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Phylogenetic analysis, pathogenesis, diagnosis and treatment: A review of Brucellosis and pig *Brucella*

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Abstract
Distinctive phenotypic characteristics are basic points to classify *Brucella* in to groups. The bacteria are also grouped into smooth and rough strains depending on the presence or absence of LPS on the O-side chain. They similarly classified according to their host preference though their host-pathogen association is not restricted. They lacks the actual pathogen-associated molecular patters (PAMPs) and classical virulence factors so they are hidden from early detection of immune system and protect themselves from *bactericid*al mechanisms. *B.suis* has wide host range with greatest phylogenetic distance between *biovar* having the most diverse strain of *Brucella* species. It is mor e generalized and chronic disease localized in the reproductive organs and the bones in pigs than bovine brucellosis. Nevertheless due to a decreased assumption and theory of reduced risk of human infection, emphasis was not given. The acquired immunity, antigen specific recognition of pathogens by the B lymphocytes has limited effect on protection of infection but is useful for diagnosis of disease. CELISA reduces reactions due to antibodies against vaccination and cross reactions subsequently during surveil lance study of pig *Brucellosis* combinations of methods ELISA of Yersinia (Ye O: 9) and Brucellosis are important. Treatment should prevent extra complication, relapse of disease and enable quick relief. Preventing is economical than to try to control diseases, it's therefore important reviewing the different characteristics/ nature of *Brucella*.

Keywords: *Brucellosis, Phylogenetic analysis, Pathogenesis, Diagnosis and Treatment*

Introduction

Phylogenetic analysis has helped better understanding of gene transfer events and evolutionary history of the organisms, discrimination of the various *Brucella* species host specificity and virulence factors (Wattam *et al*., 2014; Tan *et al*., 2015) [71, 67]. The bacteria are very similar in base composition and genome size (Bohlin *et al*., 2010) [7]. Smooth *Brucella* strains able to cross the mucosal epithelial cells in to phagocytes for replication and avoids the host immune response (Xavier *et al*., 2014; Figueiredo *et al*., 2015) [72, 24]. They adapt to the environmental conditions encountered in its intracellular replicative niche including low levels of nutrients and oxygen, acidic pH, and reactive oxygen intermediate (Glowacka *et al*., 2018) [26]. *B.suis* has wide host range and comprises five biotypes with greatest phylogenetic distance (Maiden *et al*., 2013; Wang *et al*., 2016; Sankarasubramanian *et al*., 2016) [38, 69, 58] but they have nearly identical G+C content (Suárez-Esquivel *et al*., 2020). Rate of abortion is highest in sows exposed via the genital tract at the time of breeding. *B.suis* can affect joints and tendon sheaths resulting in lameness and paralysis (Lopes *et al*., 2010; Megid *et al*., 2010) [47, 42]. In diagnostic testing especially of pig Brucellosis, false positive serological reaction (FPSR) commonly occur due to Yersinia enterocolitica (Ye O: 9) infection which possess same O-antigen lippolysaccharides to *Brucella*. as Pigs are the main reservoir of the agent (Bari *et al*. 2011; Fredriksson-Ahomaa *et al*. 2010) [6, 22]. ELISA is capable of readily identifying individual IgM and IgG antibodies allowing for a better correlation with the clinical situation (Naik *et al*., 2017) [45]. Antibiotics penetrating macrophages as well as active in acidic environment are essential to treat brucellosis successfully (Ranjbar, 2015; Solís *et al*., 2015) [55, 64].

2. Taxonomic Classification of *Brucella*

Brucella is a member of the *Brucellaceae* family in the orde r *Rhizobiales*, class *Alphaproteobacteria* of the phylum Proteo-bacteria probably evolved from Ochrobactrum-likecommon soil ancestor (Ficht, 2010; Banai and Corbel, 2 010) [23]. Nowadays the genus includes more than ten recognized species with varying host preferences, pathogenicity and epidemiology however the distribution of most of these organisms is still poorly understood (OIE, 2018; Vidal *et al*., 2018) [50, 68] (Table1).

