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MINIREVIEW

Brucella – Virulence Factors, Pathogenesis and Treatment

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Abstract

Brucellae are Gram-negative, small rods infecting mammals and capable of causing disease called brucellosis. The infection results in abortion and sterility in domestic animals (sheeps, pigs, rams etc). Especially dangerous for humans are: *Brucella melitensis*, *Brucella suis*, *Brucella abortus*, and *Brucella canis* that trigger unspecific symptoms (flu-like manifestation). *Brucella* rods are introduced via host cells, by inhalation, skin abrasions, ingestion or mucosal membranes. The most important feature of *Brucella* is the ability to survive and multiply within both phagocytic and non-phagocytic cells. *Brucella* does not produce classical virulence factors: exotoxin, cytolisins, exoenzymes, plasmids, fimbria, and drug resistant forms. Major virulence factors are: lipopolysaccharide (LPS), T4SS secretion system and BvrR/BvrS system, which allow interaction with host cell surface, formation of an early, late BCV (*Brucella* Containing Vacuole) and interaction with endoplasmic reticulum (ER) when the bacteria multiply. The treatment of brucellosis is based on two-drug therapy, the most common combinations of antibiotics are: doxycycline with rifampicin or fluoroquinolones with rifampicin. Currently, also other methods are used to disrupt *Brucella* intracellular replication (tauroursodeoxycholic acid or ginseng saponin fraction A).

Key words: *Brucella*, endoplasmic reticulum, macrophage, replication, virulence factors

Introduction

Brucella is a genus of bacteria belonging to the phylum Proteobacteria, class Alphaproteobacteria, order Rhizobiales, family Brucellaceae. Alphaproteobacteria is a very diverse group as to this class belongs both, the pathogens associated with plants: *Agrobacterium* spp., *Sinorhizobium* spp., *Mesorhizobium* spp. and the pathogens, which cause dangerous infections of animals, *e.g. Ricketsia* spp., *Bartonella* spp., *Brucella* spp. and many others (Dwight and Bowman, 2011).

Brucella genus is responsible for brucellosis, a severe febrile disease. Brucellosis is a worldwide problem, causing abortion and infertility in domestic and wild animals (Lapaque *et al.*, 2005). Infection factors are aerobic, small, Gram-negative rods. *Brucella*, a genus discovered in 1887 by David Bruce, contains the following species: *Brucella suis*, *Brucella ovis*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella neotomae, Brucella ceti*, *Brucella pinnipedialis*, *Brucella microti, Brucella inopinata*, *Brucella papionis*, *Brucella vulpis* and other strains without standing in nomenclature, that include environmental samples (Galińska and Zagórski, 2013; Whatmore *et al.*, 2014; Scholz *et al.*, 2016). Some species contain biovars, for example: *B. suis* have five biovars, *B. melitensis* contain three and *B. abortus* – nine biovars (Mizak *et al.*, 2014). Most of these species infect mainly specific hosts. *B. abortus* causes disease in cattle and infections usually lead to abortion; whereas *B. suis* is responsible for brucellosis in pigs, resulting in reproductive problems. Sheep are hosts for *B. melitensis*; infection causes impaired fertility. *B. ovis* is an etiological factor in sterility of rams (Megid *et al.*, 2010). Currently, about 500 000 cases of human brucellosis have been reported worldwide annually (Byndloss and Tsolis, 2016). Brucellosis is an endemic zoonosis with infection predominantly occurring in Middle East, Mediterranean rim (Portugal, Spain, Greece), Asia, Africa, South and Central America where the intake of dairy products is high, and protection of animal health is insufficient (Rubach *et al.*, 2013). There are single cases reported in Poland, however connected with occupational exposure or with traveling to Mediterranean countries (Galińska and Zagórski, 2013). *B. abortus* and *B. suis* are isolated not only from livestock but also from different wildlife species (bears, buffalo, bison, caribu, camelids, elk,

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ferrets, deer, foxes, rodents, rabbits, wolves) and marine mammals (dolphins, dugongs, manatees, otters, sea porpoise) (Coelho *et al*., 2015). *B. melitensis* is rarely encountered in wildlife, nonetheless individual cases have been reported in ibex and in chamois in Alps. *B. ovis* and *B. canis* have not been detected in wildlife in Europe up to date. *B. pinnipedialis* and *B. ceti* cause the most common infections in marine mammals. Birds are resistant to *Brucella* infection, whereas fish seem to be susceptible to *B. pinnipedialis* and *B. ceti* infections (Godfroid *et al.,* 2013). Infection is transmitted through close contact and during a common pasture.

Brucellosis is transferred from animals to humans, frequently human to human transmission occurs (Osman *et al.,* 2016). Especially dangerous for humans are: *B. melitensis*, *B. suis*, and *B. abortus*, *B. canis.* Brucellosis in human presents with symptoms like influenza: undulating fever, depression, weight loss, hepatomegaly, and splenomegaly (Bingöl *et al.*, 1999). Mainly human cases are connected to occupational risk or consumption of unprocessed dairy products (Boschiroli *et al.*, 2001; de Figureido *et al.*, 2015). *Brucella* rods can enter via host cells by inhalation, ingestion, skin abrasion, or mucosal membranes (Franco *et al.*, 2007). After penetration into host, rods multiply in lymph nodes; afterward, they penetrate other organs (Galińska and Zagórski, 2013). *Brucella,* can modify immune response in host cells; it has an affinity to the cells of specific tissues, *e.g.* placental trophoblast in fetal lung, pregnant females or reproductive system (de Figureido *et al.*, 2015). Brucellosis causes enlargement of lymph nodes, liver and spleen (Perkins *et al.*, 2010). Pathogenicity of *Brucella* is dependent on their ability to multiply and survive within macrophages (Sangari and Agűero, 1996; Christopher *et al.*, 2010).

