Proteomics and bioinformatics investigations to improve serological diagnosis of canine brucellosis

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January 30, 2024

Abstract

Brucella canis is pathogenic for dogs and humans. Serological diagnosis is a cost-effective approach for disease surveillance, but a major drawback of current serological tests is the cross-reactivity with other bacteria that results in false positive reactions, and development of indirect tests with improved sensitivity and specificity remain a priority. A western blotting assay was developed to define the serum antibody patterns associated to infection using a panel of positive and negative dog sera. B. canis positive sera recognized immunogenic bands ranging from 7 to 30 kDa that were then submitted to ESI–LC-MS/MS and analyzed by bioinformatics tools. A total of 398 B. canis proteins were identified.. Bioinformatics tools identified 16 non cytoplasmic immunogenic proteins predicted as non-homologous with the most important Brucella cross-reactive bacteria and 9 B. canis proteins non-homologous to B. ovis; among the latter, one resulted non-homologous to B. melitensis. The western blotting test developed was able to distinguish between infected and non-infected animals and may serve as confirmatory test for the serological diagnosis of B. canis. The mass spectrometry and in silico results lead to the identification of specific candidate antigens that pave the way for the development of more accurate indirect diagnostic tests.

Research Paper

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Abstract

Brucella canis is pathogenic for dogs and humans. Serological diagnosis is a cost-effective approach for disease surveillance, but a major drawback of current serological tests is the cross-reactivity with other bacteria that results in false positive reactions, and development of indirect tests with improved sensitivity and specificity remain a priority. A western blotting assay was developed to define the serum antibody

patterns associated to infection using a panel of positive and negative dog sera. *B. canis* positive sera recognized immunogenic bands ranging from 7 to 30 kDa that were then submitted to ESI–LC-MS/MS and analyzed by bioinformatics tools. A total of 398*B. canis* proteins were identified.. Bioinformatics tools identified 16 non cytoplasmic immunogenic proteins predicted as non-homologous with the most important *Brucella* cross-reactive bacteria and 9 *B. canis* proteins non-homologous to *B. ovis* ; among the latter, one resulted non-homologous to *B. melitensis* . The western blotting test developed was able to distinguish between infected and non-infected animals and may serve as confirmatory test for the serological diagnosis of *B. canis* . The mass spectrometry and *in silico* results lead to the identification of specific candidate antigens that pave the way for the development of more accurate indirect diagnostic tests.

Keywords: Bioinformatics, *Brucella canis* ; Western blotting; Mass spectrometry; Protein identifi-cation

Significance Statement

Diagnosis of canine brucellosis caused by *B.canis* involves both direct and indirect methods, but these tests have low specificity. Development of more sensitive and specific serological tests for the diagnosis of infections caused by *B. canis* is needed.

In the present study a western blotting assay has been developed in order to define proteomics pattern associated to *B. canis*infection using a panel of sera from dogs naturally infected and non-infected with *B. canis* . A combined immunoproteomics and bioinformatics approach was used to identify a set of immunogenic proteins. Immunogenic bands ranging from 7 to 30 kDa were submitted to ESI–LC-MS/MS and a total of 398 *B. canis* proteins were identified. These proteins were analyzed by bioinformatics and were predicted a set of *B. canis* specific candidate antigens that could be used for development of more efficient diagnostic tests. In the view to improve the diagnosis of canine brucellosis due to *B. canis* possible applications of project results are discussed.