Table 1: List of different *Brucella* species and their natural hosts*

***Brucella* species**	**Colony type**	**Natural host****	**Zoonoses**
***B. melitensis* (bv1-3)**	Smooth	Goat and sheep	+++
B.abortus (bv 1–6,7,9)	Smooth	Cattle	++
B. suis biovar***1–3	Smooth	Pig	++
2	Smooth	Wild boar &Hare	+
4	Smooth	Reindeer, Caribou	++
5	Smooth	Rodent	-
B. ovis	Rough	Sheep	–***
B. neotomae	Smooth	Desert rat	+
B. canis	Rough	Dog	+
B. ceti (B. delphini)	Smooth	Dolphins	+
B. pinnipedialis (B. phocae)	Smooth	Seals	+
B. microti	Smooth	Wild voles	?
B. inopinata	Smooth	Human	++
B. papionis	?	Baboons (*Papio* spp.)	?
B. vulpis	?	Red foxes (*Vulpesvulpes*)	?
N.N.****	Smooth	Frog	?

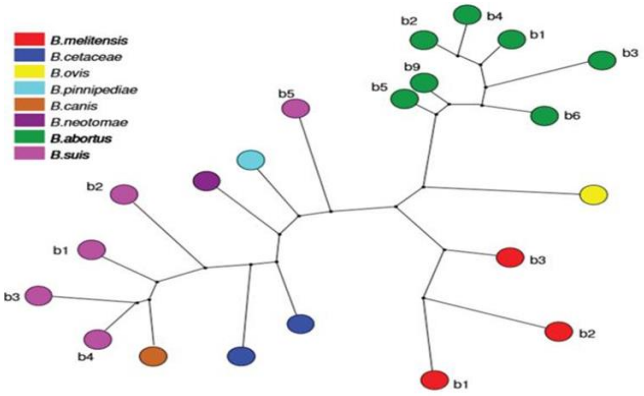
Source: El Sayed and Walid Awad (2018)

3. Morphology and Genome of the *Brucella* organisms

Brucella spp are coccoid rods in shape with a size of 0.6 to 1.5 µm in length and 0.5 to 0.7 µm in width with a microscopic appearance of 'fine sand'. They are small Gram-negative, arranged singly and less frequently in pairs, small groups, and rarely form chains (Mizak *et al*., 2014; Tan *et al*., 2015) [43, 67]. The members are generally facultative non motile non spore forming, and none encapsulated intracellular reproducing bacteria. Colonies of smooth *Brucella* strains are raised, convex circular and translucent non-pigmented and non haemolytic pale honey color. Subcultures are less transparent dull, dry, yellowish white in color. Rough colonies stains red/violet and smooth colonies do not uptake dye or stain pale yellow (OIE, 2016) [48]. The *Brucella* cell envelope is a three-layered structure and three main groups of proteins, the lipopolysaccharide, proteins, and phospholipids (Haag *et al*., 2010) [30]. The 16S and 23S rRNA operon, IS711 insertion sequence or bcsp31 are common genes among *Brucella* species.

4. Phylogenetic analysis of *Brucella Suis*

The genus *Brucella* was known by comprising two phylogenetic groups named as ''classical'' species comprising (*B.abortus, B. melitensis, B. suis, B. ovis, B. canis and B. Neotoma*) and ''non-classical'' species include (*B. ceti, microti, B. pinnipedialis, B. papionis, and B. vulpis* (Scholz *et al*., 2016; Guzmán *et al*., 2019) [60, 29]. Different techniques of analysis show dispersion between different strains of the same *B.suis* species. *B.suis* has wide host range and comprises five biotypes, 1to 4 are zoonotic but *biovar* 2 is very rarely pathogenic for human, and *biovar* 5 has not been recognized as zoonotic. There is greatest phylogenetic distance between *biovar*5 and the other *biovars*. Furthermore, separated in to two subclass one formed by the *biovar*2 and the other contains *biovars*1, 3 and 4, making this species a paraphyletic group (Maiden *et al*., 2013; Wang *et al*., 2016) [38, 69], having the most diverse strain of this bacterial genus (Le Flèche *et al*., 2006; Sankarasubramanian *et al*., 2016) [35, 58] (figure1).



Source: Le Fléche *et al.* (2006)

Fig 1: Phylogenetic analysis of *Brucella* species

5. Disease Course

5.1. Pathogenesis and clinical presentations of B. suis infection in pigs

The bacteria enter host through penetration of mucous membranes transported epithelial cells to regional draining lymph nodes where initial replication occurs then released to circulation and disseminated throughout the body to the preferred tissues in reproductive tract (Kim, 2015) [34], causing acute or chronic infection of reproductive tract that leads to abortion or still birth (Yangquan, 2012) [73]. *B. suis* usually is a more systemic generalized and chronic disease localized in the reproductive organs and the bones in pigs than bovine brucellosis. Shedding the bacteria from mucosal surfaces or in urine for long periods, prolonged bacteremia, and ability of venereal transmission are the uniqueness of *B. suis* in swine which considerably differ from characteristics of brucellosis in mammals caused by *B. abortus* or *B.melitensis* (CDC, 2017) [11]. Bacteremia and infertility are common in sow and reproductive damage in boars is irreversible, swollen/painful testicle is frequent

clinical signs of brucellosis (Moges, 2013; Herrick *et al*., 2014; Paul, 2019) [44, 31, 52].

