DETECTION OF BRUCELLOSIS IN OCCUPATIONALLY EXPOSED WORKERS

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2008

DETECTION OF BRUCELLOSIS IN OCCUPATIONALLY EXPOSED WORKERS

A DISSERTATION
Submitted to

The Department of Biological Sciences Quaid-i-Azam University, Islamabad in partial fulfillment of requirements for the degree of

MASTER OF PHILOSPHY (M.PHIL)
IN
BIOTECHNOLOGY

ISLAMABAD

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CERTIFICATE

This thesis submitted by **Muhammad Asif** is accepted in its present form by National Institute for Biotechnology and Genetic Engineering, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the degree of "Master of Philosophy in Biotechnology"

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Dated 22.05.08

Copy No: 352

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ACKNOWLEDGEMENTS

*In the name of **Allah**, The most beneficent, The most merciful.*

*All praise to almighty **Allah**, the creator, the dominant, the most supreme, the self-existing and all sustaining, whose grace and mercy enabled me to accomplish this piece of work by Sacredness of **Holy Prophet Hazrat Muhammad (S.A.W)** who is forever an entire source of guidance and knowledge for humanity.*

Most importantly I want to present tribute to my beloved parents and my brothers whose prayers, unconditional cooperation and moral support in every step of my life make me able to complete my work without which the present destination would have been my dream.

*I express my deep sense of gratitude and appreciation to National Institute of Biotechnology and Genetic Engineering and **Dr. Zafar Mahmood Khalid**, Director, NIBGE, Faisalabad, Pakistan, for providing me the best educational and research facilities and a great deal of knowledge.*

*It is a matter of great pleasure to express my special thanks and humble gratitude to my worthy supervisor, **Dr. Qaiser Mahmood Khan**, Head, Environmental Biotechnology Division, NIBGE, Faisalabad, whose presence was always a source of confidence for me. It was indeed an honour to work under his guidance. His personal interest, extremely amicable behaviour and ample support helped me in the successful completion of this work.*

I present my heartiest thanks to Mr. M. Anwar-ul-Haq, Senior Scientist, Environmental Biotechnology Division, NIBGE, for his cooperation and encouraging attitude.

It is my pleasure to thank all the respected members of Environmental Biotechnology Division, especially Dr. Samina Iqbal, Madam Ameena Mobeen, Madam Razia Tehseen, Mr. M Afzal and Mr. Aamir Jamal Hashmat for their all time valuable suggestions and sincere cooperation.

I am extremely thankful to Miss Samina Anwar, Junior Scientist, Environmental Biotechnology Division, for her brilliant technical guidance whenever needed.

My special thanks are reserved for my group fellows Dr. Rashid, Dr. Usman Waheed, M. Ismail, Tayyaba and especially Dr. Rahat Ali for their cooperation and nice company. Mr. Shahid Farooq, Mr. Sohail and Mr. Khalid for their assistance during lab work.

*I am also thankful to **Dr. Akbar Ali**, District Livestock Officer, Faisalabad, for helping me in sample collection from the veterinary professionals of Faisalabad district.*

I feel immense pleasure to express my deepest gratitude and sincere thanks to all my class fellows for their fantastic and memorable company.

At the end I would also like to say that this acknowledge is incomplete without mentioning special thanks to my best friends Muhammad Farooq, Ali Saeed, Badar, Hafiz Naseer, Arsalan and Fiaz for their kind suggestions, moral support and nice company during my study.

Muhammad Asif

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LIST OF ABBREVIATIONS

Abbreviated name	Full name
BCSP 31 gene	Gene encoding 31-KDa *Brucella* Cell Surface Protein
bp	Base pair
CSF	Cereberal Spinal Fluid
CTAB	Cetyltriethylmethylammoniumbromide
DNA	Deoxy ribonucleic acid
dNTP's	Deoxy ribonucleotidetriphosphate
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbant Assay
g	Gram
HIV	Human immunodeficiency virus
Ig	Immunoglobulins
KDa	KiloDalton
LB	Luria-Bertani (medium)
LPS	Lipopolysaccharide
mA	Milli amphere
Mb	Mega base
$MgCl_2$	Magnesium Chloride
ml	Milliliters
mM	Millimole
NARC	National Agriculture Research Council
nm	Nanometer
OD	Optical density
PCR	Polymerase chain reaction
rpm	Revolution per minute
rRNA	Ribosomal Ribonucleic acid
SAT	Serum Agglutination Test
TAE	Tris acetate EDTA buffer
V	volts

ABSTRACT

Brucellosis since the discovery of *Brucella melitensis* by Sir David Bruce in 1887 continues to pose a human health risk globally, despite the control measures undertaken by national authorities in many countries. Currently *B. melitensis* remains the principal cause of human brucellosis globally. The recent isolation of distinct strains of *Brucella* from marine mammals and humans is an indicator of an emerging zoonotic disease. Half a million of new cases of human brucellosis are reported annually worldwide, however the World Health Organization announced that these numbers are greatly underestimate the true incidence of human brucellosis as the actual number of cases is estimated to be at least 10 times the figures officially announced. In Pakistan, the disease is possibly endemic but unfortunately no data is available. A safe and effective vaccine in humans is not yet available. Prevention of human brucellosis is dependent on the control of disease in animals by vaccination, effective heat treatment of the milk and other dairy products and hygienic precautions to prevent occupational exposure.

Brucellosis in endemic and non-endemic areas remains a diagnostic puzzle due to unusual presentations and misleading non-specific manifestations. Conventional diagnostic methods (culture, serological tests etc.) are unable to diagnose the disease accurately. Therefore rapid and reliable, sensitive and specific and easy to perform assays (PCR etc.) are urgently needed to allow early diagnosis and adequate antibiotic therapy in time to decrease morbidity and mortality.

In the present study, PCR assay was developed for the diagnosis of brucellosis. The PCR assay was applied for the detection of brucellosis in different individuals occupationally exposed to animals. The results of PCR assay were also compared with that of Serum Agglutination Test. It was found that PCR assay is more sensitive and specific and establishes the diagnosis of brucellosis earlier than the conventional diagnostic methods. For confirmation of the results, the amplified products (223bp region of BCSP 31 gene of *Brucella*) were cloned and sequenced. The

BLAST analysis showed that these sequences have 100% homology with the BCSP 31 gene of *Brucella* reported from other countries.

Therefore, there is also a need to develop diagnostic facilities (especially PCR based) and national surveillance programme to combat this re-emerging zoonosis in an agricultural country like Pakistan where vast majority of population is involved in livestock farming. In the present study, confirmation of brucellosis in individuals exposed to animals, highlights the importance of further research for establishing risk of brucellosis in occupationally exposed population.

CHAPTER 1

INTRODUCTION

AND

REVIEW OF LITERATURE

INTRODUCTION AND REVIEW OF LITERATURE

Human health is inextricably linked to animal health and production. This link between human and animal populations, and with the surrounding environment, is particularly close in developing countries like Pakistan where animals provide transportation, draught power, fuel and clothing as well as nutrition (meat, eggs and milk). In both developing and industrialized countries, however, this can lead to a serious risk to public health with severe economic consequences, because number of diseases (known as zoonoses) are transmitted from animals to humans. This risk is high among the individuals who are in contact with the animals because of their occupation.

Brucellosis is one of the five common bacterial zoonoses in the world caused by organisms belonging to the genus *Brucella*, which are Gram-negative, non-sporing, facultative intracellular bacteria (Corbel, 1997). The genus *Brucella* consists of six species according to antigenic variation and host preference. These are *Brucella abortus* (cattle), *B. melitensis* (sheep and goats), *B. suis* (pigs), *B. canis* (dogs), *B. ovis* (sheep) and *B. neotome* (wood rat). Recently, *Brucella* has been discovered from a variety of marine mammals including cetaceans (e.g. dolphins), seals and otters (Jahans *et al.*, 1997).

Bang's disease, Infectious abortion, Malta fever, Mediterranean fever and Undulant fever are synonyms for brucellosis. Human contract brucellosis through contact with infected animals or from consumption of unpasteurized dairy products prepared from the milk of infected animals. In most host species, the disease primarily affects the reproductive system. In animals, the disease is characterized by abortion, retained placenta, orchitis, epididymitis and arthritis with the excretion of organism in the uterine discharge, milk and semen. In humans, the disease is associated with protean manifestations and characteristically recurrent febrile episodes that led to the description of this disease as 'undulant fever'. Other nonspecific symptoms in humans are chills, profuse sweating,

headache, arthralgia, myalgia, leg and back pain, malaise, fatigue, weight loss, inattention and depression. Fatalities are not common, though the syndrome can last for a few weeks to a year, even with treatment (Young, 1994).

Brucellosis in animals and humans is endemic in many developing countries including Pakistan. Control of brucellosis in animals is a pre-requisite for the prevention of this disease in human beings. Recently, *Brucella melitensis* has been declared by the Centers for Disease Control and Prevention to be one of the three bioterrorist agents due to the expense required for the treatment of human brucellosis patients. Also, the economic and agricultural loss caused by bovine brucellosis emphasizes the financial impact of brucellosis in the society. Human brucellosis is significant public health problem in an agricultural country like Pakistan, where the vast majority of the population is involved in land cultivation and livestock farming. Currently, *B. abortus* RB51 strain to immunize cattle and *B. melitensis* Rev.1 strain to immunize goats and sheep are used in many countries (Corbel, 1997). No vaccines are available for other animals, and a human *Brucella* vaccine does not exist. Efforts are underway to develop new vaccines for animals and human against brucellosis.

Historical perspective:

Brucellosis is a zoonosis transmitted to humans from infected animals. A type of fever characterized by fairly regular remissions or intermissions has been recognized along the Mediterranean littoral since the time of Hippocrates in 450 B.C. Much later in the 19th century, the disease was found to affect British armed forces and the local population of Malta. J.A. Marston, an assistant surgeon of the British Medical Department working in the Mediterranean in 1861, first described the symptoms of brucellosis in himself as "gastric remittent fever" (Marston, 1861). The cause of this disease was obscure until 1887 when Sir David Bruce - a Scottish physician reported numerous small coccal organisms in stained sections of spleen from a fatally infected soldier and isolated and identified organism in culture from spleen tissue of four other British soldiers stationed at Malta (Bruce, 1887). This organism, which he designated *Micrococcus melitensis*, produced a remittent fever in inoculated monkeys. One animal died from the infection

and the organism was recovered in pure culture from the liver and spleen. The organism derived its species name from Melita (honey), the Roman name for the Isle of Malta. Hughes ML, in a monograph in 1897, portrayed the findings in people in greater detail, emphasizing "undulant fever" and suggested the name undulant fever (Hughes, 1887). Wright and Smith in 1897 detected antibodies to *M. melitensis* in human and animal sera through agglutination test, which unravelled the zoonotic potential of the disease (Wright and Smith, 1897). Later, Zammit an young Maltese physician working with Mediterranean Fever Commission in 1905 confirmed it by isolating the organism from the milk and urine of goats (Zammit, 1905).Thus he concluded that the goat was the reservoir of *M. melitensis* and the consumption of the raw milk and cheese infects man.

In the same year (1897) that Hughes monograph appeared, Bang in Denmark isolated a gram negative rod from cattle, which had aborted. The third member of the group, which is also bacillary in shape, was recovered from the foetus of aborted swine by Traum in 1914 in the United States of America and implicated as an agent of brucellosis in man by Huddleson in 1943. In 1918, Alice Evans an American bacteriologist published reports which contained convincing evidence that *M. melitensis* from goats and a gram-negative rod from cows could not be differentiated morphologically or by their cultural and biochemical reactions but there were antigenic differences which could be shown by agglutination absorption test.

Meyer and Shaw further confirmed Evan's observations and suggested the generic name *Brucella* in honour of Sir David Bruce (Meyer and Shaw, 1920). The possible pathogenicity of *B. abortus* to man was suggested by Evan in 1918 and confirmed by others. In 1956, Buddle and Boyce discovered *B. ovis*, the cause of epididymitis in rams. In 1957, Stoenner and Lackman isolated *B. neotomae* from desert wood rat in Utah in USA. In 1968, Carmicheal and Bruner discovered *B. canis* as the cause of an epidemic of abortions in beagles. Human infections due to *B. canis* have been reported (Lucero et al., 2005). Two new *Brucella* species, provisionally called *B. pinnipediae* and *B. cetaceae* have been isolated from marine hosts within the past few years (Ross *et al.*, 1996). There are three reports in the literature of humans infected with marine mammal strains of

Brucella; one infection occurred in a research laboratory worker after occupational exposure (Brew *et al.*, 1999), and the other two were community-acquired infections (McDonald *et al.*, 2006).

Taxonomy:

The taxonomy of *Brucella* species is still unclear and unresolved. Based on 16S rRNA gene sequences, *Brucellae* are categorised as α-2 proteobacteria and have close phylogenetic relationships with *Agrobacterium, Rickettsia, Rhizobium* and *Rhodobacter* (Moreno *et al.*, 1990). *Brucellae* have been classified according to differences in pathogenicity and host preference, into six species: *B. melitensis, B. abortus, B. suis, B. ovis, B. canis and B. neotomae* (Jahans *et al.*, 1997). Table-1 summarizes the taxonomic characteristics of *Brucella* species.

In fact Verger and colleagues used DNA-DNA hybridization studies to investigate 51 *Brucella strains* of all species and found them to be identical (Verger *et al.*, 1985). With these results, they proposed that all species should be considered as biovars of *B. melitensis*. However, because of the differences in the animal reservoirs and in the severity of clinical disease associated with the different species, this proposal has not been widely accepted.

The scientific classification of *Brucella* is as under;

Kingdom	*Bacteria*
Phylum	*Proteobacteria*
Class	*Alpha proteobacteria*
Order	*Rhizobiales*
Family	*Brucellaceae*
Genus	*Brucella*
Species	*B. abortus, B. melitensis, B. suis, B. canis, B. ovis, B. neotome*

Table 1: Taxonomy of *Brucella* species (Pappas *et al.*, 2005).

Species	Biotype	Animal host	First described	Human virulence *
B. melitensis	1-3	sheep,goats, camels	Bruce, 1887.	++++
B. abortus	1-6, 9	cows, camels buffaloes,	Bruce, 1887.	++ to +++
B. suis	1-5	Pigs	Traum, 1914.	+
B. canis	_	canines	Carmichael and Bruner, 1968.	+
B. ovis	_	Sheep	Van Drimmelen, 1953.	_
B. neotomae	_	Rodents	Stoenner and Lackman, 1957.	_
B. pinnipediae and B. cetaceae (provisional)	_	Minke whales, dolphins, seals	Ewalt and Ross, 1994.	+

*virulence is graded on a scale from no virulence (-) to the highest degree of virulence (++++).

Cell morphology and culture characteristics:

Brucellae are small gram negative cocci or coccobacilli or short rods measuring about 0.5 to 1.5μm in length by 0.5 to 0.7μm in width. They can occur as single cells, in pairs or in short chains. They are non spore forming, uncapsulated and aflagellate and, therefore, are nonmotile (Corbel *et al.*, 1984).

The metabolism of *Brucellae* is mainly oxidative and energy is produced by utilization of amino acids and carbohydrate substrates. For many strains i-erythritol is the preferred energy source. They grow under aerobic conditions at an optimal temperature of 37°C, with many strains requiring supplemental CO_2 for growth. The optimum pH for growth ranges between 6.6 and 7.4. Growth usually results in alkalization of the medium. All strains lose viability at 56°C, however temperatures above 85°C may be required to insure complete killing of *Brucella*. They are sensitive to a wide variety of disinfectants including formaldehyde, hypochlorite, iodophores and phenols.

On Serum dextrose agar (SDA), colonies appear transparent, raised, and convex with an entire edge and have smooth, shiny surface (Corbel *et al.*, 1984). On primary isolation using SDA, *Brucella* colonies are rarely seen prior to 48 hours. At 48 hours, colonies are approximately 0.5-1.0mm in diameter. Colony variants can be classified under four morphological categories: smooth, rough, smooth-rough intermediate and mucoid. This classification is based on characteristics of bacterium's lipopolysaccharide (LPS). At the microscopic level, smooth organisms have LPS molecules containing a polysaccharide O-side chain made from a homopolymer of perosamine on their surface, while rough organisms lack this chain on their LPS (Moreno *et al.*, 1984). Rough colonies are usually less transparent than smooth variants. They have a more dull, granular surface and appear matte white, yellowish white/buff, or brown in colour. Mucoid colonies are similar to rough colonies except that they have a sticky glutinous texture.

