REVIEW ARTICLE

Canine brucellosis: Insight on Pathogenicity, zoonosis and diagnostic aspects

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Brucellosis is a severe febrile disease caused by various members of the genus *Brucella*. Canine brucellosis occurs worldwide and is endemic to America, Asia, and Africa leading to infertility and abortion in dogs. The bacterium is equipped with a battery of virulence factors like Lipopolysaccharide (LPS), T4SS secretion system and BvrR/BvrS system which enable its survival as well as spread in the host. The clinical signs in male dogs include inflammation of epididymis, testis and prostate gland where chronic epididymitis and orchitis may lead to unilateral or bilateral atrophy of testis making them sterile. The females show mid to late term abortion accompanied by inodorous, brown to yellow genital discharge. Aborted fetuses are usually partially autolyzed, edematous, congested with hemorrhages in the subcutaneous abdominal region. Females may give birth to dead or weak puppies that may die within few days. Various serological diagnostic tests have been developed but there is no standardized protocol available. Isolation of bacteria from blood samples is considered as gold-standard but has less sensitivity. Many molecular tests have also been developed with varying sensitivity and specificity. Dogs can also infect humans but the prevalence is low and infection is acquired by direct contact with infected dogs or their blood or reproductive products. The symptoms in humans are nonspecific flu like and include fever, headache, back pain, chills/night sweats, undulant fever, and weakness which are easily misdiagnosed. Unlike dogs, human do respond well to antibiotic therapy and able to clear the bacterium after long-term treatment. The disease burden can be reduced by preventing unrestricted movement of reproductively intact dogs and by continuous testing of breeding animals and their offspring before sale. Sterilization of intact stray animals and euthanasia of infected dogs may also limit the disease spread as well as the level of infection in canine population.

Keywords: Canine brucellosis; lipopolysaccharide; aborted fetuses; serological diagnostic tests

INTRODUCTION

Brucellosis is a severe febrile disease caused by various members of the genus Brucella. It is a worldwide problem, causing abortion and infertility in domestic and wild animals. Brucella are aerobic, small, Gram-negative rods and are oxidase, catalase, and urease positive [1-3]. Brucella, a genus discovered in 1887 by David Bruce, contains the following species: Brucella suis, B. ovis, B. abortus, B. canis, B. melitensis, B. neotomae, B. ceti, B. pinnipedialis, B. microti, B. inopinata, B. papionis, B. vulpis and other strains obtained from environmental samples [4,5]. Brucellosis in dogs occurs worldwide and is endemic to America, Asia, and Africa. There have been many reports of brucellosis outbreaks in the canine populations after 1966 which has led to infertility and abortion in dogs. Brucellosis can be transmitted from dogs to humans as well as from human to human also. Brucella rods enter the host cells by inhalation, ingestion, skin abrasions, through mucous membranes [6-10]. After penetration into host, the rods multiply in lymph nodes after which, they penetrate other organs. Brucella can modify immune response in host cells due to its affinity to specific tissues, e.g. placental trophoblast in fetal lung, pregnant females or reproductive system. Brucellosis causes enlargement of lymph nodes, liver and spleen. Pathogenicity of Brucella is dependent on their ability to multiply and survive within macrophages. In this review we call attention to brucellosis in dogs, highlight the Brucella canis as an unidentified pathogen and trace the present cognition regarding its zoonotic potential [10-16].

LITERATURE REVIEW

Virulence factors of *Brucella cani*

Brucella spp. is frequently called as nasty bugs based on their unusual virulence characters. *Brucella canis* has expertise to live and grow inside phagocytic and non-phagocytic cells [17]. Virulence factors of *Brucella* are not classical: Exotoxin, cytolisins, exoenzymes, plasmids, fimbriae, and drug resistant forms. The significant virulence factors are: Lipopolysaccharide (LPS), T4SS secretion system and BvrR/BvrS system, which allow association with host cell surface, formation of an early, late BCV (*Brucella* containing vacuole) and relation with Endoplasmic Reticulum (ER) when the bacteria proliferate [18-21].

Lipopolysaccharide: LPS is a crucial virulence factor of *Brucella* and consists of lipid A, an oligosaccharide core and O-antigen. The LPS is different and non-classical in *Brucella* as compared to other gram-negative bacteria like *E. coli*. The LPS is comparatively less toxic and less active than the classical LPS which causes a high fever. While non-classical LPS observed in *B. canis* causes a low fever, being a weak inducer of tumor necrosis factor [22-25].

