UNIVERSITY OF SÃO PAULO, USP

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**Immunoproteomic characterization of *Brucella canis* to identify proteins
candidates as antigens for serodiagnosis**

Pirassununga – SP
2018

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Caracterização imunoproteômica de *Brucella canis* para identificar proteínas candidatas a antígenos para o sorodiagnóstico

Tese apresentada ao Programa de Pós-Graduação em Epidemiologia Experimental Aplicada às Zoonoses da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a obtenção do título de Doutor em Ciências.

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CERTIFICADO

Certificamos que a proposta intitulada "Caracterização imunoproteômica de *Brucella canis* para a identificação de proteínas candidatas a antígenos para sorodiagnóstico", protocolada sob o CEUA nº 3113091015 (ID 001813), sob a responsabilidade de **Lara Borges Keid e equipe; David Attuy Vey da Silva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 17/11/2015.

We certify that the proposal "Immunoproteomic characterization of *Brucella canis* to the identification of proteic antigens to be used in serodiagnosis. ", utilizing 40 Dogs (40 females), protocol number CEUA 3113091015 (ID 001813), under the responsibility of **Lara Borges Keid and team; David Attuy Vey da Silva** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 11/17/2015.

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ATA DE DEFESA

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Ata de defesa pública de Tese do(a) Senhor(a) David Attuy Vey da Silva no Programa: Epidemiologia Experimental Aplicada às Zoonoses, do(a) Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo.

Aos 17 dias do mês de julho de 2018, no(a) Anfiteatro da pós-graduação realizou-se a Defesa da Tese do(a) Senhor(a) David Attuy Vey da Silva, apresentada para a obtenção do título de Doutor intitulada:

"Caracterização imunoproteômica de *Brucella canis* para a identificação de proteínas candidatas a antígenos para sorodiagnóstico"

Após declarada aberta a sessão, o(a) Sr(a) Presidente passa a palavra ao candidato para exposição e a seguir aos examinadores para as devidas arguições que se desenvolvem nos termos regimentais. Em seguida, a Comissão Julgadora proclama o resultado:

Nome dos Participantes da Banca	Função	Sigla da CPG	Resultado
Lara Borges Keid	Presidente	FZEA - USP	<u>aprovado</u>
Valéria Maria Lara Carregaro	Titular	SEMVINCULO(FZEA)	<u>aprovado</u>
Ricardo Luiz Moro de Sousa	Titular	FZEA - USP	<u>APROVADO</u>
Trícia Maria Ferreira de Sousa Oliveira	Titular	FZEA - USP	<u>Aprovada</u>
Angelica Maria Sanchez Sarmiento	Titular	Externo	<u>aprovado</u>

Resultado Final: aprovado

Parecer da Comissão Julgadora *

Angelica Sarmiento participou por videoconferência

Eu, Regina de Cássia Valbom Regina, lavrei a presente ata, que assino juntamente com os(as) Senhores(as). São Paulo, aos 17 dias do mês de julho de 2018.

Valéria Maria Lara Carregaro
Valéria Maria Lara Carregaro

Ricardo Luiz Moro de Sousa
Ricardo Luiz Moro de Sousa

Trícia Maria Ferreira de Sousa Oliveira
Trícia Maria Ferreira de Sousa Oliveira

Pl. Lara Borges Keid
Angelica Maria Sanchez Sarmiento

Lara Borges Keid
Lara Borges Keid
Presidente da Comissão Julgadora

* Obs: Se o candidato for reprovado por algum dos membros, o preenchimento do parecer é obrigatório.

A defesa foi homologada pela Comissão de Pós-Graduação em 20/07/2018 e, portanto, o(a) aluno(a) faz jus ao título de Doutor em Ciências obtido no Programa Epidemiologia Experimental Aplicada às Zoonoses.

Maria Claudia Araripe Sucupira
Presidente da Comissão de Pós-Graduação

Maria Claudia Araripe Sucupira
Presidente da CPG

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"Have I not commanded you? Be strong and courageous. Do not be afraid; do not be discouraged, for the Lord your God will be with you wherever you go."

Joshua 1:9

ABSTRACT

SILVA, D. A. V. da. Immunoproteomic characterization of *Brucella canis* to identify proteins candidates as antigens for serodiagnosis [Caracterização imunoproteômica de *Brucella canis* para identificar proteínas candidatas a antígenos para o sorodiagnóstico]. 2018. 90 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2018.

Canine brucellosis is a zoonotic disease, caused by *Brucella canis*, which is the main cause of abortion and infertility in dogs. This study had the objective to investigate a *B. canis* outbreak in a breeding kennel, to describe a multistep approach to characterize the *B. canis* isolates obtained, and to identify *B. canis* proteins specifically reacting with antibodies from naturally infected dogs. The kennel was located in São Paulo, SP, Brazil. At the time of sampling, in 2014, the kennel comprised 17 adult Pug dogs. Blood samples were used both to isolate the bacteria and to detect *Brucella* spp. DNA by the polymerase chain reaction. Serum samples were used to detect antibodies against *B. canis* using an immunocromatographic test, the rapid slide agglutination test with or without 2-mercaptoethanol and two ELISA kits. The *Brucella* isolates were characterized through the classical bacteriological tests, mass spectrometry and whole genome sequencing. The total protein content of *Brucella* isolates was extracted and separated using one and two-dimension polyacrylamide gel electrophoresis (1D and 2D, respectively), and then tested against sera collected from bacteremic, non-bacteremic and non-*B. canis* infected dogs using western immunoblotting. The reacting protein spots were identified using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). Vaginal discharge, abortion, stillbirth, conception failure and general lymphadenopathy were the clinical signs found in the infected dogs. Gram-negative, coccoid rod-shaped bacteria were isolated from 24 blood samples. Antibodies against *B. canis* were detected in all dogs at least once by the performed serological tests. Mass spectrometry analysis assigned all isolates to the genus *Brucella*. The phenotypic data clearly identified the isolates as *B. canis* with only slight differences in phage typing patterns. The 2D separated protein spots were identified by MALDI-TOF MS as 93 different proteins, out of them, 19 were identified in infected dogs (during the bacteremic and non-bacteremic phases of the infection) and were not identified in non-infected dogs. These proteins have the potential to be used as antigens in serological tests in an attempt to improve the diagnosis of the infection, since a reliable diagnosis is an essential measure for the control and prevention of canine brucellosis. The multistep approach using classical microbiological methods, mass spectrometry and whole

genome sequencing allowed the characterization of the *B. canis* with high discriminatory power, which may be useful for outbreak investigations.

Keywords: *Brucella canis*, dogs, immunoproteomic, serological tests, genotyping.

RESUMO

SILVA, D. A. V. da. Caracterização imunoproteômica de *Brucella canis* para identificar proteínas candidatas a antígenos para o sorodiagnóstico [Immunoproteomic characterization of *Brucella canis* to identify proteins candidates as antigens for serodiagnosis]. 2018. 90 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2018.

A brucelose canina é uma doença zoonótica, causada pela *Brucella canis*, que é uma das principais causas de abortamentos e infertilidade em cães. O objetivo deste estudo foi investigar um surto de *B. canis* em um canil, caracterizar os isolados de *Brucella* obtidos e identificar proteínas de *B. canis* reagentes especificamente a anticorpos de cães naturalmente infectados. O canil é localizado em São Paulo, SP, Brasil e no momento da amostragem, em 2014, o canil era composto por 17 cães adultos da raça Pug. Amostras de sangue foram utilizadas tanto para isolar as bactérias quanto para detectar o DNA de *Brucella* spp. pela reação em cadeia pela polimerase. Amostras de soro foram utilizadas para detectar anticorpos contra *B. canis* nos soros dos cães, utilizando um teste imunocromatográfico, o teste de soroaglutinação rápida em placa com ou sem 2-mercaptoetanol e dois kits de ELISA. Os isolados foram caracterizados pelos métodos bacteriológicos clássicos, espectrometria de massa e pelo sequenciamento do genoma completo. O conteúdo proteico total dos isolados de *B. canis* foi extraído e as proteínas separadas por eletroforese em gel de poliacrilamida de uma e duas dimensões (1D e 2D, respectivamente), sendo então testadas frente aos soros dos cães infectados (com ou sem bacteremia) e não infectados, utilizando *Western Immunoblotting*. Os spots proteicos reagentes foram identificados usando espectrometria de massa por ionização/dessorção a laser assistida por matriz (MALDI-TOF MS). Secreção vaginal, aborto, natimorto, falha na concepção e linfadenopatia foram os sinais clínicos observados nos cães infectados. Cocos bacilos gram-negativos foram isolados em 24 amostras de sangue. Anticorpos contra *B. canis* foram observadas em todos os cães, em pelo menos uma amostragem, pelos testes sorológicos empregados. A MS atribuiu todos os isolados ao gênero *Brucella*. Os dados fenotípicos identificaram claramente *B. canis* com apenas pequenas diferenças nos padrões de lise por fagos. Os spots de proteínas, separados por 2D, foram identificados por MALDI-TOF MS como 93 diferentes proteínas, dentre elas, 19 foram identificadas em cães infectados (com ou sem bacteremia) e não foram identificadas nos cães não infectados. Tais proteínas são candidatas a serem utilizadas como antígenos para o aprimoramento do sorodiagnóstico, uma vez que a existência de um diagnóstico confiável constitui uma medida essencial para o controle e a prevenção da brucelose canina. A abordagem múltipla utilizada, envolvendo métodos

microbiológicos clássicos, espectrometria de massa e sequenciamento completo do genoma bacteriano possibilitou a caracterização dos isolados de *B. canis* com elevado poder discriminatório, o que pode auxiliar em investigações de surtos.

Palavras-chave: *Brucella canis*, cães, imunoproteômica, testes sorológicos, genotipagem.

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1. GENERAL INTRODUCTION

Brucellosis is a widespread zoonotic infectious disease caused by facultative intracellular, small, Gram-negative coccobacillus belonging to *Brucella* genus (CORBEL & BANAI, 2005). The genus is comprised by 12 species, each one with a preferential host that may include domestic animals and wildlife. Canine brucellosis is caused by *Brucella canis* (CARMICHAEL & BRUNER, 1968), which was first isolated by Carmichael in 1966 in a kennel in United States (CARMICHAEL, 1966).

In the meanwhile, the infection has been reported in the Americas (CHIKWETO *et al.*, 2013; KAUFFMAN *et al.*, 2014; KEID *et al.*, 2017), Europe (CORRENTE *et al.*, 2010), Africa (GOUS *et al.*, 2005), Asia (DI *et al.*, 2014) and Oceania (JAMES *et al.*, 2017).

In Brazil, the infection is endemic and local studies showed a variable frequency of occurrence according to the geographic location, the diagnostic tests used, and the canine population examined (MORAES *et al.*, 2002; AZEVEDO *et al.*, 2003; FERREIRA *et al.*, 2007; DOS REIS *et al.*, 2008; MASCOLLI *et al.*, 2016). Brucellosis is particularly important in breeding kennels because it can spread rapidly in confined canine populations leading to high prevalence's and economic losses (KEID *et al.*, 2004; GYURANECZ, M. *et al.*, 2011; KEID *et al.*, 2017). Studies reported prevalence values ranging from 2.7% to 62.6% in commercial kennels in Brazil, evidenced by microbiological culturing (KEID *et al.*, 2004; KEID *et al.*, 2017).

In dogs, brucellosis induces reproductive failures due to infertility, abortion, stillbirth, orchitis, epididymitis and prostatitis (KEID *et al.*, 2004; GYURANECZ, M. *et al.*, 2011; KEID *et al.*, 2017). Furthermore, dogs may also show articular (KEID *et al.*, 2017; MORGAN, 2017) and ocular manifestations (VINAYAK *et al.*, 2004; LEDBETTER *et al.*, 2009; MOSALLANEJAD *et al.*, 2013).

The most common modes of transmission of the infection among dogs are through the oronasal and venereal routes. Dogs can contract the infection when they are exposed to or ingest infected fetal membranes, aborted fetuses and vulvar discharge (CARMICHAEL & JOUBERT, 1988). *B. canis* was also isolated from urine of infected dogs (SERIKAWA *et al.*, 1981) and from kidney (SOUZA *et al.*, 2018); therefore, the transmission among dogs and from dogs to humans via contaminated urine should be highlighted as well.

After the infection, macrophages phagocyte the bacteria, which reach lymph nodes, and a bacteremic period follows lasting several months with bacterial spread to several organs. At

the end of the bacteremic stage, *B. canis* persists indefinitely in lymphoid organs, characterizing the chronic infection (WANKE, 2004; HOLLETT, 2006).

Canine brucellosis is a zoonotic infection, but its incidence in the human population is unknown, because human cases are underreported (LUCERO *et al.*, 2005) due to unspecific clinical signs and symptoms as well as the lack of specific serological tests for the diagnosis of human infections. Most reported cases result from close contact with infected dogs (DENTINGER *et al.*, 2015) or are laboratory-acquired infections (MARZETTI *et al.*, 2013). Although human infections by *B. canis* are usually associated with mild clinical symptoms, occasionally they cause severe courses and focal complications including aneurysms, endocarditis, miscarriage and osteomyelitis (MCKEE & BALLARD, 1999; YING *et al.*, 1999; PIAMPIANO *et al.*, 2000; MARZETTI *et al.*, 2013; DENTINGER *et al.*, 2015).

Most canine infections cannot be diagnosed by physical examination, because a considerable percentage of affected dogs do not present with clinical signs (KHAMESIPOUR *et al.*, 2014; DENTINGER *et al.*, 2015). Therefore, the laboratory diagnosis is essential to the identification of the infected dogs, which is a primary measure to control and prevent infection in the canine population (WANKE, 2004; HOLLETT, 2006).

The serological tests commonly used for canine brucellosis diagnosis include the rapid slide agglutination test (RSAT) (FLORES-CASTRO & CARMICHAEL, 1978; BADA KHSH *et al.*, 1982; CARMICHAEL, 1990), the agar gel immunodiffusion test (AGID) (MYERS *et al.*, 1972) and immunochromatographic tests (ICT) (KIM *et al.*, 2007), showing variable performances regarding sensitivity and specificity for the infection diagnosis.

The performance of serological tests mainly depends on the diagnostic platform as well as the antigenic fraction used. The serological tests for brucellosis diagnosis in dogs use, in the majority, rough lipopolysaccharide (R-LPS) of *B. canis* or *B. ovis* as antigen in various platforms (e.g. agglutination, precipitation, liquid and solid phase immunoassays) (CORBEL, 1997).

False positive results have been reported mainly in the rapid slide agglutination test (RSAT) because R-LPS epitopes of *B. canis* may be shared with other Gram-negative bacteria (GEORGE & CARMICHAEL, 1984; KEID *et al.*, 2009; KEID *et al.*, 2015). Thus, it is necessary to confirm the positive results using tests that are more specific. The use of agglutination test using sera treated with 2-mercaptoethanol (2ME-RSAT) to denature IgM may enhance the specificity, but a significant reduction in the sensitivity was reported (KEID *et al.*, 2009; KEID *et al.*, 2015).

