Preparation of reagents

CHEKIT wash solution

Determined the amount of CHEKIT wash solution was needed for washing microtiter plate and diluting the samples and controls. Diluted the CHEKIT -10X wash concentrate 1:10 with water (1part concentrate with 9 parts water). It was prepared under sterile conditions, the CHEKIT wash solution was stored for one week at 2-8º C.

3.15.3.2 Test protocol

All reagents were made allowed come to room temperature (18º C- 25º C) before use. Reagents were mixed by gentle swirling or vortexing.

3.15.3.3 Short incubation

1. Dispensed 90 µl CHEKIT-wash solution into each well of the microtiter plate.
2. 10 µl of undiluted serum samples and controls were added into appropriate wells of the microtiter plate. Resulted final dilution was 1:10.
3. The contents were mixed within each well by gently shaking the microtiter plate briefly.
4. The microtiter plate was covered with a lid and incubated for 60 minutes (± 5 minutes) at 37ºC (± 2ºC) in a humid chamber.

3.15.3.4 Overnight incubation

1. Dispensed 190 µl CHEKIT-wash solution into each well of the microtiter plate.

2. 10 µl of undiluted serum samples and controls were added into appropriate wells of the microtiter plate. Final dilution = 1:20.
3. The contents were mixed within each well by gently shaking the microtiter plate briefly.
4. The microtiter plate was covered with a lid and incubated for 14 to 18 hours at 2ºC to 8ºC in a humid chamber.

3.15.3.5 After Short or Overnight Incubation

1. The plate was washed by putting 300 µl CHEKIT-wash solution per well for three times. The liquid contents of all wells were aspirated after each wash. Following the final aspiration, firmly tapped residual wash fluid from each plate onto absorbent material. The plate drying was avoided between washes and prior to the addition of the next reagent.
2. 100 µl CHEKIT-Brucellose Anti-Ruminant- IgG-PO conjugate was dispensed into each well.
3. The microtitre plate was covered and incubated for 60 minutes (± 5 minutes) at 37ºC (± 2ºC) in a humid chamber.
4. Step 5 was repeated.
5. 100 µl CHEKIT –TMB substrate was dispensed into each well.
6. The microtitre plate was incubated at room temperature (18º C - 25º C) for 15 minutes.
7. The color reaction was stoped by adding 100 µl of CHEKIT - Stop solution to each well. The stop solution was dispensed in the same order and at the same speed as the substrate.
8. The results were recorded using a phototometer at a wave length of 450 nm.

3.15.3.6 Results

To validate the assay, the OD of the positive control should not exceed 2.000 and the OD of the negative control should not exceed 0.500. The difference between the positive and negative control must be $\geq$0.300. The microtitre plate OD was recorded within two hours after the addition of stop solution.

3.15.3.7 Calculation

The OD of duplicates was averaged. The OD of the positive control (OD pos) were corrected by substracting the OD of negative control (OD neg):

Positive control: OD pos – OD neg

Sample: OD sample- OD neg

Analyze Te samples in relation to the negative and the positive controls with the formula: Value (%) = OD sample- OD neg / OD pos – OD neg X 100%

3.15.3.8 Interpretation of Results for serum samples

Value	< 80 %	≥80 %
Interpretation	Negative	Positive

3.15.4 i-ELISA using cultural filtrate antigen (i-ELISA$^{Dev.}$)

The antigen used, procedure, observation of result and interpretation was similar as mentioned above in detail (3.14).

3.15.5 Serum based PCR (*omp2a*-PCR$^{Dev.}$)

3.15.5.1 Primers

The genus specific *omp2a* gene targeting, 2A forward and 2A reverse primers were designed (Table 3.4). The gene targeting 31 kDa *Brucella* cell surface protein-31 (*bcsp31*) designed by Baily *et al.* (1992) (B4/B5) to amplify 223 bp product were procured from Biosafe and used for PCR assay. The BAF and BAR primers targeting *IS711* (Doust *et al*., 2007) were procured from Biosafe and used in PCR assay.

Table 3.4: List of primers used in PCR assay

Name of primers Sequence 5'% 3'	Product length (bp)	References
O2A (F) CGAACAGGGTGG CGAAGAC O2A (R) TCCGGCGTAATGGTGAATC	396	Designed
B4 (F) TGG CTC GGT TGC CAA TAT CAA B5 (R) CGC GCT TGC CTT TCA GGT CTG	223	Bailey *et al*. (1992)
BAF (F) GAC GAA CGG AAT TTT TCC AAT CCC BAF (R) TGC CGA TCA CTT AAG GGC CTT CAT	498	Doust *et al*. (2007)

(F) = Forward primer; (R) = Reverse primer

3.15.5.2 Isolation of genomic DNA by conventional method

DNA was isolated from serum samples using the method described by Kaushik *et al.* (2006) with minor modifications. The lysis buffer containing 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 5 mM MgCl2, 0.3 mg of gelatin per ml and 1.0% polysorbate 20 was prepared. For extraction of DNA from serum, 100 µl samples were added to 100 µl of lysis buffer to which Proteinase K was added to a final concentration of 60 mg/ml. The mixture was incubated for 60 min at 55°C in water bath. Proteinase K was inactivated by keeping vials in a water bath with temperature of 95°C for 10 min. It was followed by centrifugation at 12,000 x g for 10 min at 4°C. Collect supernatant and added an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1), mixed well by inverting, then spun for 10 min at 10,000 x g to which 0.1 volumes of sodium acetate (3 M) and 0.6 volumes of isopropanol were added. The contents were mixed gently and kept on ice for 1 h and centrifuged at 10,000 x g for 10 min. The pellet was washed with 70% alcohol twice and dried in cabinet at room temperature. Final pellet was suspended in 20 µl of Tris-EDTA buffer and stored at –20°C till further use.

3.15.5.3 DNA isolation using the ZR Serum DNA Kit (Zymo Research, USA)

Serum DNA Kit was procured from Zymo Research, USA for isolation of *Brucella* DNA from serum samples (Fig 3.2) and its components were mentioned bellow as per the manufacturer.

3.15.5.3.1 Materials

Genomic Lysis Buffer	:	1000 ml Storage at room temp.
DNA Wash Buffer	:	24 ml Storage at room temp.
DNA Elution Buffer	:	4 ml Storage at room temp.
ZymoBeads	:	1 ml Storage at room temp

3.15.5.3.2 Product description

ZR Serum DNA Kit was based on a state of the art, single buffer procedure for rapid DNA isolation from large volume serum and plasma samples. The product was optimized to yield high quality DNA from samples without RNA contamination. The ZR Serum DNA Kit was recovered genomic DNA from serum. The uniquely formulated Genomic Lysis Buffer was efficiently lysis cells, virus and cellular particles releasing DNA which was then adsorbed

directly onto the surfaces of the provided ZymoBeads. The resulted DNA/ZymoBead complexes are separated by centrifugation and then washed to remove contaminants. DNA was eluted from beads directly into the applied low-salt Elution Buffer. DNA was purified from ZR Serum DNA Kit found ideal for PCR.

b) Method

3.15.5.3.3 Buffer preparation

About 96 ml of 100% ethanol was added to the DNA Wash Buffer concentrate. Similarly, beta-mercaptoethanol was added to Genomic Lysis Buffer to get the final dilution of 0.5 ml per 1000 ml.

3.15.5.3.4 Protocol

- The following protocol was designed for serum samples. The volume (s) that can be processed can be adjusted depending on experimental needs. The standard procedure using 10 µl ZymoBeads. However, amount of ZymoBeads can be adjusted to suit any particular application.
- Ensure that the ZymoBeads are resuspended by vortexing. A 500 µl of serum was taken in a tube, to which 4 volumes of Genomic Lysis Buffer was added to make (1:4) dilution, then 10 µl of ZymoBeads was added. Finally 0.3 volumes of isopropanol was added to the mixture.
- Mixed properly by placing sample on a rotar and kept overnight at 4°C.
- Centrifuged the tubes for 2 minutes and supernatant was discarded.
- Resulted pellet was resuspend with 500 µl of DNA Wash Buffer.
- Centrifuged for 2 minute in a microcentrifuge and supernatant was discarded.
- Resulted pellet was again resuspended with 500 µl of DNA Wash Buffer.
- Recentrifuged briefly and removed any residual wash buffer. Air-dried the pellet for 15 minutes.
- 20 µl of DNA Elution Buffer was added to the ZymoBeads and resuspend by repeated pipetting.
- Centrifuged at 10,000 x g for 1 minute.
- Supernatant was collected, which contained the purified DNA. The DNA was assessed by Nanodrop method and stored at - 20°C till further use.

3.15.5.4 Quantitation and quality assessment of DNA

a) Materials

i) Agarose gel (0.8%)

ii) Gel loading buffer 6X (Appendix)

iii) 1X TBE (Appendix)

iv) Ethidium bromide

b) Method

Quality and purity of DNA were checked by submarine agarose gel electrophoresis using 0.8% agarose in 1X TBE (pH 8.0) buffer (Sambrook *et al.*, 1989), with ethidium bromide (1%) was added @ 5µl/100ml. The wells were loaded with mixture of 5µl of isolated DNA and 1µl of 6X gel loading buffer dye. Electrophoresis was carried out at 5V/cm for 20 min at room temperature and then the DNA was visualized under UV transilluminator.

Quantification of DNA was calculated by spectrophotometric method. OD at 260 and 280 nm was taken in UV spectrophotometer with eluting buffer as reference. Purity of DNA was judged on the basis of OD ratio at 260:280 nm using Nanodrop® method. The samples with acceptable purity (*i.e.* ratio 1.7-1.9) were used for PCR**.**

3.15.5.5 PCR

3.15.5.5.1 *omp2a*-PCR$^{Dev.}$

The designed OMP2A primers were used to standardize PCR technique (Table 3.4) for detection of *Brucella* in bovine serum. The protocol followed and the course of the study was as follows.

PCR mixture		**Conditions**	
Master mix	12.5 µl	Initial denaturation	94° to 95°C 5 min
Forward primer	0.5 to **1.0 µl**	Denaturation	94°C for ½ to **1 min**
Reverse primer	0.5 to **1.0 µl**	Annealing	**55°C** to 59°C for ½ to **1 min**
DNA template	1.0 to 5.0 µl **(3µl)**	Extension	72°C for ½ to **1 min**
NFW to make	25 µl	Repeated for	35 cycles
		Final extension	72°C 10 min

(The PCR mixture and the conditions boolded fitures given optimum conditions)

3.15.5.5.2 *bcsp31*-PCR

The *bcsp31* gene based (Bailey *et al.*, 1992) primers were used to detect the *Brucella* DNA in serum samples. It amplifed a PCR product size of 223 bp. This test was used to compare the developed modified *omp2a* gene based PCR technique to arrive sensitivity and specificity. The reaction was carried according to the published as mentioned below (Table 3.4 to 3.6).

Table 3.5: Components of PCR, its quantity and concentration for thermal cycling reaction.

Sr. No.	Components of PCR	Quantity
1.	PCR Master Mix (2X)	12.5 µl
2.	Forward Primer (10 pmol/µl)	1.0 µl
3.	Reverse Primer (10 pmol/µl)	1.0 µl
4.	Template DNA	3.0 µl
5.	Distilled water	7.5 µl

Table 3.6: Thermal cycling conditions and steps for B4/ B5 and BAF/BAR primer pairs in PCR

(Forward and Reverse)	Primers	
	B4/ B5	BAF/BAR
Initial denaturation	93°C, 5 min	94°C, 5 min
Denaturation	90°C, 1 min	94°C, 1 min
Annealing	64°C, 30 sec	59°C, 1 min
Extension	72°C, 1 min	72°C, 1 min
	Repeated for 35 cycles	Repeated for 35 cycles
Final extension	72°C, 10 min	72°C, 1 min

3.15.5.5.3 *IS711*-PCR

The species specific BAF and BAR primers were used to screen the *omp2a*-PCR$^{Dev.}$ and *bcsp31*-PCR positive samples to identify *B.abortus.* It amplified a product size of 498 bp.

a) Materials

i) 2X Master Mix (MBI Fermentas)

ii) Primers

iii) Extracted DNA

iv) Thin walled PCR tubes of 200 µl capacity (Bio-Rad)

v) Tubes of 500µl capacity (Axygen)

vi) Nuclease free distilled water

b) Method

PCR of DNA isolates from serum by conventional and kit methods was carried out in a final reaction volume of 25 µl in a thermal cycler (GeneAmp 9700, Applied Biosystems). Quantity and concentration of various components used for colony PCR were as per Table 3.5. Cycling conditions for different primer were as per Table 3.6.

c) Visualization of PCR amplified products by agarose gel electrophoresis

i) Materials

1. Agarose gel (2.0 %)
2. Gel loading buffer 6X
3. 1.0 X TBE (Appendix)
4. Ethidium bromide

ii) Method

The 2.0 % agarose gel containing ethidium bromide (@0.5 µg/ml) was prepared in 1.0 X TBE buffer. Each well of the gel was loaded with a mixture of 10 µl of the PCR products and 2 µl of gel loading buffer (6X). The gel was run at constant current of 80 to 100 V for 30 to 45 min. The amplified product was visualized as a single compact band of the expected size under UV light and documented by a gel documentation system (UVP /BIO-RAD, molecular imager, Gel doc XR+ imaging system).

3.16 Analysis of Data / Statistical Analysis

3.16.1 Relative sensitivity and Relative specificity

The relative sensitivity and relative specificity of the test was calculated using the method described by McDiarmid and Hellstrom (1987).

$$\text{Relative Sensitivity (\%)} = \frac{\text{Serum samples positive to both, test compared and standard test}}{\text{Serum samples positive to standard test}} \times 100$$

$$\text{Relative Specificity (\%)} = \frac{\text{Serum samples negative to both, test compared and standard test}}{\text{Serum samples negative to standard test}} \times 100$$

3.16.2 Predictive Value

When using either serological or other screening tests to determine the presence of disease in a population, it is important to know the probability that an animal 'positive' according to test is actually diseased, alternatively that a test 'negative' is actually healthy. These predictive values were calculated as follows:

$$\text{Positive predictive value} = \frac{\text{No. of animals diseased and showing a positive test result}}{\text{Total no. of animals showing a positive test results}} \times 100$$

$$\text{Negative predictive value} = \frac{\text{No. of animals diseased and showing a negative test result}}{\text{Total no. of animals showing a negative test results}} \times 100$$

3.16.3 Prevalence

The prevalence was calculated by dividing the number of animals with the disease or condition at a particular point of time by the number of individuals examined. True prevalence is the proportion of animals in the population which really do have the disease in question regardless of their test result. From a test result point of view, it includes the "true" positives and the "false" negatives. Apparent prevalence is the proportion of animals in the population giving a positive test result regardless of their true status of the disease in question. From a test result point of view, it is all the test positive animals, some of whom will be "true" positives and some which are "false" positives.

3.16.4 Accuracy of prediction

Accuracy is an indication of the extent to which a test confirms to truth. The accuracy is influenced by factors like cross-reactivity, inhibitory substances and agglutinins which frequently yield false positives. A false negative may be due to the choice of wrong test, wrong timing of the test, and presence of blocking antibodies in the serum samples.

From the data a 2x2 contingency table can be formed as bellow :

Serological test		**No. of sera tested with Gold standard /Reference Test**		**Total**
		Positive	**Negative**	
No. of sera tested With other serological tests (Index test)	Positive	a	b	a+b
	Negative	c	d	c+d
Total		**a+c**	**b+d**	**N=a+b+c+d**

The notations used above are defined as below.

a = Number of samples positive to both conventional and the gold standard tests

b = Number of samples positive for conventional but negative to the gold standard test

c = Number of samples negative for conventional but positive to the gold standard test

d = Number of samples negative for both conventional and the gold standard tests

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

From the table following parameters were calculated :

Sensitivity: It is the capacity of the test to detect diseased animals, when compared with the gold standard test (a/a+c x 100).

Specificity: It is the capacity of the test to detect non-diseased animals, when compared with the gold standard test (d/b+d x 100).

Overall agreement/ Accuracy: Is the proportional similarity of the results of both the tests (a+d/N x100).

Positive Predictive Value = a/(a+b) x100

Negative Predictive Value = d/(c+d) x100

Apparent prevalence = a+b/N x100

True prevalence = a+c/N x 100

3.16.5 Likelihood ratios (LR)

The probability before the test is carried out that the subject has the disease, known as the prior probability of disease. The positive and negative predictive values are the revised

estimates of the same probability of those subjects who are positive and negative on the test, and are known as posterior probabilities. The ratio of these probabilities is called the likelihood ratio, calculated as sensitivity /(1 - specificity). The LR is used to assess how good a diagnostic test is and to help in selecting an appropriate diagnostic test(s) or sequence of tests. They have advantages over sensitivity and specificity because they are less likely to change with the prevalence of the disease, they can be calculated for several levels of the symptom/sign or test and they can be used to combine the results of multiple diagnostic tests. A high LR may show that the test is useful, but it does not necessarily follow that a positive test is a good indicator of the presence of disease.

The LR of a positive test result (LR+) = sensitivity / (1-specificity)

The LR of a negative test result (LR-) = (1-sensitivity) / specificity

3.16.6 Odd ratio

Unfortunately, none of these indicators mentioned above in itself, validly represent the test's discriminatory performance. In addition to its global meaning of agreement between test and reference standard, accuracy in its specific sense refers to the percentage of animals correctly classified by the test under evaluation. This percentage depends on the prevalence of the target disorder in the study group whenever sensitivity and specificity are not equal, and it weights false positive and false negative findings equally. The odds ratio can be used as a single indicator of test performance. It is not prevalence dependent, and may be easier to understand, as it is a familiar epidemiologic measure (Kraemer, 1992; Korte, 1993).