Type IV Secretion System (T4SS): T4SS is a multi-protein compound involved in production of bacterial macromolecules. VirB operon encoding 12 proteins characterize this system (11, 860 bp). Expression of the virB operon is regulated by the regulator of quorum-sensing-VjbR. Where wild strains of *Brucella* can proliferate only in the endoplasmic reticulum, VirB mutants of *Brucella* cannot multiply within the endoplasmic reticulum due to its incapability to reach the ER, or multiply within [26]. In the macrophages, *Brucella* rods are localized in *Brucella*-Containing Vacuole (BCV) which interacts with the ER and is responsible for formation of specialized *Brucellae*- multiplication compartment. The attainment of endoplasmic reticulum membrane is controlled by functional virB secretion system-T4SS [26-30].

Superoxide dismutase and catalase: Macrophages containing *Brucella* produce Reactive Oxygen Intermediates (ROIs), which is a primary mechanism of destruction of the ingested bacteria and also prevents their intracellular replication. The main line of defense that prevents reactive O_2 intermediates includes superoxide dismutase and catalase [31]. SOD (metalloenzyme) is encoded by *sod* sequence and includes iron, magnesium,

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or zinc and copper at its active site [32,33]. SOD is accountable for dismutation of O^{2-} (Superoxide) to H_2O_2 (Hydrogen Peroxide) and O_2 (Oxygen)-transfer from one molecule to another ($2O^{2-}+2H^+ \rightarrow H_2O_2 + O_2$). Catalase breaks down 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. Catalase is not a 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 [34-40].

Cyclic β-1-2-Glucans (CβG): *Brucella* CβG belongs to II OPGs (Osmoregulated Periplasmic Glucans) family. These glucans engage in direction of the phagosome- lysosome fusion. Mutants are killed in phagolysosome and they are not allowed to grow. Even more, mutants treated by CβG are good to determine vacuole maturation and lysosome fusion, so they can contact the ER and replicate there [41].

Brucella has non-identical urease operons in two distinct genomes. Urease is a metalloenzyme which destroys urea to carbonic acid and ultimately breaks it down into the ammonium form, which increases the pH. This ensures it's persistent in the acidic environment. In chromosome I, there are two urease-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. Urease may preserve *Brucella* in the digestive tract when it enters the host through the mouth [42].

Cytochrome oxidase: Cytochrome oxidase helps its persistence within the macrophages, where oxygen accessibility is restricted. There are two operons in the 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) [43,44].

Nitric oxide reductase (NorD): Reduction of nitrate to dinitrogen gas is a vital activity for bacteria in case of oxygen starvation within the cell as this system permits nitrate respiration. The infected macrophages produce Nitric Oxide (NO), which the *Brucella* can use. *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. The production of this enzyme assists to defend *Brucella* against oxygen shortage inside the macrophages.

BvrR/BvrS system: The examination of *Brucella* genomic library has validated an existence 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). BvrR exhibits resemblance to response regulators proteins, as N-terminal domain is composed of highly conserved amino acids: aspartic (pos: 14, 15, 58) and lysine (pos: 107) [45-50]. C-terminal domain showed high similarity sequence to OmpR family; therefore, this protein can be included as part of this family. The protein is made up 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. BvrS contains four highly conserved regions on C-terminal domain: H, N, D/F, and G. This character results BvrS homologous to sensor proteins of the histidine protein kinase family. BvrS is located in the cell membrane. *Brucella* BvrR/BvrS are the best characterized aspect of the virulence system; mutants are impotent of invasion, prevention phagosome-lysosomefusion and intracellular replication. BvrR/BvrS system is a regulator of expression of multiple genes [51].

These proteins influence the transcription of the membrane proteins: Omp3b (Omp22) or Omp3a (Omp25a) and have the effect on other non-protein membrane molecules and hence on functional and structural membrane homeostasis. BvrR/bvrS mutants show structural changes in LPS, but O-chains seem to be undisturbed. These mutants are unable of activation of GTPase (Cdc42) before appearance into the cell, so they remain extracellularly and in consequence they do not infect the cell. BvrR/BvrS is also important for restricted lysosome fusion and intracellular trafficking.

