CHAPTER II

REVIEW OF LITERATURE

2.1 History and synonyms of the disease in animals

The history of brucellosis does not begin with the isolation and identification of *Brucella melitensis* (*Micrococcus melitensis*) in the 1880s. Many historical accounts of diseases before this time could actually be describing brucellosis including abortion epidemics in animals and fever in humans. Other than biblical references to animal abortions, one of the earliest recorded descriptions of brucellosis was made by Marston in 1859 (Vassalo 1992). He wrote of an illness, including of his own, which differed from typhoid fever. There are other recordings of how, what is now believed to have been brucellosis, disease affected the Crimean War and sailors aboard ships. Brucellosis then called Mediterranean fever was a debilitating chronic illness with the complication of rheumatism for which many Royal Naval seaman were invalidated each year (Wyatt, 1999). Captain David Bruce (1884) isolated an agent called *Micrococcus melitensis* from human spleens. Hospitalized patients were fed raw goat’s milk for many illnesses giving an early example of nosocomial infections. The early workers were convinced that brucellosis was vector borne. A shortage of monkeys for research on brucellosis led to the use of goats. It was believed that goats were not affected since they did not become ill when inoculated with cultures. Themistocles Zammit, a physician tested the blood of goats and found that as many as 50% had agglutinins. He and other workers isolated the bacteria from blood and milk of at least 10% goats.

Professor L. F. Benhard Bang, Danish veterinary pathologist and bacteriologist, described a different causative organism in cattle in 1895 called *Bacillus abortus* and in 1914 in the United States, a *Brucella* species was isolated from an aborted pig fetus and named *B. suis*. The description of isolates from cattle
and swine led to recognition of widespread distribution in other countries. In 1930, the name of the disease was changed from bovine infectious abortion to Bang’s disease. A committee of the American Veterinary Medical Association recommended a field trial of a vaccine which was developed from a strain of lower virulence named \textit{B. abortus} strain 19. This vaccine has been used for decades as the premier immunizing agent for control of bovine brucellosis. It has been studied in a variety of doses and administration methods.

In domestic animals, brucellosis has been commonly known as enzootic abortion or bovine contagious infection, epizootic abortion, infectious abortion, contagious abortion, slinking of claves, Bang’s disease, and ram epididymitis.

In the case of human brucellosis, it has been described by various names including undulant fever, Malta fever, Mediterranean fever, gastric fever, Mediterranean gastric fever, Gibraltar-Rock fever, Cyprus fever, Neapolitan fever, intermittent gastric fever or intermittent typhoid fever, pseudotyphus, febris typhomalariae, and fièvre sudorale (Ray and Steele, 1979).

2.2 **Definition of the Disease**

Brucellosis is an important zoonotic disease caused by bacteria of the genus \textit{Brucella}. In animals, the disease primarily affects cattle, sheep, goats, swine, and dogs, and is characterized by abortion or infertility and also affects people and other animal species (Ray and Steele, 1979). In human-beings, the disease is characterised by intermittent fever, chills, sweating, headache, myalgia, arthralgia, and a diversity of nonspecific symptoms (Young and Corbel, 1989).

2.3 **Morphology and characteristics of Brucella**

\textit{Brucella} are small, non-motile, non-sporing, gram-negative coccobaccilli. They grow rather slowly on ordinary nutrient media while their growth is improved
by serum or blood. The genus is a member of the $\alpha 2$ subdivision of the Proteobacteria. The *Brucella* species are intracellular parasites inside human-beings and animals and can usually be found in the reticuloendothelial and reproductive systems. Typically *Brucella* spp. occurs as small gram-negative coccobaccilli, but coccal and bacillary forms also occur. The cells are short and slender; the axis is straight; the ends are rounded; the sides may be parallel or convex outwards. In length they vary from about 0.5 - 0.7 $\mu$m, in breadth vary from 0.5 - 1.5 $\mu$m, occurring singly, in pairs or short chains (Leslie et al, 1998).

The genus *Brucella* contains a group of very closely related bacteria. The first member of the group, *Brucella melitensis*, affects primarily sheep and goats, the second member of the group, *Brucella abortus*, affects primarily cattle, the third member of the group, *Brucella suis*, affects primarily pigs, the fourth member of the group, *Brucella ovis*, affects primarily rams and ewes, the fifth member of the group, *Brucella neotomae*, affects primarily the desert wood rats, and the sixth member of the group, *Brucella canis*, affects primarily male dogs and bitches (Corbel and Macmillan, 1998).

The metabolism of *Brucellae* is mainly oxidative and energy is produced by utilization of amino acids and carbohydrate substrates. For many strains erythritol is the preferred energy source. They are aerobes but some species require an atmosphere with added CO$_2$ (5-10%) optimum temperature for growth is 37 $^\circ$C with a range of 20 – 40 $^\circ$C. the optimum pH for growth ranges between 6.6 and 7.4. Growth results in alkalization of the medium.

All *Brucella* strains are catalse positive and oxidase positive. Most of them produce nitrate reductase. However, they cannot liquefy gelatin and are ahaemolytic. *Brucella* species are slow growers and colonies become visible on suitable solid
media in 2-3 days. The colonies of smooth strains are small, round and convex but dissociation, with loss of the O chain of the LPS, occurs readily to form rough or mucoid variants. The latter forms are natural in *B. canis* and *B. ovis* as their cell wall LPS lacks O chain.