The diagnostic odds ratio (DOR) of a test is the ratio of the odds of disease in test positives relative to the odds of disease in test negatives. The value of a DOR ranges from 0 to infinity, with higher values indicating better discriminatory test performance (Afina *et al.*, 2003).

$$DOR = \frac{Se / (1 - Se)}{(1 - Sp) / Sp}$$

3.16.7 Concordance

Concordance percentage was calculated using the following formula (Perrin and Sureau, 1987):

$$\text{Concordance (\%)} = \frac{\text{Serum positive in both tests + Samples negative in both tests}}{\text{Total number of samples tested}} \times 100$$

3.16.8 Kappa statistics

The agreement between the tests was evaluated by applying kappa statistic as explained by Thrusfield (2005). The kappa values take into account the observed and expected proportion of agreement and it will give a true picture of agreement existing between the tests. Kappa values range 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

OP (Observed Prevalence) = (a+d)/n

EP (Expected Prevalence) = [(a+b)/n X (a+c)/n] + [(c+d)/n X (b+d)/n]

k = (OP – EP) / (1-EP)

3.16.9 Receiver Operator Characteristic (ROC) curve analysis

It is a plot of the true positive rate against the false positive rate for the different possible cut points of a diagnostic test. ROC curves are used in diagnostic field to determine a cutoff value for a clinical test.

An ROC curve demonstrates several things:

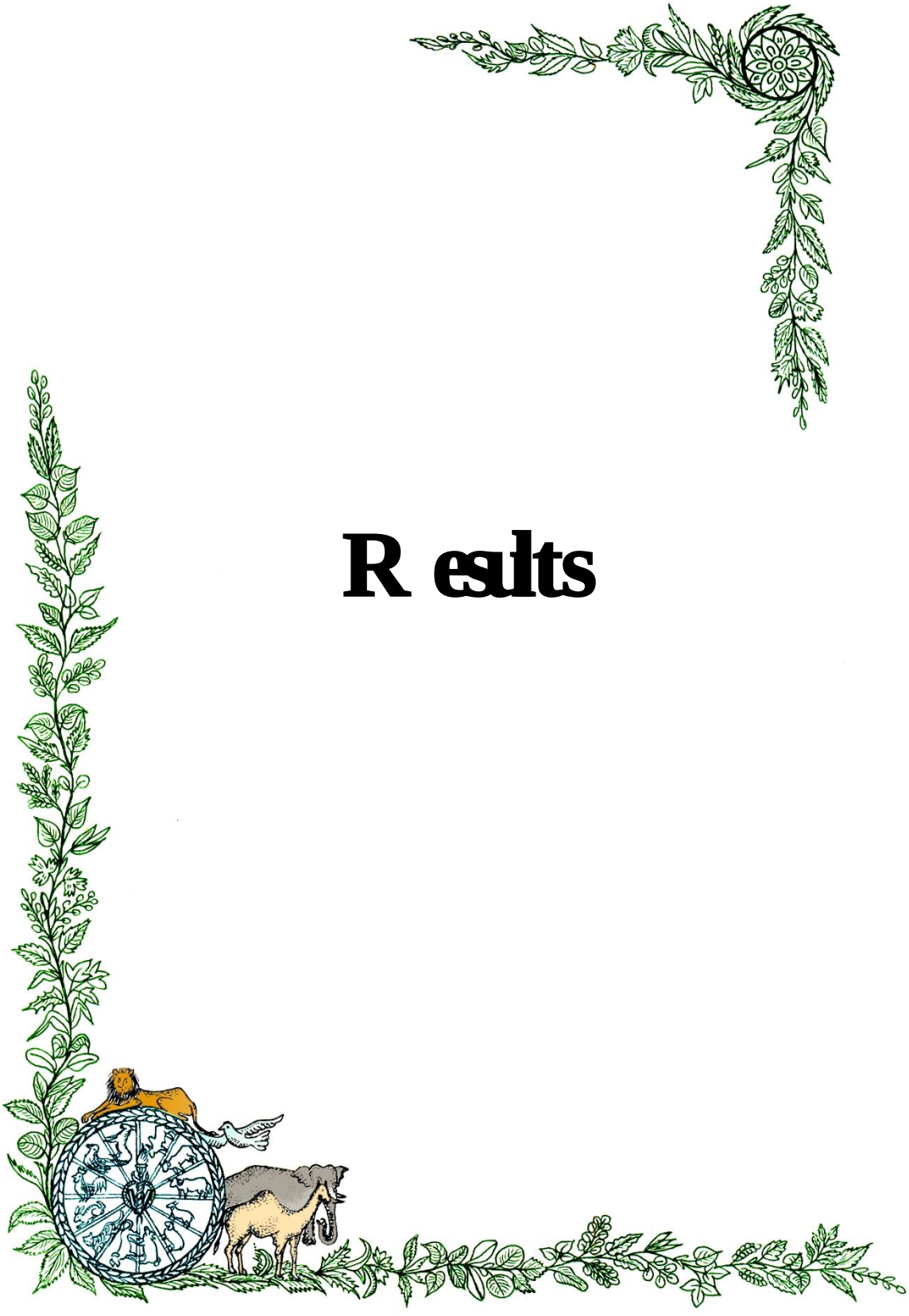
1. It shows the tradeoff between sensitivity and specificity (any increase in sensitivity will be accompanied by a decrease in specificity).
2. The closer the curve follows the left-hand border and then the top border of the ROC space, the more accurate the test.
3. The closer the curve comes to the 45-degree diagonal of the ROC space, the less accurate the test.
4. The slope of the tangent line at a cut point gives the LR for that value of the test.
5. The AUC is a measure of text accuracy.

In a ROC curve, the true positive rate (sensitivity) is plotted in function of the false positive rate (1-Specificity) for different cutoff points. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the two distributions) has a ROC curve that passes through the upper left corner (100% sensitivity, 100% specificity). Therefore the closer the ROC curve is in the upper left corner, the higher the overall accuracy of the test (Zweig and Campbell, 1993). The ROC curve in the essence is a series of likelihood ratios corresponding to each cutoff value. Since likelihood ratios are independent of disease prevalence, the ROC curve provides a standard approach to the evaluation of diagnostic test performances (Swets, 1988).

The AUC is a global assessment of a test's performance. This area equals the probability that a random individual with a disease has a higher value of the test variable than a random healthy individual. A perfect test yields an AUC of 1, whereas an uninformative test gives a value of 0.5 (Thrusfield, 2005). According to an arbitrary guideline, one could distinguish between non- informative (AUC= 0.5), less accurate (0.5< AUC= 0.7), moderately accurate (0.7 < AUC=0.9), highly accurate (0.9<AUC< 1) and perfect test (AUC=1) (Greiner *et al*., 2000). The Medcalc software was used to plot ROC curve and this program also provides the information in the form of interactive dot diagram.

3.16.10 McNemar's test

A modification of the χ 2 test – McNemar's change test- can be applied to related samples. The McNemar test is a test on a 2x2 classification table when the two classification factors are dependent, or when one wants to test the difference between paired proportions. The modified χ 2 formula only uses the values that are not in agreement between the two tests, *i.e.*, values from discordant pairs. The Medcalc software gives the difference between the proportions (expressed in %) with 95% confidence interval. When the (two-sided) p- value is less than the conventional 0.05, the conclusion is that there is a significant difference between the two proportions.



Results

4 RESULTS

4.1 Characterization of Reference Culture

The procured reference culture was confirmed as *Brucella abortus* S99 by subjecting to Gram staining and modified Ziehl-Neelsen (MZN) staining. On the basis of Gram staining, it was found to be Gram negative, coccobacillary rods, whereas by MZN staining they appeared to be red. The culture found positive by Rapid Slide Agglutination Test, in which clump was formed. The culture further identified by biochemical tests, found in smooth form by acriflavine test. Oxidase, catalase, urease and H_2S were produced but negative for indole, non-motile and reduced nitrate. By PCR, it was confirmed as *B. aborts* by obtaining 498 bp and 223 bp amlicon by *IS711* and *bcsp31*-PCR, respectively.

4.2 Protein Content of the Antigens

The protein content of culture filtrate was very less (0.02 mg/ml) in original. After dialysis and followed by PEG 8000 concentration, it increased significantly. The volume of the original was reduced 80 to 90% by PEG concentration. The protein content of cultural filtrate and killed whole cell antigen prepared from *B. abortus* S99 were estimated to be 2.5 mg/ml and 1.15 mg/ml, respectively, by Lowry method. These proteins were stored at - 20°C and - 80°C in aliquots till further use.

4.3 SDS-PAGE Profile of the Antigens

The SDS-PAGE of cultural filtrate and killed whole cell antigen on 12% polyacrylamide gel was visualized (Fig. 4.1).

4.4 Antisera Raised in Rabbits

The antisera raised in rabbits against the *B.abortus* S99 cultural filtrate antigen and killed whole cell antigen contained antibodies against antigens as revealed by i-ELISA, immuno dot blot (Fig. 4.2) and Western blot techniques (Fig. 4.3).

4.5 Immuno Dot Blot Analysis of Antigens

The culture filtrate antigen and killed whole cell antigen of *B.abortus* S99 culture were subjected to dot blot reaction using both hyperimmune sera and infected bovine sera. Both the antigens reacted with the antisera like positive control antigen. There was no reaction with negative control antigen (protein) and PBS (Fig. 4.2).

4.6 Western Blot Analysis of the Antigens

Immunodominant reactive proteins were detected in both the cultural filtrate and killed whole cell antigen, with infected bovine serum as well as hyperimmune rabbit serum, was evaluated by Western blot assay (Fig. 4.3).

4.7 Serum Samples

In the present study, a total of 1113 serum samples were collected from different sources and were subjected to four different serological tests, *viz.*, RBPT, STAT, i-ELISA^{Kit} and i-ELISA$^{Dev.}$ for detection of *Brucella* antibodies (Table 4.1). Randomly selected 100 positive serum samples in i-ELISA^{Kit} and 100 randomly selected negative samples in i-ELISA^{Kit} were tested with developed modified serum based-PCR to detect DNA in the serum samples.

4.8 RBPT

Out of 1113 bovine serum samples, 189 (16.98%) were found positive by RBPT (Table 4.1). Of the 189 RBPT positive samples, 46 (4.13%) samples were detected as negative by i-ELISA^{Kit}. Of the 924 (83.01%) RBPT negative samples, 157 (14.10%) were found positive i-ELISA^{Kit} (Table 4.2).

4.9 STAT

A total of 1113 serum samples were also tested by STAT, out of which 239 (21.47%) were detected as positive and 874 (84.78%) as negative (Table 4.1). The titer of positive

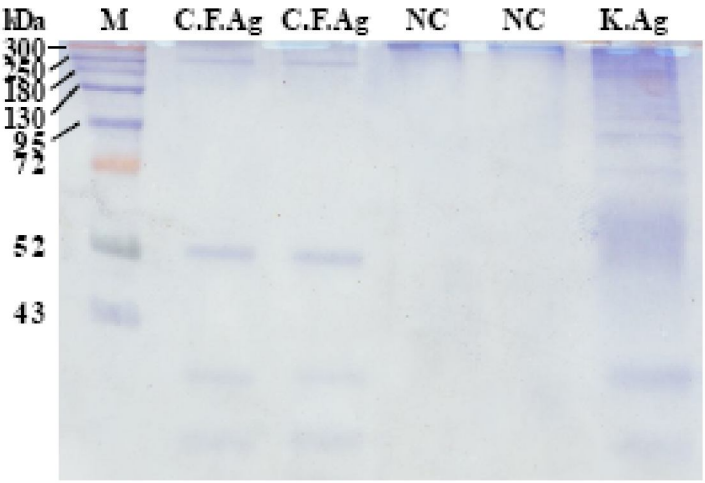
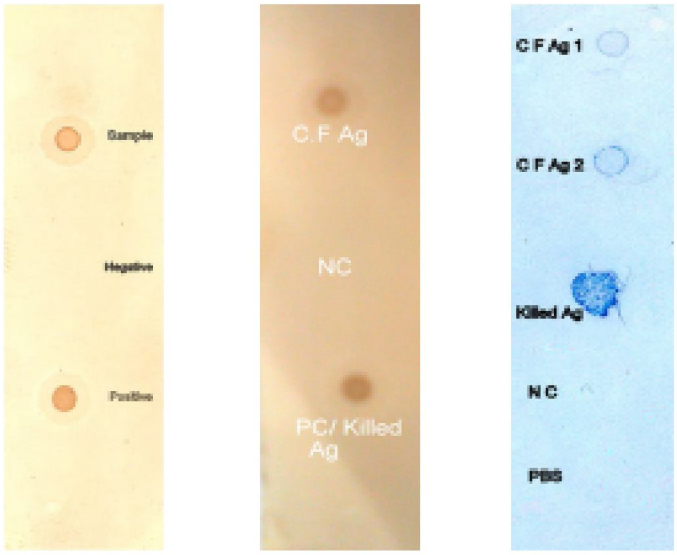


Fig. 4.1 : SDS-PAGE profile of cultural filtrate antigen of *B.abortus* S99

M : Protein marker
C.F.Ag : Cultural filtrate antigen
NC : Negative control
K.Ag : Killed whole cell antigen



a) Hyperimmune sera b) Infective sera c) Infective sera

Fig. 4.2 : Dot blot of prepared cultural filtrate antigen (*B.abortus* S99) with contols

C.F.Ag : Cultural filtrate antigen
NC : Negative control
PC : Positive control
PBS : Phosphate buffered saline

Table 4.1: Source wise seroprevalence of bovine brucellosis

Sl. No	Source of Serum Samples	RBPT	(%)	STAT	(%)	i-ELISA^{Kit}	(%)	i-LISA$^{Dev.}$	(%)
Karnataka									
1	Organized farm – A	28/78	(35.89)	34/78	(43.58)	48/78	(61.53)	57/78	(73.07)#
2	Organized farm – B	25/55	(45.45)	28/55	(50.90)	31/55	(56.36)	29/55	(52.72)
3	Organized farm – C	10/35	(28.57)	10/35	(28.57)	7/35	(20.0)	7/35	(20.0)
4	Organized farm – D	33/200	(16.50)	33/200	(16.50)	78/200	(39.0)	80/200	(40.0)
5	Unorganized farm – A	7/33	(21.21)	11/33	(33.33)	17/33	(51.51)	19/33	(57.57)
6	Unorganized farm – B	14//173	(8.09)	17/173	(9.82)	29/173	(16.76)	38/173	(21.96)
7	Unorganized farm – C	18/85	(21.17)	38/85	(44.70)	20/85	(23.52)	27/85	(31.76)
8	Unorganized farm – D	5/45	(11.11)	6/45	(13.33)	9/45	(20.0)	10/45	(22.22)
9	Unorganized farm – E	3/30	(10.0)	9/30	(30.0)	10/30	(33.33)	10/30	(33.33)
Uttar Pradesh									
10	Organized farm – A	12/41	(29.26)	12/41	(29.26)	1/41	(2.43)	2/41	(4.87)
11	Organized farm – B	2/4	(50.0)	4/4	(100.0)	3/4	(75.0)	3/4	(75.0)
12	Organized farm – C	17/164	(10.36)	22/164	(13.41)	40/164	(24.39)	43/164	(26.21)
13	Organized farm – D	0/120	(0)	0/120	(0)	0/120	(0)	4/120	(3.33)
14	Organized farm – E	13/15	(86.66)	13/15	(86.66)	4/15	(26.66)	5/15	(33.33)
Uttarakhand									
15	Organized farm – D	2/35	(5.71)	2/35	(5.71)	3/35	(8.57)	6/35	(17.14)
	Cattle	76/545***	(13.94)	114/545	(20.91)	111/545	(20.36)	130/545	(23.85)
	Buffalo	113/568	(19.89)	125/568	(22.0)	189/568	(33.27)	210/568	(36.97)
	Total (%Ve)	**189/1113****	**(16.98)**	**239/1113**	**(21.47)**	**300/1113**	**(26.95)**	**340/1113***	**(30.54)**

No. of positive samples / Total No. of samples (% positive)

* Significant difference was observed among all the tests ($p<0.05$).

**** Highly significant difference was observed between every two tests ($p<0.0001$).

***** Highly significance difference between species ($p<0.0001$) on all tests except STAT ($p>0.05$).

Table 4.2: Comparison of i-ELISA^{Kit} and RBPT in diagnosis of bovine brucellosis

Serological test		i-ELISA^{Kit}		
		Positive	**Negative**	**Total**
RBPT	Positive	143 (a)	46(b)*	189(a+b)
	Negative	157(c)	67(d)	924(c+d)
	Total	300(a+c)	813(b+d)	1113 (a+b+c+d=N)

Relative sensitivity = a / a + c × 100 = 47.66%
Relative Specificity = d / b + d × 100 = 94.34%
Positive Predictive value = a / a + b × 100 = 75.66%
Negative Predictive value = d / c + d × 100 = 83.00%
Apparent Prevalence = a + b / N × 100 = 16.98%
True Prevalence = a + c / N × 100 = 26.95%
Accuracy of prediction = a + d / N ×100 = 81.76%
LR+ = Sensitivity / (1-Specificity) = (a / a + c) / (b / b + d) = 8.42
LR- = (1 – Sensitivity) / Specificity = (c / a + c) / (d / b + d) = 0.55
Kappa Vaue = (OP-EP)/(1-EP) = 0.475 (Moderate Agreement)
DOR = Se / (1 – Se) / (1 – Sp) / Sp = 1.03
* Highly significant difference was observed between two tests for the seroprevalence (P<0.0001).

Table 4.3: Comparison of i-ELISA^{Kit} and STAT in diagnosis of bovine brucellosis

Serological test		i-ELISA^{Kit}		
		Positive	**Negative**	**Total**
STAT	Positive	167 (a)	72(b)*	239 (a+b)
	Negative	133 (c)	741(d)	874 (c+d)
	Total	300 (a+c)	813 (b+d)	1113 (a+b+c+d=N)

Relative Sensitivity = a / a + c × 100 =55.66 %
Relative Specificity = d / b + d × 100 = 91.14%
Positive Predictive value = a / a + b × 100 =69.87 %
Negative Predictive value = d / c + d × 100 = 84.78%
Apparent Prevalence = a + b / N × 100 =21.47 %
True Prevalence = a + c / N × 100 = 26.95%
Accuracy of prediction = a + d / N ×100 =81.58 %
LR+ = Sensitivity / (1-Specificity) = (a / a + c) / (b / b + d) = 6.31
LR- = (1 – Sensitivity) / Specificity = (c / a + c) / (d / b + d) = 0.48
Kappa Vaue = (OP-EP)/(1-EP) = 0.50 (Moderate Agreement)
DOR = Se / (1 – Se) / (1 – Sp) / Sp = 1.029
* Highly significant difference was observed between two tests for the seroprevalence (P<0.0001).