5.2. Virulence factors of *Brucella* infection

Brucellais devoid of the typical bacterial virulence factors such as fimbriae, toxins, capsule, functional flagella, and plasmids therefore the Organisms virulence factors functions by protecting themselves from any bactericidal mechanisms and hide from immune detection and interruption of the host immune response (Higgins *et al*., 2015) [33]. They escape immune recognition by the host then adapt long-term intracellular survival establishing and maintaining chronic infections. Smooth *Brucella* can restrain host cell apoptosis by the interaction of the O-chain with TNF-α (tumor necrosis factor) and therefore dead cells do not release specific factors thus they do not activate the immune system and the bacteria will not be recognized by the host immune system (Glowacka *et al*., 2018) [26]. In addition, because of the absence of PAMPs expression in the cell envelop of *Brucella* outer membrane the bacteria are hidden to early detection by innate immunity (Salcedo *et al*., 2013) [57]. The bacteria has multiple mechanisms to detoxify free radicals to control the oxidative killing mechanism of host phagocyte cells and are protected from oxidative killing, cationic peptides, and complement-mediated lysis by the O side chain on the LPS, and it is a key molecule for invasion (Adem. & Duguma, 2020; OIE, 2018) [4, 50]. Enzyme urease produced by all the genus *Brucella* except B. Ovis decomposes urea to carbonic acid and ammonium form, through nitrogen metabolism results in an increase of pH due to ammonia concentration and this may protect *Brucella* during passage through the digestive tract principally stomach when the bacteria enter their host through the oral route (Glowacka *et al*., 2018) [26]. In man occurs as an acute febrile state but the chronic forms of the disease are common affecting joints and causes back pain, fatigue, headaches, undulant fever, insomnia, constipation, and neuro-brucellosis or endocarditis. (Megid *et al*., 2010; Zhang *et al*., 2011; Al Dahouk S *et al*., 2013) [42, 69, 13].

6. Immunity against *Brucella* infections

Protective host immune response against intracellular pathogens is divided into the innate immune system that includes anatomical barriers, complement system and cellular populations like phagocytes, and the innate lymphocyte subsets (natural killer and γδ T cells). The other adaptive immunity composed of T lymphocytes, and B lymphocytes (Skendros and Boura, 2013; Faramarz *et al*., 2019) [62, 21].

6.1. Innate immunity

Is responsible for preventing replication, reducing the initial number and killing of the microorganism, and creating circumstances for the generation of an effective adaptive immune response (Diacovich and Gorvel, 2010) [15]. Natural immunity is the first line of protection or defense includes phagocytosis of pathogens by the neutrophils, macrophages, and DC, death by natural killer (NK)-cells, secretion of cytokines and chemokines, recognition of molecules typical of a microbe PAMPs by PRRs, and activation of the complement system. Th1 lymphocytes are characterized by the production of pro-inflammatory cytokines as IL 1β, IL6, and TNF α. Th1 cell-mediated immune response particularly IFNγ is important in protection against *Brucella* spp. infection (Byndloss *et al*., 2016) [8] by activating macrophages to increase their bactericidecapacity and cytotoxic T lymphocytes (CTL) that kill infected cells (Marco *et al*., 2012) [39].

6.2. Adaptive Immunity

Antigen specific recognition of pathogens by T and B lymphocytes and secreted antibody attempts to control *Brucella* infection through production of the CD4+ and CD8+ T cells (Skendros and Boura, 2013) [62]. CD4+ T cells exert their effect via potent cytokines such as IFN-γ and IL-10 and cytotoxic CD8+ T cells eliminate infection by lysing macrophages infected with *Brucella* spp (Cha *et al*., 2010). *Brucella* has the ability to block receptors for innate immunity inside the macrophages (Campos *et al*., 2014) [9], inhibiting phago lysosome fusion and apoptosis, and down regulating antigen presentation (Skendros *et al*., 2011) [61], which finally leads to their escape from effectors immune responses and cause chronic infection (Martirosyan *et al*., 2013) [40].