*Brucella* strains are fairly resistant to dying and can survive in biological materials for long periods, especially at low temperatures. They are sensitive to a wide variety of disinfectants including formaldehyde, hypochlorite, iodophores and phenols. They are killed by heat under pasturisation conditions.

### 2.4 Antigens of *Brucella*

Wilson and Miles (1932) reported two distinct antigens in *Brucella* species; A antigen, associated with *B. abortus* (A-dominant) and M antigen, associated with *B. melitensis* (M-dominant). Subsequently it was shown that A and M epitopes are located on the O-side chain polysaccharide of the S-LPS from the two species of *Brucella* (Bundle *et al* 1989, Cherwonogrodzky *et al* 1987). S-LPS is the immunodominant epitope and is the antigen used in most serological tests for brucellosis. Structurally, *Brucella* LPS is similar to the endotoxins of other gram-negative bacteria; however, it is less toxic than the endotoxin of the *Enterobacteriacea*. The S-LPS is composed of an outer O-polysaccharide side chain, an intermediate core oligosaccharide and an inner lipid A moiety. The structure of S-LPS is also responsible for serological cross-reactions between smooth *Brucella* species and other bacteria that possess a similar O-chain, such as *Yesinia enterocolitica* 0:9 (Weynants *et al* 1997). The relative amount of A and M epitopes in the O-chain vary among *Brucella* strains, and these epitopes are absent in rough strains (R-LPS) which lacks the O-polysaccharide (Cloeckaert *et al* 1993).
2.4.1 Native Hapten

Besides LPS, a different polysaccharide molecule on the surface of Brucellae has been termed native hapten, the second polysaccharide or polysaccharide B (Moreno et al 1981). The native hapten is strongly associated with LPS and hence, has been difficult to purify and its physical properties are similar to LPS (Moreno et al 1987). Though this molecule cross-reacts with the O-side chain, it can be differentiated from LPS by its serological and biological behavior.

2.4.2 Outer Membrane Protein

The outer membrane proteins (OMP) of Brucella species, also known as group antigens, are classified into three according to their molecular weights (Moriyon and LopezGoni 1998); group 1 (88-94 KDa), group 2 (33-39 KDa) and group 3 (25-31 KDa). In addition, a number of minor OMPs have been identified, including lipoproteins (OMP 10, 16 and 19), two homologous porin proteins (OMP 2a/OMP 2b) and OMP 1 or 89 KDa. There is diversity among the major OMP at the species, biovar and strain levels, which is of epidemiologic and taxonomic importance.

2.5 Virulence and Pathogenicity

Among the members of the group, B. abortus, B. melitensis, and B. suis species are not host-specific, and may transmit to other animal species. Cross transmission of brucellosis can occur between cattle, swine, sheep and goats and other species including dogs, horses, feral swine, bison, rein deer and camels (FAO, 2003).

2.6 Descriptions of Brucella abortus

The B. abortus is the second member group of the genus Brucella. In 1897, it was discovered by the Danish veterinarian Bernard Bang who isolated the organism from cows with an infected abortion. Cattle are the natural hosts of the organism but
it can also infect other animals. The organisms are gram-negative, coccobaccilli or short rods, in length from 0.8 - 1.5 μm long and in breadth by 0.6 - 0.8 μm wide. This species is catalase and oxidase positive and requires carbon dioxide for growth. It produces hydrogen sulphide from sulphur containing amino acids or protein (Stack and MacMillan, 2006).

2.7 Brucella infections of cattle

Brucella infection of cattle is a contagious disease characterized by abortion. It is also known as Bang’s disease, avortement épizoïtique (French) and seuchenhaftes Verwerfen (German). In acute cases in females, an inflammation of the reticuloendothelial system cells and of the placenta cells during pregnancy is followed by an expulsion of the foetus usually between the 5th and the 8th month of gestation. The organism is localised in the mammary gland and is excreted through the milk (Stableforth, 1959). The isolation of the organism from the milk of an infected cow reveals its transmission route to humans and its importance as a human pathogen (Schroeder and Cotton, 1911).

In dairy cattle, infection occurs in all ages but most commonly in sexually mature animals. Mostly, abortions occur in unvaccinated heifers after the 5th month of pregnancy (Forbes and Tessaro, 1996).

In the male animals, the organism can be found in the seminal vesicles and testes which cause an acute or chronic inflammatory process with orchitis, epididymitis, and seminal vesiculitis. It is excreted through the semen (Stableforth, 1959).

The disease is transmitted not only through the contamination of placental material and vaginal discharges of aborting animals but also through the subcutaneous injection of placental material (Woodhead and Aitken, 1889).
Cattle are the main reservoir of *B. abortus* and the introduction of pregnant, recently aborted, or recently calved animals with brucellosis from infected herds are the main source of infection for clean herds. The disease can also be introduced to clean herds via food or water contaminated with discharges. The most common way of infection is through the ingestion of contaminated material (McEwen and Peterson 1939).

