3.1. Introduction

Brucellosis is a highly infectious zoonotic and re-emerging disease for animals and humans. It poses health risk and is a neglected pathogen in developing countries including India (Hadush and Pal, 2013). The disease has been eradicated in most developed countries through the execution of several extensive control programmes. On the other hand, developing countries have continued to experience increased disease incidences because of lack of resources and properly coordinated control programmes. Infections cause significant economic losses by comparatively lowering milk production in livestock, abortion, public health and international trade implications and weak off-springs (Mathias and Mundy, 2010). The real rate of brucellosis is estimated to be 10 to 25 times more than annual reports (Bhargava, 2009). The human brucellosis incidence is directly correlated to the level of animal brucellosis in specific regions. The routes of infection are multiple: food-borne, occupational or recreational, linked to travel and even bioterrorism (Godfroid et al., 2005).

*Brucella* is non-motile, small, gram negative, non-spore forming, and strictly aerobic coccobacilli. It is positive for catalase and oxidase tests and shows variable results in urease tests (Yap *et al.*, 1992). *Brucellae* genus shows little variation genetically, eleven *Brucella* species have been recognized, they are genetically similar although each one has different host preferences (Godfroid *et al.*, 2011). *Brucellae* are highly potent pathogens in animals and humans and also effective biological agents for use in biological weapons even at very low concentration of 10 bacteria. *Brucellae* are easily transmitted to humans via aerosols and this makes the bacteria attractive for defense researchers. In notifiable Terrestrial and Aquatic Animal Diseases list of the World Organization noted for Animal Health, brucellosis disease has also been
categorized as Multiple species disease (OIE, 2014) and the main source of naturally acquired brucellosis in humans is always found to be from animal and very few cases of human to human transmission (Godfroid et al., 2005). Unpasteurized dairy foods, raw meat and carcasses are sources of infection for workers in the meat-packing industry and general population. Veterinarians may acquire brucellosis from assisting births in infected livestock, as well as through close contact and accidental inoculation (Young 1995).

The Brucella spp. has ability to successfully survive and replicate within different host cells, which explains their pathogenicity. Extensive multiplication of Brucella spp. in trophoblasts of placenta is associated with abortion in their preferential hosts, and its persistence in macrophages leads to chronic infections in both natural animal hosts and humans (Roop et al., 2009). Unfortunately, however, adequate health and safety measures are rarely observed in most developing countries, hence increasing the chances of zoonotic transmission (Swai and Schoonman, 2009). However, the fact that most animals, irrespective of where they originate, end up at the slaughter slabs or abattoirs is very important, because, apart from screening live animals at the herd level, screening slaughtered cattle at the abattoirs is also invaluable for the epidemiological investigation of bovine brucellosis.

India stands first in milk production in this context, an efficient and proper understanding of epidemiology is required to make proper control strategies as there is a growing concern that the disease may further flare up due to the intensive dairy farming. Brucellosis diagnosis is mainly based on detection of Brucella Lipopolysaccharide (LPS) specific antibodies in serum and milk samples based on serological tests. Serodiagnosis does not differentiate between an acute and chronic infection (Nirmi, 2003). Isolation of the brucella organism is considered the gold
standard diagnostic method for brucellosis since it is specific and allows biotyping of the isolate, which is relevant under an epidemiological point of view (Hadush and Pal 2013, Al Dahouk et al., 2003). However, in spite of its high specificity, culture of *Brucella* spp. is challenging. *Brucella* spp. is a fastidious bacterium and requires rich media for primary cultures.

Furthermore, its isolation requires a large number of viable bacteria in clinical samples, proper storage and quick delivery to the diagnostic laboratory (Hadush and Pal 2013; Seleem et al., 2010). Another limiting factor for culturing *Brucella* spp. is the requirement for appropriate laboratory conditions and personnel training, so the procedure can be performed safely (Nielsen and Ewalt, 2004). *Brucella* spp. is classified as a Biosafety level 3 organism, whose manipulation should be performed in biosafety level-3 laboratories (Lage et al., 2008).

