2. REVIEW OF LITERATURE

According to the World Health Organization (WHO), the Food and Agriculture Organization (FAO), and the Office International des Epizooties (OIE), Brucellosis is an important re-emerging infectious bacterial zoonotic disease and widespread in the world. Brucellosis Infections are caused by a various genera of Brucella, which has a propensity to infect a specific animal species. However, most species of Brucella are able to infect other animal species as well and some humans (Young, 1995). Brucellosis was major in the Mediterranean region and its reports are associated with military campaigns. This disease was fully elucidated by Sir David Bruce, Hughes, and Zammit working in Malta (Christopher et al., 2005). The disease is closely associated with Farmers society linked to the practice of animal husbandry and shepherding. Brucellosis is one of the most common zoonotic infections transmitted globally to humans through consumption of unpasteurized dairy products or through direct contact with infected animals, placentas or aborted fetuses. This bacterial infection shows symptoms like fever, sweating, fatigue, weight loss, headache, and joint pain persisting for weeks to months. Neurological complications such as endocarditis and testicular or bone abscess formation (Corbel et al., 2006; Ariza et al., 2007).

Bang discovered \textit{Brucella abortus}, the cause of abortion in cattle and of brucellosis (undulant fever) in human beings. \textit{B. suis} was recovered from swine by Traum and implicated as an agent of brucellosis in man by Huddleson. Evans showed that \textit{B. melitensis}, isolates of cows and pigs belonged to one genus and generic name \textit{Brucella} in honour of Sir David Bruce was suggested. Buddle and Boyce discovered \textit{B. ovis}. Stoenner and Lackman isolated \textit{B. neotomaee} from rat. Carmicheal and Bruner discovered \textit{B. canis} from dogs.
Fig.2.1: Infection or transmission of different species of *Brucella* associated with human was depicted. (Source: Raghava *et al.*, 2018)

Human infections due to *B. canis* are reported. *B. pinnipediae* and *B. cetaceae* is newly recognized marine mammal Brucellae that may also be human pathogens (Sohn *et al.*, 2003; McDonald *et al.*, 2006). The most significant clinical symptom of brucellosis is abortion at the first gestation in the cattle. Generally, females infected with brucellosis will abort only once and they may remain infected their entire life.
The clinical diagnosis of brucellosis for animals is done based on the symptom of abortion however the abortion, may be because of several other pathogens hence laboratory testing is essential. The aim of this work is to review the recent available literature on diagnostic techniques designed to diagnose brucellosis.

Given the complexity of the epidemiology of brucellosis involving various animal species, the effective control requires long lasting and carefully monitored efforts. Preventive measures are essential to minimize the risk of infection of the human population has there is no vaccination for human infection (Smits and Kadri, 2005).
2.1 Culture based methods

Conventional method used is culturing of pathogens on media, which is considered as golden standard method, which is reliable and accurate, but it’s time-consuming and cumbersome. These methods include, blending the collected sample with a selective enrichment medium to increase the population of the target organisms and plating onto a suitable media to isolate pure cultures followed by examining the cultures by phenotypic analysis or morphological characters. This process takes 3-7 days to get results and next 7-10 days for confirmation (Adzitey et al., 2013). This is an obvious problem in many industrial applications. Apart from the above disadvantages, conventional culture methods are still representing a field where progress is possible. These methods are often combined together with other nucleic acid detection methods to yield more robust results.

2.2 Serological tests

*B. abortus, B. melitensis and B. suis* are the three most important species of brucella contain O-polysaccharide on cell surface. It is a part of the lipo-polysaccharide and O-polysaccharide is lacking in other species *B. ovis and B. canis*. The major species containing O-polysaccharide which are diagnosed serologically using either whole cell antigen or smooth lipo-polysaccharide. *B. abortus* antigens were the major antigens used in majority of the serological tests, these tests can also be performed to human serology (Nielsen, 2002). The various serological tests vary in their ability to detect different isotypes.

In agglutination tests there are two commonly used tests, the Rose-bengal test (RBT) (Nicoletti, 1967; Davis, 1971) and the buffered antigen plate agglutination test.
Review of Literature

(BPAT) (Angus and Burton, 1984). The RBT uses *B. abortus* SI 119.3 whole cells, stained with rose-bengal while the BPAT uses *B. abortus* SI 119.3 whole cells stained with crystal violet or brilliant green dyes. The antigens are used at a pH of 3.65 that prevents some agglutination by IgM and encourages agglutination by IgGl thereby reducing non-specific interactions (Corbel, 1973; Allan *et al.*, 1976). Both tests are standardized, simple to perform and inexpensive; and are suitable for screening individual animals, however, false negative reactions occur mostly due to prozoning. The BPAT is an OIE prescribed test for bovine, porcine, ovine and caprine brucellosis (OIE Manual, 2004). Milk Ring test (MRT) is an adaptation of the agglutination test. If antibody is present in milk, a portion will be attached to the milk fat globules via the Fc portion of the antibody. These antibodies will agglutinate with the antigen and as the fat globules rise in milk, a purple band will appear at the top of the milk. If no antibody is present, the fat band will remain buff colored. While this is a relatively insensitive test, subject to wrong interpretation caused by various milk conditions such as mastitis, colostrums and milk at the end of lactating cycle (Nielsen, 2002), it is recommended by OIE as a screening test for bovine brucellosis (OIE Manual, 2004; Junaidu *et al.*, 2017).