In terms of antibiotic susceptibility, nearly all strains of *Brucella* are susceptible in vitro to gentamycin, tetracycline (and its derivatives), and rifampicin. Additionally, many strains are also susceptible to ampicillin, chloramphenicol, erythromycin, kanamycin,

Fig. 1: Colonies of *B. melitensis.*

(http://en.wikipedia.org/wiki/Brucella_melitensis)

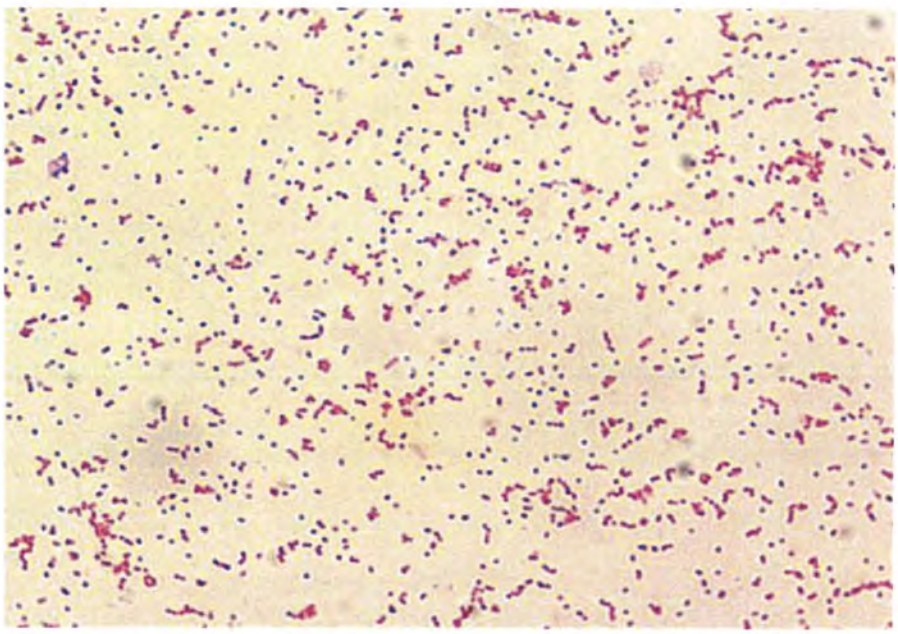


Fig. 2: *Brucella* are gram –ve in their staining morphology.

(http://commons.wikimedia.org/wiki/Image:Brucella_spp.JPG)

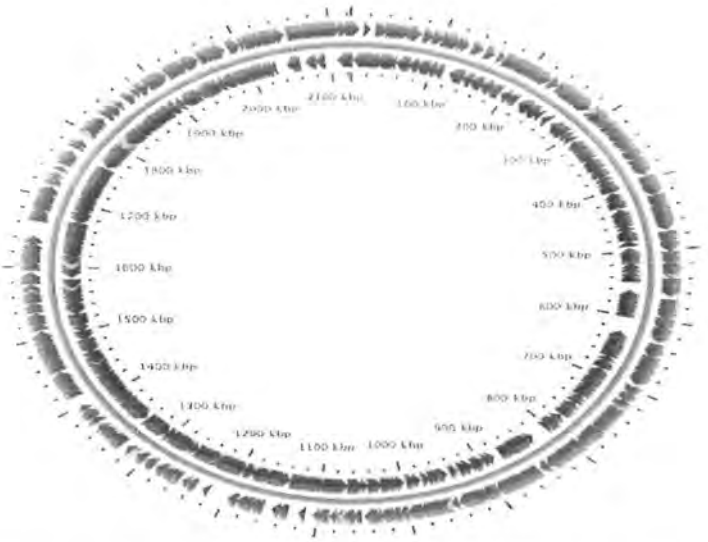
streptomycin and sulfamethoxisole/trimethoprim. Susceptibility to antibiotics can differ among species, biovars and even strains. These differences can aid in identification of specific strains of *Brucella*.

Molecular genetics:

The genome of *Brucella* contains two circular chromosomes of 2.1 and 1.5 Mb, respectively (figure 3 & 4). Both replicons encode essential metabolic and replicative functions and therefore are chromosomes, not plasmids (Jumas-Bilak *et al.*, 1995). Natural plasmids have not been detected in *Brucella*, although transformation has been effected by wide host range plasmids following conjugative transfer or electroporation (Rigby and Fraser, 1989).

The complete genomic sequence of *B. melitensis*, *B. abortus* and *B. suis* has been achieved recently (DelVecchio *et al.*, 2002). The average size of the *Brucella* genome is 2.37×10^9 daltons, with a DNA G + C content of 58-59 mol% (De Ley *et al.*, 1987). All types show > 95% homology in DNA-DNA pairing studies, justifying the nomination of *Brucella* as a monospecific genus. Restriction fragment patterns produced by infrequently cutting endonucleases support the differentiation of the nomen species (Allardet-Servent *et al.*, 1988). Restriction endonuclease analysis has generally been unsuccessful for strain differentiation, but polymerase chain amplification of selected sequences followed by restriction analysis has provided evidence of polymorphism in a number of genes including omp 2, dnaK, htr and ery (the erythrulose-1-phosphate dehydrogenase gene) (Sangari *et al.*, 1994). The omp 2 gene is believed to determine dye sensitivity, one of the traditional typing methods for biotype differentiation (Douglas *et al.*, 1984). Its polymorphism and the capacity for post-translational modification of its product may explain the tendency for variation in dye sensitivity patterns even within species and have been used as the basis for a genetic classification of *Brucella* (Ficht *et al.*, 1996). The *dnaK* gene of *B. melitensis* is cleaved into two fragments by Eco RV endonuclease, whereas the genes of the other nomen species all produce a single fragment (Cloeckaert *et al.*, 1996). The *ery* gene is reported to have undergone a 7.2 kbp deletion in *B. abortus*

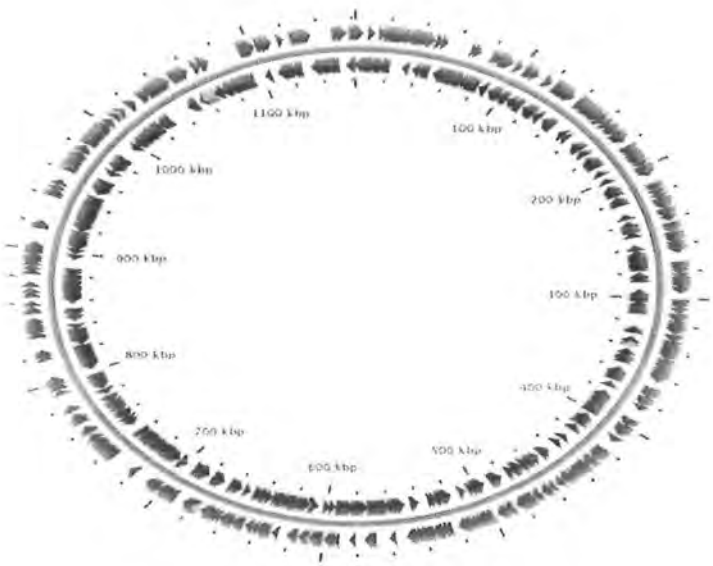
Brucella abortus biovar 1 str. 9-941 chromosome I, complete ...



Accession: NC_006932 Topology: Circular; Length: 2,124,241 bp; Genes: 2,077

Figure 3

Brucella abortus biovar 1 str. 9-941 chromosome II, complete...



Accession: NC_006933 Topology: Circular; Length: 1,162,204 bp; Genes: 1,072

Figure 4

strain 19 (Sangari *et al.*, 1994). This could explain this strain's erythritol sensitivity, a major factor in its attenuation.

Antigenic composition:

A substantial number of antigenic components of *Brucella* have been characterized. However, the antigen that dominates the antibody response is the lipopolysaccharide (LPS). LPS of rough strains (R-LPS) is similar to LPS of smooth strains (S-LPS) except that the O-chain is either absent or reduced to a few residues. Strong cross-reactions in both the agglutination and complement fixation tests have been reported between smooth species of *Brucella* and *Yersinia enterocolitica* O:9, *Escherichia hermanni, Escherichia coli* O:157, *Salmonella* O:30, *Stenotrophomonas maltophila and Vibrio cholerae* O:19 (Perry and Bundle, 1990). These have been attributed to similarities on the O-specific side chains of the lipopolysaccharide molecule of the organisms. Numerous outer and inner membrane, cytoplasmic and periplasmic protein antigens have also been characterized. Some are recognized by the immune system during infection and are potentially useful in diagnostic tests (Goldbaum *et al.*, 1993). Omp25 is an outer membrane structural protein that is highly conserved in all *Brucellae*. It is associated with both lipopolysaccharide and peptidoglycan. Recently, ribosomal proteins have emerged as immunologically important components since they confer protection against challenge with *Brucella* on account of both antibody and cell mediated responses (Corbel, 1976). One such example is L7/L12. This elicits delayed hypersensitivity response as component of brucellins (Bachrach *et al.*, 1994) and as fusion proteins, which has been shown to stimulate protective response (Oliveira *et al.*, 1996). Hence this appears to have potential as candidate vaccine component.

Virulence:

The basis for the virulence of *Brucella* can be attributed to the ability of these bacteria to escape the host defense mechanisms and to survive and replicate within the host cells. *Brucella* organisms are capable of invading and residing in professional phagocytes (Baldwin and Winter, 1994), such as macrophages, as well as non-phagocytic cells (Detilleux, 1990). The mechanism of attachment and entry into these cells by *Brucella*

has yet to be clearly elucidated. Virulence mechanisms identified so far to be associated with the ability to reside within phagocytic and/or non-phagocytic cells are; ability to inhibit phagolysosomal fusion, degranulation and activation of the myelo-peroxidase-halide system and production of tumor necrosis factor (Caron *et al.*, 1994).

In both phagocytic and non-phagocytic cells, *Brucella* has the ability to replicate within membrane-bound compartments (Pizarro-Cerda *et al.*, 1998). In non-phagocytic cells, such as HeLa cells, virulent *B. abortus* 2308 has been documented to replicate in the endoplasmic reticulum by utilizing the autophagic machinery of the HeLa cell (Pizarro-Cerda *et al.*, 1998). In professinal phagocytes, the membrane-bound compartment within which virulent *Brucella* organisms can proliferate is the phagosome. By some unknown mechanism, *Brucella* is able to block phagolysosome fusion (Frenchick *et al.*, 1985). It is now thought that the production of adenine and guanine monophosphate can inhibit phagolysosome fusion (Corbel, 1997). The ability to produce these compounds is therefore considered a virulence factor of *Brucella*. In contrast, attenuated strains of *Brucella* are unable to prevent such fusion and are thereby destroyed by the lysosomal contents (Pizarro-Cerda *et al.*, 1998).

Research on intracellular survival and replication of *Brucella* within professional phagocytes has mainly focused on macrophages. Survival within macrophages is apparently associated with the production of many different proteins. These proteins tend to be stress-induced proteins such as heat shock or acid-induced proteins. They include the 17, 24, 28, 60 and 62 kDa proteins (Lin and Ficht, 1995). Two of these proteins, the 17 and 28 kDa proteins, seem to be induced only during intracellular cohabitation of *Brucella* with macrophages.

Another stress-induced protein, HtrA, has been involved in inducing a granulomatous reaction and reduced levels of infection during the early phase of infection. However, this does not result in reduced levels in the later phases of infection. Two other types of proteins that have been put forth as possible virulence factors are siderophores and Cu-Zn superoxide dismutase (Cu-Zn SOD). Iron sequestering by siderophores may be an

integral virulence factor in intracellular survival of *Brucella* species. Low levels of iron in vivo aid the host's ability to restrict microbial growth (Corbel, 1997). Cu-Zn SOD may have a significant role in the early phase of intracellular infection, but contradictory results have been reported (Tatum, 1992).

Recently, a two-component regulatory system has been discovered in *B. abortus*. The Bvr (*Brucella* virulence related proteins) system consists of a regulatory (BvrR) and a sensory protein (BvrS). This regulatory system, BvrR-BvrS, may play a critical role in the ability of *B. abortus* to invade and multiply within the cells (Sola-Landa, 1998).

Non-protein components of *Brucella* may also contribute to its ability to survive within cells. One such cellular component is lipopolysaccharide (LPS). Smooth *Brucella* organisms are better able to survive intracellularly than do their rough counterparts. Therefore, smooth lipopolysaccharide (S-LPS) probably plays a significant role in pathogenesis.

B. abortus S-LPS is 100 times less potent than that of *E.coli* (Goldstein *et al.*, 1992) and *Salmonella* (Freer *et al.*, 1996) in inducing TNFα from macrophages as well as oxidative metabolism and lysozyme release by human neutrophils. This feature of S-LPS has been proposed to contribute to the survival of *B. abortus* within phagocytic cells. In addition, *Brucella* S-LPS is not susceptible to the actions of polycationic molecules, suggesting that smooth *Brucella* can resist the cationic bactericidal peptides of the phagocytes (Martinez de Tejada *et al.*, 1995). S-LPS has also been found to confer antiphagocytic properties to *Brucella* and does not activate the alternate pathway of complement cascade.

Epidemiology:

The epidemiology of brucellosis is complex and it changes from time to time. Wide host range and resistance of *Brucellae* to environment and host immune system facilitate its survival in the populations. Worldwide, brucellosis remains a major source of disease in humans and domesticated animals. The disease is endemic especially in countries of the

Mediterranean basin, the Arabian Gulf, the Indian subcontinent and parts of Mexico and Central and South America. Human brucellosis is found to have significant presence in rural/nomadic communities where people live in close association with animals. Worldwide, reported incidence of human brucellosis in endemic disease areas varies widely, from < 0.01 to > 200 per 100,000 population (Boschiroli *et al.*, 2001). The true incidence of human brucellosis however, is unknown for most countries including Pakistan. It has been estimated that the true incidence may be 25 times higher than the reported incidence due to misdiagnosis and underreporting. It has been shown that the incidence of human brucellosis is significantly high where ovine/caprine brucellosis caused by *B. melitensis* is endemic (WHO, 1997). Sheep and goats and their products remain the main source of infection, but *B. melitensis* in cattle has emerged as an important problem in some southern European countries, Israel, Kuwait and Saudi Arabia. *B. melitensis* infection is particularly problematic because *B. abortus* vaccines do not protect effectively against *B. melitensis* infection; the *B. melitensis* Rev.1 vaccine has not been fully evaluated for use in cattle. Despite vaccine campaigns with Rev.1 strain, *B. melitensis* remains the principal cause of human brucellosis worldwide.

Screening of household members of an index case is important epidemiological step since this picks up additional unrecognized cases (Mantur *et al.*, 2006). This must be taken into account by the family clinicians caring for these patients, so that timely diagnosis and provision of therapy occur, resulting in lower morbidity. The recent isolation of distinctive strains of *Brucella* from marine mammals (Ross *et al.*, 1996) as well as humans (McDonald *et al.*, 2006) has extended the ecological range of human brucellosis. Because new strains may emerge and existing types adapt to changing social and agricultural practices, the picture remains incomplete.

It is a well-characterized occupational disease in shepherds, abattoir workers, veterinarians, dairy industry professionals and personnel in microbiologic laboratories. Males are affected more commonly than females (Mantur *et al.*, 2006), which may be due to risk of occupational exposure. Human brucellosis affects all age groups.

