Summary and Conclusion

Brucellosis is a zoonotic disease that severely hinders livestock productivity and human health worldwide. The burden that the disease places specifically on low-income countries has led the World Health Organization (WHO) to classify it as one of the world’s leading ‘neglected zoonotic diseases (WHO, 2016). The global incidence of human brucellosis is estimated to exceed 800,000 cases per year, of which 40% are estimated to result in a chronic infection of brucellosis (Kirk et al., 2015). There are currently twelve different species described within the genus Brucella (Whatmore et al., 2014 and Scholz et al., 2016). Conventional methods used to culture the pathogen on media, which is considered as golden standard method, which is reliable and accurate, but its time-consuming and labour intensive. Blood culture is the gold standard in the diagnosis of bacterial infections including brucellosis, but success rate of this method is only 40-70% of the cases (Rubach et al., 2013).

Medicinal plants have been recognized as a part of the evolution of human healthcare for thousands of years. Medicinal components from plants play an important role in traditional as well as in modern medicine. Antimicrobial resistance is progressively becoming a serious threat to global public health. According to World Health Organization (WHO) report on antimicrobial resistance in 2014, overcoming the antibiotic resistance is the major challenge for the next millennium (ZumLa et al., 2015). In general, tetracycline/aminoglycoside combinations are the most common antibiotics used for brucellosis treatment. However, because of high rates of treatment failure or relapses due to emerging resistance, the treatment of brucellosis is still
problematic. Thus, new antibacterial compounds are becoming necessary for brucellosis treatment.

The four objectives of the study included collection and isolation of *Brucella* species from dairy samples, speciation polymerase chain reaction and genetic typing of *Brucella* species, development of SSCP-PCR and LSSP-PCR assays for identification *Brucella* species and management of Brucellosis from plant bioactive potential molecules. Chapter III concentrated on collection and isolation of *Brucella* species from Karnataka India. Total of 710 dairy samples were collected from different regions, out of which five hundred milk and blood samples from the animals with history of abortion were collected from different regions of Karnataka, India. Mysore (170), Chamarjanagara (90), Mandya (120) and Hassan (60). Two hundred ten samples were collected from the rural villages of Mandya District, Karnataka, India. Kennalu (57), Alpahalli (33), Chikkade (23), Chinakuralli (19), Hiremarali (9), Banangadi (27), Manchanahalli (24) and Basthihalli (18).

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. *Brucella* strains - *Brucella S19*, *B. melitensis*, *B. 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 was used to culture the bacteria. The Brucella species was cultured on brucella selective agar medium supplemented with Horse serum and brucella selective supplement. Cultured bacteria were maintained in Tryptic soy agar supplemented with 1 % Tryptone 0.5 % Yeast extract. The cultures were handled in Type III biosafety cabinet; cultures were incubated anaerobically for 24h to 72h.

Milk Ring Test (MRT) was performed according to Blythman *et al.* (1977) positive 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. 4.6 % seropositivity by the Rose Bengal test and 3.4 % positive prevalence by Milk ring test for samples collected in Mysore, Chamarajnagar, Mandya and Hassan was recorded. The overall seropositivity was 4.3 % for Rose Bengal Test and 3.3 % for Milk Ring Test was recorded from the samples collected from the rural villages of Mandya district, Karnataka India.

Chapter IV we have carried out the Speciation polymerase chain reaction and genetic typing of *Brucella* species. 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) according to the manufacturer's instructions. *Brucella* cultures were grown overnight in *Brucella* selective broth at 37° C, and DNA was extracted with the QIAamp DNA mini Kit (Qiagen, Germany) after inactivation for 2 h at 80° C. The purity and concentration of the genomic DNA extracted from samples was estimated by Nanodrop spectrophotometer (Thermoscientific, USA). PCR assay was carried out for gene *bcsp31* encoding an immunogenic outer membrane protein of 31 kDa of
B. abortus, which is conserved in all Brucella spp. using the specific primers BSCP31 F and BSCP31 R according to the protocol of Baily et al. (1992). ABC transporter gene was used for the designing of species specific primer, ABCT primer set was synthesized by using the primer 3 software. Amplified PCR products corroborated through 1 % agarose gel and the gel profiles were documented in Geldoc 1000 System-PC (Biorad, USA).