In complement fixation test (CFT) basis is that dilutions of serum, antigen (usually whole cell) and pre-titrated amount of complement (guinea pig serum) are added together. If antibody is present in the serum, it will bind to the antigen; and providing the antibody is IgGl isotype, complement will be activated. The indicator system consists of sheep erythrocyte sensitized with rabbit antibody. If the test serum contains antibody, complement will not be available and lysis of erythrocytes will not take place. Alternatively, if no antibody was present, the available complement activated by interaction with receptor on Fc portion of the rabbit antibody will lyse the
erythrocytes, releasing hemoglobin, which can be assessed visually or using a spectrophotometer (Nielsen, 2002).

Fig. 2.3: The Surface Plasmon Resonance imager (SPRi) label-free detection of bacteria in blood using protein microarrays and SPRi detection. Bacteria from an isolated bacterial colony were grown overnight in blood culture medium, culture was ten-fold serially diluted then, finally mixed with blood samples so that the blood-to-broth ratio was kept constant (1:5). Several dozens of antibodies can be arrayed on 1 cm². Live bacteria are captured on microarrayed specific antibodies (spotted in triplicate onto the biochip surface) during the enrichment step. SPRi data are treated and plotted as variations of light reflectivity (ΔR (%)) over time for each region-of-interest (corresponding to antibody spots arrayed on the sensor). Differential SPR images (obtained by subtracting a reference SPR image recorded at t(0) to any SPR image acquired later in the experiment) may also be displayed. (Source: Templier et al., 2017).

2.3. Enzyme immunoassays

The Indirect Enzyme linked immunosorbent assay (IELISA) was foremost developed by Carlsson et al. (1976) for diagnosis of human brucellosis. The most common system uses smooth lipopolysaccharide (SLPS) antigen coated passively onto polystyrene matrix. A strong positive, a weak positive and a negative serum control
are included to assess the assay performance and quality control. Results are frequently expressed as a percentage of reactivity of the strongly positive serum control. OIE approved version of this test utilize purified SLPS as the antigen, serum diluted 1:50 and a mouse monoclonal antibody specific for bovine IgG1 conjugated with horse radish peroxidase (OIE Manual, 2004). IELISA and Conventional serological tests and the ELISA cannot distinguish between vaccine antibody. However Competitive Enzyme Immunoassays (CELISA) can distinguish between vaccine and natural infection occurred. The CELISA is prescribed test by the OIE for international cattle trade and as an alternate test for swine brucellosis (OIE Manual, 2004; Gul et al., 2015). rOMP31- based indirect ELISA was carried out for caprine brucellosis (Singh et al., 2018). Seroprevalence of B. melitensis among small ruminants and humans in Anand region of Central Gujarat, India was carried out in the studies of Padher et al. (2018).

In common ELISA is very sensitive, accurate, reliable, cost effective and can be appropriate for screening mass or large population of livestock and human for brucellosis Infection. Though there are other serological assays for the detection of brucellosis infection they are not widely used as some of them are less sensitive and specific while a few other tests are highly cumbersome, time consuming and need expertise.

The fluorescence polarization assay (FPA) can be performed outside the diagnostic laboratory, allowing for quick and sensitive detection (Nielsen, 2002). The molecule is labelled with a fluorescent marker and is examined by plane polarized light; a small molecule will rotate through a given angle faster than a larger molecule.
Fig. 2.4: ELISA is a plate based assay technique which is used for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. Here, the indirect, direct and competitive ELISA was represented applicable for suitable applications. (Source: Alahi and Mukhopadhyay, 2017)

The time of rotation may be measured using both horizontal and vertical measurements (Nielsen et al., 2000). For diagnosis of brucellosis, a fluorescence polarization analyzer is used to obtain a background measurement of fluorescence of diluted. The FPA has been validated for large number of species, including cattle, swine, bison and a number of cervids (Nielsen et al., 2001; Marcelo et al., 2017). FPA is an alternative test for bovine and swine brucellosis diagnosis (OIE Manual, 2004).

2.4. Nucleic acid based tests

The Brucellae genome is encoded by two circular chromosomes with sizes of close to 2.05 Mb and 1.15 Mb for each one of the species (Michaux-Charachon et al., 1997). The small chromosomes of B. suis, B. canis and B. neotomae are 50 kb longer
only. The GC contents in the DNA of various members of the genus Brucella are 55-58%. Both chromosomes contain 1028 and 1035 of almost identical proportions of potential coding regions. The nucleotide sequence similarity between all Brucella species is also high and DNA-DNA homology exceeds 90%. The six species are so closely related that a mono-species genus has been recommended (Verger et al., 1985). This hypothesis was also confirmed by 16S rRNA gene sequence analysis and was experimental in the biochemical characteristics of the organisms (Moreno et al., 1990).