The RSAT, 2ME-RSAT, AGID, and ICT were evaluated for the diagnosis of canine brucellosis showing high values of diagnostic specificity, but 25%, 62.5%, 72.2%, and 10.4% of the dogs with positive blood cultures yielded negative results, respectively (KEID *et al.*, 2015). The sensitivity of these tests was even lower in dogs during the later stages of the infection; when bacteremia was no longer present (ZOHA & CARMICHAEL, 1982a; KEID *et al.*, 2009; WANKE *et al.*, 2012; KEID *et al.*, 2015).

Immunoenzymatic assays (ELISA) were also developed for the diagnosis of canine brucellosis. The sensitivity and specificity of ELISA tests using cell wall antigens as well as antigens based on cytoplasmic protein extracts (BALDI *et al.*, 1997; LUCERO *et al.*, 2002; WANKE *et al.*, 2002; BARROUIN-MELO *et al.*, 2007; ESCOBAR *et al.*, 2010; DE OLIVEIRA *et al.*, 2011), and recombinant proteins (CASSATARO *et al.*, 2002; WANKE *et al.*, 2002; CASSATARO *et al.*, 2004; TSOGTBAATAR *et al.*, 2008) for the diagnosis of *B. canis* infection in dogs were studied. A great variability was observed in the values of sensitivity and specificity obtained, but in general, higher sensitivity values were observed in dogs during the bacteremia stage.

A reliable diagnosis of the infection can be established using direct detection methods in clinical samples, such as microbiological culturing of *B. canis* (that is considered the gold standard method of diagnosis for canine brucellosis) or molecular methods proving the presence of *B. canis* DNA. However, bacterial detection rates depend on the type of biological sample used and the stage of the infection (CARMICHAEL & KENNEY, 1968; KEID *et al.*, 2004; HOLLETT, 2006; NOMURA *et al.*, 2010). Whole blood is the sample of choice for *B. canis* detection through culturing or molecular tests because of the prolonged bacteremia that characterizes the infection (KEID *et al.*, 2007a; KEID *et al.*, 2007b). Direct tests allow the accurate identification of bacteremic dogs, but chronically infected animals are not always detected (FLORES CASTRO & SEGURA, 1976; WANKE, 2004; KEID *et al.*, 2007a; KEID *et al.*, 2007b). Some studies were conducted to evaluate the performance of direct tests for *B. canis* detection in semen and vaginal discharge samples showing that the direct tests yielded higher values of sensitivity in the bacteremic dogs, probably because *Brucella* shedding occurs in higher numbers during this stage (FLORES CASTRO & SEGURA, 1976; WANKE, 2004; KEID *et al.*, 2007a; KEID *et al.*, 2007b). Moreover, direct tests are time consuming and costly especially when a high number of animals must be examined.

There is no vaccine available that may help to prevent infections in dogs. The antimicrobial treatment of canine brucellosis often fails in dogs (FLORES-CASTRO & CARMICHAEL, 1981; HINIĆ *et al.*, 2010; JAMES *et al.*, 2017), and the general

recommendations for outbreak control are usually testing and euthanizing positive animals (HOLLETT, 2006). Nevertheless, some dog owners are reluctant to euthanize their dogs, because of the strong personal relationship to their companion animal (KRUEGER *et al.*, 2014; DENTINGER *et al.*, 2015) and the lack of official regulations regarding the infection control.

The development of a reliable diagnostic method to accurately identify *B. canis* infections is of utmost importance in the context of brucellosis control, especially to overcome one of the main challenges regarding canine brucellosis that is the accurate identification of chronically infected (non-bacteremic) dogs (KEID *et al.*, 2009; WANKE *et al.*, 2012).

The era of post-genomic technologies offers exciting new opportunities to understand the biology of different *Brucella* spp. Omics data can be applied to elucidate metabolic pathways, identify therapeutic targets, virulence factors and to obtain information on immunogenic components that may be useful for serological tests and as potential immunogens (ESCHENBRENNER *et al.*, 2002; WAGNER *et al.*, 2002; AL DAHOUK *et al.*, 2006; CONNOLLY *et al.*, 2006; ESCHENBRENNER *et al.*, 2006; PAJUABA *et al.*, 2010; ZHAO *et al.*, 2011; KIM *et al.*, 2014; LEE *et al.*, 2014; WARETH *et al.*, 2015; WARETH *et al.*, 2016). For these purposes, proteomic analysis based on two-dimensional gel electrophoresis (2D) can be used. This method is based on two electrophoresis techniques, which enhance protein separation leading to a high resolution of thousands of proteins (O'FARRELL, 1975; GORG *et al.*, 2004).

The human-canine bond is becoming stronger day by day; they provide pleasure and relaxation; deep affection and steadfast loyalty; and security and constancy in our changing lives. Moreover, human-animal bonds merit greater attention in mental health research, theory, and practice, particularly in family systems and relational approaches (WALSH, 2009). However, this bond may have some deleterious consequences, especially due to the risk of transmission of zoonosis. Canine brucellosis is considered a neglected zoonosis, which is underreported in part because of the lack of standard protocols for the diagnosis and control of the infection in the canine population, as well as in human beings.

1.1.PRESENTATION OF THE CHAPTERS

The thesis is organized in five chapters, including this general introduction. Chapter 3 presents the investigation of a canine brucellosis outbreak in a Brazilian kennel and the characterization of the *B. canis* strains isolated from the infected dogs. This chapter will be submitted to the journal BMC Veterinary Research and is coauthored by Holger Brendebach,

Katarina Sobotta, Josephine Grützke, Rodrigo Martins Soares, Julia Teresa Ribeiro de Lima, Lara Borges Keid and Sascha Al Dahouk. The chapter 4 comprises the proteomic characterization of *Brucella canis* isolates to identify immunogenic proteins to be used as antigen in serological tests for canine brucellosis diagnosis. This chapter will be submitted to the Journal of Proteomics and is coauthored by Jayaseelan Murugaiyan, Lara Borges Keid, Christoph Weise, Uwe Roesler and Sascha Al Dahouk. The chapter 5 comprises the final considerations including a conclusion of the results of the chapters 3 and 4 and the presentation of relevant points for future studies.

2. OBJECTIVES

2.1. GENERAL OBJECTIVES

The objective of this study was to investigate a *B. canis* outbreak in a Brazilian breeding kennel, as well as to characterize the *B. canis* strains isolated from naturally infected dogs.

2.2. SPECIFIC OBJECTIVES

- Investigate a *B. canis* outbreak in a breeding kennel according to the clinical manifestations and to the results of serological tests and microbiological culturing and PCR of blood to classify the dogs in bacteremic or non-bacteremic.
- Characterize the *Brucella* strains isolated from the outbreak according to the classical phenotypical methods, whole genome sequencing and protein profile.
- Evaluate the protein profile of the *B. canis* isolates and search for immunogenic proteins with potential to be applied as antigen to the detection of antibodies in sera of infected dogs in different stages of the infection.

3. CHARACTERIZATION OF A *Brucella canis* OUTBREAK IN A BREEDING KENNEL

3.1. ABSTRACT

Canine brucellosis is a zoonotic infectious disease, caused by *Brucella canis* and induces reproductive failures in dogs. This study had the objective to investigate a *B. canis* outbreak in a breeding kennel and to describe a multistep approach to characterize *B. canis* isolates. The kennel was located in São Paulo, SP, Brazil. At the time of sampling, in 2014, the kennel comprised 17 adult Beagle dogs, which were sampled in three different dates. Blood samples were used for microbiological culturing and to perform the polymerase chain reaction (PCR) for *Brucella* spp. DNA detection. Sera were used for the serodiagnosis of *B. canis* using an immunocromatographic test, the rapid slide agglutination test with or without 2-mercaptoethanol and two different ELISA kits. The isolates obtained from blood samples were characterized using the classical phenotyping tests, mass spectrometry tests and genotyping was performed using whole genome sequencing. The following clinical signs were observed in the infected dogs: vaginal discharge, abortion, stillbirth, failure to conceive and lymphadenopathy. Gram-negative, coccoid rod-shaped bacteria were isolated from 24 blood samples. All dogs were positive through at least one serological test, in at least one sampling date. Mass spectrometry analysis assigned all isolates to the *Brucella* genus. According to the classical phenotypic tests, all isolates were identified as *B. canis* with only slight differences in phage typing patterns. Only seven single-nucleotide polymorphisms were detected in six isolates according to the whole genome sequencing, suggesting that a single strain was involved in the outbreak. The serological tests used showed variable performances for the detection of *B. canis* antibodies in the dogs, therefore, until a well-established standard test is available, the use of PCR and blood culturing remain essential to confirm the infection. The use of classical microbiological methods, mass spectrometry and genomic sequencing can help to characterize *B. canis* isolates, since it comprised an approach with high discriminatory power to differentiate among isolates, being useful in epidemiological investigation of outbreaks.

Keywords: *Brucella canis*, dogs, next generation sequencing, microbiological culturing, serological tests.

3.2.INTRODUCTION

The *Brucella canis*, a facultative intracellular, small, Gram-negative coccobacillus is the etiological agent of canine brucellosis (CARMICHAEL & BRUNER, 1968) that is associated with reproductive losses in dogs.

Dogs become infected generally by the intercourses or ingestion of abortion materials or reproductive secretions (CARMICHAEL & JOUBERT, 1988). Urine of infected dogs is also a route of excretion and transmission of the organism among dogs and towards humans (SERIKAWA *et al.*, 1981; SOUZA *et al.*, 2018). In view of the high number of asymptomatic dogs, the laboratory diagnosis is essential to identify the infected dogs (KHAMESIPOUR *et al.*, 2014; DENTINGER *et al.*, 2015; KEID *et al.*, 2017). However, the confirmation of the infection in a population may be a challenge because of the failures of the used tests for the diagnosis (KEID *et al.*, 2007a; KEID *et al.*, 2007b; KEID *et al.*, 2015).

Brucellosis has been reported in breeding kennels with high prevalence rates and causing losses because of abortion and infertility episodes and poses risks to public health because of the trade of infected dogs. The control of the infection in such population is difficult because of the close contact among dogs and because management practices that may favor the infection dissemination are commonly used in breeding kennels (KEID *et al.*, 2004; GYURANECZ, Miklos *et al.*, 2011; KEID *et al.*, 2017).

This study had the objective of investigate a *B. canis* outbreak in a Brazilian breeding kennel and describe a multistep approach involving phenotyping and genotyping methods to characterize the *B. canis* isolates.

3.3 MATERIALS AND METHODS

3.3.1. Study kennel and samples

The kennel was located in São Paulo, SP, Brazil. At the time of sampling the kennel comprised a total of 17 adult Pug dogs, two were males and 15 were females. The kennel had no reproductive problems until October 2013, when the first episode of abortion was observed. In the period between October 2013 and February 2014, five out of the 15 females aborted at the later third of gestation. In March 2014, the kennel was visited for the first time by veterinarians. Two more samplings were performed in all dogs of the kennel, in August and in November 2014. All dogs were clinically examined at the time of sample collection to detect

signs of brucellosis: abortion, failure to conceive, vaginal discharge, stillbirth, orchitis, epididymitis and enlargement of lymph nodes. Blood samples were aseptically collected through cephalic or jugular venipuncture using sodium citrate as anticoagulant to be used in the polymerase chain reaction and blood culturing for the direct diagnosis of *B. canis*. Blood collected without the use of anticoagulant was used to obtain serum for serodiagnosis.

3.3.2. Serodiagnosis of *B. canis* infection

Antibodies against rough *Brucella* antigens were detected using immunochromatography (Rapid Canine *Brucella* Ab Test Kit - Bionote, Hwaseong, South Korea); rapid slide agglutination test, with and without 2-mercaptoethanol, 2ME-RSAT and RSAT, respectively (Canine Brucellosis Antibody Test Kit, D-TEC® CB, Kansas City, MO, USA) and two different ELISA kits (Novateinbio kit, Cambridge-MA, USA and Cusabio kit, Wuhan, China). We have performed the tests following the manufactures instructions using sera collected in March, August and November of 2014 from the 17 dogs of the kennel. The 2ME-RSAT was conducted only in serum samples with positive results though RSAT.

3.3.3. Polymerase chain reaction (PCR) to detect *Brucella* spp. DNA in canine blood samples.

The DNA chemical extraction and amplification of whole blood samples for the detection of *Brucella* spp. DNA was performed using the protocol previously described using primers targeting the IS711 sequence of *Brucella* (BATINGA *et al.*, 2017). DNA extraction was based on mechanical and enzymatic pre-lysis of the leukocyte sediment obtained from blood samples, using zirconia/silica beads and lysozyme, respectively, followed by overnight enzymatic lysis using proteinase K and sodium dodecyl sulphate (SDS). The purification of the lysate used phenol/chloroform and alcohol precipitation.

3.3.4. Blood culturing

Blood culturing was performed as previously described (BATINGA *et al.*, 2017), using tryptose broth with 5% of fetal calf serum (FCS) for sample enrichment for 30 days and subculturing in tryptose agar with 5% of FCS each four days. The isolated bacteria were

screened using the PCR targeting the IS711 (BATINGA *et al.*, 2017) and the BCSP31 gene (BAILY *et al.*, 1992) to identify *Brucella* colonies.

3.3.5. Phenotypical characterization of the isolates

All the *Brucella* isolates were characterized using morphological, biochemical and metabolic tests (Gram and Stamp staining, CO₂ requirement, H₂S production, oxidase and catalase activity, urea hydrolysis, agglutination with monospecific sera (anti-A, anti-M, and anti-R), dye sensitivity (basic fuchsin and thionine), bacterial motility (triphenyl tetrazolium chloride solid agar), phage lysis (F1, F25, Tb, BK2, Iz, Wb, R/C) and crystal violet staining in order to identify and sub-differentiate *Brucella* spp. (ALTON *et al.*, 1975).

3.3.6. Matrix-Assisted Laser Desorption Ionization (MALDI) Time of Flight (TOF) Mass Spectrometry (MS) to characterize the *Brucella canis* isolates.

Preparations of bacterial isolates for MALDI-TOF MS were done using ethanol-formic acid extraction according to the manufacturer's instructions with alpha-cyano-4-hydroxycinnamic acid (HCCA) as matrix solution (Bruker Daltonics) and an extended >90 min bacterial inactivation step in ethanol and storage at -20 °C. Mass spectra were acquired using a Bruker Microflex LT MALDI-TOF-MS system (Bruker Daltonics). Spectra were initially analyzed using the Bruker Biotyper 3.1 software with MSP library version MBT 7311 (7,311 entries) and the Security-Relevant (SR) Database (104 entries). Since the commercially available databases contained no entries from *B. canis*, the reference database was supplemented with mass spectra from an in-house collection of *B. canis* and *B. suis* reference strains. For each sample, a set of 24 spectra was measured and a total of 20 high quality spectra were used to calculate a reference spectrum, using the automated function of the Biotyper software. Manufacturer-recommended cutoff scores were used for identification, with scores of >2.300 indicating highly probable species identification, scores between 2,000 and 2,299 indicating secure genus identification and probable species identification, scores between 1,700 and 1,999 indication probable genus identification and scores of <1700 indication no reliable identification .