### 2.8 Epidemiology

Brucellosis is an important disease among cattle and remains a major source of disease for humans and domesticated animals. If the countries do not implement high-quality and effective public health and domestic animal health programs as well as a national brucellosis control and eradication program, brucellosis will remain the most common form of an important zoonosis worldwide. Bovine brucellosis caused mainly by *B. abortus* has a major economic impact on developing countries (Michael, 1997).

From epidemiological evidence, *B. abortus*, *B. melitensis*, and *B. suis* have distinct host preferences and the organisms are capable to cause an infection in a wide range of host species, including humans. The remaining three members of the species have much greater host specificity. Typically, in all host species *Brucella* grows intracellularly, producing a variable bacteraemic phase followed by localization in the tissues of the genital tract and in the mammary gland. Abortion is typically the first clinical sign of the pregnant female, and orchitis and epididymitis are typical clinical sign of the male (Corbel, 1998). In particular, female animals that have reached sexual maturity are most susceptible to infection. It is usually detected in pregnant females through abortions (England *et al* 2004). Brucellosis in cattle occur worldwide, except in countries where it has been eradicated, including Britain,
Norway, Sweden, Finland, Denmark, Germany, Belgium, the Netherlands, Switzerland, Austria, Czech Republic, Slovakia, New Zealand, Canada, France and Italy. However, the disease is an important issue in developing countries, with biogroups of *B. abortus* usually occurring particularly in the tropical countries (Jarsen and Muller, 1982).

In India brucellosis was first recognised in 1942 and is now endemic throughout the country. The disease is reported in cattle, buffaloes, sheep, goats, pigs, dogs and humans. *B. abortus* biotype-1 in cattle and buffaloes and *B. melitensis* biotype-1 in sheep, goats and man are the predominant infective biotypes (Renukaradhya *et al.* 2002).

Biotyping can be useful for tracing sources of infection. In the field usually biogroup 1 or 3 is prevalent (Corbel, 1998). *B. abortus* biogroup 1 has a high prevalence among large farms populated by indigenous or imported cattle. However, biogroup 3 is isolated in small herds and in nomadic or semi-nomadic herds where the herds are kept together with indigenous cattle (Biajian, 1984). The prevalence surveys need definite information based on herd size, management method, vaccines used, and possible access to wild animal reservoirs of infection (Matyas and Fujikura, 1984).

Serological evidence suggests that brucellosis is highly endemic in most parts of India (Mehra *et al.*, 2000; Shringi *et al.*, 2002; Sarumathi *et al.*, 2003; Mahato *et al.*, 2004; Mittal *et al.*, 2005). The seroprevalence rate of brucellosis in cattle ranged from 0.3% in Himachal Pradesh (Renukaradhya *et al.*, 2002) to 56.2% in Assam (Chakraborty *et al.*, 2000). In the states of Uttar Pradesh and Delhi Sharma *et al.* (1979) carried out sero-epidemiologic investigation on brucellosis and reported a sero-positivity of 6.37 % in cattle and 4.9 % in buffaloes.
A random survey conducted to study the epidemiology of brucellosis in Punjab (India) using milk ELISA revealed an overall apparent prevalence of brucellosis to be 18.26%. The prevalence in the central zone of the state was significantly higher, viz. 23.2% compared to 14.2% in the sub-mountainous zone and 5.8% in the arid irrigated zone. The disease prevalence was found to be non-significantly higher in cattle (20.67%) compared to buffaloes (16.41%) and increased with age in both species. There was significant association between disease and abortion and maximum abortion cases due to brucellosis were found in > 6 month of gestation (95.7%). The disease was significantly associated with the retention of placenta; however there was no significant relationship of the disease with repeat breeding (Aulakh *et al* 2008).

2.9 **Modes of Transmission**

Though infection may occur through the skin, conjunctiva or respiratory mucosa by inhalation (Crawford *et al* 1990), the most common route of infection in cattle is the gastrointestinal tract (Crawford *et al* 1990), from where infection spreads to local lymph nodes where *Brucella* replicates intracellularly in phagocytes (Anderson *et al* 1986). Invasion of lymphatic vessels is followed by bacteraemia leading to systemic infection, favouring colonisation of the pregnant uterus, male genital organs, and mammary gland (Ko and Splitter 2003). The mammary gland is another target organ that is important in transmitting the infection through contaminated milk. *B. abortus* induces a multifocal interstitial mastitis with interstitial accumulation of macrophages and intra-acinar infiltration of neutrophils (Emminger and Schalm 1943), associated with moderate numbers of predominantly intracellular organisms. Aborted fetuses as well as fetal membranes and uterine secretions eliminated after abortion or parturition are the most important sources of
infection (Samartino and Enright, 1993). The disease can also be transmitted to calves vertically (Ray et al., 1988) and through contaminated milk (Wilesmith, 1978; Nicoletti, 1980), but these routes of infection are much less important (Crawford et al., 1990). Venereal transmission is not a major route of infection under natural conditions, but artificial insemination with contaminated semen is a potential source of infection (Rankin, 1965).

