Chapter 1

General introduction

1.1 INTRODUCTION

Brucellosis is an ancient bacterial zoonoses caused by a gram negative facultative intracellular bacterium belonging to the genus *Brucella*. It affects all forms of domestic animals and few marine mammals. Brucellosis was introduced as a disease affecting human-beings on the island of Malta during the Crimean war in the 19th centuries. The relationship between the organism and the disease was first introduced by Sir David Bruce in 1887. Both the disease and the genus *Brucella* were named after him for his discovery about the pathogen.

Even though brucellosis in livestock and mode of transmission of infection to the human population has been significantly decreased with the effective vaccination and prevention programmes in parts of the world, it remains an uncontrolled problem in regions of high endemicity such as the Mediterranean, Middle East, Africa, Latin America and parts of Asia.

Brucellosis represents a cause of health problems in a herd or flock. It is spread within the herd mainly by ingestion of contaminated material. The initial infection in the reservoir species is often followed by abortion and subsequent delayed or permanent infertility. The infected animals shed the organisms in the urine and also in the colostrum and milk (FAO, 2003).

The major route of transfer of the disease is through the consumption of unpasteurized raw milk (Nikolaos, 1998). Brucellosis can be transmitted to humans through the breaks/cut in the skin during the vaccination, contact with the infected animals or their products (Forbes et al., 2002). The chance of infection are higher for the people engaged in certain professions such as livestock handlers, slaughterhouse employees, dairy farmers, veterinarians and laboratory personnel (Kozukeev et al., 2003).
Mode of transmission of infection in human

Fig. 1.1. Major route of transmission of infection in humans from the major species of Brucella and its specific hosts

1.2 BRUCELLOSIS

Brucellosis is an infectious, contagious, and worldwide spread form of an important zoonosis disease caused by bacteria of the genus Brucella. In animals, the disease primarily affects cattle, sheep, goats, swine, and dogs, and is characterized by abortion or infertility and also affects people and other animal species (Ray and Steele, 1979). In humans, the clinical signs and symptoms of the disease includes undulant fever, weakness, sweating accompanied by pain in muscle and knee joints. The infection is caused by Brucella abortus, and it is characterized by abortion, metritis, orchitis and epididymitis among cattle (England et al., 2004, Young and Corbel, 1989a).
1.2.1 WORLDWIDE OCCURRENCE OF BRUCELLOSIS

Brucellosis is an important disease among cattle and remains a major source of disease for humans and domesticated animals. If the countries do not implement high-quality and effective public health and domestic animal health programs as well as a national brucellosis control and eradication program, brucellosis will remain the most common form of an important zoonosis disease in worldwide. Bovine brucellosis caused mainly by \textit{B. abortus} has a major economic impact on developing countries (Michael et al., 1997).

1.3 EPIDEMIOLOGY

The annual occurrence of more than 500,000 infections worldwide suggests that brucellosis is costly bacterial zoonoses (Pappas et.al, 2006). The possibilities of infection in man by all the four \textit{brucella} species assure the risk in human beings (Acha et.al, 2003). Many of the bacterial zoonoses have been known to have crossed the species barrier. The rare occurrence of brucellosis by the transfer of infection from cat to human has also been reported (Repina et al., 1993). Report of human brucellosis case and cause of infection from marine mammals has also been shown (Brew et al., 1999). The major animals grown in India are cows, buffaloes, goat and sheep in almost every village and rural areas of country. The common clinical manifestation of \textit{brucella} infection in animal results in severe reproductive loss. The reproductive loss results in abortion, birth of weak progeny or still birth in cows and small ruminants (Chahota et al., 2003). The annual rate of milk production in India is estimated to cross 110 million tonnes by 2010 based on the survey by the department of animal husbandry dairying and fisheries, Government of India. The milk from cow and buffaloes has potentially very high value for the dairy industry and contributes a major part to nations GDP.

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 (Bouza et al., 2005). The seroprevalence of brucellosis is found to be larger among dairy workers (Mathur et al., 1964). The prevalence of human brucellosis in Vellore, has been reported by (Koshi et al., 1971). Moreover, \textit{brucella} species are known to have resistance against nitric oxide and hence the organism is able to survive intracellular (Kim et al., 2006; Kikuchi et al., 2006). Most of the
human brucellosis cases are caused by *B. melitensis* but, *B. abortus* also shares equal contribution.