1. Introduction

Brucellosis is a chronic bacterial disease affecting both animals and humans caused by Gram-negative coccobacilli of the genus*Brucella* . This genus includes several species responsible for infection in livestock (*B. melitensis* , *B. abortus* ,*B. ovis* , *B. suis*) but also in companion animals (*B. canis*) [1]. Canine brucellosis is mainly caused by *B. canis* . Brucellosis due to *B. abortus* or *B. melitensis* is diagnosed sporadically in dogs living in contact with infected ruminants, but in these cases it represents only an epiphenomenon of the infection circulating in the affected farm, and the dog does not play the role of reservoir of the disease. The same applies to dog brucellosis due to *B. suis* , which is rarely identified in dogs and, in any case, always in connection with a coexisting infection in pig farms or after exposition to infected feral pigs and boars [2, 3, 4]. *B. canis* was first isolated in 1966 in USA from aborted fetuses in a kennel experiencing several cases of abortion and infertility [5]. Later, *B. canis* infection has been demonstrated by isolation or serological investigations in several countries worldwide [6]. In Italy, the presence of anti-*B. canis* antibodies in dogs was reported occasionally for a long period in the past [7-14] and, in one case [15] *B. canis* was detected by PCR in a dog with prostatitis and discospondylitis. In 2020, for the first time, *B. canis* was isolated in a commercial breeding kennel in central Italy [16].

Dogs and wild canids are thought to be the only significant hosts for*B. canis* among domesticated animals, while cattle, sheep and swine were found to be highly resistant to the infection. The natural pathways of transmission of canine brucellosis are different, but the most common is the contact with placenta, fetal tissues and vaginal discharges resulting from abortion. Infected female may transmit*B. canis* through placenta, aborted infected fetuses, or vaginal discharges following an abortion, through contact with the mucous membranes of the host organism [2].

Common symptoms are infertility, abortions, neonatal mortality, epididymitis, prostatitis, discospondylitis and uveitis [17]. However, infected but asymptomatic animals are frequently observed [5].

Due to silent symptoms, the disease spreads uncontrollably, before being diagnosed, causing big economic damages in breeding kennels and problem in assuring proper animal welfare. In dogs, therapeutic treat-

ment with antibiotics is not encouraged and antibiotic therapy does not completely eliminate the pathogen, resulting in high risk of disease transmission to other dogs and humans [5].

Humans can get *B. canis* infection through direct contact with infected dogs, in particular with aborted fetuses, and secretions and blood [18, 19], and they can develop clinical disease. The disease can be asymptomatic and chronic; the symptoms are nonspecific and may vary from fever to severe manifestations such as endocarditis, osteomyelitis, and septicemia [17]. Kennel employees, veterinarians, laboratory technicians, children and elderly and immunocompromised people have higher risk to be infected by *B. canis* [5].

Diagnosis of canine brucellosis involves both direct and indirect methods. The isolation of *B. canis* , meanly from blood culture, gives confirmation of the infection while use of serological tests may represent a more cost-effective approach for disease surveillance.*B. canis* carries rough LPS, so serological tests currently available for the diagnosis of brucellosis caused by smooth*Brucellae* (*B. melitensis* , *B. abortus* , *B. suis*) cannot be used for the diagnosis of the disease caused by*B. canis* [1, 5].

The first serological tests developed for canine brucellosis were the rapid slide agglutination test (RSAT) and rapid slide agglutination test with 2-Mercaptoethanol (2ME-RSAT) [20]. However, since the beginning it was noted a lack of specificity of these tests, counting for false positive rates that commonly range from 20% up to even 50% [21].

To increase efficacy of serological diagnosis, the use of more than one test in parallel has been suggested such as reported 2ME-RSAT as screening tests, and indirect ELISA (i-ELISA) as a confirmatory test. These tests have sensitivity ranging from 40 to 90% and specificity between 60 and 100% [17]. Other diagnostic tests reported are the agar gel immunodiffusion (AGID), the tube agglutination test (TAT), the microagglutination test (MAT) and the complement fixation test (CFT) [22].

All these tests suffer from lack of knowledge in accuracy, with only limited data available in the international literature. In addition, non-specific reactions are known with haemolysed sera or due to cross-reactions with other bacteria, such as *Pseudomonas* spp.,*Bordetella bronchiseptica* , *Streptococcus* spp.,*Staphylococcus* spp., *Salmonella* spp., *Yersinia enterocolitica* , *Escherichia coli* and *Actinobacillus equuli* [6, 22]. Finally, most of the serological tests are not available as commercial kits, raising the issue of antigen production and test standardization, especially due to the lack of international reference sera for *B. canis* .