Signs and symptoms of canine brucellosis

The clinical signs of canine brucellosis are not characteristic. Dogs may manifest the characteristic clinical signs or may remain subclinical. The male dogs show signs of inflammation of epididymis, testis and prostate gland whereas chronic epididymitis and orchitis can cause unilateral or bilateral atrophy of testis and make them sterile. In acute conditions, enlargement of testis and scrotum occurs with rashes on scrotal skin. The distinctive characteristic in females is mid to late term abortion i.e. during 45-59 days of gestation accompanied by inodorous, brown to yellow genital discharge after 42-45 days. Females also give birth to dead or weak puppies that may die within few days. Puppies which are born infected can exhibit signs of disease in succeeding life [52,53]. Another indication is early embryonic death and reabsorption of developing embryo resulting in failure of conception even after effective copulation. In the primary phase, inflammation of lymph nodes are also frequent. *B. canis* infects the intervertebral discs, eyes, kidneys, or brain. If the bacteria infects these tissues, the signs will be related to the bodily system infected. The considerable issue is that *B. canis* can cause permanent disease with irregular discharge of bacteria. If the reproduction malfunctioning/abortion is not reported then it is very hard to identify/examine [54].

Clinical manifestations in human beings: Humans get infection by direct association with contaminated reproductive secretions or blood of infected dogs. Clinical manifestation comprises of undulant fever, chills, malaise, splenomegaly, and peripheral lymphadenomegaly.

Pathological aspects of canine brucellosis

Canine brucellosis is considered to be one of the most common bacterial zoonotic infection worldwide and a cause of great economic loss in kennels. The classical signs of canine brucellosis are spontaneous abortion in a supposedly healthy pregnant bitch or failure to conceive. Carmichael reported that late abortion occurs between 30 and 57 days of gestation, and higher frequency of abortion was observed between 45 and 55 days. Aborted fetuses are usually partially autolyzed, and edema, congestion, and hemorrhage are presented in the subcutaneous abdominal region. Prolonged, viscous and serosanguinous vaginal discharge can last for 1-6 weeks after abortion [55].

Gross findings: The most common gross lesions are observed in the lymph nodes and spleen with variable degree of swelling. The testes show marked swelling with multifocal to diffuse reddish discoloration. In some male dogs, epididymal swelling and scrotal necrosis have also been observed. Non-pregnant female dogs do not show any specific gross lesions. However, an aborting bitch shows brownish vulvar discharge. Aborted fetuses are often partially autolyzed with a brown or greenish-gray placenta. There are also differences in the lungs between adult dogs and aborted foetuses where the changes in the lungs are much less prominent as compared to the findings described for adult dogs having brucellosis. Previous studies have shown that histological alterations in the lung are the most significant lesions in aborted fetus [56].

Histopathological findings: Mild to severe lymphohistiocytic interstitial inflammation is observed in the prostate glands of male dogs suffering from *B. canis*. Scrotal dermatitis characterized by the infiltration of lymphocytes and neutrophils with epidermal ulceration or crust formation has also been observed in some male dogs. The mammary gland shows multifocal interstitial lymphocytic infiltration in female dogs along with multifocal-to-diffuse lymphocytic endometritis. The most common microscopic lesion of non-reproductive organs is multifocal neutrophilic or lymphocytic hepatitis seen in the liver of affected dogs of both genders. Lymphoid tissues such as the lymph nodes and spleen usually show follicular and white pulp hyperplasia with variable degree. Placental trophoblasts are also markedly hypertrophied due to the accumulation of intra-cellular gram-negative coccobacilli [57].

Immunohistochemistry: In dogs suffering from Brucellosis, humane euthanasia has been performed with collection of tissue samples (liver, spleen, kidney, lung, lymph node, and testicle) fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, sectioned into 5 μm thick sections, and stained with Hematoxylin and Eosin (HE) following

standard procedures. These tissues have been further analyzed by IHC analysis following the method previously described. Mild-to-severe inflammatory and necrotic lesions have been observed in all affected tissues, among which lesions in the liver, kidney, and lymph nodes. Reports have shown significant necrotic changes in the splenic red pulp with few hyperplastic lesions being observed in white pulp and hyperplasia of the splenic white pulp prominent in females and not in males [58].