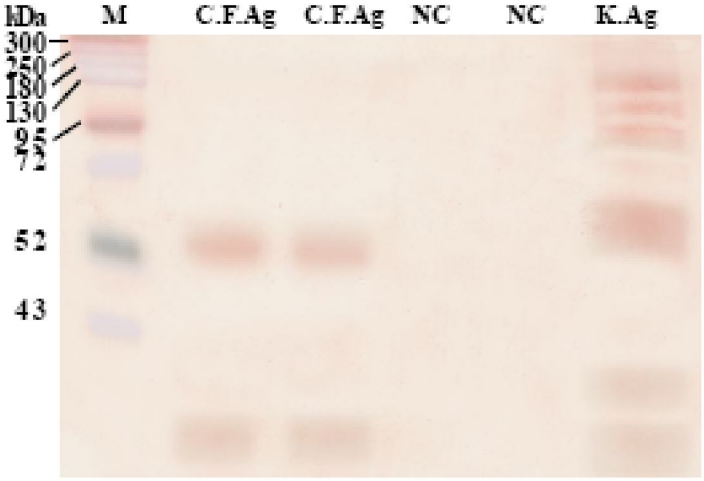


Fig. 4.3 : Western-blot of cultural filtrate antigen of *B.abortus* S99

M	:	Protein marker
C.F.Ag	:	Cultural filtrate antigen
NC	:	Negative control
K.Ag	:	Killed whole cell antigen

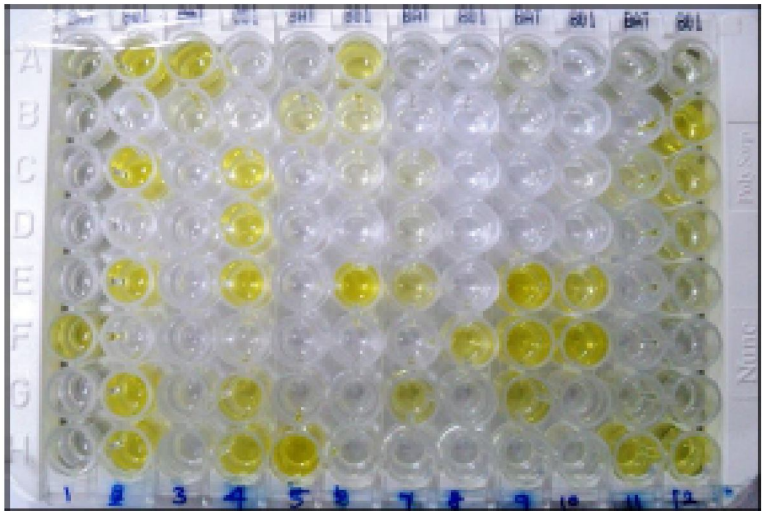


Fig. 4.4 : Screening of serum samples for bovine brucellosis using i-ELISA kit

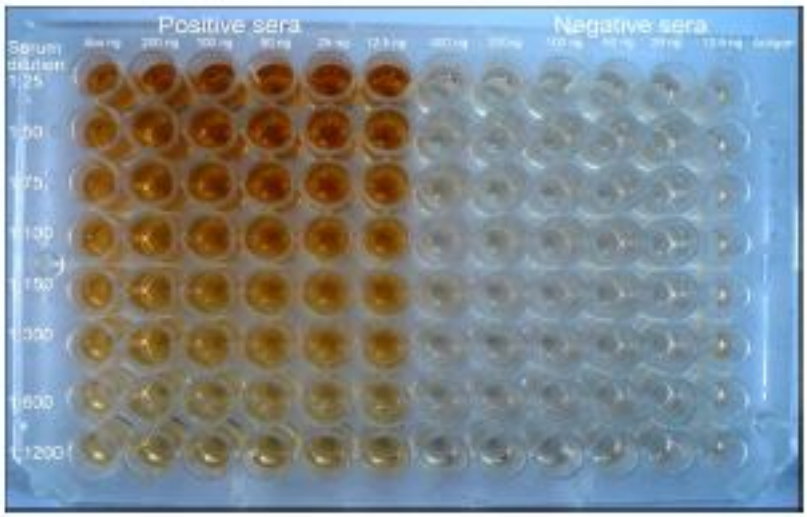


Fig. 4.5 : Optimization of cultural filtrate antigen (*B.abortus* S99) concentration and serum dilution in developing i-ELISA

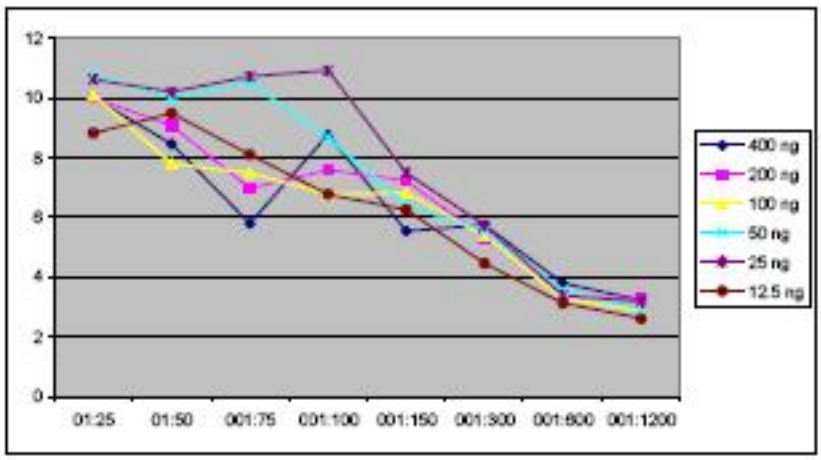


Fig. 4.6 : Line chart diagram showing the highest optimum concentration of cultural filtrate antigen in development of i-ELISA on P/N ratio ODs
Serum dilutions : 1:25 to 1:1600; Antigen concentrations : 400 to 12.5 ng/well

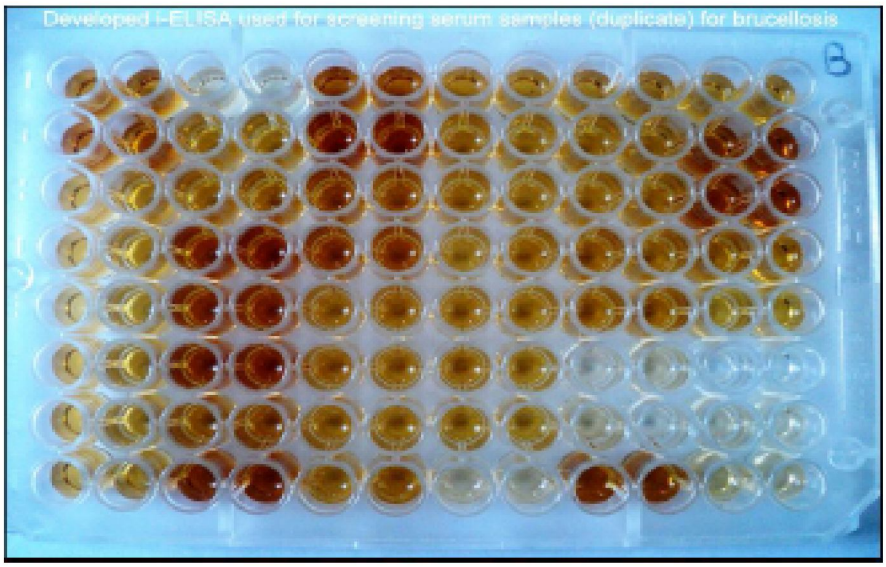


Fig. 4.7 : Screening of bovine sera for detection of brucellosis using developed i-ELISA

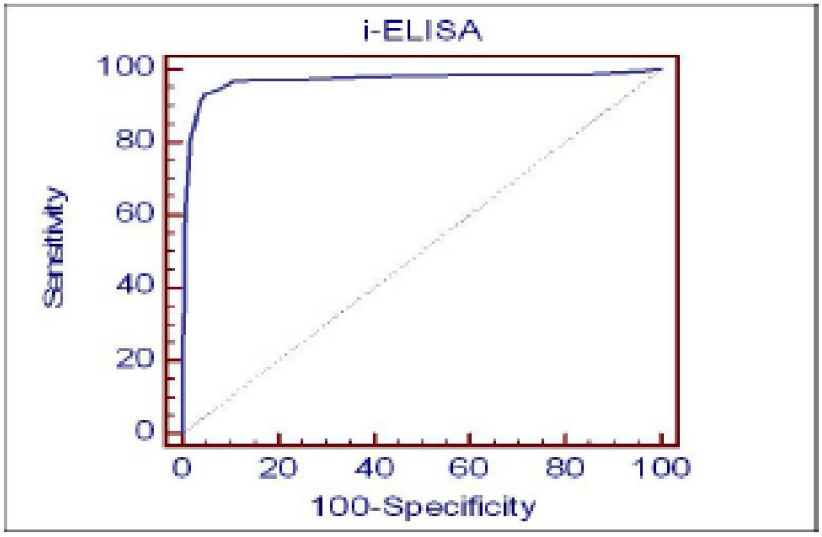


Fig. 4.8 : ROC curve for determination of cut-off value and area under curve for developed i-ELISA

samples ranged between 40 to 320 IU for both female and male animals. When tested by i-ELISA^{Kit} 72 (6.46%) of the 239 RBPT positive were found negative and out of the 874 negative samples, 133 (11.94%) were found positive (Table 4.3).

4.10 i-ELISA (Kit)

All 1113 serum samples were also tested by i-ELISA^{Kit} (IDEXX, CHEKIT Brucellose Serum – *Brucella abortus* Antibody Test Kit) and it detected maximum number by iELISA$^{Dev.}$ 300 (26.95%) samples as positive and 813 (73.04%) samples as negative (Table 4.1) (Fig. 4.4).

4.11 i-ELISA$^{Dev.}$

4.11.1 Optimization of i-ELISA$^{Dev.}$

On checkerboard titration using the cultural filtrate antigen, the optimum concentration of the test was found to be 25 ng/well of microtitre plates. The serum dilution of 1:100 µl per well was found to reproducible results (Fig. 4.5 and 4.6).

4.11.2 Screening of sera using i-ELISA$^{Dev.}$

The standardized i-ELISA$^{Dev.}$ was used to screen the collected serum samples for bovine brucellosis. The samples were given OD values above the cutoff (0.9) was considered positive, which was obtained by ROC curve in comparison to a gold standard test i-ELISA^{Kit}.

Out of 1113 bovine serum samples, 340 (30.54%) were found positive and 773 (69.45%) negative by i-ELISA$^{Dev.}$ (Table 4.1). Of the 340 positive samples, 282 (25.33%) were positive and 58 (5.21%) were found negative by i-ELISA^{Kit}. Out of the 773 negative samples, 18 (1.61%) samples were positive and 755 (67.83%) were found negative by i-ELISA^{Kit} (Table 4.4) (Fig. 4.7).

4.11.3 Receiver Operator Characteristic (ROC) curve analysis

ROC curve was calculated for all the samples tested by i-ELISA$^{Dev.}$ in comparision to golden standard test like i-ELISA^{Kit}. The AUC for i-ELISA$^{Dev.}$ was found to be 0.970, indicating the test had a maximum discriminating ability between positive and negative. An optimum cutoff of 0.9 was obtained on calculation using ROC curves (Fig. 4.8) and interactive dot diagram (Fig. 4.9).

Table 4.4: Comparison of i-ELISA^{Kit} and i-ELISA$^{Dev.}$ in diagnosis of bovine brucellosis

Serological test		i-ELISA^{Kit}		
		Positive	**Negative**	**Total**
i-ELISA$^{Dev.}$	Positive	282 (a)	58 (b)*	340 (a+b)
	Negative	18 (c)	755 (d)	773 (c+d)
	Total	300 (a+c)	813 (b+d)	1113 (a+b+c+d=N)

Relative Sensitivity = a / a + c × 100 = 94%
Relative Specificity = d / b + d × 100 = 92.86%
Positive Predictive value = a / a + b × 100 = 82.94%
Negative Predictive value = d / c + d × 100 = 97.67%
Apparent Prevalence = a + b / N × 100 =30.54 %
True Prevalence = a + c / N × 100 =26.95 %
Accuracy of prediction = a + d / N × 100= 93.17 %
LR+ = Sensitivity / (1-Specificity) = (a / a + c) / (b / b + d) = 1.01
LR- = (1 – Sensitivity) / Specificity = (c / a + c) / (d / b + d) = 0.06
Kappa Vaue = (OP-EP)/(1-EP) = 0.832 (Perfect Agreement)
DOR = Se / (1 – Se) / (1 – Sp) / Sp = 1.021
** Highly significant difference was observed between two tests for the seroprevalence (P<0.0001).

4.12 *omp2a*-PCR$^{Dev.}$ and *bcsp31*-PCR

4.12.1 Sensitivity of *omp2a*-PCR$^{Dev.}$

The threshold sensitivity of the PCR assay was determined by testing serial dilutions of *B. abortus* S99 DNA from different dilutions of DNA from 900 ng, 700 ng, 350 ng, 35 ng, 3.5 ng, 350 pg and 35 pg/µl (Fig. 4.10).

4.12.2 Detection of *Brucella* DNA by *omp2a*-PCR$^{Dev.}$ and *bcsp31*-PCR

DNA was isolated from 106, i-ELISA^{Kit} positive and 168, i-ELISA^{Kit} negative randomly selected serum samples belonging to organized-A, Unoganized-A farms of Karnataka and organized-A, C and E farms of Uttar Pradesh (Table 4.5). On DNA amplification using PCR technique both developed *omp2a* gene and *bcsp31* genes based analysis were found similar. Using both *omp2a*-PCR$^{Dev.}$ and *bcsp31*-PCR$^{Dev.}$, 10 (9.43%) (Fig. 4.11) and 12 (11.32%) (Fig. 4.12) animals were found positive by conventional and kit method respectively in i-ELISA^{Kit} positive samples. While in i-ELISA^{Kit} negative, 9 (5.35%) and 17 (10.11%) animals were also found PCR positive by using conventional and kit method of DNA isolation respectively. In total, both i-ELISA^{Kit} positive and negative serum samples, 19 (6.93%) and 29 (10.58%) animals were detected positive by using conventional and kit method (Table

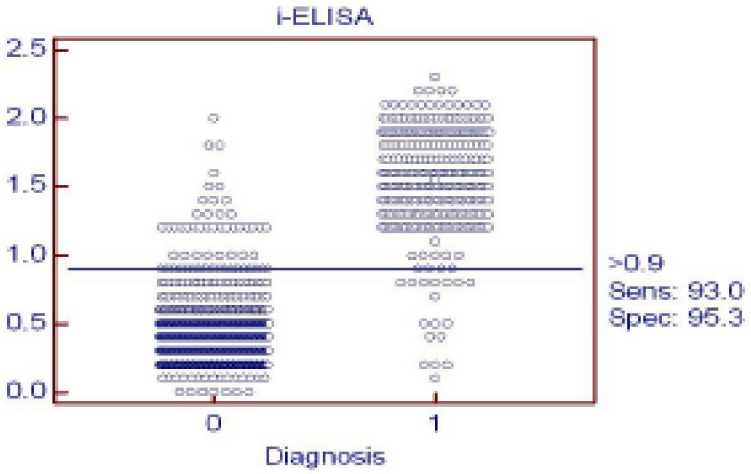


Fig. 4.9 : Interactive dot diagram for developed i-ELISA on screening of sera in diagnosis of bovine brucellosis

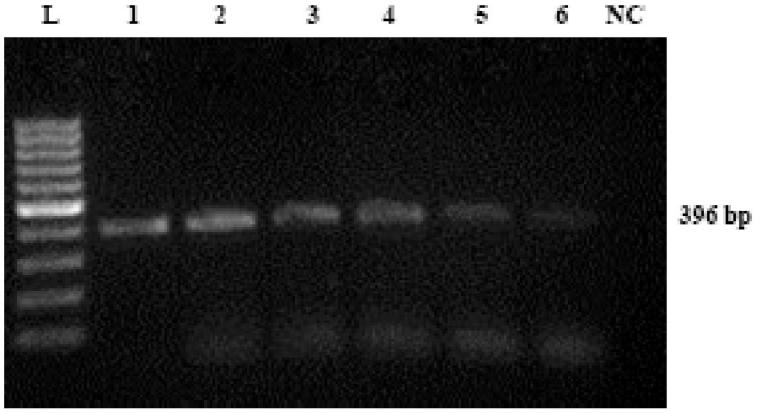


Fig. 4.10 : Standardization of developed *omp2a*-PCR with different concentration of DNA (*B.abortus* S99).