2.10 Pathogenesis

The incubation period of Brucella species may vary according to the virulence of the organism, the route of entry and infecting dose. The most common route of infection is from the oral route by licking aborted fetus, infected placentas, and vaginal discharges or by ingestion of contaminated feed and water. Fully virulent Brucella are highly invasive and capable of penetrating the mucosa or skin of the nose, throat, conjunctiva, urogenital tract, teat canal, and abraded skin (Davis, 1990). Virulent Brucella organisms can infect both nonphagocytic and phagocytic cells but the mechanism of invasion of nonphagocytic cells is not yet clearly established. While the organism invasion to nonphagocytic cells, Brucella tend to localize in the rough endoplasmic reticulum (Zhan and Cheers, 1995).

After the invasion, the Brucellae are ingested by various local phagocytic cells and multiply in mononuclear and polymorphonuclear cells and localize temporarily in the lymph nodes of the invasion site, where they cause hyperplasia and acute inflammation. This cycle is repeated by the multiplication of the Brucellae in the cytoplasm of the phagocytes. (Smith and Fitzgeorge, 1964). From the lymph nodes spreading occurs via the blood to other lymph nodes and the reticuloendothelial cells (Macrae and Smith, 1964).
In pregnant animals, the placenta and mammary gland are also invaded (Meador and Deyoe, 1989) and in acute cases, up to 85% of the bacteria are in cotyledons, placental membranes, and allantoic fluid (Radostits et al., 2000). In non-pregnant cows, localization occurs in the udder and uterus, and in cases where the animal becomes pregnant bacteremic phases occurs. Infected udders are clinically normal but they are important as a resource of infection of the uterus and also a source of infection in calves and humans by drinking the milk (Johnson, 1994).

In general, the organisms escape from the lymph nodes and set up a bacteremic phase in the cytoplasm of circulating phagocytic cells. The onset of bacteremia is variable from a few days to 2 months or up to 5 months or even more. Brucellae are dispersed throughout the body during the bacteremic phase and localize in lymph nodes (supramammary lymph nodes and mammary lymphnodes), the spleen, iliac lymph nodes, mammary gland, uterus of female, and in the epididymis and accessory sex glands of male (Alton et al., 1990).

2.11 Clinical Manifestation

Outbreaks of bovine brucellosis are associated with abortion during the last trimester of gestation, production of weak newborn calves, and infertility in cows and bulls (Enright et al., 1984). After the first episode of Brucella-induced abortion, the cow often has normal subsequent parturitions, although another abortion may occur (Nicoletti, 1980). Calves that acquire the infection vertically or by ingesting contaminated milk may remain serologically negative and not show any signs of the disease. However, heifers with latent asymptomatic infection may abort or give birth to infected calves, which are central in maintaining the disease in a herd (Wilesmith, 1978; Nicoletti, 1980). Other clinical signs of infected cows include reduced milk production, an increase in the number of somatic cells in the milk, and impaired
reproductive efficiency (Emminger and Schalm, 1943; Meador et al., 1989). Infected bulls may develop systemic signs of infection including fever, anorexia, and depression, although infection is often inapparent (Campero et al., 1990). The most significant lesion induced by *B. abortus* in bulls is orchitis (Trichard et al., 1982), which is often associated with seminal vesiculitis and epididymitis (Rankin, 1965). As a result of chronic orchitis and fibrosis of the testicular parenchyma, affected bulls may develop permanent infertility (Campero et al., 1990).

### 2.12 Diagnosis

Even in aborting animals or animals having epidydymitis, the clinical diagnosis of brucellosis in infected animals is difficult. The isolation of the organisms is the only way to make a positive diagnosis. The factors determining the diagnosis of bovine brucellosis are: absence of clinical signs other than abortion, the incubation period, the high proportion of inapparent infections, the degree of resistance, either natural or resulting from vaccination, and the presence of natural or nonspecific agglutinins (Morgan, 1982). The diagnosis should be based upon the disease history of the herd, epidemiological observations, serum antibody tests, cell mediated immunity, and the demonstration of the causal organism.

Because of the costs, difficulty of performance, and lack of sensitivity of culture procedures, there is an indirect method of diagnosis by way of serological tests. There are many serological tests for demonstrating that *Brucella* antibodies exist, which can be found in serum, milk, whey, vaginal mucus, semen, and muscle juice. The commonly used tests are the Milk Ring Test (MRT), Serum Agglutination Test (SAT), Rose Bengal Plate Test (RBT), Anti-globulin (Coombs) Test, 2-Mercaptoethanol, Rivanol, and the Enzyme-linked Immunosorbent Assay (ELISA) (Morgan, 1982).
The reliability of serological tests to detect brucellosis depends on antibodies present at the time of the examination and the fact that infected animals may escape from detection. In order to improve the detection of brucellosis, the skin-delayed-type-hypersensitivity (SDTH) (intradermal test) test appears to offer a convenient alternative to the serological tests (Fensterbank, 1984).

2.12.1 Culture

Culture when positive, provides the definitive diagnosis and is considered the gold standard in the laboratory diagnosis of brucellosis. It is essential for determining antimicrobial susceptibility and performing strain typing. The yield and recovery are dependent on the culture method used and the type and volume of specimen used.