Polymerase Chain Reaction assay employs the utilization of DNA polymerase enzyme, which amplifies specific fragments of the target DNA molecule added to the reaction (Powledge, 2004). Primers are designed to the target sequences of the target DNA molecule. Most used enzyme is Taq DNA polymerase (isolated from the bacterial species Thermus aquaticus) but the Pfu DNA polymerase isolated from Pyrococcus furiosus is also often used due to its high reliability of copying of the DNA sequence. Although these two enzymes are different they possess some similar features that make them applicable in the PCR reaction: they can generate a new fingerprint of DNA, from the template DNA sequence (Valasek and Repa, 2005). Thermal stability is compulsory due to the fact that at the start of every PCR cycle, double DNA helix is denatured to single strand at high temperature (93-96°C) in the PCR reaction tube. The temperature at which DNA molecules become single-stranded is called melting temperature (Tm). The second phase of the PCR cycle is the primer annealing to the specific complementary sequences of the target single stranded DNA molecule. Primers suppress the re-annealing of the single DNA strands and enable DNA polymerase to start the synthesis of a new strand. This is the primer annealing phase and it is performed at 52-58°C generally produce the finest results. Third stage
is the elongation stage (at approximately 72° C) which involves binding of the nucleotides from the reaction mixture to the complementary ones of the target sequence. After that, primers get displaced resulting in the creation of two copies of a target DNA segment.

The PCR primers used for amplifying BCSP 31(B4/B5) (Baily et al., 1992), 16SrRNA (F4/R2) (Romero et al., 1995), 16S-23S 16S–23S intergenic transcribed spacers (ITS) (Bru ITS-S/Br IT-S-A) (Rijpens et al., 1996; Bricker et al., 2000), 16S-23S rDNA interspace (ITS66/ITS279) (Keid et al., 2007), IS711 (IS313/IS639) (Chenby et al., 2000), per (bruc1/bruc5) (Bogdanovich et al., 2004), omp2 (JPF/JPR) (Leal Klevezas et al., 1995), outer membrane proteins (omp 2b, omp2a and omp31) (Imaoka et al., 2007), proteins of the omp25/omp31 family of Brucella spp. (Vizcaino et al., 2004), and arbitrary primers (Fekete et al., 1992). The sensitivity and specificity vary substantially for the different pairs of primers. Primers B4 and B5 targeting BCSP 31 is often used for human brucellosis diagnosis. If combined with an increase in cycle numbers, this pair of primers can provide the greatest sensitivity when testing human blood samples. Molecular detection of B. abortus using bscp31 and IS711 gene based pcr assay in cattle and buffalo (Alvarez et al., 2016; 2018), B. abortus alkB gene and B. melitensis BME11162 gene (Pobert et al., 2004). The 16S rRNA gene of Brucella was explored as a target for PCR (Herman and De Ridder, 1992). Real-time PCR with primers targeting the bscp31 gene and further characterized by the omp25 gene (Hoffman et al., 2016).

**AMOS Multiplex PCR**

Several multiplex PCRs have been described for identification of Brucella at the species level and partly at the biovar level using different primer combinations.
The first multiplex PCR, called AMOS PCR for *B. abortus*, *B. melitensis*, *B. ovis*, and *B. suis*, was published in 1994. It used five primers to identify *Brucella* at the species level (Bricker *et al.*, 1994).

**Fig.2.5: PCR method.** The PCR chain reaction contains 20–30 cycles following each other. Every cycle contains three steps: i) The double-stranded DNA is heated to 94–96 °C for separating the strands. This step is the so-called **denaturation step**, when the hydrogen bonds which holding the two strands together are broken. ii) After separating the two DNA strands the temperature is lowered, so the primers can connect to the DNA strands. This is the so-called **annealing or connecting step**. This temperature depends on the primers used in the PCR reaction (usually 5 °C lower than the melting point of the primers. iii) Finally the DNA polymerase enzyme synthesizes the complementary strand of DNA beginning from the primer. This is the so-called **elongation step**. This temperature depends on the polymerase enzyme used in the reaction. (Source: Labster theory)

This method could detect selected biovars of 4 species of *Brucella*: biovars 1, 2, and 4 of *B. abortus*; all 3 biovars of *B. melitensis*; biovar 1 of *B. suis*; and biovar 1 of *B. ovis*. This assay could not differentiate individual biovars within a species. The PCR was used to evaluate animal field samples and was found to be in 100% agreement with the conventional biotyping methods. In order to distinguish
B. abortus vaccine strains S19 and RB51 from field strain isolates, 3 additional primers were added to the original AMOS PCR (Ewalt and Bricker, 2000). The eight primer mixtures could differentiate most of Brucella strains expected to occur in the US. Based on this AMOS PCR format, another primer was designed and added. This refined AMOS PCR produced an extra band found only in B. abortus biovars 3b, 5, 6 and 9 (Ocampo et al., 2005; Shahzad Ali et al., 2014)

An improved PCR, the B. abortus species-specific PCR, was subsequently used to specifically recognize field strains of B. abortus biovars 1, 2, and 4, which were the only biovars occurring in the US. This method was also used with bovine tissue samples to differentiate the aforesaid strains from vaccine strains, other Brucella species, and Brucella-related or bacteria that might give cross reactions (Bricker et al., 2003). B. suis biovars 1, 2, and 3 were identified by a multiplex PCR, which included primers based on sequence variation of the omp2b gene. However, the use of this PCR was limited because the B. suis biovar 1 pattern produced from animal field isolates of B. suis was similar to that of B. suis biovars 2 and 3, based on identification by bacteriological methods (Ferrao et al., 2006). A multiplex PCR using 8 multi-locus variable number tandem repeat analysis (MLVA) primers was able to distinguish B. melitensis from other Brucella species and allowed strain typing (Rees et al., 2009). This method was used to identify seven epidemiologically-linked clusters of B. melitensis and the source of a laboratory-acquired infection. The assay was found to be practical for technical and economical reasons.