Lane L : DNA 100bp ladder

Lane 1-6 : Different dilutions of DNA (1-900 ng, 2-700ng, 3-350 ng, 4-35 ng, 5-3.5 ng, 6-350 pg, 7-35 pg/µl (Amplicon-396 bp)

Lane NC : Negative control

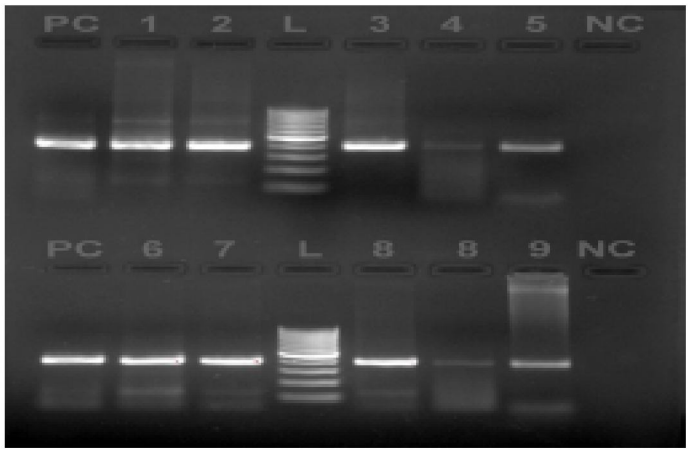


Fig. 4.11 : Detection of *Brucella* DNA on agarose gel electrophoresis and ethidium bromide staining, an amplicon of 396 bp was obtained by *omp2a*-PCR

Lane L : 100-bp DNA ladder
Lane PC : Positive Cotrol (*B. abortus S99*)
Lane NC : Negative control
Lane 1- 9 : Serum DNA from bovines

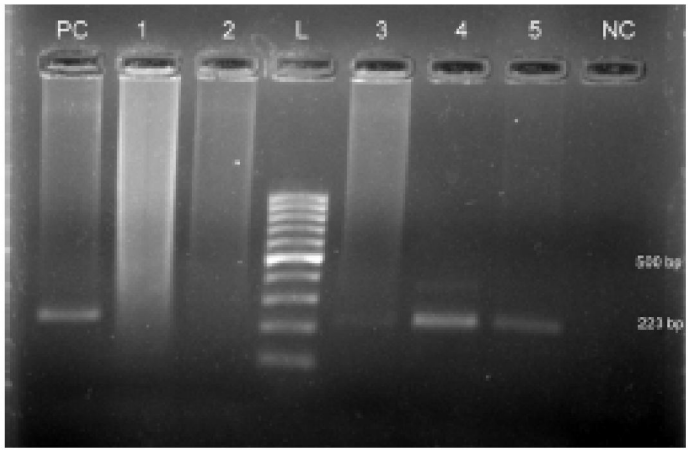


Fig. 4.12 : Detection of *Brucella* DNA on agarose gel electrophoresis and ethidium bromide staining, an amplicon of 223 bp was obtained by *bcsp31*

Lane L : 100-bp DNA ladder
Lane PC : Positive Cotrol (*B.abortus S99*)
Lane NC : Negative control
Lane 1- 5 : Serum DNA from bovines

Table 4.5: Overall prevalence of bovine brucellosis using *omp2a*-PCR$^{Dev.}$ in i-ELISA^{Kit} positive and negative population

Farm	i- ELISA^{Kit} Positive		i- ELISA^{Kit} Negative		Total	
	Conventional	KIT	Conventional	KIT	Conventional	KIT
Karnataka@						
Organized Farm -A	4/48	5/48	0/30	2/30	4/78	7/78#
Unorganized Farm-A	2/17	3/17	0/16	1/16	2/33	4/33
Uttar Pradesh						
Organized Farm -A	0/1	0/1	9/16	11/16	9/17	11/17
Organized Farm -C	4/36	4/36	0/93	3/93	4/129	7/129
Organized Farm -E	0/4	0/4	0/11	0/11	0/15	0/15
Total	10/106	12/106	9/166	17/166	19/272	29/272*
(% Positive)	(9.43%)	(11.32%)	(5.42%)	(10.24%)	(6.98%)	(10.66%)

No. of positive samples / Total No. of sample tested (% positive)

@ Overall there is no significant difference between two tests for positivity (P>0.05)

* Two methods differ highly significantly (P<0.0001).

Table 4.6: Comparison of PCR Kit and PCR conventional in diagnosis of bovine brucellosis

Test		PCR Kit		
		Positive	Negative	Total
PCR Conventional	Positive	19 (a)	0 (b)*	19 (a+b)
	Negative	10 (c)	245 (d)	255 (c+d)
	Total	29 (a+c)	245 (b+d)	274 (a+b+c+d=N)

Relative Sensitivity = a / a + c × 100 = 65.51%
Relative Specificity = d / b + d × 100 = 100%
Positive Predictive value = a / a + b × 100 = 100%
Negative Predictive value = d / c + d × 100 = 96.07%
Apparent Prevalence = a + b / N × 100 = 6.93%
True Prevalence = a + c / N × 100 = 10.58%
Accuracy of prediction = a + d / N × 100 = 96.35 %
LR+ = Sensitivity / (1-Specificity) = (a / a + c) / (b / b + d) = 6.5
LR- = (1 – Sensitivity) / Specificity = (c / a + c) / (d / b + d) = 0.34
Kappa Vaue = (OP-EP)/(1-EP) = 0.773 (Substantial agreement)
DOR = Se / (1 – Se) / (1 – Sp) / Sp = 1.025
** Highly significant difference was observed between two tests for the seroprevalence (P<0.0001).

4.6). Out of 245 PCR negative samples, 94 (34.30%) reacted positive and 151 (55.10%) were found negative by i-ELISA^{Kit} (Table 4.7).

Table 4.7: Comparison of i-ELISA^{Kit} (positive and negative) and *omp2a*-PCR$^{Dev.}$ in diagnosis of bovine brucellosis

Serological test		i-ELISA^{Kit}		
		Positive	**Negative**	**Total**
Developed PCR	Positive	12 (a)	17 (b)*	29 (a+b)
	Negative	94 (c)	151 (d)	245 (c+d)
Total		106 (a+c)	168 (b+d)	274 (a+b+c+d=N)

Relative Sensitivity = a / a + c × 100 =11.32 %
Relative Specificity = d / b + d × 100 = 89.88%
Positive Predictive value = a / a + b × 100 = 41.37 %
Negative Predictive value = d / c + d × 100 = 61.63%
Apparent Prevalence = a + b / N × 100 = 10.58%
True Prevalence = a + c / N × 100 = 38.68%
Accuracy of prediction = a + d / N × 100= 59.48%
LR+ = Sensitivity / (1-Specificity) = (a / a + c) / (b / b + d) = 1.11
LR- = (1 – Sensitivity) / Specificity = (c / a + c) / (d / b + d) = 0.98
Kappa Vaue = (OP-EP)/(1-EP) = 0.014 (Slight Agreement)
DOR = Se / (1 – Se) / (1 – Sp) / Sp = 1.109
** Highly significant difference was observed between two tests for the seroprevalence (P<0.0001)

4.12.3 *IS711*-PCR

DNA detected by *omp2a* and *bcsp31* PCR were further on *IS711*-PCR, all samples were given 498 bp amplicon on agarose gel electrophoresis, so known to be *B.abortus* species (Fig. 4.13).

4.13 Comparative Efficacy of Serological Tests

The prevalence of *Brucella abortus* antibodies in bovines *i.e.*, cattle and buffaloes showed that out of the 1113 serum samples subjected to RBPT, STAT, i-ELISA^{Kit} and i-ELISA$^{Dev.}$ found 189, 239, 300 and 340 animals positive respectively. The highest seroconversion was recorded with i-ELISA$^{Dev.}$, followed by i-ELISA^{Kit} and less sensitivity was shown by RBPT (Table 4.1).

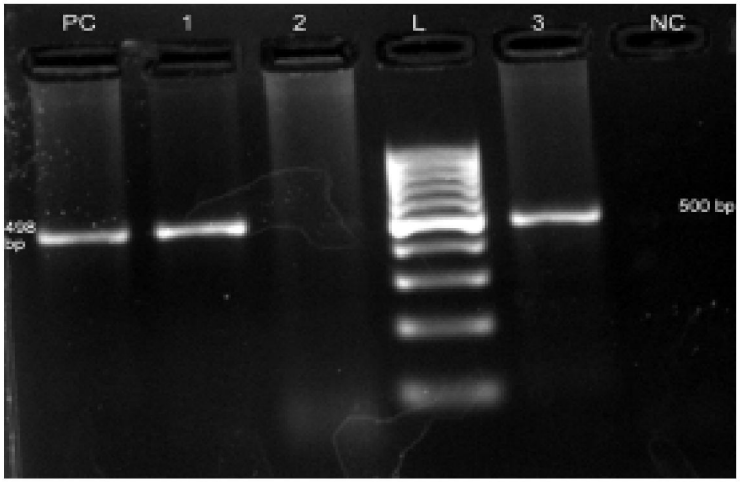


Fig.4.13 : Detection of *Brucella abortus* DNA on agarose gel electrophoresis and ethidium bromide staining, an amplicon of 498 bp was obtained by *IS711-PCR*

Lane L	:	100-bp DNA ladder
Lane PC	:	Positive Cotrol (*B.abortus S99*)
Lane NC	:	Negative control
Lane 1- 3	:	Positive DNA sample by *omp2a*-PCR.

4.14 Comparative Efficacy of Serological Tests and Serum Based PCR

Altogether 274 serum samples were tested for *Brucella* DNA amplification, 29 (10.11%) were reactive by developed-PCR technique. This result was uncomparable with RBPT, STAT and i-ELISA^{Kit} and i-ELISA$^{Dev.}$ tests gave 74 (27.00 %), 89 (32.48 %), 106 (38.68 %) and 120 (43.79 %) positive, respectively (Table 4.5).

4.15 Seroprevalence of Bovine Brucellosis

4.15.1 Overall Seroprevalence

The overall seroprevalence of bovine brucellosis, based on the 1113 samples tested by the different serological tests like RBPT, STAT, i-ELISA^{Kit} and i-ELISA$^{Dev.}$ are given in Table 4.1. The maximum numbers of serum samples detected positive by i-ELISA$^{Dev.}$ (30.54 %) followed by i-ELISA^{Kit} (26.95 %) and least by RBPT (16.98%). The PCR technique detected very less serum samples positive (10.66 %) (Table 4.5). A total of 692 negative and 139 serum samples was found positive common in all the four tests, *i.e.*, RBPT, STAT, i-ELISA^{Kit} and i-ELISA$^{Dev.}$. In the present study, 411 sera samples were positive by either of anyone above mentioned test.

4.15.2 Sex wise seroprevalence

Sex wise seroprevalence is given in Table 4.8. The seroprevalence of 11.96, 13.67, 3.41 and 5.98% was recorded in male and similarly 17.57, 22.38, 29.71 and 33.43% in female on RBPT, STAT, i-ELISA^{Kit} and i-ELISA$^{Dev.}$ tests, respectively. The STAT detected more in males, i-ELISA$^{Dev.}$ found more sensitive in females.

A highly significant differences ($p \leq 0.01$) were observed in seroprevalence rates between males and females by all tests with a greater positivity being recorded in females.

4.15.3 Breed wise seroprevalence

The sera samples collected from Surthi (60.60%) breed showed maximum positivity followed by Jersey (40.90%) and Murrah (38.93%) on i-ELISA$^{Dev.}$. Similar results were recorded with other tests employed in the present study. The minimum seropositivity was observed in HF and Sahiwal breeds (Table 4.9). On RBPT, low seroprevalence of 9.18% was recorded in Khillar, similarly 13.33% in ND (Buffaloes) and 15.53% in ND (Cattle).

Table 4.8: Sex wise seroprevalence of bovine brucellosis

Sex	RBPT	(%)	STAT	(%)	i-ELISA^{Kit}	(%)	i-LISA$^{Dev.}$	(%)
Male**	14/117	(11.96)	16/117	(13.67)	4/117	(3.41)	7/117#	(5.98)
Female	175/996	(17.57)	223/996	(22.38)	296/996	(29.71)	333/996	(33.43)
Total (%Ve)	189/1113**	(16.98)	239/1113	(21.47)	300/1113	(26.95)	340/1113*	(30.54)

No. of positive samples / Total No. of samples (% positive)
****** Highly significant difference was observed between male and female for the seroprevalence (P<0.01).

Table 4.9: Breed wise seroprevalence of bovine brucellosis

Sl. No	Breed	RBPT	(%)	STAT	(%)	i-ELISA^{Kit}	(%)	i-LISA$^{Dev.}$	(%)
A. CATTLE									
1	Jersey	7/22	(31.81)	13/22	(59.09)	7/22	(31.81)	9/22#	(40.90)
2	Khillar	9/98	(9.18)	14/98	(14.28)	26/98	(26.53)	28/98	(28.57)
3	Sahiwal	5/16	(31.25)	5/16	(31.25)	1/16	(6.25)	2/16	(12.5)
4	HF	5/73	(6.84)	5/73	(6.84)	0/73	(0)	2/73	(2.73)
5	Haryana	2/8	(25.00)	2/8	(25.00)	0/8	(0)	2/8	(25.00)
6	Vrindavani	0/1	(0)	1/1	(100.00)	0/1	(0)	0/1	(0)
7	Crossbred	32/219	(14.61)	38/219	(17.35)	48/219	(21.91)	55/219	(25.11)
8	ND (Non-descript)	16/103	(15.53)	36/103	(34.95)	29/103	(28.15)	32/103	(31.06)
B. BUFFALO									
9	Murrah	73/375	(19.46)	79/375	(21.06)	135/375	(36.00)	146/375	(38.93)
10	Surthi	18/33	(54.54)	15/33	(45.45)	24/33	(72.72)	20/33	(60.60)
11	ND (Non-descript)	2/165	(13.33)	31/165	(18.78)	30/165	(18.18)	44/165	(26.66)
	Total (%Ve)	**189/1113****	**(16.98)**	**239/1113**	**(21.47)**	**300/1113**	**(26.95)**	**340/1113***	**(30.54)**

No. of positive samples / Total No. of sample tested (% positive)

4.15.4 Age wise seroprevalence

Age wise seroprevalence is shown in Table 4.10. The maximum seropositivity was observed in above 7 year age groups, followed by 5 to 7 year group and least in bellow 5 year group on RBPT, STAT, i-ELISA^{Kit} and i-ELISA$^{Dev.}$ tests. Among different tests maximum seroprevalence was recorded by i-ELISA$^{Dev.}$ (38.45%) in above 7 years and least by RBPT (2.22%) in less than 5 years.

4.16 Evaluation of RBPT, STAT, and i-ELISA$^{Dev.}$ against i-ELISA^{Kit} and PCR (Kit)

4.16.1 Relative Sensitivity and Specificity

The most reliable test for diagnosis of bovine brucellosis is i-ELISA^{Kit}, therefore, considered as a standard test for evaluating the results of other tests used in the present study. The i-ELISA$^{Dev.}$ (94%) had a maximum relative sensitivity, followed by STAT (55.66%) and RBPT (47.66%). A different picture was observed when relative specificities were calculated. RBPT (94.34%) exhibited maximum relative specificity, followed by i-ELISA$^{Dev.}$ (92.86%), and STAT (91.14 %) (Table 4.2 to 4.4).

The *omp2a*-PCR$^{Dev.}$ in comparision to standard i-ELISA^{Kit}, showned less (11.32 %) sensitivity and moderate (89.88 %) specificity (Table 4.7). Comparison between the kit and conventional PCR recovered 65.51% relative sensitivity and 100% relative specificity, (Table 4.6).

4.16.2 Predictive Value

A higher positive predictive value was found by i-ELISA$^{Dev.}$ (82.94%), followed by RBPT (75.66%) and STAT (69.87%). The i-ELISA$^{Dev.}$ (97.67%) has high negative predictive value followed by STAT (84.78%) and RBPT (83%) in comparison to the standard i-ELISA^{Kit} test (Table 4.2 to 4.4).

Omp2a-PCR$^{Dev.}$- i-ELISA^{Kit} and kit and conventional PCR revealed 41.37% and 100% positive predictive and 61.63% and 96.07% negative predictive values (Table 4.7 and 4.6), respectively.

Table 4.10: Age wise seroprevalence of bovine brucellosis

Age group (Years)	RBPT	(%)	STAT	(%)	i-ELISA^{Kit}	(%)	i-LISA$^{Dev.}$	(%)
<5	02/90	(2.22)	03/90	(3.33)	08/90	(8.88)	10/90#	(11.11)
5 to 7	130/750	(17.33)	167/750	(22.26)	203/750	(27.06)	225/750	(30.0)
>7	57/273	(20.87)	69/273	(25.27)	89/273	(32.60)	105/273	(38.45)
Total	189/1113**	(16.98)	239/1113	(21.47)	300/1113	(26.95)	340/1113*	(30.54)

No. of positive samples / Total No. of sample tested (% positive)

****** Highly significant difference was observed between the age group for seroprevalence (P<0.0001).

4.16.3 Prevalence

A high apparent prevalence of 30.54% of bovine brucellosis was observed by i-ELISA$^{Dev.}$, followed by 21.47% in STAT, 16.98% in RBPT and 10.58% in *omp2a*-PCR$^{Dev.}$ by comparison to i-ELISA^{Kit} (Table 4.2 to 4.4 and 4.7). Between PCR kit- conventional less (6.93%) apparent prevalence was recorded (Table 4.6).

The true prevalence of 26.95% was recorded similarly in RBPT, STAT and i-ELISA$^{Dev.}$ and 38.68% in *omp2a*-PCR$^{Dev.}$ by comparision to i-ELISA^{Kit} (Table 4.2 to 4.4 and 4.7). Between PCR kit- conventional, less (10.58%) true prevalence was recorded (Table 4.6).

4.16.4 Accuracy of a test in prediction

A highest accuracy of prediction of a test was observed by i-ELISA$^{Dev.}$ (93.17%) followed by STAT (81.58%), RBPT (81.56) and least in *omp2a*-PCR$^{Dev.}$ (59.48%) when compared to i-ELISA^{Kit} (Table 4.2 to 4.4 and 4.7). A highest accuracy of prediction was observed between PCR kit and conventional (96.35%) (Table 4.6).

4.16.5 Likelihood ratios

It compares the proportion of animals with and without disease, in relation to their test results. The LR of a positive test result (LR+) is the quantitative indication of the strength of a positive result. In our study RBPT (8.42) shown maximum LR+ value followed by STAT (6.31), *omp2a*-PCR$^{Dev.}$ (1.11) and i-ELISA$^{Dev.}$ (1.01) in comparison to i-ELISA^{Kit}. But 6.5 LR+ was observed between PCR of kit and conventional tests (Table 4.2 to 4.4, 4.7 and 4.6).

While the LR of a negative test result (LR-) is the ratio of the proportion of affected individuals that test negative, and healthy individuals that test negative. In our study *omp2a*-PCR$^{Dev.}$ showed maximum (0.98) followed by RBPT (0.55), STAT (0.48) and least by i-ELISA$^{Dev.}$ (0.06) in comparison to i-ELISA^{Kit}. But 0.34 LR- was observed between PCR of kit and conventional tests (Table 4.2 to 4.4, 4.7 and 4.6).