Characteristics of *Brucella*

Species of the genus *Brucella* belong to small coccobacilli, measuring about 0,6–1,5 μm (Alton and Forsyth, 1996). They occur in single forms; rarely they create pairs or chains (Mizak *et al.*, 2014). *Brucella* are nonspore forming and non-motile Gram-negative cocobacilli (GNCB) (Alton and Forsyth, 1996). *Brucella* is an intracellular pathogen, during an infection it survives and multiplies in macrophages; the bacteria adapt to the acidic pH, low levels of oxygen, and low levels of nutrients (Kőhler *et al.*, 2002).

Lipopolisaccharide (LPS) is an essential element of structure building in each Gram-negative bacterial cell. *Brucella* is a genus that creates two forms of LPS. The smooth forms present complete LPS in the outer membrane, the rooth phenotype does not contain polisaccharide O-chain (Lapaque *et al.*, 2005; Seleem *et al.*, 2008). These infectious agents are able to produce cytochrome oxidase, catalase, and most of them are able to hydrolyze urea (Iowa State University, 2009). *Brucella* does not produce classical pathogenic factors, such as: exotoxin, cytolisins, exoensymes, exoproteins, capsules, plasmids, fimbria, and drug resistant forms (Seleem *et al.*, 2008; Baldi and Giambartolomei, 2013; Tan *et al.*, 2015). Bacterial cells are able to survive for a prolonged time in water, aborted fetus, soil, dairy products, meat, dung, and dust (Gwida *et al.*, 2010). For isolation of *Brucella* spp. the enrichment and selective media such as Thayer-Martin's medium or Farrell's medium are commonly used. The colonies mature after four to six days of incubation at temperature of 37°C. They can also grow at 28°C, but poorly and slowly. Moreover, these bacteria can grow in both aerobic atmosphere and in 10% CO_2; while, their growth is enhanced without additional CO_2 on a serum dextrose agar (Iowa State University, 2009; Whatmore *et al.*, 2014; Gupte and Kaur, 2015).

A wide range of bacterial detection methods is available. The predominatingly used culture media are: Bacto Tryptose (Difco), Triptcase soy (BBL), Tryptone soya (Oxoid), Triptic soy (Gibco). For culture of blood or body fluid a biphase medium called Castaneda should be used. Castaneda consists of two phases: liquid and solid closed in bottle. Liquid medium contains 1–2% of sodium citrate. Sample (5–10 ml) is added to the medium and incubated in 37°C in perpendiculary standing bottle in 10% carbon dioxide atmosphere (Gupte and Kaur, 2015). Serological tests are used to detect infection by examination of a specific antibodies level in serum. In the first week of *Brucella* infection the titres of IgM are dominant, but in the second week IgG class is prevalent. After four weeks, both types of antibodies reach a peak; durable, high titres of IgG can evidence failure in treatment (Al Dahouk *et al.*, 2013). Serum Agglutination Test (SAT) and Enzyme linked immunosorbent assay (ELISA) are the most common serological tests used for diagnosis of brucellosis. SAT is based on a survey of agglutination titer of different serum dilution against *Brucella* cell suspension (Alshaalan *et al.*, 2014). ELISA depends on detection of antibodies against the antigen – smooth LPS in serum (Gerasu and Kassa, 2016). The most effective methods for detection of brucellosis are molecular techniques (classical PCR, real-time PCR). The PCR method applies various pairs of primers to amplify different fragments of the genome. The examples of genes used to identification of *Brucella* spp. are: BCSP 31 (primers: B4/B5), sequence of 16S rRNA (primers: F4/R2), omp2 gene (primers: JPF/JPR) (Baddour and Alkhalifa, 2008).

Virulence factors

Lipopolisaccharide. LPS is an essential virulence factor of *Brucella*. LPS consists of lipid A, oligosaccharide core and O-antigen in Gram-negative bacteria.