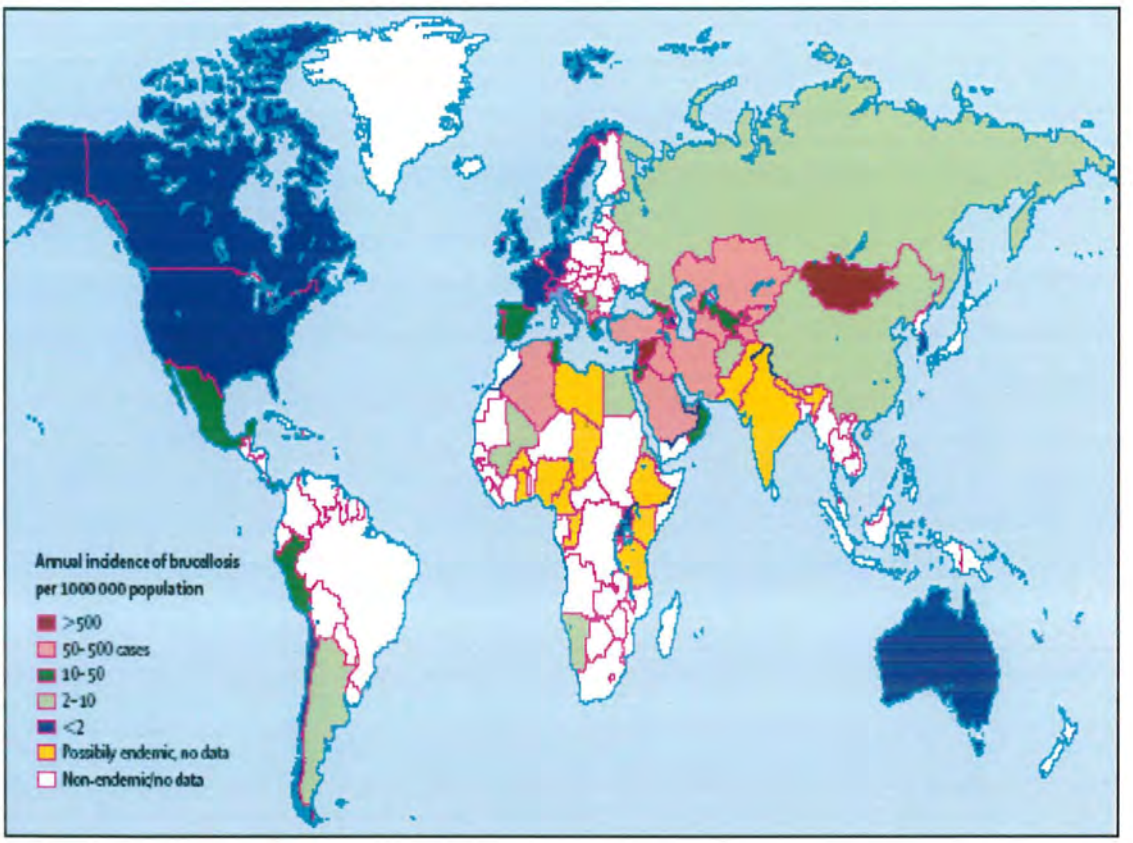


Fig. 5: World wide incidence of human brucellosis (Pappas *et al.*, 2006)

Table 2: **Global incidence of human brucellosis** (Pappas *et al.*, 2006).

Country	Annual cases per million of population	Country	Annual cases per million of population
Europe		**North America**	
Albania	63.6	Canada	0.09
Denmark	0.7	USA	0.4
France	0.5	Mexico	28.7
Germany	0.3	**Central and South America**	
Greece	20.9	Argentina	8.4
Itlay	0.9	Chile	0.6
Netherlands	0.5	Colombia	1.85
Norway	0.7	Peru	34.9
Russia	4.1	**Asia**	
Spain	15.1	Afghanistan	3.8
Switzerland	1.5	China	8
UK	0.3	India	No data, possibly endemic
Africa		Iraq	278.4
Algeria	84.3	Iran	328.6
Egypt	2.95	*Pakistan*	*No data, possibly endemic*
Ethiopia	Endemic, no data available	Saudi Arabia	214.4
Mali	2	**Oceania**	
Uganda	0.9	Australia	0.9

Modes of transmission of *Brucella*:

Brucella organisms enter the human body through several routes. These routes vary according to the endemic nature of the disease and the presence or absence of control and eradication programs.

Followings are the main routes of entry of *Brucella* organisms.

1- **Oral route:** Ingestion of unpasteurized infected milk and its products is one of the most common modes of transmission of disease in endemic countries (Busch and Parker, 1972).

2- **Respiratory route:** The risk of spread of *Brucella* organisms through inhalation depends on the traditions of animal husbandry in endemic countries. In rural areas, most of the farmers keep the animals in their houses. It is the main route of transmission in laboratory workers handling the *Brucella* cultures (Young, 2005).

3- **Cutaneous route:** Skin abrasions or accidental skin puncture during meat processing is the route of entry of *Brucella* organisms among abattoir workers (Flynn, 1983). Farmers and veterinarians involved in the process of delivering infected animals, with ungloved hands, also develop local skin lesions.

4- **Conjunctival route:** Accidental entry of *Brucella* organisms or splashing of live *Brucella* vaccine into the eyes during vaccination is a well-known route of entry among veterinarians (Williams, 1982).

5- **Blood transfusion:** Transmission of *Brucella* by blood transfusion from infected individuals, with subsequent development of disease in recipient has been well documented (Wood, 1955).

6- **Sexual transmission:** *Brucella* species have been cultured from human semen and a sexual link has been demonstrated (Wyatt, 1996).

7- **Transplacental transmission:** *Brucella* organisms can cross the placental barrier in pregnant women with active disease, causing abortion of fetus or brucellosis to the newborn (Madkour *et al.*, 1996).

8- **Transmission through breast milk:** A nursing mother with brucellosis may transmit the organisms to her infant through breast milk (Al-Abdely *et al.*, 1996).

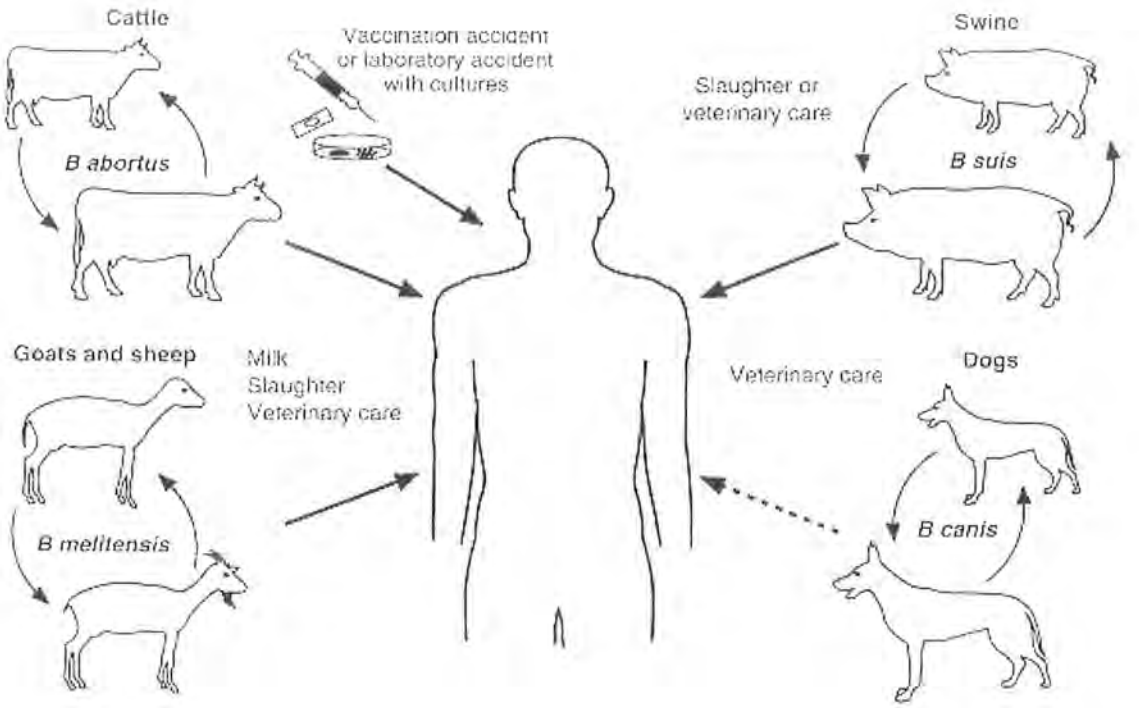


Fig. 6: Sources of transmission of brucellosis.

(http://www.gsbs.utmb.edu/microbook/ch028.htm)

Clinical manifestations in Brucellosis:

Brucellosis is a systemic disease that can involve any organ or system of the body. The onset of symptoms generally occurs within 2-3 weeks after exposure. The cardinal manifestation of human brucellosis is a fluctuating pattern of fever, due to which it is also called as undulant fever. Among other nonspecific symptoms are chills, profuse sweating, headache, arthralgia, myalgia, leg and back pain, malaise, fatigue, weight loss, inattention and depression. Fatalities are not common, though the syndrome can last for a few weeks to a year, even with treatment (Young, 1994). Most of the patients with brucellosis have complaints referable to multiple organs.

1. Osteoarticular Brucellosis:

Osteoarticular brucellosis involving bones and joints is the most common complication of brucellosis, occurring in 20-60% cases. Sacrolitis is found in most patients with back pain (Ariza *et al.*, 1993).

2. Hepatic Brucellosis:

The liver is mostly involved in brucellosis but often without giving symptoms. Hepatomegaly is present in 20-30% of cases. Brucellar hepatitis resolves completely with therapy (Ariza *et al.*, 2001).

3. Gastrointestinal Brucellosis:

This complication may mimic typhoid fever. Acute ileitis and colitis were reported in patients infected with *B. melitensis*, and in endemic areas, pancreatits has also been observed (Madkour, 2001).

4. Genitourinary Brucellosis:

Brucella are also excreted in urine but routine cultures are usually negative. Orchitis and epididymitis occur in up to 20% of men. In women, salpingitis, cervicitis and pelvic abscesses have been reported (Queipo-Ortuno *et al.*, 2006).

5. Cardiovascular Brucellosis:

Endocarditis occurs in less than 3% of cases. This complication is mostly associated with the death of the patient (Reguera *et al.*, 2003).

6. Neurobrucellosis:

Invasion of CNS occurs in less than 5% of cases. It causes neuroasthenia, encephalitis

and meningitis. In endemic areas, flaccid paralysis of upper and lower limbs has also been reported (Madkour, 2001).

7. Cutaneous Brucellosis:

In brucellosis, cutaneous lesions include dermatitis, rashes, soft tissue abscesses, ulcers and vasculitis.

8. Endocrinal Brucellosis:

Most commonly involved endocrine glands in brucellosis are testicles and epididymis. The other less frequently localization sites of *Brucella* are pituitary gland, pancreas, breast, ovary, placenta, adrenals and prostate (Vermigilio *et al.*, 1995).

9. Ocular Brucellosis:

Ocular manifestations of brucellosis is very rare but can lead to marked decreased in visual acuity, blindness, panophthalmitis and subsequent enucleation of eye (Tabbara, 1990).

10. Chronic Brucellosis:

The chronic brucellosis is defined as symptoms persisting for more than one year. Patients with chronic brucellosis fall into 3 categories.

(1) Those with bacteriologic relapse.

(2) Those with deep focus of infection.

(3) Those with nonspecific symptoms that are apparently related to active infection.

Of these, the latter appears to be the most common.

11. Pregnancy and Brucellosis:

Brucellosis can occur at any period of gestation in humans but is most commonly reported in the first trimester (Madkour *et al.*, 1996). The most commonly involved species is *B. melitensis*. The onset of symptoms in pregnant women are usually abrupt with fever, chills, sweating and generalized aches and pain with abortion in 41% of cases.

12. HIV and Brucellosis:

The HIV-patients are vulnerable to many opportunistic infections. The association between HIV and brucellosis has recently been reported (Galle *et al.*, 1997). Brucellosis in HIV-patients is oftenly misdiagnosed as an opportunistic infection caused by other organisms.

Fig. 7: Arthritis due to brucellosis.

(http://epivet.blogspot.com/2007_02_01_archive.html)

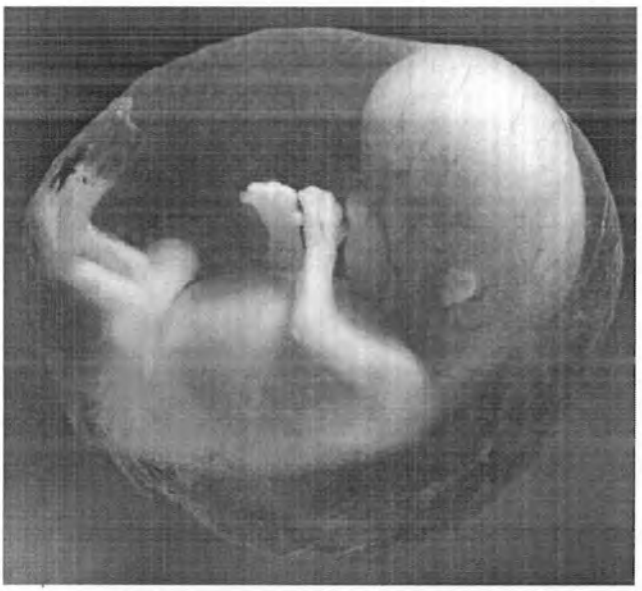


Fig. 8: Aborted Human Fetus (first trimester)

(http://www.newsbusters.org/blogs/brent-bozell/2008/01/15/bozell-column-ban-word-fetus)

Veterinarian

Mostly husbandry practices are done by females in the villages.

Farmer

Butchers

Fig. 9: Occupationally exposed workers

Diagnosis:

The patient's history is very important in diagnosis. The diagnosis of human brucellosis is based on epidemiological evidence, clinical presentation and results of laboratory investigations (Abdul-jabbar, 1994). The clinical findings are often non specific and systemic brucellosis can be misdiagnosed and confused with other diseases such as typhoid, rheumatic fever, spinal tuberculosis, pyelitis, cholecystitis, tumor of testis and thrombophlebitis (Young, 1995). In addition, overlap in the clinical features makes diagnosis of various stages of brucellosis difficult, particularly when the time of onset is unknown. Diagnosis of chronic and complicated cases becomes difficult when the symptoms are mild or atypical or when there are reasons to suspect intercurrent disease (Young *et al.*, 1985). Following techniques are used for diagnosis of brucellosis.

1. Culture:

Successful culture of *Brucella* is confirmatory, but the yield of positive culture from clinical specimens especially in chronic cases and neurobrucellosis, is very low, i.e. less than 20% (Lulu *et al.*, 1988). Moreover, culture poses a great risk for laboratory workers. For *Brucella* culturing, biosafety level 3 labs are required.

2. Serology:

Serological tests for brucellosis are more useful in diagnosis than culture (Lulu *et al.*, 1988). Conventional serological procedures e.g. Serum Agglutination Test (SAT), Rose Bengal Test (RBT), Compliment Fixation Test (CFT) and Enzyme Linked Immunosorbant Assay (ELISA) are all based on detection of anti-LPS antibodies, which remain very high even after recovery of disease (Goldbaum *et al.*, 1994).

(a) Serum Agglutination Test:

SAT developed by Wright and colleagues (Wright and Smith, 1897) remains the most popular and yet used worldwide diagnostic tool for the diagnosis of brucellosis because it is easy to perform, does not need expensive equipments and training. SAT measures the total quantity of agglutinating antibodies (IgM and IgG) (Young, 1991). SAT titres above 1:160 are considered diagnostic in conjunction with a compatible clinical presentation.

However, in areas of endemic disease, using a titre of 1:320 as cutoff may make the test more specific.

(b) Rose Bengal Test and Compliment Fixation Test:

The most widely used buffered antigen method is RBT. The RBT shows a high degree of correlation with SAT and is found to be useful screening method for large number of sera (Diaz *et al.*, 1976). CFT was first used in 1912 for the diagnosis of brucellosis (Larson, 1912). It is relatively insensitive to antibodies arising from immunization of *Brucella* vaccines. However, CFT is laborious and reagent intensive, and some antisera show anti-complimentary activity.

(c) Enzyme Linked Immunosorbant Assay:

ELISAs are more sensitive than conventional tests used in the diagnosis of human brucellosis by detecting more positive sera, higher titers and different classes of immunoglobulins (Ariza *et al.*, 1992). A close correlation between the persistence of IgG and IgA in the sera of some individuals and the chronicity of disease has been pointed out (Ariza *et al.*, 1992). Hence, IgG, IgA and IgM estimations are used to differentiate between acute and chronic human brucellosis patients. Acute cases have elevated titers of IgM alone or IgG, IgA and IgM while chronic cases have high titers of IgG and IgA.