4.16.6 Concordance

To study which test can be used in conjunction for the diagnosing bovine brucellosis, concordance between the tests was calculated and is exhibited in Table 4.11. Maximum

concordance of 93.17% was observed between i-ELISA^{Kit} and i-ELISA$^{Dev.}$. While minimum concordance of 59.48% was observed between i-ELISA^{Kit} and *omp2a*-PCR$^{Dev.}$.

Table 4.11: Concordance between different tests for bovine brucellosis

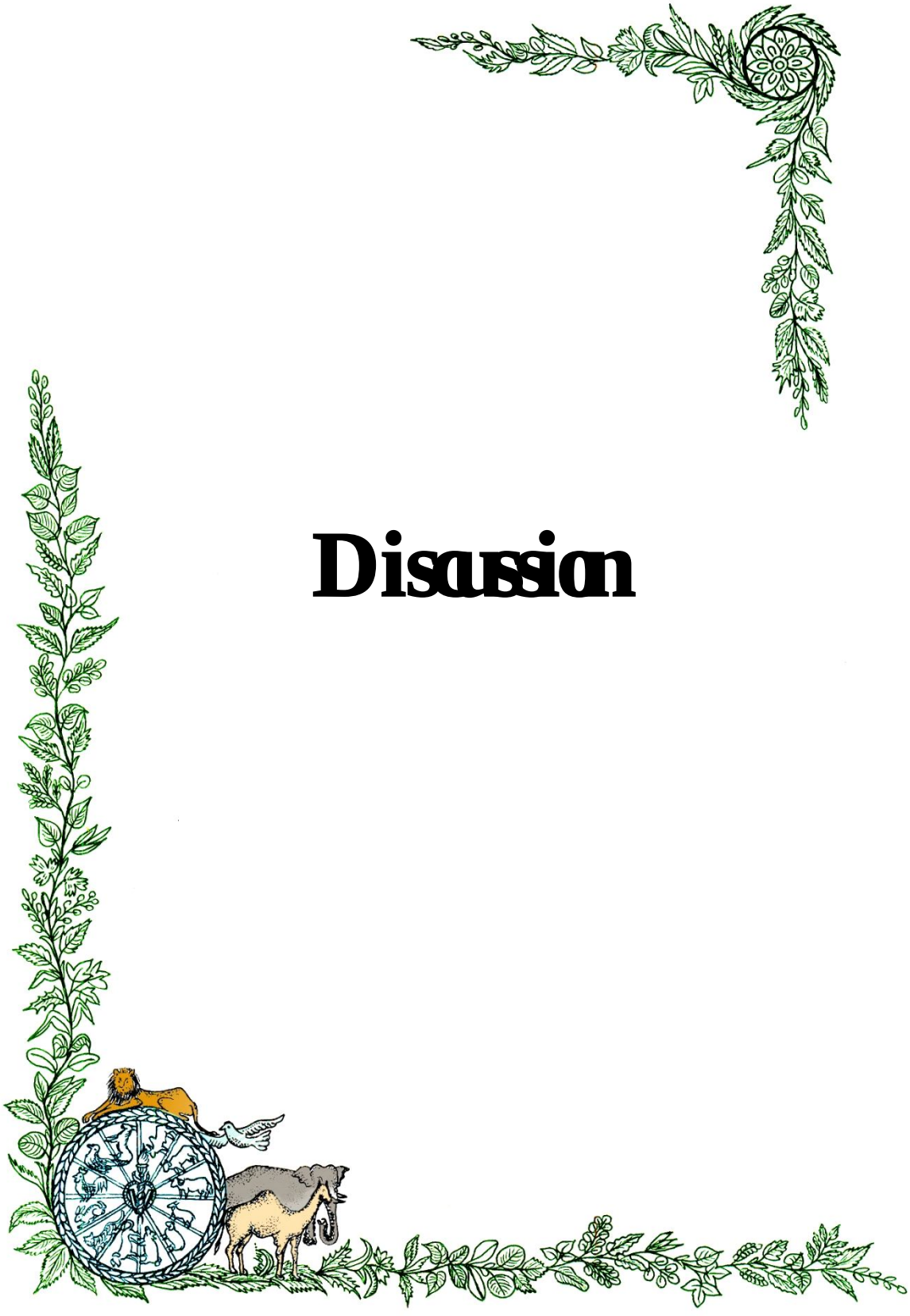
Test 1	Test 2	Concordance (%)
i-ELISA$^{Kit.}$	i-ELISA$^{Dev.}$	93.17
RBPT	STAT	92.45
i-ELISA$^{Dev.}$	STAT	82.30
i-ELISA^{Dev}	RBPT	82.21
i-ELISA^{Kit}	RBPT	81.85
i-ELISA^{Kit}	STAT	81.58
omp2a-PCR$^{Dev.}$	RBPT	64.23
omp2a-PCR$^{Dev.}$	STAT	60.58
i-ELISA^{Kit}	*omp2a*-PCR$^{Dev.}$	59.48

4.16.7 Kappa Statistic

The agreement between the two tests was calculated by kappa statistics (Table 4.2 to 4.4, 4.6 to 4.7). The Kappa value being a maximum of 0.832 between i-ELISA^{Kit} and i-ELISA$^{Dev.}$ showing almost perfect agreement. While the minimum kappa value of 0.014 observed between i-ELISA^{Kit} and *omp2a*-PCR$^{Dev.}$ showing slight agreement.

4.16.8 McNemar's test

McNemar's test gives the difference between different combination of test and its 'p' value. A highly significant difference was observed for all combinations of tests (Table 4.2 to 4.4, 4.6 to 4.7).



Discussion

5 DISCUSSION

Bovine brucellosis is one of the major zoonotic disease caused by bacteria which infects both cattle and buffaloes equally, particularly more in adults while young ones do not show infection till maturity. The bacteria commonly causing bovine brucellosis are, mainly *Brucella abortus*, less frequently *Brucella melitensis* and rarely *B. suis* (Corbel and Brinley-Morgan, 1984).

The *Brucella* are facultative intracellular parasites. They usually enter the body through cuts and abrasions in the oral mucosa, conjunctivae, nasopharynx, or genitalia and even unbroken skin. After gaining entry to the body, the organisms have been found to survive within the cells of the reticuloendothelial system, particularly within macrophages/monocytes of the immune system. They can evade the bactericidal activity of phagocyte cells and replicate within them. They are transported to the lymph nodes, where macrophages and polymorphonuclear phagocytic cells (PMNs) die, releasing more bacteria. In animals where the acute infection is not controlled, the bacteria become disseminated and eventually localize in the spleen and liver. The organisms show a marked tropism for the placenta of pregnant animals probably due to the presence of the compound erythritol. Erythritol enhances the growth of the bacteria and in many cases, fetuses will abort because of endotoxic shock and/or fetal death caused by increased numbers of bacteria and increased concentrations of endotoxin. Survival within phagocytes, cells involves inhibition of phagosome fusion with primary and secondary granules (Riley and Robertson, 1984), while survival within cells lacking phagocytic capability requires a bacterial invasion mechanism. The disease resulted in abortions, infertility, delayed heat, stillbirth interrupted lactation, loss of calves, wool, meat and milk production in animals and reduction in milk yield (Gill *et al*., 2000).

Brucellosis, an important major reproductive disease of livestock, is prevalent in most of developing countries, including India and also responsible in public health problem with zoonotic importance (Corbel, 1997; Young, 1997). Bovine brucellosis is prevalent in all states of the country and appears to be increasing in recent times, perhaps due to increased trade and rapid movement of livestock. Brucellosis is still leading to huge economic losses to dairy farmers and is a major health hazard in India. The infection in bovine has a widely distributed and so more important than infection of sheep and goats as far as economic losses are concerned.

India has a large cattle (199.08 million), buffalo (105.34 million), sheep (71.56 million), goat (140.54 million) and pig (11.13 million) population according 2007 livestock census. According to Central Statistical Organization (CSO) estimates, gross domestic product from livestock sector was about 2603 billion during 2010-11, with 23.80% share in agriculture GDP and 3.64% of National GDP (Anonymous, 2012). One of the primary aims of the livestock development program undertaken by the Government of India is to increase milk and meat production through sustainable disease control programs. The dairy sector has made rapid progress in the past two decades and now India stands first in milk production. In this context, a new prevention, control and diagnostic measures were required for containment of brucellosis. Government of India has also made it mandatory to regularly screen all the breeding bulls for brucellosis before collection semen. The bulls should be free from brucellosis for semen production and for supply to artificial insemination centers.

Historically, the credit of the first investigation of 'contagious abortion' in livestock, associated with brucellosis, goes to the Imperial Veterinary Research Institute (now Indian Veterinary Research Institute), Mukteshwar, in northern India (Anonymous, 1918). Since then, the serological evidence of infection has been reported and several studies have confirmed the widespread prevalence in different States in India (Polding, 1942; Sethi *et al.*, 1971; Sreenivasan, 1972; Nag *et al.*, 1977; Zaki *et al.*, 1981; Chandramohan *et al.*, 1992; Saini *et al.*, 1992; Kalita and Roychaudhury, 1993; Sanjay-Shakya *et al.*, 1995; Isloor *et al.*, 1998 Chakraborty *et al.*, 2000a; Hussain *et al.*, 2000; Mehra *et al.*, 2000; Chauhan *et al.*, 2000; Renukaradhya *et al.*, 2001; Jagapur *et al.*, 2013). There is growing concern that the disease may further flare up due to intensive dairy development programs, especially among the landless agricultural communities.

The procedures of serological tests are divided into two broad groups, the conventional tests and primary binding assays. Conventional tests all rely on the antibody being capable of performing a secondary function, while in primary binding assays the sole function of the antibody is to react with its antigen. Since, no serological test is 100% accurate, generally, the diagnosis is made based on the results of two or more tests. Thus the initial testing is commonly done using a screening test, a test with high sensitivity and perhaps of less specificity. The screening tests are usually relatively inexpensive, fast and simple to perform. If a positive reaction occurs in a screening test, a confirmatory test is performed. The confirmatory test is a test which provides good sensitivity but higher test specificity, thereby eliminating some false positive reactions. Examples of screening tests are RBPT and i-ELISA and a confirmatory test is the c-ELISA. The commercial kits or individually developed assays are excellent screening assays for the diagnosis of brucellosis, especially in individual animal tests or serum or milk (Poester *et al*., 2010).

The most reliable and the only unequivocal method for diagnosing animal brucellosis is the isolation of *Brucella* spp. (Alton *et al*., 1975). However, it is a time intensive and tedious process. It also puts the laboratory personnel under great risk for infection. The serological diagnosis of brucellosis was begun more than 100 years ago with Wright and Smith (1897) describing the first serological test, STAT for its diagnosis, and since then a considerable number of serological tests have been developed and modified to detect antibodies against *the Brucella organism* for diagnosing the disease, *viz*., RBPT, MRT, CFT, ELISA and RIA etc. The serological tests are faster than isolation and have sufficient sensitivity and specificity. Based on published data, it was clear that no individual test is perfect and multiple or series of tests were required to declare animal is *Brucella* positive.

Seroprevalence studies of bovine brucellosis in India

In the present study, a total of 1113 serum samples, were collected from 15 farms belongs to the states of Karnataka, Uttar Pradesh and Uttarakhand. Serum samples were used to fulfill the objectives of the study *i.e.*, seroprevalence of brucellosis using various tests (existing and developed), comparison of results between tests and to trace epidemiological factors (sex, breed, age and source of serum samples) role in the spread of infection. The aim of the present study was to develop a new assay with improved the efficiency, ease, and

effectiveness of serological and molecular testing. These samples were subjected to both direct tests like PCR and as well as indirect tests like RBPT, STAT, i-ELISA^{Kit} and i-ELISA$^{Dev.}$. In the present study, maximum seroprevalence of 50 to 100% was found to be in organized farm-A & B, unorganized farm-A of Karnataka and organized farm-B of Uttar Pradesh on many diagnostic tests. Overall, buffaloes showed more positivity over the cattle on using all serological tests. It is due to the fact that the diseased animals were housed in the farm and responsible for the massive spread among the herd.

The RBPT and STAT revealed a seroprevalence of 16.98% and 21.47%, respectively, *i.e.*, more number of sera was found positive to STAT than RBPT. Sachan and Nautiyal (2013) reported reported seroprevalence of 11.6% in cattle, 10.9% in buffaloes in Uttar Pradesh and STAT detected higher seroprevalence of 10.2% compared to 9.2% by RBPT, similalry to that repotred by Nasir *et al.* (2004), Kumar *et al.* (1999), Ghodasara *et al.* (2010) and Kanani *et al.* (2007). Many workers' (Barbuddhe *et al.*, 2004; Ghodasara *et al.*, 2010; Sundar, 2012) findings were below the range mentioned above.

High in seropositivity by STAT may due to various factors like susceptible to a false positive reaction by cross-reacting antibodies (Nielsen 2002). The antibody response to *B. abortus* in cattle consists of an early IgM isotypes response, the timing of which depends on the route of exposure, the dose of bacteria and the health status of the animal (Beh, 1973 and 1974; Allan *et al.*, 1976). The IgM response is followed almost immediately by production of IgG1 antibody and later by small amounts of IgG2 and IgA (Corbel, 1972; Beh, 1974; Allan *et al.*, 1976; Levieux, 1978; Nielsen *et al.*, 1984). IgM is the most cross reacting antibody, resulting from exposure to microorganisms other than *Brucella* Sp. or environmental antigens (Corbel, 1985).

STAT is a quantitative, less sensitive but supposed to be more specific test than RBPT. IgM isotypes of antibodies are the most active agglutinin at neutral pH in STAT (Rice and Boyes 1971, Corbel 1972, Nielsen *et al.* 1984). However, because the STAT may yield both false negative or false positive results (Corbel *et al.* 1984), it is not useful for testing individual animal. STAT has been recognized as the principal serological test widely used in diagnosis, prevention and control of bovine brucellosis. The efficacy of test is useful only when it is used at herd level. Nicoletti (1969) also recognized that not every *Brucella* infected

bovine shows a diagnostically significant titer. The presumptive diagnosis provided by the serological tests is usually accepted as an indication of brucellosis, although it can only detect IgM and IgG2 and fails to detect IgG1 (Rice and Boyes 1971). However, chronic carriers produce mainly IgG1 that block the agglutinating activities of IgG2 which may result in lower detection rates and low specificity. Since IgG2 and IgA antibodies accumulate later after exposure and are usually present in small and inconsistent amounts, the main Isotype for serological testing is an IgG1 (Allan *et al*., 1976; Lamb *et al*., 1979; Nielsen *et al*., 1984; Butler *et al*., 1986). Therefore, assays that predominately measure IgG1, *viz.*, RBPT and CFT are the most useful and recommended by the OIE as a test prescribed for international trade (OIE Mannual, 2000a).

Hafeez *et al.* (2001) reported lower detection rates by STAT in comparison to RBPT and ELISA. However, RBPT is known to be a rapid, simple and sensitive but has low specificity, but the most common test used and more sensitivity was recorded by many workers (Nasir *et al.,* 2004; Barbuddhe *et al.*,2004; Brahmabhatt *et al.,* 2009; Kaushik *et al.,* 2006; Prithiviraj, 2010) in prevalence studies of brucellosis. The seroprevalence recorded by RBPT ranges from as low as 2.90% (Upadhyay *et al*., 2007) to as high as 58.90% (Genc *et al*., 2005). Sharma and Saini (1995) reported an overall seroprevalence of 8.69 and 14.61% in cattle and buffaloes respectively in Punjab, which is less than our findings. Sukumar *et al.* (2012) was reported 23.52% prevalence in cattle and no incidence in buffalo sera in Tamilnadu using RBPT. Ghodasara *et al*. (2010) was recorded seroprevalence by RBPT and STAT were 11.21% and 16% in cows, 9.59%, 12.33% in buffaloes and overall 10.55% and 14.44% respectively.

Seroprevalence studies form the backbone of epidemiological investigations and are used to identify herds infected with *Brucella.* In the present study, i-ELISA$^{Dev.}$ showed highest seroprevalence of 30.54% followed by i-ELISA^{Kit} (26.95%), STAT (21.47%) and least by RBPT (16.98%). A similar pattern was also observed by Barbuddhe, *et al.* (2004). Chand and Sharma (2004) observed a higher overall seroprevalence in 26.50, 20.47 and 18.89% by LPS I-ELISA, RBPT and STAT, respectively. Most of the samples positive by STAT and RBPT were also positive and some more samples were positive, by i-ELISA^{Kit} and i-ELISA$^{Dev.}$. But still, 17 samples which were negative by i-ELISA^{Kit} was detected positive by serum PCR. In contrary Jagapur *et al*. (2013) reported that i-ELISA^{Kit} is highly sensitive and specific diagnostic test for screening of bovine brucellosis at low titers compared to RBPT and STAT.

Epidemiological data of bovine brucellosis based on host characteristics was evaluated by ascertaining disease association with host parameters like age, breed, and sex by chi square analysis. Sex wise analysis of serum samples showed that more number of females (29.71%) were more positive than males (3.41%) using i-ELISA^{Kit}. A highly significant difference was observed in seroprevalence rates between males and females by all tests. A similar pattern was observed by Kapur and Grewal (1974) with 33.9 and 6.25% seroprevalence in female and male, respectively (Munir *et al.*, 2011; Mohammed *et al*., 2011). Dinka and Chala (2009) observed no significant difference between male and female by RBPT. Prevalence in females is higher may be due to the fact that farm owners keep more females than males because of milk production and offspring. While less in male due to separate rearing, less movement and regular screening of all breeding bulls.

Breed wise analysis of bovine brucellosis showed more positive serum samples from Surthi (72.72%), Jersey (31.81%) and Murrah (36.0%) breeds using i-ELISA^{Kit}. But Kapur and Grewal (1974) showed 8.88% seroprevalence which is less than our finding. In case of Non-descript cattles of Karnataka, i-ELISA^{Kit} detected 28.15% and i-ELISA$^{Dev.}$ showed 31.06% positivity which found to be maximum, similarly with reports of Nawathe and Bhagwat (1984). Prithiviraj (2010) reported a higher prevalence in Murrah breed, in our study was also observed similar results. These animals were suspected for brucellosis with a history of abortions, so they may be positive to brucellosis or other infections which induce cross reacting antibodies (Nielsen and Duncan, 1990).