Lipopolisaccharide is different and non-classical in *Brucella* as compared to other Gram-negative bacteria, for example *E. coli* (Cardosos *et al.*, 2006; Christopher *et al.*, 2010). Lipopolisaccharide from *Brucella* strains is less toxic and less active than the classical LPS isolated from *E. coli*. Classical LPS causes a high pyrogenicity, while non-classical LPS shows low pyrogenicity, being a weak inducer of tumor necrosis factor (Christopher *et al.*, 2010). Three features distinguishing it from other Gram-negatives characterize lipid A found in *B. abortus*: i) the fundamental component is diaminoglucose instead glucosamine, ii) longer acyl groups, and iii) lipid A is connected to the core by amide bonds, instead ester and amide bounds (Lapaque *et al.*, 2005). In strains with smooth colonies, the smooth LPS, (S-LPS) contains: i) lipid A, that consists of two types of aminoglycose, and fatty acid besides β-hydroxymiristic acid, ii) core comprises mannose, glucose, quinovosamine, and iii) O-chains are composed of 4-formamido-4,6-dideoxymannose. The structure of the R-LPS in strains with rough colonies is similar to the S-LPS, except for O-chains, which are reduced or absent (Corbel, 1997). *B. suis* has S-LPS. The O-chain connects with lipid rafts on the macrophage surface and the bacteria enter the cell. *Brucella* strains with R-LPS, for example *B. ovis* or *B. canis* do not connect with lipid rafts and rapidly connect with lysosomes (Lapaque *et al.*, 2005). The strains with S-LPS are able to restrain host cell apoptosis by the interaction of the O-chain with TNF-α (tumor necrosis factor). Thus, dead cells do not release specific factors, therefore they do not activate the immune system and *Brucellae* are able to avoid host immune surveillance (Fernandez--Prada *et al.*, 2003).

Type IV secretion system (T4SS). T4SS is a multiprotein complex and participates in secretion of bacterial macromolecues (Cascales and Christie, 2003). This system is typified by v*irB* operon encoding 12 proteins (11 860 bp) and exhibits in *Brucella* spp. with a high degree of similarity to T4SSs found in rhizobia, for example in phytopatogenic *Agrobacterium tumefaciens* (O'Callaghan *et al.*, 1999). Expression of the *virB* operon is regulated by the regulator of quorum-sensing – VjbR (Seleem *et al.*, 2008). The wild strains of *Brucella* are able to multiply only in the endoplasmic reticulum. *VirB* mutants of *Brucella* spp. are unable to multiply within the endoplasmic reticulum, it can result from the incapability to reach the ER, or multiply within (Delure *et al.*, 2001). In macrophages, rods of *Brucella* spp. are localized in *Brucella*-containing vacuole (BCV); this organelle interacts with the ER and is responsible for formation of specialized brucellae-multiplication compartment (Kőhler *et al.*, 2002). The acquisition of endoplasmic reticulum membrane depends on a functional *virB* secretion system – T4SS (Celli *et al.*, 2003).

Superoxide dismutase and catalase. Macrophages with *Brucella* produce reactive oxygen intermediates (ROIs), this is a primary mechanism of destruction of the bacteria ingested, and it also prevents their intracellular replication (Gee *et al.*, 2005; Seleem *et al.*, 2008). The following ROIs: O^{2-} (superoxide), H_2O_2 (hydrogen peroxide), OH^- (hydroxyl radical) are very detrimental for cell structure. The production of enzymes is the main line of defense, counteracting reactive oxygen intermediates. These enzymes include superoxide dismutase (SOD) and catalase (Gee *et al.*, 2005). SOD (metalloenzyme) is encoded by *sod* sequence. An enzyme contains iron, magnesium, or zinc and copper at its active site (Benov and Fridovich, 1994). SOD is responsible for dismutation of O^{2-} (superoxide) to H_2O_2 (hydrogen peroxide) and O_2 (oxygen) – transfer from one molecule to another ($2O^{2-}+2H^+ \rightarrow H_2O_2 + O_2$) (Gopal and Elumalai, 2017). Some species possess two types of SOD (*B. abortus*, *B. melitensis*, *B. suis*). The first is cytoplasmic – a Mn cofactor – SodA. SodA neutralizes endogenously generated O^{2-} – product of aerobic metabolism. The second one, SodC is periplasmic Cu, Zn-SOD, an enzyme responsible for neutralizing exogenously generated O^{2-} and protection from the respiratory burst within macrophages (Beck *et al.*, 1990, Seleem *et al.,* 2008, Martin *et al.,* 2012).

Catalase decomposes hydrogen peroxide into oxygen and water. Catalase activity is limited to the periplasmic space, where together with Cu-Zn SOD leave external sources of ROI unchanged (Kim *et al.*, 2000). Catalase is not necessary virulence factor, the other enzymes can compensate lack of this enzyme in catalase mutants, *e.g.* alkyl hydroperoxide reductase or enzymes involved in DNA repair mechanisms (Seleem *et al.*, 2008). Catalase is encoded by a sequence similar to *katE* gene of *Escherichia coli*. *B. abortus*, *B. melitensis* and *B. suis* catalase production is regulated by an increased external level of H_2O_2 (Gee *et al.,* 2004).

Cyclic β-1-2-glucans (CβG). *Brucella* CβG belongs to II OPGs (Osmoregulated periplasmic glucans) family (Bohin, 2000). *B. abortus* CβG impacts intracellular trafficking by acting on lipid rafts on macrophage surface. These glucans participate in control of the phagosome-lysosome fusion. Mutants are destroyed in phagolysosome and they are not able to multiply. Even more, mutants treated by CβG are able to control vacuole maturation and lysosome fusion, so they can reach to the ER and replicate there (Arellano-Reynoso *et al.*, 2005).