3. Skin Test:

Brucellins are protein antigenic preparations, which are used to detect brucellosis by intradermal injection. Recent studies with ribosomal protein L7/L12 from *B. melitensis* was shown to induce strong delayed type hypersensitivity reaction in *Brucella* sensitized guinea pigs (Bacharach *et al.*, 1994), suggesting that this test might be used for diagnostic purpose in future.

4. Polymerase Chain Reaction:

The development of a specific PCR is a recent advance. PCR is fast, can be performed on any clinical specimen and can yield positive results as soon as 10 days after inoculation. PCR is used for the detection of DNA from different microorganisms (Cogswell *et al.*,

1996). Different target genes, primer pairs, PCR techniques and extraction procedures have previously been published for *Brucella* detection (Fekete *et al.*, 1990). These PCR assays have been used for the detection of both human and animal brucellosis (Leal-Klevezas *et al.*, 1995).

A number of nucleic acid sequences have been targeted for the development of *Brucella* genus-specific PCR assays, including 16S rRNA, the 16S-23S intergenic spacer region, *omp2* and *bcsp31* (Navarro *et al.*, 2002). The first published PCR based diagnostic assay was reported by Fekete and coworkers (Fekete *et al.*, 1990). This assay amplified a sequence from a gene encoding a 43-kDa outer membrane protein of *B. abortus* strain S19. Although these authors demonstrated that this assay was specific for *Brucella* and very sensitive, the primers and target sequence were never published (Bricker *et al.*, 1988). The next *Brucella* gene to be explored was the 16S rRNA gene (Herman and De Ridder, 1992). To assess specificity, the assay was applied to a panel of 17 other bacteria. No products were amplified from any non-*Brucella* species except *Ochrobactrum anthropi*, the closest known relative to *Brucella* (Bricker *et al.*, 1988).

Baily and coworkers published a new PCR assay based on the BCSP 31 gene encoding 31-kDa *Brucella abortus* protein (Baily *et al.*, 1992). This gene is conserved in all species and biovars of *Brucella* examined, however it is not expressed in *B. ovis* (Da Costa *et al.*, 1996). The specificity was only applied to *B. melitensis* and *B. abortus*. No other *Brucella* species were included in the panel of bacteria used to evaluate the procedure, nor was the close relative *Ochrobactrum anthropi* (Bricker *et al.*, 1988). However, a more thorough investigation was reported by Da Costa and coworkers in which all the *Brucella* species and biovars were tested and recognized (Bricker, 2002). This group also surveyed 98 non *Brucella* bacteria and found that all organisms except one strain of *Ochrobactrum anthropi* were negative for amplification (Bricker *et al.*, 1988). In 2001, Casanas and coworkers also studied the specificity of primers described by Baily and results obtained were the same as for Da Costa and coworkers (Casanas *et al.*, 2001).

Romero and coworkers analyzed nine different primer combinations derived from 16S rRNA of *Brucella abortus* (Romero *et al.*, 1995). All bacterial species examined were negative except *O. anthropi*. In 1995, Leal- Klevezas and coworkers described a new pair of primers homologous to regions of gene encoding an outer membrane protein (omp-2) reported to be from *B*rucella *abortus* (Leal-Klevezas *et al.*, 1995). These nucleotides amplified *Brucella* species excluded *B. suis*, biovars 2, 3 and 4, *B. canis* and *B. ovis*. No amplification was detected with DNA from closely related bacteria, including *O. anthropi*.

Matar and coworkers (Matar *et al.*, 1996) and more recently Tuncer and coworkers (Herman and De Ridder, 1992) have developed a nested PCR method for brucellosis to increase the sensitivity and specificity. In nested PCR, an aliquot of the amplification product is subjected to another round of PCR with a set of primers located internal to the first set. In this assay, it is very difficult to avoid false positive reactions due to contamination of second round of PCR with genomic DNA or amplified sequences.

Redkar and coworkers described real-time PCR assays for the detection of *B. abortus, B. melitensis and B. suis* biovar1 (Redkar *et al.*, 2001). These PCR assays target the specific integration of IS711 elements within the genome of the respective *Brucella* species or biovar. Recently, Queipo-Ortuno and coworkers described the rapid diagnosis of human brucellosis by serum quantitative real time PCR. This new method combines SYBRGreen® I technology and the LightCycler® real time detection system using primers described by Baily and coworkers, which amplify a 223bp fragment present from a gene encoding a 31-kDa *Brucella* antigen.

Currently, a real-time multiplex PCR assay has been developed for rapid confirmatory identification of *Brucella* with speciation. The genus, *B. abortus* and *B. melitensis* specific primers confirm the organism from isolates (Probert *et al.*, 2004). One case of neurobrucellosis was confirmed in laboratory with the CSF being positive by PCR but undetectable from the blood. The agglutinins were positive in CSF and blood. However,

culture of blood and CSF was negative showing the utility of molecular methods in tertiary care centers.

The advantages of PCR for the diagnosis of human brucellosis are its sensitivity and specificity. Furthermore, all types of clinical samples, even those containing an extremely low number of microorganisms, can be analyzed. There are two limitations of PCR assay: false positive and false negative results. The ability of PCR to generate many copies of a target sequence from minute quantities of template DNA necessitates that extreme care be taken to avid the generation of false positives. Although false positives can result from sample to sample contamination, a most serious source is the carry-over of DNA from a previous amplification reaction of the same target. To avoid false positives, specific precautions to control contamination must be taken.

To avoid false negatives, it is important to control the inhibitory compounds in blood samples, such as heme compounds (porphyrins and heme-blood protein complexes), anticoagulants and large amounts of total DNA of the host.

Although PCR is very promising, standardization of extraction methods, infrastructure, equipment and expertise are lacking and a better understanding of the clinical significance of the results is still needed (Navarro *et al.*, 2004). The use of molecular methods in *Brucella* endemic areas needs to be explored before they can be used in these areas to diagnose brucellosis.

Treatment:

The prerequisites for an effective therapy are: treatment should start on time, should consist of combination of drugs along with at least one drug having a good penetration into cells and should be prolonged. The treatment of human brucellosis is a controversial area because of the spectrum of disease, the possibility of chronic infection and the development of complications (Radolf, 1994). In all cases, it is important that the patient completes the full course of therapy because the risk of incomplete recovery and relapse

is otherwise increased considerably. In 1986, the World Health Organization issued guidelines for the treatment of human brucellosis. The guidelines discuss two regimens, both using doxycycline for a period of six weeks, in combination with either streptomycin for two to three weeks or rifampin for six weeks. Both combinations are the most popular treatments worldwide, although they are not used universally. The streptomycin containing regimen is slightly more efficacious in preventing relapse (Bingol and Togay-Isikay, 2006). This may be related to the fact that rifampin down-regulates serum doxycycline levels (Wise, 1980). However, parenteral administration of streptomycin mandates either hospital admission or the existence of an adequate health care network. Both of these fascilities are often absent in areas of endemic disease. On the other hand, the use of rifampin in areas in which brucellosis is endemic, where tuberculosis is also endemic, raises concern about the development of community resistance to rifampin.

Alternative drug combinations have been used, including other aminoglycosides (e.g., gentamicin and netilmicin) (Corbel, 1997). Trimethoprim–sulfamethoxazole is a popular compound in many areas, usually used in triple regimens. Quinolones are an alternative. Various combinations that incorporate ciprofloxacin and ofloxacin have been tried clinically, yielding similar efficacy to that of the classical regimens. Although quinolones have been used but the cost of this approach remains a major drawback.

Rifampin is the mainstay of treatment in cases of brucellosis during pregnancy, in various combinations. Brucellosis in children is treated with combinations that are based on rifampin and Trimethoprim–sulfamethoxazole and with aminoglycosides. In brucellosis, relapses occur at a rate of about 10% and are often milder in severity than the initial disease and can be treated with a repeated course of the usual antibiotic regimen. Table 3 summarizes information about the various antibiotics that are used to treat brucellosis.

Table 3: Antibiotics used in the treatment of Human Brucellosis (Pappas *et al.*, 2005)

Antibiotic	Dose and duration of therapy	Combinations
Doxycycline	100mg twice daily for 6 weeks	Doxycycline combined with streptomycin, with rifampin, with gentamicin or with ciprofloxacin; doxycycline and streptomycin combined with rifampin or trimethoprim-sulfamethoxazole; doxycycline combined with rifampin and trimethoprim-sulfamethoxazole.
Streptomycin	15mg/kg/day I/M for 2-3 weeks	Streptomycin and doxycycline; Streptomycin and doxycycline combined with rifampin or trimethoprim-sulfamethoxazole.
Rifampin	600-1200 mg/day for 6 weeks	Rifampin and doxycycline; rifampin and doxycycline combined with streptomycin or trimethoprim-sulfamethoxazole; rifampin and ofloxcin; rifampin and ciprofloxacin.
Trimethoprim-sulfamethoxazole	290mg twice daily for 6 weeks	Trimethoprim-sulfamethoxazole combined with doxycycline, with rifampin, or with streptomycin; Trimethoprim-sulfamethoxazole and doxycycline combined with rifampin, or with streptomycin.
Gentamicin	5mg/kg/day in 3 divided I/V doses for 5-7 days.	Gentamicin and doxycycline.
Ofloxacin	400mg twice daily for 6 weeks	Ofloxacin and rifampin.
Ciprofloxacin	500mg twice daily for 6 weeks	Ciprofloxacin with doxycycline or rifampin.

Prognosis:

The prognosis of brucellosis is generally very good. With appropriate treatment, most patients with brucellosis recover within weeks or months. However, treatment of patients with cardiac or central nervous system involvement is little difficult (Reguera *et al.*, 2003; Madkour, 2001).

Human *Brucella* Vaccines:

A vaccine has not been developed for human brucellosis. Although there are adequate scientific and financial tools for such development in some quarters, knowledge is still incomplete about the molecular pathogenesis of brucellosis. Numerous vaccines have been tested in the past, but none of them have gained wide acceptance (Caksen *et al.*, 2002).

A derivative of strain 19, strain 19-BA, given intradermally by scarification, was formerly used in the Asian republics of the former USSR. It gave limited protection for a relatively short duration and re-immunization was necessary, but was accompanied by an increasing frequency of hypersensitivity reactions (Kolar, 1989). Other attenuated strains, such as *B. abortus* 84-C and 104-M given intradermally or as aerosols were also used in China and the USSR (Lu and Zhang, 1989). Although apparently effective, these vaccines could provoke severe reactions if not administered correctly or if given to sensitized individuals, and they appear to be no longer in routine use.

Attempts have been made to develop sub-unit vaccines for use in humans. An SDS-insoluble peptidoglycan fraction of *Brucella melitensis* M15 was used in France (Roux and Serre, 1971), but conclusive evidence of efficacy based on double blind controlled clinical trials was not obtained (Hadjichristodolou *et al.*, 1994). Similarly, an acetic acid extracted polysaccharide-protein fraction developed in the USSR was reported to have low reactogenicity even in previously vaccinated individuals, but evidence of protective efficacy derived from controlled clinical trials is still awaited. It is unclear if more recent approaches such as lipopolysaccharide-protein conjugates (Jacques *et al.*, 1991) or purified protein antigens such as L7/L12 (Oliveira and Splitter, 1996) or Cu–Zn SOD (Tabatabai and Pugh, 1992) or glyceraldehyde dehydrogenase (Rosinha *et al.*, 2002)

would be effective in humans but they seem worth considering. Recent efforts have focused on developing live attenuated strains. Strain RB51 has minimal human pathogenicity and would seem to have potential but its rifampicin resistance makes it an unsatisfactory candidate for human vaccination. Other efforts have focused on *purE* mutants (Hoover *et al.*, 1999), but these may retain too much residual virulence. Other candidates such as *rfbK* mutants of *B. melitensis* may offer a useful starting point. The World Health Organization has indicated the need for further study in this field (Anon, 1997).

A major difficulty affecting the development of a vaccine against human brucellosis, is the absence of well-established correlates of protection. Although not usually a lethal infection, performance of challenge experiments in human subjects is likely to encounter ethical objections. This means that reliance will have to be placed on animal models. Both the mouse model and guinea pig model have a role to play in human vaccine development. The former is the most useful for monitoring vaccine consistency, whereas the latter may give a better indication of performance in an out-bred population. Any candidate vaccine will probably also need to be assessed for protective efficacy in non-human primates. It would seem advisable to use a vaccine with a known track record in humans, such as *B. abortus* strain 19-BA, as a baseline reference for efficacy. The cost implications and limited commercial possibilities for vaccines against human brucellosis mean that development is likely to be restricted to national defense agencies.

In animals, live, attenuated and killed vaccines against brucellosis are used. Two main live, attenuated vaccines used to control *B. abortus* infection in cattle are *B. abortus* strain S19 and *B. abortus* strain RB51. The only approved live attenuated vaccine to control *B. melitensis* infection is *B. melitensis* Rev.1. Killed vaccines against brucellosis are *B. abortus* strain 45/20 and *B. melitensis* strain H38.

Prevention and control of brucellosis:

Prevention of human brucellosis is dependent on control of the disease in domestic livestock mainly by mass vaccination (Nicoletti, 2001). In many countries, the use of *B. abortus* strain RB51 vaccine in cattle and *B. melitensis* strain Rev.1 vaccine in goats

and sheep has resulted in the elimination or near-elimination of brucellosis in these animals. 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. In spite of the clinical efficacy and cost effectiveness of vaccination, the limited availability of vaccines and lack of awareness has led to the persistence of brucellosis in most countries including Pakistan. The lack of human vaccines and effective control measures make it necessary for the doctors and other health care workers to take protective measures. Protective clothing/barriers while handling still births/products of conception and cultures can reduce occupation-related brucellosis (Madkour, 2001). The avoidance of unpasteurised dairy products will prevent infection in the general population (Busch and Parker, 1972).

Brucellosis in Pakistan and South-East Asian countries:

In the South-East Asian region, most of the countries are agricultural and majority of population is involved in land cultivation and livestock farming. The large number of human population is exposed daily to a huge number of animal population and their excreta. This is particularly the case with dairy production units which are increasing in rapidly growing cities of the SAARC (South Asian Association for Regional Cooperation) countries including Pakistan, India, Bangladesh, Sri Lanka and Nepal. Unfortunately, there is no veterinary public health unit of the World Health Organization (WHO) in most of SAARC countries (Joshi, 1991). Consequently, farmers and their families are helpless to work in the poor hygienic conditions. The occurrence of acute, often most incapacitating infection in humans caused by *B. melitensis*, usually coincides with the outbreak of brucellosis in sheep and goats (Abdussalam and Fein, 1976).

The first case of brucellosis dates back to 1942 in the Indian part of Indo-Pak subcontinent, and the disease was reported in cattle, buffaloes, sheep, goats, pigs, dogs and humans (Renukaradhaya *et al.*, 2002). Except a few published reports, there is no detailed data of brucellosis in human and animal population in Pakistan. Human cases of brucellosis have reported in mid 1980's in Multan region of Pakistan (Noor *et al.*, 1986). A sero-diagnostic survey of human brucellosis among T.B patients in Islamabad,

Pakistan revealed 19.2% seropositivity for brucellosis with slide and semi-quantitative agglutination tests (Qazilbash and Bari, 1997). According to the annual report released in the year 2000 by Ministry of Food, Agriculture and Livestock of Pakistan, prevalence of brucellosis in cattle and buffaloes ranged from 8.8 to 16.8% and 7.6 to 10.5%, respectively.

On the other hand, there are a number of published reports on human and animal brucellosis in India. The sero-epidemiological studies in India indicate that abattoir personnel are at high risk to brucellosis compared to other exposed population. Some workers have reported a prevalence of 5.3% of brucellosis in animal handlers working in urban area of India (Mudaliar *et al.*, 2003) showing presence of antibodies to *B. abortus*, while others documented 25.5% abattoir-associated positive cases for brucellosis employing Dot-ELISA (Barbuddhe *et al.*, 2000).

Brucellosis is a suspected risk factor among the unknown causes of spontaneous abortion in women. It has been reported that 6.5% of women in Gujrat state of India, who aborted spontaneously, were found to be positive for *Brucella* agglutinins (Mudaliar *et al.*, 2003).

As brucellosis is a chronic debilitating disease, resulting in severe economic losses and is a world wide re-emerging zoonoses, the present study is therefore designed to accomplish the following objectives;

1. To develop PCR based method for the diagnosis of brucellosis.
2. Application of PCR assay in occupationally exposed workers (Veterinary professionals, Farmers, Ladies involved in rearing of animals with abortion history and Butchers).
3. Comparison of the conventional serological methods (e.g. Serum Agglutination Test) with PCR.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

Materials and methods for investigation used in this study collected from different sources are given below.