3.3.7. Next Generation Sequencing (NGS) of *B. canis* isolates

Sample preparation

B. canis was grown on *Brucella* agar and DNA of the bacteria was extracted using the PureLink Genomic DNA extraction kit (Invitrogen) according to the manufacturer's protocol. The libraries for whole-genome sequencing were prepared with a Nextera XT sample preparation kit. An Illumina MiSeq platform with a 2x300 cycle, paired-end configuration was used for whole-genome sequencing.

Bioinformatics analysis

Sequence read quality was analyzed with FastQC v0.11.5 (Babraham Bioinformatics). The sequence reads were assembled with SPAdes v3.10.0 (BayesHammer read error correction) and the options *--careful* (post-processing mismatch corrector with BWA) and *--cov-cutoff auto* (BANKEVICH *et al.*, 2012). Assembly quality was analyzed using Quast v4.5 by comparison to *B. canis* type strain ATCC 23365 (GenBank file GCF_000018525.1, accession no. NC_010103.1 for chromosome 1 and accession no. NC_010104.1 for chromosome 2 with a genome size of 3,312,769 bp in total. Sequence reads were mapped with BowTie2 v2.3.0 with default parameters either against the Brazilian *B. canis* consensus draft genome or *B. canis* strain SVA13 (GenBank file GCF_000691585.1, accession no. NZ_CP007629.1 for chromosome 1 and accession no. NZ_CP007630.1 for chromosome 2, 3,310,315 bp).

Mapping statistics were analyzed with QualiMap v2.2.1 (OKONECHNIKOV *et al.*, 2016). To determine the average nucleotide identity (ANI), Gegenees v2.2.1 (ÅGREN *et al.*, 2012) was applied with a fragment size of 200. Identification of insertion sequences (IS) was performed online by BLASTn analysis against the IS database of the ISfinder website (SIGUIER *et al.*, 2006). Inspection of the De Bruijn assembly graph was done with Bandage v1.0 (WICK *et al.*, 2015).

Genetic relatedness between the Brazilian isolates and all publicly available *B. canis* genomes in the PATRIC (www.patricbrc.org) or NCBI (www.ncbi.nlm.nih.gov/genome/microbes/) databases was established with ParSNP v1.0 (TREANGEN *et al.*, 2014) and Maximum-Likelihood trees calculated by FastTree2 (PRICE *et al.*, 2010). The origin of strains was extracted from genome metadata and integrated into the phylogeny with the EMBL interactive tree of life, iTOL v4 (LETUNIC & BORK, 2016).

Single Nucleotide Polymorphism (SNP) Analysis

Whole genome single nucleotide polymorphism (wgSNP) analysis was performed using BioNumerics v7.6 (Applied Maths, Sint-Martens-Latem, Belgium). Reads were trimmed in a consecutive approach, first with the configuration: structural trimming (remove stretches: homopolymer ≤ 20 ; polyA $> 90\%$; polyGC $< 10\%$ & $> 90\%$), tail quality trimming (tail $q \geq 18$; windowed average $q \geq 15$; rolling average $q \geq 15$), length trimming (exclude < 100 bp & > 250 bp), and second with the configuration: overall quality trimming (exclude minimum q window = 5; average $q < 20$; replace with N for $q < 15$), and mapped to either the consensus draft genome of high quality samples (SPAdes assembly of concatenated read files of the isolates I15, I20, I22 and I23) or the complete genome of *Brucella canis* type strain ATCC 23365 as reference. The resulting SNP matrix was filtered for positions with a minimum coverage of five reads in total, with at least one on the forward/ leading and reverse/ lagging strand. Due to the limited filter possibilities in BioNumerics, SNP positions were extracted and manually curated in Microsoft Excel 2013 to differentiate between false base calls in the *de novo* assembly generation and SNPs at positions available in all 24 sequences or at positions with ambiguous base calls or insufficient coverage (N). Based on the final SNP matrix the pairwise distance between the isolates was calculated and clustering of isolates was performed using the Neighbor Joining algorithm in BioNumerics. A comparison of the assembled draft genome against reference sequences including variant calling was performed in Mauve v2015-02-13 (DARLING *et al.*, 2010).

3.4. RESULTS

3.4.1. Clinical manifestations

Out of 17 dogs, three showed no clinical manifestations whereas 14 suffered from at least one canine brucellosis-like symptom. No clinical manifestations were observed in the two male dogs. Vaginal discharge and abortion/stillbirth were the most frequent symptoms, followed by general lymphadenopathy and failure of conception (Table 1).

Table 1. Clinical manifestations in 17 dogs of a kennel in São Paulo-SP, Brazil.

Dog	Gender	Clinical Manifestations	Vaginal Discharge	Abortion/ Stillbirth	Infertility	Enlarged Lymph nodes	Orchitis/ Epididymitis
D08	M	0	-	-	0	0	0
D19	M	0	-	-	0	0	0
D22	F	0	0	0	0	0	-
D17	F	1	0	1	0	0	-
D09	F	1	1	1	0	1	-
D15	F	1	1	0	1	0	-
D18	F	1	0	1	0	0	-
D16	F	1	1	1	0	1	-
D21	F	1	0	1	0	0	-
D07	F	1	1	0	0	1	-
D11	F	1	0	0	0	1	-
D06	F	1	na	na	1	1	-
D13	F	1	1	0	0	0	-
D10	F	1	1	0	0	0	-
D20	F	1	0	1	0	1	-
D12	F	1	1	0	0	1	-
Total	-	14	07	06	03	07	-

0 - absence of clinical manifestations; 1 - presence of clinical manifestation; na - not available

3.4.2. Serological tests

One male dog was seronegative through the immunochromatographic test during the completely sampling period. However, the same dog revealed antibody titers towards rough *Brucella* through the RSAT in the first sampling time and ELISA (Novateinbio) kit in the first and second sampling times. The other male dog and the 15 bitches revealed antibody titers towards rough *Brucella* at least once during the sampling period when immunochromatographic test was applied (Table 2). All the dogs had antibody titers towards rough *Brucella* at least once during the sampling period in at least one of the performed serological test, such as RSAT, 2ME-RSAT and the two ELISA kits (Table 2).

3.4.3. Classical microbiology

Gram-negative, coccoid rod-shaped bacteria were isolated from 15 out of the 17 dogs of the kennel by blood culture in at least one sampling date (Table 2). A total of 24 isolates were obtained. Classical microbiological tests used to identify members of the genus *Brucella* and subtype species revealed growth both under CO₂ and O₂ atmosphere; no H₂S production;

oxidase, catalase and urease were positive; there was no agglutination with any monospecific sera; all isolates grew on thionine at all dilutions and on fuchsin at 1:100,000, but did not at 1:50,000; isolates were not motile; there were slight differences in phage typing profiles (Table 3), however, they were not essential to classify the isolates in another species of *Brucella*; and crystal violet staining identified all isolates as rough strains. In summary, despite of some different phage lysis patterns and no agglutination with monospecific sera, the phenotyping approach clearly identified all the 24 isolates as *B. canis* when compared with the reference strain of the species RM 6/66.

In one male dog (D19) the result of the blood culturing conducted on November 2014 was considered inconclusive because of the secondary contamination of the sample.

3.4.4. DNA amplification by Polymerase Chain Reaction (PCR)

All the 24 *Brucella* strains isolated from canine blood were positive in the genus specific PCR. The canine blood samples with negative results through culturing were submitted to PCR (Table 2). In three (D6 and D17, both in the third sampling and D7 in the second sampling) out of 17 dogs, no bacteria could be isolated; however, *Brucella* DNA was detected in their whole blood samples.

Table 2. Blood culturing, serological tests and *B. canis* DNA detection of 17 dogs from one kennel in São Paulo-SP, Brazil.

ID	G	March							August							November						
		BC	PCR	IC	RSAT	2ME-RSAT	ELISA Kit 1	ELISA Kit 2	BC	PCR	IC	RSAT	2ME-RSAT	ELISA Kit 1	ELISA Kit 2	BC	PCR	IC	RSAT	2ME-RSAT	ELISA Kit 1	ELISA Kit 2
D08	M	-	-	-	+	-	+	i	-	-	-	-	nc	+	i	-	-	-	nc	nc	nc	-
D19	M	+	nc	+	+	-	+	i	+	nc	+	-	nc	+	-	i	-	-	-	nc	+	-
D22	F	+	nc	+	+	-	+	+	+	nc	+	+	-	+	+	-	-	+	+	-	+	+
D17	F	+	nc	+	+	-	+	+	+	nc	+	+	-	+	+	-	+	+	+	-	+	-
D09	F	+	nc	+	+	-	+	i	+	nc	+	+	-	+	i	+	nc	+	+	-	+	i
D15	F	-	-	-	-	Nc	i	-	+	nc	+	+	+	+	i	+	nc	+	+	-	+	+
D18	F	+	nc	+	+	+	+	-	+	nc	+	+	-	+	i	+	nc	+	+	-	+	i
D16	F	+	nc	+	+	+	+	i	+	nc	+	+	-	+	+	+	nc	+	+	-	+	+
D21	F	+	nc	+	+	+	+	-	-	-	+	+	-	+	i	-	-	-	+	-	+	-
D07	F	+	nc	+	+	-	+	-	-	+	+	-	nc	+	-	-	-	+	-	nc	+	i
D11	F	-	-	+	+	-	+	i	-	-	-	-	nc	+	-	-	-	-	-	nc	+	-
D06	F	+	nc	+	+	-	+	+	+	nc	+	+	-	+	+	-	+	+	-	nc	+	-
D13	F	+	nc	+	+	-	+	-	-	-	+	-	nc	+	-	-	-	+	+	-	+	-
D10	F	+	nc	+	+	+	+	-	+	nc	+	+	-	+	i	+	nc	+	+	-	+	i
D20	F	+	nc	+	+	-	+	i	-	-	+	+	-	+	i	-	-	+	-	nc	+	-
D12	F	+	nc	+	+	-	+	-	-	-	+	+	-	+	i	-	-	+	+	-	+	i
D14	F	+	nc	+	+	-	+	-	+	nc	+	+	-	+	i	-	-	+	+	-	+	i

ID - Identification of the dogs; G - Gender; BC - Blood culture; PCR - *Brucella* spp. DNA detection by Polymerase Chain Reaction; IC - Immunocromatography; RSAT - Rapid Slide Agglutination Test; 2ME-RSAT - Rapid Slide Agglutination Test with 2-Mercapthoetanol; ELISA (Kit 1 - Novateinbio and Kit 2 - Cusabio) - Enzyme Linked Immunosorbent Assay; i – Inconclusive result (contamination); nc – Not conducted; + = Positive; - = Negative.

Table 3. Representative phage lysis patterns of the *Brucella canis* isolates.

Dog	Isolate	Phage							
		F1	F25	Wb	Fi	BK2	Iz	R/C	Tb
-	RM	NL	NL	NL	GI	GI	GI	GI	NL
	6/66								
D06	I1	NL	NL	NL	GI	NL	GI	GI	NL
D20	I12	NL	NL	GI	GI	NL	GI	GI	GI
D09	I18	NL	NL	NL	GI	NL	GI	GI	GI

NL – no lysis; GI – growth inhibition; Differences among phages lysis patterns were bolded.

3.4.5. MALDI-TOF-MS

Only genus-level identification was obtained using Bruker Biotyper 3.1 software using the available MSP and SR libraries, since only very few *Brucella* entries were present in these databases. Consequently, the dog isolates were initially misidentified as the related species *B. melitensis* with scores between 2.000 and 2.300 in most cases. By expanding the commercial database with in-house-generated reference spectra, the identification could significantly be improved with score values for the best matches of > 2.300 indicating highly probable species identification. However, *B. canis* and *B. suis* bv. 4 strains displayed highly similar spectra leading to small distances of score values so that unambiguous discrimination was sometimes difficult. Visual analysis of the spectra revealed a pair of discriminative biomarker signals that was exclusively present in *B. canis* strains and *B. suis* biovar 4, but absent from *B. suis* biovars 1, 2 and 3 (m/z 7,071 and its doubly charged ion at m/z 3,535). Based upon correspondence between the experimentally measured value and the predicted molecular weight based upon protein sequence data this signal could be assigned to ribosomal protein L35 with an average mass of 70 Da and loss of N-terminal methionine. Using this approach, the dog isolates could be successfully classified to the *B. canis*/ *B. suis* bv. 4 clade.

3.4.6. Next Generation Sequencing (NGS) Analysis

The genome of the Brazilian *B. canis* strain had an average nucleotide identity of 99.95% to the type strain *B. canis* ATCC 23365, and the GC content was 57.26%. The mean coverage of sequence reads varied between 28x (I4) and 182x (I22) and was in average 103x

over all 24 isolates. The predicted genome size was between 3,293,523 bp and 3,297,776 bp and thus 15 to 17 kbp shorter than the reference sequences ATCC 23365 and SVA13, respectively.

Mapping reads against the Brazilian consensus sequence reduced the number of unmapped reads from 3.07% to 2.27% in comparison to the complete genome of SVA13. Excluding the lower quality sequences (I3, I4 and I11), *de novo* assembly resulted in 24 to 31 contigs, while 7-9 contigs were sufficient to span 75% of the whole genome. Due to the working principles of the SPAdes algorithm, contig borders may be created at repetitive regions, e.g. insertion sequences or rRNA genes, represented by multi-edged nodes in a De Bruijn graph. Conversely, such sequences were found in small contigs below 10 kbp and the manifold coverage above average reveals the line of action of SPAdes where duplicate, repetitive sequences may be collapsed into a single one, creating assembly errors. With the applied short read sequencing technique, a completion of a contiguous, circular chromosome sequence is hampered by repetitive elements known from *Brucella* spp., for example, the insertion sequences IS711, ISBm2 and ISBm3, IS1953 and IS2020 or the rRNA operon. Sequencing techniques that over span repetitive regions are necessary for gap closing or *in silico* confirmation of repeat-based typing strategies like multilocus VNTR analysis.

To determine sequence variants between our Brazilian isolates and the *B. canis* type strain ATCC 23365, we compared two SNP analysis strategies based on either sequence read mapping (BioNumerics) or variant calling from the assembled draft genome (Mauve). First, BioNumerics' read mapping and variant calling was utilized to extract a list of SNPs that pass a coverage threshold (Table 4).

Table 4. Statistics of the BioNumerics SNP analysis. Filter A: 'Absolute Coverage' (total ≥ 5 reads, forward ≥ 1 read & reverse ≥ 1 read) and 'Remove non-informative SNP positions'. Note: all ambiguous base calls (N or non-ACTG) in any of the 24 isolates are still included.