Serological tests for the diagnosis of smooth *Brucellae* (*B. abortus* , *B. melitensis* and *B. suis*) infection use the O-polysaccharide (OPS), an immunodominant epitope in smooth lipopolysaccharide (s-LPS), as antigen; consequently, cross-reactions with other Gram-negative bacteria, such as *Y. enterocolitica* O:9, which shows analogous OPS structures, can occur [23]. *B. canis* , similarly to *B. ovis* and *B. abortus* strain RB51, has rough lipopolysaccharides (r-LPS) on its bacterial wall. The diagnosis of ovine brucellosis caused by *B. ovis* is performed using the homologous rough-specific antigen, obtained by extraction with the hot-saline method. This antigen is enriched in r-LPS [24]. Since*B. ovis* , *B. canis* and *B. abortus* strain RB51 shares similar antigenic components, each of the three species may be employed as antigen for the serological diagnosis of brucellosis caused by rough*Brucella* species [25-27]. Numerous studies have been done on smooth *Brucella* species as *B. abortus* and *B. melitensis* to identify *Brucella* unique proteins suitable as antigens for the development of more specific serological tests [17, 28-32]. Only a few studies were focused on the characterization of immunogenic proteins of rough *Brucellae* . Recently, identification of *B. canis* immunogenic proteins by proteomics and bioinformatics analyses was reported. Two recombinant cytoplasmic proteins were expressed, and tested as antigens in i-ELISA assay to detect human and canine brucellosis, but they were not able to detect canine brucellosis with high specificity and sensitivity [17].

All these considerations highlight the need for development of more sensitive and specific serological tests, as well as new protocols for the diagnosis of infections caused by *B. canis.*

In the present study a western blotting assay has been developed to define the serum antibody patterns associated to *B. canis*infection using a panel of sera from dogs naturally infected and non-infected with *B. canis* . Then LC-ESI- -MS/MS analyses and bioinformatics tools have been combined to identify a set

of immunogenic proteins predicted as *Brucella* specific. Finally, possible applications of project results are discussed in the view to improve the diagnosis of canine brucellosis due to *B. canis* .

2. Materials and Methods

2.1. Serum panel

Sera from 32 *B. canis* naturally infected dogs were collected from an outbreak occurred in a breeding kennel of Central Italy during summer 2020. The positivity to *B. canis* was confirmed by isolation of the bacterium from blood cultures. Negative sera were collected from 26 healthy dogs, which were not related to *B. canis* outbreaks. Sera were collected by local veterinary services according to Italian and European regulations for animal welfare.

2.2. Bacterial strains and growth conditions

B. canis strain RM6/66 (ATCC 23365) was grown in glycerol-dextrose agar and incubated in aerobic atmosphere for 48-72 h. Bacteria were collected, resuspended in sterile deionized water, heat-inactivated at 60 °C for 2 h and centrifuged at 3500 g for 30 min. The pellet was then washed 3 times with deionized water, dissolved in 0.2 M Tris–maleate, pH 9.0, at ratio 1:5, mixed for 2 h at room temperature (RT) with stirring and then stored at 5 ± 3 °C for 90 days before use. The antigen was then titrated and further diluted with 0.2 M Tris–maleate, pH 9.0, to obtain a ready-to-use antigen.

2.3 Western Blotting

B. canis RM6/66 (ATCC 23365) proteins were dissolved in SDS-PAGE denaturing buffer (Life Technologies), loaded into NuPAGE® 4-12% Bis-Tris gels (Life Technologies) and separated at constant voltage (200 V). Then, proteins were blotted on nitrocellulose membranes (Life Technologies) using iBlot2 Dry Blotting System (Life Technologies) at 20 V for 1 min, 23 V for 4 min and 25 V for 2 min. Membranes were blocked with 5% skim milk in 0.01 M PBS, pH 7.2, containing 0.05% Tween 20 (PBST) for 2 h at RT. Then, membranes were incubated overnight (ON) at RT with canine sera diluted 1:5000 in PBST containing 2.5% skim milk. After three washes with PBST for 10 min, membranes were incubated for 1 h at RT with Protein A-HRP (Sigma) diluted 1:5000 in PBST containing 2.5% skim milk. After three washes with PBST and one final wash with PBS for 10 min, immune complexes were detected by chemiluminescence (ECL Western Blotting Detection Kit, GE Healthcare) using the Chemidoc MP (Bio-Rad); analyses were performed using Image Lab Software version 4.0.1 (Bio-Rad).