***Brucella abortus* culture:**

Brucella abortus culture was taken from the Institute of Animal Sciences, NARC, Islamabad. This culture was used as a positive control to optimize the PCR conditions for brucellosis.

Suspected Clinical Field Samples:

A total of 90 blood samples were taken from the suspected occupationally exposed workers from different areas of Faisalabad district. They comprised of veterinary professionals (33), farmers (20), females involved in rearing of animals with abortion history (32) and butchers from the local meat shops (5).

A detailed history of these 90 occupationally exposed workers was taken which included their name, age, address, occupation, nature of work in their respective profession, history of consuming raw milk, history of fever (nature and duration) in the past and complaints of joint pain or any other complication if any.

Venous blood (5ml) was drawn from each individual aseptically. Out of this 5ml blood, 3ml was put in vacuutainers containing anticoagulant (EDTA) for DNA isolation while the rest of the blood (2ml) was put in vacuutainers without any anticoagulant for serum separation. Serum was separated out of the blood immediately by centrifugation for 2-3 minutes. This serum was used in the Serum Agglutination Test (SAT) for the detection of antibodies against *B. abortus* and *B. melitensis*. Both the serum and blood samples were stored at -20°C for further use.

Reaction Control:

Positive and negative controls were also included in this study to make it more authentic and reliable.

Positive Control:

A positive control was included in each test to ascertain the accuracy of method used for DNA isolation and reactivity of PCR amplification steps. DNA isolated from *B. abortus* culture was used as positive control in the PCR.

The positive control DNA extracted from *B. abortus* culture would react with *Brucella* genus specific primer sets (Table 4).

Negative Control:

A negative control was also included in each test to screen for the contamination of reagents and consumables used for DNA isolation and PCR amplification. The extraction of DNA was performed without any sample to assure the quality of solutions used for DNA isolation purpose. PCR amplification was carried out without adding DNA to assure that the reaction mixture is free from any contamination.

Immuno/Bactol Febrile Antigens:

Immuno/Bactol Febrile Antigens (*B. abortus* & *B. melitensis*) were purchased from Immunostics, Inc., USA. Both of these antigens are 0.5% phenol killed and are available in separate vials. These antigens were used in Serum Agglutination Test (SAT). These antigens are designed for the detection of specific febrile antibodies with increased sensitivity, specificity and overall readability.

1. DNA ISOLATION:

DNA was isolated from the *Brucella abortus* culture and all the blood samples collected from the occupationally exposed workers. The DNA from *B. abortus* culture was extracted by using Genomic DNA purification kit and CTAB methods. The DNA from all the blood samples was extracted by using Genomic DNA purification kit.

1.1 DNA isolation from *Brucella abortus* culture:

1.1.1 CTAB (Cetyltriethylmethylammoniumbromide) method:

1. Take 1.5ml bacterial (*Brucella abortus*) culture in an eppendorf; centrifuge it to make the pellet.
2. Dissolve the pellet in 567µl of TE buffer by vortexing.
3. Add 30µl of 10% SDS and 3µl of Proteinase-K (20mg/ml), mix and incubate for 1 hour at 37°C.
4. Add 100µl of 5M NaCl and 80µl of CTAB, mix thoroughly and incubate for 10-15 minutes at 65°C.
5. Add equal volume (780µl) of Chloroform/Isoamylalcohol. Mix thoroughly and centrifuge for 4-5 minutes.
6. Transfer the supernatant to a fresh tube.
7. Add equal volume of Phenol/Chloroform/Isoamylalcohol. Mix and centrifuge for 5 minutes.
8. Transfer the supernatant to a fresh tube.
9. Add 1ml of absolute ethanol and gently mix until the DNA precipitate. Keep it at -20°C for 1 hour.
10. Centrifuge for 2-3 minutes and discard the supernatant.
11. Wash the pellet with 1ml of 70% ethanol.
12. Centrifuge for 2-3 minutes and discard the supernatant.
13. Air-dry the pellet.
14. Dissolve the pellet in 30-40µl of TE buffer or PCR water.

1.1.2 Genomic DNA purification kit method:

1. Take 10-20mg of bacterial (*Brucella abortus*) culture and resuspend in 1.5ml eppendorf containing 200µl TE buffer.
2. Add 400µl of Lysis solution and mix it.
3. Incubate at 65°C for 5 minutes.
4. Add 600µl Chloroform and gently emulsify by inversion.
5. Centrifuge at 10,000 rpm for 2 minutes.
6. Transfer the upper aqueous phase containing DNA to a fresh tube.
7. Add 800µl precipitation solution (To prepare fresh working precipitation solution, mix 720µl nuclease free water with 80µl of supplied 10X concentrated precipitation solution.).
8. Mix at room temperature for 1-2 minutes.
9. Centrifuge at 10,000 rpm for 2 minutes.
10. Remove the supernatant completely.
11. Dissolve the DNA pellet in 100µl of 1.2M NaCl solution. Make sure that the pellet is completely dissolved.
12. Add 300µl cold absolute ethanol; let the DNA precipitate (10 minutes at -20°C). This time can be extended upto overnight stay at -20°C.
13. Centrifuge at 10,000 rpm for 6-8 minutes.
14. Discard the supernatant and keep the pellet.
15. Wash the pellet once with 100µl 70% cold ethanol.
16. Let the pellet dry completely.
17. Dissolve the DNA pellet in 100µl of autoclaved double distilled deionized water.

1.2 DNA Isolation from blood samples:

1.2.1 Genomic DNA purification kit method:

1. Mix 200µl sample (blood) with 400µl Lysis solution.
2. Incubate at 65°C for 5 minutes.
3. Add 600µl chloroform and gently emulsify by inversion.
4. Centrifuge at 10,000 rpm for 2 minutes.
5. Transfer the upper aqueous phase containing DNA to a fresh tube.
6. Add 800µl precipitation solution (To prepare fresh working precipitation solution, mix 720µl nuclease free water with 80µl of supplied 10X concentrated precipitation solution.).
7. Mix at room temperature for 1-2 minutes.
8. Centrifuge at 10,000 rpm for 2 minutes.
9. Remove the supernatant completely.
10. Dissolve the DNA pellet in 100µl of 1.2M NaCl solution. Make sure that the pellet is completely dissolved.
11. Add 300µl of cold absolute ethanol; let the DNA precipitate (10 minutes at -20°C). This time can be extended upto overnight stay at -20°C.
12. Centrifuge at 10,000 rpm for 6-8 minutes.
13. Discard the supernatant and keep the pellet.
14. Wash the pellet once with 100µl of 70% cold ethanol.
15. Let the pellet dry completely.
16. Dissolve the DNA pellet in 100µl of autoclaved double distilled deionized water. [Typical yield of genomic DNA is 2-10µg from 200µl of blood.]

2. Serum Agglutination Test (SAT):

The principle of this test is an immunologic reaction (agglutination) between the antibodies produced to viable bacteria (agglutinins) and their counterpart febrile antigens.

Procedure:

1. Bring antigens and sera to room temperature before use.
2. Take a clear, transparent glass slide.
3. Take a drop of serum (20-30µl) on this glass slide.
4. Shake the antigen reagent to insure uniform suspension.
5. Add a drop of antigen just below the drop of serum.
6. Mix the antigen and serum well using a piece of sterilized applicator stick or tooth pick. Use a separate applicator stick or tooth pick for each reaction.
7. Gently rock the slide back and forth for no more than one minute.
8. Observe the agglutination using any good indirect light against a dark background or we can also check agglutination under the microscope.

Interpretation of results:

1. Positive Serum Agglutination Test is indicated by the presence of agglutination/ clumping within 5 minutes (fig. 13).
2. Negative Serum Agglutination Test shows no such agglutination/clumping (fig. 14).

Precautions:

1. All sera to be tested should be clear and free from bacterial contamination.
2. Don't heat sera prior to testing.
3. Shake antigen reagent well before use to ensure smooth, uniform suspension.
4. All reagents should be refrigerated at 2-8°C when not in use.
5. Antigen reagents must not be frozen.

3. PCR amplification:

PCR amplification was carried out using *Brucella* genus specific primers. These primers amplify 223bp region of BCSP 31 gene of *Brucella* genus. The components and quantities of the PCR mix are as follows.

10X PCR buffer	5.0µl
$MgCl_2$ (25mM)	3.0µl
10mM dNTP's mix	1.0µl
Primer 1(BGS 2F)	1.0µl
Primer 2(BGS 2R)	1.0µl
Taq DNA polymerase (5U/µl)	0.5µl
ddH_2O	18.5µl
DNA	20.0µl
Total	50.0µl

Primer description (*Brucella* genus specific, BCSP 31 gene based)

Primers	F/R	Sequence	Band size
BGS 2	Forward	5′TGGCTCGGTTGCCAATATCAA3′	223bp
BGS 2	Reverse	5′CGCGCTTGCCTTTCAGGTCTG3′	

Table 4: Sequence of *Brucella*, genus-specific primers.

4. Analysis of PCR products (Agarose gel electrophoresis):

1. The amplified PCR products were analyzed by electrophoresis at 90V and 300mA in 1.5% agarose gel for one hour and twenty minutes using 1X TAE buffer.
2. Desired amount of agarose (1.5g) was taken in flask containing the electrophoresis buffer 1X TAE (100 ml), melted in microwave oven for 2-3 minutes and swirled to ensure even mixing.
3. Casting tray was prepared by wrapping meshing tape on its both sides and a suitable comb was adjusted on it.
4. The melted agarose was cooled to 45°C and ethidium bromide (5µl) was added into it, before pouring on the gel casting tray.
5. The gel thickness was kept in the range of 0.5-1.0 cm. The air bubbles were removed and it was kept at room temperature for solidification.
6. After solidification the comb was removed carefully to avoid tearing of wells. The mesh tape was also removed from the sides of the casting tray.
7. The gel casting tray containing the gel was placed in the electrophoresis tank, having 1X TAE buffer.
8. PCR products (10-15µl) were loaded on the gel in the respective wells after mixing with appropriate amount (3µl) of Invitrogen's Blue Juice (6X gel loading buffer).
9. 100 base pair DNA marker was used (fig. 11).
10. Electric current (90V, 300 mA) was applied until the blue dye was about the three quarters the way down the gel (approximately 80 minutes).
11. Gel was taken out of the electrophoresis tank when the due had reached near the anode part of the gel.
12. Gel was then photographed under the UV transilluminator at a wavelength of 254nm with Eagle Eye Gel Documentation System (Stratagene, USA).

5. Elution of DNA fragments from agarose gel using DNA Extraction Kit:

Silica powder suspension

1.5 ml specially prepared glass bead suspension in water.

Binding solution

125 ml of 6M Sodium Iodide solution.

Procedure

1. Reaction product containing DNA was loaded on agarose gel and the gel was allowed to run for the separation of reaction products. The gel slice containing DNA band was excised and approximate volume of gel slice was determined by weight (1g) and the gel slice was placed in an eppendorf tube.
2. On the basis of 1 volume of gel, 3 volumes of the binding solution were added. It was incubated at 55°C for 10 minutes to dissolve agarose gel.
3. About 6 µl of silica powder suspension was added. It was then vortexed gently and incubated at 55°C for 5 minutes. The components were mixed by vortexing after every 2 minutes to keep silica powder in suspension. Centrifuge for 5 minutes at 14000 rpm to form pellet and discard the supernatant.
4. At least 500 µl of washing buffer was added, vortexed, and spun for 10 seconds at 13500 rpm. The supernatant was again removed and this procedure was repeated for 3 times. The pellet should be resuspended completely during each washing.
5. After the supernatant from the last wash was removed, the eppendorf was spun again and the remaining liquid was removed with help of pipette. The pellet was air dried for 10-15 minutes.
6. After drying, the pellet was resuspended in 20 µl of sterile deionized H_2O or TE buffer and the eppendorf tube was incubated at 55°C for 5-10 minutes.
7. The tube was spun and the supernatant was shifted in to a new tube avoiding the pellet. The supernatant contains the eluted DNA. To determine the amount of eluted DNA, run 2 µl of this on agarose gel along with control.

6. Cloning and Sequencing of PCR product:

There are four steps in the cloning experiment.

(1) Ligation of PCR product in a suitable vector.
(2) Transformation.
(3) Plasmid isolation.
(4) Restriction analysis.

6.1 Ligation:

PCR products were ligated in TA cloning vector (pTZ57R/T) (Fig. 10) as shown below.

Vector (pTZ57R/T)	2.0µl
PCR product	5.0µl
Ligation buffer (10X)	2.0µl
PEG 4000	2.0µl
T4 DNA Ligase	1.0µl
ddH_2O	8.0µl
Total volume	20.0µl

The reaction mixture was incubated overnight at 16°C.

Preparation of Heat Shock competent cells of *Escherichia coli* (Top 10 α DH strain):

Heat shock competent cells of *E. coli* Top 10 α DH strain were prepared by the following method.

1. A selected colony from freshly grown plate of *E. coli* Top 10 α DH strain was picked and inoculated into 10ml of LB liquid medium without any antibiotic, in 100ml autoclaved conical flask using sterile toothpick. It was incubated at 37°C for overnight with vigorous shaking.
2. Next day overnight grown culture 2.5ml was re-inoculated into 250ml of the liquid LB medium (in 500ml flask) and was incubated at 37°C until OD_{600} of cells become 0.5-1.0 (10^{10} cells/ml)(approx. 3 hours).

3. Under aseptic conditions, 20ml of cultured media poured into the falcon tubes and were placed on ice.
4. The tubes were centrifuged at 5000 rpm for five minutes at 4°C.
5. The supernatant was decanted and the cells pellet was obtained. The pellet was dissolved in 10ml of $MgCl_2$ under aseptic conditions. Dissolve the pellet slowly.
6. The cells were centrifuged at 3000 rpm for 5 minutes at 4°C.
7. The supernatant was discarded; pellet was made and resuspended in 10ml $CaCl_2$ under aseptic conditions.
8. Falcon tubes were chilled for 30 minutes.
9. Centrifugation was done at 3000 rpm for 5 minutes at 4°C.
10. The supernatant was decanted and finally the cells were resuspended in 1ml of $CaCl_2$ and 0.7ml of 10% sterilized cold glycerol.
11. 200µl of cells were placed in separate aliquots and were stored at -70°C for further use.

6.2 Transformation:

Transformation of ligated PCR product in *E. coli* Top 10 α DH strain was performed by heat shock method. The following protocol was used for transformation:

1. 5µl plasmid (ligated PCR product) was added in 200µl cells aliquot and kept on ice for 30 minutes.
2. Heat shock was given on dry bath at 42°C for 2 minutes.
3. Cells were kept on ice for 2 minutes.
4. 1ml of LB (broth) was added in the mixture.
5. Cells were kept at 37°C for 1 hour.
6. Cells were pelleted by centrifugation at 13200 rpm for 2 minutes and supernatant was removed.
7. Dissolve the pellet in 100µl remaining supernatant and the whole mixture was spreaded on the selection LB plate.

Results:

White colonies that appear on the plate have insert while the blue colonies have no insert.

6.3 Plasmid isolation from *E. coli* Top 10 α DH strain:

6.3.1 Plasmid isolation by Alkaline Lysis Method:

1. A single *E. coli* colony (white colony) was cultured in 5ml liquid LB (Tryptone 2.5g, Yeast extract 1.3g, NaCl 1.3g for 250ml) medium containing ampicillin and grown overnight at 37°C with shaking.
2. The *E. coli* culture was centrifuged in 1.5ml eppendorf tube at 14000 rpm for 5-10 minutes.
3. The supernatant was discarded and the pellet was allowed to dry for 2 minutes.
4. 100µl of solution I was added to eppendorf and the pellet was suspended in the solution with the help of vortex.
5. 200µl of solution II was added to the eppendorf and mixed gently by inverting the eppendorf 5-10 times.
6. 200µl of solution III was added to eppendorf, mixed well and centrifuged at 14000 rpm for 20 minutes.
7. The supernatant was taken in fresh eppendorf and discard the pellet.
8. Equal volume of chloroform was then added and incubated at 37°C for 5 minutes.
9. Tubes were then centrifuged at 13200 rpm for 5 minutes. Clear supernatant was taken in new eppendorf tube and equal volume of Isopropanol was added.
10. Eppendorf was kept at -20°C for 20 minutes and centrifuged at 13200 rpm for 10 minutes.
11. The supernatant was discarded and the pellet was washed with 70% ethanol.
12. Air-dry the pellet.
13. Dissolve the pellet in 20µl of double distilled water.