Reference Genome	ATCC 23365	Brazilian Consensus Draft
All SNPs	206180	168610
Filter A	4127	4008

Second, after manually filtering ambiguous and low confidence SNPs, this strategy revealed 179 positions with a high percentage of presence (POP), i.e. a base call at the same position in each isolate, and 5 low POP SNPs. In the other approach, the algorithm progressive Mauve aligned the assembled contigs of the consensus draft genome against the reference sequence and yielded 183 SNPs of which 98% or 180 positions were shared with the

BioNumerics analysis. Four of the five low POP SNPs were excluded from the consensus draft assembly due to their low coverage even in the high-quality samples. Read mapping against the consensus draft genome showed two instances where the post-processing mismatch corrector of SPAdes failed to correct an assembly error (position 56.042 and 2.644.616 in the ATCC 23365 alignment). The nearest relative in the NCBI whole genome database, *B. canis* SVA13, showed a genetic distance of 177 SNPs (Mauve). The analysis of sequence variations by read mapping among the 24 isolates revealed seven SNPs in six strains (Table 5).

Table 5. SNP analysis among 24 *Brucella canis* isolates

Dog	Isolate	Position in ATCC 23365 genome						
		642523	997022	1104956	1402095	1463341	2196420	2198519
D06	I1	C	N	G	C	G	C	A
D07	I2	C	G	G	C	A	T	A
D09	I3	C	G	G	C	G	C	A
D10	I4	C	G	G	C	G	C	A
D12	I5	C	G	G	C	G	C	A
D13	I6	C	G	G	C	G	C	A
D14	I7	C	G	G	C	G	C	N
D16	I8	C	G	G	T	G	C	A
D17	I9	C	G	G	C	G	C	A
D18	I10	C	G	G	C	G	C	A
D19	I11	C	G	G	C	G	C	N
D20	I12	C	A	G	C	G	N	A
D21	I13	C	G	G	C	G	C	T
D22	I14	C	G	A	C	G	C	A
D19	I15	C	G	G	C	G	C	A
D06	I16	C	G	G	C	G	C	A
D10	I17	C	G	G	C	G	C	A
D09	I18	T	G	G	C	G	C	A
D15	I19	C	G	G	C	G	C	A
D22	I20	C	G	G	C	G	C	A
D14	I21	C	G	G	C	G	C	A
D16	I22	C	G	G	C	G	C	A
D18	I23	C	G	G	C	G	C	A
D17	I24	C	G	G	C	G	C	A
RC		05	13	32	20	80	52	05

C – Cytosin, T – Thymine, G- Guanine, A – Adenosine, RC - Read coverage in the divergent isolate.

Apart from isolate I2, which harbors two different mutations at position 1.463.341 and 2.196.420 in the ATCC 23365 alignment, five strains each differ by only one nucleotide within their observable genome sequence. Six out of seven SNPs are located in coding sequences and are nonsynonymous substitution that alter the amino acid sequence of the respective protein,

namely: GTP pyrophosphokinase (I18, SNP coverage 5x), Pyruvate dehydrogenase E1 component subunit beta (I14, 32x), N-acetylmuramyl-L-alanine amidase family protein (I8, 20x), molybdate ABC transporter modC (I2, 52x), a SH3 type 3 domain protein (I2, 80x) and one hypothetical membrane protein (I12, 13x).

Apart from SNPs found in isolates I8 and I13, all mutations were detected in later samples, which were isolated in August 2014. Interestingly, the intergenic low coverage SNP in isolate I13 (5x) was not detected in the later isolate I14 from the same dog (D16) and may showcase a post-isolation, lab-acquired mutation. The profile of the analyzed strains hint to a single outbreak.

The genetic relatedness of our Brazilian consensus draft genome was determined by a core genome multi-alignment with ParSNP, a tool from the Harvest analysis suite. We included all publicly available *B. canis* genomes with draft or complete status and extracted the geographic source of isolation from the available metadata to calculate an annotated phylogenetic dendrogram (Figure 01).

Figure 01. Phylogenetic analysis of all published *B. canis* complete and draft genomes (source: PATRIC, NCBI). Sequences were analyzed with ParSNP, FastTree2 and iTol. The branch length displays the relative genetic distance. All bootstrap support values were either below 0.5 or above 0.8.

The genetic distance between the Brazilian isolates and their nearest relative, an isolate from Argentina, is 83 SNPs (Mauve). Clearly, within the available samples, the *B. canis* subclades are geographically homogenous for the regions South America and Asia. The closest

relative in the same genus, *Brucella suis* biovar 4 strain 40, is distinctly marked-off and defines the outgroup. For better visualization of branch lengths in the phylogram, other *Brucella* species have been omitted.

3.5. DISCUSSION

Since canine brucellosis may be associated with non-pathognomonic signals and a proportion of the infected dogs may be asymptomatic (KANG *et al.*, 2009; DENTINGER *et al.*, 2015; PURVIS *et al.*, 2017), the diagnosis of the infection by the clinical manifestations may be difficult. The same clinical signs observed in this study, have been already reported (KEID *et al.*, 2015; KEID *et al.*, 2017) when describing the infection in other Brazilian breeding kennels.

Two males and one bitch, out of our 17 dogs, were asymptomatic. However, *B. canis* was isolated from the blood of one male and one bitch, and antibodies against rough *Brucella* were detected in their sera. Therefore, the diagnosis of the infection only by the clinical manifestations is not reliable; hence, the laboratory tests are required in order to confirm the infection.

The detection of antibodies against rough *Brucella* has been currently used both as screening and as confirmatory serological diagnosis of canine brucellosis (DENTINGER *et al.*, 2015; KEID *et al.*, 2015; AYOOLA *et al.*, 2016; DE OLIVEIRA *et al.*, 2016; MARÍA & SIEVER, 2016). However, misdiagnosis due to sensitivity and specificity issues may occur when serological tests are used (FLORES-CASTRO & CARMICHAEL, 1978; KEID *et al.*, 2007a; KEID *et al.*, 2009; WANKE *et al.*, 2012; KEID *et al.*, 2015). In connection with the available serological tests, the immunochromatographic assay seemed to have a better performance when compared to agglutination and precipitation tests. In this study, all the dogs with positive results in blood culturing and blood PCR were also positive though the immunochromatographic assay. Despite showing higher values of diagnostic sensitivity, a percentage of 11% and 10%, respectively, of the infected and bacteremic dogs were not detected by immunochromatographic assays in previous studies (WANKE *et al.*, 2012).

Regarding RSAT, a high sensitivity was observed when used in dogs from the kennel; however, false positive results were reported in previous studies (GEORGE & CARMICHAEL, 1974; KEID *et al.*, 2009; KEID *et al.*, 2015), therefore, the confirmation of positive results using 2ME-RSAT is recommended. However, when using the 2ME-RSAT, a low sensitivity was observed in blood culturing positive dogs, as previously reported (KEID *et*

al., 2015). The low sensitivity observed in 2ME-RSAT was similar to that observed when using the ELISA (Cusabio) for the infection diagnosis in the kennel. The ELISA (Novateinbio) test used presented a higher sensitivity compared to the remaining tests; however, it is important to consider that the ELISA tests used were not properly evaluated to determine its specificity for canine brucellosis diagnosis.

The blood culturing remains as the most reliable way to confirm the infection in a confined population because a significant proportion of the dogs may be bacteremic. However, as the infection progresses and bacteremia ceases or become intermittent, a multi-step approach may be required, comprising the clinical examinations, serological tests, blood culturing and molecular assays in routine investigations.

The high prevalence of *B. canis* infection evidenced through blood culturing in the kennel is concordant with the values reported in previous studies also conducted in commercial kennels in Brazil (KEID *et al.*, 2004; KEID *et al.*, 2017). This may reflect the use of deficient management practices that favor the dissemination of *B. canis* among dogs and the lack of official regulations to control the infection.

The isolation of the bacteria also allowed the characterization of the *Brucella* strains involved in outbreaks, which may be important for epidemiological purposes. The phenotypical characterization is the classical method to differentiate the bacteria at the species level and is an important way to identify the species of *Brucella* involved in an outbreak, since dogs can be infected by bacteria from *Brucella* genus, other than *B. canis* (KEID *et al.*, 2015; MOR *et al.*, 2016; JAMES *et al.*, 2017; WARETH *et al.*, 2017).

Despite of slight differences in the phage typing, the phenotypical profile of the isolates was consistent with the features of the reference strain *B. canis* RM6/66, as well as the recent published *B. canis* phenotypical profile performed in outbreaks involving dogs (JAMES *et al.*, 2017; KEID *et al.*, 2017; MORGAN, 2017).

The characterization of the *B. canis* isolates using mass spectrometry (MS) and the sequencing of its DNA are both more reliable, faster and less dangerous alternative to the characterization of *Brucella* isolates than classical phenotypical tests performed to differentiate the *Brucella* species. *Brucella* spp. has been identified and differentiated using MALDI-TOF-MS (AL DAHOUK *et al.*, 2006; FERREIRA *et al.*, 2010a; LASCH *et al.*, 2015; WARETH *et al.*, 2016; PURVIS *et al.*, 2017). The application of MALDI-TOF-MS to identify and differentiate *Brucella* species is a highly sensitive and specific technique (KARGER *et al.*, 2013) and allows us to speed up the specific diagnosis (PATEL, 2015), due to the possibility to use a positive blood culture when the first colonies grow (FRENCH *et al.*, 2016). MALDI-

TOF-MS was previously used for presumptive identification of *B. canis* in dogs (PURVIS *et al.*, 2017). Due to its rapidity and simplicity, MALDI-TOF-MS presents an indispensable tool for fast presumptive *Brucella* identification, especially in outbreak situations. However, due to the close genetic relationship between certain *Brucella* species and biovars, the MALDI-TOF-MS workflow does not always allow for robust and unambiguous species identification as shown for the case of the *B. canis*/*B. suis* bv. 4 clade. The rapid identification of the pathogen involved in a kennel outbreak is essential to trace out strategies for treatment and to prevent the spreading of the infection.

Since canine brucellosis is a zoonotic disease (CASTRILLÓN-SALAZAR *et al.*, 2013; MARZETTI *et al.*, 2013; JAVERI *et al.*, 2014; KRUEGER *et al.*, 2014; DENTINGER *et al.*, 2015), the detection of the bacteria, followed by the epidemiological investigation in a kennel outbreak is essential to prevent the spreading of the infection among dogs and its transmission to humans. On this purpose, the isolation of *B. canis* and its sequencing may help to identify specific genetic features of the outbreak-involved bacterial strain, its genetic proximity with strains from other outbreaks and the probable sources of infection.

The applied next generation sequencing clearly identified a single *B. canis* strain involved in the outbreak, according to the performed analyses. We also could observe a clear run-to-run quality difference; the better the coverage, the less SNPs were found. Hence, both BioNumerics and Mauve strategies have provided sufficient estimation of sequence variation when provided with high coverage sequence data and even SNP effects when compared to annotated reference sequences.

3.6. CONCLUSION

The serological tests used for *B. canis* infection diagnosis showed variable performances, thus reinforcing that the direct detection of *B. canis* in canine samples using PCR and blood culturing are essential for the confirmation of the infection.

High prevalence values of *B. canis* infection have been commonly reported in reproductive kennels in Brazil highlighting for the need of control measures to protect animal and public health. Once the infection is confirmed in a population, the characterization of the isolated strains is useful to trace back the infection. The members of *Brucella* genus have high DNA identity and clonal evolution. Therefore, high-resolution methods of typing are necessary to the proper characterization and identification of slightly differences among *Brucella* strains with epidemiological purposes such as the identification of the source of an infection. The

multistep approach used, involving classical microbiological methods, mass spectrometry and genomic sequencing allowed the characterization of the *B. canis* isolates for outbreak investigations.

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4. IDENTIFICATION OF IMMUNO-DOMINANT PROTEINS OF *Brucella canis* USING SERA COLLECTED FROM NATURALLY INFECTED DOGS

4.1.ABSTRACT

Canine brucellosis, caused by *Brucella canis*, is a zoonotic infection that has been commonly associated with abortion and other reproductive failures in dogs. Canine brucellosis is considered a neglected infection in dogs, with difficulties in the diagnosis, treatment and control. This study had the objective to characterize the protein fraction of *B. canis* isolates and to identify *B. canis* proteins reacting specifically with antibodies from naturally infected dogs. The infected dogs used came from a kennel located in São Paulo, SP, Brazil comprising 17 adult Pug dogs. The 17 dogs were sampled in three different dates when blood samples were collected and used for blood culturing and to obtain serum to be used in immunoblotting. Blood samples were also collected from five *Brucella*-free Beagle dogs to be used as negative control. The diagnosis of brucellosis was conducted using blood culturing, and according to the results, the dogs were classified as infected and bacteremic, infected and non-bacteremic or non-infected. The protein content of the *B. canis* isolates obtained was extracted and separated using one and two-dimension polyacrylamide gel electrophoresis (1D and 2D, respectively), then they were tested against the sera of dogs infected and bacteremic, infected and non-bacteremic, and non-infected by *B. canis* using western immunoblotting. The reacting protein spots were identified using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and their functions were evaluated. A total of 24 Gram-negative, coccoid rod-shaped bacteria were isolated from the dogs. The 2D separated protein spots were identified by MALDI-TOF MS as 93 different proteins, out of them, 19 were identified in infected dogs (bacteremic and non-bacteremic stage of the infection) and were not identified in non-*B. canis* infected dogs. Their functions were mainly related to the ion transport and metabolism. Infected and non-infected dogs could be differentiated by their protein patterns, which were observed through 2D followed by immunoblotting. Therefore, the immunogenic proteins that reacted with the sera from infected dogs (bacteremic and non-bacteremic) and did not reacted with the sera from non-*B. canis* infected dogs should be evaluated as antigen in serological tests in an attempt to improve the canine brucellosis diagnosis, especially regarding the identification of chronically infected and non-bacteremic dogs..

Keywords: *Brucella canis*, dogs, immunoproteomic, diagnosis.

4.2.INTRODUCTION

Canine brucellosis is a zoonotic infection, caused by *Brucella canis*, (CARMICHAEL & KENNEY, 1968) that affect dogs causing reproductive failures (KEID *et al.*, 2004; GYURANECZ, M. *et al.*, 2011; KEID *et al.*, 2017). The infection is difficult to diagnose because of the absence of pathognomonic clinical manifestations and the occurrence of asymptomatic infections (KHAMESIPOUR *et al.*, 2014; DENTINGER *et al.*, 2015). Therefore, the laboratory diagnosis is essential to identify infected dogs in a population and to establish prophylactic measures (WANKE, 2004; HOLLETT, 2006).

A variety of serological tests has been developed for the diagnosis of canine brucellosis, varying greatly in their complexity, cost, sensitivity and specificity. Thus, the results obtained are not always concordant and therefore difficult to interpret and leading to misleading results (KEID *et al.*, 2009; WANKE *et al.*, 2012; KEID *et al.*, 2015). Most serological tests rely on the use of cell wall antigens that elicit higher antibody titers during the bacteremic stage of the infection (ZOHA & CARMICHAEL, 1982a; KEID *et al.*, 2009; KEID *et al.*, 2015), thus imposing difficulties to detect the chronically infected dogs.