*Brucella* genus specific primers (synthesized by the Sigma Aldrich, India) targeting *bscp31* gene and ABCT gene showed the apparent prevalence of 4.6 %. The homology of the sequence were analyzed using BLAST analysis considering the query coverage and percentage identity of *Brucella abortus*, in which primers BSCP31 F and BSCP31 R showed 100 % homology to *B. abortus*. The gene sequences were deposited to GenBank database with the accession number KX389302, KX389303, KX389304, KX389305, KX424943, KX424944, KX424945, KX424946 and KX424947. PCR assays tend to be simple, robust and can be utilized for quick identification and confirmation of the *Brucella* species.

The gene sequences obtained from the study were compared with the sequences from the NCBI containing *B. abortus, B. melitensis, B. pinnipedalis* and *B. ceti* formed a clade and *Brucella* species, *B. canis, B. microti, B. ovis*. In the Maximum likelihood analysis *Orchobactrum* species was taken as outgroups in the analysis and observed two clades with the strong bootstrap supports >80 %. The variation within our isolates and NCBI sequences were very low and are similar. Between the clades, variation can be observed with our isolates clade as longer branch length. Since there was high genetic similarity and no variation, we sequenced only nine isolates and deposited them in the GenBank.
Bruce ladder multiplex PCR assay was performed to all the brucella strains from different animal and geographical regions. The robust bruce ladder speciation PCR can differentiate in a single step all the classical brucella species, including vaccine strains. Speciation PCR was performed in 25 μL reaction volume containing 1μL 0.4 μM of each primer eight primer sets cocktail was used according to Garcia et al., 2006. The products were analyzed by electrophoresis through ethidium bromide stained 1.5 % agarose gel and image was documented in Geldoc 1000 System-PC (Biorad, USA). All the positive isolates subjected for Bruce ladder speciation PCR confirmed that, all isolated cultures are B. abortus when compared with reference strains Brucella S19, B. melitensis, B. suis. This technique allows the characterization of the agent without performing the biochemical tests, which greatly reduces the time for the final diagnosis and provide more security for the analyst by reducing the time of exposure to microorganism (OIE, 2008). Bruce ladder speciation PCR is a low risk diagnostic technique because there is only manipulation of the genetic from the agent in laboratory. This is very important in the case of highly pathogenic microorganism to men.

In chapter V we have demonstrated the application of SSCP-PCR and LSSP-PCR assays for identification Brucella species. SSCP-PCR was carried out for the positive PCR brucellosis samples. SSCP is a rapid method for the detection of minor sequence changes in the PCR amplified sequence. From the amplified PCR product, 10 µl of individual eluted (20 ng/µl) PCR products were mixed with 25 µl of denaturing buffer [95 % formamide, 20 mM EDTA and 0. 05 % bromophenol blue]. The mixture was heated at 96 ° C for 10 min and immediately chilled with ice (Umesha et al., 2012; Chandrasekhar et al., 2012). Denatured PCR products were loaded on to 8 % acrylamide–bisacrylamide non- denaturing gel, containing 8 mL of
acrylamide/bis (40 %) and 4 mL of 10 X TBE, 40 µl of tetramethylethylenediamine, 10 % ammonium per sulfate and 28 mL water. 35 µl of each mixture was loaded and electrophoresed in pre-chilled 1 X TBE buffer at 200 V for 2 h at room temperature. An aliquot of single stranded DNA (ssDNA) ladder was also loaded on to gel to facilitate comparison of SSCP finger printing patterns, further stained with silver staining. Gels were documented using Geldoc 1000 System-PC (Bio-Rad, Gurgaon, India). In 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. The SSCP-PCR finger prints exhibited similar banding patterns and there was no variation in the banding patterns with that of reference strains B. abortus, B. melitensis and B. suis. Also SSCP-PCR did not show any genetic profile variations at intraspecies level. SSCP analysis may eliminate false diagnoses due to cross contamination (Yap et al., 1992). Which is a serious problem commonly associated with enzyme-linked immunosorbent assay (ELISA) and species-specific detection (Schots et al., 1994).