IHC staining shows bacterial antigens in the lesions of various organs. *B. canis* antigens are primarily located in the cytoplasm of macrophages and neutrophils in portal infiltrates of the liver. *Brucella* antigens are also detected in the cytoplasm of macrophages in the red splenic pulp, cytoplasm of epithelial cells of cortical and medullar tubules, and macrophages and neutrophils of the renal interstitium. IHC techniques have been widely used for the detection of *B. abortus*, *B. suis*, and *B. melitensis* antigens in many animals such as cows, sheep, goats, bovine and ovine aborted fetuses, and hares. Immunolabelling of *B. canis* antigens is stronger in the spleen, testicle, and liver than in the kidney and lymph nodes, and this was associated with the severity of inflammatory and necrotic lesions in those tissues. The detection is characterized by the observations on histopathology and IHC techniques [59-65].

DIAGNOSIS

Although several serological diagnostic tests have been developed for diagnosis of canine brucellosis but there is no standardized protocol available. However, the diagnosis always remains challenging where using a single or even different laboratory method may not be enough to attain a definitive diagnosis. Direct method is considered to be the most appropriate method for the detection of canine brucellosis and bacterial isolation from blood samples is taken as gold-standard method but it shows some sensitivity issue. Moreover, bacteria is not always present after infection as the organisms have affinity for genital tract or associated lymph nodes, hence single blood culture is not sufficient to prove the negative result. So the same diagnostic method is performed thrice at 24 hours interval for confirmative negative result. Although serological diagnostics are performed mostly but there is evidence of showing many false positive results due to cross reaction with specific as well as non-specific antigens present on the surface of other bacteria [13, 42]. The positive samples in the screening test i.e. Slide Agglutination Test (SAT) are further processed for complementary test i.e. Tube Agglutination Test (TAT) and 2-Marcaptoethanol-TAT test [66-69].

Low level of non- specific agglutinin can be removed by employing 2-Mercaptoethanol test. Rapid slide agglutination test is accurate to identify the non-infected dogs but shows false positive result due to presence of similar antigenic determinants. Although Agarose Gel Immuno Diffusion test is also employed but it shows false positive result due to use of crude SDC or PBS antigenic extracts [70]. However, Carmichael reported that *Brucella ovis* and *B. canis* surface antigens are partially identical and cross reactive hence rapid slide agglutination test combining with AGD test might be useful. Therefore, molecular techniques have been adopted nowadays for better sensitivity and accuracy. Mol did a comparative study between serological method and PCR and found variation in the positive serologic results from 6.3% by AGID to 16.5% dot ELISA where PCR showed 13.9% positive result. *B. canis* outer membrane protein 25 DNA q PCR in urine sample and vaginal swabs for early detection under field condition prior to detection of antibodies. Alfattli found that 5.76% and 12.76% dogs found to be positive for *B. canis* using rapid test and indirct ELISA respectively and 16.23% found be positive by using molecular technique i.e.16s rDNA inter-spacer PCR. PCR assay on *Brucella canis* isolated from lymph nodes and found that 91.7% negative sample for bacteriological culture showed positive result through PCR. For comparative study between molecular techniques and serological techniques sensitivity and specificity play an important role. Evaluated that compared to 2-mercaptoethanol rapid slide agglutination test PCR shows 89.2% specificity and 77.9% sensitivity however, in compared to blood culture PCR showed 92.6% sensitivity and 90% specificity. Standardized and evaluated novel PCR targeting 16S-23S rRNA inter-space in *Brucella canis* isolated from vaginal swabs of dogs [71-75].

There is lack of highly sensitive serological test concerning rapid diagnosis of Canine brucellosis as a screening test in the animals. Therefore evaluated the immunochromatographic test and found it to have greater sensitivity compared to 2-mercaptoethanol and agar gel immunodiffusion test but showed false negative result as compared to PCR as well as microbiological culture hence failed to be used as screening test due to lack of sensitivity. Guzman identified a distinctive *Brucella* spp. BCCN84.3 marker based on fatty acid methyl ester analysis, high resolution melting PCR and omp25 and omp2a/omp2b gene diversity that causes orchiepididymitis in dogs. Even cytopathology can be used as one of the diagnostic methods. Performed cytopathology using swabs and compared thr results with culture, PCR and ELISA where coccobacillary organisms as well as many immune cells were observed containing round or oval shaped bacteria in their cytoplasm. Bruce ladder multiple PCR assay using tissue samples from reproductive organs to detect *Brucella canis* but compared to tube agglutination method, it was shown to be not a definitive or reliable diagnostic method. Boeri evaluated four genes (BCSP31, 16S-23S intergenic spacer region, porins omp2a/omp2b and for insertion sequence IS711) using PCR to detect *Brucella* spp. isolated from blood and urine samples of dogs and found that gene coding for 16S-23S intergenic spacer region is the best choice in the canine clinical samples [76-78].

