Seroepidemiological study of human brucellosis among the population at risk.

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<td><em>Brucella abortus</em></td>
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<td><em>B. melitensis</em></td>
<td><em>Brucella melitensis</em></td>
</tr>
<tr>
<td>CDC</td>
<td>Centre for disease control</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra acidic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>et. al</td>
<td>et alia (and others)</td>
</tr>
<tr>
<td>GS</td>
<td>Glycine saline</td>
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<tr>
<td>HRPO</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>IFNγ</td>
<td>Interferon γ</td>
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<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
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<tr>
<td>IL-12</td>
<td>Interleukin – 12</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin – 2</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>N</td>
<td>Number</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative predictive value</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>OMPS</td>
<td>Outer membrane proteins</td>
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<tr>
<td>OPD</td>
<td>Orthophenilene diamine</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>P</td>
<td>Probability</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PPV</td>
<td>Positive predictive value</td>
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<tr>
<td>PUO</td>
<td>Pyrexia of unknown origin</td>
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<td>RBPT</td>
<td>Rose Bengal plate agglutination test</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>STAT</td>
<td>Standard tube agglutination test</td>
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<td>Th2</td>
<td>T helper cell 2</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper cell 1</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
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<tr>
<td>WHO</td>
<td>World health organization</td>
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<tr>
<td>%</td>
<td>Percentage</td>
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<tr>
<td>+ve</td>
<td>Positive</td>
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<tr>
<td>&lt;</td>
<td>Less than</td>
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<tr>
<td>&gt;</td>
<td>More than</td>
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<tr>
<td>µl</td>
<td>Microliter</td>
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<tr>
<td>°C</td>
<td>Degree of centigrade</td>
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Mymensingh, December, 2008
SUMMARY

Brucellosis is the most important zoonotic disease caused by *Brucella* species comprising gram negative, facultative, intracellular pathogens and usually is transferred to humans from infected animals. The true incidence of human brucellosis is unknown for most countries including Bangladesh. But the disease has a worldwide distribution. Brucellosis is not uncommon in our country. The present study was conducted to determine prevalence of human brucellosis among the suspected pyrexia patients and population at risk in rural area by various serological tests. This cross sectional study was carried out in the department of Microbiology, Mymensingh Medical College & department of Medicine under the faculty of veterinary science, Bangladesh Agricultural University, Mymensingh, during the period from July 2007 to June 2008. A total of 510 samples were included in this study of which 300 were from pyrexia of unknown origin group and 210 were from risk group of population. Among 300 samples from pyrexia of unknown origin group, 27 (9%), 24(8%) and 23 (7.67%) were positive by slide agglutination test, Rose Bengal plate agglutination test and Standard tube agglutination test respectively. On the other hand, 210 subjects from risk group of population, 9 (4.28%), 7(3.33%) and 7 (3.33%) were positive for brucellosis by slide agglutination test, Rose Bengal plate agglutination test and Standard tube agglutination test respectively. In pyrexia group, 22 (81.48%) out of 27 and 10(25.64%) out of 39 subjects in risk group were positive by and ELISA (IgG). The sensitivity, specificity, positive predictive value and negative predictive value of the ELISA (IgG) was found as 81.48%, 74.36%, 68.75% and 85.29% respectively.
The prevalence of brucellosis was significantly higher among female (13.46%) than male (6.63%) in pyrexia group and (8.33%) in female than male (2.63%) in risk group as detected by slide agglutination test. Seroprevalence among occupational groups in pyrexia patients were at 17.74% in animal farmers, 9.38% in house wives. Similarly seroprevalence among risk groups were at 11.11% in veterinary personnel, 6.65% in dairy workers and 4.67% in animal farmers.
**Introduction**

Brucellosis is a worldwide bacterial zoonotic disease caused by *Brucella spp* and usually transmitted to humans from infected animals. The disease may present clinically as acute, as chronic following an acute attack or as chronic and of insidious onset. Human brucellosis is known for presenting with protean manifestations (Mantur *et al*, 2006). However, the most common presenting symptom is fever. The intermittent or remittent fever may be accompanied by fatigue, malaise, anorexia, chills, sweats, headache, backache, myalgia, arthralgia and weight loss (Mantur *et al*, 2007). Human brucellosis may affect any organ or system of the body, causing osteomyelitis, hepatomegally, splenomegally, lymphadenopathy, meningitis and other central nervous system symptoms, epididymo-orchitis and endocarditis. The case fatality rate of untreated brucellosis may be up to 2% & usually result from endocarditis (Young, 1989). Many names have been applied to it, often relating to the localities in which it was particularly prevalent. Malta fever, Mediterranean fever, Gibraltar fever, Cyprus fever, Rock fever and undulant fever are probably the best known (Mantur *et al*, 2007).

Brucellosis is an ancient disease that was described more than 2000 years ago by the Romans. Sir David Bruce first isolated *Brucella melitensis* in 1887. The genus *Brucella* is currently divided into six recognized species based on phenotypic characteristics, antigenic variation and prevalence of infection in different animal hosts. *Brucella abortus* (cattle), *Brucella melitensis* (goats,sheep), *Brucella suis* (pigs, reindeer and hares) *Brucella canis* (dogs), *Brucella neotomae* (desert wood rats),
Brucella ovis (sheep) (Corbel, 1997; Moreno et al, 2002). Recently, two Brucella strains from marine mammals have been reported (Bricker et al, 2000; Cloeckaert et al, 2000) and the names Brucella pinnipediae (seal/otter) and Brucella cetaceae (porpoise/whale) have been proposed (Cloeckaert et al, 2003). There has also been a report of human infection with marine brucellae (Sohn et al, 2003). Although each species of Brucella has a preferred host, all can infect a wide range of animals, including humans. Brucellosis is a worldwide reemerging zoonosis causing high economic losses and severe human disease. Brucellosis remains an uncontrolled public health problem worldwide. In many developing countries, the problem is compounded by the absence of national surveillance programmes, diagnostic facilities and reliable data (Cooper, 1992).

The true incidence of human brucellosis however, is unknown is for most countries including India. But the disease has a worldwide distribution. Reported incidence of human brucellosis in endemic disease areas varies widely, from <0.01 to > 200 per 100,000 population (Mantur and Amarnath, 2008). The global burden of human brucellosis remains as to be more than 500,000 infections per year (Pappas et al, 2006). 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 Middle East, the Indian subcontinent and parts of the Mexico and central and South America (Corbel, 1997). Several areas traditionally considered to be endemic—e.g, France, Israel, and most of Latin America—have now achieved control of the disease. On the other hand, new foci of human brucellosis have emerged, particularly in central Asia. The situation in Syria has been rapidly
worsening. Furthermore, the disease is still present, in varying trends, both in European countries and in the USA (Pappas et al, 2006).

In Bangladesh, there are sporadic reports of prevalence of animal & human brucellosis. The true incidence of human brucellosis in our country is not known. But a study by Ahmed et al in 2006 revealed that brucellosis is not uncommon among the risky population of rural area. Serological tests done among cattle handlers of some villages in southwest part of the country showed 13% positive cases of brucellosis. Antibody titer against *Brucella abortus* was found to be significantly higher than *Brucella melitensis* (Ahmed et al, 2006).

The brucellosis is usually transmitted from infected animal to man by direct contact via skin abrasion, ingestion of contaminated animals products such as unpasteurized milk, milk products and raw meat. Aerosol transmission in abattoirs and laboratories also can occur (Salari, 2002).

*Brucella* are small aerobic gram-negative coccobacilli, nonmotile, non-spore-forming, unencapsulated, facultative intracellular bacteria (Young, 1995). These are opsonized by normal human serum which promotes their phagocytosis by polymorphonuclear leukocytes and activated macrophages. *Burcellae* resist intracellular phagocytic killing by suppression of myeloperoxide-hydrogen peroxide-halide system and the production of superoxide dismutase (Canning et al, 1986). The pathogen-phagocyte interaction plays a key role in determining the severity and outcome of the disease. The organisms surviving within and escaping from the phagocytes multiply and reach the bloodstream via the lymphatics, subsequently localizing in the liver, spleen,
bones, kidneys, lymph nodes, heart valves, nervous system and testes. In these organs, the bacteria are ingested by macrophages and survive by inhibition of phagosome-lysosome fusion. In infected tissues, inflammatory responses as noncaseating or caseating granulomas and abscesses have typically been found (Madkour and Kasper, 2005). Cytokines, including IL-1, IL-12, TNF, and gamma interferon, appear to be important in host defense against *Burcella* species infection. The S-LPS is a major determinant of virulence and dominates the antibody response. *Brucella* LPS is a relatively poor inducer of gamma-interferon and tumor necrosis factor-α, both of which are essential for the elimination of the organism (Zhan et al, 1995). Other important virulence factors include: production of inhibitors of phagolysosome fusion such as adenine and guanine monophosphate levels, outer membrane protein 25 which has been identified as the down regulator of TNF- alpha. All these molecular events lead to persistence or recurrence of the infection (Jubier et al, 2001).

**Brucellosis** is diagnosed either by isolation of *Brucella* in culture (isolation of organism by culture is gold standard method) or by combination of serological test and clinical finding consistent with brucellosis. Isolation of the *Brucella* organism is the definitive means of diagnosis but in practice it is difficult due to early tissue localization and the exacting culture requirement of the organism. In practice, blood cultures are positive in 10-30% of brucellosis and remainder is diagnosed serologically (young, 1995).

A variety of serological test has been developed for detection of brucellosis of which Rose Bengal plate test, Standard tube agglutination test are the most widely used,
more recently the *Brucella* ELISA (enzyme linked immunosorbent assay) test was introduced for detection of *Brucella* specific IgG and IgM antibodies (Ertek *et al.*, 2006). Although no single test provides 100% sensitivity and specificity, standard tube agglutination test (STAT) still remains the test of choice in diagnosis. In the presence of appropriate signs and symptoms, a presumptive diagnosis of brucellosis is usually defined serologically as STAT titer of 1:160 or greater (Bettelheim *et al.* 1983).

**Brucellosis** is a disease of human as well as animal and distributed throughout the world including Bangladesh. But true prevalence of human brucellosis in our country is not known. Having the described background, any study to see seroprevalence of human brucellosis would have seemed to be rational. Therefore, the objectives of the present study were to determine *Brucella* antibody titer (*B. abortus* and *B. melitensis*) among the risky population at Mymensingh region of Bangladesh.
OBJECTIVES

General objective:
To determine prevalence of human brucellosis by various serological tests among the suspected patients and population at risk.

Specific objectives:
(a) To determine antibody titer among the suspected patients of brucellosis.
(b) To determine antibody titer among the population at risk of brucellosis.
(c) To identify risk factors of brucellosis.
(d) To compare among different serological methods for diagnosis of brucellosis.

Hypothesis:
Seroconversion against Brucella species is present among the risk group of population.
REVIEW OF LITERATURE

2.1 HISTORICAL BACKGROUND

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 region since the time of Hippocrates in 450 B.C. Much later in the 19\textsuperscript{th} centuries, 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 responsible organism, \textit{Micrococcus melitensis} was isolated and identified in culture from spleen tissue of fatally infected British soldiers at Malta in 1887 by Sir David Bruce, a British Army physician. The organism derived its species name from Melita (honey), the Roman name for the Isle of Malta; where the disease was first recognized (Bruce, 1887) The classical description of the clinical illness by Hughes in 1897 altered its designation to the more frequent term “undulant fever” and suggested the name undulant fever (Hughes, 1887).

A Danish physician and veterinarian, Bernhard Bang discovered \textit{Brucella abortus} 1897 while investigating contagious abortion that had been affecting Cattle in Denmark for over a century. He also discovered the organism that affected Sheep and goats. Thus the disease becomes known as “Bang’s disease” (Bang, 1897). Brucellosis has also many synonyms derived from the geographical regions in which disease occurs e.g., Mediterranean fever, Malta fever, Gibraltar fever, Cyprus fever and typhomalarial fever (Mantur \textit{et al}, 2007).
The third member of the organism named *Brucella suis* which also is bacillary in shape was recovered from the fetus of aborted swine by Traum in 1914 in the United States of America (Traum, 1914). In 1918, Alice Evans an American bacteriologist published reports which contained convincing evidence that *B 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. She also showed in 1920 that *B. melitensis* was also a bacillus. She showed that *B. melitensis*, isolates of cows and pigs belonged to one genus (Evans, 1918). Meyer and Shaw further confirmed Evan's observations and suggested the generic name *Brucella* in honour of Sir David Bruce. (Meyer and Shaw, 1920).