**Bruce ladder Multiplex PCR**

The Multiplex PCR (mPCR) represents the method 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 lower cost. The
primary advantage is, less reagent and enzyme (TaqDNA polymerase) utilization. Another advantage is that like pathogens are analyzed individually 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). Concentration of magnesium influences the reaction specificity which is one of the most important factors in the PCR reaction (McPherson and Moller, 2000). Generally, MgCl₂ concentration in mPCR is higher than that used in the conventional PCR reaction. Anthony et al. (2000) used universal primer for the detection of multiple pathogens at the same time. In this method a single set of primer is used to amplify conserved stretches of DNA from 16S rDNA. A most important advantage of the Bruce-ladder PCR assay over earlier described multiplex PCR tests is that it can identify and distinguish for the first time all of the Brucella species and the vaccine strains in the same test (Stevic et al., 2017).

Fig. 2.6: Development of technologies for rapid and accurate detection of hazards and hygiene management in the food chain. The conventional culture based and multiplex PCR detection advantages was represented with respect to sensitivity, cost and rapidity (A) and the bruce ladder was compared to the convetional drawbacks to overcome the advantage of the PCR through bruce ladder PCR in identification of the Brucella sp. Pathogens. (Source; Yasuhiro Inatsu, NARO).
Real time PCR or quantitative PCR (qPCR)

Real time PCR or quantitative PCR (qPCR) is one more adaptation of the PCR method to enumerate the number of copies of nucleic acids during PCR. Thus, qPCR is used to quantify DNA and cDNA, determining gene numbers present within different samples (Lobert et al., 2010). qPCR offers advantages such as speediness in getting the result, reduced risk of contamination and the ease in handling (Mackay et al., 2002; Maibach and Altwegg, 2003). The real-time PCR machine is a thermal cycler which is able to stimulate the fluorescent dye with a laser for quantifying amplification product each minute of cycle and show exponential and plateau phases. Real-time PCR has been used to quantify the amount of Phytophthora DNA with specific gene primer methods of detection and identification has proved to be inherently more specific and sensitive than traditional methods (Martin et al., 2009).

Most bacterial detection is DNA-based because the genomes of bacteria are DNA, rather than RNA as with some viruses. However, reverse-transcriptase PCR (RT-PCR)-based tests for bacteria have been published such as the one for the detection of hemolysin from V. parahaemolyticus described by Nakaguchi et al. (2004). RT-PCR is a method used for studying gene expression and uses RNA as its template to produce complementary DNA (cDNA). This PCR was designed to amplify RNA sequences (especially mRNA) through synthesis of cDNA by reverse transcriptase (RT). Later, this cDNA is amplified using PCR. This type of PCR has been useful for diagnosis of RNA viruses, as well as for evaluation of antimicrobial analysis. It has also been used to study gene expression in-vitro, because obtained cDNA retains the original RNA sequence. The main challenge of using this technique is in mRNA of test samples especially handling of the low level mRNA of interest,
coupled with low stability at room temperature and sensitivity to ribonucleases and change in pH (Kaynak-onurdag et al., 2016; Gwida et al., 2016). Evaluated the capacity of the *Brucella* spp. eryC gene as a diagnostic marker for brucellosis by quantitative real-time PCR. *eryC* gene encodes the enzyme d-erythulose-1-phosphate dehydrogenase that plays an important role in the erythritol metabolism (Rodriguez-Lazaro, 2017).

![Diagram of RT-PCR methods](image)

**Fig. 2.7: One step and two step methods of RT-PCR.** There are two primary ways to carried out RT-PCR; In one-step, all the components including specific primers are put into a single tube as same as the PCR reaction. In a two-step method, the first reaction involves the formation of cDNA with the help of a separate reverse transcription reaction and then addition of cDNA to the PCR reaction (Source; Ifeanyi, 2015).

**Ligase Chain Reaction PCR**

LCR is a recent technique used to detect nucleic acid sequence of microbes by DNA amplification. It is similar to PCR but, only probe molecules amplify through polymerization of nucleotides. Two probes for each DNA strand are ligated together to form a single probe. LCR uses thermostable DNA polymerase and a DNA ligase
enzyme to drive the reaction. A major drawback of this technique is the detection of food pathogen as, it can detect DNA from dead organisms. LCR employs specificity of ligase enzymes to achieve allelic discrimination. It is one of the novel amplification techniques for the detection of point mutation in microbial pathogens. The sole characteristic of LCR is the second primer set, mirror to the first pair, which is designed with the nucleotide at the 3’ end of the upstream primer denoting the sequence difference. In the presence of target DNA, the adjacent probes are ligated by thermostable DNA ligase. If there is a mismatch at the primer junction, it will be discriminated against by thermostable ligase and the lack of PCR product. The absence of the ligated product indicates at least a single base-pair change in the target sequence; the ligated products can provide as templates and be amplified exponentially by thermal cycling (Wiedmann et al., 1994).

Fig. 2.8: The ligase chain reaction. The LCR consists of a series of repeated cycles of oligonucleotide hybridization and ligation, to generate multiple copies of the nucleic acid sequence chosen as a target. Once produced ligation, the resulting product can be separated by heat denaturation (Source: King, 1996).
A new high-throughput PCR-ligase detection reaction-capillary electrophoresis (PCR-LDR-CE) assay for the multiplexed identification of 20 bloodborne pathogens including *B. abortus* biothreat agent (Pingle *et al*., 2007). (LCR)-based method that simultaneously assesses multiple genetic markers at the single-nucleotide level (Wattiau *et al*., 2011). One drawback is that, the target is amplified but contamination risk and variation in copy number of the plasmid containing the LCR target is also a source of error. LCR has a very well discriminating power at a target nucleotide site, such as single nucleotide base change.