*Brucella* species have been recovered from foetal membranes, vaginal secretions, milk, semen, arthritis or hygroma fluids and the stomach contents, spleen and lungs from aborted foetuses. From the carcasses, the bacteria could sometimes be isolated from the lymph nodes, spleen, uterus, udder, testes, epididymes, joint exudate, abscesses and other tissues (Alton *et al*, 1988). A wide range of selective media can be used for cultivation. The suitable media include *Brucella* agar medium (BAM) base, trypticase soy agar, modified Thayer-Martin medium, Farrell’s medium, serum dextrose agar (SDA), glycerol dextrose agar and Castaneda’s medium (OIE, 2004).

In a four day old culture, colonies of *Brucella* appear in a smooth form and are pale honey in colour when viewed through a transparent medium, 1-2 mm in diameter, translucent and round, with smooth margins. The colonies are convex and pearly white when seen from the above. In rough form, the colonies are much less transparent and have a more granular, dull, matte white to brown surface. In nature, *B. abortus, B. melitensis, B. suis* and *B. neotomae* usually occur in smooth form.
while *B. ovis* and *B. canis* are found in rough form. Identification up to the genus level is done by biochemical tests and slide agglutination test (OIE, 2004). In India, Polding (1942) first reported the recovery of 46 isolates of *Brucella* from cattle, buffalo, goat, horse and man. Mathur (1963) isolated 16 strains of *B. abortus* from placenta of 14 cows having history of abortion as well as from 2 buffaloes and 8 strains were recovered from 23 milk samples.

### 2.12.2 Serology

Serological tests for brucellosis have been more useful in diagnosis when compared with direct demonstration of the infectious organism by culture (Lulu *et al* 1988) but serologically positive animals with no active *Brucella* infection complicate the diagnosis (Goldbaum *et al* 1992). Conventional serological procedures e.g. Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT), Compliment Fixation Test (CFT), and Indirect Fluorescent Antibody Test (IFAT) are all based on detection of anti-lipopolysaccharide (LPS) antibodies, which remain very high even after the recovery from the disease (Lulu *et al* 1988).

Historically STAT has been recognised as the principal serological test used for the diagnosis of brucellosis. IgM isotypes of antibody is the most active agglutinin at neutral pH (Nielsen *et al* 1984). Therefore the STAT is susceptible to false positive reaction by cross-reacting antibodies (Corbel 1998; Nielsen 2002). Due to low specificity by original tube agglutination test, a large number of modifications have been made to destroy or inactivate IgM agglutinins. Of these modifications, the Acidified antigen, Rivanol precipitation and 2-Mercaptoethanol are in common use in various laboratories for inactivating IgM. Grewal *et al* (2009a) developed an improved micro-titre plate agglutination test with advantages over the conventional STAT for diagnosis of *B abortus* infection in cattle and buffaloes. Grewal *et al* (2009b) used RBPT for detection of *Brucella* infection in aborted bovine foetus.
At present mainly serological methods are used for diagnosis of this infection in India. The long-term serological studies at national level have indicated that 5% of cattle and 3% of buffaloes could be infected with brucellosis (Renukaradhya et al, 2002). The serological tests depend on a reaction between *Brucella* antigen and antibodies produced in response to the infection. A number of classes and subclasses of antibody (isotypes) may occur in positive sera. The various serological tests vary in their ability to detect different isotypes.

In Rose Bengal Plate Test (RBPT) antigen is used at a pH of 3.65. The low pH prevents some agglutination by IgM and encourages agglutination by IgG1 thereby reducing non-specific interactions (Corbel, 1972 and 1973; Allan et al, 1976). RBPT is considered to be suitable for screening of individual animals, however, some cross-reacting antibodies have been detected by this test and false negative reaction may occur mostly due to prozoning (OIE, 2004). Numerous variations of the Indirect Enzyme Linked Immunosorbent Assay (I-ELISA) have been described employing different antigen preparations, antiglobulin-enzyme conjugates and substrates. Several commercial I-ELISAs are available that have been validated in extensive field trials and are in wide use. I-ELISA is a highly sensitive test but sometimes not capable of differentiating between antibody resulting from S19 vaccination or other false positive serological reactions (FPSR) and that induced by pathogenic *Brucella* strains. I-ELISA should therefore be considered more as a screening test rather than a confirmatory test for testing of vaccinated cattle or herds affected by FPSR problems (OIE, 2004). ELISA is comparable to the CFT and Rivanol test, but less sensitive than the STAT (Byrd et al 1979).

ELISA is more sensitive, more rapid and simpler method than the other tests (Magee 1980). Agreement between ELISA and other tests was found to be 100% (STAT), 75.7% (ME), 97.8% (MCF) and 95.2% (ACF). (Ruppanner et al 1980).
Specificity and sensitivity of ELISA for detection of brucellosis is comparable to those of STAT and CFT (Turilli et al 1986). The agreement between plate agglutination test (PAT) and STAT was found to be 61.7% (Kim et al 1988). ELISA gave more positive reactions than that of CFT when both these tests were comparatively evaluated for detection of brucellosis on sera of unvaccinated brucellosis free population, B. abortus strain 19 vaccinated brucellosis free population and a brucellosis positive population of unknown vaccination status (Kerby et al 1997) and sensitivity of BPAT, STAT, CFT, was found to be markedly lower than the ELISA (Molnar et al 1998).

Fifty nine (59) breedable murrah buffaloes were tested Sharma et al (1990) by applying various serological tests for brucellosis in Vietnam having history of abortion in buffaloes at 6-9 months of pregnancy and suspected for brucellosis. Of the 59 animals examined, 33 were found positive reactors.

