1. INTRODUCTION

India is an agrarian nation and a large extent of the population, particularly in the rural areas depends on agriculture and primarily on animal production. Brucellosis is an important zoonotic disease which is naturally transmitted between animals and humans. Zoonotic infections represent an important group of occupationally-acquired infectious diseases in agrarian and meat industry workers. The different occupational groups exposed to animals include veterinarians, cattle inspectors, workers in slaughter house, butchers, meat industry workers, health care workers and laboratory workers. They are at high risk of acquiring these infections than the general population. Veterinarians and abattoirs are having high occupational risk, because of direct exposure to infected body fluids and tissues of brucellosis infected animals.

Brucellosis is caused by acid fast gram-negative coccobacillus belonging to the genus Brucella named after Sir David Bruce, who first isolated this microbe. The etiological agent, mode of transmission, clinical manifestations and infection severity were known in the late nineteenth and mid 20th century because of pioneering work by several investigators. The cause of the disease was difficult to understand until Bruce reported numerous small coccoid organisms in stained sections of spleen from a seriously infected soldier. He put forth the name *Micrococcus melitensis* (Madkour and Kasper, 2001). Brucellosis has been known by a few names like undulant fever, Mediterranean fever, Gibraltar fever, Malta fever and so forth (Wright, 2000).

Asia is the world's largest milk producing region according to the Food and Agricultural Organization (FAO, 2012); Within the Asian region, India stands number one in the milk production globally. India contributes a whopping 9.5 percent of
global milk production of cow’s milk alone; majority of the milk in India is from buffaloes. From unorganized farms of small farmers over eighty percent of the milk production comes. There are more than 130000 dairy cooperative societies at the Village level (Dairy technology catalogue, 2018). India also exports milk to other countries. Hence we can understand how important the dairy sector is in adding growth to the global economy. Animal husbandry and dairying plays a prominent role in the rural economy in supplementing the income of rural households, particularly, the landless, small and marginal farmers. Several measures initiated by the government to increase the productivity of huge livestock have resulted in significant increase in the raw cow milk output.

Brucellosis is a zoonosis caused by facultative intracellular bacteria of the genus *Brucella*. *Brucellae* are a small non motile aerobic, coccobacilli belong to α-2 subdivision of the proteobacteria. Other members in this group include *Ochrobactrum, Rhizobium, Rhodobacter, Agrobacterium, Bartonella* and *Rickettsia*. This disease is caused by a single or several *Brucella* species and biovars in different geographical region. Currently, the genus *Brucella* contains eleven species: *B. melitensis, B. abortus, B. suis, B. ovis, B. canis* and *B. neotomae*. *B. pinnipedae, B. cetacean, B. innopinata* and *B. microti* (Whatmore, 2009). *Brucella melitensis, B. abortus, some B. suis* biovars are the most commonly reported in domestic animals and *B. canis* also known to be zoonotic (Al Dahouk et al., 2013). Though *Brucella* exhibits host species preference, cross-infections to other animal species is also possible to occur (Corbel, 2006).

Brucellosis is found around the world, though it has been eradicated from many developed countries, it is one of the most neglected serious diseases in
developing countries. The rate of contamination fluctuates from one country to another and between regions and within the country, with the highest prevalence in dairy cattle. In India, brucellosis was first reported in 1942 and is currently endemic all through the nation (Renukaradhya et al., 2002). The disease has been reported in cattle, buffaloes, sheep, goats, pigs, dogs and human beings. In general, risk factors such as unhindered trade and movements of infected animals, recreational, utilization of semen from unscreened bulls for manual sperm injection and poor farmhouse cleanliness presumably ascribe to the spread and transmission of the disease. Grazing of the animals in the yards and association with a frequent assimilation of flocks of sheep and goats also feature in the high prevalence and wide distribution of Brucellosis.

The counteractive action of development or re-emergence of brucellosis also requires the knowledge about magnitude of problem in a given geographical area. People infected with Brucella species, more often will not have signs and manifestations; predictable with a flu like or septicemia ailment, frequently with tricky on set. The manifestations and clinical signs most usually revealed are, fever, exhaustion, discomfort, chills, sweats, migraines, myalgia, arthralgia, weight reduction (Young, 1983; Young, 1989).

The development of a definitive diagnostic test for brucellosis remains a tricky target. From the infected animal brucellosis is identified either by isolation of Brucella organisms in culture or by a combination of serological tests and clinical findings consistent of brucellosis. Gold standard method is always isolation of the Brucella pathogens which is one of the perfect means of diagnosis but in practice it is difficult due to the early tissue localization, exacting culture needs of the organism.
and also cumbersome. In practice blood cultures are positive in 10-30% of brucellosis and the remaining is analysed serologically (Young, 1983).