Serological tests are crucial for laboratory diagnosis of brucellosis since most of control and eradication programs rely on these methods. Inactivated whole bacteria or purified fractions (i.e., lipopolysaccharide or membrane proteins) are used as antigens for detecting antibodies generated by the host during the infection. Antibodies against smooth *Brucella* species (*viz.*, *B. abortus*, *B. melitensis*, and *B. suis*) cross react with antigen preparations from *B. abortus*, whereas antibodies against rough *Brucella* species (*viz.*, *B. ovis and B. canis*) cross react with antigen preparations from *B. ovis* (Nielsen 2002). Although several serological methods are currently available, these tests can be classified as screening tests, monitoring or epidemiological surveillance tests like milk ring test and Rose Bengal Test.
3.2. Materials and Methods

3.2.1 Brucella reference strains and culture condition

*Brucella* strains - *Brucella S19, Brucella melitensis, Brucella suis* were procured from Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, Uttar Pradesh, India. *Brucella S99* from Indian Council of Agricultural Research - National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI), Bangalore, India. They were tested for the purity, biochemical and molecular characteristics before use. Type III Biosafety containment facility was used to culture the bacteria.

Tryptose agar medium supplemented with 10 % Peptone and 5 % Yeast extract was used for routine sub culturing and maintenance of the *Brucella* strains. Tryptose agar media plates were streaked with the cultures and incubated for three days at 37±1° C. After three to seven days of incubation, plates were held towards light source and colony morphology was studied.

3.2.2 Biochemical tests

**Gram’s staining**

A loopful of bacterial suspension was smeared onto a glass slide, air-dried and heat fixed by passing the slide rapidly two to three times using a Bunsen burner. The smear was flooded with crystal violet solution for 1 min. The slide was washed with a gentle stream of tap water and flooded with Lugol’s iodine for 1 min. Again the slide was washed with water and blot dried, and decolorized by washing with a gentle stream of 95 % ethyl alcohol for 30 s is to remove excess stain that will easily wash away, then blot dried and counter stained by flooding with safranin for 20 sec. The slide was again washed with tap water and blot dried. The preparation was observed
under a compound microscope at different magnifications for pink-red or blue-violet stained bacteria representing gram negative or gram-positive nature respectively.

**Oxidase test**

In a freshly prepared one percent solution of tetra methyl-p-phenylene-diamine dihydrochloride strips of Whatman No.1 filter paper were soaked for about 45 seconds, dried and then stored in a dark bottle. The cultures were used for oxidase test. Before use strip was moistened in distilled water and the colony to be tested was picked up and smeared over the moist area. A positive reaction was indicated by development of an intense deep purple colour, appearing with in 5-10 seconds. If there is no change in the colour of the reagent, the test result is negative.

**Urease test**

A loopful of freshly grown suspected culture were prepared in 0.3 mL of sterile distilled water in serological test tubes and homogenized in a vortex mixer, Brucella culture was inoculated in to the urease tubes and incubated at 37° C and observed for development of colour for every 10 min. The urease production was indicated by the development of purple pink colour.

**3.2.3 Study Population**

Five hundred milk and blood samples from the animals with history of abortion were collected from different districts of Karnataka, India. Two hundred ten samples were collected from villages of Mandya district, Karnataka, India. Blood was aspirated from jugular veins in plain vacutainer tubes without anticoagulant using aseptic techniques. The samples were kept on ice at about 4° C and transported immediately to the laboratory. Tubes were centrifuged at 3000 rpm for 3 min to
separate the serum and stored at -20°C till further use. Milk samples were aseptically obtained from all 4 quarters of the animal’s mammary gland during their routine milking time and stored at -20°C till further use. The blood and milk samples collected from different districts of Karnataka and villages of Mandya district, Karnataka, India

3.2.4. Presumptive test for brucellosis - Milk ring Test and Rose Bengal Test

**Rose Bengal test**

The RBT antigen consisted of standardised *B. abortus* antigen sourced from the IVRI, Izatnagar, India. According to Alton *et al.* (1975) the standard technique suggested was adopted. The test was carried out by mixing 0.02 mL of serum and 0.02 mL of *B. abortus* Rose Bengal coloured antigen on a slide and mixed by a spreader and observed for reaction up to four min. The results were recorded. Definite clumping/ agglutination was considered as positive reaction, whereas no clumping/ agglutination was considered negative. Experiments were conducted in triplicates and repeated three times.