Seroprevalence of 12.98% and 2.40% was reported by Lodhi et al (1995) from 208 sera samples collected from adult buffaloes and cows in and around Faisalabad when tested against RBPT and STAT respectively.

A serological survey of brucellosis in cattle and buffalo carried Isloor et al (1998) across 23 States of India using RBPT as screening test reported an overall prevalence rate of infection as 1.9% in cattle and 1.8% in buffaloes.

Seroprevalence of brucellosis as reported by Prahlad et al (1999) was found to be 7.09%, 2.70%, 11.14% and 8.10% when 296 buffalo sera samples were tested against RBPT, STAT, CFT and dot-ELISA, respectively. Rao et al (1999) reported that Dot-ELISA gave a higher percentage of positive results followed by RBPT and STAT.

Seroprevalence of infection as reported by Mehra et al (2000) among cows, heifers and buffaloes was 9.6%, 12.6% and 11.4%, respectively in organized farms.
and it was 2.2% cows vs. 9.4% in buffaloes of unorganized farms, when 877 cow,
349 heifer, 70 buffalo sera samples, from organized farms and, 135 cow and 95
buffaloe sera samples, from unorganized farms were tested by STAT.

The relative sensitivity and specificity of STAT and RBPT classified on basis
of ELISA results was found to be 88.61% and 98.59% for STAT, respectively and
56.96% and 96.77% for RBPT, respectively when 141 bovine sera were screened for
brucellosis using these tests (Chakraborty et al 2000).

Seroprevalance of brucellosis in Punjab as reported by Sandhu et al (2001)
was found to be 10.06 and 9.33% when 666 cows and 750 buffaloes were screened
for brucellosis, respectively.

Diagnostic sensitivity of I-ELISA and CFT was found to be 100% and 83%
respectively when 4803 cattle sera were tested against them, where as their
specificities were 99.8% and 100%, respectively (Paweska et al 2002). Further I-
ELISA performed on an automated ELISA wo rk station provided a rapid, simple,
highly sensitive and specific diagnostic system for large-scale detection of antibodies
against *B. abortus*.

Using RBPT Rajesh et al (2003) reported an overall 1.95% sero-prevalence
of brucellosis in 719cattle from Kerala.

In a seroprevalence study of brucellosis carried out by Varasada (2003) in
cattle (344) and buffaloes (251) of central Gujarat, 16.80%, 14.03% and 22.01% of
animals were found positive by RBPT, STAT and I-ELISA, respectively.

Sensitivities of avidin-biotin ELISA (AB-ELISA), RBPT and STAT in
detecting antibodies to *B. abortus* from cattle with a history of reproductive failures
and in healthy cattle were 100%, 88.22% and 90.59%, respectively (Sarumathi et al
2003). Further AB-ELISA proved to be a reliable screening test for detecting
antibodies to *Brucella* in cattle.
Prevalence of brucellosis in organized farms with abortion storms in Goa region as assessed by Barbuddhe et al (2004) was 37.38%, 36.45% and 40.18% when 107 sera samples were screened by RBPT, STAT and AB-ELISA, respectively.

Based on the findings of a study on prevalence of brucellosis in cattle, Chand and Sharma (2004) suggested use of ELISA in comparison to RBPT and STAT for assessing the situation of brucellosis in cattle, to have better results because chances of non-detection of an infected animal in ELISA is minimum. Erdenebaatar et al (2004) reported that ELISA can be used to eliminate false positive results amongst RBPT positive sera.

Seroprevalence study of brucellosis carried out by Nasir et al (2004) in 1473 cattle and 481 buffaloes from various Government and 286 cattle and 223 buffaloes from different private livestock farms was carried out by performing Rose Bengal plate test (RBPT) and serum agglutination test (SAT). RBPT recorded the seroprevalence as 14.70% in cattle and 15.38% in buffaloes at Government and 18.53% in cattle and 35.40% in buffaloes at various private livestock farms. Out of these RBPT positive animals, 7.19% cattle and 2.91% buffaloes at Government farms, whereas 9% cattle and 23.70% buffaloes at private livestock farms were found sero-positive using SAT.

A serological survey carried out by Singh et al (2004) in 6 organized dairy farms in Punjab using RBPT, STAT and AB-ELISA. To compare the sensitivity and specificity of RBPT and STAT, AB-ELISA was used as the gold standard. The study revealed that the sensitivity of RBPT (88.46%) was higher when compared with STAT (46.15%), while specificity of STAT (98.31%) was slightly higher than RBPT (97.75%).

A comparative study for detection of Brucella antibodies by Bhattacharya et al (2005), reported that AB-ELISA detected antibodies in more samples (43),
followed by RBPT (37) and the least by STAT (29) out of 360 bovine serum samples tested.

A total of 859 cattle and 133 buffaloes of organized sector and unorganized sector of Jodhpur region were screened by Kachhawaha et al (2005) using RBPT. The positive samples were tested by STAT. The prevalence of brucellosis was found much higher in cattle (41.79%) than in buffaloes (25.56%) and also more in cattle of organized sector in comparison to unorganized sector.