Serological tests are generally utilized for the diagnosis of brucellosis and they are exclusively utilized as a part of annihilation programs. Particular antibodies to Brucella in serum and other body liquids are identified by a consistently expanding assortment of procedures; some of the tests have been developed with a view to differentiating antibodies resulting from infection and those from vaccination, and others aim to detect the chronic carrier animal in areas or herds with a very low incidence (Morgan et al., 1967). Serum, uterine discharge, vaginal mucus, milk, or semen such body fluids from suspected cattle may contain different quantities of antibodies of the IgM, IgG1, IgG2 and IgA types directed against Brucella (Beh, 1974). Brucellosis infected animal may or may not make all antibody types in detectable quantity; Several tests are used to detect brucellosis, for blood tests are the complement fixation test (CFT), Rose Bengal test (RBT), serum agglutination test (SAT) and the enzyme-linked immunosorbent assay (ELISA). Anti-globulin (Coombs) test and the 2-β mercaptopethanol or rivanol daptions of the SAT are performed very rarely. The milk ring test and ELISA is used for detecting antibodies to Brucella infection in milk. The MRT test is inexpensive, effortless and requires no particular or specialized equipment to carry out. It detects anti-Brucella IgM and IgA bound to milk fat globules. Though, false positive reactions occur when milk that contains colostrum or milk at the end of the lactation period is used. As well as milk from cows suffering from a hormonal disorder or cows with mastitis may produce false reactions (Bercovich and Moerman, 1979). The RBT is a basic agglutination technique. Because the test does not need any special laboratory facilities and is easy and simple to perform, it is used to screen sera for antibodies to Brucella. The test
may yield false negative results, although rarely in infected cattle that give positive results with the CFT (Tanwani and Pathak, 1971). Although the low pH (3.6) of the antigen enhances the specificity of the test, the temperature of the antigen and the ambient temperature at which the reaction takes place may influence the sensitivity and specificity of the tests (MacMillan, 1990).

Conventional species identification and biotyping involves the gold standard isolation, phage susceptibility tests, agglutination tests, and biochemical tests such as growth on media containing dye and hydrogen sulphide production tests. These techniques are lengthy and time consuming, involve risk of handling live cultures, and require highly expertise personnel for interpretation. PCR-based species identification is fast, easy to perform and interpret, and offers a good alternative to conventional techniques.

Multiplex PCR (mPCR) is an analytical technique that is used for simultaneous identification of several gene sequences belonging to the same pathogen. The main advantage of the mPCR as compared to the conventional method is its cost effectiveness. The primary advantage is, less reagent and enzyme (Taq DNA polymerase) utilization. Another advantage is that like pathogens are analyzed together in addition, short time is required for sample preparation and getting the results. The only limitation is that the amplified fragments of same length cannot be detected and lower quantity of amplified product may not be visible on agarose gel. This could be overcome by, designing the primers longer than those used in conventional PCR having a higher melting temperature (Tm). Bruce-ladder was species specific PCR and all the strains and biovars from the same Brucella species gave the same profile. The practical interest of Bruce-ladder for typing purposes is
evi
dent since some of the cumbersome and long-lasting microbiological procedures currently used could be avoided.

Single-strand conformation polymorphism (SSCP) analysis is a rapid highly sensitive and cost effective approach for characterizing DNA sequences. This method was first described by Orita et al. (1989) and SSCP has since been successfully used for detecting various alterations in amplified DNA base sequences, which includes substitutions, deletions, insertions, and rearrangements. Polymerase chain reaction (PCR) products are now routinely used for SSCP analysis (PCR-SSCP) (Fedorko et al., 2001). After PCR amplification of the targeted sequences, the amplified DNA product is denatured to form single stranded DNA and subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE). Under non denaturing conditions, Single stranded DNA (ssDNA) has a secondary structure that is determined by the nucleotide base sequence. The mobility of the ssDNA depends on the secondary structure of the amplified DNA product. Different banding pattern of ssDNA at different positions on the gel indicate different sequences. PCR-SSCP is capable of detecting at a higher degree of about more than 90% of all single base substitutions in 200 bp fragments.