**Low-stringency Single-Specific-Primer PCR**

Low-stringency Single-Specific-Primer PCR (LSSP-PCR) is a tremendously simple PCR based technique; to facilitate the detection of single or multiple mutations in gene sized DNA fragments. It consists of two steps, the first step is amplification of the template using the specific PCR primers followed by the second step LSSP-PCR, which uses low-stringency conditions and only one primer, usually used in the sPCR. Briefly, template DNA fragments are subjected to PCR using high concentrations of a single specific oligonucleotide primer, with large amounts of Taq DNA polymerase and under low annealing temperature. Under these conditions, the primer hybridizes specifically to its complementary region and nonspecifically 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). Thus the reaction yields a large number of products that can be resolved by electrophoresis to give rise to a multiband DNA signature that reflects the DNA template sequence. Small change in the single base mutation could drastically alter the signature band pattern, producing new signatures as a diagnostic tool (Vago *et al*.,
LSSP-PCR has been broadly used in the detection of mutations in human genetic diseases (Vago et al., 1996), sequence variations in human mitochondrial DNA (Barreto et al., 1996) and for gene banding pattern of infectious agents such as Human Papilloma Virus (HPV) (Villa et al., 1995), Trypanosoma cruzi (Vago et al., 1996), Trypanosoma rangeli and Leishmania infantum (Alvarenga et al., 2012). LSSP-PCR gene signatures were used to study polymorphic variations of Brucella species and identification of the B. abortus (Raghava et al., 2017).

**Single-Strand Conformation Polymorphism PCR**

Single-Strand Conformation Polymorphism (SSCP) analysis is generally considered as the most suitable method for the detection of mutations in short stretches of DNA. The SSCP process involves PCR amplification of the target fragment, denaturation of PCR product with heat and formamide (or other denaturants like sodium hydroxide, urea and methyl mercury hydroxide) and electrophoresis on a non-denaturing polyacrylamide gel described by Chandrashekar et al. (2012). Single stranded DNA (ssDNA) fragments fall into unique conformations determined by their primary sequence whose structures are stabilized by intramolecular interactions (Lee et al., 2000). As a result, even a single base alteration can result in a conformational change, which can be detected by the altered mobility of the ssDNA molecule in SSCP. PCR--SSCP has also been used to locate mutated sequences in bacteria Bacillus subtilis, Hepatitis C virus (Lareu et al., 1997), Human Papiloma Virus type 16 (Van, 1995) and also searching for mitochondrial DNA point mutations (Jaksch, 1995). Moreover, the subtraction of mitochondrial cytochrome C oxidase subunit I (Hu et al., 2002) and identification of a Ralstonia solanacearum in tomato (Umesha
et al., 2012), the diagnosis of Leptospira (Chandan and Umesha, 2013) were accomplished. PCR—SSCP was used for the detection of mutation in Brucella species (Raghava et al., 2017).

Fig. 2.9: The Single-stranded conformation polymorphism (SSCP) analysis. SSCP analysis detects sequence variations (single-point mutations and other small-scale changes) through electrophoretic mobility differences. DNA that contains a sequence mutation (even a single base pair change) has a measurable mobility difference compared to wild type DNA when subjected to nondenaturing (or partially denaturing) conditions. (Source; https://www.youtube.com/watch?v=L8C7qqIwR7g)
**Restriction Fragment Length Polymorphism**

Restriction Fragment Length Polymorphism (RFLP) is a very simple method that uses particular restriction enzyme digestion of the genomic DNA. It is used for the comparison of the number and size (mass) of the fragments produced by restriction endonucleases cutting at a specific recognition site of the target DNA (Ueda et al., 2005). The resulting DNA fragments are examined by electrophoretic separation. Presence, absence, or changes in the mass of the resulting DNA fragments are evidences of changing DNA sequences. This method requires pure culture for the discrimination of bacteria at the species level.

![Restriction Fragment Length Polymorphism (RFLP)](image)

**Fig. 2.10: The principle of restriction fragment length polymorphism (RFLP).** The suspected blood sample was used to extract the DNA and treated with restriction enzymes, then separated the fragments in gel electrophoresis. Using southern blotting, the DNA fragments were transferred on to nylon membrane, using radioactive specific DNA probes used to bind and film was washed to remove unbound then autoradiograph was made to decode the results. (Source; Heal force)

RFLP is a method used to follow a specific DNA sequence as it passes on to other cells. The function of RFLP is to match a certain DNA with the DNA a
individual has record of. RFLP is used to measure recombination rates which lead to a genetic map. Also RFLP is used in paternity cases or criminal cases; another reason why RFLP is used is to determine the disease status of a person. This technique has generally been unsuccessful for typing of Brucella when applied to the whole genome (Isloor et al., 2001) but polymerase chain amplification of selected sequences followed by restriction analysis has provided evidence of polymorphism in a number of Brucellae genes (Cloeckaert et al., 1995). RFLPs of selected genes display sufficient polymorphism to distinguish Brucella species and biovars. PCR-RFLP analysis shows excellent typeability, reproducibility, stability, and epidemiological concordance. Consequently, PCR-RFLP assays of specific gene loci can serve as tools for diagnostic, epidemiological, taxonomic, and evolutionary studies (Al Dahouk et al., 2005; Piranfar et al., 2015; Shariatpanahi et al., 2016; Bahmani et al., 2017).

