# "EVALUATION OF CONVENTIONAL CASTANEDA BIPHASIC AND LYSIS CENTRIFUGATION BLOOD CULTURE TECHNIQUES AND POLYMERASE CHAIN REACTION IN THE DIAGNOSIS OF HUMAN BRUCELLOSIS"

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This is to certify that the dissertation entitled **"EVALUATION OF CONVENTIONAL CASTANEDA BIPHASIC AND LYSIS CENTRIFUGATION BLOOD CULTURE TECHNIQUES AND POLYMERASE CHAIN REACTION IN THE DIAGNOSIS OF HUMAN BRUCELLOSIS"** is a bonafide research work done by **Registration No. BI0111003.**

**Dr. M. B NAGAMOTI** **MD, PhD**
Professor and Head,
Department of Microbiology,
J. N. Medical College,
Nehru Nagar
Belgaum – 590 010
**Date:**
**Place: Belgaum**

**Dr. A. S. GODHI** **MS,FICS**
Principal,
J. N. Medical College,
Nehru Nagar,
Belgaum – 590 010

**Date:**
**Place: Belgaum**

# LIST OF ABBREVIATIONS USED

| | | |
|---|---|---|
| BHI | – | Brain Heart Infusion Agar/Broth |
| CFT | – | Complement Fixation Test |
| CELISA | – | Competitive Enzyme Linked Immuosorbent Assay |
| FPA | – | Fluorescent Polarization Assay |
| 2-ME | – | 2 - Mercaptoethanol |
| PCR | – | Polymerase Chain Reaction |
| RBPT | – | Rose Bengal Plate Test |
| SAT | – | Standard Agglutination Test |
| Spp | – | Species |
| I.U./ml | – | International Units/ millilitre |
| IVRI | – | Indian Veterinary Research Institute |
| WHO | – | World Health Organization |

# ABSTRACT

**BACKGROUND:**

Brucellosis is an endemic zoonotic disease with a wide spectrum of non-specific symptoms, Serological methods being the mainstay of diagnosis, are characterized by low specificity especially in areas with high base line titres. Though blood culture is gold standard of diagnosis, low rate of culture yield and long doubling time of the organism are the main limitations .Molecular diagnostic modalities like PCR for detection of DNA are being increasingly used. Hence this study was undertaken to evaluate the blood culture techniques – Conventional Castaneda Biphasic Medium and Lysis Centrifugation Technique and PCR for diagnosis of human Brucellosis.

**Objective:**

To evaluate Conventional blood culture methods (Castaneda Biphasic Medium and Lysis Centrifugation) and PCR in diagnosis of human brucellosis

**Material and Methods:**

The study was a cross sectional study carried out in in the serology section of department of Microbiology, Jawaharlal Nehru Medical College, Belgaum. A total of 1545 serum samples were screened. 50 serum samples were positive for Brucellosis by slide agglutination test using Rose Bengal Card Test and were included in the study.

10-12 ml of blood was collected for both the blood culture methods while serum was used as the sample for PCR.

The culture isolates of *Brucella* were identified by colony morphology, staining and biochemical reactions. DNA extraction was done from serum samples and PCR was done targeting the IS711 genome of *Brucella.*

**Results:**

Out of the 50 samples positive by RBPT, blood culture was positive in 10 cases (20%) by the Conventional Castaneda Biphasic Medium and in 21 cases (42%) by the Lysis Centrifugation Technique. The mean duration of culture positivity by Conventional method was 6 days while the mean duration of culture positivity by Lysis Centrifugation was 3 days.

Sensitivity of the Castaneda Biphasic Medium compared to the Lysis Centrifugation Technique was 47.6% while the specificity was 100%.

PCR was positive in 47 cases (94%). The sensitivity of PCR compared to Lysis Centrifugation Technique was 100%.

**Conclusion:**

Isolation of the organism by blood culture though being the gold standard, has a low sensitivity and also the duration of incubation required is long. Blood culture by the Lysis Centrifugation Technique not only increases the rate of isolation but also decreases the duration of incubation.

PCR by amplifying even the intracellular DNA can detect the presence of the organism in the body within 24 hours thus giving conclusive evidence of infection especially in chronic cases and focal complications. However PCR can detect even non-viable bacilli. Also the cost, technical expertise and infrastructure required may preclude its use at all centres.

**Keywords:** Brucellosis, Castaneda Biphasic Medium, Lysis Centrifugation Technique, PCR

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF GRAPHS

# LIST OF FIGURES

# INTRODUCTION

Brucellosis is a zoonotic disease widely distributed throughout the world and is endemic in many developing countries[1]. Around 5,00,000 cases are reported worldwide annually according to the World Health Organization report[2]. It is an important public health problem in the Mediterranean region, the Middle East, India and South America. It is known by various other names like Malta fever, Undulant fever, Mediterranean fever, Gibraltar fever, Danube fever, Rock fever, Crimean fever, Bang's disease and Cyprus fever[3].

Brucellosis is caused by *Brucella* which is a slow growing fastidious gram negative intracellular coccobacillus producing infections in humans and animals. Out of the 8 species of *Brucella (B. melitensis, B. abortus, B. suis, B. canis, B. ovis, B. ceti, B. neotomae* and *B. pinnipedialis),* the commonest[4] species causing human Brucellosis is *B. melitensis* (83%) followed by *B.abortus* (16-17%) and *B.* suis(1%). These 3 species preferentially infect goats & sheep, cattle and pigs respectively. However they are not host specific. Each species can infect any of the hosts.

The disease is transmitted to humans from infected animals and their products. Infections in humans results from direct or indirect contact with infected animals. This includes consumption of raw milk and milk products, maintenance of livestock and handling of their birth products. Person to person transmission has been recorded rarely implicating either sexual contact[5], or by the transfer of tissue, blood and bone marrow[6]and also by breast feeding[7]. Laboratory acquired infection is usually by accidental ingestion, inhalation or contamination of skin and mucous membranes while handling *Brucella* cultures. The greatest risk of transmission in laboratory is by aerosol generation while handling cultures. People at risk of Brucellosis are

agriculturists, people who consume raw milk and milk products, abattoirs, veterinarians and laboratory workers.

Clinically human Brucellosis presents with protean manifestations[8] which pose a diagnostic dilemna. The wide spectrum of clinical manifestations may range from asymptomatic state to complications of various organ systems and a chronic state. The symptoms may be very non-specific, although focal forms occur in 20-40% cases[9]. Most of the cases present as fever of varying duration and severity. Any organ system may be affected with the musculoskeletal system[10,11] being the most common. The complications are seen in the following systems - musculoskeletal system (sacroilitis, septic arthritis, spondylitis), central nervous system (meningitis, meningoencephalitis), cardiovascular system (infective endocarditis), respiratory system(pneumonia, pleural effusion), genito-urinary system (orchitis, epididymitis) and other viscera(hepatitis, splenic abscess).

Accurate early diagnosis is very important for treatment and good patient care. Diagnosis is based on serological and microbiological methods[12]. Serological tests which are usually used for screening may not always be specific especially in endemic areas due to the presence of endemic titres. The commonly used serological tests for screening of human Brucellosis are

a) Rose Bengal Plate Test (RBPT)[Sensitivity-99%, Specificity-94.3%] [13]

b) Standard Tube Agglutination Test(SAT) [Sensitivity-100%, Specificity-97%][14],

Other serological tests for Brucellosis are

a) 2-mercapto-ethanol(2-ME)

b) Complement Fixation Test(CFT)[15]

c) Competitive Enzyme Linked Immunosorbent Assay(CELISA)[16]

d) Fluorescence Polarization Assay(FPA)[16]

Isolation of the organism from clinical samples remains the "Gold Standard" of laboratory diagnosis. However the problem with culture is that it is time consuming, has low sensitivity in patients with chronic Brucellosis, duration of incubation required is longer and poses a risk to laboratory personnel as *Brucella* is a class III pathogen. Conventional blood culture methods like Castaneda biphasic medium takes a long time (minimum of 7-10 days) to yield a visible growth. Lysis centrifugation blood culture technique increases the isolation rate by 25% and growth is seen in 2 to 4 days[17].

The role of Polymerase Chain Reaction (PCR) in the diagnosis of human Brucellosis is being analysed. The PCR technique is being increasingly used as a more rapid and confirmatory method in diagnosis of human Brucellosis. PCR provides an early evidence of the disease by detecting DNA, thus confirming the presence of organism in the body in less than 24 hours[18,19].

Thus this study is undertaken to evaluate the blood culture methods – Conventional Castaneda Biphasic Medium & Lysis Centrifugation Technique and PCR for early diagnosis of human Brucellosis which will help in appropriate treatment as per WHO guidelines and thereby prevent complications.

# OBJECTIVE OF THE STUDY

To evaluate Conventional blood culture methods (Castaneda Biphasic Medium and Lysis Centrifugation) and Polymerase Chain Reaction in diagnosis of human Brucellosis.

# REVIEW OF LITERATURE

**History:**

The history of Brucellosis dates back to 450 BC when it was described by Hippocrates. There is mention of Brucellosis-type illnesses in his '*Epidemics'* writing.

The present epidemiology of Brucellosis dates back to 1886when Sir David Bruce, a British army surgeon, found a coccobacillus in the spleen of an infected soldier who had died of "Malta fever" in the Crimean war. He isolated the organism in pure culture in 1887 and named it "*Micrococcus melitensis*". He transmitted the disease to monkeys and recovered it from their liver and spleen. The disease was named 'Mediterranean fever'.

In 1897, Bernard Bang, a Danish physician, isolated the organism causing contagious abortion in cattle. He named it *Bacillus abortus*. He isolated the organism in pure culture and reproduced the disease in healthy heifers. The infection in cattle came to be known as 'Bang's disease'.

In 1905, the Maltese physician, Themistockles Zammit tried to infect goats with *Micrococcus melitensis*. Agglutination tests prior to the experiment showed that many goats were already infected and raw goat milk was found to be the source of infection.

In 1920s Alice Evans, an American bacteriologist, reported the presence of *Bacillus abortus* in fresh milk. She also reported that the morphology and pathology of *Bacillus abortus* and *Micrococcus melitensis* were quite similar. These findings were confirmed by Meyer and Shaw in 1920 and the generic name *Brucella* was proposed for these organisms in honour of Sir David Bruce.

*Brucella suis* was reported in 1934 and *Brucella canis* in 1966. *Brucella ovis* was reported in 2003 and *Brucella neotomae* in 2007. The Marine *Brucella* species have been discovered in the last 15 years.

In India the first case of Brucellosis was reported in 1942. It is now endemic throughout the country. *B. melitensis* bio type-I in sheep and goats and *B.abortus* biotype-I in cattle are the main infective serotypes. Seroprevelance studies have shown that 5% of cattle and 3% of buffaloes are infected[20].

**EPIDEMIOLOGY:**

**Global Scenario:**

Though Brucellosis has been eradicated in many developed countries, worldwide it is a major source of disease in humans and domesticated animals. The disease is endemic in countries of the Mediterranean basin, the Arabian gulf, the Indian subcontinent and parts of Mexico and Central and South America. Worldwide the reported incidence of human Brucellosis in endemic disease areas varies widely from <0.01 to >200 per 1,00,00 population[21]. *B.melitensis* as the etiological agent accounts for the highest number of Brucellosis in most of the areas. It has been found that the incidence of human Brucellosis is significantly high where bovine/ caprine Brucellosis caused by *B.melitensis* is endemic[22]. *B.melitensis* infection in cattle is emerging as a major problem in many countries. The ecological range of Brucellosis has extended after isolation of distinctive strains of *Brucella* from marine animals as well as humans[23,24].

The mode of transmission is by ingestion, inhalation and inoculation. Consumption of raw milk and milk products, unpasteurized milk, contact with infected animals and their birth products or their body discharges are the common

sources of infection. It is a well-known occupational disease and carries risk for shepherds, abattoir workers, veterinarians, dairy industry workers and laboratory personnel. Rare modes of transmission include-sexual transmission, transplacental transmission, blood transfusion, bone marrow or tissue transplant and breast feeding. It affects males more than females, probably due to the occupational exposure. All age groups including paediatric population can be affected by human Brucellosis.

**Indian Scenario:**

Brucellosis is endemic in the Indian subcontinent. After the report of the first case in 1942, it has been now reported from almost all the states[20,25].

In a study done by Mathur, the seroprevalence of Brucellosis among dairy personnel in contact with infected animals was 8.5%[26]. In another study conducted by Thakur *et al,* prevalence of Brucellosis in animal contacts was found to be 4.97%, with a high prevalence of 17.39% among field veterinarians[27]. A study done in Gujarat showed the prevalence of *Brucella* antibodies as 8.5%[28]. In a study done in the department of Microbiology, J. N. Medical College, Belgaum, Karnataka the seroprevalence of Brucellosis was found to be 8.5%[29].

**ETIOLOGICAL AGENT:**

**Taxonomy, Morphology and Biochemical Characteristics:**

The taxonomical profile of the etiological agent *Brucella* is as follows-$\alpha_2$ subgroup of *Proteobacteria* of the family *Brucellaceae* in the order *Rhizobiales.*

It is a fastidious gram negative facultative intracellular coccobacillus measuring 0.5-0.7µ by 0.8-1.0µ. It is unencapsulated, nonsporing and non-motile. Its growth is enhanced by 10%$CO_2$. It is Catalase, Oxidase and Urease positive. It is

partially acid fast and produces $H_2S$. Certain dyes like thionin, basic fuschin have a bacteriostatic effect on *Brucella* when they are incorporated into media that support the growth of *Brucella*. *Brucella* can grow on Chocolate agar, Trypticase soy broth/agar and Brain Heart Infusion broth/agar.

It is sensitive to sunlight, ionizing radiation and moderate heat, is killed by boiling and pasteurization, but resists freezing and drying. Hence it is stable in aerosol form which causes airborne transmission. Effective disinfectants against *Brucella* are phenols, ethanol, hypochlorite, iso-propanol, iodophors, ethylene oxide and formaldehyde[30].

*Brucella* can survive for 2hours in soft cheese made of goat and sheep milk, 6 weeks in dry soil contaminated with infected urine, vaginal discharge or placental and fetal tissues of animals, and for 6 months in damp soil and manure.

**Virulence Factors:**

*Brucella* which are facultative intracellular organisms can multiply inside the phagocytic cells and evade intracellular killing by various mechanisms. The virulence factors which help the organism to evade host defences, penetrate host cells , escape intracellular lysosomal killing and allow long term intracellular survival and multiplication are as follows:

- Lipopolysaccharides
- The two component BvrR/BvrS system
- VirB operon
- Cyclic β-1,2 glucans
- Phosphatidylcholine

- Brucella host factor I(HF-I) protein
- Superoxide dismutase
- PrpA
- **Lipopolysaccharides:**

The lipopolysaccharide (LPS) is the major virulence factor of *Brucella.* It is a non endotoxic lipopolysaccharide unlike the lipopolysaccharide of enterobacteriaceae. It may be smooth or rough depending on the presence or absence of surface exposed O- polysaccharide chain respectively. O-polysaccharide plays a major role in virulence associated with smooth LPS since mutant rough strains are unable to survive in macrophages[31,32]. Some rough strains may be naturally virulent[33].*Brucella* can block maturation of the organism containing phagosome through the interaction of smooth LPS with lipid rafts, which contributes to inhibition of phagosome-lysosome fusion[34]. Also smooth LPS provides resistance to complement and antimicrobial peptides such as α-defensins and lactoferrins. It also confers resistance against nitric oxide, free radicals and lysozyme--important antibacterial mechanisms of macrophages and neutrophils. Hence smooth LPS may be considered a virulence factor required for resistance against both extra- and intracellular antimicrobial mechanisms of the host.