In Europe, the highest prevalence of human brucellosis occurs in the countries of the Iberian Peninsula and the Mediterranean littoral or basin region (Portugal, Spain, Southern France, Italy, Greece, Turkey, North Africa). In the Mediterranean and Middle East countries, the annual incidence of human brucellosis varies from 1 to 78 cases per 100,000 and in South European countries reported up to 77 cases per 100,000 people (Mousa and Elhag, 1988).

1.4 METHODS IN SURVEILLANCE

In humans, surveillance can be done through hospitals and public health centers. The sudden death of the animals should mandatorily be reported to municipalities and safe disposal of animal carcasses are some of the methods needed to be implemented (Chugh, 2008). The number of reported cases showing symptoms of undulant fever should be monitored carefully and complete background details about a patient’s history of illness and occupation has to be noted. 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, socioeconomic status, and environmental hygiene. The details collected based on the surveillance reports should be sent to the municipalities, corporation and hospitals. The advertisements in newspaper, radio and television about brucellosis and other bacterial zoonoses along with other government policies should be mandatorily announced.

1.5 CHARACTERISTICS OF *Brucella*

*Brucellae* are small, non-motile, non-sporing, gram-negative coccobaccilli short rods. They grow rather slowly on ordinary nutrient media while their growth is improved by serum or blood. They are aerobic; there is no growth under strictly anaerobic conditions. The genus is a member of the α2 subdivision of the Proteobacteria. The *Brucella* species are intracellular parasites inside human-beings and animals and can usually be found in the reticuloendothelial and reproductive systems. Typically *Brucella* spp. occurs as small gram-negative coccobaccilli, but coccal and bacillary forms also occur. The cells are short and slender; the axis is straight; the ends are rounded; the sides may be parallel or convex outwards. In length they vary from about 0.5 - 0.7
µm, in breadth vary from 0.5 - 1.5 µm, occurring singly, in pairs or short chains (Leslie et al., 1998).

The genus *Brucella* contains a group of very closely related bacteria. The first member of the group, *Brucella melitensis*, affects primarily sheep and goats, the second member of the group, *Brucella abortus*, affects primarily cattle, the third member of the group, *Brucella suis*, affects primarily pigs, the fourth member of the group, *Brucella ovis*, affects primarily rams and ewes, the fifth member of the group, *Brucella neotomae*, affects primarily the desert wood rats, and the sixth member of the group, *Brucella canis*, affects primarily male dogs and bitches (Corbel and Macmillan, 1998b).

Among the above members of the group, *B. abortus*, *B. melitensis*, and *B. suis* species are not host-specific, and may transmit to other animal species. Cross transmission of brucellosis can occur between cattle, swine, sheep and goats and other species including dogs, horses, feral swine, bison, rein deer and camels (FAO, 2003).

From epidemiological evidence, *B. abortus*, *B. melitensis*, and *B. suis* have distinct host preferences and the organisms are capable to cause an infection in a wide range of host species, including humans. The remaining three members of the species have much greater host specificity. Typically, in all host species *Brucella* grows intracellularly, producing a variable bacteraemic phase followed by localization in the tissues of the genital tract and in the mammary gland. Abortion is typically the first clinical sign of the pregnant female, and orchitis and epididymitis are typical clinical sign of the male (Corbel, 1998a). In particular, female animals that have reached sexual maturity are most susceptible to infection. It is usually detected in pregnant females through abortions (England et al., 2004).

1.6 IMMUNOGENS OF *Brucella*

Unlike other gram negative bacteria, *Brucella* lacks spores, flagella, pilli, capsule/ specified exotoxins and instead it has lipopolysaccharides, outer membrane proteins, cytosolic or ribosomal proteins which are considered as the potential immunogens of this bacterium (Goldbaum et al., 1993; Tabatabai and Pugh, 1994; Onate and Folch, 1995; Oliveira et al., 1996; Rebecca et al., 1998; Vemulapalli et al., 2000). The recombinant outer membrane proteins like
Omp31 has also been reported for their seroaignostic potential in goats and sheep brucellosis (Gupta et al., 2007).