Low-stringency single specific primer PCR (LSSP-PCR) is an extremely easy and simple technique that permits the detection of single or multiple mutations in gene-sized DNA fragments. Briefly, amplified DNA bases are subjected to PCR using high concentrations of a single specific oligonucleotide primer, larger amount of Taq DNA polymerase, and a very low annealing temperature. Under these conditions, the primer hybridizes specifically to its complementary region and non-specifically to multiple sites within the DNA fragments in a sequence-dependent manner, producing a heterogeneous set of reaction products that constitutes a unique “gene signature
profile” (Pena et al., 1994). LSSP-PCR has been widely used in many microorganisms and in the fields of genetics and molecular medicine to achieve rapid, economical and sensitive detection of mutations in the gene sized fragments as well as sequence variations in the DNA fragments.

Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents (Cohen, 1992). For brucellosis treatment, a combination of antibiotics that penetrate the macrophage should be used to treat the infection of brucellosis which is intracellular infection. The preferred treatment for human brucellosis caused by B. melitensis field strains is a combination of long-acting tetracyclines and streptomycin, a gentamicin/doxycycline combination may be the first choice (Grilló et al., 2006). In general, tetracycline/aminoglycoside combinations are the most widely given antibiotics for brucellosis treatment. However, because of high rates of treatment failure or relapses due to emerging resistance, the treatment of brucellosis is still problematic as the disease is pathognomonic. Therefore, novel antibacterial compounds are becoming essential for brucellosis treatment. Medicinal plants have always been vital sources for new drug discovery. Plants readily synthesize substances for their defense against insects, herbivores, and microorganisms (Aboaba et al., 2006). Furthermore, they may also produce secondary antimicrobial metabolites as a part of their normal growth and development or in response to stress (Mirjana et al., 2004). Many people in rural region still depend on the traditional medicine for the treatment of their ailment and since ancient era various parts of plants has been used in the treatment and prevention of various diseases (Tanaka et al., 2002). Recent theories state that secondary
metabolites produced by plants extensively depend on the environmental circumstance, period and intensity of stress, genetic plasticity and composition of plants. Based on these theories, area of plant growth can influence chemical composition of secondary metabolites in plants (Zhao et al., 2005).

Hence the present study was taken up with the following objectives,

1. **Collection and isolation of** *Brucella* **species from dairy samples.**

2. **Speciation polymerase chain reaction and genetic typing of** *Brucella* **species.**

3. **Development of SSCP-PCR and LSSP-PCR assays for identification** *Brucella* **species.**

4. **Management of brucellosis from plant bioactive potential molecules.**

**Chapter I: General introduction**

This thesis comprises of different chapters and chapter 1 includes the general introduction.

**Chapter II: Review of literature**

A comprehensive review of literature pertaining to the topic of the study is provided in this chapter.

**Chapter III: Collection and Isolation of** *Brucella* **species from dairy samples.**

Samples were collected from different regions of Karnataka, India, and also samples were collected from the rural villages of Mandya District, Karnataka. The collected samples were screened by the presumptive tests. Milk samples were
aseptically obtained from all four quarters of the animal’s mammary gland during their routine milking time and stored at -20°C till further use. 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. Reference Strains were procured from Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, Uttar Pradesh, India, and 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 was used to culture the bacteria. The *Brucella* species was cultured in Type III biosafety cabinet; Ming Test (MRT) was performed according to Blythman *et al.* (1977) positive samples were based on the purple coloured ring formation in the milk positive and negative samples were differentiated. The serum samples collected were tested with Rose Bengal Test (RBT) as described by Alton *et al.* (1988). The appearance of agglutination was recorded.

**Chapter IV: Speciation polymerase chain reaction and genetic typing of* Brucella* species.**

As the brucellosis infection varies from mild to severe fatal stage, laboratory based techniques gain importance for the accurate diagnosis. Culturing of the organism in the laboratory is the most demonstrative approach, but due to its lengthy work and prolonged time, alternatively Polymerase Chain Reaction (PCR) based molecular technique can be used as a rapid and specific for the diagnosis of brucellosis especially in situations of outbreaks. This problem was overcome by
developing molecular techniques that can rapidly detect and characterize microorganisms in a shorter time. Detection of bacteria by polymerase chain reaction (PCR) amplification using 16S rRNA and specific oligonucleotide primers offers many advantages over classical techniques; neither purification nor cultivation of the pathogen is required and the specificity, sensitivity and response time of tests are improved. In this contrast, PCR technique is highly sensitive, less laborious method to detect pathogen by specific primers. Recent advances in the field of molecular genetics have led to new possibilities for pathogen detection that depend on the recognition of DNA sequences that are specific only to the pathogen genome that is to be detected.