Wright and Smith in 1897 detected antibodies to *B.melitensis* in human and animal sera through agglutination test, which unravelled the zoonotic potential of the disease (Wright and Smith, 1897). Later, Zammit a young Maltese physician working with Mediterranean Fever Commission in 1905 confirmed it by isolating the organism from the milk and urine of goats. Thus he concluded that the goat was the reservoir of *B.melitensis* and the consumption of the raw milk and cheese infects man. (Zammit, 1905).

In 1956, Buddle and Boyce discovered *B. ovis*, the cause of epididymitis in rams (Buddle, 1956). In 1957, Stoenner and Lackman isolated *B. neotomae* from desert wood rat in Utah in USA (Stoenner and Lackman, 1957). 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 (Ewalt et al, 1994 and Ross et al, 1996).

2.2 EPIDEMIOLOGY

2.2.1 PREVALENCE OF INFECTION

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 (Mantur et al, 2007).

Brucellosis is the most common zoonotic infection worldwide (Corbel, 1997). It is endemic in the Mediterranean region, the Middle East, Latin America and parts of Asia and Africa, but the epidemiology is changing over the last decades due to socioeconomic changes, improved disease recognition and eradication programmes (Pappas et al, 2005). Human brucellosis is found to have significant presence in rural 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 (Corbel, 1997). According to the World Health Organization, half a million new human cases each year are reported worldwide (WHO, 1997). The true incidence of human brucellosis however, is unknown for most countries including India. 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).

In the United States the CDC reports fewer than 0.05 cases per 100,000 populations, with most being reported from Texas, California, and Illinois. During the past 10
years, approximately 100 cases per year have been reported in the United States (CDC, 2001). The occurrence of brucellosis in India was first established early in the previous century and since then has been reported from almost all states. (Sehgal and Bhatia, 1990; Renukaradhya et al, 2002). Several studies and publications indicate that human brucellosis can be a fairly common disease in India. A study by Agasthya et al in 2007 revealed that, the disease of prevalence was at 41.23% in veterinary inspectors, 30.92% in veterinary assistants, 12.37% in veterinary officers, 6.18% in veterinary supervisors, 2.06% in shepherds and 1.03% in butchers (Agasthya et al, 2007). Mathur reported 8.5% seroprevalence of brucellosis among dairy personnel in contact with infected animals (Mathur, 1964). Another study conducted by Thakur and Thapliyar revealed a prevalence rate of 4.97% in samples obtained from persons exposed to animals (Thakur and Thapliyar, 2002). In Bangladesh, there are sporadic reports of prevalence of human & animal brucellosis. The true incidence of human brucellosis in our country is not known. But a study conducted by Ahmed et al in 2006 revealed that brucellosis is not uncommon among the risky population of rural area (Ahmed et al, 2006).

Many nations have deployed eradication campaigns, with some countries such as the UK and several other northern European countries successfully gaining brucellosis-free status. Even in these countries, however, comprehensive surveillance is essential to maintain this status. Human brucellosis is rare in countries where eradication programmes against brucellosis in cattle, sheep and goats have been implemented successfully. For example, in France, a country with one of the largest farming communities in Europe, less than 50 brucellosis cases are reported annually (Pappas et al, 2006). But recent re-emergence in Malta and Oman indicates the difficulty of
eradicating this infection (Amato, 1995). 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. In some South American countries, particularly Brazil and Colombia *B. suis* biovar 1 has become established in cattle (Lopez, 1989). Screening of household members of an index case is important epidemiological step since this picks up additional unrecognized cases. (Almuneef *et al*, 2004 and Mantur *et al*, 2006). The recent isolation of distinctive strains of *Brucella* from marine mammals as well as humans 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. (Brew *et al*, 1999; Sohn *et al*, 2003 and McDonald *et al*, 2006)

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, which may be due to risk of occupational exposure. Human brucellosis affects all age groups (Mantur *et al*, 2006).
2.2.2 Transmission:

The transmission of *Brucella* infection and its prevalence in a region depends upon several factors like food habits, methods of processing milk and milk products, social customs, husbandry practices, climatic conditions, socio-economic status and environment hygiene. Environmental sanitation is particularly important in the context of air borne transmission (WHO, 1986) *Brucellosis* is almost invariably transmitted to man from infected domestic animals. Transmission of *B. melitensis* from person to person has also been reported in the literature (Mantur *et al*, 1996; Wyatt, 1996).

Transmission of brucellosis to humans occurs through the consumption of infected, unpasteurized animal milk and milk products, through direct contact with infected animal parts (such as the placenta, fetus, fetal fluids and vaginal discharges from infected animals) through ruptures of skin and mucous membranes and through the inhalation of infected aerosolized particles (Pappas *et al*, 2005). Brucellosis is an occupational disease in shepherds, abattoir workers, veterinarians, dairy-industry professionals, and personnel in microbiologic laboratories. Consumption of unpasteurized dairy products, especially raw milk, soft cheese, butter, and ice cream are the most common means of transmission (Eckman, 1975). Bacterial load in animal muscle tissues is low, but consumption of undercooked traditional delicacies such as liver and spleen has been implicated in human infection (Malik, 1997). In addition, laboratory acquired *Brucella* infection due to accidental ingestion, inhalation and mucosal or skin contact is a major health hazard for the laboratory workers handling the cultures of the virulent or attenuated *Brucella* strains. The disease has been recognized as one of the common laboratory-transmitted infections and has been
reported to occur in clinical, research, and production laboratories. (Arlett, 1997; Grammont *et al*, 1996; Noviello *et al*, 2004 and Yagupsky and Baron, 2005)

**Bioterrorism:**

Bioterrorism and its potential for mass destruction have been subjects of increasing international concern. Production costs of biological weapons are low, and aerosol dispersal equipment from commercial sources can be adapted for biological weapon dissemination. As far as brucellosis is concerned, inhalation of only a few organisms is sufficient to cause a significant likelihood of infection. In a theoretical model of a bioterrorist attack and in the absence of an intervention program for 100,000 persons exposed, a *B. melitensis* cloud would result in 82,500 cases of brucellosis requiring extended therapy, with 413 deaths. The economic impact of such a brucellosis bioterrorist attack would cost $477.7 million per 100,000 persons exposed (Kaufmann *et al*, 1997)

**2.2.3 Reservoirs of Brucella species:**

Brucellosis is a worldwide zoonotic disease caused by *Brucella spp.* in which domestic animals such as cattle, goats, sheep, pigs, camel, buffalo and dogs serve as a reservoir hosts (Corbel, 1997; Moreno *et al*, 2002). Fresh milk and dairy products prepared from unpasteurized milk such as soft cheeses, yoghurts and ice creams contain high concentration of the bacteria and consumption of these is an important cause of human brucellosis (Bikas *et al*, 2003). *Brucella* species can survive in proper environmental condition, damp soil and seawater and can be a source of infection. Notably abortion materials such as placenta, fetal parts, fetal membranes, amniotic fluid and vaginal discharges of infected animals may contain high amounts of the bacterium and act as source of brucellosis (Henk *et al*, 2005).
2.2.4 Risk factors of Brucella infection:

Risk factors for human brucellosis include the handling of infected animals, ingestion of contaminated animal products such as unpasteurized milk and milk products (including cow, goat, and camel milk), meat, history of travel to endemic areas and handling of cultures of Brucella spp. in laboratories. Other risk factors include: abattoir workers, veterinarians, slaughterhouse workers and dairy workers (Buchanan et al, 1974; Corbel, 1997).

2.2.5 Strategy for prevention and control of Brucella infection:

Prevention of human brucellosis is dependent on control of the disease in domestic livestock mainly by mass vaccination. In many countries, the use of B. abortus strain S19 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. Studies are ongoing to develop an effective vaccine against B. suis (Nicoletti, 2001). No vaccine is available for the prevention of brucellosis in human. Reporting of brucellosis to health authorities is extremely important and knowledge of the disease prevalence can be used to prioritize a disease control policy for brucellosis and to alert health staff (Henk et al, 2005). Furthermore, health education programs regarding environmental hygiene and food hygiene should be adopted that aim at stopping the spread of infection among animals and then to humans, and also adoption of hygienic measures among high-risk population (Salari, 2002). Animal handlers should wear appropriate protective clothing/barriers when working with infected animals. Meat should be well cooked. Milk should be pasteurized and unpasteurized dairy products should be avoided. Laboratory workers should culture the organism only with appropriate biosafety containment (Young, 1995).
2.3 MICROBIOLOGY OF BRUCELLA

2.3.1 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, Ochrobactrum, 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*, of which four are recognized human zoonoses. The presence of rough or smooth lipopolysaccharide correlates with the virulence of the disease in humans. Two new *brucella* species, provisionally called *Brucella pinnipediae* and *B. cetaceae*, have been isolated from marine hosts within the past few years (Pappas et al, 2005)

**Scientific classification:**

- Kingdom: Bacteria.
- Phylum: Proteobacteria.
- Class: Alpha Proteobacteria.
- Order: Rhizobiales.
- Family: Brucelloceae.
- Genus: Brucella.

**Species:** *B melitensis*, *B abortus*, *B Suis*, *B canis*,

*B ovis B neotomae*, *B pinnipediae*, *B cetaceae*
2.3.2 Morphology:

*Brucellae* are facultative intracellular, small aerobic gram-negative coccobacilli or short rods, partially acid-fast that lack capsules, flagellae, endospores or native plasmids. The bacterium is of 0.5-0.7µ in diameter and 0.6-1.5µ in length. They are oxidase, catalase and urease positive. They occur singly, in groups or short chains (Young, 1995)

2.3.3 Cell envelope, outer membrane and LPS:

The *brucellae* are gram negative eubacteria endowed with cell envelopes composed of inner and outer membranes enclosing a periplasm with a peptidoglycan mesh and soluble components. The outer membrane contains the lipopolysaccharide (LPS) that is the *Brucella* major virulence factor. Lipopolysaccharide is vital to both the structural and functional integrity of the gram negative bacterial outer membrane. Ubiquitously expressed by all Gram-negative bacteria, and containing several well-conserved domains, LPS also serves as one of the primary targets of the innate arm of the mammalian immune system. LPS has three domains: lipid A, the core oligosaccharide, and the O-antigen or O side chain. *Brucella* strains may occur as either smooth or rough, expressing smooth LPS (S-LPS) or rough LPS (R-LPS) as major surface antigen. This bacterium possesses an unconventional non-endotoxic lipopolysaccharide that confers resistance to anti-microbial attacks and modulates the host immune response. The strains that are pathogenic for humans (*B. abortus, B. suis, B. melitensis*) carry a smooth LPS involved in the virulence of these bacteria. The LPS O-chain protects the bacteria from cellular cationic peptides, oxygen metabolites and complement-mediated lysis and it is a key molecule for *Brucella*
survival and replication in the host (Lapaque et al, 2005). Pathogenicity is also related to production of lipopolysaccharides containing a poly N-formyl perosamine O chain, superoxide dismutase, erythrlose phosphate dehydrogenase, stress-induced proteins related to intracellular survival, and adenine and guanine monophosphate inhibitors of phagocyte functions. Protective immunity is conferred by antibody to lipopolysaccharide and T-cell-mediated macrophage activation triggered by protein antigens (Corbel, 1997).

2.3.4 Cultural characteristics:

*Brucella* spp. are aerobic with *B. abortus* requiring a carbon dioxide (5-10%) enriched atmosphere. All strains grow over a temperature range 20\(^0\)C-40\(^0\)C with best at 37\(^0\)C in a medium enriched with animal serum and glucose. Tryptone soya (tryptic soy) biphasic medium (Castaneda) is recommended for isolation *Brucella* species. Several commercially produced blood cultural systems are also suitable and some provide rapid isolation (Cheesbrough, 2000).

Culture should be kept for 4 weeks with subculture every few days. When subculture in solid media, colonies usually appears 2-3 days after inoculation. A variety of colonial forms are produced by Brucella strains. *B. melitensis*, *B. abortus* and *B. suis* are normally form smooth, moist, convex, transparent and glistening (honey droplet). The colonies may be colorless or grey-white. All colonies are non-pigmented and non-hemolytic. But *B. ovis* and *B. canis* strains produced permanently rough colonies. However, the organism such as *B. melitensis*, *B. abortus* and *B. suis* can mutate, specially in liquid media, forming rough colonies in subculture. There is corresponding loss in virulence and an antigenic change, so that they are no longer readily agglutinated by homologous antisera prepared against normal smooth strains.
All suspicious colonies should be examined by Gram stain, oxidase and urease test (Corbel, 1997)

2.3.5 Antigenic Components:

A substantial number of antigenic components of *Brucella* have been characterized.

