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Review Article

Outer Membrane Proteins: Its Role in *Brucella* Virulence and Immunogenicity

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ABSTRACT

Members of the genus *Brucella* are facultative intracellular bacterial pathogens that have the ability to survive and multiply in the phagocytes and cause abortion in cattle and undulant fever in humans. *Brucella* spp. particularly *Brucella melitensis, Brucella abortus*, and *Brucella suis* represent a significant public health concern. The ability of *Brucella* to invade and replicate in host cells which is being linked to its outer membrane properties as well as to structures found within the cell envelope continue to be a major challenge with regards to treatment and control of the disease. The *Brucella* outer membrane proteins (OMPs) has been proposed to be involved in virulence (i.e., resistance to bactericidal cationic peptides and polycations), permeability to hydrophobic agents, resistance to divalent cation chelators, and poor activation of bactericidal mechanisms by LPS. Studies on a molecular level have now highlighted the mechanisms that are involved surrounding the pathogenesis of *Brucella* particularly involving OMPs.

Key words: *Brucella*, Brucellosis, Outer membrane proteins, Virulence, Immunogenic

INTRODUCTION

Brucellosis is a well-known domestic animal infection which survives within a broad range of eukaryotic cells as a small gram-negative, facultative intracellular *coccobacillus.* This disease is characterized by abortion and reduced fertility in animals, and also by chronic infections with symptoms such as undulant fever, arthritis and osteomyelitis in humans (Pappas *et al*., 2006).

Brucella can be divided into two types, *smooth* (S) and *rough* (R), according to the presence or absence of the O-chain in *lipopolysaccharide* (LPS). The *S-type Brucella* LPS contains O-chains, in which most of the antigenic sites on the *Brucella* surface are present; the *R-type Brucella* LPS lacks O-chains. The outer membrane proteins (OMP) cannot be sufficiently exposed because the surface of the *S-type Brucella* is covered by LPS. The OMP can be exposed on the surface of the *R-type Brucella*. *Brucella* OMPs show very strong immunogenicity, which may be associated with the survival of *Brucella* in macrophages (Zheng *et al*., 2015).

The main worldwide pathogenic species are *Brucella abortus (B. abortus)* and *Brucella melitensis (B. melitensis)*, which are involved in bovine and ovine brucellosis, respectively. Brucellosis causes severe economic losses for livestock farms worldwide. Due to serious economic and medical consequences of this disease, many efforts have been made to prevent the infection through the use of recombinant vaccines based on *Brucella* OMP antigens (Yousefi *et al*., 2015). Test-and- slaughter programs in conjunction with vaccination are the most important methods of control of animal brucellosis. Thus, prevention of human brucellosis depends predominantly on the control of the disease in animals (Pasquevich *et al*., 2009).

The *Brucella* OMP has been proposed to be involved in virulence (i.e., resistance to bactericidal cationic peptides and polycations), permeability to hydrophobic agents, resistance to divalent cation chelators, and poor activation of bactericidal mechanisms by LPS (Tibor *et al*., 2002).

Study on the interaction between *Brucella* spp. and the host revealed unique properties, which are mainly focused on the low activation profile of the immune response. The two main factors responsible are the *Brucella* spp. LPS and the residence of the pathogenic bacteria in safe intracellular compartments, such as the endoplasmic reticulum. The direct consequence is that these properties affect treatment options (Fugier *et al*., 2007).

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Although considered as the standard method for detecting *Brucella*, microbiological detection is characterized by strong subjectivity, low sensitivity and specificity, the presence of cross- reactions with other Gram-negative bacteria, requires a long period of time, poor universality, and existence of certain risks, thus making it difficult to test a larger number of samples. Although vaccination is considered to be the most effective method for controlling the brucellosis epidemic, it is difficult to distinguish natural infection from vaccine immunity. These factors make brucellosis an urgent problem (Zheng *et al*., 2015). The purpose of this review is to provide an overview on the properties of *Brucella* OMPs and its role in virulence and immunogenicity.