A random survey was conducted by Dhand et al (2005) to study the epidemiology of brucellosis in Punjab, using the ‘Survey Toolbox’ sampling software. Serum samples collected from the animals were screened for *Brucella* antibodies by an avidin-biotin enzyme-linked immunosorbent assay, which revealed an apparent overall prevalence of brucellosis to be 12.09%. The prevalence varied from a low of 0% to a high of 24.3% in various districts. The prevalence rates among buffaloes and cattle were 13.4% and 9.9%, respectively.

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

A serological survey of brucellosis in livestock animals and workers was conducted by Cadmus et al (2006) in Ibadan, Southwestern Nigeria between. A total of 1,210 cattle, 54 sheep, 496 goats, 200 pigs and 21 humans were screened using the Rose Bengal test (RBT). Prevalence in trade cattle was 5.82% while 0.86% was recorded in goats. None of the sheep and pigs was positive to the test. Out of the 11
samples taken from butchers, seven were positive and none of the ten herdmens were sero-positive.

A total of 194 serum samples from breeding bulls of different AI Centres of Gujarat were screened for presence of *Brucella* antibodies by Kanani (2007) using ELISA and reported an overall seroprevalence of 8.25%. They also found much higher seroprevalence in cattle than in buffalo bulls. Further sensitivity of RBPT and STAT was found to be of 50% and 62.5%, respectively when ELISA was considered gold standard test while specificity was found to be of 98.31% and 97.75%, respectively.

Serological test results from a study to determine sero-prevalence were used by Muma *et al* (2007) to compare the performance of RBPT, competitive-ELISA (c-ELISA) and fluorescent polarization assay (FPA) in diagnosing brucellosis in cattle using latent class analysis. The highest Sensitivity was achieved by the c-ELISA (97%) and the highest Specificity by the FPA (93%), conversely these tests also had the lowest Specificity and Sensitivity, respectively, with the RBT performing well in both the Sensitivity (93%) and Specificity (81%).

A second generation competitive enzyme immunoassay (c-ELISA) for detection of bovine antibody to *B. abortus* was developed by Nielson *et al* (2008) to eliminate reagent variables in the assay. The performance characteristics of the new c-ELISA, sensitivity, specificity and exclusion of antibody of *B. abortus* S19 vaccinated animals, were very similar to those of the classical c-ELISA and to the indirect enzyme immunoassay (I-ELISA) when using sera deemed positive by isolation of the bacterium.

Early detection of brucellosis is essential for its control and eradication. An Indirect Enzyme Linked Immunosorbent Assay (I-ELISA) was developed by Munir
et al (2008) and compared with the commercial kit using one hundred negative and positive sera each from buffaloes. The agreement for the positive result between the developed and commercial I-ELISA was 78% and for the negative it was 100%.

Serum samples (1106) of cattle from pastoral and agro-pastoral farming system were screened for antibodies against Brucella spp., by Dinka and Chala (2009) using the RBPT. Brucella antibody was detected in all study districts and an overall herd seroprevalence of 11.2% was recorded from the study areas.

Combinations of conventional serological methods and new ELISA procedures were evaluated to develop the most efficient and effective diagnostics for the detection of brucellosis in humans and animals by Grushina et al (2010). Sera from humans (n = 249) and animals (n = 99) were collected from brucellosis endemic areas (Zhambyl district and Enbekshi-Kazakh district of Almaty region in Kazakhstan) for serologic analysis. Sera from the humans reacted positively in the RBPT (38.5%), SAT (43.3%), I-ELISA (42.5%) while sera from the animals reacted positively in RBPT (79.8%), SAT (89.9%), CF (87.8%) and I-ELISA (100%).

Milk samples from cattle (n = 86) and buffalo (n = 114) were screened by Shafee et al (2011) using milk ring test (MRT) and indirect enzyme-linked immunosorbent assay (I-ELISA). An overall prevalence was found to be 3% and 8.5% in cattle and buffaloes using MRT and I-ELISA, respectively. The prevalence was 4.6% and 1.7% in cattle and buffalo using MRT, respectively, while I-ELISA exhibited 20% and 0% prevalence in cattle and buffalo, respectively. The prevalence was higher in government dairy farm, compared to privately owned dairy farm.

2.12.3 Polymerase Chain Reaction

The use of nucleic acid-based diagnostics in veterinary medicine has increased exponentially in recent years. These techniques have redefined the level of
information available for animal disease control programmes. In addition, modifications of nucleic acid detection techniques such as polymerase chain reaction (PCR) have led to the development of rapid, specific assays. The high sensitivity and specificity of PCR combined with its simplicity and speed has made it an assay of choice for detection of an analyte in a given sample in various infections. PCR has facilitated detection of bacteria particularly slow growing, difficult or time consuming to grow, and differentiation of of species or strains. In recent years this technique has been applied for the diagnosis of Brucella species and genus or species specific several PCR assays using primers derived from different gene sequences on Brucella genome such as insertion sequence designated as IS711 (Bricker and Halling 1994), 16S rRNA spacer region (Rijpens et al 1996), 31 KDa outer membrane protein (Baily et al 1992, Serpe et al 1999 Casanas et al 2001), 43 KDa outer membrane protein (Fekte et al 1992) and omp-2 outer membrane protein (Leal-Klevezas et al 1995), have been developed.