2.4 Mass spectrometry analysis (nLC-ESI-MS/MS)

B. canis RM6/66 (ATCC 23365) proteins were separated using a NuPage 4-12% Bis-Tris pre-cast gel (Life Technologies) at 200 V. Then proteins were stained with SimplyBlue SafeStain (Life Technologies). Stained gel was stored in 0.5% acetic acid at 4°C until protein analysis. Based on pattern profile identified by western blotting, two slices in the range 7-30 kDa were excised from the gel and the proteins were identified by nLC-MS/MS (Orbitrap QExactive-HF, Thermo Fisher) as previously reported [33-35].

Briefly, reduction with 10 mM DTT, alkylation with 55 mM IAA and trypsin digestion overnight at 37 °C were carried out as previously reported [36]. Five µl of peptides were injected on an UPLC EASY-nLC 1000 (Thermo Scientific) and separated on a homemade fused silica capillary column (75 µm i.d., length 25 cm), packed in house with ReproSil-Pur C18-AQ 1.9 µm beads (Dr. Maisch, Ammerbuch-Entringen, Germany). A gradient of eluents A (2% acetonitrile, 0.1% formic acid) and B (80% acetonitrile with 0.1% formic acid) was used to achieve separation, from 5% to 100% B (in 30 min, 250 nL/min flow rate). The nLC system was connected to a quadrupole Orbitrap QExactive-HF mass spectrometer (Thermo Fisher) equipped with a nano-electrospray ion source (Proxeon Biosystems). Top 15 method was applied. Raw data were processed with Proteome Discoverer (version 1.4.1.14, Thermo Scientific) and Mascot (version 2.6.0, Matrix Science) searching against B. canis, assuming a fragment ion mass tolerance of 20 ppm and a parent ion tolerance of 10 ppm; specified enzyme was trypsin; carbamidomethylation of cysteine was set as a fixed modification; oxidation of methionine and acetylation of the N-terminus of proteins were set as variable

modifications. Scaffold (version 4.8.9, Proteome Software Inc.) was used to validate MS/MS based peptide and protein identifications. Only proteins with greater than 99.0% probability and containing at least 3 peptides (greater than 95% probability) were accepted. Therefore, only proteins detected in at least 2 out of 3 biological replicates were included in bioinformatics analysis.

2.5. Bioinformatics analysis

Data generated by mass spectrometry analyses were then submitted for bioinformatics analysis for protein identification and selection. As first step a combination of softwares were applied to identify cytosolic and non-cytosolic proteins. LipoP 1.0 Server [37] was used for prediction of lipoprotein signal peptides; TMHMM Server version 2.0 was predictive of transmembrane helices [38, 39] and SignalP 4.1 Server [40] was applied for signal peptides prediction. PSORTb version 3.0.2 [41] and CELLO version 2.5 [42, 43] predicted subcellular localization. The data obtained from the above software analyses were combined to discard cytosolic proteins.

Non–cytosolic proteins were further analyzed to predict B-cell linear epitopes by BepiPred version 1.0 Server [44] by setting a threshold equal or higher than 0.35 and a minimum length of 4 residues. NetSurfP version 1.1 server [45] was used to predict the surface accessibility of an amino acid and protein secondary structure. The epitopes not exposed to the solvent were discarded. The proteins with B-cell solvent-exposed epitopes were further analyzed by Vaxign [46-47] and VaxiJen tools [49] for prediction of protective antigens. Only the proteins with adhesion score greater than 0.5 (Vaxign tool) and those with threshold greater than 0.4 (VaxiJen) were considered as candidate antigens.