In a recent study, Golshani et al., (2016) have showed that that membrane protein (Omp2b) has a potential to induce both B-cell and T-cell-mediated immune responses and it can be evaluated as a new subunit vaccine candidate.

1.6.1 LIPOPOLYSACCHARIDES

*Brucella* LPS is a biologically active component present in the cell membrane and generally comprised of three domains: 1. Polysaccharide / O-side chains or O-antigen (non toxic portion)
2. Core polysaccharide 3. Lipid A (toxic portion) O-antigen is a homopolymer consisting of 96-100 units of α 1-2 linked perosamine. It is considered the immunodominant subunit of LPS (Caroff et al., 1984; Ugalde et al., 2003). Structurally, the O-antigen resembles with that of *Vibrio cholera* LPS, but is totally different from other enterobacteriaceae LPS (Duenas et al., 2004). The middle portion of the LPS, the core polysaccharide, consists of the trisaccharide 2-ketodeoxy 3-octonate (Kdo). The embedded part of lipid A is composed of long chain saturated fatty acids and small amounts of hydroxylated fatty acids, but lack β-OH mystic acid-linked fatty acids (Aragon et al., 1996). Wild type and attenuated *Brucella* strains present smooth or rough types of LPS based on the presence or absence of an O-chain. The virulence of *Brucella* species has been linked with the type of LPS present (Zygmunt et al., 2009). *Brucella* LPS is released into body fluids where it is readily ingested by phagocytic cells through pinocytosis or receptor-mediated endocytosis (Poussin et al., 1998). Two days after ingestion, *Brucella* LPS molecules are found inside macrophages in small vacuoles that coalesce together. The lipid A moiety of LPS is deacylated and dephosphorylated (Wuorela et al., 1993; Leyva-Cobian et al., 1997).

*Brucella* LPS is designated as a non-classical endotoxin that plays a pivotal role in pathogenesis and modifies phagocytosis, phagolysosome fusion, cytokine secretion, and apoptosis (Beninati et al., 2009). In contrast to most endotoxins, it is nonpyrogenic, does not induce a localized Shwartzman reaction, does not increase the susceptibility to histamine and does not activate complement to any significant level. Despite these properties, it is reported as the major
antibody-inducing antigen present in *Brucella* infections (Zaitseva et al., 1996). Invading Brucellae are mainly found in and metabolized by short-lived neutrophils and long-lived macrophages. Two mechanisms are being used, non-oxidative or oxidative pathways. Under non-oxidative conditions, antimicrobial proteins such as lysozyme (LZ) and peptides are released, while under oxidative conditions, brucelcidal oxidants are formed. These oxidants are recognized as reactive oxygen species (ROS), including superoxide anions, hydrogen peroxide, chloramines, hydroxyl radicals and hydrochlorous acid (Liautard et al., 1996) and reactive nitrogen intermediates (RNI) (Jinkyung and Splitter, 2003).