6.3.2 Plasmid isolation by Gene JET™ Plasmid Miniprep Kit:

1. Make pellet of the whole culture.
2. Add to the pelleted cells:
    a. 250µl of Resuspension solution and vortex.
    b. 250µl of Lysis solution and invert the tube 4-6 times.
    c. 350µl of Neutralization solution and invert the tube 4-6 times.
3. Centrifuge for 5 minutes to pellet cell debris and chromosomal DNA.
4. Transfer the supernatant to the supplied Gene JET spin column and avoid disturbing or transferring the white precipitate.
5. Centrifuge for 1 minute.
6. Discard the flow-through and place the column back into the same collection tube.
7. Add 500µl of wash solution to the Gene JET spin column and centrifuge for 30-60 seconds.
8. Discard the flow through and place the column back in to the same collection tube and repeat the wash procedure (steps # 7).
9. Discard the flow through and centrifuge for an additional 1 minute to remove residual wash solution. This centrifugation is important to remove residual ethanol in plasmid preps.
10. Transfer the Gene JET spin column into a fresh 1.5ml microcentrifuge tube.
11. Add 50µl of the elution buffer to the center of the Gene JET spin column membrane to elute the plasmid DNA. Take care not to contact membrane with pipette tip.
12. Incubate for 2 min at room temperature and centrifuge for 2 minutes.
13. Discard the column and store the purified plasmid DNA at -20°C.

6.4 Screening of clones / Restriction analysis:

The isolated miniprep DNA was digested with BamH1 and Xba1 enzymes to confirm 223 base pair clone of BCSP 31 gene of *Brucella*. The reaction reagents were as follows.

Plasmid DNA	5µl
10X Reaction buffer (Y^+)	2µl
BamH1	0.5µl
Xba1	0.5µl
RNase	1µl
Double distilled H_2O	11µl
Total volume	20.0µl

The reaction mixture was incubated at 37°C for 1 hour. The digested DNA samples were run on 1% agarose gel along with the standard 100 base pair DNA ladder (Fig. 11) and recombinant clone was marked. The samples having the correct inserted fragment i.e. of 223 base pair (portion of BCSP 31 gene of *Brucella*) were selected and marked as recombinant clones (fig. 17). After screening, the selected clones were sequenced and BLAST analysis was performed.

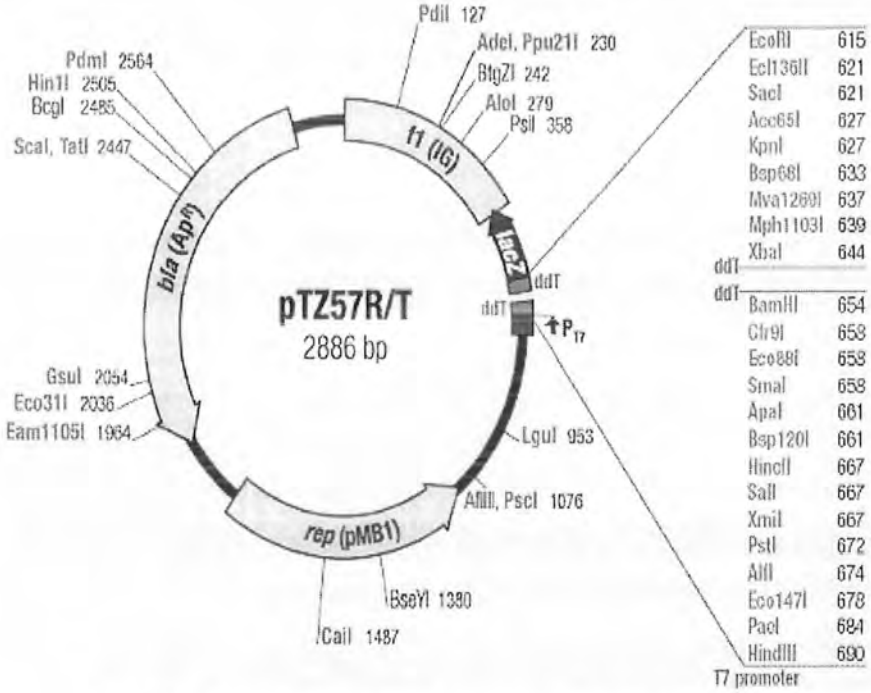


Fig. 10: Map of TA cloning vector (pTZ57R/T).

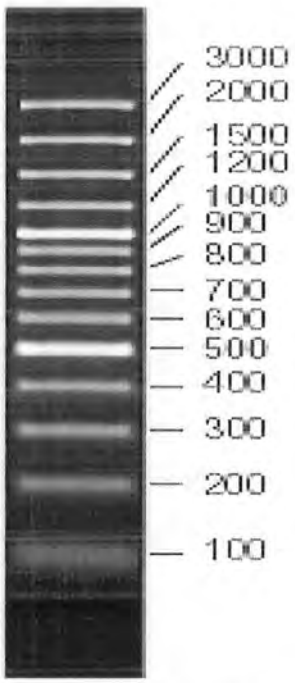


Fig. 11: 100 base pair DNA ladder.

CHAPTER 3

RESULTS

RESULTS

In this study, an attempt has been made to develop PCR test for the diagnosis of brucellosis in humans. Cloning and sequencing of BCSP 31 gene of *Brucella* has also been done. A positive control was included in each test to ascertain the accuracy of methods used for DNA isolation and reactivity of the amplification steps. Similarly, a negative control was also included in each test to screen for the contamination of reagents. Finally, the amplified PCR products were run on 1.5% agarose gel in TAE buffer. Gel was stained with ethidium bromide and visualized in UV light for the presence of specific band of amplified products.

For the extraction of DNA from *Brucella abortus* culture, two protocols i.e. CTAB and Genomic DNA purification kit were adopted. The quantity of extracted DNA was determined using spectrophotometer at wavelength of 260 and 280nm. CTAB method was found to be better for DNA extraction from culture because it gives higher yield of DNA. For DNA isolation from blood samples, many methods were tried (e.g. DNA Zol method, Organic method, TT Lysis method and Genomic DNA purification kit method). Results indicated that Genomic DNA purification kit method is better as far as the purity of the DNA is concerned.

Serum Agglutination Test (SAT) for the detection of antibodies against *B. abortus* and *B. melitensis* was also performed in this study. Positive Serum Agglutination Test was indicated by the presence of agglutination/clumping (antigen-antibody complexes) within 5 minutes, whereas negative Serum Agglutination Test was indicated by absence of agglutination. Agglutination was observed by seeing the slides against the light source. For confirmation of the presence/absence of agglutination, slides were also observed under the light microscope. Figure 13 indicates a positive Serum Agglutination Test, while figure 14 indicates a negative Serum Agglutination Test. Table 6 represents the summary of the results of Serum Agglutination Test on field samples.

For sequencing, the amplified PCR product of 223bp (BCSP 31 gene) was ligated in pTZ57R/T vector and transformed in *E. coli* top 10 α DH strain. The isolated miniprep DNA from E. coli was digested with BamH1 and Xba1 enzymes and run on 1.5% agarose gel along with 100bp DNA ladder to see the clone having correct insert of 223bp fragment of BCSP 31 gene of *Brucella* (fig. 17). After screening, the recombinant clone was sequenced and BLAST analysis was performed.

Amplification of BCSP 31 gene of *Brucella* by PCR:

Figure 15 represents the analysis of amplified product of BCSP 31 gene of *Brucella* DNA isolated from the *B. abortus* culture, along with negative control and 100bp DNA ladder. The amplified product of 223bp was observed in all the samples (*B. abortus* culture) as judged from 1.5% agarose gel electrophoresis. No amplification was observed in negative control.

Figure 16 shows the analysis of the amplified product of BCSP 31 gene of *Brucella* DNA isolated from blood samples, along with positive control, negative control and 100bp DNA ladder. The amplified product of 223bp was observed in positive control and in some blood samples as judged by 1.5% agarose gel electrophoresis. No amplification was observed in the negative control.

This experiment showed that the PCR test for brucellosis has been successfully established in the present laboratory conditions. Table 7 represents the summary of results of PCR test on field samples.

Field samples:

The field samples analyzed for the presence or absence of brucellosis are listed in table 7. The table 7 shows the number of positive samples detected by PCR using *Brucella* genus specific primers. Each sample was tested thrice in order to get reliable results. Out of 90 blood samples, 13 were positive by PCR for brucellosis. The temperature profile optimized for PCR amplification for brucellosis is shown in the table 5.

Sr. No.	Step	Temperature		Time
1	Initial denaturation	93°C		5 minutes
2	Denaturation	90°C	For 35 cycles	1 minute
3	Annealing	60°C		30 seconds
4	Extension	72°C		1 minute
5	Final extension	72°C		8 minutes
Then was held at 4°C for further use.				

Table 5: PCR profile optimized for brucellosis.

Cloning of PCR product of *Brucella* (BCSP 31 gene) in TA cloning (pTZ57R/T) vector:

PCR product of 223bp region of BCSP 31 gene of *Brucella* was eluted from the gel using DNA extraction kit and then ligated in TA cloning vector (pTZ57R/T) and transformed in *E. coli* top 10 α DH strain. Plasmids were isolated from the *E. coli* top 10 α DH strain by Alkaline Lysis method and Gene Jet™ Plasmid Miniprep kit. The clones were confirmed by restriction analysis with BamH1 and Xba1 enzymes. Clones of 223bp were confirmed on 1.5% agarose gel electrophoresis (figure 17).

The cloned PCR product of *Brucella* was then sequenced and BLAST analysis was performed. It was found that our sequence has 100% similarity with the BCSP 31 gene of *Brucella* (Assession No. M20404.1).

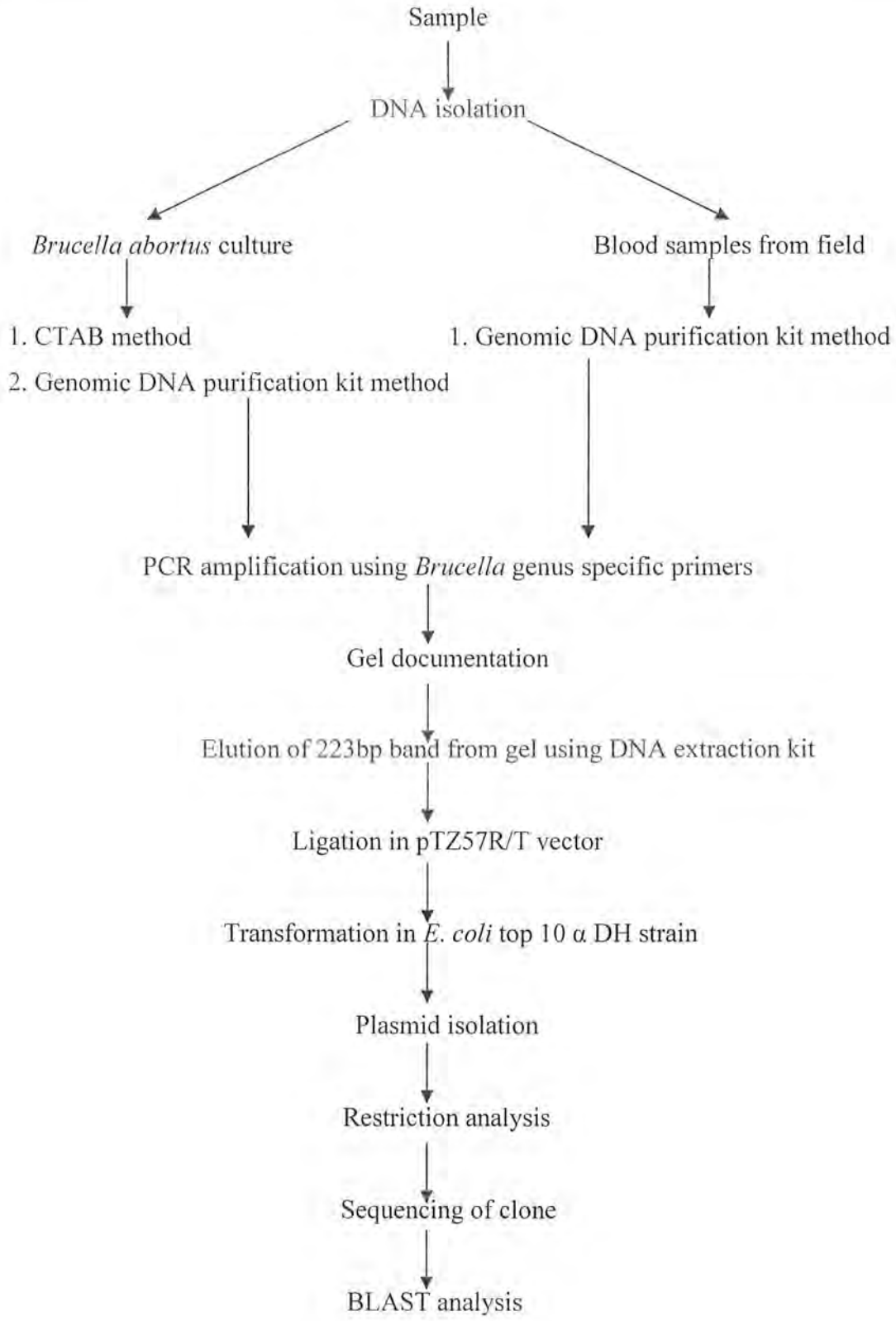


Fig. 12: Schematic representation of different steps used in this study.

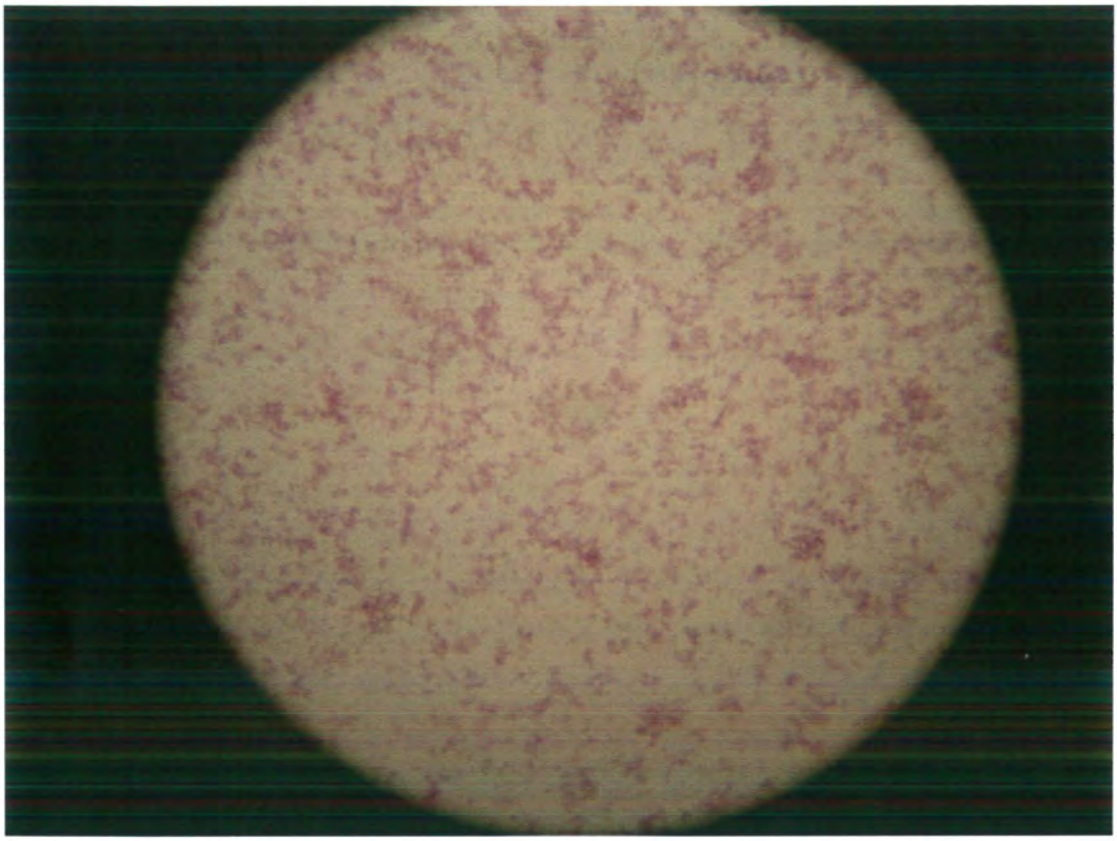


Figure 13: Positive Serum Agglutination Test. Visible agglutination/clumping as seen through Light Microscope (20X).