- **The two component BvrR/BvrS system:**

*Brucella* relies on a two- component regulator system, BvrR/BvrS while entering into the cell. This system regulates the expression of outer membrane proteins (OMPs) involved in invasion of host cells[35]. The two components of this system are BvrR, a regulator protein, and BvrS, a sensor protein with histidine-kinase activity. It is needed for recruitment of GTPases and act in filaments, and for

maintaining the integrity of the bacterial cell wall. This system helps in the intracellular survival of the organism and thus acts as a virulence factor.

- **VirB operon:**

The VirB operon consists of the VirB1-VirB12 genes which code for the *Brucella* type IV secretion system (T4SS). It is required for intracellular growth of *Brucella* in phagocytic and non-phagocytic cells[36].The T4SS is a virulence factor characterized by a transporter apparatus localized in the outer membrane. It is able to translocate bacterial DNA or effector proteins into target host cells. Effector molecules secreted by the T4SS possibly play a role in phagosome maturation and trafficking of the *Brucella*-containing vacuole towards its replication niche[37].The T4SS is absolutely required for intracellular survival and replication.

- **Cyclic β-1,2 glucans:**

Glucans are constituents of the bacterial periplasm with osmoregulatory and cholesterol sequestering activity and are required for survival of *Brucella* in non-phagocytic cells. Cyclic 1,2-glucans of *Brucella* prevent phagosome maturation by interfering with lipid rafts, thus altering protein expression in the vacuolar membrane and excluding lysosomal proteins from the BCV[38].

- **Phosphatidylcholine:**

Phosphatidylcholine is present in the cell envelope of *Brucella*. It is synthesized with the help of choline present in the medium. It is necessary to maintain a chronic infectious process[39].

- ***Brucella*host factor I(HF-I) protein:**

It is an abundant RNA binding protein also known as *Hpq*. It contributes to generalized stress resistance during stationary phase growth in the organism, and is essential for survival in macrophages and virulence[40].

- **Superoxide dismutase:**

The periplasmic Cu, Zn cofactored Superoxide Dismutases are coded by the *sodC*gene. They are a family of metalloenzymes containing either iron, manganese, or copper and zinc at their active sites. These enzymes catalyze the dismutation of superoxide ($O_2^-$) to hydrogen peroxide ($H_2O_2$) which can then be further detoxified through the action of catalases and peroxidases. The superoxide dismutase protects the intracellular *Brucellae* from the respiratory burst of host macrophages[41].

- **PrpA:**

PrpA (Proline Racemase Protein A) is a *Brucella* B-cell lymphoproliferative virulence factor. It induces a transient non-responsive state of spleenocytes, acts as a potent IL-10 inducer and participates in the efficient establishment of a chronic infection. It acts during the acute phase of the infectious process, inducing a transient non-responsive state of the immune system which delays or hampers the immune response facilitating the establishment of a chronic infection[42].

**IMMUNITY AND PATHOGENESIS[43,44,45]:**

Exposure to infection activates both humoral and cell mediated immunity. Antibodies produced have a bactericidal action on the organisms helped by polymorphonuclear phagocytes which phagocytose the organism. However the antibody response is unable to eradicate the infection. The organisms taken up by the

macrophages and other cells lead to a persistent intracellular infection. Infection leads to release of cytokines like IL-2 which promote the release of Interferonγ (INFγ). INF γ directs the $T_H$-2 responses and stimulates macrophage activation. Activated macrophages lead to killing of intracellular *Brucella* through the activation of oxygen intermediates. INF γ also induces apoptosis, enhances cell differentiation and cytokine production. Tumor Necrosis Factor-α (TNF-α) produced in response to immune responses leads to activation of cytotoxic lymphocytes which may cause partial killing of the organisms. But virulent *Brucella* have the ability to suppress the TNF-α response and hence this mechanism has a limited role in protection. Inflammatory cytokines like IL-6 and IL-10 down regulate the protective response. The replication of the organism takes place intracellularly, first in the lymph nodes draining the point of entry and then through haematogenous route spread to almost any site causing a chronic localizing infection. Most commonly involved sites are the reticuloendothelial system, musculoskeletal system and the genitourinary system. Both acute and chronic inflammatory responses develop in Brucellosis. Local immune response leads to granuloma formation, with or without necrosis and caseation leading to abscess formation.

IgM immunoglobulins against lipopolysaccharides appear during the first 7-10 days of infection followed by decline in the level of IgM and appearance of IgG immunolobulins after $2^{nd}$ week. Treatment leads to a decrease in the titres of both the immunoglobulins. Failure of decrease of IgG titres is indicative of chronicity or relapse. The antibody response in Brucellosis helps in clearing the extracellular infection as well as in serological diagnosis. However it does not eradicate the infection due to the presence of the intracellular organisms.

**THE DISEASE[45]:**

Brucellosis is an acute or sub-acute febrile illness usually marked by an intermittent or remittent fever accompanied by malaise, anorexia and prostration, and which, in the absence of specific treatment, may persist for weeks or months. The incubation period is usually 1-2 weeks, but may extend upto3 months depending upon the virulence of the infecting strain, the size of the inoculum, the route of infection and the resistance of the host. Brucellosis invariably causes fever associated with night sweats. It shows an undulating pattern that persists for weeks followed by afebrile period which may be followed by a relapse. In more than half the cases the fever is accompanied by musculoskeletal symptoms and signs. In addition to fever, patients also become increasingly lethargic and fatigued; lose appetite and weight; and have non-specific myalgia, headache and chills. Focal features include musculoskeletal pains, osteomyelitis, bursitis and monoarticular or polyarticular septic arthritis. 25% of the patients develop hepatospleenomegaly and 10-20% patients have generalized lymphadenopathy. 10% of male patients have epididymoorchitis. Neurological involvement leads to lethargy and depression, and rarely lymphocytic meningoencephalitis. Endocarditis occurs in ~ 1% patients affecting mainly the aortic valve. Other cardiovascular complications include myocarditis, pericarditis andseptic embolization. Maculopapular rashes, papulonodular lesions and other skin manifestations are rare, but may occur.

The diagnosis of the disease which indicates infection with a *Brucella* strain must be supported by laboratory tests which indicate the presence of the organism or a specific immune response to its antigens.

## LABORATORY DIAGNOSIS:

A high degree of clinical suspicion should be maintained based on the epidemiological information. Travel history, history of exposure to animals and exotic foods are important[46]. Laboratory diagnosis includes isolation and identification of *Brucellae* from clinical samples, detection of antigen, demonstration of *Brucella* specific antibodies and demonstration of the genome.

**I) Isolation of the organism**

**II) Serological Diagnosis**

**III) Molecular Techniques for Diagnosis**

### I) Isolation of the organism:

The isolation of the organism from the clinical specimen in a suspected case of Brucellosis confirms the diagnosis. Hence culture is considered the gold standard for diagnosis of Brucellosis.

As *Brucellae* primarily infect the reticuloendothelial system, the most common specimens from which the organism is isolated are blood and bone marrow. In a few studies the culture positivity rate from blood and bone marrow was found to be 70% and 92% respectively[47]. It can be isolated from various other clinical specimens like CSF, urine, synovial fluid, tissue biopsy etc.

However the doubling time of *Brucella* is long and hence culture gives delayed results, even if positive. Also due to the long duration of incubation there may be contamination of the cultures. The rate of positive blood cultures by conventional Castaneda Biphasic Medium in acute cases is 70-80%[48] which may be reduced to 30-50%[49] in chronic cases and focal complications.

The methods[50] of culturing *Brucella* are

1. **Monophasic method**
2. **Biphasic method**
3. **Rapid Methods**

1. **Monophasic method:**

Detection of *Brucella* from clinical samples by routine culture techniques is hampered due to its slow growth. As the most common sample used for isolation of *Brucella* is blood, around 5-10 ml of blood is inoculated aseptically into the culture broth and is incubated at 37$^{o}$C in the presence of 10%$CO_2$. Blind subcultures are made every 3$^{rd}$ day to isolate the organism. Cultures are incubated for atleast 4weeks before being reported negative[45,50].

Disadvantages:

- Time consuming due to repeated subculture
- Repeated subculture may lead to contamination of the culture
- Risk to the laboratory personnel handling the cultures
- Incubation for longer period in case of negative cultures
- Diagnosis is delayed

2. **Biphasic Method:**

The biphasic method of culture developed by Castaneda in which both solid and liquid media are contained within the same bottle obviates the need of repeated subculture and thus minimizes the chances of contamination and laboratory acquired

infection. The solid medium is layered along one of the broad inner surfaces of the bottle, while a certain amount of liquid medium is added to the bottle.

Like monophasic method 5 to 10 ml of blood is drawn and inoculated into the broth phase of the medium; the contents are mixed well and the mixture is tipped daily to allow the blood-broth mixture to flow over the agar slant. The bottle is then incubated in the upright position in the incubator at 37$^{o}$C in the presence of 10% $CO_2$ and examined at 48 hour intervals. Any colonies that appear on the solid media are subcultured and identified. If no colonies are observed the bottles are tilted and re-incubated, the cycle is repeated for at least 30 days[50,51] .

3. **Rapid isolation methods:**

Techniques for rapid isolation of *Brucella* from blood are divided into 2 groups

A) Non Automated culture systems

a) Clot culture

b) Lysis-Centrifugation Technique

B) Automated culture systems

**A) Non Automated Culture Systems**

**a) Clot culture[52]:**

The clot culture is a technique where in serum, which may contain antibacterial activity, is removed from a blood specimen and the remaining clot is disrupted using streptokinase or with the help of a sterile glass rod. The clot fragments obtained are then added into a broth medium for culture.

Blood clot culture system has shown a substantial increase in rate of isolation over that of whole blood culture. Bacterial growth is significantly faster in cultures of blood clot compared to whole blood. But *Brucellae* are best isolated by blood clot cultures only in acute brucellosis.

**b) Lysis-centrifugation culture:**

Lysis-centrifugation can be carried out in two ways

- In-house lysis-centrifugation
- Isolator lysis-centrifugation

**In-house lysis-centrifugation[17]:** In this method, 5 ml of blood is collected in 50 ml screw-capped sterile centrifugation tube containing 20 ml of sterile distilled water and 1.5 ml of 4% sodium citrate. The contents are gently mixed and centrifuged at 2000 x g for 30 minutes. The supernatant is discarded, and the sediment is inoculated onto agar plates in duplicate. The plates are incubated at $37^{o}$ C with $CO_2$ for 7 days.

The lysis-centrifugation technique has shown a substantial increase in rate of isolation over that of conventional culture technique. It can also increase the recovery of *Brucella* from clinical specimens like bone marrow and cerebrospinal fluid. By employing this technique *Brucella* can be recovered within three days of incubation.

**Isolator lysis-centrifugation:** The Isolator blood culture system is a commercial lysis-based method. The blood specimen collected is inoculated into an Isolator Microbial tube and processed. The blood lysate obtained is dispersed on the surface of the agar medium and incubated. The plates are examined for bacterial growth once a day for 10 days[53].The Isolator blood culture system to detects *Brucella* within 2 to 5 days.

Disadvantages of lysis-centrifugation method

- Contamination rates are higher due to excessive manipulation.
- Due to the manipulation it poses a substantial risk to laboratory personnel.

**B) Automated culture systems:**

Over the last few years various automated blood culture systems are being used for isolation of *Brucella* species like BACTEC 460, BACTEC NR 730, BACTEC NR 660, BACTEC 9240 e[54,55].

Development of automated blood culture systems and technical improvement has resulted in increase in the isolation rates and shortening of detection time. Nowadays, with the use of the BACTEC 9000 series it is possible to recover more than 95% of cultures within the routine 7-day blood culture protocol and sub-culturing of negative media is no longer necessary.

As Brucellosis is prevalent in developing countries, the use of modern bacteriologic techniques is limited and hence conventional techniques are commonly used. The blood culture positivity in Brucellosis depends on the stage of the disease, the infecting species, culture medium used, antibiotic treatment and blood culture technique employed.

**Identification of *Brucella* Isolates*:***

*Brucella* spp are identified by their morphology in gram staining, modified ZN staining, colony morphology and biochemical reactions. In gram stain they appear as gram negative coccobacilli. Modified ZN staining (using 1% $H_2SO_4$ as decolouriser) demonstrates their acid fastness and they appear as weakly acid fast(i.e red).

On Chocolate agar or BHI agar they show greyish translucent circular colonies. *Brucellae* are catalase positive, oxidase positive, strongly urease positive, reduce nitrates to nitrites, produce $H_2S$ and require 10% $CO_2$for optimum growth.

II) **Serological Diagnosis**:

Though culture is the gold standard for diagnosis of Brucellosis, it is not always possible to isolate the organism as culture positivity in Brucellosis is very low, especially in chronic cases and relapses. Hence serological techniques are the ones most commonly used in the routine laboratory diagnosis of Brucellosis. The major *Brucella* antigens that are useful for diagnosing human Brucellosis are the smooth(S) lipopolysaccharide (LPS) of the outer cell membrane and the internal (cytosolic) proteins. The LPS is the immunodominant antigen. However it is also the molecule carrying the epitopes that may cross-react with other Gram-negative bacteria including *Yersinia enterocolitica O:9, Escherichia coli O:157, Francisella tularensis, Salmonella urbana O:30, Vibrio cholerae*, and others.

The various serological techniques used for diagnosis of Brucellosis are

(a) Rose Bengal Plate Test (RBPT)

(b) Standard Tube Agglutination Test (SAT)

(c) 2-mercapto-ethanol (2ME)

(d) Complement Fixation Test (CFT)

(e) Coombs antiglobulin test

(f) Competitive Enzyme Linked Immunosorbent Assay(CELISA)

(g) Fluorescence Polarization Assay (FPA)

**(a) Rose Bengal Plate Test(RBPT):**

The RBPT is currently the recommended rapid screening test, but the results should always be confirmed by other tests particularly in areas where there is a high incidence of animal brucellosis. The sensitivity of RBPT is about 99%[13], but it can give false positive reactions with sera from patients infected with *Y. enterocolitica 0:9* or other cross reactive organisms and from healthy individuals that have had contact with *Smooth-Brucella* without developing disease.

It is a slide agglutination test. The antigen is prepared from a concentrated cell suspension of smooth strain of *B.abortus* (strain 99). The cells are stained with Rose Bengal stain and suspended in buffered *Brucella* antigen diluent (pH3.65±0.05). The test is very sensitive and positive samples should be checked by the CFT or by an IgG specific procedure such as ELISA. False-negative reactions occur especially in the early stages of acute infection.