**Amplified Fragment Length Polymorphism**

Amplified Fragment Length Polymorphism (AFLP) represents another genotyping technique based on selective amplification of restriction fragments of DNA molecule (Vos et al., 1995). The method involves restriction endonucleases digestion of total purified genomic DNA followed by ligation of the resulting fragments by a double-stranded oligonucleotide adapter complementary to the base sequence of the restriction site. The adapters are designed such that the original restriction site is not restored after ligation, thus preventing further restriction digestion. Selective amplification of sets of these fragments in PCR is achieved with primers corresponding to the contiguous base sequences in the adapter, the restriction site plus one or more nucleotides in the original target DNA. The resulting PCR-amplified DNA fragments are then analyzed by gel electrophoresis.
AFLP can be applied for the determination of sources of contamination, especially in cases such as live stocks (Siemer et al., 2005). A notable AFLP study examined 78 different isolates from the six classical Brucella species and produced an unrooted dendrogram that found that each of the species fell into distinct clusters aside from the grouping of *B. suis* and *B. canis* (Whatmore et al., 2007).

**Random Amplified Polymorphic DNA Technique**

The Random Amplified Polymorphic DNA (RAPD) technique is a PCR assay that uses arbitrary primers and it can be applied to differentiate races, strains and pathogenic or non-pathogenic isolates (Williams et al., 1990). The primers engaged in this technique are very short fragments (ten or fewer bases) of DNA from a known source. It is highly probable that these primers may be able to find some
complementary sequences in the target DNA, producing a mixture of DNA fragments of various sizes. When the products of such a reaction are analyzed by gel electrophoresis, distinct banding patterns are produced and some of these patterns may prove to be specific to certain species or varieties or strains.

![Diagram](image)

**Fig. 2.12: The RAPD analysis general model.** The use of a single decamer oligonucleotide promotes the generation of several discrete DNA products and these are considered to originate from different genetic loci. Polymorphisms result from mutations or rearrangements either at or between the primer binding sites and are detected as the presence or absence of a particular RAPD band. This means that RAPDs are dominant markers and, therefore, cannot be used to identify heterozygotes (Kumar and Gurusubramanian, 2011).

The patterns themselves may be useful for detection and diagnosis of some pathogenic fungi, but some of the bands in certain cases, may be cut out of a gel and sequenced to produce specific primers for more precise PCR analysis or probes for dot hybridization and other detection procedures. RAPD analysis can be used as rapid method for identification, typing and discrimination of closely related phages. RAPD-PCR has been used to generate fingerprints and assess the genetic diversity of phages infecting Brucella abortus (Zhu et al., 2009; Jain et al., 2014; Qasem et al., 2015).
Fluorescent In-Situ Hybridization

Fluorescent In-Situ Hybridization (FISH) with oligonucleotide probes directed at rRNA is the most common method among molecular techniques not based on PCR. The probes used in FISH are 15–25 nucleotides in length, and are covalently labeled at their 5’ end with fluorescent labels. It uses fluorescently labeled 16S rRNA probes and fluorescent microscopy to detect. After hybridization, the specifically stained cells are detected using epifluorescence microscopy (Wagner et al., 2003). FISH in combination with flow cytometry has been used for rapid culture independent detection of *Salmonella* spp., (Bisha and Brehm, 2010). *Pseudomonas aeruginosa*, *Helicobacter* spp., (Husted et al., 2000), and *Streptococcus* spp., (Kempf et al., 2000).

**Fig. 2.13: The Fluorescence In Situ Hybridization (FISH).** It is a cytogenetic technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes. This technique is based on the mechanism of nucleic acid base pairing; only those parts of the chromosome with high degree of sequence complementarity will be recognized and bound by fluorescent probes. Scientists can find out the position of the fluorescent probe bound to the chromosomes by using fluorescence microscopy. (Source; Abnova)

Binding of short fluorescence-labeled DNA or nucleic acid-mimicking PNA probes to ribosomes of infectious agents with consecutive analysis by fluorescence microscopy allows identification of bacterial and eukaryotic pathogens at genus or species level. FISH analysis leads to instant differentiation of infectious agents.
without any delay due to the need for microbial culture. As a microscopic technique, FISH has the unique potential to provide information about spatial resolution, morphology and identification of key pathogens in mixed species samples (Frickmann et al., 2017). This powerful technique enables researchers to rapidly identify a range of chromosomal aberrations across the genome, including those causing mental retardation, various cancers, birth defects, etc.

2.5 Management of brucellosis using the medicinal plants

Brucellosis, a bacterial zoonosis and major public health concern due to its high morbidity rate. The prevalence of infection in humans is directly associated with occurrence in animals, particularly in domestic ruminants (Grillo et al., 2006; Raghava et al., 2017). Infection of brucellosis causes significant economic losses by comparatively low milk production in livestock, abortion, weak off-springs, public health and international trade implications (FAO, 2010). Brucellae are highly potent pathogen in animals, 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 these make bacteria most attractive for defence researchers (Doganay et al., 2013). Infectious diseases pose a severe health concern worldwide. The development of drug resistant pathogens due to haphazard use of antibiotics has increased the need for new source of antimicrobial agents. This has encouraged screening of new plant species for potential medicinal and antioxidant properties (Gulcin et al., 2002; Jayaprakasha et al., 2006).