PCR assay was found far more sensitive than conventional cultural method. This coupled with its speed and reduction in risk to the laboratory workers, made this technique a very useful tool for the diagnosis of focal complications of brucellosis Morata et al (2001).

Three pairs of primers corresponding to (i) gene encoding 31 kDa B. abortus antigen (primers B4/B5), (ii) a sequence 16S rRNA of B. abortus (primers F4/R2) and (iii) a gene encoding an omp2 (primers JPF/JPR) were compared by Navarro et al (2002) for their sensitivities, for detecting Brucella DNA and reported that the most sensitive primers were F4/R2 as they amplified 8 fg of purified B. melitensis Rev 1 DNA.
PCR technique provides a promising option in *Brucella* diagnosis with high sensitivity in detecting *Brucella* from pure cultures. Vaid *et al* (2004) applied PCR for detection of brucellosis using primers derived from the 43 kDa outer membrane protein gene of *B. abortus*, the 16S rRNA gene, insertion sequence IS711, BCSP31 (*Brucella* Cell Surface Protein) gene.

PCR assay developed by Evangelista *et al* (2005) for detection of *Brucella* DNA from milk by using the eryD and wboA genes was reported to be more sensitive as compared to bacteriological method, but less sensitive than the serological methods.

PCR for detection of *B. abortus* infection in blood, milk and lymph tissues by using different primers that amplify various regions of the *Brucella* genome, IS711 genetic element, 31 kDa outer membrane protein and 16S rRNA was used by Leary *et al* (2006). They found that there was no amplification when PCR assays was applied to the blood samples, but obtained amplicons in a proportion of the culture positive milk (44%) and lymph tissue samples by the same methods.

A genetic marker for molecular detection of *B. abortus* targeting a conserved region of *Brucella* cell surface protein-31 (BCSp31) gene was developed by Asif *et al* (2009) and they reported 100% amplification efficiency of this assay in detection of *B. abortus*.

### 2.12.4 Comparison of serological, cultural and molecular methods

A diagnostic assay should be sensitive, specific, fast and easy to perform. Though there are a number of assays for diagnostic of brucellosis but none is 100% sensitive and specific. To overcome this weakness various authors have comparatively evaluated the efficacy of serological, cultural and molecular assays in diagnosing brucellosis. Ferris *et al* (1995) compared results of 6 serological tests viz


### 2.13 Prevention and Control

Until now, reliable cure for brucellosis in animals has not yet been devised because of its intracellular characteristics. The course of the disease may be modified by tetracycline alone or in combination with streptomycin. However, effective controls must be based on minimizing the potential for infection by sanitary methods, the factors dominant for the spread of the disease, and vaccination (Fensterbank, 1976). An effective control of animal brucellosis requires the following basic elements: (1) surveillance to find all the infected animals and herds, (2) controlling
the transmission of the infection to new animals or herds, and (3) the eradication of
the reservoir to eliminate the sources of the infection in order to protect susceptible
animals or herds (Metcalf, 1986).

The use of control measures for animals has a beneficial effect on the
incidence of the disease in humans. The prevention of disease transmissions within
the herd must include: the identification and subsequent slaughtering of infected
animals, the isolation of recently calved or aborted animals the cleaning and
disinfection of the quarantine area before other animals are reintroduced, the disposal
of all products of abortion by deep burial under a covering of lime, the purchase of
herd replacements from disease-free sources, and the isolation of pregnant
replacement animals until they have calved and passed a serological test.

The prevention of disease transmissions between several herds requires the
restriction of the movement of the animals and the quarantine of purchased animals.
Prevention and control are conducted by the best country basis, national rules
governing; diagnosis; vaccination; surveillance; the movement of animals, and by the
compulsory slaughtering of infected and potentially infected animals providing
financial compensation. The restriction of the sale and movement of infected and
disease-exposed animals by laws and regulations plays a major role in any control
program (Leslie et al, 1998).

The most beneficial control method in bovine brucellosis is vaccination at an
early age. The vaccine, which consists of a live suspension of a smooth-intermediate
attenuated strain of *B. abortus* (strain 19), has found worldwide use in cattle. It fully
protects 65 – 75 % of the animals, while the remaining animals are at least partly
protected (Alton, 1988).
The killing of infected animals is the most effective method of eliminating the reservoir of *Brucella* in individual herds because the organisms do not survive outside of the host. The slaughtering of infected animals while rescuing the meat is a satisfactory method of destroying *Brucella* infected host animals since the organism will not survive in the meat after the slaughtering. The depopulation of infected herds in the final phase of an eradication program is a convenient method of eliminating brucellosis from an area (Metcalf, 1986).

A study by Kumar *et al* (2005) reported that abortions in *Brucella* positive pregnant animals could be successfully controlled by using long acting tetracyclines. Such type of treatment is effective in Indian conditions where slaughter of animals is banned due to religious sentiments.

As education is an obligatory part of any disease control programme to be successful, therefore, it is mandatory for those who are concerned with the implementation of disease control programmes to make public aware of the disease i.e. about its etiology, epidemiology modes of transmission, economic, and public health importance and the modalities by which they are able to restrict the entry of the infectious agent into a healthy herd.