***Brucella* OMPs**

The *Brucella* cell membrane is comprised of three layers; the innermost layer is the cytoplasmic membrane, the intermediate layer is the peripheral cytoplasmic membrane, and the outermost layer is the outer membrane (OM). The outer membrane binds closely to the *peptidoglycan* layer to constitute the cell wall containing LPS, *protein* and the *phospholipid layer* (Zheng *et al*., 2015).

Three families of *Brucella* OMPs have been identified: OMP2 family, *OMP25*/*OMP31* family, and another consisting of all OMPs excluding those in the two families above. The first two families have been widely examined and mainly include protective antibodies and antigens related to *Brucella* virulence. Members of the *Brucella OMP25/OMP31* family show strong antigenicity and can be used to diagnose brucellosis. In addition, the family members are associated with bacterial virulence and are potential protective antigens (Zheng *et al*., 2015).

Among the *Brucella* OMPs, the *OMP2a* and *OMP2b* porin proteins behave biochemically much as the classical nonspecific trimeric *Escherichia coli (E. coli)* porins, but they are only remotely related to other known porins by their sequence. The pore-forming activities of *OMP2a* and *OMP2b* differs slightly, *B. melitensis* 16M *Omp2a* showing characteristics of a larger pore than *B. melitensis* 16M *OMP2b* (Paquet *et al*., 2001).

OMPs are synthesized in the cytoplasm as precursors with an *N*-terminal signal sequence, which is essential for translocation across the intracellular membrane via a translocation apparatus that is composed of the Sec proteins. It has been demonstrated that the acquisition of tertiary structure precedes, at least partially, the insertion of the proteins into the OM (Robert *et al*., 2006).

The notion that the *Brucella* OM plays a key role in virulence has been reinforced by the identification of the two-component regulatory system BvrR/BvrS, which controls the expression of at least two OMPs, *OMP3a* and *OMP3b*, (also known as *OMP25* and *OMP22*, respectively) as well as the structure of the LPS (Manterola *et al*., 2007).

OMP31, a *BrucellaOMP25/OMP3*1 family, does not only play a role in protective humoral immunity but also in the induction of specific cellular immunity (Snyder *et al*., 2013). Antibacterial immunity is dominated by cellular immunity partly due to the complexity of the organism being an intracellular and ability to evade host immune system, the variation in biological structure and activity of lipoproteins, combined with the complex nature of the translocation machinery (Goolab *et al*., 2015).

Virulence and immunogenicity

To be a successful infectious agent, *Brucella* requires four steps: adherence, invasion, establishment, and dissemination within the host. This pathogen gains entry to the host via the mucosal surfaces and is phagocytized by macrophages and neutrophils in an effort by the host to eliminate the organism. However, once inside the phagocyte, *Brucella* is able to survive and replicate. Mutant strains that lose intracellular survival cannot carry out infection of their host; therefore, the virulence of *Brucella* depends upon its ability to survive and replicate within host cells (Wang *et al*., 2009).

The phagocyte migrates via the lymphatic system to the draining lymph node, where *brucellae* infection causes cell lysis and eventual lymph node hemorrhage approximately 2 to 3 weeks following exposure. Because of vascular damage, some of the bacteria enter the bloodstream, and subsequent bacteremia develops, which results in dissemination of the pathogen throughout the host (Edmonds *et al*., 2000).

In the mouse model, *Brucella* can be found in multiple tissues including the spleen and liver, forming microgranulomas during the chronic stages of the infection. Using bioluminescent *Brucella* strains (Rajashekara *et al*., 2005), also found the bacteria to target the salivary glands, which could be of significance in relation to human infection, where infection is acquired through the ingestion of contaminated food.

Pathogenic *Brucella* spp. cause abortion in female animals by colonization of placental trophoblasts, and can cause sterility in male animals (Fugier *et al*., 2007). The resulting tissue necrosis of the fetal membranes allows transmission of the bacteria to the fetus. The net effect of chorionic and fetal colonization is the premature delivery of a dead calf during the last trimester of pregnancy. Evidence does exist that hormones and possibly the sugar erythritol may play an important role in the tropism of *brucellae* to the reproductive tract (Enright, 1990).