Medicinal plants are traditionally used across the globe for the treatment of various diseases, including asthma, gastrointestinal symptoms, skin disorders, respiratory and urinary problems, and hepatic and cardiovascular disease and other
infectious disease (Van Wyk and Wink, 2004; Tian et al., 2014). Plants have the ability to synthesize diverse array of biologically active compounds (Bajguz, 2007; Cushnie et al., 2014) that are key for them to survive and thrive in the natural environment, including protective functions with respect to abiotic stresses derived from temperature, water availability, mineral nutrients supply (Simmonds, 2003; Treutter, 2006; Vardhini and Anjum, 2015). Observations on the use and efficacy of medicinal plants significantly add to the discovery of their therapeutic properties, with the intention that they are frequently prescribed, even if their chemical constituents are not always completely known. All over the globe, the use of medicinal plants has considerably supported primary health care (Maciel MAM et al., 2002). The composition of biologically active compounds of medicinal plants varies widely depending on the plant species, soil type and on their association with microbes (Zhang et al., 2017; Morsi, 2017).

The genus *Callistemon* R. Br. (commonly known as bottlebrush) belongs to the family of Myrtaceae and comprises over thirty species. *C. citrinus* are woody aromatic trees or shrubs broadly spread in the wet tropics, Australia, South America and tropical Asia, but are now broaden all over the world. The leaves of *Callistemon* species are lanceolate in arrangement and very aromatic, Lot of research has been carried out on medical properties of different species of *C. citrinus*. Antioxidant and antimicrobial activities of methanolic extract obtained from *Callistemon linearis* DC. Leaf has been reported. Methanolic extract exhibited potential antimicrobial activity against both gram positive as well as gram negative bacteria and moderate activity against fungal species.

In the studies of Karaman, (2003) aqueous and methanol extracts of the leaves of *Juniperus oxycedrus* were studied for their *in vitro* antimicrobial properties. The
antimicrobial activity of the extracts was evaluated based on the inhibition zone using the minimal inhibition concentration (MIC), disc-diffusion assay and minimal bactericidal concentration (MBC) values. Among the extracts methanol extract showed inhibitory effects on genera *Acinetobacter, Bacillus, Brevundimonas, Brucella, Enterobacter, Escherichia, Micrococcus, Pseudomonas, Staphylococcus*, and *Xanthomonas*.

Adiguzel *et al.* (2007) reported that in vitro antimicrobial activities of the essential oil and methanol extract from *Satureja hortensis* and its composition in essential oil. The chemical composition of hydrodistilled essential oil of *S. hortensis* was analysed by GC-MS. The main constituents of the oil were thymol (40.54 %), γ-terpinene (18.56 %), carvacrol (13.98 %), and p-cymene (8.97).

In the studies of Darabpour (2009) *T. polium* medicinal plant was studied for its antimicrobial activity. The ethanolic and Methanolic extract of *T. polium* aerial parts showed potential activity against both gram positive and gram negative bacteria and plant bioactive metabolites were studied. Lone major constituents of essential oil extracted from *C. zeylanicum* named (E)-cinnamaldehyde has an antityrosinase activity, Cinnamon is well-known oldest spices, which has been widely used for centuries (Gruenwald *et al.*, 2010)

*T. peruviana* plant species constitutes glycosides, whose toxicity against snails, slugs, bacteria, insects (Panigrahi and Raut, 1994), (Obasi and Igboechi, 1991) (McLaughlin *et al.*, 1980) and humans (Langford and Boor, 1966) has been documented. *T. peruviana* plant extracts have also been reported to have antifungal properties against *Cladosporium cucumerinum* (Gata *et al.*, 2003)
Studies of Tsevalmaa, (2018) showed that the *Caryopteris mongolica* root extract may be valuable in the treatment of brucellosis infected patients, along with doxycycline or other antibiotics, to decrease the toxicity of high-dosage antibiotics and to prevent the development of antibiotic resistance and to prevent *Brucella* infection. They have reported the phytochemical analysis of root extract of *Caryopteris mongolica* and isolated three new compounds and five known diterpene derivatives, namely, demethylcryptojaponol, iconone, 6α-hydroxydemethylcRYPTOJAPONOL, cytrophyllone B, and 14-deoxycoleon U. Among them a new compound of abietane diterpene derivatives showed strong antibacterial activity.

*Acacia nilotica* commonly known has Acacia; it belongs to the family of Mimosaceae. The powdered bark of the plant with minute salt is used for treating acute diarrhea (Gill *et al*, 1992). Many countries in the developing world are now regaining interest in making use of their indigenous resources and considering the possibilities of including synthetic medicine with traditional medicinal elements, particularly at the primary health care level (Akerele *et al*, 1984). *Eugenia jambolana* belongs to family Myrtaceae, which is commonly called as Jamun, Black plum or Indian Black berry. In modern medicine satisfactory effective therapy is not yet available to cure diabetes mellitus. It is well known for its antidiabetic activity.