**Lipopolysaccharide (LPS):** Lipopolysaccharide (LPS) is the antigen that dominates the antibody response. Non-smooth strains (R-LPS) are similar to LPS of smooth strains (S-LPS) except that the O-chain is either absent or reduced to a few residues. There is a some antigenic cross-reaction between smooth species of *Brucella* with other organisms such as *Yersinia enterocolitica* O:9, Francisella tularensis, *Escherichia coli* O:157, *Salmonella* O:30 and *Vibrio cholerae* O:1 (Perry and Bundle, 1990). These have been attributed to similarities on the O-specific side chains of the lipopolysaccharide molecule of the organisms.

**Outer membrane proteins:** 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. Omp 25 is an outer membrane structural protein that is highly conserved in all *Brucellae*. It is associated with both lipopolysaccharide and peptidoglycan (Goldbaum *et al.*, 1993).

**Ribosomal proteins:** 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. Hence this appears to have potential as candidate vaccine component (Oliveira and Splitter, 1996).
2.3.6 **Genome of Brucella species:**

In the initial years of this decade, the complete genomic sequence of *B. melitensis*, *B. abortus* and *B. suis* has been achieved (DelVecchio *et al.*, 2002 and Sanchez *et al.*, 2001)). The average size of the genome is $2.37 \times 10^9$ daltons, with a DNA G + C content of 58-59 mol %.( DelVecchio *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 *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 erythulose-1-phosphate dehydrogenase gene) (Ficht *et al.*, 1989). The omp 2 gene is believed to determine dye sensitivity, one of the traditional typing methods for biotype differentiation. 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* (Cloeckaert *et al.*, 1995). A 7.2 kbp deletion in the ery gene in *B. abortus* strain 19 may explain the erythritol sensitivity of this strain which is a major factor in its attenuation (Sangari *et al.*, 1994) The genome of *Brucella* contains two circular chromosomes of 2.1 and 1.5 Mb, respectively. Both replicons encode essential metabolic and replicative functions and therefore are chromosomes, not plasmids (Michaux *et al.*, 1993) 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).
2.3.7 Determinants of virulence:

The pathogenicity in human brucellosis is related to various factors.

**Lipopolysaccharides of Brucella species:** The S-LPS is a major determinant of virulence and dominates the antibody response. The elimination of virulent *Brucella* depends upon activated macrophages and hence requires development of Th1 type cell-mediated immunity. *Brucella* LPS is a relatively poor inducer of gamma-interferon and tumour necrosis factor-α, both of which are essential for the elimination of the organism (Zhan *et al.*, 1995 and Caron *et al.*, 1994). Unusually, it is an effective inducer of interleukin 12, which stimulates Th1 type response and is closely correlated with gamma interferon production. Lipopolysaccharide of *Brucella* has two forms, smooth and rough. In general, rough strains, containing less or no O polysaccharide (OPS), are less virulent than smooth strains and less resistant to complement attack (Ko and Splitter, 2003).

**Intracellular survival of Brucella species:** The Intracellular survival and multiplication of *Brucella* species are a property associated with virulence, since it is essential to the organism’s ability to gain access to nodes and tissues. Production of inhibitors of phagolysosome fusion such as adenine and guanine monophosphate levels which are capable of inhibiting the degranulation of peroxidase-positive granules of polymorphnuclears leukocytes. This would decrease the release of myeloperoxidase enzyme into phagolysosome and reduce the iodination proteins. The myeloperoxidase-hydrogen peroxide-halide antibacterial system is inhibited and the Intracellular survival of organism is facilitated (Canning *et al.*, 1986). Survival within macrophages is also associated with the synthesis of stress induced proteins of different molecular weight, notably 24 kDa which induces acid environment of pH<
4. This acid environment is also responsible for limiting antibiotic action (Pizarro et al, 1998)

**Outer membrane protein 25 of Brucella species:** It is also associated with virulence of organism. It has been identified as the down regulator of TNF alpha especially in an early stage of infection. This leads to impaired activation and cytotoxic function of natural killer cells (Jubier et al, 2001).

**Urease:** Recently urease enzyme has been identified as an important determinant of virulence. Urease has the role to protect *Brucellae* in their passage through the stomach when acquired by the oral route, which is the major way of infection in human brucellosis (Sangari et al, 2007).

All these factors probably play a substantial role in the intracellular survival of at least 15 to 30% of *Brucellae* ingested and these *Brucellae* start replicating in the endoplasmic reticulum. After entering the human body and being taken up by local tissue lymphocytes, *Brucellae* are transferred through regional lymphnodes into the circulation and are subsequently seeded throughout the body, with tropism for the reticuloendothelial system (Mantur et al, 2007).

**2.3.8 Pathogenesis:**

Brucellosis is almost invariably transmitted to man from infected domestic animals (Henk et al, 2005). *Brucellae* can enter mammalian hosts through skin abrasions or cuts, the conjunctiva, the respiratory tract, and the gastrointestinal tract (Buchanan et al, 1974). Following penetration of skin or mucosal epithelium, organisms are rapidly ingested by polymorphonuclear leukocytes, which generally fail to kill them (Elsbach, 1980) and are also phagocytosed by macrophages. Bacteria transported in macrophages, which travel to lymphoid tissue draining the infection site, may
eventually localize in lymph nodes, liver, spleen, mammary glands, joints, kidneys, and bone marrow with the formation of abscesses or granulomatous lesions, resulting in complications of disease (Ko and Splitter, 2003). In macrophages, *brucellae* inhibit fusion of phagosomes and lysosomes (Harmon *et al.*, 1988). They reside in specialized compartments with acidic environments and replication of bacterium takes place in the endoplasmic reticulum without affecting host cell integrity via a process facilitated by the type IV secretion system (Boschiroli *et al.*, 2002). After replication, *brucellae* are released with the help of hemolysins and induced cell necrosis (Gorvel and Moreno, 2002). On the contrary they are apoptosis inhibitors, thus creating a frame for eternal survival and replication (Pappas *et al.*, 2006) and allowing the bacteria to escape the extracellular immune mechanisms of the host (i.e., complement and antibodies)(Young, 1983).

The host response against *Brucella* spp. involves the whole gamut of the immune system, from innate to adaptive immunity (Golding *et al.*, 2001). Cytokines including IL-1, IL-12, IFN-gamma and TNF-alpha appear to have an important role in the pathogenesis of brucellosis and the Th1/Th2 balance may be involved in the susceptibility or resistance to the disease (Pasquali *et al.*, 2001 and Galanakis *et al.*, 2002). Th1 cells are mediators of the effector mechanisms required for resistance to intracellular pathogens, while a Th2 cell response is detrimental in combating this type of infection (Yingst and Hoover, 2003). A Th1 response is essential for resolution of the primary infection caused by *Brucella* and the essential aspect of this response appears to be IFN-γ production (Mielke *et al.*, 1998). On the other hand, Th2 cytokine such as IL-4 evoke strong antibody responses (Romagnani, 2000). It has
been long postulated that the outcome of the disease reflects the equilibrium developed between the bacterium and the human immune response (Pappas et al., 2006).

2.3.9 The Host Response in Humans:

The host response in humans reflects unique features of *brucella*. Smooth lipopolysaccharide does not activate the alternative complement pathway. *Brucella* is resistant to damage from polymorphonuclear cells owing to suppression of the myeloperoxidase–hydrogen peroxide–halide system and copper–zinc superoxide dismutase and the production of inhibitors of adenylate monophosphate and guanyl monophosphate. Impaired activity of natural killer cells and impaired macrophage generation of reactive oxygen intermediates and interferon regulatory factors have been documented (Salmeron et al., 1992; Rodriguez et al., 1997 and Ko et al., 2002). CD4^+^ T lymphocytes play a limited role, acting either by facilitating clonal expansion of other cytolytic cells, as CD8^+^, or by functioning as cytolytic effectors. An increase of CD4^+^ and CD8^+^ lymphocytes is characteristic in brucellosis (Bertotto et al, 1993) as is the importance of a Vγ9Vδ T-cell receptor (Ottones et al, 2000)

IgM against lipopolysaccharide appeared during the first week of infection, followed by class IgG as early as the second week. Both classes of immunoglobulin peaked during the fourth week, and the use of antibiotics was associated with a decline in both IgM and IgG titers. IgM titers persisted at levels that were higher than those of IgG titers for more than six months, and both classes were present for almost a year. The appearance of IgA immunoglobulin in conjunction with IgG immunoglobulin for longer than six months was consistent with the presence of chronic disease. Antibody
response in brucellosis, although extremely useful diagnostically, plays a limited part in the overall host response (Pappas et al, 2005).

Interferon-\(\gamma\) has a central role in the pathogenesis of brucellosis by activating macrophages, producing reactive oxygen species and nitrogen intermediates; by inducing apoptosis, enhancing cell differentiation and cytokine production; by converting immunoglobulin G to immunoglobulin G2a; and by increasing the expression of antigen-presenting molecules (Yingst and Hoover 2003; Zhan and Cheers, 1993). That interferon-\(\gamma\) has a central role in the evolution of brucellosis is highlighted by the effect of a genetic polymorphism in interferon-\(\gamma\) (the +874A allele). Patients who are homozygous for the +847 allele may be relatively more susceptible to brucellosis (Bravo et al, 2003). Typically, serum interferon-\(\gamma\) levels in patients with brucellosis are increased. (Ahmed et al, 1999; Demirdag et al, 2003).

In contrast, the importance of tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)) in human brucellosis is the subject of debate. The inhibition of TNF-\(\alpha\) in human disease is an early, crucial step in infection. This inhibition may also be involved in the impaired activation and cytotoxic function of natural killer cells owing to an active bacterial mechanism that involves outer-membrane protein 25, which has been identified as the down-regulator of TNF-\(\alpha\) (Jubier et al, 2001). Serum levels of TNF-\(\alpha\) were undetectable in patients with active brucellosis in one study (Ahmed et al, 1999) but another study reported that serum levels were increased in a linear fashion with serum levels of interferon-\(\gamma\) and other inflammatory markers (Demirdag et al, 2003). The role of interleukin-12, mainly as a regulator of interferon-\(\gamma\) production, has been extensively studied in and humans (Ahmed et al, 1999; Zhan and Cheers, 1995).
2.4 Clinical manifestations:

Brucellosis is a systemic disease that can involve any organ or system of the body. Four species of Brucella can cause human disease. B. melitensis (found in sheep and goats), B. abortus (found in cattle), B. suis (found in swine) and B. canis (found in dogs). Disease from marine species has also emerged (Sohn et al, 2003; McDonald et al, 2006). B. melitensis remains the principal cause of human brucellosis worldwide. A recent study did not report any clinical difference between cases caused by B. melitensis and B. abortus (Dokuzoguz et al, 2005). The infective dose of Brucella, especially B. melitensis is very low (10 organisms). The incubation period lasts for about 1 to 3 weeks but may be as long as several months, depending on the virulence of the organisms, there route of entry, the infecting dose and the host’s preexisting health status (Madkour and Kasper, 2005).

Human brucellosis is known for presenting with protean manifestations (Mantur et al, 2006). The disease usually manifests as an acute (< 2 months) or subacute (2-12 months) febrile illness, which may persist and progress to a chronically (> 1 year) incapacitating disease with severe complications (Mantur et al, 2007). The onset of symptoms may be either abrupt or gradual. The most common symptoms are fever, fatigue, malaise, chills, sweats, headaches, myalgia, arthralgia and weight loss (Mantur et al, 2006; Dames et al, 2005 and Young, 1989). In some cases the patient presented with only joint pain, low back ache, (Mantur et al, 2006), involuntary movements of limbs, burning feet (Mantur et al, 2006; Mantur et al, 2004). Physical examination of the patient often reveals no abnormalities and deceptively well. But some patients, in contrast, are acutely ill with pallor, lymphadenopathy,
hepatosplenomegally, arthritis, spinal tenderness, epididymoorchitis, rash, meningitis, cardiac murmurs, or pneumonia (Madkour and Kasper et al, 2005).