Therefore, direct detection methods such as microbiological culturing or detection of *B. canis* DNA are usually necessary to confirm the infection in a population. However, bacterial detection rates are largely influenced by the nature of the biological samples used and the stage of the infection (CARMICHAEL & KENNEY, 1968; KEID *et al.*, 2004; HOLLETT, 2006; NOMURA *et al.*, 2010). Direct tests allow accurate identification of bacteremic dogs, but chronically infected animals are not easily detected (FLORES CASTRO & SEGURA, 1976; WANKE, 2004; KEID *et al.*, 2007a; KEID *et al.*, 2007b). Moreover, these tests are time-consuming and pose risk because of the laboratory manipulation of *B. canis* isolates.

The post-genome approach of proteomics using two-dimensional gel electrophoresis (2D) can be used to evaluate the protein pattern of *Brucella* isolates to discover, isolate, and identify new proteins with several purposes, including the search for immunogenic proteins that can be used as antigens for serological tests (WELDINGH *et al.*, 1998; LAMONTAGNE *et al.*, 2010; POMASTOWSKI & BUSZEWSKI, 2014); (ESCHENBRENNER *et al.*, 2002; WAGNER *et al.*, 2002; AL DAHOUK *et al.*, 2006; CONNOLLY *et al.*, 2006; ESCHENBRENNER *et al.*, 2006; FERREIRA *et al.*, 2010b; ZHAO *et al.*, 2011; KIM *et al.*, 2014; LAUER *et al.*, 2014; LEE *et al.*, 2014).

A comprehensive analysis of *B. canis* may help to identify immunogenic proteins, which can be used as antigens in diagnostic tests for canine brucellosis. Therefore, the aim of the

present study was to identify *B. canis* proteins specifically reacting with antibodies of naturally infected dogs during several stages of the infection.

4.3.MATERIAL AND METHODS

4.3.1. Bacterial Strains and Sera

Blood and serum samples were collected from dogs of a brucellosis outbreak in a kennel located in São Paulo, SP, Brazil comprising 17 Pug dogs (15 females and two males). The animals were sampled three times during the year of 2014; in March, August and November. Twenty-four *B. canis* strains were isolated by hemoculture and characterized in a previous study indicating that a single strain was associated with the outbreak.

The 17 dogs were classified into four groups according to the presence of bacteremia in each sampling time. The fifth group consisted of five non-*B. canis* infected Beagle dogs, which had no canine brucellosis-like clinical signs, no *B. canis* strains were cultured, DNA of *B. canis* was not detected in their blood and antibodies against *Brucella* were not detected in their sera using an immunocromatographic test (Figure 1). The samples identification is show in the Table 1 (Supplementary material).

Figure 1. Classification of the 17 dogs from the infected kennel according to the results of blood culturing for *Brucella canis* isolation, conducted in three different occasions (March, August, and November, 2014). The Group I comprised five dogs with positive hemoculture only in the first sampling; Group II comprised five dogs with positive hemoculture in the first and the second sampling time; group III comprised five dogs with positive results in the three sampling times; and the Group IV is consisted of two dogs with negative results through blood culturing in the samplings, however with close contact with positive dogs. The five non-infected dogs from the kennel considered free of brucellosis were included in group V (Control Group).

4.3.2. Bacterial Culture and Protein Extraction

The cell culture and whole cell protein extraction of the 24 *B. canis* isolates was performed as described previously (MURUGAIYAN *et al.*, 2013a). In brief, the bacterial strains were cultured in Tryptic Soy media for 48h at 37° C; the cells were harvested by centrifugation, washed twice with phosphate buffered saline (PBS pH 7,2), spun down, and the resulting pellets were resuspended in 80% ethanol (v/v). Following centrifugation, the ethanol was discarded and the cell pellet air dried to ensure the removal of traces of ethanol. The cell pellets were then resuspended in 250 µL of HEPES buffer (20 mM, pH 7.4) and sonicated (duty cycle: 1.0, amplitude: 100%, UP100H; Hielscher Ultrasound Technology, Teltow, Germany) for 45 seconds on ice. After centrifugation at 11,290×g for 10 minutes at 4°C, the clear supernatant was collected and stored in aliquots at -18°C until further use. The protein content was determined using the modified Bradford method (BRADFORD, 1976).

4.3.3. One and Two-Dimensional Electrophoresis (1D and 2D, respectively)

For 1D electrophoresis, we used 5 µg of the protein extract for each sample, acetone precipitated, plus 5 µl of sodium dodecyl sulfate (SDS) and loaded in 12 % polyacrylamide gel for electrophoresis (SDS-PAGE). For staining, we used the Blue Silver staining (modified colloidal Coomassie staining) overnight while shaking (CANDIANO *et al.*, 2004). For destaining, we washed the gels multiple times in distilled water.

The 2D electrophoresis was conducted as previously described (MURUGAIYAN *et al.*, 2013b). Briefly, each protein extract containing 100 µg of proteins were acetone-precipitated, resuspended with 135 µL of DeStreak Rehydration solution and added for overnight rehydration of IPG dried strips (Immobiline™ Dry Strip, pH 3 to 10 NL, 7 cm, GE Healthcare Bio-sciences AB; Uppsala, Sweden). The isoelectric focusing (IEF) was performed by using the Ettan™ IPGphor3™ Unit (GE Healthcare Europe; Freiburg, Germany) and carried out at 20°C for 6.5 h; at 300 V for 2 h, 1000 V for 1 h and 5000 V for 3.5 h and 50 µA/strip. Then, the strips were reduced for 20 min in 2 mL equilibration buffer 1 (0.05 M trichloroethylene HCl pH 8.8, 6 M urea, 30 % glycerol, 4 % SDS, 2 % dithioerythritol, 0.002 % bromophenol blue), washed with water and alkylated with equilibration buffer 2 (0.05 M trichloroethylene HCl pH 8.8, 6 M urea, 30 % glycerol, 4 % SDS, 2.5 % iodoacetamid, 0.002 % bromophenol blue).

Standard molecular weight prestained protein ladder marker (10-250 kDa; Page Ruler™ Plus, Thermo Scientific; Germany) and IPG strips were loaded onto the top of a homogeneous 12 % polyacrylamide gels and sealed with 1 % agarose solution. Electrophoresis was carried out at room temperature and 10 mA/gel for first 15 minutes and thereafter, 15 mA/gel until the tracking dye reached the bottom of the gels (~1.5 h). 2D gels were subjected to Western blotting using dogs' sera for identification of immunoreactive proteins and another set of gels were visualized using Coomassie blue stain as previously described (CANDIANO *et al.*, 2004) for protein identification using mass spectrometry. The procedure was repeated with the bacterial protein solution obtained from dogs of groups I to III and the respective sera. Regarding the dogs from Groups IV and V, of which no *B. canis* was isolated, the canine sera were tested using the protein extract of two pools containing five *B. canis* isolates each, which were randomly chosen among the isolates. Preliminary gels were repeated until getting a triplicate with the same pattern. One representative gel from each group was chosen for further analysis.

4.3.4. Western Immunoblotting

1D and 2D gel immunoblotting were performed as previously described (KO *et al.*, 2012) with minor modifications. Briefly, proteins were separated on 1D and 2D gels and transferred at 80 mA/gel for 90 min to nitrocellulose membranes (Bio-Rad laboratories; Munich, Germany) using Towbin transfer buffer (0.025 M Tris, 0.192 M glycine, 2.33% SDS, 20 % (v/v) methanol, pH 8.3). The nitrocellulose membrane was blocked overnight at room temperature with gentle shaking in 2.5 % dried skimmed milk powder (70166 Sigma-Aldrich, Germany) in Tris buffered saline (TBS). The membrane was washed twice using Tween-TBS (TBST; 20 mM Tris, pH 7.5; 500 mM NaCl; 0.05 % Tween-20; 10 min).

Then, the membrane was incubated shaking for 90 min at room temperature with dog's sera diluted 1:100 with Tween-TBS. The diluted sera was discarded, the membrane was washed with Tween-TBS, then incubated with anti-dog IgG (H and L) peroxidase-conjugated produced in goat, diluted 1:1000 for 1h. After washing the nitrocellulose membrane twice with Tween-TBS for 10 min, the detection of signals was carried out using the TMB kit™ (3,3',5,5'-tetramethylbenzidine liquid substrate; Sigma-Aldrich; Steinheim, Germany) according to the manufacturer's protocol. The canine sera collected in third season (November 2014) from each group were confronted with the representative isolates obtained from dogs of groups I to III. Randomly chosen sera from dogs of groups IV and V were used to evaluate the immunogenic reaction and compare with the infected dogs, using the protein extract of a pool of isolates.

4.3.5. In-gel trypsin digestion and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS)

The protein spots that matched to the western blotting signals were excised from two-dimensional dodecyl sulphate sodium polyacrylamide gels (2D gels) and subjected to trypsin in-gel digestion as described previously (MURUGAIYAN *et al.*, 2013b). Following trypsin digestion, the resultant peptides were extracted and dried completely using vacuum centrifugation. Then, the precipitates were reconstituted with 5 µl of 33 % acetonitrile in 0.1 % trifluoro-acetic acid (Merck, Darmstadt, Germany). A total of 0.5 µl of this extract was mixed with 0.5 µl of saturated solution of HCCA (α -cyano-4-hydroxycinnamic acid, Sigma-Aldrich, Steinheim, Germany) matrix in 50% acetonitrile and 2.5 % tri-fluoro-acetic-acid and spotted on a ground steel MTP 384 target plate (Bruker Daltonics, Bremen, Germany).

The spots were allowed to dry at room temperature and MALDI-TOF-MS was carried out using Ultraflex III TOF/TOF MS machine (Bruker Daltonics, Bremen, Germany) equipped with nitrogen laser source, LIFT cell for fragment ion post acceleration and ion reflector. The peptide mass fingerprint (PMF) product ion spectra were acquired in positive reflection mode with the following parametric setting: 50 Hz laser, ion source I: 25, ion source 2: 21.60 kV; lens: 9.8 kV; 100 laser shots with 50 shot steps from different positions of the target spot (random walk movement) and the laser irradiation was set at 30 to 40%; pulsed ion extraction time: 150 ns; matrix suppression, deflection, suppress up to 700 Da; mass range of 900-4000 Da.

The external calibration was carried out in the mass range of 700 to 4000 m/z using peptide Calibration Standard II (Bruker Daltonics, Bremen, Germany). The autoXecute acquisition option of the software Flex Control 3.0 and Flex Analysis 3.0 was used to acquire PMF spectra and to select seven MALDI-TOF-MS product ion spectra from each spot. The parameter settings were: laser-induced dissociation (LID) mode, initial acceleration potential of 8 kV in the primary ion source, re-acceleration potential of 19 kV in the LIFT cell, laser irradiation: 35 to 45 %, 500 laser shots with 100 shot steps in random movement, primary choice range: 1000-25000 m/z and secondary choice range: 900 to 3000 m/z.

4.3.6 Protein identification

The software Biotools 3.2 (Bruker Daltonics, Bremen, Germany) was used to combine PMF and MS/MS ions and protein identification was carried out through a database search (MASCOT, www.matrixscience.com) against all entries of NCBIprot database. The parameters setting was: trypsin digestion; up to three missed cleavage; fixed modifications — carbamidomethyl (C); variable modifications — oxidation (M); peptide tol.: ± 0.6 Da; MS/MS tol.: ± 0.7 Da; peptide charge: + 1, + 2 and + 3. The protein identification was considered valid if matched to proteins specific for *Brucella* spp., more than 2 peptides and the MOlecular Weight SEarch (MOWSE) score was significant ($p < 0.05$).

4.4. RESULTS AND DISCUSSION

4.4.1. 1D and 2D electrophoresis

Whole-cell protein extracts of *B. canis* were separated using 1D and 2D gel electrophoresis. Several preliminary gels were prepared; hence, we achieve the best quality of representative images. The representative 1D and 2D gels are shown in Figures 2 and 3, respectively. The 1D gel image (Figure 2) revealed comparable pattern of protein for all 24 isolates (I1-I24) showing no visible differences in the protein pattern among the isolates. The 2D gel image (Figure 3) shows the protein profile of *B. canis* isolate I2.

Figure 2. Representative SDS-PAGE (1D gel) of 24 *Brucella canis* isolates (Isolates: I1 – I24).

Figure 3. Representative *Brucella canis* 2D gel (Isolate: I2).

4.4.2. Western Immunoblotting

The representatives 1D and 2D gel blotted and immunodetected images on cellulose membranes are shown on Figures 4 to 8. The 1D images of figures 4A to 6A reveal the protein profile recognized by canine sera from two dogs of each group (I to IV) comprising infected dogs in different stages of infection regarding bacteremia (obtained in March, August and November, 2014) and from two non-infected dogs (group V) that were sampled once. The 2D gel images of figures 4B to 6B show the protein profile recognized by sera obtained from infected dogs at the last sampling time (November, 2014), comprising samples from dogs in bacteremic (Figures 4B and 5B) and non-bacteremic (Figure 6B) stages of infection.

Figure 4. 1D and 2D electrophoretic and immunogenic profiles of *Brucella canis* whole protein extract. (A) 1D immunoblots showing major antigenic bands of two *B. canis* isolates protein preparation: protein preparation of isolate I2 recognized by serum samples of non-infected dog D1 (group V) (S01), and infected dog D7 (group I) sampled on March, 2014 (S07), August 2014 (S78) and November 2014 (S112); and protein preparation of *B. canis* isolate I5 recognized by serum samples of non-infected dog D2 (group V) (S02), and infected dog D12 (group I) sampled on March, 2014 (S12), August 2014 (S86) and November 2014 (S120). Dogs D7 and D12 showed bacteremia only in the first sampling. Molecular marker are indicated on the right and left in kiloDalton (kDa). (B) 2D immunoblots showing major antigenic spots from *B. canis* isolate I5 protein preparation recognized by serum sample of infected dog D12 (group I) sampled on November 2014 (S120). Molecular marker is indicated on the right in kDa.

Figure 5. 1D and 2D electrophoretic and immunogenic profiles of *Brucella canis* whole protein extract. (A) 1D immunoblots showing major antigenic bands of two *B. canis* isolates protein preparation: protein preparation of isolate I1 recognized by serum samples of non-infected dog D1 (group V) (S03), and infected dog D6 (group II) sampled on March, 2014 (S06), August 2014 (S73) and November 2014 (S107); and protein preparation of *B. canis* isolate I7

recognized by serum samples of non-infected dog D4 (group V) (S04), and infected dog D14 (group II) sampled on March, 2014 (S14), August 2014 (S81) and November 2014 (S115). Dogs D6 and D14 showed bacteremia in the first and second samplings. Molecular marker are indicated on the right and left in kDa. **(B)** 2D immunoblots showing major antigenic spots from *B. canis* isolate I7 protein preparation recognized by serum sample of infected dog D14 (group II) sampled on November 2014 (S115). Molecular marker is indicated on the right in kDa.