The LSSP-PCR analysis was carried out in 20 µl reaction mixture containing 3 µl of eluted PCR product (amplified DNA template) 20 ng/µl, 100 mM dNTPs, 5 µL of 10 X buffer, 2 U of Taq DNA, and 50 pmol of the forward primer in 0.2 mL PCR tubes. After denaturation step at 95 ºC for 10 min, annealing at 35 ºC for 1 min and extension at 72 ºC for 1 min, the same experimental conditions were repeated with respect to reverse primer (Labnet, Multigene gradient, CA. USA). LSSP-PCR profiles were visualized on silver staining in 8 % polyacramide gels [(10 mL of acrylamide/bis, 4 mL of 10 X TBE (Tris base 89 mM, boric acid 89 mM, 20 mM EDTA at pH 8.0)] after electrophoresed in pre-chilled 1 X TBE buffer at 200 V for 2 h at room temperature. Gels were documented using Geldoc 1000 System-PC (Bio-
Rad, Gurgaon, India). 5’-TGGCTCGGTGTTGCCAATATAA-3’ F primer and 5’-GCGCTTTGCTTTCAGGTCTG-3’ R primers were used in the LSSP-PCR assay resulting in similar banding pattern. *B. abortus* showed there was no interspecific variability among the positive isolates. *B. abortus* showed variable number of banding pattern but it showed similar banding patterns in all the isolated PCR positive samples. The phylogenetic dendrogram obtained from the LSSP-PCR also exhibited that there was no inter-specific variability among the PCR positive samples.

The SSCP-PCR and LSSP-PCR analysis were carried out for all the samples to find out if there is any genetic diversity. So only the mutated sequences or polymorphic variation samples can be further sequenced, which will be significant in large scale screening of the infected animals. LSSP-PCR technique for the characterization of *Brucella*, specially designed primers from the sequence of the *BSCP31* protein was used for the first time. Present study showed similar banding pattern because of sequence similarity matching to Segatto *et al.* (2013) studies. Significantly, results also showed sequence similarity while using specific primers in LSSP-PCR. Although the LSSP-PCR is used for strain typing techniques. One of the main advantages of using LSSP-PCR for genetic typing of brucellosis from biological samples is the possibility of detecting *Brucella* first by means of a PCR reaction and subsequently identifies them through the comparison of their genetic signatures with known LSSP-PCR profiles. This is clearly exemplified by our results, which showed specific signatures with no variation at intra species level. Thus, LSSP-PCR may be very useful in screening test for genetic identity and establishment of matrilineal relationships, prior to the more definitive study of this region by DNA sequencing. The major limitation of SSCP technology for the analysis of community DNA is the high rate of re-annealing of DNA strands after an initial denaturation during
electrophoresis. SSCP analysis would be ideal tool to identify clones with DNA products identical to those originally extracted from the amplified community DNA. LSSP-PCR presents almost unlimited molecular applications where rapid and sensitive detection of mutation and sequence variation is important. Due to, low cost, sensitivity, specificity, simplicity of execution and high reproducible genetic profile, the use of SSCP-PCR, LSSP-PCR technique could be extended to other pathogenic bacteria for identification and can be considered as a valuable microbiological tool to study genetic diversity of quarantine pathogens along with their epidemiology in developing countries.