As a final step, the potential antigenic proteins of *B. canis*resulting from the above bioinformatics analyses were screened by BLASTp to check for sequence similarity with other *Brucella* species and cross-reactive bacteria. Among the genus *Brucella* , *B. melitensis* , *B. ovis* , *B. abortus* and *B. suis* were considered. Cross-reactive bacteria included *Pseudomonas aeruginosa* , *Bordetella bronchiseptica* , *Actinobacillus equuli* , *Streptococcus* spp., *Staphyloccus* spp.,*Moraxella* type, *Salmonella* spp. and *Campylobacter*spp. [50], the environmental bacterium *Ochrobactrum anthropi*and the plant pathogens or symbionts *Rhizobium leguminosarum* ,*Rhizobium /Agrobacterium* group and *Rhizobium tropici*[1]. The criteria used to identify non-homologous proteins were: identity and/or coverage lower than 95% for *Brucella* species and 35% for cross reactive bacteria.

3. Results

3.1 Western blotting

Serum antibodies from 31 out of 32 B. canis infected animals identified common bands ranging from 7 to 30 kDa, in contrast to serum antibodies from non-infected animals, where no bands or bands ranging from 40-200 kDa (3 animals only) were observed (Figure 1).

3.2 Mass spectrometry (nLC-ESI-MS/MS) and bioinformatics analysis

Two gel slices containing B. canis proteins ranging from 7 to 30 kDa were excised and analyzed by mass spectrometry analysis (Figure 1) and 398 B. canis proteins were identified. Some proteins were present in more than one band, therefore the repeated proteins were discarded. The workflow adopted for the prediction of protein candidates is shown in Figure 2.

Among the 398 identified proteins, 245 (61.3%) proteins were cytoplasmic and 153 (38.7%) non-cytoplasmic. Hence, the study focused on non-cytoplasmic proteins, as they are involved in pathogenesis and survival of Brucella in macrophages [51].

These proteins were examined to identify B-cell solvent-exposed epitopes (Supplementary Table 1): 145 proteins were identified and further investigated by Vaxign and VaxiJen tools to predict antigens. Forty-seven proteins had adhesion score greater than 0.5 when analyzed by Vaxign tool, and 123 proteins had threshold greater than 0.4 by VaxiJen tool. Overall, 126 proteins were predicted as potential antigens: 44 proteins were predicted as protective antigens by both softwares, 3 proteins only by Vaxign and 79 proteins only by VaxiJen tool.

Then BLAST was used to verify similarity among the 126 B. canis potential antigen proteins and proteins of other species of Brucella as well as cross-reactive bacteria.

As expected, all B. canis proteins resulted homologous to B. abortus and B. suis. Nine B. canis proteins are non-homologous to B. ovis and, among them, one was found non-homologous to B. melitensis. As the sequence homology present among the Brucella species is very high, the criterion used to identify non-homologous proteins were 95% identity.

Sixteen B. canis proteins were found to be non-homologous to all cross-reactive bacteria examined (P. aeruginosa, B. bronchiseptica, A. equuli, Streptococcus spp., Staphyloccus spp., Moraxella type, Salmonella spp. and Campylobacter spp,). According to Uniprot, 7 proteins are included in the following categories: one is an integral component of membrane, one has oxidoreductase activity, one is mitochondrial respiratory chain complex I assembly, one is a membrane protein, one has phosphatidylserine decarboxylase activity and phosphatidylethanolamine biosynthetic process and for two proteins no category was assigned. Nine proteins are uncharacterized, even if for two of them it was possible to assign gene ontology (integral membrane components). Regarding environmental and plant pathogens/symbionts cross-reactive bacteria (Rhizobium and Agrobacterium), 2 proteins are non-homologous to all cross-reactive bacteria examinated and among them one is also non-homologous to all cross-reactive bacteria; the other one is uncharacterized protein.

4. Discussion

In this study a western blotting assay was set up in order to identify the *B. canis* protein pattern recognized by serum antibodies from infected dogs. The test clearly showed that IgGs of infected animals selectively bind to some *B. canis* proteins of low molecular weight (7-30 KDa) not recognized by antibodies of non-infected dogs, so the western blotting may serve to distinguish infected from non-infected animals.