**(b)Standard Tube Agglutination Test(SAT)[30]:**

This test is the most commonly used quantitative test for the diagnosis of Brucellosis. It was first performed by Wright in 1897. The serum (tube) agglutination test (SAT), or micro-titre plate variants of this, using heat/phenol-killed whole Smooth-cells, detects antibodies to the S-LPS. It measures the agglutinating antibodies IgG and IgM. The antigen used for this test is the smooth strain of *B. abortus* 99 procured from IVRI (Indian Veterinary Research Institute), Izzatnagar, U.P. Significant titres depend on the endemic titre. Usually patients of acute Brucellosis have titres of >160. However no single titre is diagnostic and all results have to be interpreted according to the epidemiology and clinical findings.

Disadvantages:

- The test does not distinguish between IgG and IgM antibodies.
- Also it is useful in acute cases and of not much use in chronic cases and relapses.
- Prozone phenomenon may give false negative reactions
- Lack of seroconversion in the early stages of the disease may give a negative result
- Cannot detect *B. canis* infection
- The IgM immunoglobulins may cross-react with *Yersinia enterocolitica O:9, Escherichia coli O:157, Francisella tularensis, Salmonella urbana O:30, Vibrio cholerae,* and others.

**(c) 2-mercapto-ethanol(2ME):**

This test is performed similar to SAT, except that in place of phenol saline, 2ME is used in a concentration of 0.05M. The 2ME inactivates the IgM antibodies by disrupting the disulphide bonds. The IgG antibodies are resistant to inactivation by 2ME and hence agglutinate.2ME test is a better indicator of recent infection than the SAT. Spink[56]has suggested that a positive 2ME test is evidence of an active infection and the need for antibiotic therapy. In a study by Buchanan[57]a more extensive evaluation of the 2ME test was done. About 15 to 25 sera were collected from 92 patients with Brucellosis during a 18 month period after their diagnosis and initiation of therapy. Each serum specimen was tested by the SAT and 2ME tests. The results indicate that the 2ME test is superior to the SAT in evaluating the effectiveness of treatment and also to rule out a diagnosis of chronic brucellosis.

**(d) Complement Fixation Test(CFT):**

The principle of the test is that IgG antibody fixes the complement well while IgM does not. In the early stages of Brucellosis when only IgM are present, CFT is usually negative. In acute brucellosis CFT titres appears in the 2nd month and reach the maximum at the 4th month of the disease. CFT is usually positive in sub- acute and chronic Brucellosis. High titres persist for about 12 months. Sera are diluted with doubling dilutions. The test is negative if 100% haemolysis appears in the serum with the lowest dilution[14].

**(e) Coombs antiglobulin test[58]:**

This test has a high diagnostic value. It is mainly used in retrospective studies. It helps in the detection of incomplete antibodies in the cases of chronic brucellosis. The titres of incomplete antibodies persist longer than the titres of complete antibodies. Coombs antiglobulin reaction is performed with sera which react negatively in Wright's agglutination reaction.

**(f) Competitive Enzyme Linked Immunosorbent Assay (CELISA):**

It is a method for the qualitative and quantitative detection of antibodies in the serum. The competitive enzyme immunoassay (CELISA) for the detection of serum antibody to *Brucella* is a multispecies assay[59]. The monoclonal antibody used in this assay is specific for a common epitope of smooth lipopolysaccharide (S-LPS). The most commonly used format of CELISA utilizes smooth LPS from *B. abortus* as antigen, passively attached to a polystyrene matrix[14]. In a study conducted by Araj et al, the specificity of ELISA varied from 98-99% and sensitivity from 94-98% for different immunoglobulins.

**(g) Fluorescence Polarization Assay (FPA):**

FPA was developed as a test that can be performed under field conditions allowing rapid and accurate diagnosis of Brucellosis in animals. The basis of the test is that a molecule in solution rotates randomly at a rate inversely proportional to its size. For diagnosis of Brucellosis, a fluorescence polarization analyzer is used to obtain a background measurement of the fluorescence of diluted serum. An antigen consisting of an OPS fragment prepared from *B.abortus* strain 1119- 3, approximately 22 k-Da in size, labelled with FITC(Fluorescein isothyocianate) is added and incubated for 2 min, followed by final reading. The result is presented in milipolarization units/mP[60]

To test the usefulness of this test in humans, a study conducted by Konstantinidis et al showed specificity of 96.1% and sensitivity of 93.5% for this test[61]. Another study by Lucero et al showed specificity of FPA to be 97.1% and sensitivity of 96.1%[62].

**III) Molecular Diagnosis:**

Genome of *Brucella:*

Molecular diagnosis of *Brucella* is based on the use of polymerase chain reaction (PCR) and real-time PCR methods. Nucleic acid amplification techniques, like PCR, characterized by high sensitivity and specificity and short turnaround time can overcome the limitations of conventional methodology. These methods are used for

a) Detection of *Brucella* in clinical samples,

b) Control of disease progress and

c) Pathogen typing.

The samples used for molecular diagnosis include blood, serum and tissues. Serum is the most preferred sample for the diagnosis of Brucellosis by PCR[63].The most commonly targeted genes are 16S and 23SrRNA, omp25, IS711 and BSCP31.

***16SrRNA:***

The 16S rRNA in most *Brucella* species is highly conserved and, as a distinct marker of the species, enables rapid and accurate diagnosis of Brucellosis without the need of routine diagnostic methods[64]. In a study by Gee et al,[65] *Brucella* strains along with 17 related strains were tested for the 16SrRNA gene sequence. All *Brucella* 16S rRNA gene sequences were determined to be identical and were clearly different from the 17 related strains, suggesting that 16S rRNA gene sequencing is a reliable tool for rapid genus-level identification of *Brucella* spp. and their differentiation from closely related organisms[65].

***23SrRNA:***

The *23SrRNA* operon is also quite specific for *Brucella* spp. and is sequenced by PCR.

***IS711:***

The *Brucella* genome contains an insertion sequence (IS) element called IS*711* or IS*6501*, which is specific to the genus. The copy number of IS*711* varies in the genome of the different *Brucella* species, ranging from 7 in *B. abortus, B. melitensis* and *B. suis*to more than 30 in *B. ovis* and in *Brucella* strains isolated from marine mammals[66].

***OMP2:***

*OMP2* is a gene encoding for a major cell envelope protein in *Brucella*. A DNA sequence analysis of the *OMP*2 locus of *Brucella* type strains revealed nucleotide differences that can be used for species identification[67].

***BSCP31:***

This is the most commonly targeted gene. It is a 31 K-Dalton, *B.abortus* antigen.

**Treatment:**

The essential element in the treatment of all forms of human Brucellosis is the use of effective antibiotics for an appropriate length of time. Antibiotic treatment should be started as early as possible, even in patients who show a spontaneous improvement. In patients with complications, additional treatment, including surgical intervention, may be necessary.

Uncomplicated acute Brucellosis invariably responds well to adequate and appropriate antibiotic treatment.

A variety of antimicrobial drugs have shown good activity in vitro against *Brucella* species; But the results of routine susceptibility tests do not always correlate with clinical efficacy. Hence beta-lactam antibiotics, such as penicillins and cephalosporins, and macrolide antibiotics, such as erythromycin, are associated with unacceptably high rates of relapse when used to treat patients with Brucellosis. Although newer macrolides, such as azithromycin and clarithromycin are more active in vitro than erythromycin, they have not shown superiority over current regimens for

treatment of patients with brucellosis, and their role in therapy remains to be determined.

**Treatment regimen:**

The treatment of Brucellosis according to WHO standard regimen[68] is as follows

1) Treatment in uncomplicated cases in adults and children above 8years of age

Tetracycline (500mg 6$^{th}$ hourly for 6 weeks) has been the standard regimen for the treatment of human Brucellosis. Doxycyline (an analogue of Tetracycline) is now the drug of choice as it is given once or twice daily and also has fewer gastrointestinal side effects. It is given in a dose of 100mg 12$^{th}$ hourly for 6 weeks.

Single drug therapy is known to have a relapse rate of 10-20%. Hence it is recommended to add an aminoglycoside along with the Doxycycline in the first few weeks of therapy. Streptomycin 1gm daily intramuscularly for the first 2-3 weeks is added to the regimen. The synergestic effect of these 2 drugs is more bactericidal to *Brucella* spp. than either of the drugs used alone.

2) Principal Alternative Therapy.

Rifampicin is active in vitro against *Brucella spp.* It is used in a combination regimen of Doxycycline (200mg once daily orally) and Rifampicin (600-900mg daily orally) for 6 weeks. Its efficacy has been found to be equal to the standard regimen of Doxycycline and Streptomycin.

3) Secondary Alternative Therapy:

- Fluoroquinolones:

  Fluoroquinolones have a high activity in vitro against *Brucella* species. They are well absorbed orally and achieve high concentrations within phagocytic cells. However bactericidal activity at intracellular pH is low and when used as monotherapy relapse rate is high. Hence quinolones should always be used in combination with doxycycline or rifampicin.

- Trimethoprim-sulfamethoxazole (TMP-SMZ/Co-trimoxazole): TMP-SMZ in a fixed ratio of 1:5 (80 mg TMP/400 mg SMZ) is active in vitro against *Brucella* species. But due to high rates of relapse it is used in combination with doxycycline, rifampicin or streptomycin.

4) Treatment of complications of Brucellosis like Spondylitis and Neurobrucellosis, Brucellosis in pregnancy and Brucellosis in children of age less than 8 years require special consideration with regard to the choice of antibiotics and duration of treatment.

# MATERIAL AND METHODS

**Source of Data**: All serum samples received in the serology section of Microbiology department, Jawaharlal Nehru Medical College, Belgaum, positive for Brucellosis by Rose Bengal Card Test.

**Study Type**: Cross Sectional Study

**Sample Size**: Sample size(n) is calculated using following formula-

$n=4\times z^2\times p\times q/d^2$ where

n-Sample size

p- Sensitivity of PCR(97%)

q- 100-p (100-97)

d-absolute error which is 10%

z-1.96~2

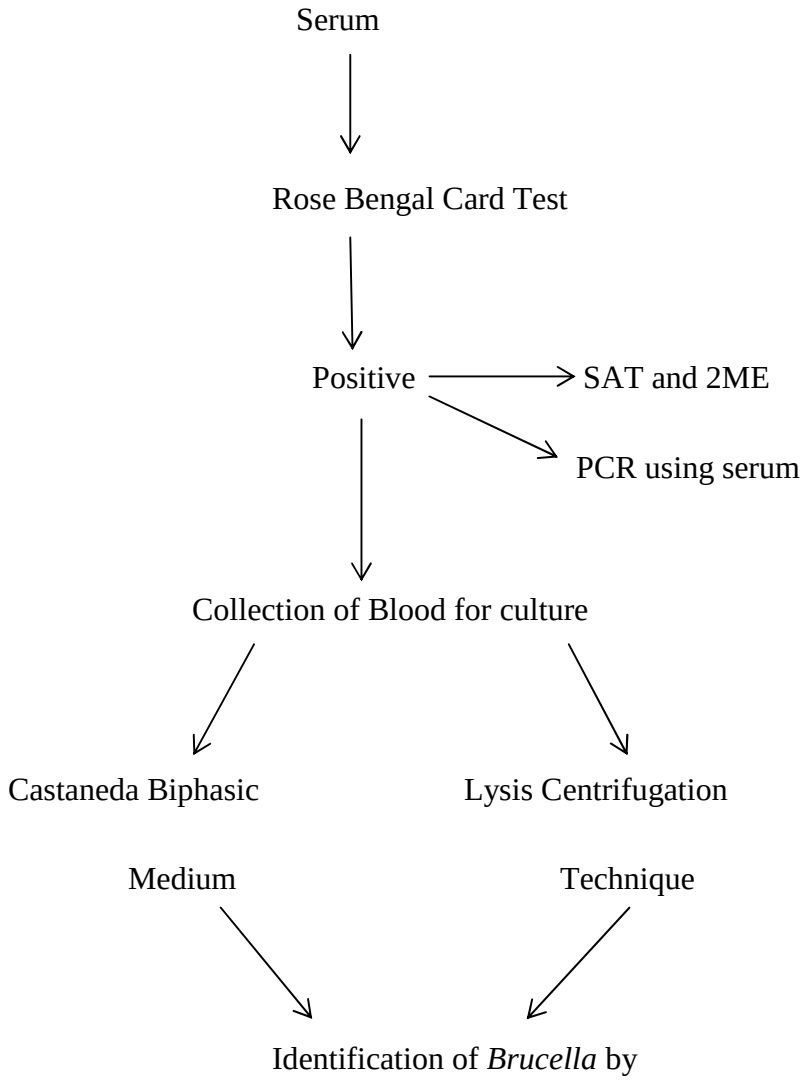
Hence sample size is $n=4\times(2)^2\times97\times3/(10)^2$

$n=46.46\sim50$

n=50

**Inclusion criteria**: All serum samples positive for Brucellosis by slide agglutination test using Rose Bengal card test.

**Methodology:**




1) Colony Morphology
2) Gram Stain
3) Modified ZN Stain
4) Biochemical Characteristics
    a) Urease Test
    b) Catalase Test
    c) Oxidase Test
    d) $H_2S$ Production
    e) $CO_2$Requirement

**Rose Bengal Plate Test:**

Rose Bengal Plate Test is performed with Rose Bengal Stain obtained from the Biological Products Division, Indian Veterinary Research Institute, Izzatnagar, U.P, India.

Rose Bengal antigen is a concentrated cell suspension of smooth suspension of *Br. abortus* (strain 99). The cells are stained with Rose Bengal stain and suspended in buffered *Brucella* antigen diluents (pH 3.65+0.05) and is stored at 4°C.

**Procedure**: On a clean ceramic tile one drop (30µl) of serum to be tested and one drop(30µl) of the Rose Bengal antigen are added side by side. The antigen and the serum are mixed thoroughly and spread over a circle of 2.5cm. The tile is then gently rocked for 4min.

**Positive Control**: A known seropositive sample of Brucellosis

**Negative Control**: Serum sample from healthy control.

**Interpretation of Result**: The result is interpreted as positive if there is visible agglutination in both the test sample and the positive control with no agglutination in the negative control. Agglutination in the positive control, with no agglutination in the test sample and the negative control is interpreted as negative.

**Standard Tube Agglutination Test (SAT)[26]:**

It is a quantitative test for IgG and IgM. It uses plain *Brucella abortus* antigen procured from the Division of Biological Products, Indian Veterinary Research Institute(IVRI), Izzatnagar, Uttar Pradesh, India. The antigen is a suspension of pure smooth culture of *Brucella abortus* strain 99 in phenol saline. It is stored at $4^{o}$C.

**Reagents and Material Required**:

- Phenol saline(Normal saline containing 0.5% phenol)
- *Brucella abortus 99* antigen
- Kahn tubes
- Measuring pipettes(1ml capacity)

**Procedure**: 10 Kahn tubes were kept in a rack. To the 1st tube, 0.8ml of phenol saline and to the remaining 9 tubes 0.5ml of phenol saline was added. Then to the first tube 0.2ml of the test serum was added and was mixed thoroughly. Then 0.5 ml of this mixture was transferred to the 2nd tube containing 0.5ml of phenol saline , mixed well and 0.5ml was transferred to the 3rdtube. This doubling dilution was continued till the last tube and 0.5ml from the 10th tube was discarded. This gave dilutions of 1:5, 1:10, 1:20 and so on. Then to each tube, 0.5ml of the *Brucella abortus* plain antigen was added and mixed well. This gave a final dilution of 1:10, 1:20, 1:40 and so on.