In Chapter VI was designed to study the management of brucellosis by using the potential plant bioactive metabolites. Different medicinal plants were collected from Western Ghats of Karnataka, India. The taxonomic identification of these plants was done by Prof. G. R. Shivamurthy, former Professor, Department of Botany, University of Mysore, Karnataka, India. *Acacia nelotica, Withania somnifera, Eugenia jambolana, Callistemon citrinus, Clerodendrum inerme, Terminalia arjuna, Lucas aspera, Thevetia peruviana, Hemidesmus indicus, Gloriosa superba, Cymbapogon citrates, Acorus calamus, Rhamnus cathartica, Cinnamomum verum, Thuja occidentalis* and *Santalum album* were used in the present study. Plant leaves were dried leaves was 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.

*Brucella* cultures were grown in Muller-Hinton Broth (Merck, USA) medium at and also in Tryptic soya agar medium supplemented with Peptone and beef extract were incubated at 37°C for 22 h. 0.1 mL of bacterial suspension (10cfu/mL) was poured on each plate containing Muller-Hinton Agar (MHA). The lawn culture was prepared by sterile cotton swab and allowed to remain in contact for 1 min. Different concentrations of ethanolic extracts (1, 5, 10, 25, 50, 100, 200, and 300 mg/mL) from each plant were prepared and used for the assay. The sterile filter paper discs (6-mm diameter) were saturated by 50 μL of different concentrations of each extract and then were placed on lawn cultures. (Mau *et al*., 2001; Cermelli *et al*., 2008) The Petri dishes were subsequently incubated at 37°C for 24 h and the inhibition zone around each disc was measured in mm. As positive controls, discs (Difco, USA) containing streptomycin 10 μg, gentamicin 10 μg and Ciprafloxin 10 μg were used.

*Acacia nelotica, T. arjuna, E. jambolana* and *C. citrinus* showed antibrucellosis activity, *C. citrinus* showed strong antibrucellosis activity compared to that of *Acacia nelotica, T. arjuna, E. jambolana*. Hence Further the TLC profiling was carried out for the extract with highest antibrucellosis activity. The TLC plates were developed in a twin trough glass chamber containing mixture of chloroform and methanol (99:1; v/v) as the mobile phase. The retention factor values for the plants extracted with ethanol ranged from Rf value of 0.27, 0.39, 0.83 to 0.97. The Rf value 0.97 spot showed antibrucellosis activity and antibacterial activity against other human pathogens. This indicates presence of bioactive metabolites were concentrated.
in spot D $R_f$ value of 0.97. The spot D was further studied using the Gas chromatography and Mass Spectroscopy (GC-MS). The identification of the compounds present in the TLC purified ethanolic extracts were based on direct comparison of the retention times and mass spectral data with those for standard compounds, and by computer matching with the Wiley 229, Nist MS Library. The individual compounds were screened for the antimicrobial activity but the activity was not observed. This might be due to the separation of the constituents, which were showing activity at the synergistic level. The antibacterial activity showed by the TLC purified ethanolic extract of *C. citrinus* could be attributed to the presence of bioactive metabolites. *C. citrinus* is an effective antimicrobial plant which can be used as antibrucellosis agent.

The salient features of the present study are:

- This study showed 4.6 % prevalence of bovine brucellosis in Karnataka, India. 3.3 % in rural regions of Mandya district.

- Specific primers designed from this study can be used for the detection of *Brucella* species

- SSCP-PCR analysis would be ideal tool to identify clones with DNA products identical to those originally extracted from the amplified DNA. Also it can inevitably deal with large number of samples

- LSSP-PCR in this study presents rapid and sensitive detection of mutation and sequence variation.

- From the present study SSCP-PCR, LSSP-PCR gene signatures developed can be used for rapid detection of pathogens and quickly quarantine before the spread of infection.
• For the first time the different medicinal plants from Western Ghats were screened for the antibrucellosis activity.

• The crude and TLC purified *Callistemon citrinus* ethanolic extract exhibited strong antibrucellosis activity.

• The bioactive compounds identified were reported for the first time and the bioactive metabolites identified exhibited as potential antibacterial agents against brucellosis and other Human pathogens.