Figure 6. 1D and 2D electrophoretic and immunogenic profiles of *Brucella canis* whole protein extract. **(A)** 1D immunoblots showing major antigenic bands of two *B. canis* isolates protein preparation: protein preparation of isolate I3 recognized by serum samples of non-infected dog D5 (group V) (S05), and infected dog D9 (group III) sampled on March, 2014 (S09), August 2014 (S76) and November 2014 (S110); and protein preparation of *B. canis* isolate I4 recognized by serum samples of non-infected dog D1 (group V) (S01), and infected dog D10 (group III) sampled on March, 2014 (S10), August 2014 (S75) and November 2014 (S109). Dogs D9 and D10 showed bacteremia in the first, second and third sampling time. Molecular marker are indicated on the right and left in kDa. **(B)** 2D immunoblots showing major antigenic spots from *B. canis* isolate I3 protein preparation recognized by serum sample of infected dog D9 (group III) sampled on November 2014 (S110). Molecular marker is indicated on the right in kDa.

The 1D gel image of figure 7A reveals the protein profile recognized by canine sera from group IV comprising dogs with negative results through blood culturing in the three sampling times (March, August and November, 2014) but that had close contact with infected dogs, and from two non-infected dogs (group V) that were sampled once. The 2D gel image of figure 7B show the protein profile recognized by sera obtained from one dog of the infected kennel at the last sampling time (November, 2014).

Figure 7. 1D and 2D gel electrophoretic and immunogenic profiles of *Brucella canis* whole protein extract. **(A)** 1D immunoblots showing major antigenic bands of the pool of five *B. canis* isolates protein preparation (I6, I7, I11, I16, and I19) recognized by serum samples of non-infected dogs (D2 and D3 from group V) (S02 and S03), and infected dogs D8 and D11 (group IV) sampled on March, 2014 (S08 and S11, respectively), August 2014 (S85 and S74, respectively) and November 2014 (S119 and 108, respectively); Dogs D9 and D10 showed negative results through blood culturing in the first, second and third sampling time, although they had close contact with infected and bacteremic dogs. Molecular marker are indicated on the right and left in kDa. **(B)** 2D gel immunoblots showing major antigenic spots from the pool of five *B. canis* isolates protein preparation (I6, I7, I11, I16, and I19) recognized by serum sample of infected dog D11 (group IV) sampled on November 2014 (S108). Molecular marker is indicated on the right in kDa.

The 1D gel image of the figure 7A reveals the protein profile recognized by canine sera from group V comprising the non-infected dogs; and the 2D gel image show the protein profile recognized by sera obtained of a non-infected dog of group V.

Figure 8. 1D and 2D electrophoretic and immunogenic profiles of *Brucella canis* whole protein extract. (A) 1D immunoblots showing major antigenic bands of the pool of five *B. canis* isolates protein preparation (I6, I7, I11, I16, and I19) recognized by serum samples (S01, S02, S03, S04, and S05) of non-infected dogs (D1 to D5, from group V). Molecular marker are indicated on the right and left in kDa. (B) 2D gel immunoblots showing major antigenic spots from the pool of five *B. canis* isolates protein preparation (I6, I7, I11, I16, and I19) recognized by serum sample (S02) of infected dog D2 (group V). Molecular marker is indicated on the right in kDa.

The dogs of the groups I, II and III showed very similar patterns of immunogenic bands or spots. Although the dogs of group IV revealed no bacteremia at all sampling times, their sera reacted against bacterial proteins, mainly those of high molecular weight. Although these dogs showed no bacteremia during the samplings, we cannot assume that they were free of infection, because they also developed antibodies against the proteins extracted from *B. canis* and they had close contact with the infected dogs.

The 1D and 2D immunoblots of sera of non-infected dogs (group V) revealed the recognition of *B. canis* proteins. Theoretically, these dogs were healthy and showed no clinical signs of canine brucellosis. Hence, we did not expect immunoreactive antibodies in their serum. However, these dogs are not supposed to be pathogen free. They might suffer from infections with potentially cross-reacting antibodies which can lead to an unspecific immune response. Therefore, protein functions have to be characterized to identify *B. canis* specific proteins.

4.4.3. Protein identification

Immunogenic *B. canis* proteins recognized by the sera of 22 dogs (15 infected, two contact animals without bacteremia at any sampling time, and five controls) were identified by MALDI-TOF-MS. We were able to identify 93 different proteins from 185 spots excised from the 2D gel linked to the 92 spots that reacted in the 2D Western Blotting membrane (Table 7 – Supplementary material). The representative immunoreactive protein spots found in sera of dogs from group III that were excised from the 2D gel and used for protein identification by MS can be observed in Figure 10.

Figure 9. 2D Western Blotting with 92 labelled immunoreactive protein spots. The 24 highlighted spots (red box) are those that reacted with bacteremic (GI, GII and GIII) and non-bacteremic (GIV) dogs and did not reacted with non-*Brucella canis* infected dogs (GV). Image from Group III.

In Figure 10, we summarized all immunoreactive protein spots found by Western Blotting in the groups I to V, represented by dogs in different stages of infection regarding

bacteremia. The spots were represented according to the groups (Figure 10) using the InteractiVenn (Figure 11) web-based tool (HEBERLE *et al.*, 2015).

Group I - D3; D5; D6; D7; D9; D10; D11; D12; D13; D14; D15; D16; D17; D18; D19; D22; D23; D24; D25; D26; D27; D31; D32; D33; D34; D36; D37; D40; D41; D42; D43; D44; D50; D51; D52; D54; D55; D56; D57; D63; D64; D65; D66; D67; D68; D69; D71; D72; D73; D74; D75; D77; D78; D79; D81; D82; D83; D84; D85; D86; D89; D90; D95; D99; D100; D105; D106; D110; D111; D112; D113; D114; D115; D117; D118; D120; D121; D122; D123; D124; D127; D130; D133; D140; D141; D142; D143; D144; D145; D146; D171; D182
Group II - D3; D5; D6; D7; D9; D10; D11; D12; D13; D14; D15; D16; D17; D18; D19; D22; D23; D24; D25; D26; D27; D31; D32; D33; D34; D36; D37; D40; D41; D42; D43; D44; D50; D51; D52; D54; D55; D56; D57; D63; D64; D65; D66; D67; D68; D69; D71; D72; D73; D74; D75; D77; D78; D79; D81; D82; D83; D84; D85; D86; D89; D90; D95; D99; D100; D105; D106; D110; D111; D112; D113; D114; D115; D117; D118; D120; D121; D122; D123; D124; D127; D130; D133; D140; D141; D142; D143; D144; D145; D146; D171; D182
Group III - D3; D5; D6; D7; D9; D10; D11; D12; D13; D14; D15; D16; D17; D18; D19; D22; D23; D24; D25; D26; D27; D31; D32; D33; D34; D36; D37; D40; D41; D42; D43; D44; D50; D51; D52; D54; D55; D56; D57; D63; D64; D65; D66; D67; D68; D69; D71; D72; D73; D74; D75; D77; D78; D79; D81; D82; D83; D84; D85; D86; D89; D90; D95; D99; D100; D105; D106; D110; D111; D112; D113; D114; D115; D117; D118; D120; D121; D122; D123; D124; D127; D130; D133; D140; D141; D142; D143; D144; D145; D146; D171; D182
Group IV - D3; D5; D6; D7; D9; D10; D11; D12; D13; D14; D15; D16; D17; D18; D19; D22; D23; D24; D25; D26; D27; D31; D32; D33; D34; D36; D37; D40; D41; D42; D43; D44; D50; D51; D52; D54; D55; D56; D57; D63; D64; D65; D66; D67; D68; D78; D79; D81; D82; D130; D133; D140; D141; D142; D143; D144; D145; D146; D171; D182
Group V - D3; D5; D6; D7; D9; D10; D11; D12; D13; D14; D15; D16; D17; D18; D19; D22; D23; D24; D25; D31; D32; D36; D37; D40; D41; D42; D43; D44

Figure 10. Immunoreactive protein spots recognized by canine serum samples of dogs from the infected kennel sampled on November, 2014, representing each one of the five groups: serum sample (S) 120 of dog (D) 12 (infected and bacteremic) representing group I; S115 of D14 (infected and bacteremic) representing group II; S110 of D9 (infected and bacteremic), representing group III; S108 of D11 (suspected of canine brucellosis and non-bacteremic) representing group IV; and S02 of D2 (non-infected dog from brucellosis free kennel) representing group V.

Figure 11. Venn diagram of the immunoreactive protein spots found by Wester Blotting in canine sera of dogs representing the various groups (I to V).

The sera from bacteremic dogs (groups I, II and III) showed a comparable pattern of reactive protein spots and their identification. The most identified proteins were the single-stranded DNA-binding protein (06 spots), followed by catalase (05 spots), chain A Cu/Zn superoxide dismutase (05 spots), dihydroorotase multifunctional complex type (04 spots), peptidylprolyl isomerase (04 spots), amino acid ABC transporter substrate-binding protein (04 spots), iron ABC transporter substrate-binding protein (03 spots), lysine-arginine-ornithine-binding periplasmic protein (03 spots) and 3-oxoacyl-ACP reductase (03 spots). Their functions are related mainly to the inorganic ion transport and metabolism, besides of DNA replication, recombination and repair; post-translational modification, protein turnover and chaperones; transport and metabolism of nucleotides and amino-acids (HUERTA-CEPAS *et al.*, 2016). Catalase, dihydroorotase multifunctional complex type and iron ABC transporter substrate-binding protein were identified in the infected (bacteremic or not) and in non-*Brucella canis* infected dogs.

Out of 185 spots, 27 immunoblotted spots, 24 distinct plus 3 double spots (78-2, 79-2 and 81-2) have reacted in bacteremic (groups I, II and III) and non-bacteremic dogs (group IV) and did not react in non-*B. canis* infected dogs (group V), when the dog's sera were applied in the 2D Western Blotting membrane. Out of 93 proteins identified (Table 2 - Supplementary

material), 19 different proteins were related to these 27 spots (Table 3 - Supplementary material). Their functions are mainly related to amino-acid transport and metabolism, translational, ribosomal structure and biogenesis and transcription. Some of these proteins were also identified in *Brucella abortus* and *Brucella melitensis* also by MALDI-TOF-MS in different hosts, such as cow, buffalo, sheep and goat (WARETH *et al.*, 2016).

Some antigenic proteins from *Brucella* have been used as recombinant antigens in serological tests (MOHAMMADI & GOLCHIN, 2018), mainly in ELISA assays (THEPSURIYANONT *et al.*, 2014; HOP *et al.*, 2016; KOYUNCU *et al.*, 2018). *B. canis* recombinant immunogenic antigens, such as recombinant Cu-Zn superoxide dismutase, heat shock protein 60, rhizopine-binding protein and Cu-Zn superoxide dismutase (TSOGTBAATAR *et al.*, 2008); and hypothetical protein - Ag 4 (CASTILLO *et al.*, 2014), were also tested as antigens in serological tests. However, the panel of canine sera used to evaluate the performance of the serological tests developed using those recombinant antigens did not allow a proper evaluation of the diagnostic sensitivity, since positive dogs confirmed through culturing were not used.

The development of a vaccine for canine brucellosis was tested using recombinant outer membrane protein 31 antigen formulated in different adjuvants (CLAUSSE *et al.*, 2014); the authors have concluded that Omp31 could be a useful candidate for the development of a subcellular vaccine against *B. canis* infection.

The immunogenic protein Cu-Zn superoxide dismutase, was one of the most frequent identified protein among the found spots. Its function is related to the inorganic ion transport and metabolism. However, it is not a specific *B. canis* protein and it was identified only in spots from bacteremic dogs (groups I, II and III).

During the bacteremia, the infection is easier identified by direct or indirect tests of diagnosis, because *B. canis* is easily isolated from blood and antibodies directed to R-LPS antigens predominate during bacteremia (ZOHA & CARMICHAEL, 1982b). However, during the chronic stage of the infection, the bacteria may be restricted to lymphoid tissues, therefore, false-negative results in culturing and in serological tests using R-LPS as antigen were commonly observed (KEID *et al.*, 2007a; KEID *et al.*, 2007b; KEID *et al.*, 2009; BOERI *et al.*, 2018).

Immunoreactive proteins free of R-LPS can be applied as more specific antigens in the serological diagnosis of the infection. In addition, it has been proposed that the antibody response to protein antigens is delayed when compared to that of LPS, thus increasing the antibody response in chronic infections (ZOHA & CARMICHAEL, 1982a; LETESSON *et al.*,

1997). Therefore, the 19 immunogenic proteins identified, which have reacted to the sera from the suspected but non-bacteremic dogs from this study (group IV) and had not reacted to the sera from non-*B. canis* infected dogs, should be tested as specific immunogenic antigens for canine brucellosis serodiagnosis, since they have the potential to detect both, bacteremic and non-bacteremic infected dogs.

4.5.CONCLUSIONS

The use of 2D gel electrophoresis followed by immunoblotting was firstly used to determine the protein pattern of *B. canis* strains yielding the identification of immunogenic proteins able to differentiate infected and non-infected dogs.

The immunogenic *B. canis* proteins identified through MALDI-ToF-MS may be used as antigens in serological tests with the potential to increase the sensitivity and specificity of canine brucellosis diagnosis. A reliable diagnosis of canine brucellosis is important to avoid economic losses in kennels and to prevent infection in dogs as well as the transmission to humans, mainly due to the close contact between humans and dogs.

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4.6. SUPPLEMENTARY MATERIAL

Table 1. Sample identification

Group	Dog identification	Serum sample identification	Date of sampling	Blood culturing	<i>Brucella canis</i> isolate identification
V	D1	S01	12 March 2014	0	xxxx
	D2	S02	12 March 2014	0	xxxx
	D3	S03	12 March 2014	0	xxxx
	D4	S04	12 March 2014	0	xxxx
	D5	S05	12 March 2014	0	xxxx
II	D6	S06	22 March 2014	1	I1
		S73	2 August 2014	1	I16
		S107	8 November 2014	0	xxxx
I	D7	S07	22 March 2014	1	I2
		S78	2 August 2014	0	xxxx
		S112	8 November 2014	0	xxxx
IV	D8	S08	22 March 2014	0	xxxx
		S85	2 August 2014	0	xxxx
		S119	8 November 2014	0	xxxx
III	D9	S09	22 March 2014	1	I3
		S76	2 August 2014	1	I18
		S110	8 November 2014	1	xxxx
III	D10	S10	22 March 2014	1	I4
		S75	2 August 2014	1	I17
		S109	8 November 2014	1	xxxx
IV	D11	S11	22 March 2014	0	xxxx
		S74	2 August 2014	0	xxxx
		S108	8 November 2014	0	xxxx
I	D12	S12	22 March 2014	1	I5
		S86	2 August 2014	0	xxxx
		S120	8 November 2014	0	xxxx
I	D13	S13	22 March 2014	1	I6
		S80	2 August 2014	0	xxxx
		S114	8 November 2014	0	xxxx
II	D14	S14	22 March 2014	1	I7
		S81	2 August 2014	1	I21
		S115	8 November 2014	0	xxxx
III	D15	S15	22 March 2014	0	xxxx
		S77	2 August 2014	1	I19
		S111	8 November 2014	1	xxxx
III	D16	S16	22 March 2014	1	I8
		S82	2 August 2014	1	I22
		S116	8 November 2014	1	xxxx
II	D17	S17	22 March 2014	1	I9
		S84	2 August 2014	1	I24

III	D18	S118	8 November 2014	0	xxxx
		S18	22 March 2014	1	I10
		S83	2 August 2014	1	I23
II	D19	S117	8 November 2014	1	xxxx
		S19	22 March 2014	1	I11
		S72	2 August 2014	1	I15
I	D20	S106	8 November 2014	I	xxxx
		S20	22 March 2014	1	I12
		S87	2 August 2014	0	xxxx
I	D21	S121	8 November 2014	0	xxxx
		S21	22 March 2014	1	I13
		S88	2 August 2014	0	xxxx
II	D22	S122	8 November 2014	0	xxxx
		S22	22 March 2014	1	I14
		S79	2 August 2014	1	I20
		S113	8 November 2014	0	xxxx

Table 2. Immunoreactive proteins of *Brucella canis* identified by 2D gel electrophoresis, Western Blotting and MALDI-ToF-MS.