Late-gestational chorionic trophoblasts, compared with trophoblasts obtained from early-gestational placentas, produce higher amounts of erythritol and different concentrations of hormones. *B. abortus* is also known to invade and replicate poorly in early-gestational trophoblasts, compared with late-gestational cells. The predilection of *B. abortus* to invade and replicate inside bovine trophoblasts is an important step in the abortion process (Edmonds *et al*., 2000).

To replicate in macrophage cells, *Brucella* have evolved mechanisms such as inhibition of apoptosis to modulate cell death machinery which in turn favors pathogen survival and replication cell death by apoptosis is a common response of mammalian cells to a wide variety of bacterial infections (Zhang *et al*., 2016).

The clinical management of brucellosis is of particular concern because of high initial treatment failure and relapse rates. Direct detection of the organisms is time consuming method since it takes about 8-10 days, besides *Brucella* is a biosafety level III pathogen and requires level III facilities to handle it. For these reasons,

molecular techniques have been pursued in the recent past by various researchers throughout the globe. In the recent past, a number of recombinant OMPs (*rOMPs*) have been characterized and evaluated immunologically. A few *Brucella* OMPs have been cloned, expressed and identified from different species of *Brucella* species (Kaur *et al*., 2015).

Studies utilizing two-dimensional gel electrophoresis showed differences in various OMPs between the wild type and both *B. abortus bvrR* and *bvrS* mutants. *OMP25* (also known as OMP3a) is not expressed in *bvrR* and *bvrS* mutants and *OMP22* (also known as *OMP3b*) is absent in the *bvrS* mutant only (Lamontagne *et al*., 2007). Both *bvrR* and *bvrS* mutants are less invasive than the wild-type strain and both mutants fail to replicate within phagocytic or nonphagocytic cells and are targeted to lysosomes for degradation (Fugier *et al*., 2007).

Some reports mentioned that *B.abortus bvrS* and *bvrR* mutants are poor invaders and fails to survive and replicate within professional and nonprofessional phagocytes. To test whether their deficiency in *OMP3a* and *OMP3b* proteins could explain this phenotype, both OMP mutants were evaluated with HeLa cells by using the gentamicin survival assay and double immuno-fluorescence microscopy. Result demonstrated that absolute number of bacteria per cell was higher in all mutants as compared to that of the wild type. The number of intracellular bacteria per cell was significantly higher for the *OMP3b* and *OMP3a* mutants and significantly lower for the *bvrS* mutant than for the wild type (Manterola *et al*., 2007).

OMP31, which is 34% homologous with *Brucella OMP25* was reported to be an important immunogenic major outer membrane protein and antigen, which is present in all *Brucella* species, with the exception of*B. abortus*. Dendritic cells are antigen-presenting cells of the mammalian immune system. Their main function is to process antigen material and present it on the cell surface to the T-cells of the immune system. They act as messengers between the innate and the adaptive immune systems (Billard *et al*., 2007).

It was hypothesized that *OMP31* from *B. melitensis* benefits survival and replication in macrophages by inhibiting apoptosis through tumor necrosis factor-alpha (TNF-α) signaling following *Brucella* infection. To confirm the above theory, a flow cytometric analysis was conducted to evaluate the apoptosis of RAW264.7 cells induced by *B. melitensis* 16M or the mutant. The results demonstrated that the deletion of *OMP31* triggered an increased apoptosis in mutant strain than the parent strain. Furthermore, deletion of *OMP31* increased the levels of TNF-α for the mutant strain in comparison with those for *B. melitensis* 16M from 4 hr post- infection for RNA (Fig. 1) and 8 hr post infection for protein. At 2-12 hr post-infection, the concentration and transcriptional level of TNF-α increased rapidly, triggered by both mutant stain and parent strain, although the mutant stain induced greater TNF-α expression (Zhang *et al*., 2016).