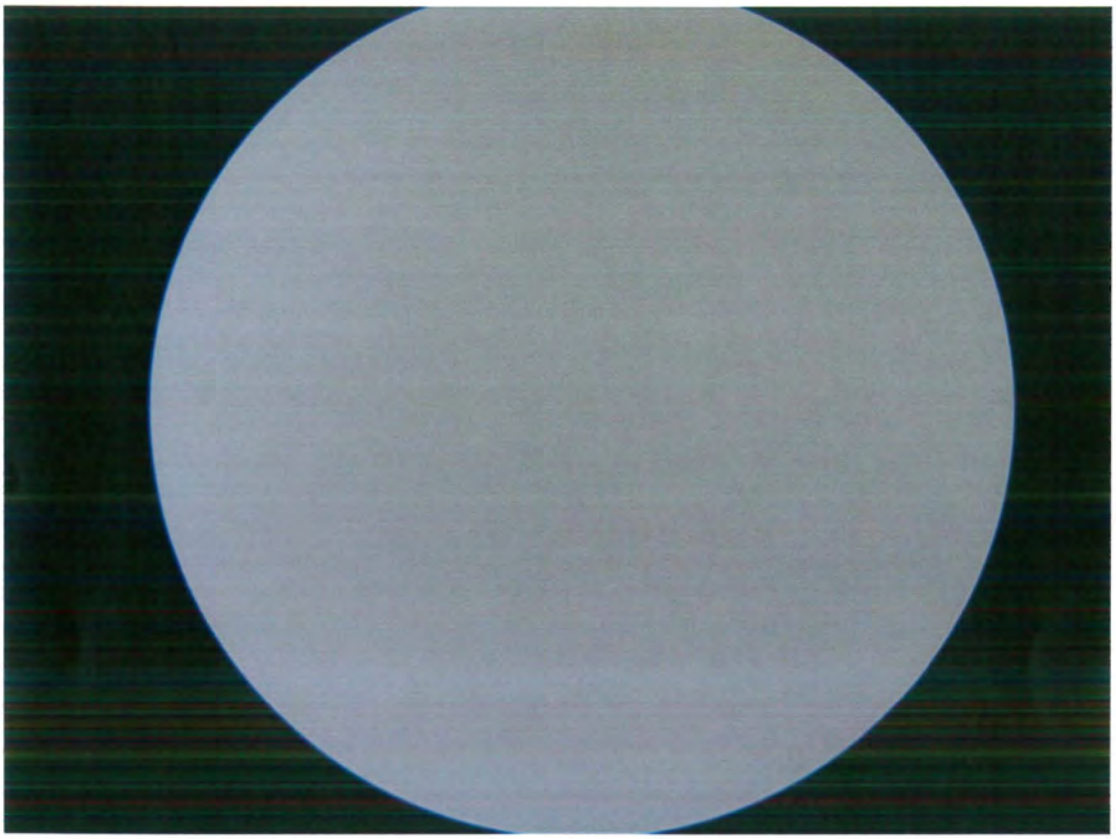


Figure 14: Negative Serum Agglutination Test. No agglutination/clumping as seen through Light Microscope (20X).

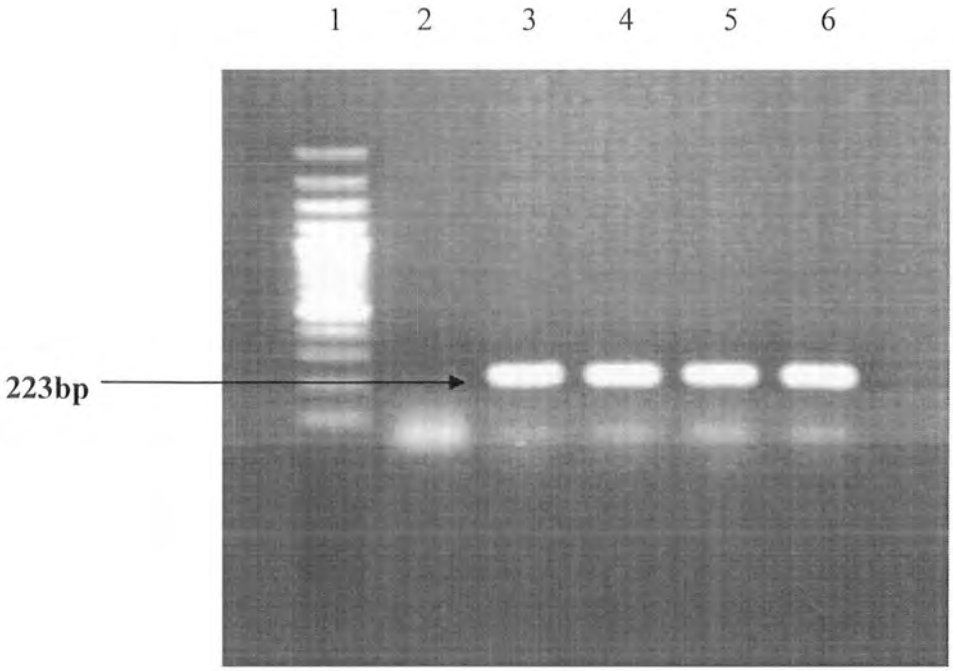


Figure 15: PCR amplification (223bp) of BCSP 31 gene of *Brucella* DNA isolated from *Brucella abortus* culture.

Lane 1: 100bp DNA size marker.

Lane 2: Negative control.

Lane 3-6: Amplified DNA products of 223bp size.

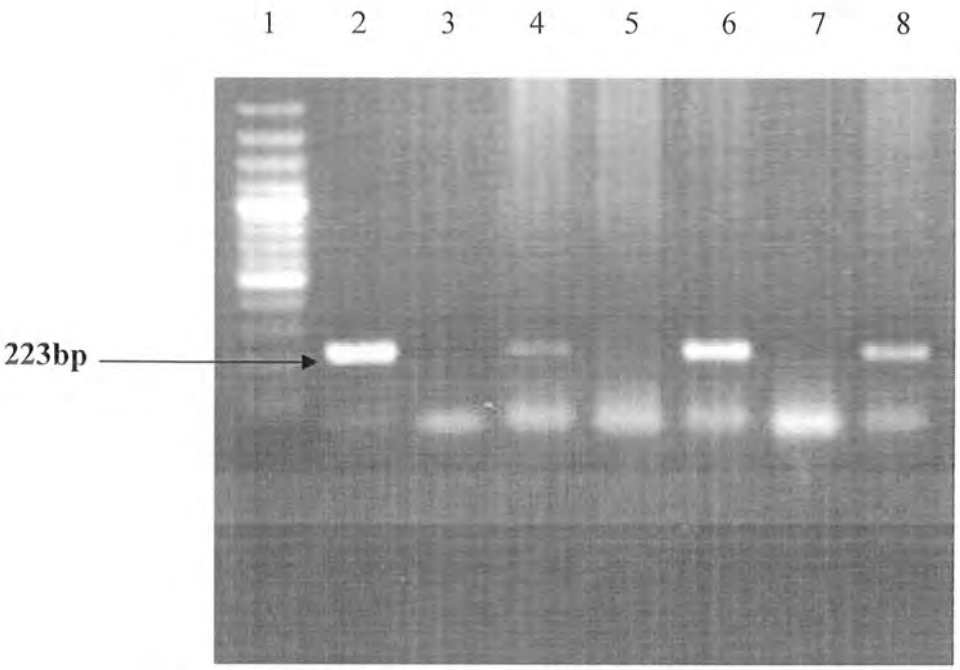


Figure 16: PCR amplification (223bp) of BCSP 31 gene of *Brucella* DNA isolated from the field (blood) samples.

Lane 1: 100bp DNA size marker.

Lane 2: Positive control.

Lane 3: Negative control.

Lane 4: Blood sample positive for brucellosis.

Lane 5: Blood sample negative for brucellosis.

Lane 6: Blood sample positive for brucellosis.

Lane 7: Blood sample negative for brucellosis.

Lane 8: Blood sample positive for brucellosis.

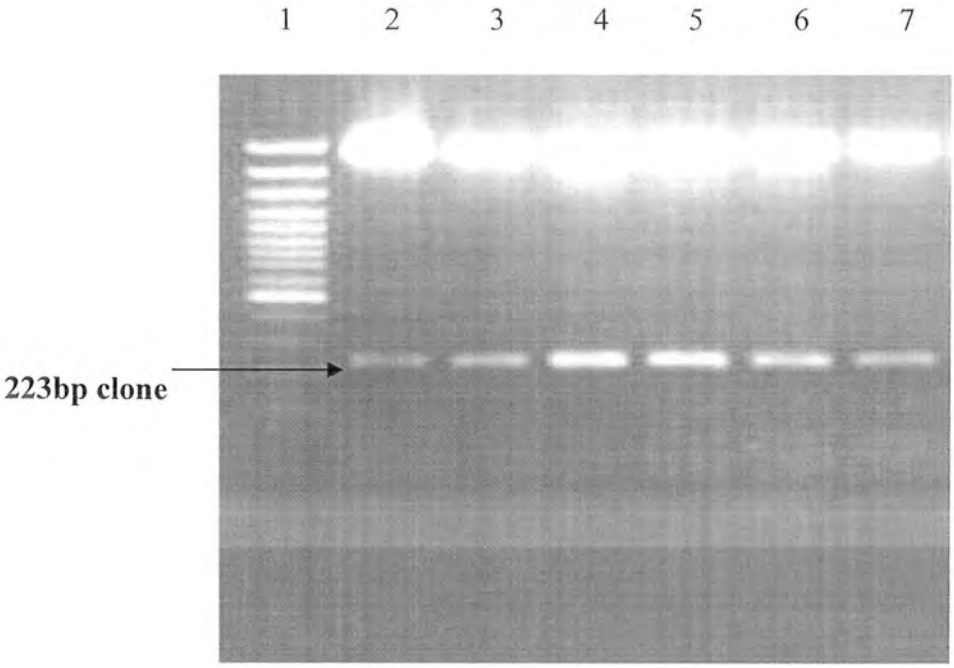


Figure 17: Restriction analysis of *Brucella* (223bp region of BCSP 31 gene) clone in pTZ57R/T vector.

Lane 1: 100bp DNA size marker.

Lane 2-7: 223bp clone of *Brucella* (BCSP 31 gene) in pTZ57R/T vector.

Sequence Analysis:

The clones were sequenced by Macrogen, Korea from both forward and reverse sides. The sequence has 100% similarity with the *B. abortus* BCSP31 gene (Accession no. **M20404.1**).

5'CGCGCTTGCCTTTCAGGTCTGCGACCGATTTGATGTTTGCATCCTTACGCGC

AACGATATGGATCGTTTCCGGGTAAAGCGTCGCCAGAAGGCGCAAATCTTCC

ACCTTGCCCTTGCCATCATAAAGGCCGGTGCCGTTATAGGCCCAATAGGCAA

CGTCTGACTGCGTAAAGCCGGACTCCAGAGCGCCCGACTTGATCGCATTGAT

ATTGGCAACCGAGCCAAA **3'**

Blast analysis has shown the comparativity with the following sequences reported from other parts of the world.

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP000911.1	*Brucella suis* ATCC 23445 chromosome I, complete sequence	414	414	99%	6e-113	100%
CP000872.1	*Brucella canis* ATCC 23365 chromosome I, complete sequence	414	414	99%	6e-113	100%
CP000708.1	*Brucella ovis* ATCC 25840 chromosome I, complete sequence	414	414	99%	6e-113	100%
DQ229169.1	*Brucella melitensis* biovar Abortus 31 kDa cell surface protein gene, partial cds	414	414	99%	6e-113	100%
AE014291.4	*Brucella suis* 1330 chromosome I, complete sequence	414	414	99%	6e-113	100%
AE009521.1	*Brucella melitensis* 16M chromosome I, section 78 of 195 of the complete sequence	414	414	99%	6e-113	100%
AE017223.1	*Brucella abortus* biovar 1 str. 9-941 chromosome I, complete sequence	414	414	99%	6e-113	100%
AM040264.1	*Brucella melitensis* biovar Abortus 2308 chromosome I, complete sequence, strain 2308	414	414	99%	6e-113	100%
M20404.1	***B. abortus* BCSP31 gene encoding a 31-KDa cell surface protein, complete cds**	**414**	**414**	**99%**	**6e-113**	**100%**
AM747718.1	*Brucella abortus* partial csp31 gene	329	329	87%	2e-87	96%
AM773653.1	*Brucella suis* partial csp31 gene	309	309	87%	3e-81	95%

Table 6: Summary of the results of Serum Agglutination Test (SAT) on field samples and the percentage of positive samples.

Occupationally exposed group.	No. of samples screened	*B. abortus* +ve	*B. melitensis* +ve	*B. abortus* +ve, *B. melitensis* –ve	*B. abortus* –ve, *B. melitensis* +ve	Both +ve	Net +ve	Both -ve	% age
Veterinary professionals	33	5	6	1	2	4	7	26	21.2
Farmers	20	4	10	1	6	3	10	10	50
Abortion cases	32	9	12	1	4	8	13	19	40.6
Butchers	5	2	3	0	1	2	3	2	60
Total	**90**	**20**	**31**	**3**	**13**	**17**	**33**	**57**	**36.66**

Table 7: List of blood samples, showing number of samples collected from each occupationally exposed group and the number of positives by PCR and their percentage.

Occupationally exposed group	No. of blood samples screened	Positive by PCR	Percentage
Veterinary Professionals	33	2	6.06
Farmers	20	5	20
Abortion cases	32	4	15.62
Butchers	5	2	40
Total	**90**	**13**	**14.44**

Table 8: Comparison of the results of Serum Agglutination Test and PCR.