Kang for the first time developed a species specific((BcSS) PCR against *B. canis* infection with a detection limit of 6pg/µl and by using the buffy coat which was 100 times more sensitive than whole blood. Potency of molecular techniques comparing between PCR and LAMP (Loop-Mediated Isothermal Amplification) assay targeting IS711 insertion sequence to detect *B. canis* and found to have 100% specificity for both techniques but with 100% and 44.44% sensitivity in PCR and LAMP. Even scientists have tried using related antigen to detect anti-*Brucella* antibodies in canine blood as sero prevalence study. Determined the genetic similarity between *Rhizobium tropici* CIAT 899 strain and *Brucella canis* NCTC 10854 strain using RAPD-PCR and evaluated feasibility of using *R. tropici* to detect anti-*Brucella* antibodies but showed elevated result for false positive and false negative sera as compared to Indirect ELISA using *Brucella* antigen itself, hence proved to be not feasible [79].

Cortina developed enzyme (iELISA) and Lateral Flow Immunoassay (LFIA) using rough Lipopolysaccharide antigens of B. CANIS which was a rapid and easy test that could be used as screening test with high specificity and sensitivity. For both of the developed tests iELISA as well as LFIA, the sensitivity was found to be 98.6%, and the specificity was 99.5% and 100%, respectively. Although now a days Matrix-assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is being performed mostly for identification of bacteria but it is limited to genus level only. But with combination of genotypic characterization, the species level also can be identified for the same. Genetic characterization and performed MALDI-TOF MS to identify *B. canis* in blood culture.

Zoonotic aspect of Canine brucellosis

B. abortus, *B. melitensis* and biovars 1, 3, and 4 of *B. suis* are associated with zoonoses whereas *B. canis* is less regarded with zoonosis because of various reasons. First, cross species transmission has been seen in different species of *Brucella*. Second, the disease in humans is under reported and misdiagnosed due to the nonspecific nature of clinical signs produced and due to inability of the commercially available serological tests to detect rough *B. canis* bacteria. Third, confirmation of the disease is challenging due to intermittent bacteremia observed in the affected patients making diagnosis extremely challenging.

Human infection has a low prevalence and is acquired by direct contact with infected dogs or their blood or reproductive products *viz.* aborted material, seminal fluid, vaginal discharge, urine etc. Among different samples, faeces and vaginal discharge after abortion contain the highest bacterial load. Pregnant women, children, and immunosuppressed patients among general public and Veterinarians, laboratory workers, dog breeders and animal caretakers/kennel workers constitute the high risk group. High burden of canine brucellosis in the stray dog population could lead to spill over in humans in areas where intact, stray dogs are taken into shelters or adopted. Pet owners which adopt an infected dog may also be at high risk of

contracting the diseases as neutered dogs can still shed the bacteria in secretions and urine.

The symptoms in humans are nonspecific flu like and include fever, headache, back pain, chills/night sweats, recurrent/undulant fever, and overall weakness which are easily misdiagnosed. Polyarthritis, meningitis, endocarditis, hepatomegaly, and splenomegaly may be observed in severe cases. Peripheral lymphadenomegaly may also be seen with *B. canis* infection. Treatment in humans consists of prolonged antibiotic use by combining two or more drugs depending on age, pregnancy and immune status of the patient. Maintaining good hygiene standards when handling dogs and its urine, feces, or reproductive products may be practised.

The disease burden can be reduced by preventing unrestricted movement of reproductively intact dogs by continuous testing of breeding animals and their offspring before sale. Sterilization of intact stray animals and euthanasia of infected dogs may also limit the disease spread as well as the level of infection in canine population. The general public must be made aware about the importance of proper diagnosis and methods to limit the further spread of infection in canine and humans by following treatment and control strategies such as sterilization, antimicrobial drug therapy, and repeat testing, or euthanasia. The incidence of canine brucellosis may be reduced by improving diagnostic tests and developing vaccines which would decrease the disease incidence in the canine population and thus ultimately reduce the risk for humans.

CONCLUSION

B. canis infection needs to be considered in dogs and molecular diagnostic technique can be included in the routine work up of dogs with clinical symptoms. As the organism is of zoonotic concern, currently control of canine brucellosis within kennel typically depends on

preventive measures and euthanasia of infected dogs. Unlike dogs, human do respond well to antibiotic therapy and able to clear the bacterium after long-term treatment.

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