Use of western blotting method as diagnostic test, mainly confirmatory test, has been reported for serological diagnosis of other animal diseases, such as Contagious Bovine Pleuropneumonia in cattle [52, 53] or Dourine in horses [54]. The use of western blotting to characterize antibody response against *B. canis* antigen has been described in the past [55] and more recently Barkha et al. (2011) [56] showed that dog anti-*B. canis* hyperimmune sera identified low molecular weight immune reactive bands of *B. canis*external (12, 28, 39 and 45 kDa) and internal antigens fractions (20-24 kDa). Results obtained in the present work also support these findings with some differences in the molecular range of the immune reactive bands identified that in our case was restricted to 7-30 kDa. The difference in *B. canis* strain, the antigen preparation procedure used in this study together with the application of chemiluminescence to reveal immunoreactivity might have contributed to the observed variations. Though these encouraging results, western blotting was never applied for serological diagnosis of *B. canis* on a large scale. Our results, in addition to previous findings, encourage a field applicability of western blotting, mainly as confirmatory test of doubtful cases, where epidemiological evidences of *B. canis*infection do not support serological positivity to other indirect tests.

The second step of this study was focused on characterizing the protein composition of immunodominant bands identified by IgGs antibodies of*B. canis* infected dogs, in order to find potential diagnostic antigenic biomarkers to be used as antigens for new recombinant diagnostic tests specific for canine brucellosis. The low molecular weight protein pattern specifically recognized by sera of infected dogs was then characterized by mass spectrometry, identifying 398 *B. canis* proteins. Among them, an *ad hoc* developed bionformatics pipeline identified 126 potential antigens and then 16 *B. canis*potential specific targets were selected after screening for non-cytosolic, immunogenic, non-cross-reactive proteins.

In a recent study, Jimenez and coworkers (2020) [17] carried out identification and characterization of immunoreactive proteins focusing on the cytoplasmic (internal) fraction of *B. canis* that led to the expression of two recombinant target antigens with limited sensitivity and specificity. In our study, we targeted non-cytosolic proteins located on the membrane/external part of the bacteria that have higher chance to be involved in host-pathogen interactions and to be immunogenic. Starting from the set of proteins identified by mass spectrometry, bioinformatics analyses recognized 126 non-cytosolic proteins potentially immunogenic, with some proteins already describe in the literature. One of the protein identified was the outer

membrane protein assembly factor BamD (A9M681), a conserved multi-component protein complex that is responsible for the biogenesis of β-barrel outer membrane proteins (OMPs) in Gram-negative bacteria. BamD deletion causes lethality in *E. coli* and *Neisseria meningitidis* , and Bam has a role in the production of OMPs for survival and pathogenesis [57]. Proteins Omp25, Omp31 and SodC were also identified: these proteins have been well characterized as virulence factors or immunogenic proteins in *Brucella* ; further these proteins were identified in outer membrane vesicles (OMVs) in *B. canis*[58]. The protein Sod (Superoxide dismutase [Cu-Zn]) is associated to virulence in a number of microorganisms [31]. Omp31 appears as an immunodominant antigen in the course of "rough" (R)*B. ovis* infection in rams and as important protective antigen for*B. ovis* infection in a mouse model. Omp25 is involved in virulence of *B. melitensis* [59], moreover *B. suis*Omp25 suppresses production of TNFα, crucial to clear *B. suis*infection [60]. It was shown that Omp25 and Omp31 induce protection against *Brucella in vivo* and could be a potential subunit brucellosis vaccines candidate [61]. The proteins SodC, Omp25 and Omp31 were also identified on membrane blebs isolated from *B. abortus* 2308 and RB51. Mice vaccinated with membrane blebs from rough or smooth *B. abortus* showed a protective immune response similar to the one elicited by vaccine *B. abortus* RB51 after the challenge with virulent strain *B. abortus* 2308, suggesting that these proteins could be good candidate for vaccine against brucellosis [62]. In another study in mice, Clausse *et al.* (2014) [63] showed that immunization with Omp31 is effective against*B. canis* infection.

Recently, in a study of Paci *et al.* (2020) [34] *B. ovis* Omp31 and *B. melitensis* Omp25 were indicated as good candidate antigens for development of *Brucella* specific serological tests and vaccines.