Age wise analysis revealed maximum seroprevalence in the age group of 4-7 years (Sundar, 2012) as these animals were sexually mature, which corroborate the findings of Nielsen and Duncan (1990) and Sukumar *et al.* (2012). The analysis of the data also showed a significant difference in the seroprevalence of brucellosis between <5 years, 5-7 years and >7 years of age groups. Similar study was done by Kapur and Grewal (1974) and other workers also showed a higher seroprevalence in adult animals than young ones (Dinka and Chala, 2009; Prithiviraj, 2010; Mohammed *et al*., 2011 and Sundar, 2012). No or less prevalence was found in < 4years and more in > 4 year age groups (Nicoletti, 1990; Sukumar *et. al.*, 2012). It showed that the aged animal has more chances of exposure to the bacteria and contracting disease. Younger animals have maternal immunity which explains the low prevalence of brucellosis.

In Karnataka, 45.80% (191/417) animals were found positive by i-ELISA^{Kit} among 5 organized farms. The 5 unorganized farms or villages of Karnataka showed 22.71% (82/361) animals positive. In Uttar Pradesh, 13.94% (48/344) animals of 5 organized farms were found positive for brucellosis. Similarly a single organized farm of Uttarakhand showed 8.57% (3/35) positivity for brucellosis (Jagapur *et al*., 2013).

Gill *et al.* (2000) recorded seroprevalence of 11.80% in cattle and 10.67% in buffaloes during 1990-99 in Punjab. The seroprevalence rate ranged from 6.65% (123/1860) in the central states of Madhya Pradesh (Mehra *et al*., 2000) to 60% in a north-eastern state of Assam (Chakraborty *et al*., 2000a).

Disease prevalence according to the source of samples collected, indicated that the organized farm-B (75%) and C (24.39%) of Uttar Pradesh showed maximum prevalence of brucellosis, similarly organized farm-A (61.53%) of Karnataka. These samples predominately originated from large herds with a history of abortion and infertility. This is due to inefficient maintenance of farm premises and poor management practices. None of the animals are reported as positive in organized farm-D of Uttar Pradesh. This could be due to their good husbandry practices and adopted hygienic measures.

The higher prevalence of bovine brucellosis in organized farms as compared to unorganized farms, may be due to spread of infection from one animal to another by contact between the females or during natural service with infected bull (Jagapur *et al*., 2013).

Patel *et al*. (2011) reported lesser positivity of 0.94% and 2.85% by RBPT and i-ELISA respectively and 3.63% prevalence was reported in Mehsana district, followed by 2.80% in Gandhinagar and 1.64% in Patan districts of Gujarat using i- ELISA.

Relative sensitivity and specificity of different tests were calculated, by considering i-ELISA^{Kit} as standard test. The maximum sensitivity was shown by i-ELISA$^{Dev.}$ (94%) followed by STAT (55.66%), RBPT (47.66%) and PCR (11.32%). Agarwal and Batra (1999) reported similar findings, i.e. higher sensitivity of 92.04% by LPS-ELISA taking CFT as a standard test. While in case of specificity, it was maximum by RBPT (94.34%), followed by i-ELISA$^{Dev.}$ (92.86%), STAT (91.14%) and PCR (89.88%). Paweska *et al*. (2002) reported higher sensitivity and specificity for i-ELISA, that is 100% and 99.8%, respectively in bovines.

But in contrast Sundar (2012) reported higher sensitivity in RBPT (80.21%) and less sensitivity by STAT (62.50%) and serum PCR (54.50%). Pruthiviraj (2010) reported maximum relative specificity by STAT (99.57%) followed by RBPT (98.71%) and less in LPS I-ELISA (65.17%). By considering i-ELISA as the gold standard test, many workers have compared the results of other tests in case of brucellosis (Nielsen *et al.*, 1996). The lower relative sensitivity of RBPT and STAT was also reported by other researchers (Ghodasara *et al*. 2010; Patel, 2007). In contrary, Singh *et al.* (2004) revealed high sensitivity by RBPT (88.46%) than STAT (46.15%). Similarly other researcher (Brahmabhatt *et al.*, 2009; Kumar *et al.*, 1999) reported lower relative of RBPT than STAT. Chakraborty *et al*. (2000) also found a higher sensitivity of STAT (88.61%) over RBPT (56.96%) and contrary higher specificity of the STAT (98.59%) than that of RBPT (96.77%).

The probability that an animal, 'positive' according to the test, is actually positive; alternatively that a test-negative animals is a true negative. These probabilities are the predictive values of the test. The positive predictive value is calculated to know the true positive and negative predictive value for true negative. In the present study, highest positive predictive value was found for i-ELISA^{Dev} (82.94%) followed by RBPT (75.66%) and STAT (69.87%). The i-ELISA$^{Dev.}$ (97.67%) showed high negative predictive value followed by STAT (84.78%) and RBPT (83%) in comparison to the standard i-ELISA (Kit) test.

In the present study high apparent prevalence by i-ELISA$^{Dev.}$ (30.54%) and STAT (21.47%) and less by RRPT (16.98%) was recorded while the true prevalence of 26.95% was recorded in all the tests. Similarly, Trangadia *et al.* (2012) reported higher prevalence of bovine brucellosis with apparent and true prevalence of 7.57% (2443) and 5.66% in Gujarat and 12.27% (1687) and 10.60% in Andhra Pradesh respectively. The variation in prevalence between states is due to several factors *i.e.*, method of sampling, source of samples, intensive dairy farming, mixed farming, unrestricted movement of infected animals and extent of control measure adopted.

Accuracy is the degree to which an individual measurement represents the true value of the attribute that is being measured: the greater the accuracy, the greater the degree. In the present study i-ELISA$^{Dev.}$ (93.17%) showed maximum accuracy of prediction followed by RBPT (81.76%), STAT (81.58%), and PCR (59.48%). So here, i-ELISA$^{Dev.}$ was found to

be more accurate for the detection of true results than PCR. PCR which is less accurate detects more false positive and false negative results compare to RBPT.

The perfect diagnostic test would have LR^+ equal to infinity (detecting all true positive, and generating no false positive), and the best test for ruling in a disease is, therefore, the one with the highest LR^+. The perfect diagnostic test would have an LR^- equal to zero (producing no false negative, but detecting all true negative), and the best test for ruling out a disease, is therefore, the one with the lowest LR^-. In present study maximum LR^+ was shown by RBPT (8.42) followed by STAT (6.31) detecting maximum true positive, while i-ELISA$^{Dev.}$ showed a minimum LR^- (0.06), thus detecting maximum true negative.

Concordance between the two tests is calculated to have an idea about the agreement between the tests, so as to know what combinations of serological tests can be used for diagnosing the disease correctly. However, the concordance is greatly affected by the sensitivity and specificity of the tests under consideration. Strengthening the findings of relative sensitivities and specificities, concordance percentages calculated for different tests revealed the maximum concordance of 93.17% between i-ELISA^{Kit} and i-ELISA$^{Dev.}$ showing that these tests may be used in conjunction in serial testing. Less concordance was observed between i-ELISA^{Kit} - STAT (81.58%) and i-ELISA^{Kit} - RBPT (81.85%). Similarly, Ghodasara *et al*. (2010) recorded 88.88% agreement between STAT and ELISA and 84.44% agreement between RBPT and ELISA. This may be due to the higher sensitivity of i-ELISA^{Kit} and less specificity of STAT due to cross reacting antibodies. The minimum concordance of 59.48 per cent was found between i-ELISA^{Kit} and serum PCR.

In the absence of gold standard test, kappa statistic is widely used to study the agreement between the two tests. In this study, kappa values were calculated for all the tests taken pair wise. It was seen that there were almost 'perfect' agreement between i-ELISA^{Kit} and i-ELISA$^{Dev.}$ (0.832) indicating that these tests were able to discriminate between positive and negative cases perfectly to an equal extent. A 'moderate' agreement was seen between i-ELISA (Kit) and RBPT (0.475), i-ELISA^{Kit} and STAT (0.5), while Sundar (2012) reported 'perfect' agreement between i-ELISA and RBPT. Slight agreement observed between i-ELISA^{Kit} and serum PCR (0.014), but Sundar (2012) reported 'fair' agreement. Mahajan *et al*. (2011) reported a very good agreement between RBPT and STAT.

ROC curve was calculated for all the samples tested by i-ELISA$^{Dev.}$ in comparison to standard test *i.e.*, i-ELISA^{Kit}. The AUC for i-ELISA was found to be 0.970, indicating the test had a maximum discriminating ability between positive and negative samples. An optimum cutoff of 0.9 was calculated and the samples gave OD of 0.9 and above were considered positive.

Development of i-ELISA using cultural filtrate antigen

The LPS of smooth *Brucella* species when compared to other antigenic molecules, has been considered the most important antigen during immune response in brucellosis. LPS elicits long lasting serological response in both vaccinated and infected animals (Dubray, 1985; Baldi *et al.*, 1996). The common serological tests like CFT, RBPT, STAT, MRT and ELISA are mainly based on the detection of antibodies directed against the LPS portion of the cell membrane (Nielsen, 2002). Therefore, it is difficult to differentiate between vaccinated and infected animals using LPS-based serological tests (Dubray, 1985). In addition, tests based on anti-LPS antibodies give false positives because of cross-reactivity with other Gram-negative bacteria like *Yersinia enterocolitica* O:9, *Salmonella* species, *Escherichia coli* etc. (Corbel, 1985; Weynants *et al.*, 1996; Kittelberger *et al.*, 1997).

Therefore, a major goal in immunological studies of brucellosis has been the identification of non-lipopolysaccharide antigens, which could be useful to circumvent drawbacks of LPS antigen. The second problem is that a diagnosis cannot be established on the basis of single one antibody titer. The polyclonal antibodies against *Brucella* surface antigens , in order to identify different or common antigenic structures. If employed in serodiagnostic tests by whole cells bacteria or cultural filtrate antigens, consequently increase specificity and decreases the cross reactions.

The cultural filtrate antigen of *B.abortus* S99 constitutes immunodominant secretory antigens released into the media (Ellwood *et al.*, 1967) when it is grown and this cultural filtrate protein has been a target molecule for detection of many anti-*Brucella* antibodies. The recombinant OMP proteins have been used for the detection of antibodies (Cloeckaert *et al.*, 2001a), produced in natural infection.

The i-ELISA$^{Dev.}$ was found to be the most sensitive among the tests used in the present study as it detected (30.54%) sera samples positive for brucellosis, which is similar to a high of 40.18% prevalence reported by Barbuddhe *et al*. (2004) and Magee (1980). It may be due to higher sensitivity and its ability to detect all types of immunoglobulin (Quinn *et al.*, 1994).

A high seroprevalence recorded by i-ELISA$^{Dev.}$ may be because of the fact that this is a primary binding assay and has a high sensitivity (Tizard, 1982) compared to conventional tests (Cargill *et al.*, 1985; Sutherland, 1985). A lower sensitivity of i-ELISA^{Kit} compare to i-ELISA^{Dev} may be explained by the fact that all the OMPs are not surface exposed (Cloeckaert *et al.*, 1992b), but many antibodies were targeted in i-ELISA$^{Dev.}$ by using cultural filtrate antigen containing different immunoreactive proteins.

Serum based PCR in the prevalence studies of bovine brucellosis

The control and eradication of animal brucellosis are closely linked with diagnosis and eliminate the disease. Diagnostic methods include direct tests, involving bacterial isolation or DNA detection by PCR-based methods. The indirect test includes serological tests, which are applied *in vitro* (milk or blood) and allergic test applied *in vivo*.

For diagnosis of brucellosis various serological tests are employed with varying degree of sensitivity and specificity. Because of broad sensitivity and low specificity in areas of endemicity, lack of usefulness in diagnosing chronic disease and relapse, the presence of cross-reacting antibodies and lack of timelines constitute problems associated with brucellosis serology (Young, 1997).

Isolation bacteria or detection DNA of *Brucella* spp. by PCR is the only method that allows certainty of diagnosis (Godfroid *et al*., 2010). Isolation of organisms is tedious, cumbersome and time consuming thus it is generally not being followed in routine diagnostic laboratories. Moreover, attempts to isolate *Brucella* from individual animals may not be always successful. Even isolation is the gold standard test for diagnosis of brucellosis, but it is not easy, resource-intensive, requires highly skilled technical personnel to handle samples in level 3 biocontainment facilities. However, the sensitivity of this technique is low, ranging from 15 to 70% (Young, 1997). Detection and identification of *Brucella* spp. in clinical specimens by

culturing is a difficult task with significant delays and hazards to laboratory personnel (Yagupsky, 1999).

Extensive efforts have been expended on the development of molecular diagnostic assays based on amplification of different genomic targets by the PCR for the identification of *Brucella* spp. as recently reviewed by Bricker (2002b). But molecular methods are not considered to be confirmatory tests due to limited specificity (Bricker and Halling, 1995). This technique applied for direct detection of *Brucella* DNA in clinical specimens and can overcome the limitations of conventional methodology. Molecular methodology offers an alternative way of diagnosing brucellosis. The widespread successes of PCR as a technique comes from the fact that it is rapid, automate, efficient, sensitive and specific (Gee *et al*., 2004; Bricker, 2002b).

PCR has been applied to various clinical samples such as tissues (Fekete *et al.*, 1992; Gallien *et al*., 1998; Cetinkaya *et al*., 1999; Cortez *et al.*, 2001), blood (Queipo-Ortuno *et al*., 1997), milk (Rijpens *et al*., 1996; Romero and Lopez-Goni, 1999; Tantillo *et al.*, 2001) and nasal secretion (Sreevatsan *et al*., 2000). Elfaki *et al*. (2005) used serum samples for PCR and reported handling of sera that are positive for brucellosis is safer than handling blood specimens with confirmed brucellosis. Reports were suggested the test was specifically avoiding cross-reactivity encountered in most serological assays for brucellosis (MacMillan, *et al*., 1990). It is advantageous to handle serum over the whole blood due to inhibition by anti-coagulants, hemoglobin, host DNA or any other substance present in whole blood but not in serum (Zerva *et al*., 2001). In addition, red blood cell lysis and washings by centrifugation were not required. The serum DNA isolation procedure has been simplified with shortely turn around time and increased sensitivity.

This study aimed to develop a genetic marker for molecular detection of *B. abortus.* A pair of primers/genetic marker was designed for a conserved region of *Brucella omp2a* gene of *B. abortus*. This genetic marker was applied for PCR qualitative assay to detect isolated serum DNA with 100% amplification-efficiency. Similarly, primers were designed to amplify the gene of *bcsp31* and obtained 224 bp product (Asif *et al*., 2009).

In the present study both, i-ELISA^{Kit} positive (106) and negative (166) serum samples were subjected to *omp2a*-PCR$^{Dev.}$. DNA was isolated from serum using the method described by Tokimatsu *et al.* (1995) and Kaushik *et al.* (2006) with minor modifications. Several studies have documented the presence of circulating pathogen DNA in serum samples (Brown *et al.*, 1995; Bougnoux *et al.*, 1999). The use of serum instead of whole-blood samples offers several advantages for nucleic acid amplification methods, measurement and adjustment of isolated DNA concentrations are not required.

omp2a-PCR$^{Dev.}$ and *bcsp31*-PCR for the conventional method of DNA isolation, detected 9.43% of i-ELISA^{Kit} positive and 5.42% of i-ELISA^{Kit} negative serum samples, over-all diction is 6.98%. But kit isolated samples gave positivity of about 11.32% in i-ELISA^{Kit} positive and 10.24% in i-ELISA^{Kit} negative, on total it was 10.66%. *omp2a*-PCR$^{Dev.}$ and *bcsp31*-PCR were found to be equally sensitive and gave 396 bp and 223 bp band respectively on agarose gel electrophoresis. There are no amplified products with closely related bacteria with *Brucella*: *Agrobacterium* spp., *Y. enterocolitica* O:9, *S. aureus*, *Streptococcus* spp. or *E. coli*. Similarly, Sharifi *et al.* (2008) reported 100% agreement between *omp2a*-PCR$^{Dev.}$ and *bcsp31*-PCR for the diagnosis of *B. abortus* vaccine or *Brucella* field strains.

Moreover, *omp2a*-PCR$^{Dev.}$ was able to identify an additional number of positive samples which were serologically negative. This makes it a valuable tool in the diagnosis of bovine brucellosis especially in the early stages of infection. The kit method of DNA isolation found superior over conventional methods, it might be due purity of the chemicals and standardized protocol used in kit method. On contrary, Sunder (2012) reported 35.13% in seropostive and 7.4% in seronegative samples positive on *BCSP31*-PCR. Keid *et al.* (2010) reported a more diagnostic sensitivity of 27.71% in serum PCR for the diagnosis of canine brucellosis. However, higher sensitivity has been reported by the many authors. Zerva *et al.* (2001) reported 94% diagnostic sensitivity for serum PCR, while Elfaki *et al.* (2001) reported for still higher diagnostic sensitivity (96%) serum PCR.

Mukherjee *et al.* (2007), reported that *bcsp31*-PCR was the most sensitive (92.72%) followed by *omp2*-PCR (61.81%) and ELISA (55.55%) and stated that PCR did not permit any consideration of false positive. Thus both PCRs exhibited a specificity and positive predictive value of 100% while ELISA showed 81.8% specificity and 83.3% positive predictive value.

The *bcsp31*-PCR also gave a higher negative predictive value (88.88%) than the *omp2a*-PCR$^{Dev.}$ (61.81 %) and ELISA (55.55%).

Serum DNA samples that were positive by *bcsp31*-PCR / *omp2a*-PCR$^{Dev.}$ assay were reexamined by the species specific *IS711*-PCR assay. Results showed that all samples gave an amplicon of the 498 bp segment with reference *B. abortus* S99 strain and etiology found to be *B.abortus*.

Similarly, two PCR assays were used: one for the detection of *Brucella* genus (*bcsp31*-PCR) and the other for the identification of *Brucella* species involved (*IS711*-PCR). The study reported that primers detected all *Brucella* spp. and do not cross-react with other bacteria, including those that are phylogenetically related to *Brucella* spp. (Baily *et al*., 1992).