2.4.1 Complication of brucellosis: Human brucellosis is known for complications. Complications can be very diverse depending on the specific site of infection. Osteoarticular, genitourinary, gastrointestinal, nervous, cardiovascular, skin and mucous membranes and respiratory complications are observed (Hasanjani et al, 2004).

Bones and joints: Osteoarticular disease: Bone and joint involvement is the most frequent complication of brucellosis and occurs in up to 40% of cases (Mousa et al, 1987). Three distinct forms exist; peripheral arthritis, sacroilitis and spondylitis. Peripheral arthritis is the most common and is non-erosive and it usually involves the knees, hips, shoulders, sacroiliac, sternoclavicular ankles and wrists joints in the context of acute infection (Mantur et al, 2006; Tsolia et al, 2002 and Bosilkovski et al, 2004).

Genitourinary tract: Epididymoorchitis is the second most genitourinary complication in men and is often difficult to differentiate from other testicular lesions (Navarro et al, 2001). Brucellosis in pregnancy poses a substantial risk of spontaneous abortion (Khan et al, 2001).

Central Nervous System: Central nervous system (CNS) is involved in about 5-7% of the cases of brucellosis especially in B. melitensis infection. Meningitis, encephalitis, meningoencephalitis, meningovascular disease, brain abscesses and demyelinating syndromes have all been reported (Mantur et al, 2006; Tsolia et al, 2002 and Shakir et al, 1987).
Heart: *Brucella* endocarditis occurs in less than 2% of cases and remains the principal cause of mortality in the course of brucellosis. It usually involves the aortic valve and typically requires immediate surgical valve replacement. Early recognition, adequate antibiotic treatment and the absence of signs of heart failure can guide the practitioner toward prolonged, conservative treatment (Reguera et al, 2003).

Gastrointestinal tract and hepatobiliary system: Manifestations of *Brucella* infection are generally mild and include nausea, vomiting, constipation, acute abdominal pain and/or diarrhea. Acute ileitis with inflammation of payer’s patches and colitis has been reported (Madkour and Kasper, 2005). Hepatitis with enlargement of liver and spleen may be documented in 15 to 20 percent of cases. But liver abscess and jaundice are rare (Ariza et al, 2001). Granulomas can be present in liver in case of both *B abortus* and *B melitensis* infection (Cervantes et al, 1982).

Respiratory Tract: Respiratory tract complications such as pneumonia pleural effusion may be seen in abattoir workers and are thought to be caused by the inhalation of *Brucellae*. Respiratory complications of brucella are rare (Pappas et al, 2003).

Skin and Mucous membrane: Complications involving the skin, although rare, are reported in the literature (Mantur et al, 2006).

2.4.2 Differential diagnosis of brucellosis:

Differential diagnosis for brucellosis includes enteric fever, malaria, rheumatic fever, tuberculosis, cholecystitis, thrombophlebitis fungal infection, autoimmune disease and tumors (Mantur et al, 2006; Lulu et al, 1998 and Young, 1989).
2.5 Laboratory Diagnosis:

The clinical illness in brucellosis is often non specific when considered in the individual patient. Therefore evaluation of patients often includes a number of tests dictated by differential diagnosis. Clinician must develop a high degree of clinical suspicion based on epidemiological information. A thorough travel history as well as history of exposure to animals and animal products is usually critical to making the clinical diagnosis (Young, 1995). When a patient is suspected of having brucellosis, at least a blood sample should be collected from the patient for culture, because isolation of *Brucella* organisms provides the definitive diagnosis of brucellosis. A proper and prompt diagnosis is important, as the treatment requires specific and prolonged antibiotics (Solera *et al*, 1997).

Diagnosed of brucellosis is based on isolation of *Brucella* organism in culture, demonstration of *Brucella* genome by PCR or by a combination of serological tests and clinical findings consistent with brucellosis. Isolation of the *Brucella* organism is the definitive means of diagnosis, but in practice it is difficult due to the early tissue localization and the exacting culture requirements of the organism. In practice, blood cultures are positive in only 10 -30% of brucellosis and the remainder is diagnosed serologically (Young, 1995).

Identification of *Brucella* strains is done using standard classification tests, including Gram stain, a modified Ziehl-Neelsen (ZN) stain, growth characteristics, oxidase activity, urease activity, H₂S production (four days), dye tolerance such as basic fuchsin (1: 50000 and 1: 100000) and thionin (1:25000, 1:50000 and 1:100000) and seroagglutination. Mantur and colleagues have recommended Gram stain morphology and modified ZN staining, coupled with the urease test, for rapid identification of
Brucella to the level of genus where facilities for further identification are not available (Mantur et al, 2006).

2.5.1 Blood culture: The absolute diagnosis of brucellosis requires isolation of bacterium from blood, bone marrow, or tissue sample. Brucella are adapted to an intracellular habitant and their nutrition requirements are complex. Some strains have been cultured on defined media containing amino acids, vitamins, salts and glucose. Brucella are cultured in standard biphasic (solid and liquid) media or with Castaneda bottle, which incorporates both solid and liquid media in same container (Pappas et al, 2005). Fresh specimens from human sources are usually inoculated aseptically into the broth phage of Castaneda’s biphasic media. The media were incubated at 37°C with or without 5-10% Co2 and examined for bacterial growth once a day for 30 days, before being discarded as negative. Tilting broth/blood mixtures over the solid phase every day. Automated blood culture systems (BACTEC 9240) are also reliable in isolating Brucella. Even with Automated system, subculture should be performed for at least four weeks (Bannatyne et al, 1997). When subculture in solid media, colonies usually appear 2-3 days after incubation. A variety of colonial forms are produced by Brucella strains including small, convex, smooth colonies, mucoid and rough colonies also, they may be colorless or grey-white (Cheesbrough, 2000). Brucellae are small, gram negative coccobacilli with oxidase and unease positive that resemble fine grains of sand. Species identification performed on the basis of particulars characteristics (Pappas et al, 2005).

Brucellas are highly infectious pathogens. Laboratory-acquired infection can occur following accidental inoculation or inhalation of organism (Cheesbrough, 2000). Brucellae are relatively slow growing organism and the culture result may not become
available for several days or weeks. The percentage of cases with positive cultures range from 15-70% (Memish et al, 2000). Recently, higher rates of positive blood cultures (91% in acute brucellosis and 74% in chronic brucellosis) along with the rapid confirmation of clinical diagnosis have been reported by lysis centrifugation technique (Mantzur and Mangalgi, 2004). The modern automated blood culture systems (BACTEC-9240) have somewhat improved the speed of detection but are still too slow to make a rapid diagnosis (Bannatyne et al, 1997). *Brucella* species are difficult to isolate, particularly *B abortus* (rarely isolate from blood culture). But cultures are more commonly positive in *B melitensis* and *B suis* infection. The organisms are more likely to be isolate from the blood in acute brucellosis during the time of fever and isolation is extremely rare in chronic brucellosis (Cheesbrough, 2000). Bone marrow cultures are considered the gold standard for the diagnosis of brucellosis, since the relatively high concentration of *brucella* in the reticuloendothelial system makes it easier to detect the organism. Bone marrow cultures have been positive more often than blood culture specially when patients have taken antibiotics Furthermore; bacterial elimination from the bone marrow is equivalent to microbial eradication (Gotozzo et al, 1986). However, harvesting bone marrow for culture remains an invasive, painful technique, and results have not been universally reproducible (Iseri et al, 2006).
2.5.2 Serological diagnosis of human brucellosis:

Unequivocal diagnosis of brucellosis requires isolation of the causal agent. Blood culture is the method of choice, but specimens need to be obtained early prior to antibiotic administration and need prolonged periods of incubation. In addition, failure to detect the pathogen is a frequent occurrence. Although in the last few years PCR-based laboratory tests have been proposed, they cannot be considered a routine diagnostic method yet. These limitations make serology for antibody detection the most useful tool for the laboratory diagnosis of brucellosis (Mantur et al, 2007). Antibodies usually begin to appear in the blood at the end of the first week of the disease, IgM appearing first followed by IgG. The serological tests like Rose Bengal Plate Agglutination Test (RBPT), Standard tube agglutination test (SAT), Coombs test, immunocapture agglutination test (Orduna et al, 2000) latex agglutination, complement fixation test, ELISA, lateral flow assay—a simplified version of ELISA, dipstick assay, fluorescence polarization assay (FPA) have all been applied in the diagnosis of human brucellosis (Lucero et al, 2003)

**Rose Bengal Plate agglutination:** The RBPT is often used as a rapid screening test (Ruiz-Mesa et al, 2005). RB is based on the agglutination of serum antibodies with a stained whole cell preparation of killed *Brucella*. RBPT is performed by mixing on a glass plate a drop of RB reagent with an equal volume of serum and agglutination is read after 2 to 4 minutes (Henk et al, 2005). The sensitivity is very high (>99%) but the specificity is disappointingly low (Mantur et al, 2006 and Barroso et al, 2002). However, this is of value as a screening test in high risk rural areas where it is not always possible to perform the tube agglutination titration test. To increase the specificity and the positive predictive value of the RBPT, the test may
be applied to a serial dilution (1:2 through 1:64) of the serum sample. The specificity of the test increases when higher dilutions agglutinate and titres of 1:8 or 1:16 and above may be regarded as positive (Smits and Kadri, 2005). This approach may result in a lower sensitivity. Whenever possible, a serum that gives a positive result should be confirmed by a more specific test. The RBPT is also of value in the rapid confirmation of neurobrucellosis, arthritis, epididymoorchitis, hydrocele due to *Brucella* if the neat is positive in CSF, synovial fluid, testicular fluid /semen and hydrocele fluid respectively The RBPT is not affected by prozones or immunoglobulin switching (Mantur *et al*, 2006).

**Standart tube agglutination test**: SAT developed by Wright and Smith 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 (Wright and Smith, 1897). SAT measures the total quantity of agglutinating antibodies (IgM and IgG) (Young, 1991). Although no single serological test provides 100% sensitivity and specificity, STAT still remains the test of choice in diagnosis of brucellosis. In the presence of appropriate signs and symptoms, a presumptive diagnosis of brucellosis is usually defined serologically as a standard tube agglutination titer of 1: 160 or greater (Bettelheim *et al*, 1984). However, in areas of endemic disease, using a titre of 1:320 as cuttoff may make the test more specific. The differentiation in the type of antibody is also important, as IgG antibodies are considered a better indicator of active infection than IgM and the rapid fall in the level of IgG antibodies is said to be prognostic of successful therapy (Buchanan and Faber, 1980).
The serological diagnosis of infection by the STAT is sensitive and reproducible. The success of the agglutination test has depended lastly on the selection and standardization of the antigen. The antigen from *B. abortus* has been used to diagnose disease with any one of the three commonly encountered species of *Brucella*. For the diagnosis of disease caused by *B. canis*, it is necessary to use specific antigen or antigen from *B. canis*. Usually two-fold dilations of serum from 1 in 20 to 1 in 640 are tested. Most test tubes are read after 24 hours incubated at 37°C. Control positive and negative *Brucella* sera must be included with each batch of test (Cheesbrough, 2000).

Drawbacks of the serum agglutination test include the inability to diagnose *B. canis* infections; false-positive reactions can also be seen in the SAT test and they occasionally result from cross reactions with antibodies to *Yersinia enterocolitica* O:9, *Francisella tularensis*, *Escherichia O157*, *Salmonella O:30*, *Vibrio cholerae O:1* (Pappas *et al*, 2005). On the other hand, sometimes false-negative reactions can also be seen in the SAT tests in early in the course of infection due to presence of blocking antibodies, or the so called "prozone" phenomenon (i.e., the inhibition of agglutination at low dilutions due to an excess of antibodies or to nonspecific serum factors) (Young, 1991). Some of these shortcomings can be overcome by modifications such as the addition of EDTA, 2-mercaptoethanol, or antihuman globulin (Pappas *et al*, 2005).

**Enzyme-linked immunosorbent assay:** Indirect enzyme-linked immunosorbent assay typically uses cytoplasmic proteins as antigens. ELISA measures IgM, IgG and IgA, which allows for a better interpretation of the clinical situation and overcomes some of the shortcomings of serum agglutination test. A comparison with the SAT,
ELISA yields higher sensitivity and specificity. (Almuneef and Memish, 2003) Among the newer serologic tests, the ELISA appears to be the most sensitive; however, more experience is needed before it replaces the SAT as the test of choice for brucellosis (Mantur et al, 2007). In patients with neurobrucellosis, ELISA offers significant diagnostic advantages over conventional agglutination methods (Araj et al, 1986).