Occupationally exposed group	**No. of samples**	**SAT +ve**	**PCR +ve**	**SAT+ve, PCR-ve.**	**PCR+ve, SAT-ve**	**Both +ve**	**Both -ve**
Veterinary professionals	33	7	2	5	0	2	26
Farmers	20	10	4	6	0	5	10
Abortion cases	32	13	5	6	2	2	21
Butchers	5	3	2	1	0	2	2
Total	**90**	**33**	**13**	**18**	**2**	**11**	**59**

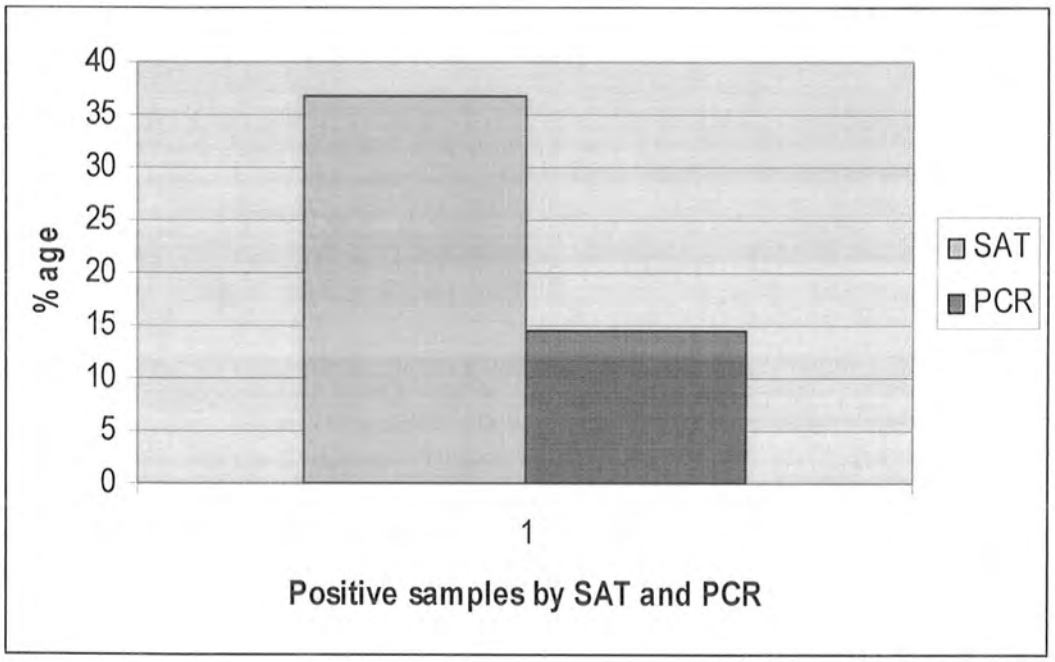


Fig.18: Percentage of positive samples by SAT and PCR analysis

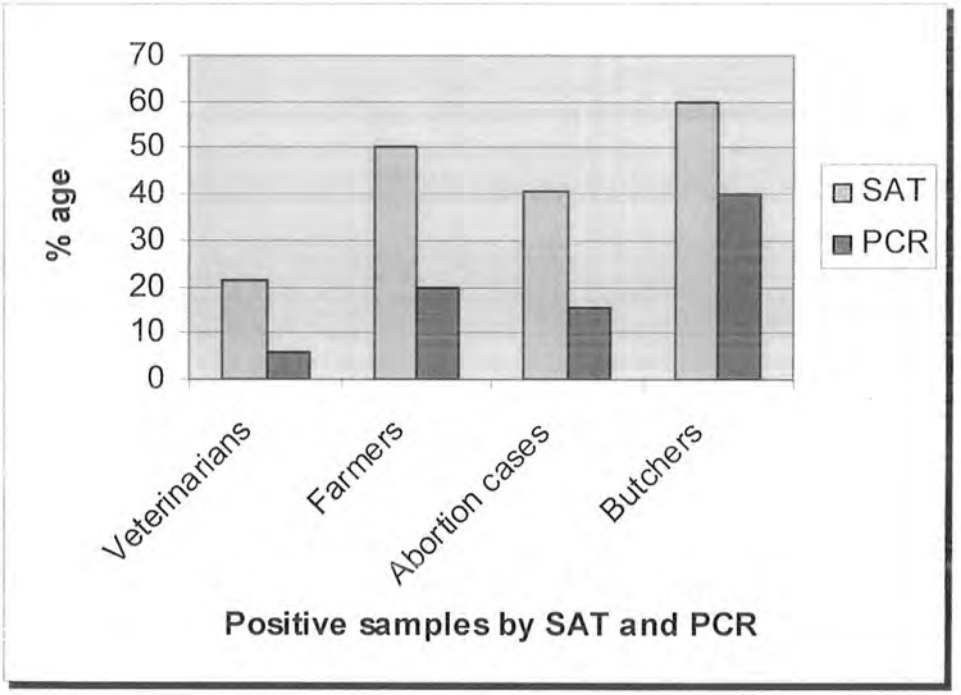


Fig.19: Percentage of positive samples in each occupationally exposed group by SAT and PCR analysis.

CHAPTER 4

DISCUSSION

DISCUSSION

Brucellosis is a major health problem all over the world. It is primarily a disease of animals transmitted directly or indirectly from infected animals to man. Brucellosis is an occupational hazard. Dairy workers, shepherds, veterinarians, abattoir workers and animal husbandry personnel are particularly at risk. It constitutes an uncontrolled public health problem in many developing countries like Pakistan. The problem is compounded by an absence of national surveillance programme, diagnostic facilities or reliable data.

Brucella organisms are shed in milk, urine and vaginal discharges and they thereby contaminate the environment. The infection occurs through the ingestion of unboiled milk of infected animals, contact with vaginal discharge, urine, faeces and blood of infected animals, through unbreached skin and mucous membrane of conjunctiva and also by inhalation (Young, 1983). Brucellosis is of particular concern in Pakistan because nearly 70% of Pakistani population resides in rural areas in close contact with livestock. The global picture of Brucellosis has shown resurgence especially in USA (Wise, 1980). Brucellosis has become a major public health concern in the Saudi Arabia and the Middle East countries, as they are a traditional bedouin pastoralist society (Cooper, 1991).

As brucellosis is a chronic debilitating disease resulting in severe economic losses and is worldwide re-emerging zoonosis. Therefore, it is important to diagnose it promptly and accurately for better prognosis and treatment of disease. The clinical picture of brucellosis alone cannot always lead to diagnosis since the symptoms are nonspecific and often atypical; therefore, diagnosis needs to be supported by laboratory tests. Although many serological tests and new automated blood culture techniques have been developed to diagnose brucellosis, there are still significant problems in the diagnosis of the disease. Blood cultures are time-consuming, since the average time required for growth has been reported to be 6-7 days (Gotuzo *et al.*, 1986). Most recent studies report that incubation period for the isolation of *Brucella* from BACTEC bottles range from 2.5 to 5 days, with 94 to 97% of *Brucella* strains growing within this time period (Yagupsky *et al.*, 1997). In

addition, handling of such organisms pose a high risk to laboratory personnel, as *Brucella* species are class III pathogens. Due to this, brucellosis is one of the most commonly recognized causes of laboratory-transmitted infections, and 2% of all brucellosis cases are laboratory acquired (Young, 2005). The sensitivity of blood cultures ranges from 53 to 90% (Ariza *et al.*, 1995), whereas it is significantly reduced in focal and chronic forms of the disease (Lulu *et al.*, 1986; Mousa *et al.*, 1987).

On the other hand, the serological tests lack specificity, because cross-reactions of *Brucella* antigen have been reported with the antibodies directed against other gram-negative bacteria e.g. *Francisella tularensis* (Francis and Evans, 1926), *Yersinia enterocolitica* (Feeley, 1969), *Vibrio cholerae* (Ahvonen *et al.*, 1969), *Salmonella* and *Escherichia coli* (Stuart and Corbel, 1982). The present study confirms this fact. Moreover, the antibody titers often remain positive for a protracted period after therapy even in cases of complete recovery (Ariza *et al.*, 1992; Gazapo *et al.*, 1989; Pellicer *et al.*, 1988). One of the main characteristics of brucellosis is that the risk of relapse remains high even after appropriate antibiotic treatment (Ariza *et al.*, 1995). This problem results from the intracellular location of *Brucella* species, which protects the bacteria from some of the basic mechanisms of the host immune system, as well as therapy (Young, 1975; Ariza *et al.*, 1995). For this reason, patients who have completed a full antimicrobial therapy should be strictly followed up for 1 year in order to detect any relapse and to provide adequate treatment. SAT is not used for the diagnosis of relapses because SAT detects only the antibodies and in both cases i.e. after complete recovery and in case of relapse, the antibody titer remain high for extended period of time. Therefore, SAT is unable to diagnose relapse and cannot differentiate between complete recovery and relapse.

Pakistan is an agricultural country and exposure of human beings to animals is quite high. In spite of this, very limited studies on brucellosis have been undertaken in both animals and humans. All these studies involve conventional methods (culture, serological tests etc.) for the diagnosis of brucellosis which are less sensitive and specific than modern molecular methods. Therefore, it is a need of time to develop more sensitive and specific molecular tests like PCR etc, for accurate diagnosis and favorable prognosis of the

disease. In the present study, PCR based method for diagnosis of brucellosis was developed. The present work is the first of its kind on brucellosis in Pakistan.

In the present study, SAT and PCR assay were used for the diagnosis of brucellosis in individuals who are in contact with animals because of their occupation. For DNA isolation, simple commercial methods (i.e. CTAB and Genomic DNA purification kit) that provide a very good DNA purification were used. The presence of high concentrations of leukocyte DNA, which has been previously reported to inhibit PCR (Morata *et al.*, 1998), did not affect the amplification of *Brucella* DNA in PCR assay used in this study .This is in accordance with the findings of Mitka *et al.* (2007) who reported that high concentration of human genomic DNA did not interfere with the amplification of *Brucella* DNA in the PCR.

According to Zerva *et al.* (2001) serum is the preferred clinical sample for the diagnosis of brucellosis. However, the results of the present study show that instead of serum, whole-blood is the preferred clinical sample for the diagnosis of brucellosis. This is in accordance with the findings of Mitka *et al.* (2007), who reported that buffy coat and whole blood samples were the preferred clinical samples for the diagnosis of brucellosis. The false-negative results in serum might be due to the intracellular residence of *Brucella* in leukocytes, which results in low numbers of bacteria in serum, and also to the small size of the inoculum.

In the present study, a total of 90 blood samples were collected from the occupationally exposed individuals. Among these samples, 33 belonged to veterinary professionals (including veterinary officers, veterinary assistants, and A.I technicians), 32 belonged to the females with abortion history, 20 belonged to the farmers (including animal owners, dairy workers, and shepherds) and 5 belonged to the butchers. Serum Agglutination Test and PCR were applied on these samples. Results of Serum Agglutination Test and PCR are shown in table 6 & 7 respectively. Statistical analysis of these 90 cases indicated a significant variation in the percentage of prevalence of brucellosis among different category of occupationally exposed workers. It can be observed from table 6 that out of 90 individuals, 33 (36.66%) were positive by Serum Agglutination Test. Among these 33

individuals, 7 (21.2%) belonged to veterinary professionals, 13 (40.6%) belonged to the ladies with abortion history, 10 (50%) belonged to the farmers and 3 (60%) belonged to the butchers.

Results of PCR, as shown in table 7, indicate that out of 90 individuals, 13 (14.44%) were found to be positive for brucellosis by PCR. Among these 13 cases positive by PCR, 2 (6.06%) belonged to the veterinary professionals, 5 (15.62%) belonged to the ladies with abortion history, 4 (20%) belonged to the farmers and 2 (40%) belonged to the butchers. Each sample was checked thrice in order to avoid false positive and false negative results.

This higher number of individuals positive by SAT as compared to PCR may be due to the cross reactivity of antibodies raised against other gram negative bacteria, with the *Brucella* antigen (Stuart and Corbel, 1982). Out of 13 individuals positive by PCR, 11 (84.61%) were also positive by Serum Agglutination Test, while the remaining 2 (15.38%) were negative by Serum Agglutination Test.

Queipo-Ortuno *et al.*, (1997) achieved similar results. He found that out of 50 samples positive by PCR, 8 (16%) were negative by Serum Agglutination Test. The negative Serum agglutination test may occur early in the course of disease (Wicher *et al.*, 1981), or in case of focal infection. This clearly indicates that PCR establishes the diagnosis of brucellosis earlier and is more sensitive and specific than Serum Agglutination Test.

The present study, in the light of both SAT and PCR results, indicates higher prevalence of disease in butchers among the occupationally exposed workers. This is in accordance with the findings of Cadmus *et al.* (2006) and Araj, G.F. and Azzam, R.A. (1996) who reported the seroprevalence of brucellosis 63.3% and 54% respectively in butchers, which was the highest among occupationally exposed workers.

The fundamental reasons for the high infection rate recorded among the butchers may be due to the poor state of meat inspection services and the unhealthy practices by butchers during slaughtering, skinning and cutting of the meat. Generally, the butchers do not wear any protective clothing, leaving them exposed to infected material such as blood, urine,

vaginal discharges, aborted fetuses and especially placentas from infected animals. These butchers are constantly exposed on a daily basis to infected material and because of cuts on their bodies (especially hands and feet) they are at great risk of exposure to the disease. As a result of little or no access to detailed medical care, those who develop symptoms like fever, joint ache and weakness, always associate them with other diseases like malaria etc. Moreover, in Pakistan, especially in rural areas, mostly diseased animals were slaughtered. Most of these diseased animals have problems relating to the reproductive system (e.g. repeat breeding, abortion, metritis etc.) and *Brucella* is one of the major pathogen affecting the reproductive system.

In conclusion, the results of the present study suggest that the PCR assay has several advantages over the conventional methods for the diagnosis of brucellosis including speed, safety, high sensitivity and specificity. PCR assay establishes the diagnosis of brucellosis earlier than does the conventional methods. This is very significant since starting the antibiotic treatment earlier may reduce the chances of complications associated with the disease. PCR is also a method of choice for the diagnosis of relapses (Morata *et al.*, 1999), which are difficult to diagnose by serological methods.

The present study showed that the brucellosis is an occupational hazard in the veterinary practitioners, farmers and abattoir workers and the cases of brucellosis may be easily misdiagnosed because of the deceptive nature of the clinical signs and symptoms. All the cases that were positive by PCR had varied clinical manifestations of brucellosis. The clinicians miss many cases of brucellosis because it is not considered a common disease. The clinicians should keep in mind the possibility of an occupational or environmental exposure in cases of fever, arthritis, and orchitis or any other clinical manifestation of brucellosis. It would also be worthwhile to create awareness about the disease so that necessary precautions and periodic screening of such occupationally exposed people can be done. Elimination of the infection in animals by vaccination to produce *Brucella* free animals/animal products can prevent the infection in humans. The practice of universal precautions among high-risk population cannot be overemphasized.

CHAPTER 5

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REFERENCES

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CHAPTER 6

APPENDIX

APPENDIX

50X Tris-acetate EDTA (TAE) buffer

Glycial Acetic acid	57.1ml
0.5M EDTA (pH= 8.0)	100ml
Tris base	242g

Make final volume with distilled water upto1000ml.

6X Gel loading Buffer

Glycerol	30.0% (w/v)
Xylene cyanol FF0.	25% (w/v)
Bromophenol Blue	0.25% (w/v)

L.B (Lauria-Bertini) Medium

Yeast extract	0.5%
NaCl	0.5%
Tryptophan	1.0%
Bectoagar	1.5%

Adjust pH to 7.5 and then autoclave

Solution I (Suspension buffer)

EDTA	1mM
RNase	100µg/µl
Tris (pH= 7.4-7.6)	50mM

Solution II (Denaturation solution)

SDS	1%
NaOH	0.2N

Solution III (Neutralization solution) (pH= 4.8-5.0)

Glycial acetic acid	11.5ml/100ml
Potassium acetate	3M

0.5M EDTA

To 150ml of water in a beaker, add 46.525g of EDTA- Na-$2H_2O$.
Mix well.
Add NaOH pellets to adjust the pH at 8.0.
Add water to make the final volume 250ml.

Antibiotics used in Cloning experiment

Antibiotics	**Stock solution**	**Working solution**	**Solvent**
Ampicilline	100 mg/ml	100μg/ml	water
Kanamycine	50 mg/ml	50μg/ml	water
Tetracycline	10 mg/ml	10 μg/ml	90% ethanol

5M NaCl
NaCl 73.125g
Add water to make the final volume 250ml

Chloroform/Isoamylalcohol (24:1)
Chloroform 24ml
Isoamylalcohol 1ml

Phenol/Chloroform/Isoamylalcohol (25:24:1)
Phenol 25ml
Chloroform 24ml
Isoamylalcohol 1ml

Ethanol 70%
Absolute ethanol 70ml
Distilled water 30ml

10% SDS
Dissolve 5g SDS in 40ml of distilled water.
Adjust pH to 7.2 by few drops of HCl.
Adjust the final volume to 50ml with distilled water

Washing Buffer for DNA elution
Concentrated buffer 6ml
Double distilled deionized water 95ml
Absolute ethanol 100ml

Precipitation solution

10X supplied conc. solution	80µl
Double distilled deionized autoclaved water	720µl

PCR Reaction

Reagent	Quantity used	Final concentration
10X Buffer	5µl	1.0X
$MgCl_2$ (25mM)	3µl	1.5mM
Forward Primer	1µl	10pmol/µl
Reverse Primer	1µl	10pmol/µl
dNTPs (10mM)	1µl	0.2mM
Taq polymerase (5U/µl)	0.5µl	2.5U
Template DNA	20µl	
Water	18.75µl	

CTAB-NaCl solution (10% CTAB in 0.7 M NaCl)

Dissolve 4.1g NaCl in 80ml water and slowly add 10g CTAB while heating (65°C) to dissolve. Adjust final volume 100ml.

Ethidium bromide (10mg/ml)

Add 1g of ethidium bromide to 100ml of water. Stir on magnetic stirrer for several hours to ensure that the dye is dissolved. Wrap the container in aluminium foil or transfer the solution to dark bottle and store at room temperature.

TE Buffer

Tris HCl (pH 8.0)	10mM
EDTA	1mM