The specificity of *bcsp31*-PCR is superior over routine serological tests used for the diagnosis of brucellosis, because of LPS cross-reactivity with other bacteria (Corbel, 1985; Elfaki *et al*., 2005).

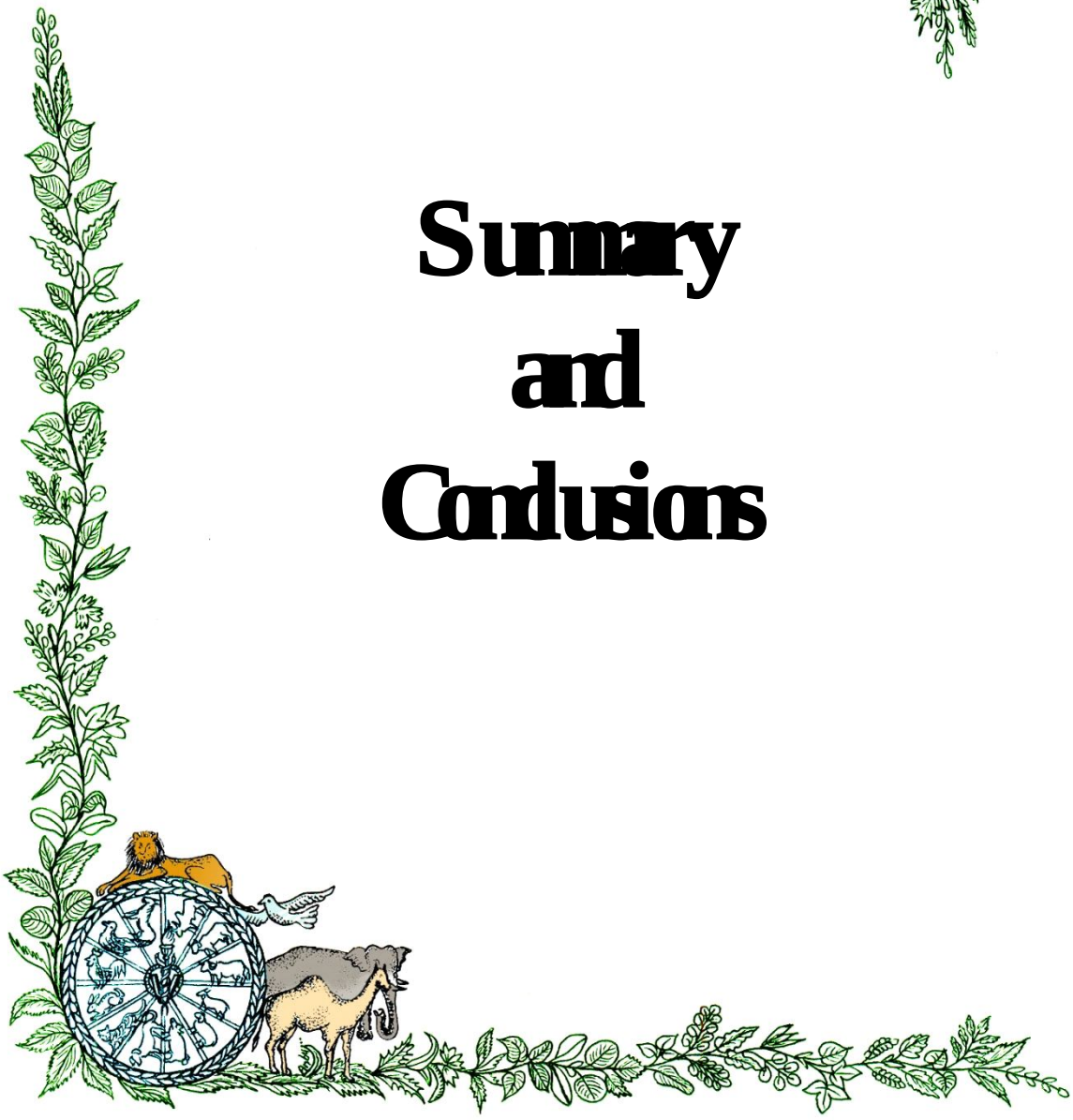
Low diagnostic sensitivity of *omp2a*-PCR$^{Dev.}$ in our study may be due to later/chronic stages of infection, where circulating DNA could be presumably low/absent as opened by Takele *et al*. (2009). Pappas and Papadimitriou (2007) indicated that bacteraemia may be transient, initial event in human disease, followed by macrophage invasion, which is the central pathological event. Following intracellular replication, bacteraemia may reappear continuously or intermittently. As the disease evolves over time, bacteraemia tends to be absent, as is true for the majority of chronic brucellosis cases. Moreover, it has been emphasized that in brucellosis extremely low bacterial load is needed to induce infection. This means that the initial bacteraemic course may run undetected due to the low number of circulating bacteria which may be a presumable reason for the low assay sensitivity of serum PCR. Other hypotheses to account for those false-negative PCR results are a number of organisms below the detection limit, the degradation of target DNA in the samples, and inefficient DNA extraction by conventional method over kit.

Apart from seropositive samples, some of the sero negative samples are also detected as positive by PCR. This may be due to that these animals may be in the early stage of infection when *Brucella* antibodies are below the detection limit of serological tests. Other hypotheses

for an increase in sensitivity of serological tests may cross react with other gram negative organisms especially *Y. enterocolitica* O:9 (Garin- Bastuji *et al.*, 2006).

The finding of the present study is that none of the serological tests used in the diagnosis of brucellosis is perfect, therefore, a battery of tests either in series or in parallel need to be used. In serial testing, serum samples that are positive to all tests are considered positive. Similarly, Gill *et al*. (2000) reported none of screening tests was 100% accurate to detect positive animal correctly, agreement varies between tests and among populations. Parallel testing involves conducting two or more tests on serum at the same time and declared positive if found positive to any of the tests. Thus, maximizes sensitivity.

In the present study 139 serum samples were positive to all tests and giving a seroprevalence of 12.48%. Sharma and Bist (2012) found 16 (28.57%) cattle positive in all the tests (RBPT, STAT and dot-ELISA) from the Mathura district of western UP. A total of 441 serum samples were found positive for any one of the tests applied and a seroprevalence of 39.62% of brucellosis was observed among the collected bovine serum samples.



Summary and Conclusions

6 SUMMARY AND CONCLUSIONS

Brucellosis in bovines is an infectious disease caused by Gram negative facultative intracellular bacteria belonging the genus *Brucella* and causing reproductive disorders in male and female animals. The disease has a public health impacts, with socioeconomic importance. It is disease of sexually matured animals and transmitted by direct or indirect contact with infected sources.

The present study was undertaken to develop i-ELISA$^{Dev.}$ using cultural filtrate antigens. The antigen prepared from PIA was found better over Brucella broth. The latter was highly sensitive but lacked specificity. Similarly in synthetic glucose media, no growth was observed. The i-ELISA$^{Dev.}$ was evaluated to detect *Brucella* antibodies in serum and was compared with other existing serological tests. Since the bacteria remain intracellular in early infection and settle in some organs at the later starges and eventiually serological tests are not useful in these stages. Alternative is the detection of DNA of *Brucella* in blood, serum, secretions, semen, milk and other tissues. In the present study, highly conserved, *omp2a* gene targeted primers were designed to develop PCR to detect DNA in serum samples and compared with *bcsp31*-PCR. A total of 1113 serum samples (cattle and buffaloes) were collected from 15 farms of Karnataka, Uttar Pradesh and Uttarakhand and screened for the presence of *Brucella* antibodies using developed and existing tests. At present many serological tests of *Brucella* detect antibodies against SLPS of LPS. However, due to substantial similarity with other Gram-negative bacteria with sharing epitopes SLPS antigen based tests may yield false positive reaction. Because of this major diagnostic problem attempts were made to develop i-ELISA with cultural filtrate antigen of *Brucella abortus* S-99 and *omp2a*-PCR for detection of *Brucella* DNA from non-infectious serum samples in seroprevalence studies of bovine brucellosis.

Using checker board titration, the i-ELISA$^{Dev.}$ developed and optimized with 25 ng of cultural filtrate antigen of *B.abortus* S99 per well of microtitre plate and 1:100 dilution of serum gave reproducible results. This test was used to screen all the collected 1113 serum samples and O.D values were compared with gold standard test *i.e.*, i-ELISA^{Kit}. The cutoff e"0.9 and 0.970 AUC were obtained by ROC curve in comparison to a gold standard test i-ELISA^{Kit}. Thus, the developed test was found to have maximum discriminating ability between positive and negative samples and perfect agreement with the gold standard test.

In the present study, i-ELISA$^{Dev.}$ showed the highest seroprevalence of 30.54% followed by i-ELISA^{Kit} (26.95%), STAT (21.47%) and the least by RBPT (16.98%). The PCR technique detected very less seropositivity (10.66 %). The positive samples identified by i-ELISA$^{Dev.}$, included 113 (23.85%) cattle and 210 (36.97%) buffalo samples. As many as 282 (25.33%) were found to be positive and 58 (5.21%) as negative by i-ELISA^{Kit} and i-ELISA^{Div}. Of the 773 negative samples by i-ELISA^{Div} 18 (1.61%) samples were positive and 755 (67.83%) were found negative by i-ELISA^{Kit}. Thus there was high agreement between the two tests in identifying positive and negative for brucellosis. A common of 692 (62.17%) negative and 139 (12.48%) positive serum samples were found in all the four serological tests used. In the present study, 411 (36.92%) serum samples were found positive by at least one of the serological tests employed.

The buffalo serum samples gave more seropositivity of 19.89%, 22.%, 33.27% and 36.97% compared to cattle of 13.94%, 20.91%, 20.36% and 23.85% on using RBPT, STAT, i-ELISA^{Kit} and i-ELISA^{Dev} respectively.

Sex wise seroprevalence of 11.96%, 13.67%, 3.41% and 5.98% was recorded in males and 17.57%, 22.38%, 29.71% and 33.43% in females by RBPT, STAT, i-ELISA^{Kit} and i-ELISA$^{Dev.}$ tests, respectively. Thus, greater positivity was recorded in females than males ($p \leq 0.01$) by using all tests, except STAT ($p > 0.05$).

Breed wise, the highest seroprevalence was recorded in Surthi (60.60%) next in Jersey (40.90%) and Murrah (38.93%) on i-ELISA$^{Dev.}$and other tests. The less seropositivity was observed in HF and Sahiwal breeds. Less seroprevalence (9.18%) was also recorded in Khillar, in ND buffaloes (13.33%) ND cattle (15.53%) by using RBPT.

Age wise seropositivity was observed in the descending order between >7 year >5 to 7 years and below >5 year groups on using all serological tests. The maximum seroprevalence was recorded by i-ELISA$^{Dev.}$ (38.45%) in above 7 years and least by RBPT (2.22%) in less than 5 years with highly significance difference between age groups ($p \leq 0.0001$).

Comparison between the tests indicated a maximum relative sensitivity by i-ELISA$^{Dev.}$ (94%) followed by STAT (55.66%), RBPT (47.66%) and least by serum-PCR (11.32%). While, relative specificities was found to be the highest by RBPT (94.34%) followed by i-ELISA$^{Dev.}$ (92.86%), STAT (91.14%) and least by serum-PCR (89.88%) in comparison to standard I-ELISA^{Kit}.

The maximum concordance of 93.17% was observed between i-ELISA^{Kit} and i-ELISA$^{Dev.}$and minimum of 59.48% was recorded between i-ELISA^{Kit} and serum-PCR. The i-ELISA^{Kit} and i-ELISA$^{Dev.}$ tests gave maximum of 0.832 kappa value. This showed almost perfect agreement between the tests, so this combination can be used for screening serum samples for bovine brucellosis. While the minimum kappa value of 0.014 was observed between i-ELISA^{Kit} and serum-PCR showing slight agreement. The developed *omp2a*-PCR$^{Dev.}$ and *bcsp31*-PCR showed similar sensitivity. The detected DNA samples beloned to *B.abortus* as confirmed by performing *IS711*-PCR. Of the 106 randomly selected i-ELISA^{Kit} positive serum samples, 10 (9.43%) and 12 (11.32%) animals were detected positive for *Brucella* DNA isolated by conventional and ZR Serum DNA Kit method, respectively. Similarly, out of 168, i-ELISA^{Kit} negative serum samples, 9 (5.35%) and 17 (10.11%) showed positivity for DNA isolated by conventional and kit method respectively. In total, 19 (6.93%) and 29 (10.58%) animals were detected positive for *Brucella* DNA recovered using conventional and kit method respectively. Only 27 (9.81%) animals were found positive by both PCR and i-ELISA^{Kit}. Out of 245 PCR negative samples, 94 (34.30%) reacted positive and 151 (55.10%) were found negative on i-ELISA^{Kit}. This result suggest that in i-ELISA^{Kit} negative samples gave minimum positivity by PCR and maximum PCR negative samples found positive by i-ELISA^{Kit}. This variation might be due to lots of factors: an incubation period of infection, age of the animal collection and storage serum samples and DNA isolation methods/kits. Very less in younger and more in adults, sex, farm management it varies between farms. In our study females showed more seroprevalence and it varied between farms,

The study indicated the developed i-ELISA was more sensitive, so it can be used as a screening test. The variability among the different tests was observed for detection of bovine brucellosis. However, the study revealed the prevalence of *Brucella* infection in organized and unorganized cattle and buffalo farms of Karnataka, Uttar Pradesh and Uttarakhand states of India.

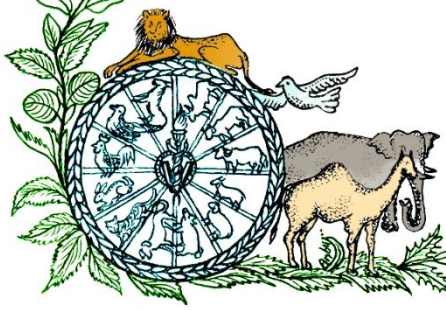
CONCLUSIONS

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

- Developed i-ELISA (i-ELISA$^{Dev.}$) was found to be more sensitive as compared to golden standard test (i-ELISA^{Kit}) and other serological tests (RBPT and STAT) in detection of brucellosis.
- The overall seroprevalence of brucellosis was found to be 16.98%, 21.47%, 26.95% and 30.54% by RBPT, STAT, i-ELISA^{Kit} and i-ELISA^{Dev}, respectively.
- Lesser seroprevalence was observed in cattle than buffaloes on all serological detection tests due to difference in animal husbandry practices.
- Much higher seroprevalence was observed in females compared to males in all serological detection tests, this may be due to periodic changing of males but not with the females.
- Breed wise, more seroprevallence was found in Surthi, followed by Jersey, Murrah and the minimum in HF and Sahiwal breeds.
- The maximum seropositivity was observed in >7 year, next in 5 to 7 year and least in < 5 years group on all serological studies, which may be due to persistence of infection in sexually matured animals.
- While considering i-ELISA^{Kit} as gold standard test, i-ELISA$^{Dev.}$ showed maximum sensitivity, specificity and agreement while less by *omp2a*-PCR$^{Dev.}$.
- ZR Serum DNA Kit (10.58%) for DNA extraction was found better in comparison to other conventional method (6.93%) indicating that inefficient extraction of DNA by conventional method.
- The *omp2a*-PCR$^{Dev.}$ was found to be similar in sensitivity with *bcsp31*-PCR. But overall serum sensitivity was found very low. It ranged from 5.35% to 9.43% and 10.1% to 11.32% in i-ELISA^{Kit} positive and negative, respectively.

- Species specific *IS711*-PCR, declares the etiology of brucellosis in bovine was *B.abortus*.
- However, i-ELISA^{Kit} negative animals revealed the presence of *Brucella* DNA and vice versa. Thus in control programs, it is necessary to test the animals for both the presence of antibody and detection of *Brucella* DNA.
- A lot of variation was found in sreropositivity between serological tests for detection of bovine brucellosis. Only common reactive animals to be declared as positive, while others to be subjected to a confirmatory test to declare positive.
- The validation of i-ELISA$^{Dev.}$ is further required by screening large numbers of serum samples and comparing with LPS based i-ELISA to check false positive reactions.
- Finally, the study revealed the presence of *Brucella* antibodies in the serum samples of cattle and buffaloes belonging to Karnataka, Uttar Pradesh and Uttarakhand states of India.

Mini Abstract



7 MINI ABSTRACT

Bovine brucellosis is an important zoonotic disease, which causes heavy economic losses in India. The present study was undertaken to develop serological and molecular diagnostic tests *i.e.*, i-ELISA$^{Dev.}$ and serum based *omp2a*-PCR$^{Dev.}$ to compare the diagnostic potential with i-ELISA^{Kit} and *bcsp31*-PCR by a seroprevalence survey of bovine brucellosis. The sensitivity and specificity of these diagnostic tests were compared between them and even with basic good old tests like RBPT and STAT. The cultural filtrate antigen of *B. aborts* S99, was found suitable for developing i-ELISA with immunoreactive proteins. A total of 1113 bovine serum samples were collected from 15 different farms located in Karnataka, Uttar Pradesh and Uttarakhand. The seroprevalence was found to be 16.98%, 21.47%, 26.95% and 30.54% by RBPT, STAT, i-ELISA^{Kit} and i-ELISA$^{Dev.}$ respectively. Seroprevalence of brucellosis was found to be lesser in cattle as compared to buffaloes by all serological tests. Higher seroprevalence was also observed in females than males using all serological tests. Maximum seroprevalence was found in Surthi, Jersey and Murrah, minimum in HF and Sahiwal breeds. Age wise, more seropositivity was observed in animals ageing >7 years, next in 5 to 7 years and least in < 5 years group of bovines. The *omp2a* gene targeted serum based PCR technique was found to have a similar sensitivity as to *bcsp31*-PCR. The detection of DNA ranged from 5.35% to 9.43% (106) in i-ELISA^{Kit} positive and 10.1% to 11.32% (168) in i-ELISA^{Kit} negative serum samples by conventional and ZR Serum DNA kit methods of isolation. The detected DNA samples were found to be of *B.abortus* by *IS711*-PCR. The i-ELISA$^{Dev.}$ showed maximum relative sensitivity, relative specificity and agreement in comparison to gold standard i-ELISA^{Kit} but reverse with *omp2a*-PCR$^{Dev.}$. A lot of variation was observed in sensitivity of tests, so battery of tests can be used to increase the efficiency of diagnosing the brucellosis. According to this study a combination of direct test and indirect test can be used for screening bovine brucellosis.