A set of antigen control tubes were set up to compare the test samples.

| Antigen Control Tubes | 0.5%Phenol saline(ml) | *B.abortus* antigen(ml) | Degree of agglutination |
|---|---|---|---|
| **1** | Nil | 2.0 | Nil |
| **2** | 1.25 | 0.75 | 25% |
| **3** | 1.5 | 0.5 | 50% |
| **4** | 1.75 | 0.25 | 75% |
| **5** | 2.0 | Nil | 100% |

All the tubes were incubated at $37^{o}$ C for 48 hours.

**Observation and Interpretation**: The tubes were kept at room temperature for 1 hour and then interpreted. The tubes were examined with a light source against a dark background. The highest serum dilution showing 50% agglutination was considered as the end point. The titres were converted into International Units(I.U) as per the joint FAO/WHO committee. Titres of ≥80I.U/ml (1:40) were considered as significant.

**2-Mercaptoethanol test**:

The 2ME test was performed similar to the SAT, except for the addition of 2ME instead of phenol saline to a final concentration of 0.05M in each tube. The results were expressed in titres. The 2ME test breaks the disulphide bonds of IgM and helps to distinguish acute and chronic Brucellosis.

**Culture:**

A total of 10ml of blood was collected under aseptic precautions

**1) Castaneda Biphasic Medium:** 5 ml of blood was drawn under aseptic precautions and inoculated into the BHI broth phase of the Castaneda Biphasic Medium containing BHI agar and broth; the contents were mixed well and the mixture was tipped daily to allow the blood-broth mixture to flow over the agar slant. The bottle was then incubated in the upright position in the incubator at $37^0$C in the presence of 10% $CO_2$ and examined at 48 hour intervals. The colonies that appeared on the solid media were identified with the help of morphology on staining and biochemical reactions. If no colonies were observed the bottles were tilted and re-incubated, the cycle was repeated for at least 30 days to report culture as negative

**2) Lysis Centrifugation Technique:**

5ml venous blood+20ml sterile distilled water +1.5ml of4% Sodium citrate

↓

Centrifugation at 2000×g for 30 min

↓

Inoculation of sediment onto Brain Heart Infusion medium and incubation at 37˚Cin the presence of 10%$CO_2$

**Identification of Isolates:**

The culture isolates were identified by

1**) Colony Morphology**: Colonies of *Brucella* were circular, convex and greyish translucent measuring 1-2mm in diameter

2) **Gram Staining**: On gram staining *Brucella* appeared as gram negative coccobacilli measuring 0.5-0.7µ by 0.8-1.0µ .

3) **Modified ZN Staining**: On modified ZN staining using 1%$H_2SO_4$ as decolouriser, *Brucella* appeared as weakly acid fast (red) coccobacilli.

4) **Biochemical Reactions**:

a. Catalase Test: *Brucella* were catalase positive using 3% $H_2O_2$

b. Oxidase Test: *Brucella* were oxidase positive using N,N Tetramethyl para Phenylene Diamine Dihydrochloride

c. Urease Test: *Brucella* gave a positive urease test in 15-20minutes with Christensen's Urea Medium .

d. $H_2S$ production: A lead acetate paper was inserted into the bottle containing BHI agar slant inoculated with the test culture and observed for $H_2S$ production.

e. $CO_2$ Requirement: The test isolates were subcultured on BHI agar in duplicates and tested for $CO_2$ requirement by incubating one plate under 10%$CO_2$ and the other without $CO_2$.

**Polymerase Chain Reaction**:

**Gene Targeted**: IS711 element of *Brucell spp.*

**Primer Sequences:**

Forward Primer- AAC AAG CGG CAC CCC TAA AA

Reverse Primer- CAT GCG CTA TGA TCT GGT TACG

**Positive Control:** A known strain of *Brucella melitensis*

**Negative Control:** PCR master mix consisting of 2µl of 1X Taq bufferA,0.3µl of 1Unit of Taq polymerase, 0.2µl of 2mMdNTP and 14.5µl of molecular graded water.

**Molecular Weight Marker:** 100bp size

All reagents were obtained from Bangalore Genei (Lot No. 1562083 612115600031730)

**Protocol for DNA extraction from serum samples:**

**Lysis:**

1) 200µl of serum + 50µl of Proteinase K +200µl of Lysis buffer
2) Vortexed for10sec
3) Incubated at 70$^{o}$C for 15minutes with vortexing once during incubation
4) Added 4µl of RNAse A (100mg/ml), vortexed briefly and incubated at room temperature for 5minutes.
5) Added 210µl of absolute ethanol and vortexed again
6) One Genei Pure column was placed in a 2ml collection tube and was loaded with the sample ethanol mixture
7) Centrifuged at 11000 rpm for 1min and collection tube with flow through was discarded

**Wash**:

8) 1 volume of wash buffer I(125µl) was diluted with 3 volumes of ethanol(375µl) and mixed thoroughly.
9) The Genei Pure column was placed in a fresh 2ml collection tube and 500µl of wash buffer I was added to it.

10) Column was centrifuged at 11000rpm for 1min and collection tube with flow through was discarded

11) The column was placed in a fresh collection tube and 500µl of wash buffer II (125µl wash buffer II diluted in 375µl of absolute ethanol) was added to it and centrifuged at 11000rpm for 1 min.

12) The flow through was discarded and the column was again centrifuged at 11000 rpm for 1 min in the same collection tube to remove traces of wash buffer.

13) The Genei Pure column was placed in a fresh 1.5ml vial and incubated at 70$^{o}$C for 2min in a dry bath for complete removal of ethanol.

**Elution:**

14) 100µl of elution buffer was prewarmed in a dry bath set at 70$^{o}$C for 5 min.

15) The column was placed in a fresh sterile 1.5ml vial and 100µl of the prewarmed elution buffer was added to the centre of the Genei Pure column

16) It was incubated at room temperature for 1min and then centrifuged at 11000 rpm for 1min.

17) The eluted DNA was stored at -20$^{O}$C.

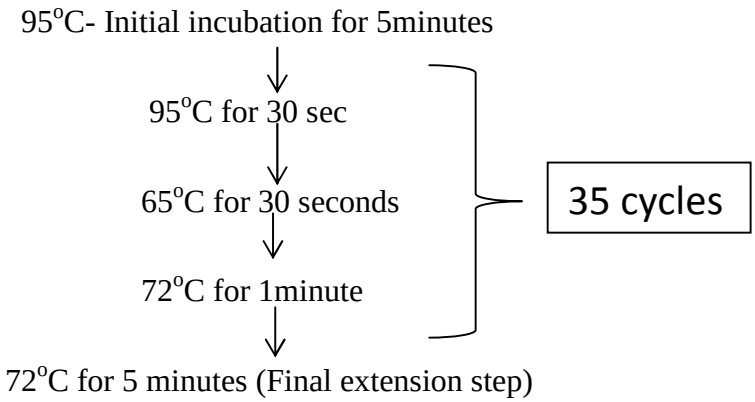
**DNA Extraction from known strain of *Brucella melitensis*:**

DNA extract for use as positive control was prepared by boiling a loopful of growth culture of known strain of *B.melitensis* grown on BHI agar in 0.5ml of TE buffer for 5min. The suspension was then centrifuged at 11000 rpm for 1 min. The supernatant was collected as DNA extract.

**PCR Protocol:**

PCR mixture consists of 17µl of PCR master mix, 1µl of template DNA (100ng) and 1µl each of forward and reverse primer(5pm). Total PCR master mix-20µl.

**PCR cycling conditions**:




**Detection of Amplicon:**

After DNA amplification, the amplified products along with positive and negative controls and the molecular weight ladder, were run on 1.5% agarose gel electrophoresis in the presence of ethidium bromide. The amplified products were read on gel documentation system.