In many studies performed with ELISA, it was determined that IgG positivity and the increasing of the antibody titers were considerably valuable in relapsed cases and in patients with chronic infections (Ariza et al, 1992 and Araj, 1986). In studies reported that the IgM antibody may be detected by ELISA after the first week following the entry of the organism. The peak level is reached 4 weeks later. The IgG antibody has a delayed appearance, although it is found together with IgM 4 weeks after the initial antigenic stimulus. In acute brucellosis, very high IgM levels may be found (Osoba et al, 2001). Following recovery, IgM levels fall slowly and gradually disappear from the blood over a period of several months, whereas IgG levels fall rapidly and disappear from the blood with in a few weeks of infection. The persistance, therefore of IgG antibodies by ELISA in the blood is an indicator of chronic or relapse brucellosis (Cheesbrough, 2000). Variation of ELISA exists such as competitive ELISA and sandwich ELISA which may prove useful as a follow-up tool.

**Mercaptoethanol test:** Low titre agglutinins due to residual IgM may persist for many years or even years after the infection has cleared. The mercaptoethanol test is carried out simultaneously and in same manner as the standard agglutination test except that the saline dilution contain 0.05M 2-mercaptoethanol (2ME). The agglutinating ability of IgM is destroyed by mercaptoethanol test and therefore
agglutination in this test is indication of continuous present of IgM and likelihood of persisting and follow-up of brucellosis by 2ME test is importance in conjunction with SAT. So 2ME agglutination test is a useful assay, as it is inexpensive and technologically simple with stable reagents (Mantur et al, 2006)

**Other serological test:**

A dipstick assay offers a rapid and reliable diagnostic alternative in acute brucellosis (Cacao et al, 2003). The rapid and simple assays like *Brucella* IgM and IgG lateral flow and latex agglutination have been developed recently. The sensitivity and specificity of lateral flow assay for culture confirmed brucellosis is >95% (Smits et al, 2003). The sensitivity of the latex agglutination assay was determined to be 89.1% for the initial serum samples collected for the patients with culture confirmed brucellosis and the specificity was 98.2% (Abdoel and Smits, 2007).

**2.5.3 Molecular diagnosis:**

The development of a specific polymerase chain reaction (PCR) is a recent advance. Polymerase chain reaction (PCR) is fast and can be performed on any clinical specimen (Queipo-Ortuno et al, 2006). Two major gene sequences have been used as targets: the 16S rRNA gene sequence which presents total genus-specific homology and has been satisfactory in clinical settings (Nimri, 2003), Another *BCSP31* gene, which encodes an immunogenic protein of the external membrane of *B. abortus* and has been extensively studied in clinical practice (Morato et al, 1999). Cross-reactivity with *Ochrobactrum* is noticed sporadically with both techniques. A comparison of the two techniques showed superiority of the 16S rRNA target in terms of sensitivity (Navarro et al, 2002).
Real-time PCR is another diagnostic tool for detection of *B. abortus, B. melitensis* and *B. suis* biovar1. These PCR assays target the specific integration of IS711 elements within the genome of the respective *Brucella* species or biovar (Redkar *et al*, 2001). 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 and Gee *et al*, 2004). 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).

**Hematological examination:** The blood count is often characterized by mild leukopenia and relative lymphocytosis, along with mild anemia and thrombocytopenia. Pancytopenia in brucellosis is multifactorial and is attributed to hypersplenism and bone marrow involvement. Rarely, marked pancytopenia or isolated deficits can be attributed to diffuse intravascular coagulation, hemophagocytosis, or immunologically mediated cellular destruction (Young *et al*, 2000 and Pappas *et al*, 2004).

**2.6 Treatment of Brucellosis:**

Treatment of human brucellosis should involve antibiotics that can penetrate macrophages and can act in the acidic intracellular environment. There is a general need for combined treatment, since all monotherapies are characterized by unacceptably high relapse rates. Practitioners must weigh such questions as the optimal duration of treatment, cost-effective and conveniently administered regimens,
favorable pharmacokinetics and pharmacodynamics, and attention to local virulence factors (Solera et al, 2004; Pappas et al, 2005)

The treatment of human brucellosis is a controversial because of the spectrum of disease, the possibility of chronic infection and the development of complications (Radolf, 1994). In 1986, the World Health Organization issued guidelines for the treatment of human brucellosis. The guidelines discuss two regimens, both using doxycycline 100 mg twice daily for a period of six weeks, in combination with either streptomycin (1 gm/day intramuscular) for two to three weeks treatment or rifampin 600 to 900 mg once daily for six weeks. Both combinations are the most popular treatments worldwide, although they are not used universally (WHO, 1986). The streptomycin containing regimen is slightly more efficacious in preventing relapse (Mantur et al, 2006; Ariza et al, 1985). This may be related to the fact that rifampin down-regulates serum doxycycline levels (Colmenero et al, 1994) However, parenteral administration of streptomycin mandates either hospital admission or the existence of an adequate health care network- both of which 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 usually endemic, raises concern about the development of community resistance to rifampin (Pappas et al, 2005).

Alternative drug combinations have been used, including other aminoglycosides (e.g., gentamicin) (Solera et al, 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 classic regimens (Karabay
et al, 2004). Although quinolones have been used and will continue to be used, the cost of this approach remains a major drawback. The action of macrolides is attenuated in the acidic phagolysosomal environment, and thus these agents are not useful in brucellosis (Solera et al, 2001).

Childhood brucellosis can be successfully treated with a combination of two drugs; doxycycline 4 mg / kg / day and rifampicin 10 mg/kg /day orally for six weeks. Some authors advise that gentamicin (5 mg/kg/day intramuscularly) be administered concomitantly for the initial five to seven days of therapy in order to prevent relapse. Co-trimoxazole (TMP/SMX) 8 mg / 40 mg/kg/day can be used for children < 6 years of age (Mantur et al, 2004). Rifampicin with or without a combination of cotrimoxazole has proved safe to treat brucellosis during pregnancy. 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 regimens (Pappas et al, 2005; Ozbay and Inanmis, 2006).

Most complications of brucellosis can be adequately treated with standard regimens. Treatment of some complications like spondylitis, osteomyelitis, neurobrucellosis and endocarditis also require combination therapy but longer duration for 8 to12 weeks. For neurobrucellosis, combination therapy with two or three drugs - that is doxycycline, rifampicin and trimethoprim-sulfamethoxazole that penetrate CNS and are active against the infecting isolate is recommended (McLean et al, 1992). The combination of doxycycline with rifampicin and trimethoprim-sulfamethoxazole has been used successfully in the treatment of brucellar endocarditis. Although cases of endocarditis caused by Brucellae have been cured with antimicrobial chemotherapy alone, it is generally believed that surgical intervention (valve replacement) combined
with antibiotic therapy is the best approach (Young, 1995; Mantur et al, 2006 and Cisneros et al, 1989).

A human vaccine has not been developed for 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 have gained wide acceptance (Schurig et al, 2002). Vaccines derived from the B. abortus strain 19 have been used in the former Soviet Union, and strains of B. abortus 104M have been used in China. A phenol-insoluble peptidoglycan fraction of B. melitensis strain M15 was used in France (Hadjichristodoulou et al, 1994). Theoretical vaccine targets for the future might use rfbK mutations of B. melitensis, outer-membrane protein 25, and the cytoplasmic protein BP26 (Ko and Splitter, 2003)

Eradication of brucellosis depends largely on socioeconomic and political circumstances. Progress in understanding the molecular pathogenesis of the disease, vaccine engineering, and postgenomic approaches may lead to new preventive interventions Furthermore, the discovery of new pathways in modifying the acidic intracellular environment in which the microbe moves might be used in adjuvant pharmacotherapy. Determination of microbial load might modify treatment planning and the potential for complications (Pappas et al, 2005).
MATERIALS AND METHODS

3.1 Place and period of the study:
The study was carried out in the Department of Microbiology, Mymensingh Medical College in collaboration with department of Medicine under the faculty of veterinary science, Bangladesh Agricultural University, during the period from July 2007 to June 2008. The suspected cases of brucellosis were selected from out door and in door department of Mymensingh Medical College Hospital and population at risk of brucellosis also were selected from field level of Mymensingh district by using questionnaires based data sheet about hypothesized risk factors. The distributions of human brucellosis at field level were determined by using Geographical Information System [GIS] software and spatial analysis.

3.2 Study design: Cross-sectional descriptive study.

3.3 Study population: The present study which included at least 510 cases of pyrexia of unknown origin (PUO) and population at risk. The subjects were selected into two groups according to following criteria.

a). Fever group: Patients having clinical suspicion of brucellosis irrespective of age and sex were selected on the basis of following criteria.
   i. Pyrexia of unknown origin: It was defined by febrile illness for more than 2-3 weeks duration having the temperature exceeding 38.3°C [101°F] where no diagnosis is established. (Petersdorf & Beeson, 1961).
   ii. Malaise, profuse sweating, weight lose.
   iii. Arthralgia, depression.
   iv. Splenomegally /Hepatomegally/ lymphadnopathy (Mantur et al, 2007)
b) **Risk group:** This group was included as per the following criteria:

(i) Animal farmers (Rearing of animals: cattle, goat, sheep, buffalo, pig, and dog).

(ii) Veterinarian workers.

(iii) Slaughter house workers.

(iv) Shepherds.

(v) Dairy workers/Dairy farm/who had a history of raw milk consumption,

   (Agasthya *et al*, 2007)

3.4 Data collection and recording:

All relevant data were systematically recorded in predesigned data sheets for future analysis by appropriate computer programme.

3.5 **LABORATORY PROCEDURE**

3.5.1 **Sample:** Blood was used for serological tests.

3.5.2 **Sample collection:**

Written consents were taken from the patients or from their attendants before collection of samples. 10 ml of venous blood were collected aseptically and centrifuged at 1500 rpm for 5 minutes. Serum was stored in a sterile eppendorfs tube at – 20 °c until uses.

3.5.3 **Serological tests:** The following four serological tests were performed for diagnosis of brucellosis.

   A. Slide agglutination test:

   B. Rose Bengal plate test.

   C. Standard tube agglutination test.

   D. Enzyme-linked immunosorbent assay (ELISA).
All the collected samples were tested by slide agglutination test, Rose Bengal slide agglutination test and standard tube agglutination test. Titration of 1/80 were accepted as exposure to Brucella species and 1/160 were accepted as Brucella infection (Sumer et al, 2003). All the positive samples were examined by ELISA to detect IgG antibodies against Brucella species.

3.5.3.1 A. Slide agglutination test

Slide agglutination test is commonly used rapid and simple test for serological detection of brucellosis.

**Principle of the test:** Brucella abortus and B melitensis used as antigen for detection of brucella specific IgG and IgM antibodies.

**Procedure of slide agglutination test**

**Rapid slide titration methods**

1. Using micropipette, dispensed 0.08ml, 0.04ml, 0.02ml, 0.01ml, 0.005ml of undiluted serum onto a row 3 cm diameter circles on white tile.

2. Shake the reagents bottle well and added one drop of the undiluted antigen to each serum aliquot.

3. Mixed well by using a stirring stick and spreading the contents to fill the circles.

4. Rotated the slide slowly and agglutination was read at one minute.
Reading and interpretation

Agglutination seen in any circle was indicated of following results.

0.08ml=1:20, 0.04ml=1:40, 0.02ml=1:80, 0.01ml=1:160 and 0.005ml=1:320.

In this way the rapid slide test provided an approximation of the expected results from corresponding tube test.

3.5.3.2 B. Rose Bengal test

The Rose Bengal is a rapid plate agglutination test for diagnosis of brucellosis. It is very useful screening test and show similar result to standard tube agglutination test (Agasthya et al, 2007)

Principle of the test

The Rose Bengal test is used for the qualitative detection of anti-Brucella antibodies in human serum. The bacterial suspension of Brucella abortus (strain 544) stained with Rose Bengal dye is used for detection of Brucella specific IgG and IgM antibodies.

Clinical significance of the test:

The reagent, because of its formulation in an acid buffer, is reactive with both IgG and IgM antibodies and very useful for the diagnosis of chronic individuals, which present a very high level of IgG antibody.

Reagents:

a) Rose Bengal: Brucella abortus suspension, strain 544, in lactate buffer 1mol/L, phenol 5 g/L, Rose Bengal, PH 3.6.
b) Positive control+: Animal serum, with an anti-Br abortus antibody, Sodium azide 0.95 g/L.

c) Negative control-: Animal serum, Sodium azide 0.95 g/L.