TNF-α is important for the autocrine stimulation of macrophage functions, which is important for triggering specific immunity against a number of intracellular pathogens. TNF-α is one of many physiological factors that are able to trigger apoptosis. Furthermore, TNF-α-induced apoptosis has been indicated to play a role in a variety of pathologies linked to chronic inflammation and auto-immune diseases (Zhang *et al*., 2016).

In vitro study was conducted to measure TNF-α secretion by human dendritic cells (DCs) infected with *Brucella* or *E. coli*. It was reported that in contrast to the results obtained with *E. coli*, which induced remarkable secretion of TNF-α, DC invasion by *Brucella* did not promote any comparable production of this cytokine. The concentrations of TNF-α in *Brucella*-infected DC supernatants remained very low and were just above those observed in immature DC supernatants. The absence of interleukin (IL)-10 production by infected DCs suggested that *Brucella* could affect the production of TNF-α in an IL-10-independent way that might contribute to the development of a chronic infection (Billard *et al*., 2007).

Some reports stated that there are possibilities that *Brucella OMP25* and/or *OMP31* could be involved in regulating the production of TNF-α, a pro-inflammatory cytokine that activates the antibacterial activities of macrophages, stimulates antigen-presenting cells, and participates in the initiation of a specific immune response by infected macrophages. To analyze the effect of *OMP25* and *OMP31* on TNF-α production by infected macrophages, *OMP25* and *OMP31* null mutants of *B. suis* was constructed. Result demonstrated that pre-treatment of wild type *B. suis* with an anti-*OMP25* monoclonal antibody directed against an epitope exposed at the surface of the bacteria resulted in substantial TNF-α production during macrophage infection (Fig. 2). These observations demonstrated that *OMP25* of *B. suis* is involved in the negative regulation of TNF-α production upon infection of human macrophages (Jubier-Maurin *et al*., 2001).

Brucella is resistant to bactericidal polycations such as polymyxin B. as compared to other gram-negative bacteria, a property that is being linked to OM. Using *Bacillus subtilis sac*B gene *counter-selectable marker* (Tibor *et al*., 2002), evaluated the the loss of both *OMP10* and *OMP19*s and its effect on bacterial growth, and virulence, as assessed in appropriate *in vitro* and animal models. They reported that *OMP10* mutants showed no increase in sensitivity to *polymyxin B*. Survival of the *OMP19* mutant was reduced relative to that of 544 Nal^r over a polymyxin B concentration (Fig. 3). In addition, minimum inhibitory concentration assays showed that *OMP10* and *OMP19* mutants were more sensitive to *sodium deoxycholate* than the parent strain, suggesting altered OM properties (Tibor *et al*., 2002).

Study on the role of the outer membrane proteins of the *OMP25/OMP31* family in invasiveness and intracellular survival of virulent *B. ovis* in phagocytes was conducted. Findings demonstrated that the absence of *OMP25d* from the OM of *B. ovis* protective antigen (PA) did not significantly affect the viability of the bacteria. However, it did negatively affect their intracellular multiplication capacity, since no significant increase in the number of intracellular bacteria was observed at 72 h post infection. In contrast, the absence of *OMP22* from the outer membrane of *B. ovis* PA dramatically reduced the intracellular multiplication capacity of the microorganism, which was completely cleared at 24 h post-infection (Martin-Martin *et al*., 2008).

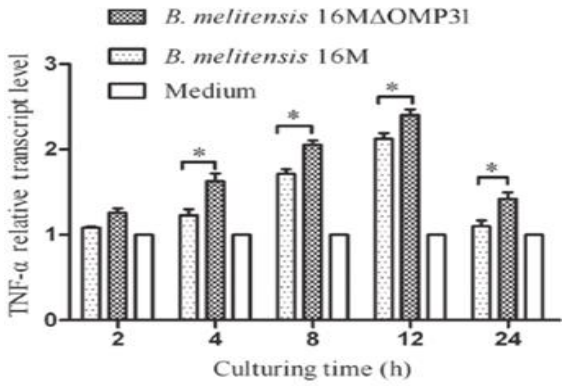

Fig. 1: TNF-α levels for RAW264.7 macrophages infected with *B. melitensis* 16MΔOMP31 mutant or parent strain. Transcriptional levels (Zhang *et al*., 2016).