No.	Spot No.	NCBI Acc. No	Protein name	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
1	D070	ABD65310.1	31 kDa cell surface protein, partial [<i>Brucella abortus</i>]	517	31084	5,50	45	9
	D075	WP_006253138.1	3-oxoacyl-ACP reductase [<i>Brucella canis</i>]	342	26174	5,53	52	11
2	D077	WP_006253138.1	3-oxoacyl-ACP reductase [<i>Brucella canis</i>]	219	26174	5,53	43	7
	D084	WP_008935419.1	3-oxoacyl-[acyl-carrier-protein] reductase [<i>Brucella</i>] MULTISPECIES	249	26026	6,44	45	11
3	D041	WP_038415769.1	ABC transporter substrate-binding protein [<i>Brucella canis</i>]	258	37914	6,03	28	12
4	D124	WP_008936123.1	acyl-CoA thioesterase [<i>Brucella</i>] MULTISPECIES	115	14315	5,84	27	4
5	D129	AAL51684.1	acyl-CoA hydrolase [<i>Brucella melitensis</i> bv. 1 str. 16M]	76	14602	6,59	40	6
6	D100	WP_006177141.1	acetyl-CoA carboxylase biotin carboxyl carrier protein subunit [<i>Brucella</i>] MULTISPECIES	80	16306	4,84	26	3
7	D032	WP_002964773.1	adenylosuccinate synthetase [<i>Brucella</i>] MULTISPECIES	481	46646	5,84	41	17
8	D033	AAA75455.1	adenylosuccinate synthetase [<i>Brucella abortus</i>]	513	43634	7,14	34	13
9	D040	WP_002965452.1	alcohol dehydrogenase AdhP [<i>Brucella</i>] MULTISPECIES	297	36566	5,76	23	10
10	D044	EXU82522.1	alcohol dehydrogenase [<i>Brucella melitensis</i> 548]	129	34808	5,43	27	5

No.	Spot No.	NCBI Acc. No	Protein name	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
11	D022	WP_008933719.1	aldehyde dehydrogenase family protein [<i>Brucella</i> spp. NVSL 07-0026]	258	51458	5,99	33	14
12	D023	WP_006173682.1	aldehyde dehydrogenase family protein [<i>Brucella</i>] MULTISPECIES	154	51429	5,99	25	11
13	D063	EEY29746.1	cationic amino acid ABC transporter [<i>Brucella suis</i> bv. 5 str. 513]	386	30547	5,25	36	9
14	D079-2	WP_006072682.1	amino acid ABC transporter substrate-binding protein [<i>Brucella</i>] MULTISPECIES	391	29067	5,57	53	12
	D081-2	WP_006072682.1	amino acid ABC transporter substrate-binding protein [<i>Brucella</i>] MULTISPECIES	328	29067	5,57	42	8
	D159	WP_004685893.1	amino acid ABC transporter substrate-binding protein [<i>Brucella</i>] MULTISPECIES	411	41888	5,31	34	11
	D166	WP_004689042.1	amino acid ABC transporter substrate-binding protein [<i>Brucella</i>] MULTISPECIES	374	27705	6,77	40	9
15	D085	WP_015799895.1	amino acid ABC transporter substrate-binding protein [<i>Brucella microti</i>]	666	27706	6,20	45	10
16	D061	WP_002965970.1	aminotransferase [<i>Brucella</i>] MULTISPECIES	189	31568	5,71	28	8
17	D062	WP_025199207.1	aminotransferase [<i>Brucella</i> spp. BO2]	327	31489	5,82	33	9

No.	Spot No.	NCBI Acc. No	Protein name	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
18	D029	EEX59830.1	carboxyl-terminal protease [<i>Brucella abortus</i> bv. 2 str. 86/8/59]	72	33281	7,77	12	3
	D030	EEX59830.1	carboxyl-terminal protease [<i>Brucella abortus</i> bv. 2 str. 86/8/59]	82	33281	7,77	12	3
	D119	4L05_A	Chain A, Cu/zn Superoxide Dismutase From <i>Brucella Abortus</i>	272	16176	6,11	47	6
19	D120	4L05_A	Chain A, Cu/zn Superoxide Dismutase From <i>Brucella Abortus</i>	255	16176	6,11	63	7
	D121	4L05_A	Chain A, Cu/zn Superoxide Dismutase From <i>Brucella Abortus</i>	294	16176	6,11	63	7
	D122	4L05_A	Chain A, Cu/zn Superoxide Dismutase From <i>Brucella Abortus</i>	271	16176	6,11	63	7
	D123	4L05_A	Chain A, Cu/zn Superoxide Dismutase From <i>Brucella Abortus</i>	270	16176	6,11	63	7
20	D008	EEH15197.1	chaperone protein DnaK [<i>Brucella ceti</i> str. Cudo]	201	68796	4,86	13	7
21	D009	WP_006149711.1	chaperonin GroEL [<i>Brucella melitensis</i>]	303	57480	5,01	23	11
	D010	WP_006149711.1	chaperonin GroEL [<i>Brucella melitensis</i>]	445	57480	5,01	23	11
22	D011	WP_011005794.1	molecular chaperone GroEL [<i>Brucella melitensis</i>]	137	57483	5,04	26	10
23	D143	WP_008936403.1	molecular chaperone GroES [<i>Brucella</i>] MULTISPECIES	138	10401	5,41	50	5
	D146	WP_008936403.1	molecular chaperone GroES [<i>Brucella</i>] MULTISPECIES	104	10401	5,41	30	3
24	D024	WP_006109403.1	dihydroorotase multifunctional complex type [<i>Brucella abortus</i>]	476	49040	6,17	25	11

No.	Spot No.	NCBI Acc. No	Protein name	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
24	D025	WP_006109403.1	dihydroorotase multifunctional complex type [<i>Brucella abortus</i>]	300	49040	6,17	22	11
	D007	WP_006209480.1	dihydroorotase multifunctional complex type [<i>Brucella abortus</i>]	64	49066	6,24	29	10
	D150	EEP64169.1	dihydroorotase multifunctional complex type [<i>Brucella abortus</i> str. 2308 A]	285	51844	6,41	18	9
25	D001	WP_006014582.1	DNA starvation/stationary phase protection protein Dps [<i>Brucella ovis</i>]	123	18269	5,25	17	3
26	D002	EFG36962.1	starvation-inducible DNA-binding protein [<i>Brucella</i> sp. NVSL 07-0026]	365	17672	4,98	27	4
27	D067	WP_004689099.1	D-ribose ABC transporter substrate-binding protein [<i>Brucella suis</i>]	625	33297	5,11	38	9
	D068	WP_004689099.1	D-ribose ABC transporter substrate-binding protein [<i>Brucella suis</i>]	660	33297	5,11	38	9
28	D136	WP_002965776.1	ribose 5-phosphate isomerase B [<i>Brucella</i> MULTISPECIES]	382	15826	5,33	67	8
	D137	WP_002965776.1	ribose 5-phosphate isomerase B [<i>Brucella</i>] MULTISPECIES	351	15826	5,33	52	8
29	D083	EFG36466.1	ribose transport system substrate-binding protein [<i>Brucella</i> spp. NVSL 07-0026]	199	20383	5,21	30	4
30	D175	WP_009364167.1	ribose 5-phosphate isomerase B [<i>Brucella</i> spp. BO2]	150	15825	5,33	35	4

No.	Spot No.	NCBI Acc. No	Protein name	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
31	D098	EEX86951.1	DSBA oxidoreductase [<i>Brucella ceti</i> B1/94]	266	27532	7,57	30	7
32	D130	WP_006132662.1	DUF2059 domain-containing protein [<i>Brucella canis</i>]	221	18820	6,75	42	6
	D135	WP_006132662.1	DUF2059 domain-containing protein [<i>Brucella canis</i>]	291	18820	6,75	36	5
33	D144	WP_004683653.1	DUF1476 domain-containing protein [<i>Brucella</i>] MULTISPECIES	196	12185	4,84	80	12
	D145	WP_008505508.1	DUF1476 domain-containing protein [<i>Brucella</i>] MULTISPECIES	206	12155	4,84	51	9
34	D183	WP_002964697.1	DUF1134 domain-containing protein [<i>Brucella</i>] MULTISPECIES	111	21266	7,85	24	4
35	D046	EEH15264.1	Extracellular ligand-binding receptor [<i>Brucella ceti</i> str. Cudo]	264	40473	5,78	22	6
36	D050	EEW86339.1	extracellular solute-binding protein family 1 [<i>Brucella melitensis</i> bv. 1 str. 16M]	261	41956	4,97	21	8
37	D078-1	EEW91094.1	extracellular solute-binding protein family 3 [<i>Brucella suis</i> bv. 4 str. 40]	395	28698	5,38	55	12
37	D079-1	EEW91094.1	extracellular solute-binding protein family 3 [<i>Brucella suis</i> bv. 4 str. 40]	391	28698	5,38	54	12
38	D111	AIJ50948.1	fe2+ transport family protein [<i>Brucella abortus</i>]	126	18636	5,67	26	3
39	D058	WP_022576198.1	formate transporter [<i>Brucella abortus</i>]	112	13025	4,78	17	2

No.	Spot No.	NCBI Acc. No	Protein name	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
40	D093	WP_008507507.1	fructose-6-phosphate aldolase [<i>Brucella inopinata</i>]	272	23554	5,47	17	4
41	D076	WP_069713965.1	glycine/betaine ABC transporter substrate-binding protein [<i>Brucella</i> spp. B13-0095]	274	31933	5,57	47	14
	D171	WP_069713965.1	glycine/betaine ABC transporter substrate-binding protein [<i>Brucella</i> spp. B13-0095]	100	31933	5,57	21	4
42	D057	WP_006134463.1	hypothetical protein [<i>Brucella canis</i>]	108	33284	5,32	31	6
43	D116	WP_005973423.1	hypothetical protein [<i>Brucella melitensis</i>]	124	14407	5,11	37	3
44	D179	AAL53934.1	hypothetical membrane associated protein [<i>Brucella melitensis</i> bv. 1 str. 16M]	144	20645	9,00	20	4
45	D184	WP_002967744.1	hypothetical protein [<i>Brucella</i>] MULTISPECIES	163	17990	6,74	35	7
	D108	WP_002964319.1	hypothetical protein [<i>Brucella</i>] MULTISPECIES	78	13262	4,61	52	6
46	D047	AAB50572.1	immunogenic 39-kDa protein [<i>Brucella abortus</i>]	325	43216	5,10	34	15
47	D069	WP_076781250.1	immunogenic protein [<i>Brucella</i> spp. 09RB8471]	341	34280	6,92	33	8
48	D095	KDZ97930.1	invasion protein B, partial [<i>Brucella melitensis</i>]	234	19299	5,16	34	4
49	D015-2	WP_049595885.1	leucyl aminopeptidase [<i>Brucella canis</i>]	351	52542	5,75	23	12
50	D064	AEW13370.1	lysine-arginine-ornithine-binding periplasmic protein [<i>Brucella</i> <i>canis</i> HSK A52141]	265	36678	5,09	26	8

No.	Spot No.	NCBI Acc. No	Protein name	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
	D078-2	WP_006165687.1	lysine-arginine-ornithine-binding periplasmic protein [Brucella spp. 63/311]	394	29085	5,39	54	12
50	D080	WP_006165687.1	lysine-arginine-ornithine-binding periplasmic protein [Brucella spp. 63/311]	507	29085	5,39	67	14
	D081-1	WP_006165687.1	lysine-arginine-ornithine-binding periplasmic protein [Brucella spp. 63/311]	328	29085	5,39	42	8
51	D086	WP_014315855.1	metal-dependent hydrolase [Brucella canis]	372	24889	5,69	43	8
	D087	WP_014315855.1	metal-dependent hydrolase [Brucella canis]	440	24889	5,69	32	6
52	D082	WP_012219776.1	MetQ/NlpA family lipoprotein [Brucella canis]	325	30535	5,32	39	10
53	D109	WP_002968422.1	bacterioferritin [Brucella] MULTISPECIES	98	18685	4,69	14	2
	D117	WP_002968422.1	bacterioferritin [Brucella] MULTISPECIES	235	18685	4,69	24	3
53	D118	WP_002968422.1	bacterioferritin [Brucella] MULTISPECIES	269	18685	4,69	24	3
54	D038	WP_004689573.1	4-hydroxy-tetrahydrodipicolinate synthase [Brucella] MULTISPECIES	440	31892	6,26	46	10
	D161	WP_004689573.1	4-hydroxy-tetrahydrodipicolinate synthase [Brucella] MULTISPECIES	209	31892	6,26	22	5
55	D012	WP_004690104.1	catalase [Brucella] MULTISPECIES	428	56541	6,52	58	23

No.	Spot No.	NCBI Acc. No	Protein name	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
55	D013	WP_004690104.1	catalase [<i>Brucella</i>] MULTISPECIES	372	56541	6,52	62	24
	D019	WP_004690104.1	catalase [<i>Brucella</i>] MULTISPECIES	231	56541	6,52	33	15
	D149	WP_004690104.1	catalase [<i>Brucella</i>] MULTISPECIES	296	56541	6,52	24	12
	D006	WP_008936945.1	catalase [<i>Brucella</i>] MULTISPECIES	165	56521	6,63	50	7
56	D014	WP_004690711.1	cytosol aminopeptidase [<i>Brucella</i>] MULTISPECIES	368	52600	5,64	23	12
	D015-1	WP_004690711.1	cytosol aminopeptidase [<i>Brucella</i>] MULTISPECIES	351	52600	5,64	23	12
57	D181	WP_002964766.1	deoxyuridine 5'-triphosphate nucleotidohydrolase [<i>Brucella</i>] MULTISPECIES	150	16707	6,59	57	9
58	D065	WP_006641394.1	electron transfer flavoprotein subunit alpha/FixB family protein, partial [<i>Brucella</i>] MULTISPECIES	68	29705	4,86	21	3
59	D185	WP_008503580.1	electron transfer flavoprotein subunit beta/FixA family protein [<i>Brucella</i>] MULTISPECIES	64	26404	7,77	12	2
60	D034	WP_004683843.1	enolase [<i>Brucella</i>] MULTISPECIES	902	45462	4,99	43	15
61	D096	WP_006180596.1	gamma carbonic anhydrase family protein [<i>Brucella</i>] MULTISPECIES	201	18780	6,05	39	6
62	D132	WP_008507451.1	heme-degrading domain- containing protein [<i>Brucella</i>] MULTISPECIES	315	14423	5,63	53	7