7. Diagnosis of Swine Brucellosis

Usually, combinations of methods are used to diagnosis brucellosis. Currently based on the detection of antibodies against the (SLPS) strains some validated serological tests are available for brucellosis diagnosis in swine (McGiven *et al*., 2012; OIE, 2017) [41, 49]. Although it is difficult to isolates the bacteria due to the delays in symptoms, resulting in incorrect sample types and low bacterial loads in specimens such as blood, milk, and/or tissues, isolation of *Brucellae* is still the gold standard for diagnosis (Godfroid J *et al*., 2011) [28].

7.1. Serological Methods

The methods are relatively easily accessible, and comparatively cheap, and sensitive (Godfroid *et al*., 2010; Smirnova *et al*., 2013) [27]. Smooth *Brucella* species have common epitopes in their SLPS two surface antigens 'A' and 'M'. The 'A' antigen predominates in B. abortus and B. suis, while 'M' is the major antigen in B. melitensis (OIE, 2009; David and John, 2017) [46, 14]. *Brucella* oligo polysaccharide (OPS) antigens shared with other Gram negative bacteria such as Escherichia coli O: 157, Salmonella and particularly Yersinia enterocolitica O:9 as a result serological assays show variable levels of cross-reactivity therefore combinations of methods are used to diagnosis brucellosis (Al Dahouk *et al*., 2013; Poester *et al*., 2010) [13, 14].

7.1.1. Buffered antigen plate agglutination tests

The Rose Bengal plate agglutination test (RBPT) is one of a group of tests known as the buffered *Brucella* antigen tests performed with a stained *B. abortus* suspension at pH of 3.6 to 3.7 and serum. RBPT is an agglutination test based on the reactivity of antibodies against SLPS where drops of stained *brucella* antigen and serum mixed on a plate and any resulting agglutination signifies a positive reaction. Its simplicity made it an ideal screening test for small laboratories with limited resources. The drawbacks of RBT include low sensitivity particularly in chronic cases,

relatively low specificity in endemic areas, and prozones that make strongly positive sera appear negative in RBT (Díaz *et al*., 2011) [16].

7.1.2. Complement fixation test (CFT)

Throughout the world regarded as being the confirmatory test for the serological detection of *Brucella* in infected animals (OIE, 2012) [47]. The test detects specific antibodies of the IgM and IgG1 type that fix complement mainly the IgG1isotype antibody, as the IgM antibodies are partially destroyed during the inactivation process. It may test false negative when antibodies of the IgG2 type hinder complement fixation because of the occurrence of the prozone phenomenon whereby low dilutions of some sera from infected animals do not fix complement. This is due to the presence of high levels of non-complement fixing antibody isotopes competing for binding to the antigen which are

diluted out at higher dilutions and fix the complement. Cont aminating bacteria over warming during deactivating causes a positive or "anti-complementary" reactions make the test invalid (OIE, 2016) [48]. The test can detect cross reactions caused by bacteria that share common chain of lipo polysaccharide (LPS) antigen such as Yersinia enterocolitica O: 9, Escherichia coli O157, Salmonella serotype, Vibrio cholera, and Pseudomonas mallophilia (Perrett *et al*., 2010) [53]. In the case of swine sera, Interactions between pig complement and guinea pig complement of CFT produce a pro-complementary activity that interferes with the sensitivity of the assay (Ducrotoy *et al*., 2016; OIE, 2018) [17, 49].

7.1.3. Enzyme linked immune sorbent assays (ELISA)

Are very sensitive and good assays for detecting dormant carriers, relatively simple and easily automated (Kauffman *et al*., 2014) [33]. Compared to other Gram-negative bacteria, the LPS of *Brucella* strains induce very small inborn immune response, and a lesser rate of maturation and activation of dendrite cells but the LPS of *B. suis* is a highly immunogenic, T-cell independent antigen that can directly activate B-cells and elicit antibody responses in infected swine (Olsen and Tatum, 2016) [51]. Though there is some disparity among strains IELISA has become the standard for diagnosis of swine brucellosis (Fabien *et al*., 2012) [20]. It has been used for serologic diagnosis of brucellosis in sheep, goats, and pigs for smooth LPS *Brucella* species. But it is not capable of differentiating antibodies induced by the vaccine strains S19 or Rev1 (Eoh *et al.*, 2010; Lim J *et al.*, 2012) [19, 36]. In CELISA sample antibody competes for binding to antigen-coated to microliter plate wells with a limited amount of labeled antibody. The higher the sample antibody concentration, the weaker the output signal, indicating that the signal output inversely correlates with the amount of antigen in the sample (OIE, 2016). Sensitivity varies from 92 to 100%, whereas the specificity ranges from 90 and 99%. CELISA is used for the detection of anti-*Brucella* antibodies in serum samples from cattle, sheep, goats, and pigs with smooth *Brucella* LPS as antigens (Godfroid *et al*., 2010; Perrett *et al*., 2010) [27, 53].