Test procedure

a) Samples and all the reagents were kept at room temperature before uses.

b) The reagents were shaken.

c) The reagents were checked against positive and negative control as (follows).

d) 50 µl of serum was placed onto a circle of slide and then a drop of reagent was kept next to the sample.

e) Both drops were mixed by a stirrer, spread over the full surface of the circle.

f) The plate was rotated by a mechanical rotator at 80-100rpm for 4 minutes.

Reading and interpretation:

a) The presence of agglutination was indicating positive result (+ve) (presence of specific antibodies)

b) The absence of agglutination were indicate negative result (-ve) (absence of specific antibodies).

3.5.3.3 C. Standard tube agglutination test (STAT):

Standard tube agglutination test is most widely used of all serological tests for brucellosis and simple to perform (Memish et al, 2002)

Principle of the test:

Smooth whole cells of B. abortus were used as antigen for detection of brucella specific IgG and IgM antibodies.
Test procedure

a) Standard tube agglutination test was done by using previous dilution of serum sample in saline.

b) A raw of test tubes was prepared for antigen from 1 in 20 to 1 in 320 dilutions.

c) Two tubes were prepared for positive and negative control.

d) A drop of antigen suspension was added to each tube.

e) Both the antigen and serum were mixed thoroughly and was incubate at 37°C degree centigrade for 24 hours.

Reading and interpretation:

After the indicated period of time, presence or absences of agglutination were observed. In positive serums for Brucella antigens a clear granular agglutination was appear. The titer of highest dilution giving positive result.

3.5.3.4 D. Enzyme-linked immunosorbent assay (ELISA):

The test has recently introduced for detection of Brucella specific IgG and IgM antibodies by using smooth lipopolysaccharide of Brucella abortus 99. The indirect ELISA has more sensitive and specific than Rose Bengal Plate Test (RBPT) and Standard Tube Agglutination Test (STAT) (Agasthya et al, 2007). The IgM antibody may be detected after the first week following the entry of organism. The peak level of IgM antibody will be reached 4 weeks later (Memish et al, 2002). On the other hand, IgG positivity and the increasing of the antibody titers were considerably valuable in the relapse cases and in the patient with chronic infection (Ariza et al, 1992). All the positive sera samples from both groups were tested for Brucella specific IgG antibodies by ELISA using a commercial kit. The test were performed and evaluated according to the kit procedure.
ELISA test for detection of anti-Brucella IgG:

Serum anti-Brucella IgG was detected by ELISA Brucella IgG kit by using smooth LPS Brucella spp. The test procedure was as per manual.

**Principle of the test:**

*Brucella* ELISA is an indirect immuno-enzymatic technique allowing the detection of antibodies against smooth *Brucella* sp SPS (Limet et al., 1988; Godfroid et al, 1994).

**The reaction was made of 3 steps.**

a) Samples were transferred in a smooth *Brucella* sp LPS coated well. Antibodies present in the sample binded to the bacterial antigen.

b) After washing the wells, a protein-G/peroxydase conjugate was added. It binds to the immunoglobulins bound to the antigen.

c) The excess of conjugate was removed by washing. The enzyme linked to the complex was revealed by the addition of a substrate that was transformed in a coloured product. Optical densities were measured in a spectrophotometer after stopping the enzymatic reaction.

**Test procedure:**

a) Coated the ELISA plates with 0.1 ml per well of 1 µg/ml of LPS (stock solution 10 mg/ml) in GS buffer (stock solution) diluted 50 x, pH 9, 2 ± 0, 1. Close the wells with an adhesive cover and incubate for 3 hours at 37 °C ± 3°C and, then, for 18 hours at 5°C ± 3°C. The plates can be stored as such for up to 4 weeks at 5°C ± 3°C.

The plates must be washed 5x with the washing solution before use.

b) The reagents and serum samples to be tested were taken out of the refrigerator and were allow warming to room temperature.
c) Transfer, always in duplicate, 0.05 ml of liquid:

1. Standard curve: 6 two-step serial dilutions (from 1/270 to 1/8640) of the reference positive control (Belgian reference positive control 1121) in GS-EDTA-Tw buffer with 2 % negative serum (the last dilution (1/8640), the estimated OD of which was used as a cut-off, is tested 4 times on each plate)

2. Serum samples 1/50 diluted in GS-EDTA-Tw (10µl serum + 500 µl GS-EDTA-Tw)

3. A negative control 1/50 diluted in GS-EDTA-Tw( 10µl FCS + 500 µl GS-EDTA-Tw)

4. A blank: GS-EDTA-Tw

**Framework**

74µl serum 1121 $\rightarrow$ 2 ml serum buffer (1/270)

1ml 1/270 $\rightarrow$ 1ml serum buffer (1/540)

1ml 1/540 $\rightarrow$ 1ml serum buffer (1/1080)

1ml 1/1080 $\rightarrow$ 1ml serum buffer (1/2160)

1ml 1/2160 $\rightarrow$ 1ml serum buffer (1/4320)

1ml 1/4320 $\rightarrow$ 1ml serum buffer (1/8640)

d) Incubated the microplates for $\pm$ 1 hour at room temperature ($20 \pm 5^\circ$C)

Washed the plates 5 times with the washing solution

e) Transferred in each well 0.05 ml of the protein G-HRPO conjugate solution

f) Incubated the microplates for $\pm$ 1 hour at room temperature ($20 \pm 5^\circ$C),

Wash the plates 5 times with the washing solution

g) Transferred in each well 0.1 ml loaf the substrate solution
h) Incubated the microplates at room temperature (20 ± 5 °C) in the dark for 5 to 15 minutes depending on the pre-lecture of the dilution 1/8640. When it gives a value of 0.050, you must stop the reaction.

i) Stopped the reaction by adding 0.025 ml of the stopping solution in each well.

**Reading:**

Read the optical densities (OD) with 492 nm and a 620 nm filter. The OD measurement was the difference between the two. ELISA units were defined using the cubic spline of the standard curve (OD in function of dilution) as a scale. The scale was multiplied by 16 200 to obtain ELISA units (so the standard curves ranges between 60 and 2).

**Interpretation of the results:**

In most species, sera with a titre greater than 2 were considered positive and sera with a titre lower than 2 were considered negative.
Results

The Present study was conducted on a total of 510 subjects, among them, 300 were pyrexia of unknown origin group and 210 were risk group of population of either sex from 2 to 80 years of age.

Table no.1 & Figure no.1 shows age and sex distribution of the pyrexia of unknown origin group. Among them, 196 were male and 104 were female. Majority of the subjects 154 (51.33%) were in the age group of <20 years. Minimum numbers of subjects 39 (13%) were in the age group of >41 years. Male to female ratio was 1.88:1. Figure no. 2 and 3 shows the distribution of male and female respondents in pyrexia group according to age. Total male were 196 (65.33%) and female were 104 (34.67%) in pyrexia group. Among 196 male respondents majority of the subjects were 94 (47.96%) and age group were <20 years. Among 104 female respondents, majority of the subjects were 60 (57.69%) and age group were <20 years.

Table no. 2 & Figure no. 4 shows age and sex distribution of risk group of Population. Among them, 150 were male and 60 were female. Majority of the subjects 120 (57.14%) were in the age group between 21-40 years Minimum numbers of subjects 19 (9.05%) were in age group of < 20 years. Male to female ratio was 2.5:1.
Table 1. Age and sex distribution of the pyrexia of unknown origin group.

<table>
<thead>
<tr>
<th>Age group (Yrs)</th>
<th>Pyrexia of unknown origin group (n-300)</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;20</td>
<td></td>
<td>94 (61.04%)</td>
<td>60 (38.96%)</td>
<td>154 (51.33%)</td>
</tr>
<tr>
<td>21 – 40</td>
<td></td>
<td>74 (69.16%)</td>
<td>33 (30.84%)</td>
<td>107 (35.67%)</td>
</tr>
<tr>
<td>&gt;41</td>
<td></td>
<td>28 (71.79%)</td>
<td>11 (28.21%)</td>
<td>39 (13%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>196 (65.33%)</td>
<td>104 (34.67%)</td>
<td>300 (100%)</td>
</tr>
<tr>
<td>Ratio</td>
<td></td>
<td>Male : Female = 1.88 : 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate percentage

- Minimum age- 2 years, maximum age-80 years
- Majority of the subjects in the age group of < 20 years – 51.33%
- Minimum numbers of subjects in the age group of > 41 years – 13%
- Male – 65.33%
- Female – 34.67%
Figure 1: Age-Sex distribution of Pyrexia group

Age-Sex distribution of Pyrexia group

- <20 years: 94 (Male 60, Female 34)
- 21–40 years: 74 (Male 33, Female 41)
- >41 years: 28 (Male 11, Female 17)
Figure 2. Distribution of male & female respondents according to age in pyrexia group.
Table 2. Age and sex distribution of the risk group of population.

<table>
<thead>
<tr>
<th>Age group (Yrs)</th>
<th>Risk group of population (n=210)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>&lt;20</td>
<td>16 (84.21%)</td>
</tr>
<tr>
<td>21–40</td>
<td>76 (63.33%)</td>
</tr>
<tr>
<td>&gt;41</td>
<td>58 (81.69%)</td>
</tr>
<tr>
<td>Total</td>
<td>150 (71.43%)</td>
</tr>
<tr>
<td>Ratio</td>
<td></td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate percentage

- Minimum age - 9 years, maximum age -70 years.
- Majority of the subjects in the age group of between 21 – 40 years – 57.14%
- Minimum numbers of subjects in the age group of <20 years – 9.05%
- Male – 71.43%
- Female – 28.57%
Figure 3: Age & Sex distribution of the risk group of population
Table no.3 shows seroprevalence of brucellosis among pyrexia group. Prevalence of brucellosis detected by slide agglutination test, Rose Bengal plate agglutination test and Standard tube agglutination test were 27(9%), 24(8%), 23(7.67%) respectively.

Table no.4 shows prevalence of brucellosis among risk group. Seroprevalence of brucellosis were 9(4.28%), 7(3.33%), 7(3.33%) as examined slide agglutination test, Rose Bengal plate agglutination test and Standard tube agglutination test respectively.

Table no.5 shows occupational wise seroprevalence of brucellosis of pyrexia of unknown origin. Highest prevalence of brucellosis among occupational group were animal farmers was 17.74% followed by house wives were 9.38%.

Table no.6 shows occupational wise seroprevalence of brucellosis of risk group. Highest prevalence of brucellosis among occupational group was veterinaries workers were 11.11% followed by dairy workers were 6.45% & then animal farmers were 4.67%.

Table no. 7 shows the prevalence of brucellosis was significantly (here chi-square = 3.87, p<.05 & df = 1) affected by sex, where the rate of detection was higher among female (13.46%) than male (6.63%) as detected slide agglutination test.

Table no. 8 shows that 63.33% cases were from rural areas and 36.67% were from urban area in pyrexia of unknown group. The prevalence of brucellosis was slightly higher in rural area than in urban area as detected by standard tube agglutination test.
Table 3. Seroprevalence of brucellosis among pyrexia group.

<table>
<thead>
<tr>
<th>Total sample (n=300)</th>
<th>Slide agglutination test</th>
<th>Rose Bengal plate agglutination test</th>
<th>Standard tube agglutination test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>27(9%)</td>
<td>24(8%)</td>
<td>23(7.67%)</td>
</tr>
<tr>
<td>Negative</td>
<td>273(91%)</td>
<td>276(92%)</td>
<td>277(92.33%)</td>
</tr>
</tbody>
</table>
Table 4. Seroprevalence of brucellosis among risk group.