Urease. In *Brucella* there are non-identical urease operons in two separate genomes. Urease is a metalloenzyme, that decomposes urea to carbonic acid and to ammonium form, and it results in pH increase. This feature enables its survival in acid environment (Seleem *et al.*, 2008). In I chromosome, there are two

urea-operons: *ure-1* and *ure-2*, separated by 1 Mb of DNA. *Ure-1* and *ure-2* encode structural genes: *ureA*, *ureB*, *ureC* and accessory genes: *ureD*, *ureE*, *ureF*, *ureG* (Mobley *et al.*, 1995). That urease may protect *Brucella* during passage through the digestive tract (stomach), when the bacteria access their host through the oral route (Bandara *et al.*, 2007). Urease is produced by all bacteria belonging to the genus *Brucella* but *B. ovis* (Sangari *et al.*, 2007).

Cytochrome oxidase. Cytochrome oxidase is an enzyme facilitating *Brucella*'s survival inside the macrophages, where oxygen availability is limited. There are two operons in genome encoding two types of high oxygen-affinity oxidases: cytochrome cbb3-type and cytochrome bd (ubiquinol oxidases) oxidases. Cytochrome cbb3 oxidase is expressed *in vitro* and allows for colonization of anoxic tissues (maximal action in microaerobiosis). Cytochrome bd oxidase is expressed during intracellular multiplication and enables adjustment to the replicative niche (Loiser-Meyer *et al.*, 2005), by restraining the creation of oxidative free radicals and detoxification of compartment inside the cell (Endley *et al.*, 2001).

Alkyl hydroperoxide reductase (AhpC, AhpD). These enzymes attempt protection against oxygen radical and reactive nitrogen (Chen *et al.*, 1998). *AhpC* and *ahpD* are organized in an operon under one promoter control. *AhpC* mutants are more sensitive to peroxide killing and are vulnerable to spontaneous mutagenesis (DelVecchio *et al.*, 2002; Seleem *et al.*, 2008).

Nitric oxide reductase (NorD). Reduction of nitrate to dinitrogen gas is an essential process for bacteria in case of oxygen deficiency inside the cell; this process allows for respiration of nitrate (Stevanin *et al.*, 2005). The infected macrophages produce nitric oxide (NO), and *Brucella* can use it for own purposes. *Brucella* NorD consists of four types of reductases: Nir – nitrite reductase, Nar – nitrate reductase, Nor – nitric oxide reductase and Nos – nitrous oxide reductase, called the nitrification island. Possibility concerning productions of this enzymes helps to protect *Brucella* against low-oxygen conditions inside macrophages (Seleem *et al.*, 2008).

***Brucella* virulence factor A (BvfA).** Periplasmic protein that occurs only in genus *Brucella*; there are no homologous sequences in Gen Bank. The *bvfA* expression is induced in macrophages, through phagosome acidification. Presumably this protein is involved in forming the replication intracellular niche. BvfA function is not precisely identified (Lavigne *et al.*, 2005).

Base excision repair (BER). *XthA* gene encodes exonuclease III, which takes part in the base excision repair of DNA. Two different sequences of *xthA* occur in the *Brucella* genome: *xthA-1* and *xthA-2*. *XthA-1* mutants exhibit increased sensitivity to reactive oxygen species (ROS), so this enzyme is responsible for protection against oxidative destruction (Seleem *et al.*, 2008).

BvrR/BvrS system. The analysis of *Brucella* genomic library has confirmed an occurrence of two open reading frames: *bvrR* and *bvrS*. The *bvrR* encodes BvrR proteins (237 amino acid) and *bvrS* encodes BvrS (601 amino acid). There are two potential promoters (–10 and 35 seq. located 50 bp upstream ORF of *bvrR*), and ribosome-binding sequence (9 bp upstream of the first codon) (Sola-Landa *et al.*, 1998). BvrR exhibits resemblance to response regulators proteins, as N-terminal domain is composed of highly conserved aminoacids: aspartic (pos: 14, 15, 58) and lysine (pos: 107). C-terminal domain showed high similarity sequence to OmpR family; therefore, this protein can be included as part of this family (Mizuno and Tanaka, 1997; Martínez-Nūñez *et al.*, 2010). The protein is composed of three highly conserved domains: N-terminal sensing, periplasmic domain together with transmembrane component, cytoplasmic domain with distinctive histidine residue and C-terminal ATP-binding domain (Viadas *et al.*, 2010). BvrS includes four highly conserved regions on C-terminal domain: H, N, D/F, and G. This feature causes BvrS homologous to sensor proteins of the histidine protein kinase family (Stock *et al.*, 1995). BvrS is located in the cell membrane (Martínez-Nūñez *et al.*, 2010). Brucella BvrR/BvrS are the best characterized components of the virulence system; mutants are incapable of invasion, prevention phagosome-lysosome fusion and intracellular replication. BvrR/BvrS system is a regulator of expression of multiple genes (Viadas *et al.*, 2010). These proteins affect the transcription of the membrane proteins: Omp3b (Omp22) or Omp3a (Omp25a) and have the influence on other non-protein membrane molecules and hence on functional and structural membrane homeostasis (Manterola *et al.*, 2007). *BvrR*/*bvrS* mutants show structural changes in LPS, but O-chains seem to be undisturbed. These mutants are incapable of activation of GTPase (Cdc42) before entry into the cell, so they persist extracellularly and in consequence they do not infect the cell (Guzmán-Verri *et al.*, 2001). BvrR/BvrS is also responsible for limited lysosome fusion and intracellular trafficking (López-Goñi *et al.*, 2002).