No.	Spot No.	NCBI Acc. No	Protein name	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
	D048	WP_006181169.1	lysine [<i>Brucella</i>] MULTISPECIES	233	39170	4,88	25	6
63	D049	WP_006192075.1	iron ABC transporter substrate- binding protein [<i>Brucella</i>] MULTISPECIES	228	36484	5,20	35	11
	D039	WP_004692206.1	iron ABC transporter substrate- binding protein [<i>Brucella</i>] MULTISPECIES	301	37396	6,47	44	22
64	D089	WP_004689974.1	molybdate ABC transporter substrate-binding protein [<i>Brucella</i>] MULTISPECIES	290	26630	5,48	41	10
65	D110	WP_004691793.1	peptidylprolyl isomerase [<i>Brucella</i>] MULTISPECIES	507	20796	6,74	47	10
	D112	WP_004691793.1	peptidylprolyl isomerase [<i>Brucella</i>] MULTISPECIES	268	20796	6,74	37	6
	D113	WP_002964221.1	peptidylprolyl isomerase [<i>Brucella</i>] MULTISPECIES	475	18249	5,54	75	12
65	D115	WP_006174256.1	peptidylprolyl isomerase [<i>Brucella</i>] MULTISPECIES	198	18275	5,54	54	5
	D020	WP_004691793.1	peptidylprolyl isomerase [<i>Brucella</i>] MULTISPECIES	104	20796	6,74	26	3
66	D134	WP_008935432.1	peroxiredoxin [<i>Brucella</i>] MULTISPECIES	292	16700	5,35	24	3
67	D056	WP_004692061.1	phosphonate ABC transporter substrate-binding protein [<i>Brucella</i>] MULTISPECIES	116	37395	5,17	23	7

No.	Spot No.	NCBI Acc. No	Protein name	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
67	D055	WP_004692061.1	phosphonate ABC transporter substrate-binding protein [Brucella] MULTISPECIES	137	37395	5,17	16	4
68	D073	WP_006641989.1	polyphenol oxidase [Brucella] MULTISPECIES	144	29127	6,00	16	6
69	D157	WP_004684149.1	protein TolB [Brucella] MULTISPECIES	62	48585	8,75	21	8
70	D074	WP_002968829.1	protein-methionine-sulfoxide reductase catalytic subunit MsrP [Brucella] MULTISPECIES	323	31752	5,60	36	8
71	D090	WP_004691908.1	riboflavin synthase [Brucella] MULTISPECIES	251	22291	5,02	30	9
	D174	WP_006174040.1	riboflavin synthase [Brucella] MULTISPECIES	161	22292	4,95	37	7
72	D091	WP_006165928.1	ribosome-recycling factor [Brucella spp. 63/311]	189	20655	5,71	25	5
	D092	WP_006165928.1	ribosome-recycling factor [Brucella spp. 63/311]	227	20655	5,71	25	5
73	D168	WP_006173662.1	ribosomal subunit interface protein [Brucella] MULTISPECIES	259	21618	5,23	28	5
74	D017	WP_002964286.1	ribosome-recycling factor [Brucella] MULTISPECIES	60	20651	5,98	10	2
75	D140	WP_006172154.1	RidA family protein [Brucella] MULTISPECIES	128	12320	5,63	50	5
75	D142	WP_009363205.1	RidA family protein [Brucella] MULTISPECIES	87	12352	5,63	43	3

No.	Spot No.	NCBI Acc. No	Protein name	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
76	D102	WP_002967690.1	single-stranded DNA-binding protein [<i>Brucella</i>] MULTISPECIES	253	18360	5,54	54	12
	D105	WP_002967690.1	single-stranded DNA-binding protein [<i>Brucella</i>] MULTISPECIES	325	18360	5,54	68	16
	D106	WP_002967690.1	single-stranded DNA-binding protein [<i>Brucella</i>] MULTISPECIES	264	18360	5,54	72	15
76	D021	WP_002967690.1	single-stranded DNA-binding protein [<i>Brucella</i>] MULTISPECIES	126	18360	5,54	65	14
	D107	WP_002967690.1	single-stranded DNA-binding protein [<i>Brucella</i>] MULTISPECIES	187	18360	5,54	76	18
	D180	WP_008935844.1	single-stranded DNA-binding protein [<i>Brucella</i>] MULTISPECIES	115	18443	5,54	39	8
77	D158	WP_086440147.1	sn-glycerol-3-phosphate ABC transporter substrate-binding protein UgpB [<i>Brucella</i>] MULTISPECIES	158	46563	5,31	10	5
78	D045	KFH21654.1	thiosulfate transporter subunit [<i>Brucella abortus</i> 544]	306	36769	5,22	25	7
79	D156	WP_004692030.1	sulfate ABC transporter substrate-binding protein [<i>Brucella</i>] MULTISPECIES	171	37731	5,71	16	5

No.	Spot No.	NCBI Acc. No	Protein name	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
80	D005-2	WP_004692460.1	TAT-dependent nitrous-oxide reductase [<i>Brucella</i>] MULTISPECIES	136	71567	5,54	26	14
81	D099	WP_002964610.1	transcription elongation factor GreA [<i>Brucella</i>] MULTISPECIES	229	17518	4,83	63	10
82	D060	WP_006133687.1	trehalose utilization protein ThuA [<i>Brucella</i>] MULTISPECIES	186	29571	5,85	30	6
83	D037	WP_076771217.1	type I glyceraldehyde-3-phosphate dehydrogenase [<i>Brucella</i> spp. 09RB8910]	109	36413	6,26	9	4
84	D169	WP_008936144.1	ubiquinol-cytochrome c reductase iron-sulfur subunit [<i>Brucella</i>] MULTISPECIES	143	20082	5,35	36	6
85	D125	WP_005974306.1	VOC family protein [<i>Brucella melitensis</i>]	236	13540	5,93	34	4
86	D127	WP_006173413.1	VOC family protein [<i>Brucella</i>] MULTISPECIES	105	13582	5,93	34	4
87	D005-1	EEX88562.1	nitrous-oxide reductase [<i>Brucella ceti</i> M13/05/1]	148	59396	6,12	31	14
88	D035	WP_006135904.1	phosphopyruvate hydratase [<i>Brucella melitensis</i>]	237	45535	4,99	15	5
89	D152	WP_008933882.1	serine peptidase [<i>Brucella</i> spp. NVSL 07-0026]	84	53534	5,81	16	8
90	D097	AAL52548.1	superoxide dismutase (mn) [<i>Brucella melitensis</i> bv. 1 str. 16M]	118	27778	8,65	7	2
91	D043	WP_006132888.1	thiamine ABC transporter substrate binding subunit [<i>Brucella canis</i>]	660	36875	6,06	51	14

No.	Spot No.	NCBI Acc. No	Protein name	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
91	D042	WP_006132888.1	thiamine ABC transporter substrate binding subunit [<i>Brucella canis</i>]	497	36875	6,06	52	14
92	D054	EFG36498.1	thioredoxin [Brucella spp. NVSL 07-0026]	138	21288	4,78	31	8
93	D126	KFJ56240.1	universal stress family protein [<i>Brucella neotomae</i> 5K33]	149	13172	5,57	33	4
	D128	KFJ56240.1	universal stress family protein [<i>Brucella neotomae</i> 5K33]	142	13172	5,57	33	4

Table 3. Immunoreactive proteins of *Brucella canis* identified by 2D gel electrophoresis, Western Blotting and MALDI-ToF-MS, which have reacted front of bacteremic (GI, GII and GIII) and non-bacteremic dogs (GIV) and have not reacted front non- *Brucella canis* infected dogs (GV).

Protein No.	Spot No.	NCBI Acc. No	Protein name	Protein function	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
01	D033	AAA75455.1	adenylosuccinate synthetase [<i>Brucella abortus</i>]	Nucleotide transport and metabolism	513	43634	7,14	34	13
02	D034	WP_004683843.1	enolase [<i>Brucella</i>] Multispecies	Carbohydrate transport and metabolism	902	45462	4,99	43	15
03	D046	EEH15264.1	Extracellular ligand-binding receptor [<i>Brucella ceti</i> str. Cudo]	-	264	40473	5,78	22	6
04	D047	AAB50572.1	immunogenic 39-kDa protein [<i>Brucella abortus</i>]	-	325	43216	5,10	34	15
05	D050	EEW86339.1	extracellular solute-binding protein family 1 [<i>Brucella melitensis</i> bv. 1 str. 16M]	-	261	41956	4,97	21	8
06	D054	EFG36498.1	thioredoxin [<i>Brucella</i> spp. NVSL 07-0026]	Post-translational modification, protein turnover, and chaperones	138	21288	4,78	31	8
07	D055	WP_004692061.1	phosphonate ABC transporter substrate-binding protein [<i>Brucella</i>] MULTISPECIES	-	137	37395	5,17	16	4
	D056	WP_004692061.1	phosphonate ABC transporter substrate-binding protein [<i>Brucella</i>] MULTISPECIES	-	116	37395	5,17	23	7
08	D057	WP_006134463.1	hypothetical protein [<i>Brucella canis</i>]	-	108	33284	5,32	31	6

Protein No.	Spot No.	NCBI Acc. No	Protein name	Protein function	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
09	D063	EEY29746.1	cationic amino acid ABC transporter [<i>Brucella suis</i> bv. 5 str. 513]	Amino acid transport and metabolism	386	30547	5,25	36	9
10	D065	WP_006641394.1	electron transfer flavoprotein subunit alpha/FixB family protein, partial [<i>Brucella</i>] MULTISPECIES	Energy production and conversion	68	29705	4,86	21	3
11	D067	WP_004689099.1	D-ribose ABC transporter substrate-binding protein [<i>Brucella suis</i>]	Carbohydrate transport and metabolism	625	33297	5,11	38	9
	D068	WP_004689099.1	D-ribose ABC transporter substrate-binding protein [<i>Brucella suis</i>]		660	33297	5,11	38	9
12	D078-1	EEW91094.1	extracellular solute-binding protein family 3 [<i>Brucella suis</i> bv. 4 str. 40]	-	395	28698	5,38	55	12
	D064	AEW13370.1	lysine-arginine-ornithine-binding periplasmic protein [<i>Brucella canis</i> HSK A52141]	-	265	36678	5,09	26	8
13	D078-2	WP_006165687.1	lysine-arginine-ornithine-binding periplasmic protein [<i>Brucella</i> spp. 63/311]	-	394	29085	5,39	54	12
	D081-1	WP_006165687.1	lysine-arginine-ornithine-binding periplasmic protein [<i>Brucella</i> spp. 63/311]	-	328	29085	5,39	42	8
14	D079-1	EEW91094.1	extracellular solute-binding protein family 3 [<i>Brucella suis</i> bv. 4 str. 40]	-	391	28698	5,38	54	12

Protein No.	Spot No.	NCBI Acc. No	Protein name	Protein function	MOWSE Score	MW (Da)	pI	% seq. Coverage	No of peptides matching
15	D079-2	WP_006072682.1	amino acid ABC transporter substrate-binding protein [Brucella] MULTISPECIES	-	391	29067	5,57	53	12
	D081-2	WP_006072682.1	amino acid ABC transporter substrate-binding protein [Brucella] MULTISPECIES	-	328	29067	5,57	42	8
16	D082	WP_012219776.1	MetQ/NlpA family lipoprotein [Brucella canis]	Inorganic ion transport and metabolism	325	30535	5,32	39	10
17	D130	WP_006132662.1	DUF2059 domain-containing protein [Brucella canis]	Unknown function	221	18820	6,75	42	6
18	D140	WP_006172154.1	RidA family protein [Brucella] MULTISPECIES	-	128	12320	5,63	50	5
	D142	WP_009363205.1	RidA family protein [Brucella] MULTISPECIES	-	87	12352	5,63	43	3
19	D143	WP_008936403.1	molecular chaperone GroES [Brucella] MULTISPECIES	Translation, ribosomal structure and biogenesis	138	10401	5,41	50	5
	D146	WP_008936403.1	molecular chaperone GroES [Brucella] MULTISPECIES	Translation, ribosomal structure and biogenesis	104	10401	5,41	30	3
20	D144	WP_004683653.1	DUF1476 domain-containing protein [Brucella] MULTISPECIES	Transcription	196	12185	4,84	80	12
	D145	WP_008505508.1	DUF1476 domain-containing protein [Brucella] MULTISPECIES		206	12155	4,84	51	9
21	D171	WP_069713965.1	glycine/betaine ABC transporter substrate-binding protein [Brucella spp. B13-0095]	-	100	31933	5,57	21	4

5. FINAL COMMENTS

Canine brucellosis is considered a neglected zoonotic infection that is endemic in Brazil. The majority of the studies that have been conducted on this theme are directed to the improvement of the laboratory diagnosis, since the accurate detection of the infected dogs is the primary measure to control the infection. Besides the improvement of the diagnosis, epidemiological studies are necessary to better understand the dynamic of the infection in a population.

The identification of infected dogs is an important tool for the public health, since brucellosis is a zoonotic infection (CASTRILLÓN-SALAZAR *et al.*, 2013; MARZETTI *et al.*, 2013; JAVERI *et al.*, 2014; KRUEGER *et al.*, 2014; DENTINGER *et al.*, 2015) and the human-canine bond is becoming stronger day by day (WALSH, 2009).

In this study, a description of a brucellosis outbreak in a Brazilian kennel was conducted including a multi-approach characterization of the bacteria that could help to better understand the epidemiological features of the infection. The identification, followed by the characterization of the bacteria involved in an outbreak are important actions in order to draw up measures to control the infection and to prevent its transmission among dogs and towards humans.

An important contribution of this study was the evaluation of the protein profile of *B. canis* isolates and the identification of immunogenic proteins potentially able to differentiate infected from healthy dogs, including the identification of chronically infected (non-bacteremic) dogs. The immunogenic proteins found both in bacteremic and non-bacteremic dogs and not found in non-*B. canis* infected dogs, are candidates as specific antigens for serodiagnosis and should be tested in serological platforms for canine brucellosis diagnosis in order to overcome one of the main drawbacks regarding brucellosis control that relies on the lack of accurate diagnostic tests. Moreover, they could be tested for the development of a vaccine against canine brucellosis.

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