<table>
<thead>
<tr>
<th>Total sample (n=210)</th>
<th>Slide agglutination test</th>
<th>Rose Bengal plate agglutination test</th>
<th>Standard tube agglutination test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>9 (4.28%)</td>
<td>7 (3.33%)</td>
<td>7 (3.33%)</td>
</tr>
<tr>
<td>Negative</td>
<td>201 (95.72%)</td>
<td>203 (96.67%)</td>
<td>203 (96.67%)</td>
</tr>
</tbody>
</table>
Table 5. Seroprevalance of Brucellosis among occupational groups of patients with pyrexia of unknown origin. (n=300)

<table>
<thead>
<tr>
<th>Occupational groups</th>
<th>Number of samples</th>
<th>Positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal farmers</td>
<td>62</td>
<td>11</td>
<td>17.74</td>
</tr>
<tr>
<td>Housewives</td>
<td>32</td>
<td>03</td>
<td>9.38</td>
</tr>
<tr>
<td>Businessman</td>
<td>11</td>
<td>01</td>
<td>9.09</td>
</tr>
<tr>
<td>GO &amp; NGO personnel</td>
<td>35</td>
<td>02</td>
<td>5.71</td>
</tr>
<tr>
<td>Workers</td>
<td>19</td>
<td>01</td>
<td>5.26</td>
</tr>
<tr>
<td>Students</td>
<td>90</td>
<td>04</td>
<td>4.44</td>
</tr>
<tr>
<td>Others</td>
<td>51</td>
<td>01</td>
<td>1.96</td>
</tr>
<tr>
<td>Total</td>
<td>300</td>
<td>23</td>
<td>7.67</td>
</tr>
</tbody>
</table>
Table 6. Seroprevalance of Brucellosis among occupational groups in risk population. (n=210)

<table>
<thead>
<tr>
<th>Occupational group</th>
<th>Number of samples</th>
<th>Positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veterinary supervisor, Veterinary assistant, Veterinary physicians</td>
<td>9</td>
<td>01</td>
<td>11.11</td>
</tr>
<tr>
<td>Milker or dairy workers</td>
<td>31</td>
<td>02</td>
<td>6.45</td>
</tr>
<tr>
<td>Animal farmer (Cattle, Goat, Sheep, Pig, Buffalo etc.)</td>
<td>150</td>
<td>07</td>
<td>4.67</td>
</tr>
<tr>
<td>Slaughter house workers</td>
<td>20</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>Total</td>
<td>210</td>
<td>10</td>
<td>4.76</td>
</tr>
</tbody>
</table>
Table 7. Occurrence of brucellosis by slide agglutination test in PUO according to sex.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Slide agglutination test</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
</tr>
<tr>
<td>Male</td>
<td>13 (6.63%)</td>
<td>183 (93.37%)</td>
<td>196</td>
</tr>
<tr>
<td>Female</td>
<td>14 (13.46%)</td>
<td>90 (86.54%)</td>
<td>104</td>
</tr>
<tr>
<td>Total</td>
<td>27 (9%)</td>
<td>273 (91%)</td>
<td>300</td>
</tr>
</tbody>
</table>

(P< 0.05 by chi-square test)
Table 8. Prevalence of brucellosis among pyrexia group in rural and urban area by standard tube agglutination test

<table>
<thead>
<tr>
<th>Residence</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rural</td>
<td>16 (8.42%)</td>
<td>174 (91.58%)</td>
<td>190</td>
</tr>
<tr>
<td>Urban</td>
<td>7 (6.36%)</td>
<td>103 (93.64%)</td>
<td>110</td>
</tr>
<tr>
<td>Total</td>
<td>23 (7.67%)</td>
<td>277 (92.33%)</td>
<td>300</td>
</tr>
</tbody>
</table>
Table no.9 shows result of the slide agglutination test in paired sample of pyrexia of unknown origin group. 9 cases of pyrexia group shows no rising titre by slide agglutination in paired sera between 2 – 3 weeks interval.

Table no.10 shows sensitivity and specificity of ELISA (IgG) in brucellosis cases and risk group of population. The sensitivity and specificity was 81.48% and 74.36% respectively. Positive predictive and negative predictive value was 68.75% and 85.29% respectively.

Table no.11 shows comparison between brucellosis cases and risk group by ELISA. 27 brucellosis cases diagnosed by slide agglutination test, Rose Bengal plate agglutination test and Standard tube agglutination test. Among them 22 (81.48%) were positive by ELISA IgG. In another group, 39 sera sample (10 were positive by slide agglutination, RBPA and STAT and 29 were negative sera by other serological tests) from risk group also tested by ELISA IgG. Among them 10 (25.64%) were positive by ELISA IgG.
Table 9. Results of the slide agglutination test of paired sample of pyrexia group. (n=9).

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>First collection</th>
<th>Result</th>
<th>Second collection</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>B127</td>
<td>27/12/2007</td>
<td><em>B. abortus</em> (1:160)</td>
<td>7/1/2008</td>
<td><em>B. abortus</em> (1:160)</td>
</tr>
<tr>
<td>B130</td>
<td>27/12/2007</td>
<td><em>B. abortus</em> (1:160)</td>
<td>30/1/2008</td>
<td><em>B. abortus</em> (1:80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>B. melitensis</em> (1:320)</td>
<td></td>
<td><em>B. melitensis</em> (1:160)</td>
</tr>
<tr>
<td>B185</td>
<td>30/01/2008</td>
<td><em>B. melitensis</em> (1:160)</td>
<td>20/2/2008</td>
<td><em>B. melitensis</em> (1:80)</td>
</tr>
<tr>
<td>B 269</td>
<td>27/03/2008</td>
<td><em>B. abortus</em> (1:320)</td>
<td>29/6/2008</td>
<td><em>B. abortus</em> (1:160)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>B. melitensis</em> (1:160)</td>
<td></td>
<td><em>B. melitensis</em> (1:80)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table no.9 shows no rising titer in paired sera sample.
Table 10. Sensitivity and specificity of ELISA IgG in brucellosis cases and risk group of population.

<table>
<thead>
<tr>
<th>ELISA test (IgG)</th>
<th>Brucellosis cases (n=27)</th>
<th>Risk group (n=39)</th>
<th>Total</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>(a) 22 (81.48%)</td>
<td>(b) 10 (25.64%)</td>
<td>(a+b) 32</td>
<td>81.48%</td>
<td>74.36%</td>
</tr>
<tr>
<td>Negative</td>
<td>(c) 05 (18.52%)</td>
<td>(d) 29 (74.36%)</td>
<td>(c+d) 34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>39</td>
<td>66</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N: B: Sensitivity and specificity was calculated by following formula.

- a = True positive, b = False positive, c= False negative & d = True negative
- Sensitivity = \( \frac{a}{a+c} \times 100 \)
- Specificity = \( \frac{d}{b+d} \times 100 \)
- Positive predictive value = \( \frac{a}{a+b} \times 100 = 68.75 \)
- Negative predictive value = \( \frac{d}{c+bd} \times 100 = 85.29 \)
Table 11. Comparison of ELISA IgG between brucellosis cases and risk group of population.

<table>
<thead>
<tr>
<th>ELISA test (IgG)</th>
<th>Brucellosis cases</th>
<th>Risk group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>22 (81.48)</td>
<td>10 (25.64%)</td>
<td>32</td>
</tr>
<tr>
<td>Negative</td>
<td>05(18.52%)</td>
<td>29 (74.36%)</td>
<td>34</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>39</td>
<td>66</td>
</tr>
</tbody>
</table>

(P< 0.001 by chi-square test)
Worldwide, millions of individuals are at risk of acquiring brucellosis especially in developing countries, where the infection in animals has not been brought under control, which may be due to mismanagement on animal quarantine, eradication of infected animals or vaccination in the poor areas (Wang et al., 1998). Clinical picture of brucellosis in man is very heterogenous and non specific which may be represented either by subclinical or atypical infection in both the acute and chronic stages. This makes the diagnosis of brucellosis; always require laboratory confirmation, either by isolation of Brucella organism in a culture, or by demonstration of specific antibodies by a combination of serological tests and clinical finding consistent with brucellosis (Colmenero et al., 1996; Morata et al., 2003). Isolation of the Brucella organism is the definitive mean of diagnosis but in practice it is difficult due to the early tissue localization and the exacting culture requirements of the organism. In practice, blood culture are positive in 10%-30% a brucellosis and the remainder is diagnosed serologically (Young, 1995). Although no single test Provides 100% sensitivity and specificity, a variety of serologic tests have been applied to brucellosis, STAT is the most widely used, however indirect enzyme linked immunosorbent assay (ELISA) was documented as the most sensitive test (Corbel and MacMillan, 1998). Keeping the described in mind we studied seroprevalence of human brucellosis among pyrexia and risk group in our country by slide agglutination test, Rose Bengal plate agglutination test, Standard tube agglutination and ELISA (IgG) test.

In the present study, in the pyrexia of unknown origin group, majority of the cases were in the age group of < 20 years and between 21-40 years in risk group of
population (Table 1 and Table 2). We found 51.33% cases in the age group below 20 years in pyrexia group. A study by Asmaa et al (2005) reported most of the clinically suspected cases (43.30%) in the age group between 1-15 years having very close correlation with present study. In risk group of population, we found 57.14% of subject in the age group between 21-40 years. A study by Ahmed et al (2006) from Bangladesh showed 43% subjects in the age group between 21-40 years are more susceptible to infection.

In the Present study, in the pyrexia of unknown origin group, out of 300 cases 65.33% were male and 34.67% were female giving a male to female ratio of 1.88 :1 (table 1). This finding were similar with Asmaa et al (2005) who reported 66.66% male and 33.33% female and found male to female ratio of 1.99:1. In the risk group of population, we found 71.43% were male and 28.57% were female with a male to female ratio a 2.5: 1. Similar findings of male dominance (86.92%) over female (13.08%) subjects were reported in study by Salari (2002) from Iran and male to female ratio 6.65:1. The increase member of male patients over female in this study might be due to occupational exposure to animals. Male are the active and main earning member of the most of the family still now, so they are more privileged to visit physician chamber for treatment.

In the present study, in pyrexia of unknown origin group, 300 febrile patients clinically suggestive of brucellosis were screened by slide agglutination test, Rose Bengal plate agglutination test and Standard tube agglutination test. Among them, 27 (9%), 24 (8%) and 23 (7.67%) were positive for brucellosis respectively (Table no.3). Almost similar finding was found in a study by Handa et al (1998) from north India identified 12 (9.9%) brucellosis cases (4 acute,8 chronic) in a group of 121 patients
with pyrexia of unknown origin. Another study by Panjarathinam and Jhala (1986) from Gujarat in India 8.5% prevalence of Brucella agglutinins was recorded in human cases that were also similar with our results. A study by Kadri et al (2000) from Kashmir (India) reported 28 (.8%) of 3532 patients of pyrexia of unknown origin that does not correlate with our study. Another similar finding was also reported by Sen et al (2002) in Varanasi found 28 (6.8%) positive patients of 414 with pyrexia of unknown origin. A study from Egypt by Asmaa et al (2005) reported 92 (1.29%) sero positive brucellosis patients out of 7154 cases with PUO which does not correlate with our study. Another study done by Mantur et al (2006) from India reported 495 (1.8%) positive cases out of 26948 cases that results also does not correspond with our study. The increase prevalence rates in our country may be due to existence of disease among animals, no vaccination programme in animals, occupational contact and social habits of different population.

In our study, in risk group of population, 210 subjects were examined by slide agglutination test, Rose Bengal plate agglutination test and Standard agglutination test. Among them, 9 (4.28%), 7 (3.33%) and 7 (3.33%) were positive for brucellosis respectively (Table no.4). This finding was almost similar with Centinkaya et al (2005) and reported 62 (3.35%) positive cases out of 1850 in the rural area of Kayseri, central Anatolia, Turkey. Another study by Ahmed et al (2006) from Bangladesh revealed that brucellosis in not uncommon among the risk population of rural area and showed 13% positive cases of brucellosis among the cattle handlers of some village in the southwest part of the Bangladesh which is higher than our finding. The variation in prevalence rates of brucellosis among population in different geographical location
may be due to variation in existence of disease among animals, close contact with animals, lack of personal hygiene practice and social habits.

When seroprevalence of different occupational groups compared, out of 300 clinically suspected brucellosis cases, 23(7.67%) were positive (Table no.5) of human brucellosis varied significantly which rationale with relation of farmers where infection was more in animal farmers (17.74%), followed by house wives (9.38%).

In our study, in risk group, 10 (4.76%) out of 210 subjects became positive when tested by slide agglutination test, Rose Bengal plate agglutination test, Standard tube agglutination test and ELISA (Table no. 6). Regarding occupational groups, our study reported seroprevalence rate 4.67% among animal farmers, 6.45% among milker/dairy workers and 11.11% in veterinary personnel such as veterinary inspector, veterinary assistant and veterinary supervisors. This finding was almost similar with salari (2002) from Iran and reported 35 (3.75%) seropositive out of 933 subjects among animal farmers. A study conducted by Thakur and Thapliyal (2002) from India revealed a Prevalence rate of 4.97% in samples obtained from persons exposed to animals that is also correlate with our study. Another study by Mathur (1964) from north India reported seroprevalence of 8.5% among dairy workers in contact with infected animals that was slightly higher than our result. Another study conducted by Agasthya et al (2007) examined 618 serum samples from veterinary Personnel and found 15.69% tested positive. Another similar study conducted by Hemashettar and Patil from India (1991) reported 24 (8.2%) Brucella specific antibodies in significant titer among veterinary workers.