Brucella BvrR/BvrS regulatory system action activate sensor domain of the BvrS protein by environmental signals through kinase activity. Additionally, BvrS causes phosphorylation and activation of BvrR protein. BvrR activates transcription of *omp3a*, *omp3b* and other genes responsible for lipid A structure and perhaps core of LPS. In consequence, *bvrS*/*bvrR* mutants are more sensitive to cationic peptides and display increased permeability for surfactants (López-Goñi *et al.*, 2002; Seleem *et al.*, 2008). The influence of Omp3a and Omp3b on virulence remains unexplained in details (Manterola *et al.*, 2007).

It has been proven that BvrR/BvrS two-component system regulate the expression of *virB* by positive stimulation of *vjbR* transcription. *VjbR* transcriptional factor interacts with *virB* promoter (Martínez-Nūñez *et al.*, 2002).

Role of virulence factors in chronic persistence. Evading an immunological response to *Brucella* antigens depends on LPS structure. The appearance of elongated fatty acid on the lipid A (*Brucella*-C_{28} compared to others Enterobacteriaceae $C_{12} - C_{16}$) leads to poor activation of TLR4 (Tool-like receptor 4) (de Figueiredo *et al.*, 2015; Byndloss and Tsolis, 2016). The other feature of *Brucella* spp. LPS is core oligosaccharide glycosylation pattern that prevents a connection of the bacteria with TLR4 (co-receptor MD-2) (Byndloss and Tsolis, 2016). Toll-like receptors, the transmembrane proteins, act as PRRs (Pattern recognition receptors) and initiate the innate immune responses. TLRs are responsible for the recognition of components of microorganism (Uemattsu *et al.*, 2008). The TLR protein is composed of two domains: extracellular domain that is rich in leucine repeats and it is responsible for recognition of microbial components, and cytoplasmic domain – TIR, involved in signal transmission, activation of intermediate proteins and finally an activation of NF-κβ and cytokines (Radhakrishnan *et al.*, 2009). TLR5 detects flagellin, but flagellin in *Brucella* spp. can avoid interaction with TLR5 as it lacks the domain recognized by TLR. (Andersen-Nissen *et al.*, 2005; Kim, 2015). *Brucella* encoded TcpB/BtpA protein that acts as a following virulence factor (TcpB – *B. melitensis*, Btp1/BtpA – *B. abortus*). Three pathogenic microorganism can produce similar proteins: *Salmonella* spp., *E. coli* and *Brucella* (Radhakrishnan *et al.*, 2009). These proteins contain TIR domain and show similarity to TIRAP (MAL) – a TLR adaptor protein. TcpB promotes a degradation of TIRAP and disrupts TLR4 signalling that result in inhibition of proinflammatory cytokines production and dendritic cell maturation (Newman *et al.*, 2006; Oliveira *et al.*, 2008; Radhakrishnan *et al.*, 2009; Sengupta *et al.*, 2010; Byndloss and Tsolis, 2016). TIRAP triggers recruitment of MyD88 and hence mediates TLR4 and TLR2 signalling (Kagan and Medzhitov, 2006). It seems to be likely that TcpB is able to interact with Death Domain of MyD88 and affect the signalling pathway (Chaudhary *et al.*, 2012). Another group of researchers has proven that *Brucella* encodes another TIR domain-containing protein, called BtpB (present in all *Brucella* strains). This protein reacts with MyD88, inhibits TLR signalling, and disrupts activation of dendritic cells BtpB, restraining TLR2, TLR4, TLR5 and TLR9 signalling; and together with BtpA affects DC maturation and inflects host inflammatory responses (Salcedo *et al.*, 2013).

B. abortus has proline racemase and thus is able to produce anti-inflammatory cytokine IL-10. This cytokine modulates macrophage activity during early phase of infection and leads to persistence and long-term survival of microorganism inside host cells (Byndloss and Tsolis, 2016).

Role of virulence factors in reproductive disease. The investigations performed on bovine placental explants have proven that in early infection with *B. aborus* the suppression of proinflammatory cytokines occured. This process is dependent on BtpB and T4SS proteins (use of mutants of the *virB* and *btpB* genes results in the reverse effect by enhancement of the proinflammatory cytokines production) (Mol *et al.,* 2014). In later phases, 12 h after infection with *B. abortus*, stimulation of proinflammatory cytokines and CXC chemokines production – CXCL6 (GCP-2) and CXCL8 (interleukin 8) takes place. CXCL6 and IL-8 known as neutrophil chemoattractants cause neutrophil influx and have been reported to cause necrotizing placentis after infecting a pregnant cow (Carvalho Neta *et al.*, 2008).

Pathogenesis

Invasion to cell. The *Brucella* strains survive and multiply within both phagocytic and non-phagocytic cells. The main targets for this bacterium are macrophages, dendritic cells and trophoblast cells. However, *Brucella* can also multiply within other cells, for example epithelioid cell (HeLa) or murine fibroblast (NIH3T3) (Pizarro-Cerdá *et al.,* 2000; Celli, 2006; Xavier *et al.*, 2010). *Brucella* translocates across the mucosal epithelial cells layer, where the professional phagocytes (macrophages and DC cells) engulf the bacteria. *Brucella* survives within non-phagocytic cells up to 72 hours after infection, overcomes the epithelial barrier and then penetrates the phagocytic cells. Approximately 10% of these bacteria survive this initial phase. In macrophages the pathogen avoids the host immune response; therefore, it can multiply and spread to other tissues using cellular tropism. The *Brucella* strains penetrate the host cells through a zipper-like mechanism (Gorvel and Moreno, 2002; de Figureido *et al.*, 2015). Bacteria can spread in a host through the lymph nodes and then translocate to the preferred tissues in reproductive tract (Kim, 2015). There, *Brucella* induces acute or chronic infection of reproductive tract that leads to abortion or severe reproductive tract diseases. (He, 2012).