**Milk Ring test**

According to the Blythman and Forman (1977) Milk ring test was performed by adding 30 μL of *B. abortus* bang ring antigen (IVRI, Izatnagar, India). The milk and antigen mixtures were incubated at 37°C for 1 h, together with positive and negative control samples. Experiments were conducted in triplicates and repeated three times.
3.3. Results

Cultural and biochemical confirmation of *Brucella* species

The *Brucellae* studied were Gram-negative coccobacilli and non-motile organisms. All the *Brucella* strains grew well on Tryptic soy agar supplemented with 10 % peptone and 5 % yeast extract without the need for CO₂ atmosphere at 37°C. The colony morphology of this group of organisms was typical for each species described. Broadly, the colonies were pinpointed, translucent, pale honey colored and convex from above after three days of incubation.

**Gram’s staining**

The reagents of Gram’s staining *i.e.* crystal violet, Gram’s iodine, Safranine were procured locally. The cultures were stained by the standard procedures described by Cruickshank *et al.* (1975) to study the Gram’s reaction of the organisms.

**Oxidase test**

Strips of What man, No.1 filter paper were soaked in a freshly prepared one percent solution of tetra methyl-p-phenylene-diamine dihydrochloreide for about 30 sec, dried and then stored in a dark bottle. The cultures were used for oxidase test. Before use strip was moistened in distilled water and the colony to be tested was picked up and smeared over the moist area. A positive reaction was indicated by development of an intense deep purple colour, appearing with in 5-10 sec.

**Urease test**

A loopful of each *Brucella* strain was inoculated in to the urease tubes and incubated at room temperature and checked for development of colour. The production of urease was indicated by the development of purple pink colour.
Presumptive test for brucellosis - Milk ring Test and Rose Bengal Test

From the collected total samples of 710, 500 Milk and blood samples were collected from the different districts of Karnataka, India (Table 3.1), and 210 blood and milk samples were collected from the rural villages of Mandya district (Table 3.2). Samples were screened for brucellosis infection by MRT (Fig. 3.1) and RBT (Fig. 3.2). Out of which Using Rose Bengal test (RBT) 4.6 % and Milk ring test (MRT) 3.4 % prevalence was observed in Mysore, Chamarjanagara, Mandya and Hassan. In rural village of Mandya district Rose Bengal test (RBT) 3.3 % and Milk ring test (MRT) 3.3 % prevalence was observed in Kennalu, Alpahalli, Chikkade, Chinakuralli, Hiremarali, Banangadi, Manchanahalli, and Basthihalli.

Table 3.1. Screening of different milk and blood samples for brucellosis collected from different regions of Karnataka, India.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Total no. of samples</th>
<th>Milk Ring Test</th>
<th>Rose Bengal Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Milk</td>
<td>Blood</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Mysore</td>
<td>170</td>
<td>170</td>
</tr>
<tr>
<td>2</td>
<td>Chamarjanagara</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>Mandya</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>4</td>
<td>Hassan</td>
<td>90</td>
<td>90</td>
</tr>
</tbody>
</table>

Samples collected from Different regions of Karnataka were screened for Brucellosis infection in bovines by Milk ring test and Rose Bengal test.

Table 3.2. Screening of different milk and blood samples for brucellosis collected from villages of Mandya district, Karnataka, India.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Total no. of samples</th>
<th>MRT</th>
<th>RBT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Milk</td>
<td>Blood</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Kennalu</td>
<td>57</td>
<td>57</td>
</tr>
<tr>
<td>2</td>
<td>Alpahalli</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>Chikkade</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>Chinakuralli</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>Hiremarali</td>
<td>09</td>
<td>09</td>
</tr>
<tr>
<td>6</td>
<td>Banangadi</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>7</td>
<td>Manchanahalli</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>8</td>
<td>Basthihalli</td>
<td>18</td>
<td>18</td>
</tr>
</tbody>
</table>

Samples collected from Different villages of Mandya districts were screened for Brucellosis infection in bovines by Milk ring test and Rose Bengal test.
Fig. 3.1: Presumptive test for the detection of Brucellosis- Rose Bengal test. Rose Bengal test showing negative (left) and Positive (right) results for Brucellosis. Agglutination is observed in the positive for Rose Bengal test.