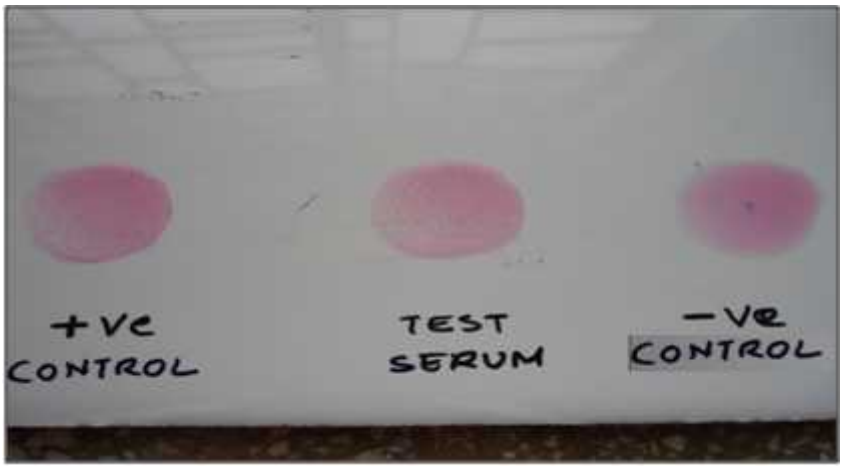



**Figure 1: Rose Bengal Card Test**

**Figure 2: Standard Tube Agglutination Test**

**Figure 3: Standard Tube Agglutination Test Showing Agglutination**

**Figure 4: Centrifugation Machine**


**Figure 5: Lysis Centrifugation Tube**

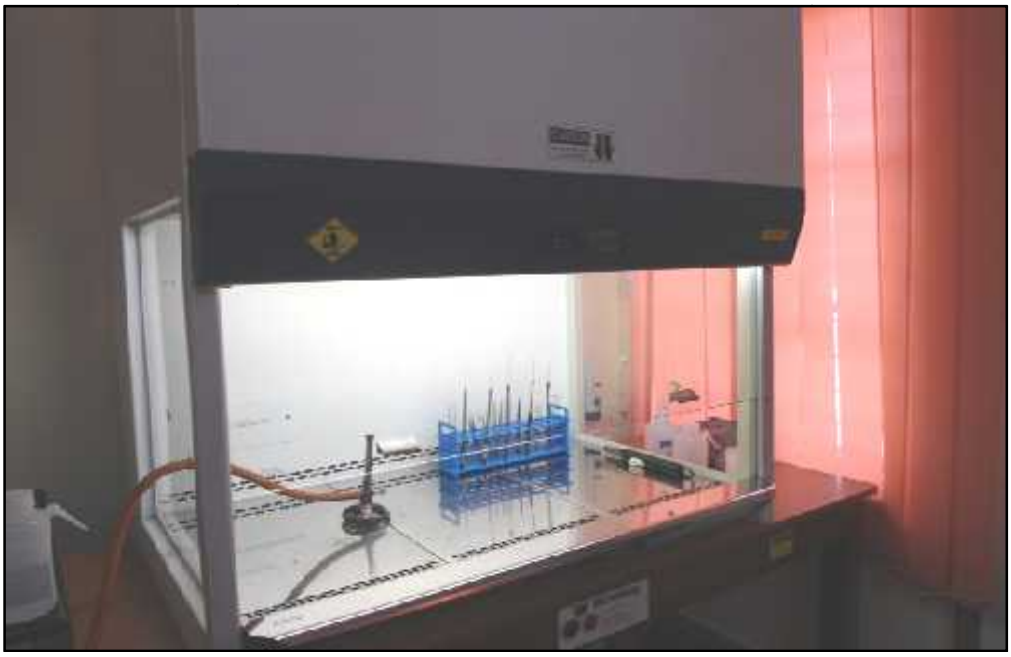


**Figure 6: Biosafety Cabinet**

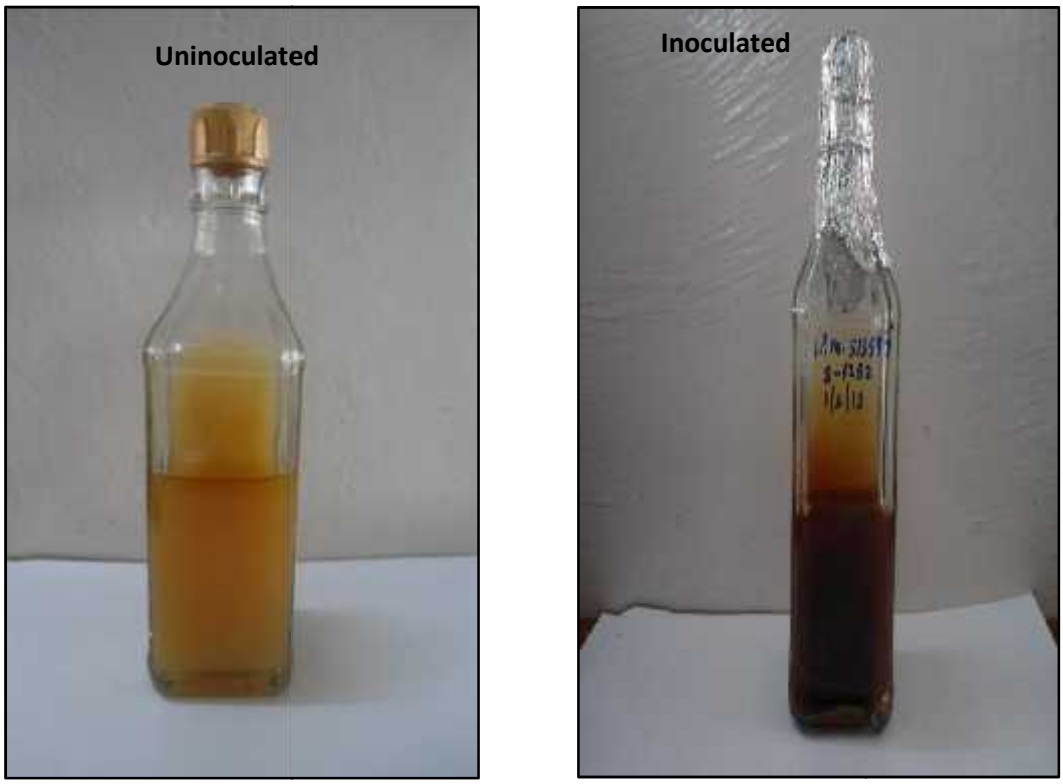



**Figure 7: Castaneda Biphasic Medium**

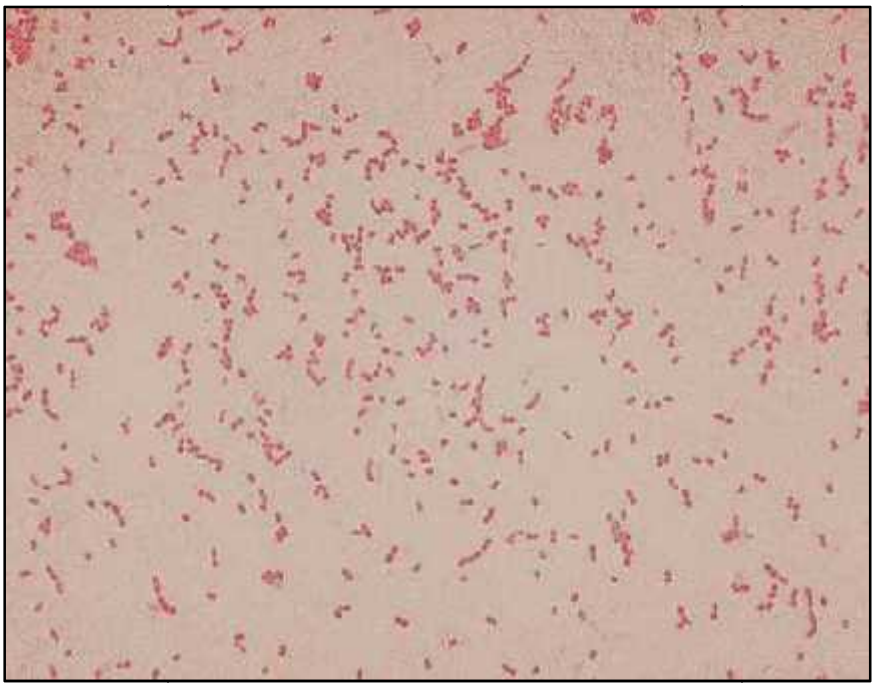


**Figure 8: Gram Staining of *Brucella***


**Figure 9: Urease Test for *Brucella***

**Figure 10: $H_2S$ Production**


**Figure 11: Oxidase Test**

**Figure 12: Thermalcycler**

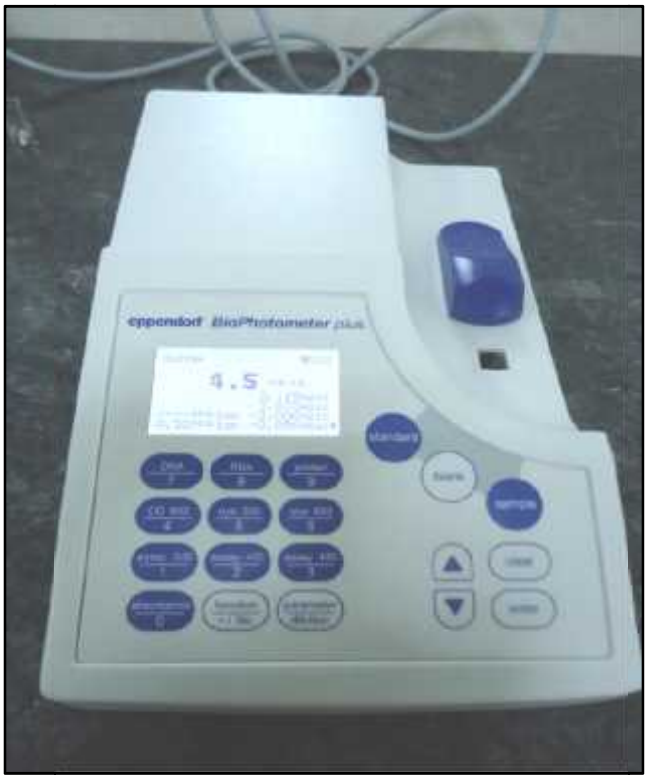



**Figure 13: Biophotometer**


**Figure 14: Electrophoresis**

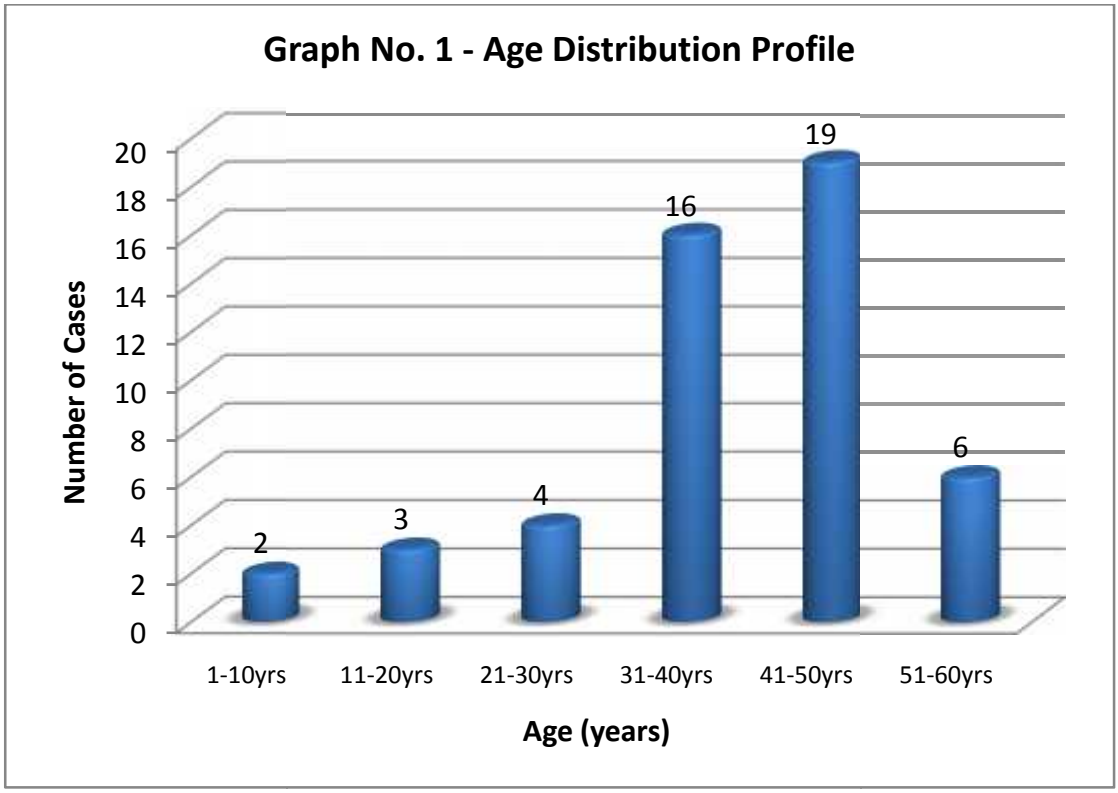
**Figure 15: U.V Transilluminator**

**Figure 16: Gel Documentation System**

# RESULTS

**Table 1: Age Wise Distribution of cases**

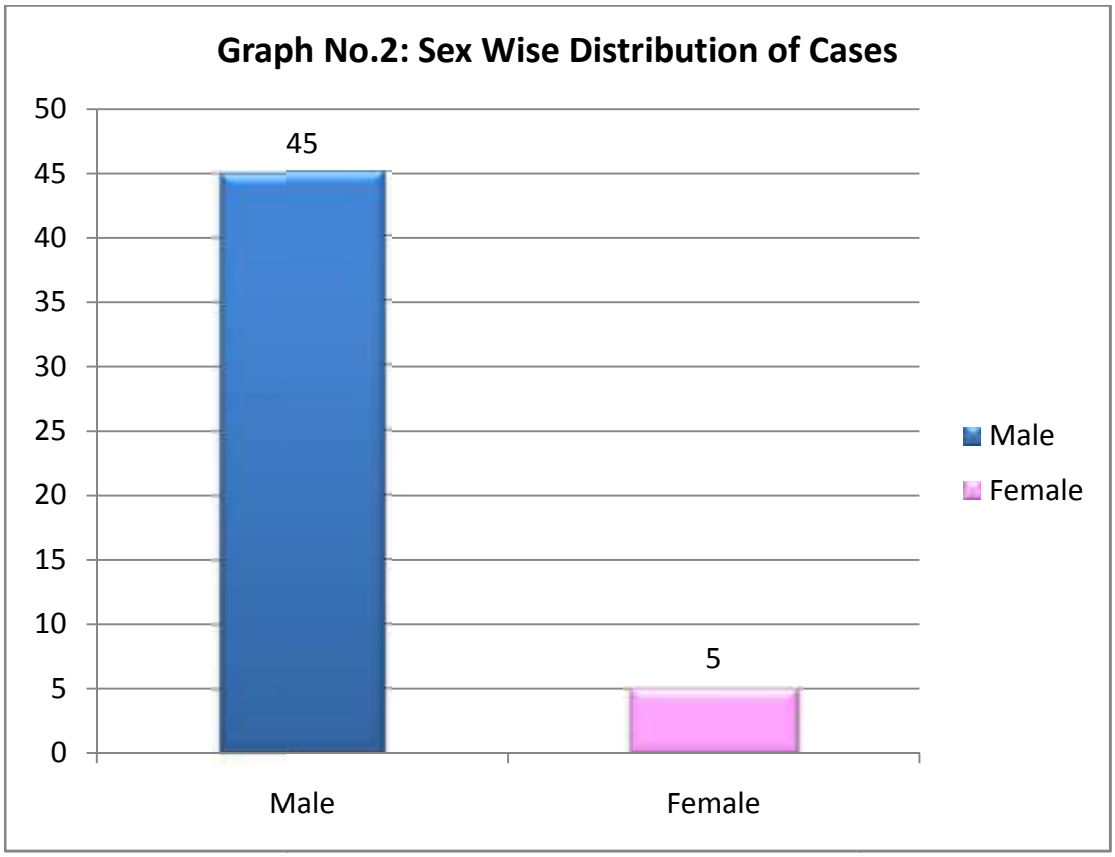
| Age(yrs) | Number of Cases |
|---|---|
| 1-10 | 2 |
| 11-20 | 3 |
| 21-30 | 4 |
| 31-40 | 16 |
| 41-50 | 19 |
| 51-60 | 6 |
| Total | 50 |




Maximum number of cases were in the age group of 31-50 yrs which is the age group usually involved in agricultural and veterinary work.

**Table 2: Sex Wise Distribution of Cases**

| | Male | Female |
|---|---|---|
| Number | 45 | 5 |
| Percentage | 90% | 10% |

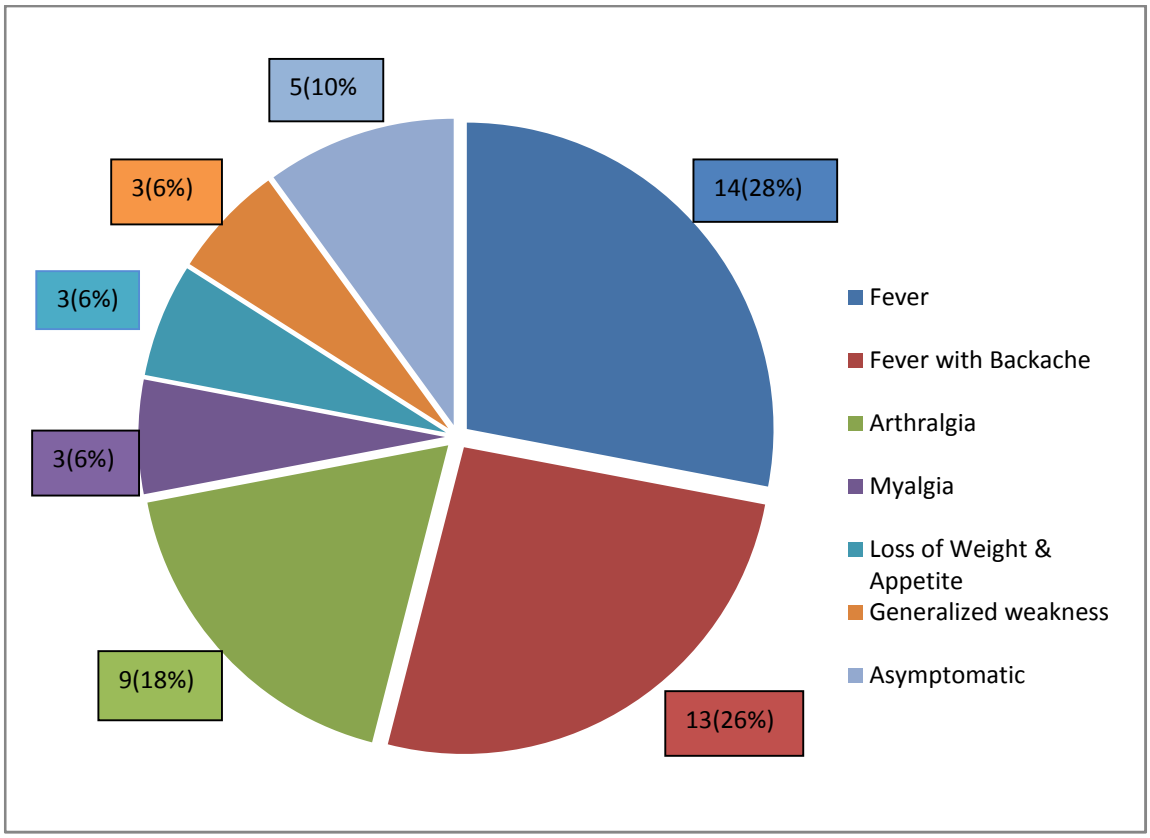



The male to female ratio was found to be 9:1.

**Table 3: Clinical Profile of Cases**

| Clinical Features | No. of Cases | Percentage |
|---|---|---|
| Fever | 1 4 | 28 |
| Fever with backache | 13 | 26 |
| Arthralgia | 9 | 18 |
| Myalgia | 3 | 6 |
| Loss of appetite & weight | 3 | 6 |
| Generalised weakness | 3 | 6 |
| Asymptomatic | 5 | 10 |

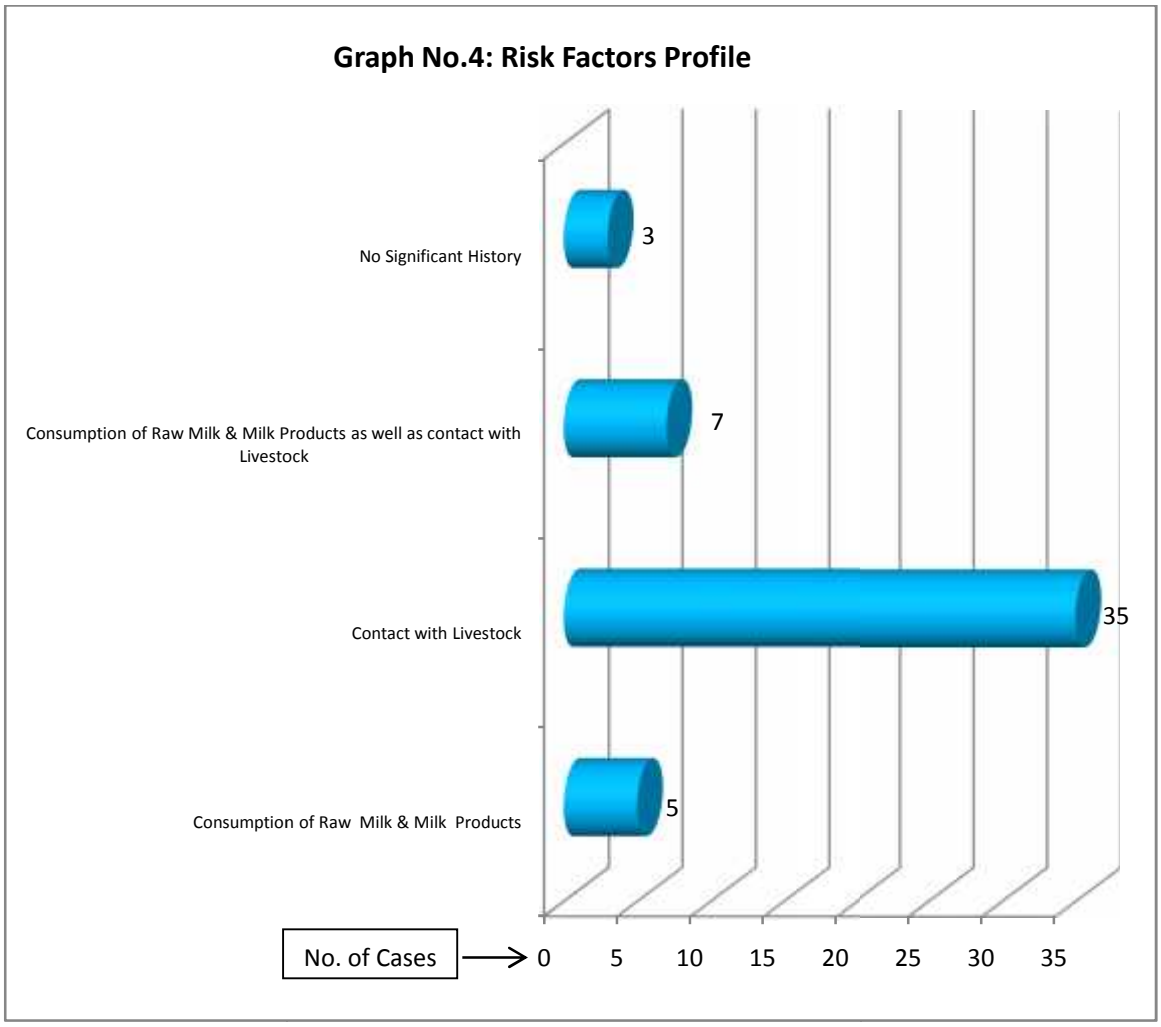
**Graph No. 3: Clinical Profile of Cases**




Fever was the main symptom in 14 cases (28%) while fever with backache was also equally common with 13 cases (26%). Arthralgia commonly involved the sacroiliac and knee joints.