Killing *Brucella* requires enhanced macrophage and neutrophil stimulation that augment the release of the LZ, ROS, and RNI and ultimately cell mediated immunity. Lysozyme has the ability to degrade *Brucella* cell walls by hydrolyzing constituent peptidoglycan molecule and cleaving the glycosidic bonds between NAG and NAM (Mc-Ghee and Freeman, 1970; Maria et al., 2003), ROS damages fatty acid side chains of the *Brucella* cell wall (Jinkyung and Splitter, 2003). RNI inhibits cellular respiration (Gross et al., 1998). Therefore, taken together these metabolic functions (LZ, ROS, RNI) are of importance for antibrucella activity. Additional support for this statement comes from the observation that treatment of macrophages with methylene blue (an electron carrier) enhances killing of intracellular Brucellae, indicating their susceptibility to ROS. Similarly, inhibition of RNI by NG monomethyl L-arginine resulted in blocking of macrophage anti-*Brucella* activity (Jiang and Baldwin, 1993). Activation of immune cells (macrophages and neutrophils) can be achieved with a number of stimulators including bacterial cell-wall components, lipopolysaccharides (LPS), cytokines, interferon-gamma (IFN-γ) and tumor necrosis factor (TNF-α). Each of these factors acts independently or in combination to elicit various states of activation (Connelly et al., 2003). Evidence (Goldstein et al., 1992; Rasool et al., 1992) exists that of the immune cells stimulators listed, *Brucella* LPS is of particularly important to study, because it exhibits minimal endotoxic activity compared to other stimulators (10,000 times less toxic than E. coli LPS and 1000 times less toxic than *Salmonella typhimurium* LPS). This property may contribute to its potential use for immune cell stimulation studies and as an adjuvant in the *Brucella* vaccine (Goldstein et al., 1992). Attention has been focused on the fact that *Brucella* LPS can serve as a potential cell stimulator without adverse
Previous studies on *Brucella* smooth and rough LPS have emphasized extraction procedures (Alina et al., 2007; Salmani et al., 2009), biological properties (Schurig et al., 1991; Aragon et al., 1996), anti-LPS antibodies detection (Khatun et al., 2009), vaccine design (Apurba et al., 2002), immunogenic mimicking of LPS epitopes (Benninati et al., 2009), macrophage activation in an artificial metastasis model (Schultz et al., 1978) and comparison of the LPS properties of *Brucella* with the LPSs of other species (*Escherichia coli* and *Salmonella*) (Jarvis et al., 2002). However, the precise role of LPS in induction of anti-Brucella immunity remains unresolved (Billard et al., 2007).

### 1.7 MEMBRANE PROTEINS OF *Brucella*

#### 1.7.1 GROUP (1, 2 AND 3) Outer membrane proteins

The major outer membrane proteins (Omps) of *Brucella* were classified according to their apparent molecular mass in SDS-PAGE as group 1 (94 or 88 kDa), group 2 (36–38 kDa), and group 3 (31–34 and 25–27 kDa). This was first identified in the 1980s based on detergent extraction (Winter, 1987). It was observed that several outer membrane proteins have shown resistance to non-ionic detergents and are also known to be tightly associated with LPS and peptidoglycan. In general, the specific role of group 1 outer membrane was unknown and studies are done to understand the structure and function of membrane proteins.

The *Brucella* genus has a two closely related genes (Omp2a and Omp2b) which, encode porin proteins with a high degree (>85%) of homology (Ficht et al., 1989). Interestingly, only Omp2b is expressed in laboratory grown *B. abortus*. Association in a native trimeric SDS-resistant state and dissociation in to monomeric state under boiled conditions are the typical properties of bacterial porins in gel electrophoresis. The group 3 outer membrane proteins sharing only 34% identity with a molecular mass of 25 and 31 kDa are coded by the genes *omp* 25 and *omp* 31 (Cloeckaert et al., 1996).
Fig 1.2. General structure of *E.coli* cell envelope (Natividad et al., 2006).

1.8 DIAGNOSIS

Because of variable symptoms, non-distinctive clinical signs, and subclinical and atypical infections the clinical diagnosis of brucellosis in humans is particularly difficult. A wide variety of symptoms were revealed by persons acquiring the disease in a slaughter plant, on a farm or ranch, or from the consumption of raw milk or cheese made from raw milk, many of which did not result in an initial diagnosis of brucellosis (Young, 1983). The confirmation of the diagnosis came mostly after detecting *Brucella* antibodies by bacteriological tests and serological tests. However, the results remain unsatisfactory in the chronic phase of the disease.