7.3. Bacterial isolation

Bacterial isolation from abortion contents, semen, Spleen, lymph nodes, uterine fluid, vaginal swabs, and in humans blood often is an accurate and sensitive methods to identify the organism on selective medium (Poester *et al*., 2010; OIE, 2018) [54, 50]. They come into view as coccobacilli/ short rods, arranged singly but sometimes in pairs or small groups under microscope using modified Ziehl Neelsen and/or Koster's staining from tissues, secretions, and exudates (OIE, 2018) [50]. Chlamydophila *abortus*, Chlamydia psittaci, and Coxiellaburnetti nevertheless can mislead the diagnosis because of their superficial similarity (Poester *et al*., 2010) [54].

7.4. Molecular Techniques

Amplification of specific DNA regions been successfully used for *Brucella* identification and typing (Çiftci *et al*., 2017) [12]. The *Brucella* species are very similar to the genetic level at which all species share about 97% DNA homology (Scholz *et al*., 2013; Suárez-Esquivel *et al*., 2020) [59]. Multiplex PCR specifically identify B. suis from other brucellae and differentiate among *B. suis biovars* 1 to 5(Suis ladder) (Albert *et al.*, 2011; Wattiau *et al.*, 2011) [2, 71]. The "real-time" PCR can monitor the progress of the amplification at the same time quantifies amount of PCR product (DNA or RNA) through its detector in contrast to the conventional PCR method where analysis is possible only after the process is completed. Is faster, sensitive and specific than normal PCR and less complexity at the quantification of sample. It allows the success of multiple PCR reaction to be determined

automatically after only a few cycles, without separate analy sis of each reaction, and avoids the problem of "false negati ves", contamination and save time (Garibyan & Avashia, 2013; Sagar, 2020) [25, 56].

8. Treatment control and prevention

Treatment of brucellosis in animals is not very effective; however, multi drug regimens could be tried (Al-Anazi and Al Jasser, 2014) [3]. The incubation period of the bacteria ranges from 1 week to 2 months, however is usually 2 to 4weeks. Principally in acute and localized forms of brucellosis, combination of doxycycline with streptomycin (DS) is good therapeutic option with less side effects and less relapses (Sriranganathan *et al.*, 2010; FAO & WHO, 2020) [65]. Doxycycline and aminoglycoside therapy is recommend ed in uncomplicated chronic or acute cases. Streptomycin and doxycycline, or gentamicins are also advocated (Alavi and Alavi, 2013) [1]. Good husbandry management such as disinfection of contaminated areas, Prevention of contacts with the herds or flocks of unknown status or those with brucellosis and Pre-purchase tests, movement controls, surveillance, and tracing of infected animals are good preventive measures. Good bio security and double fencing have been used to protect pigs raised outdoors (OIE, 2018) [50]. Semen for artificial insemination should only be collected from *Brucella* negative animals that are tested regularly. Possibly due to a decreased emphasis on *B. suis* as compared to *B. abortus* and *B.melitensis* and an assumption of reduced risk of human infection commercially vaccines had not provided to protect swine from *B. suis* infection (Olsen and Tatum, 2016) [26]. Lack of standardized repeatable challenge model that replicates infection and clinical disease in swine under field conditions was the other problem to produce vaccine (Stoffregen*et al*., 2013). Vaccination with selective depopulation is one preferred regulatory method to control swine brucellosis (Olsen and Tatum, 2016) [26].

9. Conclusion

Brucellosis is worldwide zoonosis can infect any organ like tuberculosis causing granuloma. Though the genus *Brucella* contains many species with varying epidemiology, studies focused on the ‘‘classical’’ phylogenetic groups. The bacteria have multiple mechanisms to protect themselves from *bactericid*al and immune systems even adopt the acidic PH in the digestive tract. The disease could appear with various clinical presentations. The intracellular nature of the organism makes difficult to eliminate it in host cellular responses or by antimicrobials. In PCR because of the high sensitivity of the technique, there should be proper controls to ensure that positive responses are not due to contamination. Though the tendency of reduced recovery of isolates from chronically infected animals, slow growth of *Brucella* in vitro, high costs associated with microbiological assays and biosafety concerns makes difficult, isolation is assumed as the gold standard methods for detection of *Brucella*.

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