*Adhatoda vasica* Nees belongs to family Acanthaceae, it is an evergreen shrub and its common name is Malabar nut. It is well known for medicinal properties for bronchitis, asthma and other pulmonary infections. It is also known for its anti-arthritis, antiseptic, antimicrobial, expectorant, sedative and antituberculosis properties (Dey, 1980; Singh and Jain, 1987). *Thuja occidentalis* belongs to family Cupressaceae, which is commonly known as Arbor vitae or white cedar. it is an ornamental tree.
grown in Europe. It is used as a medicinal plant in various forms of traditional medicines like folk medicine, homeopathy, and so forth. for treatment of bronchial catarrh, enuresis, cystitis, psoriasis, uterine carcinomas, amenorrhea and rheumatism (Chang et al., 2000) Thuja is also occasionally used for treating diseases of skin, blood, gastrointestinal tract, kidney, brain, warty excrescences, spongy tumors, and so forth, and claimed to have pronounced remedial effects. The extract has been reported to enhance the antibody response to sheep blood cells (Bodinet et al., 1999).

*Gloriosa superba* L. a flowering plant belongs to the family Colchicaceae. It is best an ornamental plant which is also known for medical properties, it is also a poisonous plant as a toxic weed. it has rich alkaloid content hence, it . It has been used in the treatment of gout, infertility, open wounds, snakebite, ulcers, art is used as a traditional medicine for arthritis and sexually transmitted diseases

Infectious diseases signify an important cause of morbidity and mortality among the general population, predominantly in developing countries. Consequently, pharmaceutical companies have been stirred to develop new antimicrobial drugs in recent duration, especially due to the constant emergence of microorganisms resistant to conventional antimicrobials. it appears that, bacterial species shows the genetic ability to acquire and transmit resistance against currently available antibacterials as there are frequent reports on the isolation of bacteria that are known to be sensitive to routinely used drugs and became multiresistant to other medications available on the market (Nascimento et al., 2000; Sakagami et al., 2002). So common strategy adopted by pharmaceutical companies to provide the market with new antimicrobial drugs include altering the molecular structure of the existing medicines in order to make them more efficient or recover the activity lost due to bacterial resistance mechanisms
(Chartone-Souza et al., 1998). 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.

**Table 2.1. List of Medicinal plants showing the antibrucellosis activity.**

<table>
<thead>
<tr>
<th>Plant Name</th>
<th>Plant part</th>
<th>Active extract</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Juniperus oxycedrus L.</td>
<td>leaf</td>
<td>Methanolic extract</td>
<td>Karaman et al., 2003</td>
</tr>
<tr>
<td>Satureja hortensis</td>
<td>leaf</td>
<td>Ethanolic extract</td>
<td>Adiguzel et al., 2007</td>
</tr>
<tr>
<td>Petroselinum crispum</td>
<td>seed</td>
<td>Ethanolic extract</td>
<td>Seyyednejad et al., 2008</td>
</tr>
<tr>
<td>Oliveria decumbens</td>
<td>leaf</td>
<td>Ethanolic extract</td>
<td>Motamedi et al., 2010</td>
</tr>
<tr>
<td>Cinnamomum zeylanicum</td>
<td>bark</td>
<td>Methanolic extract</td>
<td>Gruenwald et al., 2010</td>
</tr>
<tr>
<td>Moringa oleifera</td>
<td>leaf</td>
<td>Methanolic extract</td>
<td>Alawad et al., 2012</td>
</tr>
<tr>
<td>Scrophularia deserti</td>
<td>leaf</td>
<td>Ethanolic extract</td>
<td>Pouya et al., 2012</td>
</tr>
<tr>
<td>Origanum syriacum</td>
<td>leaf</td>
<td>Ethanolic extract</td>
<td>Ayman et al., 2013</td>
</tr>
<tr>
<td>Crocus sativus</td>
<td>leaf</td>
<td>Methanolic extract</td>
<td>Monte et al., 2015</td>
</tr>
<tr>
<td>Cyathula uncinulata</td>
<td>leaf</td>
<td>Methanolic extract</td>
<td>Bisi-Johnson et al., 2015</td>
</tr>
<tr>
<td>Callistemon Citrinus</td>
<td>leaf</td>
<td>Ethanolic extract</td>
<td>Sri Raghava et al., 2017</td>
</tr>
<tr>
<td>Caryopteris mongolica Bunge</td>
<td>root</td>
<td>Acetone extract</td>
<td>Tseveelmaa et al., 2018</td>
</tr>
</tbody>
</table>

The diagnosis of brucellosis in livestock and wildlife is complex and serological results need to be carefully analyzed. The *B. abortus* S19 and *B. melitensis* Rev. 1 vaccines are the cornerstone of control programs in cattle and small ruminants, respectively. In the absence of a human brucellosis vaccine, prevention of human brucellosis depends on the control of the disease in animals currently, the treatment of brucellosis remains a major public health concern, especially in developing countries in order to increase the treatment efficacy and avoid disease relapse. Serology is the first tool in detecting subclinical infections. Better tests and testing strategies should be developed but the “gold standard” in brucellosis remains the isolation of *Brucella spp.*, and is thus mandatory. Brucellosis is not a sustainable disease in humans. The
source of human infection resides always in domestic or wild animal reservoirs. Therefore, as a general rule, prevention of human zoonotic brucellosis depends predominantly on the control of the disease in animals.

*Fig. 2.14: Medicinal plants showing the antibrucellosis activity.*
The epidemiology of brucellosis involving various animal species, the effective control requires long lasting and carefully monitored efforts. Preventive measures are essential to minimize the risk of infection of the human population as there is no Vaccination for human infection (Smits and Kadri, 2005). Since the treatment of animal brucellosis is very expensive, one should encourage the mass vaccination of livestock. Animal owners should be taught about the importance of vaccination of their animals.