1.9 AVAILABLE DIAGNOSTIC METHODS

1.9.1 BACTERIAL CULTURE METHOD

The culture of blood or tissue samples from the suspected patients or animal sample in solid agar is a gold standard method of brucellosis diagnosis still followed in several countries. But, culture of blood samples in solid agar plates is very tedious due to the slow growing nature of the organism and also requires 5 to 10 % CO₂ for its growth in the nutrient media. The blood cultures from the suspected humans are not predicatively positive all the time. Delay in the diagnosis of the disease results in the complication of treatment in man. However, blood culture of *Brucella* with some improvement has given some good results as mentioned by the reporters (Mantur et al., 2004; Mantur et al., 2007). Detection of *Brucella melitensis* from humans by
some new blood culture systems are also been carried in the past decades (Yagupsky, 1994). Some studies on bone marrow culture have also been tried out but the results are uncommon and lacked reproducibility (Gotuzzo et al., 1986; Shehabi et al., 1990; Mantur and Amarnath, 2008). Bacteriological tests in a laboratory found *Brucella* organisms in blood or bone marrow, lymph nodes, cerebrospinal fluid, urine and abscesses and was occasionally isolated from sputum, placenta, mother’s milk, vaginal discharge and seminal fluid (Castaneda, 1947; Finegold and White, 1969; Gotuzzo et al., 1986).

1.9.2 GENOME BASED DETECTION METHOD

The polymerase chain reaction (PCR) is a modern technology having a wide application in the field of molecular biology. Using this technology, a single or few copies of DNA nucleotides can be amplified into several higher order of a particular DNA nucleotide sequence. This technology was introduced in to our modern biology field in the year 1983 by Kary Mullis. Following ten years afterwards, Kary Mulllis and his friend Micheal Smith were awarded Nobel Prize in chemistry in the year 1893 for their breakthrough work on PCR.

The use of PCR for the detection of *Brucella* genome has shown a lot of success in many cases and this method is a rapid and specific for diagnosing brucellosis. The detection of *Brucella* in infected animal milk using PCR has also been reported (Leal-Klevezas et al., 1995). The main advantages with PCR is its sensitivity with which a trace amount of *Brucella* DNA can be detected with a protein coding gene specific primers (Romero et al., 1995; Matar et al., 1996; Debeaumont et al., 2005). The PCR is still considered a better choice compared to MRT and RBPT as per the study in Pakistan (Akhtar et al., 2010). The use of PCR to quantitatively determine the genome of *Brucella melitensis* in patient samples have showed the extended application of PCR (Navarro et al., 2002). The advantage of using PCR is that this technique can easily differentiate species with specific designed primers (Bricker and Halling, 1994; Cloeckaert et al., 1996). Reasonable use of PCR with the combination of ELISA has been tested by Al-Dahouk et al., (2004) to detect the species of *Brucella*. 
1.9.3 SEROLOGY BASED METHOD

The detection of antibodies in an animal due to \textit{Brucella} infection is a most widely used method which is similar to agglutination but there are other methods like using the principle of ELISA (Renukaradhy et al., 2002) have also been studied. For detecting antibodies like IgM & IgG, the whole organism is used in ELISA, brucella milk ring test (MRT), Rose Bengal Plate Test (RBPT) which results in lot of false positive results due to the structural similarities of lipo polysaccharides of \textit{Brucella} species with other gram negative bacteria (Corbel et al., 1983 and 1985). The cross reactions seen in conventional tests are due to the lack in sensitivity (Weynants, et al., 1996) and more importantly lack in differentiating vaccinated and non-vaccinated animals (Ana et al., 2010).

The brucella milk ring test (MRT) is a preliminary screening test done on cattle based on the principle of agglutination and the animals reacting positive in the tests are isolated and confirmed by blood culture or PCR detection. The MRT is highly cross reactive due to the common structural similarities seen in other gram negative bacteria.

In diagnosing human brucellosis, serum agglutination test (SAT) has been shown to be sensitive and specific (Ertek et al., 2006). Similarly, \textit{Brucella} Rose Bengal Plate Test (RBPT) has been shown to be effective in screening \textit{Brucella} infection in humans (Oomen and Waghela, 1974; Hasanjani Roushan et al., 2005). The study of Jordi Serra and Miquel Vinas (2003) showed less significance in terms of specificity with the use of RBPT in the sera diagnosis of human brucellosis. The disadvantages in agglutinations are overcome by new methods for detecting \textit{Brucella} IgG & IgM (Smits et al., 2003). Latex agglutination is also tried for the diagnosis of brucellosis to improve sensitivity (Abdoel and Smith, 2007) in the recent decade.
Table 1. Some of the currently available commercial serodiagnostic tests for brucellosis in the market.