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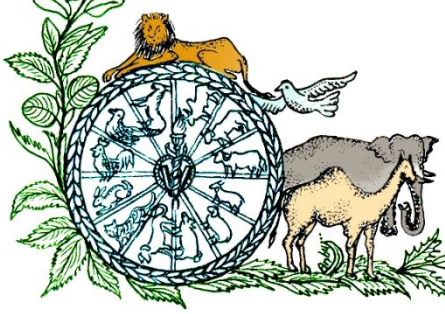

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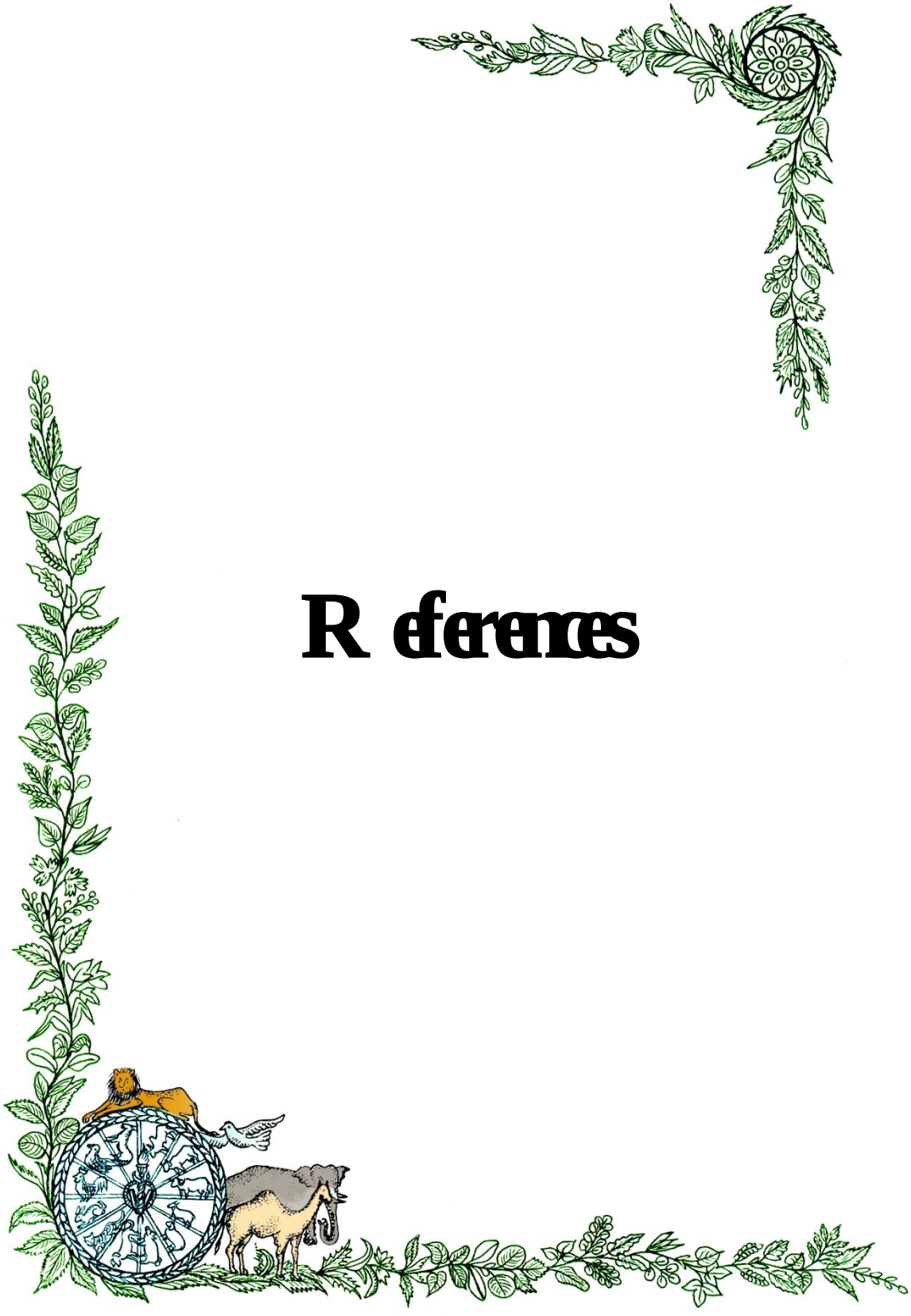

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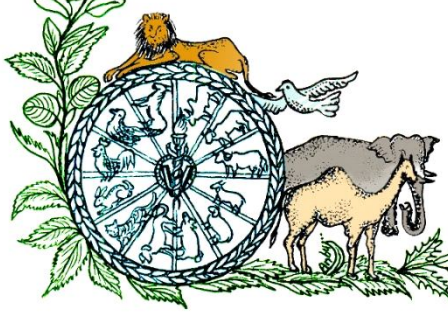

Appendix

APPENDIX

Media, reagents and stains for bacterial culture and identification:

Brucella Agar Medium (BAM)

Brucella agar base (Dehydrated, HiMedia)

Ingredients	Grams/liter
Casein enzyme hydrolysate	10.00
Dextrose	1.00
Peptic digest of animal tissue	10.00
Sodium bisulphite	0.10
Sodium chloride	5.00
Yeast extract	2.00
Agar	15.0

Brucella selective supplement (HiMedia) : Ingredients /1 Vial

Cycloheximide	50.00 mg
Nalidixic acid	2.50 mg
Vancomycin	10.00 mg
Nystatin	50000.00 I.U.
Bacitracin	12500.00 I.U.
Polymyxin B sulphate	2500.00 I.U

Rehydrated the contents of 1 vial with 5ml of 50% methanol. Brucella Agar medium was prepared by suspending 21.55 gm of dehydrated Brucella agar base in 500 ml of distilled water and sterilized by autoclaving at 15 psi pressure, 121°C temperature for 15 minutes. The molten medium was cooled to about 45°C temperature and aseptically added 5% v/v sterile inactivated horse serum (HiMedia) and rehydrated contents of one vial of Brucella selective supplement. The above medium was mixed well and poured into sterile petri plates.

Brucella Broth Base (BBB)

Ingredients	Grams/liter
Casein enzyme hydrolysate	10.00
Dextrose	1.00
Peptic digest of animal tissue	10.00
Sodium bisulphite	0.10
Sodium chloride	5.00
Yeast extract	2.00

Brucella Broth Base was prepared by suspending 14.05 gm of dehydrated media in 500 ml of distilled water and sterilized by autoclaving at 15 psi pressure, 121°C temperature for 15 minutes. The molten medium was cooled to about 45-50°C temperature and aseptically added 5% v/v sterile inactivated horse serum (HiMedia) and rehydrated contents of one vial of Brucella selective supplement. The above medium was mixed well and poured into sterile petri plates.

MacConkey Agar (MA) (Dehydrated, HiMedia)

Ingredients	Grams/liter
Peptic digest of animal tissue	20.00
Lactose	10.00
Bile salt	5.00
Sodium chloride	5.00
Neutral red	0.07
Agar	15.00
Final pH (at 25°C)	7.5 + 0.2

Suspended 55.07 gm of dehydrated MCA in 1000 ml distilled water and sterilized by autoclaving at 15 psi pressure, 121°C for 20 minutes. The molten medium was cooled to about 50°C temperature and poured into sterile petri plates.

Blood Agar (BA)

Blood agar base (Dehydrated, HiMedia)

Ingredients	Grams/liter
Beef heart, infusion form	500.00
Tryptose	10.00
Sodium chloride	5.00
Agar	15.00
Final pH (at 25°C)	7.3 + 0.2

Suspended 40 gm of dehydrated blood agar base in 1000 ml distilled water and sterilized by autoclaving at 15 psi pressure, 121°C temperature for 20 minutes. The molten medium was cooled to about 50°C temperature and aseptically 5% v/v sterile defibrinated sheep blood was added. The above medium was mixed well and poured into sterile petri plates.

Nitrate Reduction Test

Test medium	
Peptone	10.00 gm
Sodium chloride	5.00 gm
Distilled water	1000.00 ml
Adjusted pH to 7.4 then added	
Potassium nitrate	1.00 gm

The above medium was sterilized by autoclaving at 10 psi pressure, 115°C temperature for 30 minutes.

Test reagents

a) Sulfanilic acid: 8.0 gm of sulphanilic acid was dissolved in 1000 ml of 5M acetic acid.

b) Alpha naphthylamine: 5 gm of alpha naphthylamine was dissolved in 1000 ml of 5M acetic acid.

Urea Agar

Solution: 1

Peptone	1.0 gm
NaCl	1.5 gm
Glucose	1.0 gm
Potassium dihydrogen phosphate	2.0 gm
Phenol red	0.012 gm (or 0.2% sol_n 6ml)

Agar	15.0 gm
Distilled Water	900.0 ml

pH 6.8, Autoclave at, 15 psi pressure for 15 min

Solution: 2

Urea	2.0 gm
Distilled water	100 ml
Total	1000 ml

pH 6.8 to 6.9, Sterilize by Seitz filter

Note: To constitute the medium Solution 2 was added in Solution 1, temperature of Solution 1 was brought down to 50°C in a water bath maintained at 50°C and then distributed in test tubes

Indole test

a) Kovac's reagents

Paradimethylaminobenzaldehyde	50 gm
Pure amyl or Isoamyl alcohol	75 ml
Concentrated pure hydrochloric acid	25 ml

The aldehyde was dissolved in the alcohol by gentle warming in water bath, cooled and then hydrochloric acid was added. Protected from light and was stored at 4°C temperature.

b) Tryptone water

Tryptone	10 gm
Sodium chloride	5 gm
Distilled water	1000 ml

The ingredients were dissolved in distilled water by gentle warming and then sterilized at 15 psi pressure, 121°C temperature for 20 minutes.

Motility Sulphide Medium (MSM)

Ingredients	Grams/liter
Proteose peptone	10.00
Beef extract	3.00
L-Cystine	0.20
Ferrous ammonium citrate	0.20
Sodium citrate	2.00
Sodium chloride	5.00
Gelatine	80.00
Agar	4.00
Final pH (at 25°C)	7.3 + 0.2

Suspended 10.44 gm of dehydrated MSM in 100 ml distilled water. The medium was boiled with constant agitation and dissolved completely. The 4 ml medium was dispensed in tubes and sterilized by autoclaving at 15 psi pressure, 121°C for 20 minutes. The tubes were cooled in an upright position.

Modified Ziehl-Neelsen (MZN) stain

a) Diluted carbol fuchsin (HiMedia)

b) Acetic acid (decolourizer)

Concentrated acetic acid	1 ml
Distilled water	100 ml

c) Methylene blue (counter stain)

Methylene blue	8.0 gm
Ethanol 95% (v/v)	300 ml
Distilled water	1300 ml
Potassium hydroxide	0.13 gm

Gram's stain

a) Ammonium oxalate crystal violet

Solution 1: Crystal violet	2.0 gm
Ethyl alcohol (95 per cent)	20.0 ml
Solution 2: Ammonium oxalate	0.8 gm
Distilled water	80.0 ml

Solution 1 and 2 was mixed well and then filtered.

b) Gram's iodine solution

Iodine	1.0 gm
Potassium iodide	2.0 gm

The ingredients were dissolved in distilled water to make total volume 300 ml and then filtered.

c) Acetone or Ethyl alcohol (decolorizer)

d) Safranin (counter stain)

Safranin-O (2.5 per cent solution) in 95 per cent alcohol	10 ml
Distilled water	100 ml

Buffers for serological and molecular assay :

Phosphate Buffer Saline Solution (pH 7.2)

Na_2HPO_4	1.19 gm
NaH_2PO_4	0.22 gm
NaCl	8.55 gm
DI/dH_2O Q.S. to	1 liter

Phosphate Buffer Saline Solution (pH 6.4)

Na_2HPO_4	3.0 gm
KH_2PO_4	6.7 gm
NaCl	8.5 gm
DI/dH_2O Q.S. to	1 liter

Phenol saline

NaCl	8.55 gm
DI/dH_2	
O Q.S. to	1 liter

Mixed well than add 0.5% phenol.

Extraction Buffer

1M Tris	10.0 ml
0.5M EDTA	20.0 ml
10% SDS	100.0 ml

5M NaCl	20.0 ml
DI/dH_2O	850.0 ml

Peptone saline (pH 7.0)

Peptone	10.0 gm
NaCl	5.0 gm
DI/dH_2 O Q.S. to	1 liter

The ingredients were dissolved and sterilized by autoclaving at 15 psi pressure, 121ºC for 20 minutes.

Chloroform:isoamyl alcohol

Chloroform	24 ml
Isoamyl alcohol	1 ml

Phenol:Chloroform:isoamyl alcohol

Phenol	25 ml
Chloroform	24 ml
Isoamyl alcohol	1 ml

TAE (50X)

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
Deionized water up to	1000 ml

TBE (5X)

Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
Deionized water up to	1000 ml

Agarose gel loading buffer (6X)

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)
Ficoll (Type 400; Pharmacia)	15% (w/v)

Dissolved in appropriate volume of deionized water

TE buffer (pH 8.0)

Tris base	0.06 g
EDTA	0.0075 g
Deionized water up to	50 ml

Ethidium bromide (1%)

Ethidium bromide	10 mg
Distilled water	1.0 ml

Lysis buffer (1 X) for Genomic DNA

KCl	100 mM
Tris HCl (pH-8.3)	20 mM
$MgCl_2$	5 mM
Gelatin	0.3 mg
Polysorbate 10%	

Reagents for ELISA :

Coating buffer (Carbonate-bicarbonate buffer, 0.05M, pH-9.6)

Sodium carbonate (anhydrous)	0.159 g
Sodium bicarbonate	0.293 g
Distilled water to	100 ml
Store at 4°C for a maximum of 14 days.	

Blocking solution

Skimmed milk powder	1 g
PBS-Tween 20	20 ml

Washing buffer (PBS-Tween 20, 0.01M, pH-7.4)

Sodium chloride	8.00 g
Na_2HPO_4. 2H2O	1.15 g
KH_2PO_4	0.20 g
Potassium chloride	0.20 g
Tween-20	0.5 ml
Made upto 1000 ml with distilled water	

Substrate buffer (citrate buffer; pH-4.6)

Citric acid	0.452 g
Tri-sodium citrate	0.670 g
Distilled Water to	100 ml

Substrate solution

Orthophenyl diamine	6 mg
Citrate buffer	10 ml
Hydrogen peroxide (30%)	4 µl
Substrate solution was prepared just before use	

3M H2SO4 solution

H_2SO_4 (97-98% purity)	16.1 ml
Distilled water	100 ml

Reagents for SDS–PAGE :

30% Acrylamide : Bis-acrylamide mix

Acrylamide	29.0 gm
Bis-acrylamide	1.0 gm
Distilled water	100 ml

1M Tris (pH 6.8)

Tris	6.05 gm
Distilled water	65 ml

Heat, adjust pH 6.8 by 1M HCl
Make 100 ml with DW, autoclave, store at 4°C

1.5 M Tris (pH 8.8)

Tris	18.15 gm
Distilled water	65 ml

Heat, adjust pH 8.8 by 1M HCl
Make 100 ml with DW, autoclave, store at 4°C

10% SDS

SDS	10 gm
Distilled water	100 ml

10% APS

APS	10 gm
Distilled water	100 ml

Tris – Glysine Buffer (5X)

Tris base	15.1 gm
Glysine	54.0 gm
Distilled water	900 ml
10% SDS	50 ml

Make 1000 ml with DW and pH 8.2.

Sample loading buffer (2X)

Tris HCl (1 M, pH 6.8)	3.12 ml
Glycerol	5 ml
10% SDS	10 ml
2-Mercapto-ethanol	1 ml
Bromophenol blue	1 mg

Make 25 ml with DW, store at 4°C.

Staining solution

Coomassie brilliant blue	1 gm
Methanol	250 ml
Acetic acid	50 ml
Distilled water	200 ml

Destaining solution

Methanol	150 ml
Glacial acetic acid	50 ml
Distilled water	300 ml

Reagents for Western blotting :

Transfer buffer

25 mM Tris base	1.5 gm	
150 mM Glycine	5.65 gm	
Methanol (10%)	50 ml	
Distilled water - make	500 ml	(pH 8.8)

Blocking solution: mentioned above in ELISA reagents.
Washing buffer: mentioned above in ELISA reagents.
Substrate solution

DAB	1 tab
Distilled water	10 ml
Hydrogen peroxide (30%)	10 µl

Substrate solution was prepared just before use

Reagents for STAT :

Normal Saline Solution (NSS)

Sodium chloride	8.5 g
Distilled water	1000 ml

Phenol Saline (Carbol saline)

Phenol	5 ml
NSS to	1000 ml

STAT

For interpretation, 5 standards were prepared as shown in table:

Test Tube	Antigen	Phenol saline	Agglutination
1	1ml	—	No agglutination
2	0.75ml	0.25 ml	25% agglutination
3	0.5 ml	0.5 ml	50% agglutination
4	0.25ml	0.75ml	75% agglutination
5	—	1ml	100% agglutination

The reciprocal of dilution showing color opacity as that of 50% agglutination standard tube was considered as end titre.

VITAE

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Educational Qualifications (Graduation onwards):

Name of the exam passed	Year of Passing	University	Marks
B.V.Sc	1989-94	Veterinary College Bidar	84.10
M.V.Sc. (Micro.)	1995-98	Veterinary College Bidar (UAS, Dharwad)	88.80

Scholarship, award, exams and services

1. Best boy award given by KVSR Jr. College, Gadag in the year 1985.
2. Best Cadet and 2nd in Cross country by NCC, UAS - Dharwad in 1989.
3. UAS merit scholarship during M.V.Sc. studies during 1995-97.
4. National Eligibility Test by ASRB, New Delhi in Dec. 1998.
5. Department of AH & VS, Karnataka was sponsored for Ph.D. studies and serving as Veterinary Officer in the department since 1999.

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