The non-opsonized *Brucella* organisms are internalized through lectin or fibronectin receptors but opsonized by complement and Fc receptors. The opsonized bacteria are more prone to be destroyed within macrophages than non-opsonized ones. The

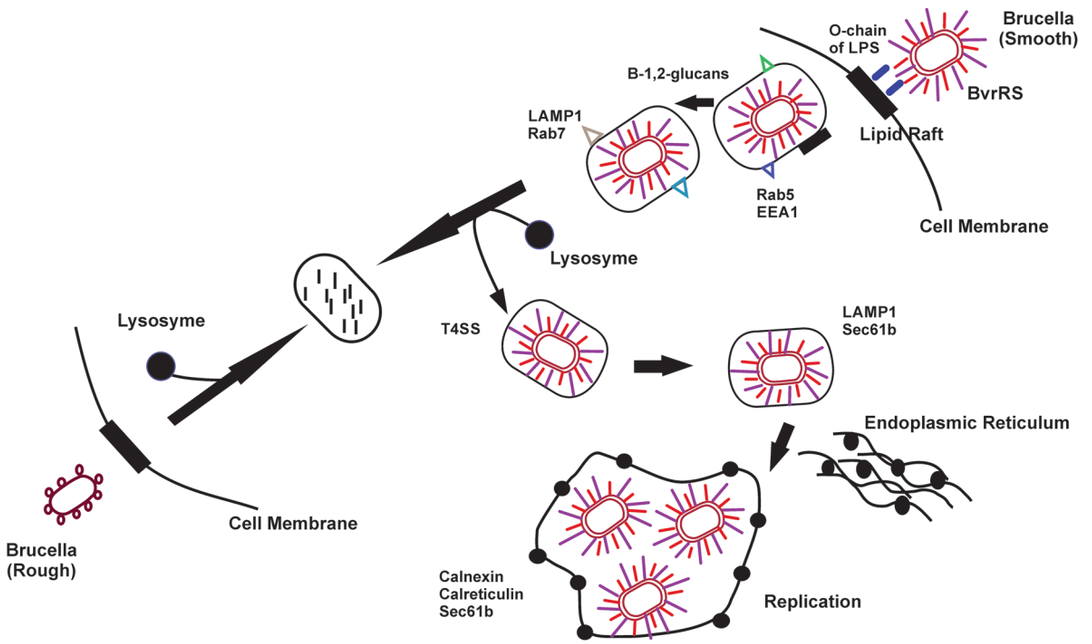

Fig. 1. Mammalian cell invasion and intracellular trafficking.

Smooth *Brucella* invasion into a cell by lipid rafts and acquisition of Rab5 and EEA1 markers – early BCV. β-1,2-glucans present in mature BCV and modification of lipid rafts. Then, the transient BCV interacts with lysosomes, T4SS is activated and it regulates intracellular trafficking from autophagosome to endoplasmatic reticulum (BCV acquires LAMP1 and Sec61β markers – late BCV – occurs only in epithelial cells). BCV acquires the endoplasmic reticulum markers (calnexin, calreticulin and Sec61-β) and the *Brucella* replicates. Rough *Brucella* organisms do not penetrate cell by lipid rafts, and therefore is exterminated.

pathogen binds to receptors containing of sulfated residues and sialic acid on surfaces of epithelial cells (de Figureido *et al.*, 2015).

Penetration into the epithelial cell requires actin polymerization (Kim, 2015). The adhesion of *B. abortus* to the cell surface leads to activation of GTPases of Rho subfamily, *e.g.* Rho, Rac, and Cdc42 (Guzmán-Verri *et al.*, 2001). These proteins are involved in cytoskeletal regulation and have an impact on parasitic bacterial internalization. Cdc42 is the only GTPase activated directly by *B. abortus* during the contact with a non-phagocytic cell. It seems that other GTP-ases (Rho or Rac) are activated indirectly, because their inhibition impede invasion into host cells (Waterman-Storer *et al.*, 1999). Other protein acting as second messengers, *i.e.* cGMP, PIP3-kinase, MAP-kinase and tyrosine kinase are involved in adhesion of bacteria to the host cell surface (Guzmán-Verri *et al.*, 2001).