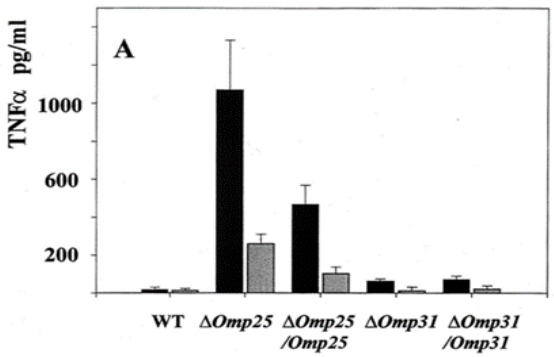

Fig. 2: The Δomp25 *B. suis* mutant induces TNF-α production in human macrophages. (**A**) THP-1 cells were infected with WT or mutant *B. suis* and cultured for 7 h in complete culture medium supplemented with gentamicin at 30 µg/ml. The cell supernatants were then harvested, and their TNF-α contents were determined by ELISA (black bar) or a bioassay (grey bar). Each experiment included infection with WT *B. suis* and the following mutants: Δomp25 *B. suis*, the complemented Δomp25 strain of *B. suis* (Δomp25-omp25 *B. suis*), Δomp31 *B. suis*, and the complemented Δomp31 strain of *B. suis* (Δomp31-omp31 *B. suis*). Each experiment also included a control cell activation with *E. coli* LPS at 100 ng/ml. In these experiments, LPS-stimulated cells produced 3,000±400 pg of TNF-α per ml. Values represent the mean±SD of 10 different experiments (Jubier-Maurin *et al*., 2001).

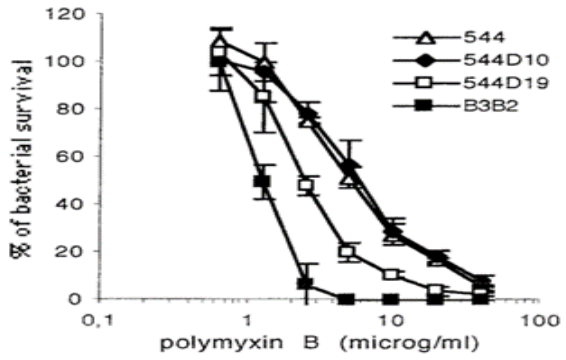

Fig. 3: Bactericidal effect of polymyxin B on *B. abortus* 544 Nal^r, 544D19.1, 544D10.1, and B3B2. The data presented are the results of a representative experiment and are means± standard deviations (error bars) of plate counts from three wells. Results are expressed as percentages of the *brucellae* surviving in wells incubated in the absence of the peptide (Tibor *et al*., 2002).

Pei and Ficht (2004) reported that rough mutants of *B. abortus* and *B. melitensis* are cytotoxic for the J774 murine macrophage cell line. The cytotoxicity is macrophage-specific and resembles oncosis and necrosis, but not apoptosis. In contrast, *B. ovis* PA, a natural rough strain, is not cytotoxic for cultured *HeLa* or J774. A1 cells, and that the bacterium multiplies actively inside these cells. These observations reveal that the rough character of *B. ovis* PA is not associated with the cytotoxicity of the bacteria for macrophages (Martin-Martin *et al*., 2008).

Conclusions

The greatest challenge in the treatment and control of *Brucella* pathogen is due to their ability to survive and multiply within the host cells. Studies using recombinant protein technology lead to the understanding of the mechanisms that underlie the pathogenesis of *Brucella.*

Proteomic characterization of the OM composition revealed some important host factors that are involved in *Brucella* intracellular replication. Moreover, the recent identification of several *Brucella*-translocated effector proteins will greatly advance the study of how *Brucella* manipulates and interacts with host cell pathways at the molecular level.

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