One of the major drawbacks of current serological tests for *B. canis* is the cross-reactivity with other bacteria that results in false positive reactions in the course of serological testing [6, 22]. Thus, it is important to assess the cross-reactivity of potential target antigens. In theory, an experimental laboratory approach would have required the screening of all candidate antigens identified, expressed as recombinant antigens, against cross-reactive sera. However, the high number of antigens identified and the lack of reference hyperimmune sera against the different cross-reactive bacteria imposed an alternative, time-saving and economically sustainable strategy. Thus, bioinformatics analyses were used to discard all the non-cytosolic immunogenic proteins showing an identity higher than 35% with any of the cross-reactive bacteria. This led to the exclusion of 87% of potential candidate proteins ascertained, narrowing the number of optimal targets but also, confirming the high homology of several *B. canis* proteins with the bacteria responsible for cross-reactive immunity. Among the 16*B. canis* specific proteins finally predicted, chaperone surA protein was identified, that is reported to be a protective antigen of*B. abortus* 104M [64]. For the remaining proteins, no functional information are described in the literature and some of them resulted uncharacterized. One of the major limitations of the *in silico* approach described in this study is that, despite the accuracy adopted in combining the different bioinformatics softwares, results generated are predictive and requires subsequent laboratory confirmation.Canine brucellosis caused by *B. canis* is nowadays considered an emerging and zoonotic disease and the increased trade and movement of dogs worldwide is imposing the application of measures to prevent, monitor and control disease spread within and across Countries. Diagnosis of *B. canis* relies on the analysis and interpretation of epidemiological data and together with laboratory results of direct and indirect tests. However, serological tests still represent the most cost/effective tools for disease surveillance and the diagnosis of*B. canis* in humans are lacking. Based on the results of the present study the western blotting test is able to distinguish between infected and uninfected animals and could be used as confirmatory test for the serological diagnosis of *B. canis* . The mass spectrometry and *in silico* results lead to the identification of a set of*B. canis* specific candidate antigens that pave the way for the development of more efficient diagnostic tests.

Author Contributions: Conceptualization, L.M. and I.K.; methodology, L.M., T.D.F., F.D.O. and I.K.; software, I.K.; validation, I.K.; formal analysis and investigation, L.M., T.D.F., F.D.O.., F.P. and I.K.; resources, F.S., F.D.M. and M.T.; data curation, L.M., T.D.F., F.P. and I.K.; writing—original draft preparation, M.L., I.K., T.D.F.; writing—review and editing, F.S., M.T. and F.D.M.; supervision, F.S., M.T. and F.D.M.; project administration, F.S., M.T. N.D.A. and F.D.M.; funding acquisition, F.S., M.T. N.D.A. and F.D.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Italian Ministry of Health, grant number IZSAM 02/20 RC (BruM-Life project).

Data Availability Statement: The data presented in this study are openly available in UniProt.

Conflicts of Interest: The authors declare no conflict of interest.

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Figure 1. SDS-PAGE and western blot analyses. Representative image of SDS-PAGE and Coomassie stain of *B. canis* total proteins (lane 1) and immunoblot using sera from *B. canis* infected (lane 2) and non-infected dogs (lane 3). The proteins were separated on a 4–12% Bis-tris gel (Life Technologies). M: molecular weight marker 10-260 KDa (Novex Sharp Prestained Protein Standard, Life Technologies).

Figure 2. Overview of bioinformatics tools used for prediction of protein candidates.

Supplementary Materials

Supplementary Table S1 : *Brucella canis* proteins identified by LC-ESI_MS/MS and subcellular localization prediction. Non cytoplasmic proteins are presented in bold.

Supplementary Table S2 : Detection of homologs of*Brucella canis* potential antigens with the most important*Brucella* cross-reactive bacteria and *Brucella* species by BLAST. Non homologous proteins to *Brucella* cross-reactive bacteria are highlited in grey, while to the *Brucella* cross reactive are presented in bold. The criteria used to identify non-homologous proteins were: identity lower than 95% for*Brucella* species and 35% for cross reactive bacteria.