Adhesion to macrophage surface is also associated with small GTPases activation (Guzmán-Verri *et al.*, 2001) and F-actin polymerization (transient and rapid F-actin accumulation). In the early stages of adhesion Annexin I is also involved, a protein that is implicated in membrane fusion (Kusumawati *et al.*, 2000). Bacterial internalization occurs also by the lipid rafts – microdomains that occur in the macrophage cell membrane. These structure contribute to intracellular trafficking of *Brucella* (Fig.1) (Xavier *et al.*, 2010). Non-opsonized *Brucella* strains internalize in human monocytes and murine macrophages by lipid rafts. This process requires activation of TLR4 and PI3-kinase. However, this process in human dendritic cells is only partly dependent on lipid rafts (von Bargen *et al.*, 2012). *Brucella* strains with lack of O-polysaccharides in LPS (R-LPS) do not penetrate eukaryotic cells by lipid rafts, and thus are exterminated by macrophages (Porte *et al.*, 2003; Gomez *et al.*, 2013). Lipid rafts are rich in cholesterol, GPI (glycosylphosphatidylinositol) and GM1 (gangliosides) (Brown and London, 1998). Lipid rafts-associated proteins: GPI and GM1 as well as cholesterol inosculate with *Brucella*-contain macropinosomes and facilitate internalization with macrophage (Naroeni and Porte, 2002).

Brucella can be recognized by TLRs, but owing to modifications its interaction with TLRs is 10-fold lesser than for Enterobacteria. Hence, the activation of NF-κβ and production of inflammatory cytokines is weaker (de Figureido *et al.*, 2015).

Intracellular trafficking. It has been supposed that intracellular trafficking is not essentially different in professional and non-professional phagocytes (Gorvel and Moreno, 2002). After several minutes of invasion, the bacteria interact with early endosomal network-related compartment – early BCV – *Bru-*

cella Containing Vacuole. This compartment is characterized by Rab5 (GTP-binding protein) and EEA1 (early endosomal antigen 1) markers (Pizarro-Cerdá *et al.*, 1998; Pizarro-Cerdá *et al.*, 2000). β-1,2-glucans are necessary for the regulation of BCV maturation in macrophages as well as in epithelial cells. Additional function of β-1,2-glucan is the modification of rich in cholesterol lipid rafts, which are located on BCV membrane surface (Arellano-Reynoso *et al.*, 2005). The interaction with early endocytic network last about 10 minutes (Pizarro-Cerdá *et al.*, 1998). At this stage, acidification of BCV takes place leading to changes on the bacterial genes expression and enabling intracellular survival (Carvalho Neta *et al.*, 2010). BCV does not interact with late endosome and it avoids fusion with lysosomes (by β-glucans and LPS occurrence) (Gorvel and Moreno, 2002; Celli, 2006). However, early BCV is transformed to intermediate BCV that is LAMP1 and Rab-7 marked (late endosomal/lysosomal markers), indicating that interactions with late endosomal compartments and lysosomes become necessary. What is more, in this step BCV acquires also Rab-interacting lysosomal protein (RILP) that is Rab-7 effector (Starr *et al.*, 2008; von Bargen *et al.*, 2012; Gomez *et al.*, 2013). The interaction between BCV and late endosomes/lysosomes is transitional and controlled. This event allows for acidification of BCV and expression of acidic-contingent bacterial factors, *e.g.*, *virB*; simultaneously, the cathepsin D action does not take place (Boschiroli *et al.*, 2001; Starr *et al.*, 2008; von Bargen *et al.*, 2012). *Brucella* type IV secretion (T4SS encoded by *virB* operon) is responsible for regulation of intracellular trafficking from autophagosome to endoplasmatic reticulum (ER) (Fig.1) (Gorvel and Moreno, 2002).

About 1 hour after internalization, *Brucella* organisms are located within multimembranous autophagosome with LAMP1 and Sec61β (calreculin). This structure is also called as a late BCV and occurs only in epithelial cells (Pizarro-Cerdá *et al.*, 1998). LAMP1 function is not accurately described; however, it presumably participates in the pathogen intracellular survival (Gorvel and Moreno, 2002). The final step of *Brucella* intercellular trafficking is an acquisition of the markers characteristic of the endoplasmic reticulum: calnexin, calreticulin and Sec61β, although in this step BCV lose the LAMP-1 (Pizarro-Cerdá *et al.*, 1998). However, this protein is constantly present in large vacuole only in human monocytes, in which opsonized *Brucella* multiply (Bellaire *et al.*, 2005). ER is the only compartment that is suitable for *Brucella* replication (Pizarro-Cerdá *et al.*, 1998). The mechanism of BCV-ER connection remains unclear. In this process there are involved small GTPases Rab2, GAPDH – glyceraldehyde-3-phosphate dehydrogenase, the COPI complex (Coat Protein Complex I) and protein kinase C (PKC_I). COPI and PKC_I control vesicular trafficking to the ER from Golgi. GAPDH/COPI/Rab2/PKC_I complex is responsible for *Brucella* replication within the ER (Fugier *et al.*, 2009).

Treatment of brucellosis

Currently, there are no effective vaccines for human, although several *Brucella* vaccines are accessible for livestock. Live, attenuated vaccines bereft of virulence factors, still present residual virulence (*e.g.*, Live *B. abortus* vaccine strain 19, Live *B. abortus* vaccine strain RB51, Live *B. melitensis* vaccine strain Rev-1). Subunit vaccines are proven to be relatively safe and they raise less concerns compared to live vaccines. They do not cause infection, as they present purified proteins or DNA to stimulate immune response. Researchers are still working on the improvement in livestock vaccines and their application in preventing human infections (Yang *et al.*, 2013).