Fig. 3.2: Presumptive test for the detection of Brucellosis-Milk ring test. Milk ring test showing Positive (left) and negative (right) results for Brucellosis. Purple color ring formation is observed for positive for MRT.

3.4 Discussion

Bovine brucellosis is one of the most re-emerging infectious diseases causing difficulties for veterinary authorities that are confronted, not only with animal but also
with major public health implications. In animals, *B. abortus* is usually transmitted by contact with the placenta, fetus, fetal fluids and vaginal discharges from infected animals. Animals are infectious after either abortion or full-term parturition. *B. abortus* may also be found in the milk, urine, semen, feces and hygroma fluids. Shedding in milk can be prolonged or lifelong, and may be intermittent. Many infected cattle become chronic carriers. Infection usually occurs by ingestion and through mucous membranes, but *B. abortus* can be transmitted through broken skin. Although the mammary gland is usually colonized during the course of an infection, it can also be infected by direct contact, with subsequent shedding of the organisms in the milk. Other species can be infected with *B. abortus* after contact with infected cattle or other maintenance hosts. Carnivores do not seem to be a significant source of infection for other animals. Dogs and coyotes can be infected with *B. abortus*, shed bacteria in reproductive discharges, and can infect cattle if these species are kept in close confinement under experimental conditions. In cattle, abortions and stillbirths usually occur two weeks to five months after infection. Reproductive losses typically occur during the second half of gestation; thus, the incubation period is longer when animals are infected early in gestation.

Here in, for the first time, a large population based brucellosis survey covering diverse cattle populations in different regions of Karnataka, India is reported. According to Corbel, (2006) globally brucellosis is under-reported because of its unclear clinical flu like symptoms, difficult in laboratory diagnosis and lack of understanding by medical professionals. In the present study 3.4 % of brucellosis positive for MRT test showed concordant matches to study of Islam et al. (2013) in Bangladesh. Since MRT is a presumptive test, negative results do not mean animals are not infected with *Brucella* spp. Several possible reasons have been taken into account to explain the relatively inconsistent performance of *Brucella* detection. The
Brucella could be located only in the lymph nodes and not yet reached the milk at the sampling time. The infection stage also influences the antibody level and the number of the bacteria (Alton et al., 1988). Some serological tests lack sensitivity and it is impossible to differentiate antibodies produced after vaccination from those produced after infection (Godfroid et al., 2010). Gram negative bacteria that have antigenic similarities with Brucella LPS can lead to false results by cross reactivity with Yersinia enterocolitica O:9, Escherichia coli and some Salmonella spp. (Nielsen et al., 2004).

Clinicians have to rely on serological tests to confirm the diagnosis of brucellosis in humans since the disease manifests with a variety of symptoms. The SA test is the most used assay, however its results can be confusing where the person does not have the disease but has been repeatedly exposed to an infectious source. Frequently the test is negative or inconclusive during the incubation stage or in the late chronic stage of the disease, since following an infection the IgM declines more rapidly than IgG levels, and in chronic stages of the disease the predominant immunoglobulin present is IgG (Morgan, et al., 1969). The use of anti-human gamma globulin serum (AHG) in the test was found to be more reliable and sensitive in detecting such non-agglutinating antibodies (Kerr, et al., 1969) The CF test has also been found to be more reliable in diagnosing brucellosis. Sirmatel et al. (2002) sampled sera of 184 humans diagnosed clinically with either acute or chronic brucellosis and found the SA test with a higher rate of positivity (83.7 %) compared to 61.9 % for both the RBP test and the IgG detecting ELISA. In the sera from healthy control individuals, the RBP test was positive in 25 % of the individuals negative with both the SA test and the ELISA. Merta et al. (2003) tested, with SA and RBP tests, serum samples from 310 patients with clinical signs, which would evoke a differential diagnosis including brucellosis. Both the tests showed a 100 % specificity and
sensitivity compared with culture positive individuals. The RBP test is considered highly sensitive and specific, but it remains positive for long periods of time in patients who have received treatment for brucellosis (Awad, 1998; Diaz and moriyon, 1989). Relative to the diagnosis with the SA test, the RBP test had a higher sensitivity compared with the CF test.