**Table 4: Risk Factors Profile of Cases**

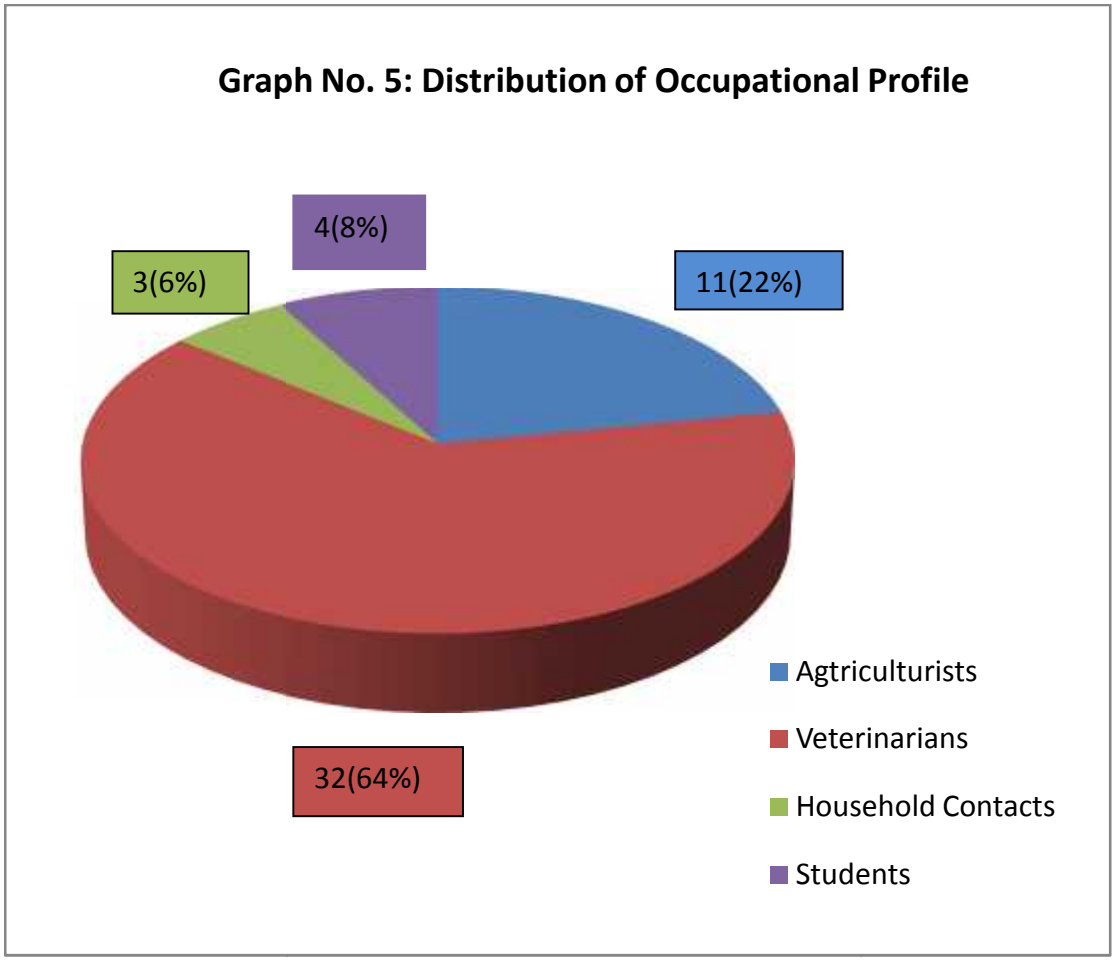
| No. of Patients | Consumption of raw milk and milk products | Contact with Livestock | Consumption of raw milk and milk products/ Contact with Livestock | No Significant History |
|---|---|---|---|---|
| 50 | 05 | 35 | 07 | 03 |




Majority of patients gave history of contact with livestock (70%), while 10% gave history of consumption of raw milk and milk products. There were 3 cases with no significant history.

**Table 5: Occupational Profile**

| Occupation | No. of Patients |
|---|---|
| Agriculturists | 11 |
| Veterinarians | 32 |
| Household Contacts | 3 |
| Students | 4 |




More than 80% of the cases were in the high risk occupational group of veterinary work and agriculture which pose a high risk of contracting the disease.

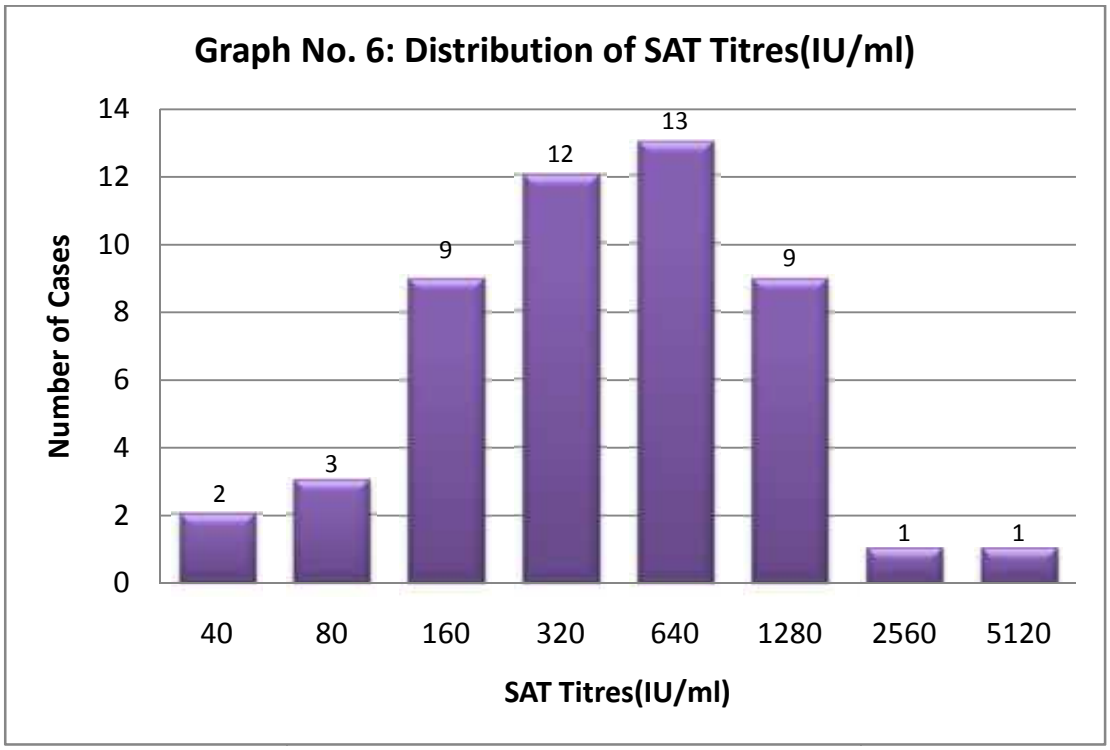
**Table 6: SAT and 2ME Titres**

| SL. No. | Lab No. | SAT(I.U/ml) | 2ME |
|---|---|---|---|
| 1 | S-686-12 | 1280 | 1:40 |
| 2 | S-2976-12 | 640 | 1:320 |
| 3 | S-3412-12 | 640 | 1:40 |
| 4 | S-5803-12 | 320 | 1:40 |
| 5 | S-6442-12 | 160 | 1:40 |
| 6 | S-7019-12 | 1280 | 1:40 |
| 7 | S-8034-12 | 320 | 1:80 |
| 8 | S-8564-12 | 160 | 1:40 |
| 9 | S-8608-12 | 640 | 1:20 |
| 10 | S-8609-12 | 1280 | 1:20 |
| 11 | S-8610-12 | 320 | 1:80 |
| 12 | S-8611-12 | 640 | 1:20 |
| 13 | S-9480-12 | 160 | Neg |
| 14 | S-9979-12 | 320 | 1:40 |
| 15 | S-9980-12 | 640 | 1:40 |
| 16 | S-9981-12 | 160 | Neg |
| 17 | S-9982-12 | 320 | 1:40 |
| 18 | S-9983-12 | 320 | 1:20 |
| 19 | S-9984-12 | 80 | 1:20 |
| 20 | S-9986-12 | 2560 | 1:80 |
| 21 | S-11338-12 | 1280 | 1:20 |
| 22 | S-11532-12 | 640 | 1:160 |
| 23 | S-11691-12 | 1280 | 1:20 |
| 24 | S-12255-12 | 640 | 1:20 |
| 25 | S-12333-12 | 5120 | 1:160 |

| | | | |
|---|---|---|---|
| 26 | S-12468-12 | 640 | 1:80 |
| 27 | S-1252-13 | 1280 | 1:40 |
| 28 | S-1747-13 | 1280 | 1:80 |
| 29 | S-2012-13 | 640 | 1:20 |
| 30 | S-2017-13 | 160 | 1:40 |
| 31 | S-2021-13 | 160 | 1:80 |
| 32 | S-2022-13 | 640 | Neg |
| 33 | S-2023-13 | 80 | 1:20 |
| 34 | S-2025-13 | 40 | 1:20 |
| 35 | S-2026-13 | 160 | 1:40 |
| 36 | S-2028-13 | 320 | 1:20 |
| 37 | S-2030-13 | 320 | 1:40 |
| 38 | S-2031-13 | 640 | 1:20 |
| 39 | S-2032-13 | 160 | 1:20 |
| 40 | S-2035-13 | 640 | 1:20 |
| 41 | S-2036-13 | 320 | 1:40 |
| 42 | S-2040-13 | 40 | Neg |
| 43 | S-2044-13 | 1280 | 1:40 |
| 44 | S-2053-13 | 160 | 1:20 |
| 45 | S-2055-13 | 320 | 1:40 |
| 46 | S-2314-13 | 320 | 1:40 |
| 47 | S-2325-13 | 640 | 1:20 |
| 48 | S-2826-13 | 320 | 1:80 |
| 49 | S-2829-13 | 80 | 1:20 |
| 50 | S-3336-13 | 1280 | 1:20 |

**Table 7: Distribution of Significant SAT Titres**

| SAT Titres(IU/ml) | No. of Cases |
|---|---|
| 40 | 2 |
| 80 | 3 |
| 160 | 9 |
| 320 | 12 |
| 640 | 13 |
| 1280 | 9 |
| 2560 | 1 |
| 5120 | 1 |




Majority of the cases had significant SAT titres between 160 IU/ml and 1280 IU/ml.

**Table 8: Blood Culture Positivity**

| S.L. No. | I.P. No. | Castaneda Biphasic Medium | Day of culture positivity | Lysis Centrifugation Technique | Day of culture positivity | SAT Titre (I.U/ml) | 2ME Titre |
|---|---|---|---|---|---|---|---|
| 1 | S-686-12 | Positive | 5 | Positive | 4 | 1280 | 1:40 |
| 2 | S-3412-12 | Negative | -- | Positive | 3 | 640 | 1:40 |
| 3 | S-7019-12 | Negative | -- | Positive | 5 | 1280 | 1:40 |
| 4 | S-8608-12 | Negative | -- | Positive | 4 | 640 | 1:20 |
| 5 | S-8609-12 | Positive | 6 | Positive | 2 | 1280 | 1:20 |
| 6 | S-8611-12 | Negative | -- | Positive | 3 | 640 | 1:20 |
| 7 | S-9980-12 | Negative | -- | Positive | 4 | 640 | 1:40 |
| 8 | S-9986-12 | Positive | 5 | Positive | 3 | 2560 | 1:80 |
| 9 | S-11338-12 | Positive | 8 | Positive | 2 | 1280 | 1:20 |
| 10 | S-11691-12 | Negative | -- | Positive | 4 | 1280 | 1:40 |
| 11 | S-12255-12 | Positive | 6 | Positive | 3 | 640 | 1:20 |
| 12 | S-12333-12 | Positive | 4 | Positive | 4 | 5120 | 1:160 |
| 13 | S-1252-13 | Positive | 9 | Positive | 3 | 1280 | 1:40 |
| 14 | S-1747-13 | Negative | -- | Positive | 4 | 1280 | 1:80 |
| 15 | S-2012-13 | Negative | -- | Positive | 3 | 640 | 1:20 |
| 16 | S- 2022-13 | Negative | -- | Positive | 5 | 640 | Neg |
| 17 | S- 2031-13 | Negative | - | Positive | 2 | 640 | 1:20 |
| 18 | S- 2035-13 | Positive | 7 | Positive | 3 | 640 | 1:20 |
| 19 | S- 2044-13 | Positive | 5 | Positive | 3 | 1280 | 1:40 |
| 20 | S-2325-13 | Negative | -- | Positive | 4 | 640 | 1:20 |
| 21 | S-3336-13 | Positive | 6 | Positive | 2 | 1280 | 1:20 |

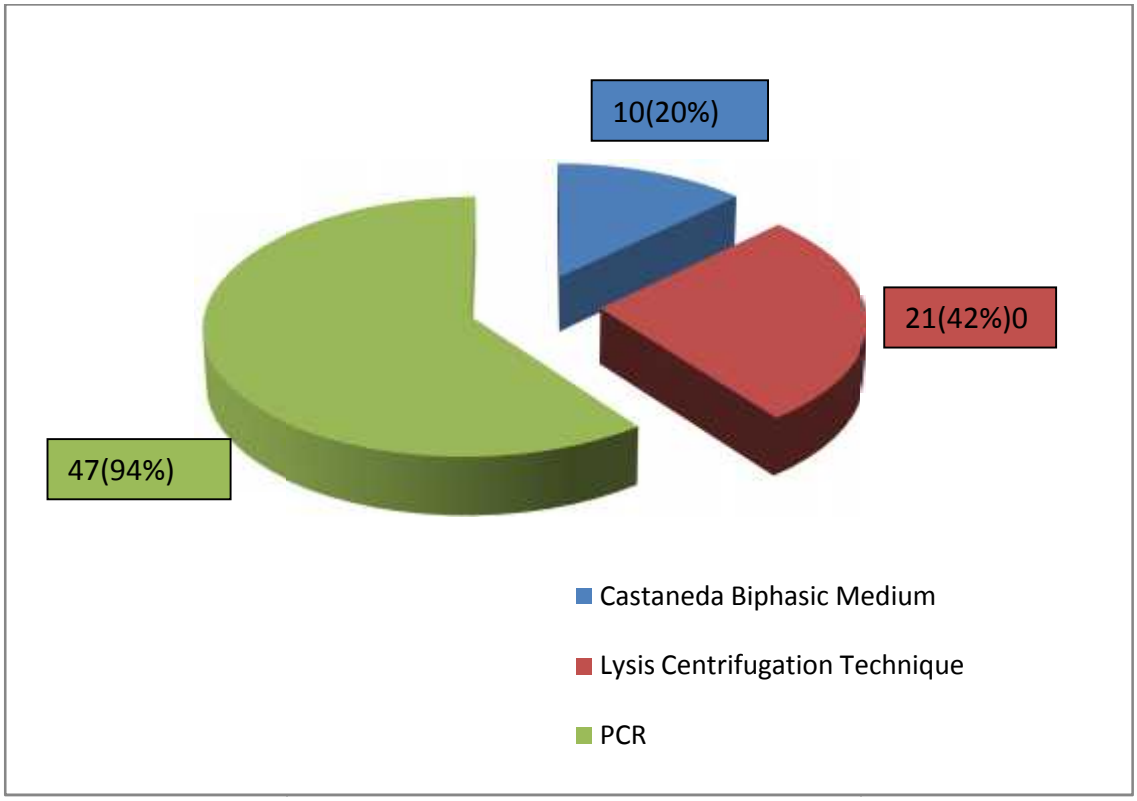
Blood culture by Conventional Castaneda Biphasic Medium was positive in 10 cases while the culture was positive in 21 cases by the Lysis Centrifugation Technique. All the cultured cases positive by Conventional Castaneda Biphasic Medium were positive by Lysis Centrifugation Technique also.