To implement a successful treatment against brucellosis, antibiotics penetrating into macrophages as well as active in acidic environment are essential (Ranjbar, 2015). Brucellosis is a disease, that rarely leads to death and responds well to diverse therapeutic strategies (Solís *et al.*, 2015). However, single-antibiotics therapy is inadequate in brucellosis, as it leads to relapse of disease (Pappas *et al.*, 2006). Similarly, the therapy with single agent like: oxytetracycline, rifampin or doxycycline, causes high rate of relapses (9–25%) and prolongation of therapy does not provide satisfying effects. The treatment with trimethoprim-sulfamethoxazole or ciprofloxacin results in relapse in 30% and 83% of cases, respectively (Ranjbar, 2015). Treatment should prevent relapse of disease, further complications (arthritis, spondylitis, sacroilitis *etc.*) and enable quick relief of symptoms. The combination of two antibiotics in therapy of infections caused by *Brucella* is more effective than monotherapy. The WHO in 1986 recommended doxycycline with rifampicin for six weeks, replaced with tetracycline in combination with streptomycin. Currently, the combinations of other antibiotics or chemotherapeutics in therapy of brucellosis are used, such as fluoroquinolones or co-trimoxazole with rifampicin, doxycycline-streptomycin and doxycycline-rifampicin (Skalsky *et al.*, 2008). During the treatment of brucellosis with streptomycin and doxycycline (SD), a failure of treatment and relapse rates at 7.4% and 4.8%, respectively were noted. Almost similar results of therapy were observed during therapy with doxycycline and rifampin (DR) or streptomycin together with tetracycline (ST); however, their relapse rates were higher than in SD treatment. Another dual therapies of brucellosis are known, for example doxycycline and gentamicin (DG) with the average failure rate of 5.2% and the relapse rate

of 5.9%, or cotrimoxazole and rifampicin (RCTM) used in children brucellosis with the failure of treatment and relapse rates at 0–16.4% and 3.1–10%, respectively. The treatment with ciprofloxacin or ofloxacin with doxycycline, cotrimoxazole, rifampicin brought about the relapse rate between 3.2 to 26% (average 11.4%) and the failure rate between 3.2% to 26% (12.2%) (Alavi and Alavi, 2013). There were three clinical trials that used triple-drug therapy with doxycycline, rifampicin and aminoglycoside. There is no clear evidence on the superiority of triple-drug therapy when compared to the two-drug therapy. Nevertheless, it seems that triple-drug therapy is more effective in preventing relapses, but less successful in short-term treatment than two-drug therapy (Solís *et al.*, 2015). The research by Alavi and Alavi (2013) suggested triple therapy for eight weeks in complicated cases (with spondylitis, or arthritis) due to lower treatment failure rates than two-drug therapy. Doxycycline and aminoglycoside therapy is recommended in uncomplicated chronic, or acute cases and in complicated cases without endocarditis, spondylitis, arthritis. In uncomplicated cases, streptomycin and doxycycline, or gentamicin are also advocated (Alavi and Alavi, 2013).

Smith and colleagues (2013) found a new strategy to treat brucellosis. The connection of BCV with the ER requires remodeling of endoplasmatic reticulum, which is necessary in modification of the ER structure during host stress response, which is called the Unfolded Protein Response (UPR). The disruption of UPR, through tauroursodeoxycholic acid drug can inhibit *Brucella* replication. UPR can be a novel target in the of brucellosis (Smith *et al.*, 2013).

There are studies concerning the influence of ginseng saponin fraction A (RGSF-A) for combating brucellosis. Ginseng is a valued plant in Asia, considered a panacea to variety range of diseases. Arayan *et al.* (2015) examined the influence of RGSF-A for eradication of bacterial infection in RAW 264.7 cells. In this study, the bacterial internalization and adhesion were reduced in the cells treated when compared to the control cells without treatment. RGSF-A takes part in downregulation of MAPKs (mitogen-activated protein kinases) and hence, limits polymerization of F-actin and inhibits bacterial penetration into the cells. RGSF-A influences also intracellular trafficking of *B. abortus* and favors interaction of *B. abortus*-containing phagosomes (BCPs) with LAMP-1. LAMP-1 is transmembrane protein, that is responsible for the fusion of lysosomes with phagosomes, enabling the connection of BCPs with lysosome and elimination of bacteria (Arayan *et al.*, 2015; Reyes *et al.*, 2016; Huy *et al.*, 2017). Huy *et al.* (2017) have proven that ginsenoside Rg3 – panaxadiol saponin components of RGSF-A have been the major factor controlling brucellosis.

Undoubtedly, there are also other promising herbal plants *e.g. Teucrium polium, Scophularia deserti, Alhagi, Eucalyptus*, garlic and roots of barberry that contain bioactive ingredients (flavones, flavonoids, anthocyanins and tanins) that can be effecive in preventing or even combat brucellosis (Naghadi *et al.*, 2016).

Conclusions

Brucella is an intracellular pathogen, especially dangerous for domestic animals, which causes massive infections and thus significant economic losses. Moreover, people who work with infected animals comprise a risk group, *e.g.*, farmers, veterinarians, or laboratorians and they are most endangered of being exposed to the pathogen. Brucellosis in human causes non-specific symptoms, therefore no plausible estimation can be managed to detect the number of infected people. *Brucella* is an inquisitive etiological agent, as does not produce classical virulence factors. The process of infection is a complex one, and there are many unexplained issues associated with it. Therefore, further studies of infection mechanisms are required.

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