DNA from all dairy and blood samples and bacterial strains was extracted using a commercial purification system with columns (QIAamp Blood Midi; QIAGEN GmbH, Hilden, Germany). The purity and concentration of the genomic DNA extracted from samples was estimated by Nanodrop spectrophotometer. PCR assay was carried out for gene bscp31 encoding an immunogenic outer membrane protein of 31 kDa of B. abortus, which is conserved in all Brucella spp. ABC transporter gene was used for the designing of species specific primer, ABCT primer set was synthesized by using the primer 3 software. Bruce ladder multiplex PCR assay was performed to all the brucella strains from different animal and geographical regions. All the positive isolates subjected for Bruce ladder speciation PCR Bruce ladder speciation PCR is a low risk diagnostic technique because there is no manipulation of the genetic form the agent in laboratory. This is very important in the case of highly pathogenic microorganism to men.
Chapter V: Development of SSCP-PCR and LSSP-PCR assays for identification of *Brucella* species.

Many arrays of molecular approaches have been investigated by the researchers in search for alternative techniques for more efficient, rapid identification of zoonotic pathogens. This chapter includes the development of Single-strand conformation polymorphism (SSCP) and Low-stringency single specific primer (LSSP) for discrimination of *Brucella* species isolates. Species-specific PCR based methods are commonly used detection methods for identification and genetic diversity studies of pathogenic bacteria. LSSP and SSCP-PCR amplification provides reliable pathogen detection in routine testing by amplification PCR reaction mixture.

Here, we developed a new, general approach for directly determining the identity of bacteria based on the principle of SSCP and LSSP, electrophoresis of PCR-amplified specific primers. SSCP and LSSP PCR were assessed for genetic typing of *Brucella* species isolates from the different regions of karnataka. SSCP allowed *Brucella* species specific patterns to distinguish pathogen up to species level. The individual genetic profiles of *B. abortus* isolates were obtained by LSSP-PCR; the polymorphic variation was detected by LSSP polymorphic banding patterns. The genetic variation of LSSP was confirmed by sequence analysis of isolated strains. SSCP and LSSP-PCR techniques were most effective for preliminary screening of genetic variability in *brucella* pathogen which since the early specific and rapid identification of carriers/reservoirs of pathogens plays a crucial role in prevention of further spread of brucellosis infection. The SSCP-PCR species specific Brucella surface cell protein of 31KDa (*BSCP31*) gene was used to identify and discriminate the *B. abortus* at species level for the first time. SSCP analysis may eliminate false
diagnoses due to cross contamination (Yap et al., 1992). Cross contamination is a serious problem commonly associated with enzyme-linked immunosorbent assay (ELISA) and species-specific detection (Schots et al., 1994).

**Chapter VI: Management of Brucellosis from plant bioactive potential molecules.**

The present study comprises the use of traditional medicines to overcome the side effects caused by chemotherapeutics. In the present study an attempt has been made to use the herbal medicine to cure brucellosis infection either by direct killing or by inhibiting the growth of brucellosis. In this study Different medicinal plants were collected from Western Ghats of Karnataka, India and screened for the antibrucellosis activity. *Acacia nelotica, Withania somnifera, Eugenia jambolana, Callistemon citrinus, Clerodendrum inerme, Terminalia arjuna, Lucas aspera, Thevetia peruviana, Hemidesmus indicus, Gloriosa superba, Cymbapogan citrates, Acorus calamus, Rhamnus cathartica, Cinnamomum verum, Thuja occidentalis* and *Sant. album*. Plant leaves were dried, weighed, grounded into fine powder and extracted using different solvents. *Brucella* strains - *Brucella abortus, Brucella melitensis, Brucella suis* were procured from Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, Uttar Pradesh, India. *Escherichia coli* (1610), *Salmonella typhimurium* (98) *Bacillus cereus* (430), *Shigella flexneri* (1457), *Vibrio cholera* (3904), *Pseudomonas aeruginosa* (1688), *Vibrio paraheamolyticus* (451), *Bacillus subtilis* (6939) and *Enterobacter aerogenes* (13048) bacterial strains were obtained from Microbial Typing Culture Collection (MTCC), IMTECH, Chandigarh, India and American Tissue Culture Collection (ATCC) and were cultured as per the protocol prescribed by MTCC and ATCC respectively and were used in the study. Human
brucellosis still presents scientists and clinicians with several challenges, with regard to the understanding of its pathogenic mechanism, severity, progression, and development of improved treatment regimes (Bosilkovski et al., 2015). The treatment of brucellosis is still problematic. Thus, new antibacterial compounds are becoming necessary for brucellosis treatment.