The mean duration of culture positivity by Conventional method was 6 days while the mean duration of culture positivity by Lysis Centrifugation was 3 days

**Table 9: Comparison of Blood Culture Methods and PCR**

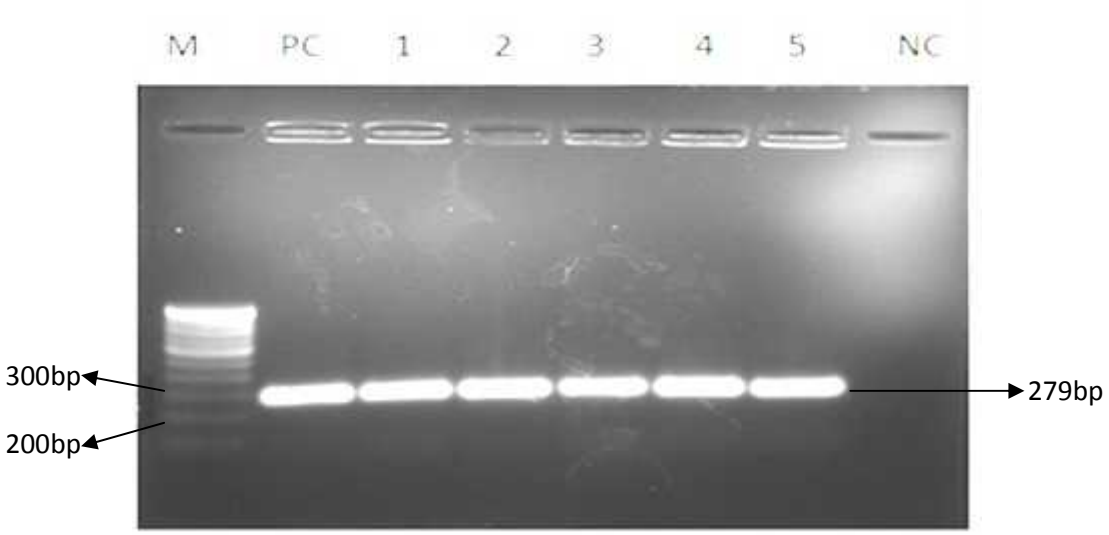
| No. of cases positive by Rose Bengal Card Test | No. of Case with Significant SAT titres | No. of Cases positive by Castaneda Biphasic Medium | No. of Cases positive by Lysis Centrifugation Technique | No. of Cases positive by PCR |
|---|---|---|---|---|
| 50 | 45 | 10 | 21 | 47 |

**Graph No.7: Comparison of Blood Culture Methods and PCR**




The sensitivity of the Castaneda Biphasic Medium compared to the Lysis Centrifugation Technique[65] was found to be 100% while specificity was found to be 47.6%.

The Sensitivity of PCR compared to the Lysis Centrifugation Technique was 100%.




M – 100bp ladder

PC – Positive control

1

2

3

4

5

Samples

NC - Negative control

**Result Summary:**

- Total number of cases screened -1545
- Total number of cases positive by Rose Bengal Plate Test -50
- Total number of positive blood cultures by Castaneda Biphasic Medium- 10
- Total number of positive blood cultures by Lysis Centrifugation Technique – 21
- Mean No. of Days for Positive Blood Culture in Castaneda Biphasic Medium – 6
- Mean No. of Days for Positive Blood Culture by Lysis Centrifugation Method – 3
- Total number of cases positive by PCR -47

# DISCUSSION

Human Brucellosis being a zoonotic disease has a wide spectrum of clinical manifestations. The spectrum ranges from an asymptomatic state to complications affecting various organ systems in the body like hepato-spleenomegaly, septic arthritis, endocarditis, pneumonia, orchitis, epididymitis, neurobrucellosis causing meningitis and meningoencephalitis, etc.

Though it is an endemic disease in India, there is a gross lacuna with regard to the awareness about the disease, its transmission and prevention. Also clinicians do not maintain a high degree of suspicion, especially in non- endemic areas.

Serological techniques are the most common methods of diagnosis of human Brucellosis. But they lack specificity and titres may not distinguish between acute and chronic cases especially in endemic areas. Culture being positive in only 30-50% of cases and due to the longer duration of incubation required cannot be relied upon. Also in chronic cases and focal complications it is very difficult to isolate the organism. Lysis centrifugation has been shown to increase the culture yield by about 25% and also the duration of incubation required is less. Molecular diagnosis is highly sensitive but may not be available in all laboratory setups.

An effort was made in this study to evaluate the Conventional Castaneda Biphasic Medium and Lysis Centrifugation Technique – the two blood culture methods and PCR in diagnosis of human Brucellosis.

**Age and Sex**:

Although wide variation in the age group was seen in individuals with Brucellosis, the majority of patients were in the age group of 30-50 years(64%). This is the age group which is actively involved in agricultural activities and maintenance of livestock and thus are at risk of contracting the disease. In a study done by Elbeltagy KE[69] the mean age of prevalence of Brucellosis was found to be 33.8% years. In this study Brucellosis was found to be less prevalent over the age of 60yrs.

The male to female ratio in this study was found to be 9:1 and majority (3) of the females were in the age group of 41-50. The differences in gender distribution are due to the differences in the work pattern leading to exposure to the risk factors. Male predominance has also been reported by many other authors[70]. There was no significant difference in the SAT titres according to the age or sex.

**Occupational Profile**:

Majority of the patients in this study were involved in veterinary work which is one of the risk factors for acquiring the infection. Also a large number of patients were agriculturists with maintenance of livestock which is again a risk factor. There were 4 children below the age of 18 years with a history of consumption of raw milk and milk products. Out of the 5 female patients, 4 were housewives who handled the livestock at home and 3 of them gave a history of consumption of raw milk.

Thus the known risk factors for transmission of Brucellosis i.e contact with infected animals and their birth products and consumption of raw milk and milk products were present in most of the cases. Contact with infected animals and their products was thus found to be the major risk factor for Brucellosis followed by

consumption of raw milk and milk products which has been reported by other authors[71].

**Clinical Profile**:

Majority of the patients presented with on and off fever of more than 15 days. 14 out of 50 cases (28%) came with a history of fever. These included the 5 paediatric patients. 13 out of 50 cases (26%) presented with fever along with backache as the chief complaints. In majority of the cases the backache was severe enough to restrict the activity of the patient. The other chief complaint with which the patients presented were arthralgia mainly involving the sacroiliac and knee joints (18%). Thus most of the cases presented with fever and symptoms involving the musculoskeletal system. In a study done by Sathyanarayanan V et al, fever was found to be the main presenting symptom along with other musculoskeletal symptoms[72]. In another study by Kochar DK et al, fever and joint pain were found to be the major presenting clinical features[73].

On clinical examination 8 out of 50 patients had palpable hepatospleenomegaly. 8 out of the 9 cases having arthralgia had radiological evidence of septic arthritis involving either the sacroiliac or the knee joint. Thus arthritis was found to be the commonest focal complication. A study by Colmenero JD[11] et al showed the prevalence of osteoarticular complications in Brucellosis to be around 25%. In another study by Bosilkovski M[74] et al, 59.2% of the patients had osteoarticular complications mainly involving the peripheral joints. Though epididymoorchitis, endocarditis and cutaneous lesions are other known focal complications of Brucellosis, they were not seen in our study.

Other than fever and osteo-articular complications, constitutional symptoms like generalized weakness, malaise and loss of weight along with fever were present in 36% of the cases.

**Serological Diagnosis:**

In the present study RBPT was positive in 50 patients out of 1545 patients screened. Majority of the cases had SAT titres ranging from 160IU/ml to 5120IU/ml. However 2 of the cases had SAT titres of 40 IU/ml. As this is an endemic area, a SAT titre of ≥80IU/ml is taken as cut off titre for diagnosis of Brucellosis by serology.

However titres do not correlate with the burden of disease as the antibodies are not protective and do not clear the infection. Immune response does not have any effect on the intracellular pathogen. PCR assay was positive in cases with titres as low as 160 IU/ml to high titres of 5120 IU/ml.

**Culture Methods**:

Though isolation of *Brucella* is conclusive evidence of infection, the rate of recovery of the organism in culture is very low. This may be due to the fastidious, slow growing, intracellular location of the organism or due to antibiotic therapy given prior to the collection of blood samples for culture like cephalosporins, macrolide antibiotics and fluoroquinolones which are known to be effective against *Brucella* in vitro and are also to be used as secondary alternative therapy according to the WHO regimen[68].

In this study blood culture by conventional Castaneda Biphasic Medium showed growth in 10 out of 50 cases(20%) and blood culture by lysis centrifugation technique showed growth in 21 out of 50 cases(42%). The sensitivity of conventional

blood culture using Castaneda Biphasic Medium compared to the Lysis Centrifugation Technique was 47.6% while specificity was 100%.

A study done by Espinosa BJ et al[75] showed isolation rate by Castaneda biphasic medium to be 35.2% and by lysis centrifugation to be 43.2%. In a study by Mantur et al[17],lysis centrifugation yielded a higher culture positivity rate of 20% and 40% more than conventional Castaneda Biphasic medium in acute and chronic cases respectively.

Also the mean duration of time required for isolation by conventional method in this study was 7 days and for lysis centrifugation the mean duration for isolation was3 days. In the study done by Mantur et al[17] the mean duration of incubation for culture positivity by lysis centrifugation was 2.4 and 2.7 days in acute and chronic cases respectively while the mean duration of incubation for culture positivity by conventional Castaneda method was 6.7 and 7.2 days in acute and chronic cases respectively.

The blood culture positivity correlated with cases having high SAT titres which is an indicator of acute infection and bacteremia. Clinically also the blood culture positive cases had fever as the most common presenting symptom indicating bacteremia. Hence in chronic cases and focal complications blood culture may not detect the organism.

**PCR:**

PCR targeting the IS711 genome of the *Brucella* spp which is an insertion sequence showed positive results in 47 out of 50 cases (94%).

Serum was used as the sample for PCR. A study by Zerva et al has shown that serum is the preferred sample than blood for PCR[63]. Serum contains less *Taq* polymerase inhibitors compared to blood and also DNA extraction from serum is easier than DNA extraction from blood. Heme is a potent inhibitor of PCR. Hence repeated washing steps are needed to remove heme if blood sample is used for PCR. The DNA in the serum is due to the breakdown products during bacteremia.

In the 3 cases negative by PCR, the culture results were also negative. Out of the 3 cases, 2 cases had SAT titres of 40IU/ml while 1 case had a SAT titre of 80 IU/ml. All the 3 cases were clinically asymptomatic. As this is an endemic area a baseline titre of ≥80IU/ml is taken as the cut off titre. Thus these 3 cases negative by PCR could be due to the presence of endemic titres or due to cross reactivity.

Other 2 cases which had SAT titres of 80IU/ml and were clinically asymptomatic were positive by PCR. This could be due to subclinical infection or early stage of disease where significant seroconversion has not taken place. Hence PCR was able to detect both these asymptomatic cases.

The sensitivity of PCR when compared to lysis centrifugation culture technique was 100%. In cases where the patient has been started on antibiotics culture may be negative while PCR may be positive as PCR can detect DNA of damaged and non viable bacilli. PCR can detect DNA in amounts as small as 15fg.

In a study done by Elfaki et al[76]PCR was found to be positive in 70% of the cases compared to 40% positive results by culture. In a study done by Al-Attas RA[77] et al the sensitivity of PCR was found to be 100%.

PCR assay is thus more sensitive compared to culture and also it is more useful as a diagnostic tool in cases of Brucellosis with focal complications and

chronicity. Also in endemic areas where SAT titres may be difficult to interpret PCR gives a conclusive evidence of infection. The results of PCR are available within 24 hours and also the risk to laboratory personnel is considerably reduced as handling of the sample is less. Serum which was used as the sample for PCR can be stored at -20°C without any problems till PCR is done.

PCR has its own limitations. It doesn't differentiate between live and dead organisms and amplifies DNA from viable and non-viable bacilli. Cost, infrastructure and technical expertise requirement hamper the availability of PCR at all centres.

# CONCLUSION

Brucellosis which is a zoonotic disease is endemic in many countries including India. Risk factors like contact with infected animals and their birth products, consumption of raw milk and milk products are the major source of infection in majority of the cases.

Clinical diagnosis of Brucellosis is difficult unless a high degree of suspicion is maintained and also the spectrum of clinical manifestations which are seen in Brucellosis are very non-specific.

Laboratory diagnosis is mainly based on serological techniques. Though RBPT is a rapid screening test with high sensitivity it can give false positive reactions and hence specificity is low. SAT titres may be difficult to interpret especially in endemic areas, chronic cases and relapses.

Isolation of the organism by blood culture is the gold standard of diagnosis. However due to the fastidious nature of the organism, long doubling time and its intracellular location, culture yield is low and duration of incubation required may also be prolonged by the conventional Castaneda Biphasic Medium.

Hence in this study blood culture by Conventional Castaneda Biphasic Medium and Lysis centrifugation technique were evaluated. The number of positive blood cultures by Lysis Centrifugation technique were 21 out of 50 (42%), while the number of positive blood cultures by using Conventional Castaneda Biphasic Medium were 10 out of 50(20%). The sensitivity of Castaneda Biphasic Medium blood culture technique compared to the Lysis Centrifugation technique was 47.6% and specificity was 100%.