<table>
<thead>
<tr>
<th>Product name/company</th>
<th>Detection method/Antigen used</th>
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<tbody>
<tr>
<td>ImmunoComb® Canine <em>Brucella</em> Antibody Test Kit. <strong>Biogal-Galed Labs</strong></td>
<td>Antibodies specific for <em>B.canis</em></td>
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<tr>
<td></td>
<td><strong>Antigen used</strong>: not mentioned</td>
</tr>
<tr>
<td>ID SCREEN® Brucellosis Serum Indirect Multi-Species. <strong>ID VET innovative diagnostics</strong></td>
<td>Anti-LPS antibodies specific for <em>B.abortus/B.melitensis</em></td>
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<td></td>
<td><strong>Antigen used</strong>: Lipopolysaccharides</td>
</tr>
<tr>
<td>ROSE BENGAL Kit (Rapid slide Agglutination antigen). <strong>ID VET innovative diagnostics</strong></td>
<td>Detection of antibodies against <em>B.abortus/B.melitensis</em> in milk of bovine, caprine, ovine</td>
</tr>
<tr>
<td></td>
<td><strong>Antigen used</strong>: phenol and heat inactivated rose Bengal stained <em>Brucella abortus</em> biovar 1 strain</td>
</tr>
<tr>
<td>Anti-<em>Brucella</em> IgG Human ELISA Kit. <strong>Abcam</strong></td>
<td>IgG and IgM class Antibodies specific for <em>Brucella</em> sp. <strong>Antigen used</strong>: not mentioned</td>
</tr>
<tr>
<td>ubio quickVET Bovine <em>Brucella abortus</em> Antibody Rapid Test Kit (Rapid card test).<strong>Ubio</strong></td>
<td>IgG class Antibodies specific for LPS of <em>Brucella</em> sp.</td>
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<td><strong>Antigen used</strong>: Lipopolysaccharides</td>
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1.10 AIM AND OBJECTIVES OF THE PRESENT WORK

Until the beginning of the present century, only a small number of human diseases had been recognized as animal origin, but at the present time, far more than 300 diseases are proved to be of zoonotic origin. Eventhough, brucellosis is a significant and increasing veterinary and public health problem in India, it is been considered as a highly neglected disease. Serological tests that detect the presence of anti-lipopolysaccharide (LPS) antibodies are the main tests used for the diagnosis of brucellosis. But, the use of LPS as antigen may lead to false positive reaction, if the animals have been exposed to other Gram-negative bacteria with LPS similar to those of brucellae (Godfroid et al., 2002; See et al., 2012; Adone and Pasquali, 2013) causes cross reactivity with organisms such as Yersenia enterocolitica, and Vibrio that share common features of the LPS (Hurvell and Lindberg, 1973; Munoz et al., 2005). These antigenic relationships between Brucella LPS and LPS expressed by other Gram-negative bacteria may lead to false-positive serological reaction (Corbel 1985). Although many membrane proteins from Brucella have been studied for utilization in antibody detection as well as generation of monoclonal antibodies (Cloeckaert et al., 1990 and 2001; Kumar et al., 2007; Thavaselvam et al., 2010;), Group-2 OMPs (particularly porins) from Brucella have not been studied as a potential antigen marker for the diagnostic purposes. Moreover, application of anti-porin monoclonal antibodies in the detection of specific antigen as a sera marker is currently not available as practice in case of brucellosis. Hence, the production of highly specific and sensitive monoclonal antibodies to Brucella proteins (porins) devoid of LPS molecules would help in the improvement of brucellosis diagnosis in human and animals. In the present study, we generated anti-porin monoclonal antibody and was utilized to develop an ELISA system for detection of porin antigen in both human and bovine serum. Also, we expressed the recombinant porin antigen to explore its possibility in developing an immunoassay for the detection of the anti-porin antibodies in the serum of both human and bovine samples.

1.11 THE OBJECTIVES OF THIS STUDY WERE

i. Extraction, purification and characterization of group2 outer membrane protein (porins) of B.abortus.

ii. Production, characterization and purification of anti-porin murine monoclonal antibodies.
iii. Development of an immunoassay for porin antigen detection in human / bovine serum.

iv. Production and purification of recombinant porins