Clavijo et al. (2003) reported that all 87 individuals that had acute (<3 months) disease were positive on culture for Brucella, whereas only 23 of 46 that had chronic (>3 months) disease were positive. The RBP test was more sensitive, since out of 133 patients, 128 patients were positive with RBP test compared to 120 and 90 with the SA and IgM detecting ELISA tests, respectively. The RBP test may be an initial test of choice, if adequate standards of performance of the RBP test and the reagent quality are maintained, in rural hospitals and clinics. This is especially true for developing countries where the population is at a greater risk of infection and clinics are not properly equipped for performing ELISA or CF test (Oomen and Waghela, 1974; Maichomo, et al., 1998 and Glanville, et al., 2017). Our study shows that RBP test, a comparatively rapid test, has good sensitivity and specificity relative to the routinely used SA test supporting the finding of Yohannes et al. (2012), who recommended use of at least two tests coupled with clinical history of the patient to confirm the diagnosis of brucellosis.

The milk ring test (MRT) is a satisfactory inexpensive test for the surveillance of dairy herds for brucellosis and may serve as an alternative to serum or milk based tests. Single MRT would have a 65% probability of detecting one reactor cow in more than 95 % of the herds in most dairy states if the test was conducted on bulk tank samples (Roepke and Stiles, 1970). Popova and Arakelyan (2011) concluded that the MRT has good sensitivity particularly for early stages of brucellosis in animals. The
MRT works on the principle that antibodies against *B. abortus* attach themselves to fat globule which rise to the surface of the milk, clustering in the cream layer and bind with tetrazolium/haematoxylin stained *B. abortus* antigen to form a ring in the creamy layer of milk (Sutra *et al.* 1986; Huber and Nicoletti, 1986).

In a study in Vindhya region of Madhya Pradesh, 4.58 % milk samples of cows were found positive with milk ring test (Singh *et al.*, 2016). Kumar *et al.* (2016) screened milk samples from selected districts of Tamil Nadu and found 4.35 % of milk samples positive for brucellosis using MRT. Shome *et al.* (2014) in a detailed study found overall positivity of 2.55 % among 64818 pooled milk. Dubey and Mathur (1980) found prevalence of brucellosis to be 4.51 % in cows and 3.4 % in buffaloes Milk samples in the Ajmer and Tonk districts of Rajasthan by the milk ring test. In various earlier studies, there has been a variation in prevalence of brucellosis. The prevalence of brucellosis has been recorded both high and lower than that observed in the present study. This indicated the spatial variation among the prevalence of brucellosis in bovines. Such a high prevalence as recorded in this study is a serious public health concern and there is a need to create awareness among the masses for disease prevention.

Each year, half a million cases of brucellosis are reported worldwide. Recently, many countries have eradicated brucellosis from their herds, and many other countries significantly reduced the prevalence of the infection among their livestock. In Karnataka, in spite of the number of research works on Seroprevalence of brucellosis in cattle and humans, there are no reports on bacteriological isolation and identification of *Brucella* spp. from serologically negative dairy cattle. In the present study, *Brucella* spp. was isolated from seronegative dairy cattle with a history of abortion, repeat breeding, retention of the placenta, and stillbirth. Hence, it is very
important to isolate *Brucella* isolates to design an effective control measure for brucellosis in Karnataka. So, it is advisable to detect or eradicate brucellosis; a bacteriological test and a PCR technique should be performed in addition to serological test of milk sample.

In conclusion these findings also portend significant public health implications following great economic losses to poor people particularly in the rural areas. This study also connected with the background of nationwide epidemiological surveillance, required to quantify extent of economic loss on farm animal and there control strategies.