Blood culture may not be positive in all cases of Brucellosis especially in chronic cases, relapses and those cases presenting with focal complications. In such cases PCR can be used as the confirmatory diagnostic tool.PCR can detect the presence of bacilli in the body and confirm the diagnosis within 24 hours. Other samples like CSF, Synovial fluid, Semen can also be used for PCR in place of blood and serum, especially in patients presenting with focal complications like Neurobrucellosis, Septic arthritis and Epididymoorchitis.

PCR was positive in 47 out of 50 cases (94%) with a sensitivity of 100% compared to the Lysis Centrifugation Technique. The specificity of PCR was low (10%) as it detects non-viable bacilli also.

PCR can be used as a definitive test for ruling out Brucellosis in a clinically suspected case. Positive findings by PCR can be supported by culture and serological tests along with the knowledge of the endemicity of the disease in the area.

**Limitations of PCR :**

- Technical Expertise, Infrastructure and cost may prevent its availability at all centres.
- Monitoring the effectiveness of therapy in Brucellosis may not be possible with PCR as it can detect DNA of non viable bacilli also.

# SUMMARY

This study was aimed at evaluating the conventional blood culture techniques using Castaneda Biphasic Medium and Lysis Centrifugation Technique and Polymerase Chain Reaction in the diagnosis of human Brucellosis.

Serology is the mainstay of diagnosis, but this being an endemic area, titres may be difficult to interpret. Though isolation of organism is gold standard of diagnosis, low culture yield and prolonged incubation pose diagnostic problems. PCR has emerged as a rapid and conclusive diagnostic tool. Hence an attempt was made to evaluate blood culture methods and PCR.

All the serum samples received in the serology section of department of Microbiology, Jawaharlal Nehru Medical College, Belgaum which were positive by the Rose Bengal Card Test were included in the study. 10-12 ml of blood was collected for both the blood culture methods while serum was used as the sample for PCR. The study revealed the following findings:

- Age group commonly affected was between 31-50 years
- 4 cases belonged to paediatric age group
- Brucellosis was more common in males, male to female ratio being 9:1
- Fever was the main symptom in 14 cases(28%) while fever with backache was also equally common with 13 cases(26%). Fever with other constitutional symptoms was found in 36% cases.
- Arthralgia commonly involved the sacroiliac and knee joints
- Majority of patients gave history of contact with livestock while consumption of raw milk and milk products was the next commonest risk factor observed

- More than 80% of the cases were in the high risk occupational group of veterinary work and agriculture
- The SAT titres of the 50 cases studied were between 40 IU/ml and 5120 IU/ml with most cases having significant titres between 160 IU/ml and1280 IU/ml
- Blood culture by Conventional Castaneda Biphasic Medium was positive in 10(20%) cases while the culture was positive in 21(42%) cases by the Lysis Centrifugation Technique
- The mean duration of culture positivity by Conventional method was 6 days while the mean duration of culture positivity by Lysis Centrifugation was 3 days
- The sensitivity of Castaneda Biphasic Medium compared to the Lysis Centrifugation technique was 47.6% while specificity was 100%.
- PCR with serum as the sample was positive in 47 out of 50 cases (94%).
- The sensitivity of PCR compared to Lysis Centrifugation was 100%.

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# ANNEXURE - I

## CONSENT FOR PARTICIPATION IN RESEARCH

**TITLE: Evaluation of Conventional Castaneda Biphasic and Lysis Centrifugation blood culture techniques and Polymerase Chain Reaction in diagnosis of human Brucellosis**

**Study Investigator Dr. _____**

**Guide : Dr. _____**

The purpose of research is Evaluation of Conventional Castaneda Biphasic and Lysis Centrifugation blood culture techniques and Polymerase Chain Reaction in diagnosis of human Brucellosis

You are requested to participate in the study which will help to provide appropriate and effective treatment. During the study you will be asked some questions and you are supposed to answer to the best of your knowledge.

Your participation in research is voluntary. Your decision whether or not to participate in the study will not affect your relationship with Jawaharlal Nehru Medical College. If you decide to participate you are free to withdraw at any time.

**PROCEDURE INVOLVED**:

10-12ml of blood is collected from serologically positive cases of Brucellosis and subjected for investigation.

**RISKS AND BENEFITS**:

There are no risks/minimal risks involved and benefits are to be evaluated.

**PRIVACY AND CONFIDENTIALITY:**

The only people to know that you are a research subject are members of the research team. No information about you or provided by you during research will be disclosed to others without your written permission, except in emergency to protect your rights and welfare.

**AUTHORIZATION TO PUBLISH RESULTS:**

When the results of research are published or discussed, in a conference no information will be displaced that would disclose your identity. Any information that is obtained in connection with this study and that can be identified with you will remain confidential.

**FINANCIAL INCENTIVES FOR PARTICIPATION:**

You will not have to pay/offer any gifts for participating in the research. You will not be reimbursed for expenses.

If you have any queries about your rights as a study subject, you may call

Dr. _____, Professor & Head of Department of Pathology, Chairman, J.N. Medical College Institutional Ethical Committee for Human Subjects Research, J. N. Medical College, Belgaum, Ph. 0831-2473777, Ext. 1527.

In case you have any questions related to the study, you can contact Dr. _____, Professor, Department of Microbiology, J. N. Medical College, Belgaum, (Mobile _____) or Dr. _____ (Mobile no. _____). In case you have any questions about your rights as a study participant you can contact Dr_____ (_____).

**CONSENT STATEMENT**

I undersigned _____have been explained in my vernacular language about the study and my participation in the study is voluntary. If I want, I can withdraw at any time. Also I have been given enough time to clear my doubts and rights as study participant.

Signature or left hand thumb print of participant or legally authorized representative.

Participants Name _____ signature_____

Witness Name _____ signature_____

Experimenters Name _____ signature_____

Date: Place:

# ANNEXURE - II

# PROFORMA

| | | | |
|---|---|---|---|
| Name | : | Age | : |
| Sex | : | Occupation | : |
| Hospital No. | : | Lab. No. | : |

**Presenting complaints:**

**History of present illness:**

Fever

Myalgia

Night Sweats

Joint pains

Others

**Past history:**

**Personal history:**

**Family History:**

**Treatment History:**

**General Physical Examination:**

| | |
|---|---|
| **Pulse** | **Temp.** |
| **Resp. Rate** | **Weight** |
| **B.P** | |

**Systemic Examination:**

**Clinical Diagnosis:**

**Investigations:**

1. Slide agglutination test
2. Standard agglutination test
    a. SAT
    b. 2 ME
3. Blood Culture
    a. Castaneda Biphasic Method
    b. Lysis centrifugation Technique

    Identification of Isolates

    a. Gram Staining
    b. ZN Staining
    c. Catalase Test
    d. Oxidase Test
    e. Urease Test
    f. $H_2S$ Production
    g. $CO_2$ Requirement
4. PCR

# ANNEXURE – III: MASTER CHART

| SL. No. | Lab No. | Age (yrs) | Sex | Duration of Fever | H/O Contact With Livestock | H/O Raw Milk consumption | RBPT | SAT (I.U/ml) | 2ME | Castaneda Biphasic Medium | Day of Culture Positivity | Lysis Centrifugation | Day of Culture Positivity | PCR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | S-686-12 | 5 | M | 10 days | -- | + | + | 1280 | 1:40 | + | 5 | + | 4 | + |
| 2 | S-2976-12 | 36 | M | 2 months | + | + | + | 640 | 1:320 | -- | -- | -- | -- | + |
| 3 | S-3412-12 | 45 | F | 20 days | + | + | + | 640 | 1:40 | -- | -- | + | 3 | + |
| 4 | S-5803-12 | 23 | M | 15 days | + | + | + | 320 | 1:40 | -- | -- | -- | -- | + |
| 5 | S-6442-12 | 26 | M | 1 month | + | -- | + | 160 | 1:40 | -- | -- | -- | -- | + |
| 6 | S-7019-12 | 48 | M | 15 days | + | -- | + | 1280 | 1:40 | -- | -- | + | 5 | + |
| 7 | S-8034-12 | 11 | F | 2 months | -- | + | + | 320 | 1:80 | -- | -- | -- | -- | + |
| 8 | S-8564-12 | 34 | M | 15 days | + | -- | + | 160 | 1:40 | -- | -- | -- | -- | + |
| 9 | S-8608-12 | 38 | M | 1 month | + | -- | + | 640 | 1:20 | -- | -- | + | 4 | + |
| 10 | S-8609-12 | 38 | M | 10 days | + | -- | + | 1280 | 1:20 | + | 6 | + | 2 | + |
| 11 | S-8610-12 | 42 | M | 2 months | + | -- | + | 320 | 1:80 | -- | -- | -- | -- | + |
| 12 | S-8611-12 | 40 | M | 20 days | + | -- | + | 640 | 1:20 | -- | -- | + | 3 | + |
| 13 | S-9480-12 | 60 | M | 3 months | + | + | + | 160 | Neg | -- | -- | -- | -- | + |
| 14 | S-9979-12 | 40 | M | 1 month | + | -- | + | 320 | 1:40 | -- | -- | -- | -- | + |
| 15 | S-9980-12 | 43 | M | 15 days | + | -- | + | 640 | 1:40 | -- | -- | + | 4 | + |
| 16 | S-9981-12 | 36 | M | 2 months | + | -- | + | 160 | Neg | -- | -- | -- | -- | + |
| 17 | S-9982-12 | 40 | M | 6 months | + | -- | + | 320 | 1:40 | -- | -- | -- | -- | + |
| 18 | S-9983-12 | 33 | M | 2 months | + | -- | + | 320 | 1:20 | -- | -- | -- | -- | + |
| 19 | S-9984-12 | 33 | M | -- | + | -- | + | 80 | 1:20 | -- | -- | -- | -- | + |
| 20 | S-9986-12 | 39 | M | 10 days | + | -- | + | 2560 | 1:80 | + | 5 | + | 3 | + |
| 21 | S-11338-12 | 18 | M | 15 days | + | + | + | 1280 | 1:20 | + | 8 | + | 2 | + |
| 22 | S-11532-12 | 8 | M | 1 month | -- | + | + | 640 | 1:160 | -- | -- | -- | -- | + |
| 23 | S-11691-12 | 12 | M | 15 days | -- | + | + | 1280 | 1:20 | -- | -- | + | 4 | + |
| 24 | S-12255-12 | 50 | M | 10 days | + | -- | + | 640 | 1:20 | + | 6 | + | 3 | + |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | S-12333-12 | 60 | F | 20 days | + | + | + | 5120 | 1:160 | + | 4 | + | 4 | + |
| 26 | S-12468-12 | 50 | F | 20 days | + | + | + | 640 | 1:80 | -- | -- | -- | -- | + |
| 27 | S-1252-13 | 60 | M | 15 days | -- | + | + | 1280 | 1:40 | + | 9 | + | 3 | + |
| 28 | S-1747-13 | 48 | F | 2 months | + | -- | + | 1280 | 1:80 | -- | -- | + | 4 | + |
| 29 | S-2012-13 | 28 | M | 20 days | + | -- | + | 640 | 1:20 | -- | -- | + | 3 | + |
| 30 | S-2017-13 | 45 | M | 1 month | + | -- | + | 160 | 1:40 | -- | -- | -- | -- | + |
| 31 | S-2021-13 | 47 | M | 2 months | + | -- | + | 160 | 1:80 | -- | -- | -- | -- | + |
| 32 | S-2022-13 | 40 | M | 1 month | + | -- | + | 640 | Neg | -- | -- | + | 5 | + |
| 33 | S-2023-13 | 43 | M | -- | -- | -- | + | 80 | 1:20 | -- | -- | -- | -- | -- |
| 34 | S-2025-13 | 41 | M | -- | -- | -- | + | 40 | 1:20 | -- | -- | -- | -- | -- |
| 35 | S-2026-13 | 49 | M | 20 days | + | -- | + | 160 | 1:40 | -- | -- | -- | -- | + |
| 36 | S-2028-13 | 50 | M | 1 month | + | -- | + | 320 | 1:20 | -- | -- | -- | -- | + |
| 37 | S-2030-13 | 40 | M | 1 month | + | -- | + | 320 | 1:40 | -- | -- | -- | -- | + |
| 38 | S-2031-13 | 42 | M | 20 days | + | -- | + | 640 | 1:20 | -- | -- | + | 2 | + |
| 39 | S-2032-13 | 35 | M | 1 month | + | -- | + | 160 | 1:20 | -- | -- | -- | -- | + |
| 40 | S-2035-13 | 42 | M | 15 days | + | -- | + | 640 | 1:20 | + | 7 | + | 3 | + |
| 41 | S-2036-13 | 48 | M | 1 month | + | -- | + | 320 | 1:40 | -- | -- | -- | -- | + |
| 42 | S-2040-13 | 43 | M | -- | -- | -- | + | 40 | Neg | -- | -- | -- | -- | -- |
| 43 | S-2044-13 | 28 | M | 10 days | + | -- | + | 1280 | 1:40 | + | 5 | + | 3 | + |
| 44 | S-2053-13 | 44 | M | 1 month | + | -- | + | 160 | 1:20 | -- | -- | -- | -- | + |
| 45 | S-2055-13 | 41 | M | 20 days | + | -- | + | 320 | 1:40 | -- | -- | -- | -- | + |
| 46 | S-2314-13 | 39 | M | 15 days | + | -- | + | 320 | 1:40 | -- | -- | -- | -- | + |
| 47 | S-2325-13 | 54 | M | 1 month | + | -- | + | 640 | 1:20 | -- | -- | + | 4 | + |
| 48 | S-2826-13 | 56 | M | 1 month | + | -- | + | 320 | 1:80 | -- | -- | -- | -- | + |
| 49 | S-2829-13 | 56 | M | -- | + | -- | + | 80 | 1:20 | -- | -- | -- | -- | + |
| 50 | S-3336-13 | 35 | M | 20 days | + | - | + | 1280 | 1:20 | + | 6 | + | 2 | + |

# KEY TO MASTER CHART

M-Male

F - Female

RBPT – Rose Bengal  Plate Test

SAT – Standard Agglutination Test

2 ME – 2 Mercaptoethanol

PCR – Polymerase Chain Reaction

# Introduction

# Objectives

# Review of Literature

# Material & Methods

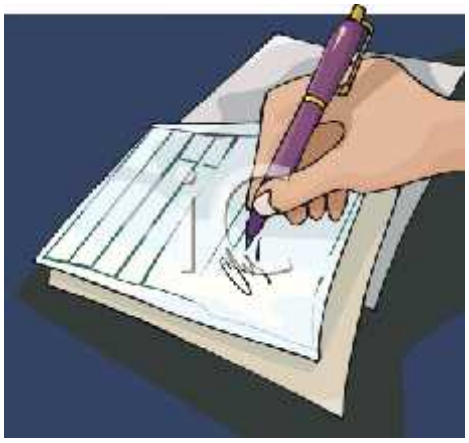
# Results

# Discussion

# Conclusion

# Summary

# Bibliography



# Annexure I:

# Consent Form

# Annexure II:

## Proforma

# Annexure III:

## Master Chart