In our study, in pyrexia of unknown origin group, the prevalence of brucellosis was significantly ($\chi^2$=3.87, $P<.05$ and df=1) affected by sex (table no. 7), where the female
infection was higher than male detected by slide agglutination test. Similar findings were also reported by Asmaa et al (2005) from Egypt. They found the prevalence of brucellosis was higher among female than male as detected by agglutination test. Another study by Zafer et al (2005) from Turkey showed that the prevalence of brucellosis was higher female (6.3%) than in male (3.1%). In all studies, female are infected more often than males, most likely because of their increased exposure to domestic animals in rural area.

In the present study, in pyrexia group, 63.33% cases were from rural areas and 36.67% were from urban areas (Table no. 8). The result showed that the prevalence of brucellosis is higher in rural area (8.42%) than in urban areas (6.36%) as detected by serological tests. Though results were not statistical significant, it is obviously fact that existing peoples are in more contact with animals. This finding is in agreement with Asmaa et al (2005) from Egypt who concluded that the higher prevalence in rural areas may be due to close contact of individuals with livestock. However, the occurrence of brucellosis in urban setting may be explained by the role of consumption of raw infected milk and milk products.

In our study, in pyrexia group, 9 paired samples were collected at 2-3 weeks interval that were serologically detected cases and found no rising titers but most cases remained same. This firmly can explain in the fact that all the patients treated by antibiotics (Table 9). Closely similar findings also reported by Mantur et al, (2006) followed-up 79 diagnosed brucellosis patients and monitored by Brucella antibodies by STAT for different lengths of time and found measurable titers ranging from 1:160 to 1:640.
In our study, in both pyrexia and risk group of population, we found ELISA IgG tests were positive in 22 (81.48%) out of 27 cases in pyrexia group and 10 (25.64%) were positive out of 39 subjects in risk group of population. We calculated sensitivity and specificity of the ELISA (IgG) tests were 81.48% and 74.36% respectively as per table no.10. In this study, we found positive predictive value of 68.75% and negative predictive value 85.29% respectively (Table no.10). Our results are remarkably consistent with the findings of Ertek et al (2006) from Turkey who reported a sensitivity (81.3%) of the Patients but Ertek et al (2006) showed 95.0% specificity by ELISA (IgG) test in confirmed brucellosis cases which does not support our study. Different studies found sensitivity and specificity values as follows: 91% & 100% by Araj et al (2005), 84% and 100% by Gomex et al (2008). Above mentioned studies showed more than 80% sensitivity and above 95% specificity by ELISA (IgG) test. Our finding was showing good correlation with sensitivity of the test but not corresponded with specificity in the tests. This difference was probably due to the fact that in those studies included culture positive brucellosis cases used in one group and healthy individuals were used in another group. But in our study, all subjects belonging as cases group were positive by slide agglutination test, RBPT and STAT test and another group belonging as control groups was risk group of population. There was no culture positive brucellosis cases included in our study.

In the present study, 22 (81.48%) were positive out of 27 cases by ELISA (IgG) in pyrexia group. In risk group, 10 (25.64%) were positive out of 39 subjects by ELISA (Table no.11). The rate of ELISA positivity was significantly higher in pyrexia group than risk group (Here, chi-square value is $19.92$, df =1 and $p \leq 0.001$). In agreement with our finding Asmaa et al (2005) from Egypt has reported 78 (84.7%) ELISA (IgG) out
of 92 brucellosis cases that was detected by STAT. Another study conducted by Agasthya et al., (2007) from India reported 97(15.69%) positive cases out of 618 by ELISA in the risk group of individuals. This result does not correspond with our finding.
Conclusion and Recommendations

There is no available data regarding brucellosis in our country. However, the present study revealed that a considerable number of brucellosis cases were present in pyrexia of unknown origin and also in risk group of population such as animal farmers, dairy workers and veterinary personnel in rural area of Mymensingh district. Brucellosis can be diagnosed by variety of serological tests such as slide agglutination test, Rose Bengal plate agglutination test, Standard tube agglutination and ELISA test. Although serological tests are not the test of gold standard for detection of cases of brucellosis but all tests showed satisfactory results. Other tests like culture, PCR based technique and large scale study would be better alternates for detection of brucellosis in future.

The following Recommendations are suggested.

1. The prevention of human brucellosis is based on occupational hygiene and food hygiene.
2. All dairy products should be prepared from heat treated milk and consumption of raw milk or products made from raw milk should be avoided.
3. Meat should be adequately cooked.
4. Special precaution should be taken by laboratory workers.
5. Public health education should be emphasized on food hygiene and occupational hygiene.
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Appendix – 1

Clinical case record sheet for pyrexia of unknown origin patient in MMC hospital, Mymensingh.

A. S.L. no: Identification number: Date:

B. Particulars:


4. Father/Mother/Husbands name:

5. Address:
   a. Community: Village: P.O: Union: Thana: Dist:
   b. Hospital: Word no: Unit no: Bed no:
      Date of admission: Reg. no:

6. Occupation:

7. Education: Primary/Secondary/College/Diploma/University.

c. Clinical Symptoms/Signs:

1. Fever: Yes/no. Duration of fever:

   Nature: Quotidian/Tertian/Irregular/Rising and falling.
5. Arthralgia: Yes /no          6. Fatigue: Yes /no          7. Malaise: Yes /no
8. Sweating: Yes /no          9. Mental status:
10. Splenomegally / Hepatomegally / Lymphadenopathy.

D. Hypothesized risk factors:


2. Veterinarians workers: Veterinary officer, Veterinary supervisor, Veterinary inspector, Veterinary field assistant,

3. Slaughter house workers/Abattoirs/Butcher.

4. Contact with animal for: Milking/Bathing/Traction/Herding/ feeding with duration.

5. Dairy workers/Dairy farm:

6. Drinking of raw milk: Yes/ no, Yoghurt: Yes/ no, sour milk: Yes/ no.

E. Do you please agree to take your blood sample for diagnosis of brucellosis: Yes /no

F. If yes please provide your signature here:

G. Results of the diagnostic tests:

1. Rose Bengal Test: positive/negative/inconclusive

2. Standard Tube Agglutination Test: positive/negative/inconclusive

3. IgG or IgM by ELISA: positive /negative/inconclusive.
Appendix – II

Rose Bengal Plate agglutination test:

(1) Reagents:

d) Rose Bengal: Brucella abortus suspension, strain 544, in lactate buffer 1mol/L, phenol 5 g/L, Rose Bengal, PH 3.6.

e) Positive control+: Animal serum, with an anti-Br abortus antibody, Sodium azide 0.95 g/L.

f) Negative control-: Animal serum, Sodium azide 0.95 g/L.

(2) Equipment:

Mechanical rotators:

Micropipette

Plastic plate

Plastic tube

Centrifuge machine

Eppendorfs tube

Interpretation of the results:

Macroscopically the presence or absence of visible agglutination immediately after removing of rotators. The presence of agglutination indicated positive result. The absences of agglutination indicated negative result.
Appendix – IV.

Standard tube agglutination test:

1. **Reagent**
   a. Bacterial antigen – Suspension of *Brucella* in glycine buffer pH 8.2.
      
      Sodium azide – 0.95g/l
   b. Positive control – Animal serum with an antibody of *anti Brucella*.
      
      Sodium azide – 0.95g/l
   c. Negative control - Animal serum. Sodium azide – 0.95g/l.

**Equipment:**

   Mechanical rotators:
   - Micropipette
   - Plastic plate
   - Plastic tube
   - Centrifuge machine
   - Eppendorfs tube

**Interpretation of the result:**

**Positive result:**

Positive: A clear granular agglutination appears after 24 hours incubators.

Negative: No clear granular appearance appears after 24 hours.
ELISA Test (Brucellosis):

Reagents (preparation of reagent):

Buffers

GS buffer (glycine saline) 10 fold concentrated =Stock solution

- Glycine (75.07 g/mol) 1M 75g
- Natrium chloride (58.44 g/mol) 1.7M 99.3g
- Demineralised water
- pH 9.2 ± 0.1 (adjust with NaOH)

The solution can be stored for up to 6 months at 5°C ± 3°C. 1L

GS-EDTA-Tw buffer

Dilute the GS buffer stock solution in demineralised water (final solution = 1/10).

- EDTA (372.24 g/mol) 50 mM 18.6g
- Tween 80 1/10 10ml
- pH 9.2 ± 0.1 (adjust with NaOH)

The solution can be stored for up to 2 months at 5°C ± 3°C. 1L

Because of its viscosity, Tween 80 is first diluted tenfold in demineralised water before it is added to the buffer (1ml Tween 80 + 9 ml demineralised water 1/10).
Protein G – HRPO buffer with 0.1 % Tween 80

- Dinatrium hydrogenophosphate (141.96 g/mol of $+ 2 \text{H}_2\text{O}$ 177.99 g/mol)  
  10mM 1.4g of 2.2g
- Natrium chloride  
  150mM 8.8g
- Tween 80  
  1/10 10ml
- Demineralised water

pH 7.2 ± 0.1 (adjust with HCl)

The solution can be stored for up to 2 months at 5°C ± 3°C. 1L

Serum buffer

- Foetal calf serum  
  2% 200µl
- GS-EDTA-Tw buffer  
  10 ml

Substrate buffer

Dissolve one tablet in 100ml demineralised water.

Solutions

Washing solution NaCl-Tw:

- Sodium chloride  
  0.15M 18g
- Tween 20  
  0.01% (volume) 200µl
- Demineralised water.

The solution can be stored for 1 week at 20°C ± 5°C. 2L
**Protein G – HRPO solution**

The conjugate is lyophilised and kept at -20°C. Before use you have to add 0.250 ml demineralised water and 0.250 ml glycerol to dissolve the conjugate. Shake gently and try to not keep de conjugate longer than necessary out of the freezer.

The protein G – HRPO solution is prepared on the spot as follows (for 10 ml):

- Protein G - HRPO diluted 1/2500 4μl
- Foetal calf serum 2% 200μl
- Protein G – HRPO buffer 10ml

**Stopping solution**

Sulfuric acid 2M (= 4N)

**Substrate solution**

The substrate that is transformed in the presence of peroxydase is ortho-phenylenediamine (OPD). The solution is prepared on the spot, as follows (for 25 ml):

- OPD 10 mg 1 tablet
- Substrate buffer 25ml
- H₂O₂ 30% 5μl
Equipment

- Fridge at 5°C +/- 3°C.
- Freezer at -20°C +/- 3°C.
- Incubator at 37°C +/- 3°C.
- Centrifuge
- Water bath at 37 °C ± 1°C
- Microplate washing system (manual or automatic)
- Spectrophotometer for microplates (492 and 620 nm filters)
- If available, sample dilution and distribution system
- PH meter.
Reading

Read the optical densities (OD) with 492 nm and à 620 nm filters. The OD measurement is the difference between the two. ELISA units are defined using the cubic spline of the standard curve (OD in function of dilution) as a scale (see attached excel sheet). The scale was multiplied by 16 200 to obtained ELISA units (so the standard curves ranges between 60 and 2).

Interpretation of the results

In most species, sera with a titre greater than 2 were considered positive and sera with a titre lower than 2 were considered negative.

Appendix – VIII.

STATISTICAL FORMULA

a. Formula for mean

\[
\bar{x} = \frac{\sum x}{n}
\]

\(\bar{x}\) = mean of observations

x = individual observations

n = number of observation
\[ \Sigma = \text{summation} \]

**b. Formula for Standard Deviation**

\[ SD = \sqrt{\frac{\sum (x - \overline{x})^2}{n-1}} \]

- \( SD \) = Standard deviation
- \( x \) = Individual observation of a series
- \( \overline{x} \) = mean of observations
- \( n \) = number of observation
- (n-1) = applicable for Sample
c. Formula for Chi-square test ($\chi^2$):

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

Here,

**O** = Observed frequencies

**E** = Expected frequencies

**df** = Degrees of freedom = $(c-1) (r-1)$

**\Sigma** = Summation.