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Research article

# Diagnosis of human and canine *Brucella canis* infection: development and evaluation of indirect enzyme-linked immunosorbent assays using recombinant *Brucella* proteins

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ABSTRACT

*Brucella canis*, a Gram-negative coccobacilli belonging to the genus *Brucellae*, is a pathogenic bacterium that can produce infections in dogs and humans. Multiple studies have been carried out to develop diagnostic techniques to detect all zoonotic *Brucellae*. Diagnosis of *Brucella canis* infection is challenging due to the lack of highly specific and sensitive diagnostic assays. This work was divided in two phases: in the first one, were identified antigenic proteins in *B. canis* that could potentially be used for serological diagnosis of brucellosis. Human sera positive for canine brucellosis infection was used to recognize immunoreactive proteins that were then identified by performing 2D-GEL and immunoblot assays. These spots were analyzed using MALDI TOF MS and predicted proteins were identified. Of the 35 protein spots analyzed, 14 proteins were identified and subsequently characterized using bioinformatics, two of this were selected for the next phase. In the second phase, we developed and validated an indirect enzyme-linked immunosorbent assays using those recombinant proteins: inosine 5′ phosphate dehydrogenase, pyruvate dehydrogenase E1 subunit beta (PdhB) and elongation factor Tu (Tuf). These genes were PCR-amplified from genomic DNA of *B. canis* strain Oliveri, cloned, and expressed in *Escherichia coli*. Recombinant proteins were purified by metal affinity chromatography, and used as antigens in indirect ELISA. Serum samples from healthy and *B. canis*-infected humans and dogs were used to evaluate the performance of indirect ELISAs. Our results suggest that PdhB and Tuf proteins could be used as antigens for serologic detection of *B. canis* infection in humans, but not in dogs. The use of recombinant antigens in iELISA assays to detect *B. canis*-specific antibodies in human serum could be a valuable tool to improve diagnosis of human brucellosis caused by *B. canis*.

## 1. Introduction

Brucellosis, one of the most widespread zoonosis in the world [1], is caused by several generally accepted species of Gram-negative coccobacilli that belong to the genus *Brucella* [2]. These facultative intracellular pathogens [3] can infect a wide range of mammals; however, their host preference and pathogenicity may vary [4]. The most studied zoonotic *Brucella* species are *Brucella melitensis, B. abortus* and *B. suis* [5]. Nonetheless, there are additional species of zoonotic concern, such as *Brucella canis* whose main reservoir are dogs. Canine brucellosis can be venereally or orally transmitted by contact with infected secretions [6]. In dogs, its symptoms, which are not as severe as other *Brucella* infections, include embryonic mortality, abortions, neonatal morbidity and mortality, epididymitis, prostatitis, discospondylitis and uveitis, infertility in both genders, among others [7, 8, 9]. Canine brucellosis is also a zoonotic disease. In humans, *B. canis* infection occurs through contact with contaminated secretions from infected dogs, or as result of bad laboratory handling [6, 10, 11]. Similar to brucellosis caused by

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*B. abortus* or *B. melitensis* infection, in humans, the disease can be asymptomatic [10] or chronic; it may take months, even years, before symptoms appear [11]. In humans, its symptoms are nonspecific and may vary from undulant or persistent fever, to severe manifestations such as endocarditis, osteomyelitis and septicemia [6, 12, 13, 14]. In addition, some cases are under-reported likely due to lack of specific symptoms and of accurate diagnostic techniques [15].

*B. canis* infection is initially diagnosed using a rapid slide agglutination test with 2-Mercaptoethanol (2ME-RSAT), which is a screening test that detects total antibodies against the bacterium. An indirect ELISA test (iELISA) to detect the level of antigen-specific IgG or IgM antibodies [16] is recommended as a confirmatory test. The gold standard, however, is blood culture, but this test has reduced sensitivity, as the bacteria can be isolated most often from acute, but not from chronic cases of infection [16]. Additional tests such as Polymerase Chain Reaction (PCR) [17], are often used to confirm the species of the *Brucella* isolates. Currently available serological tests, such as 2ME-RSAT and iELISA, exhibit some issues as they have variable sensitivity (ranging from 40 to 90%) and specificity (between 60-100%) [16, 18, 19, 20, 21]. This could be explained by the difficulties to obtain specific immunogenic antigens to be used to detect IgG or IgM antibodies in serum of infected hosts.

Humoral immunity induced by *B. canis* infection in humans is poorly characterized, representing a challenge for the development of diagnostic tests. While this type of immunity has been mainly studied in *B. melitensis* and *B. abortus* infections [22], the conclusions of these studies cannot be extrapolated to *B. canis* infections, since this bacteria is a rough species, while the former two are phenotypically smooth.

Diagnostic tests that detect infection with smooth *Brucella* species mainly utilize smooth LPS as the antigen. Infection with rough species cannot be detected by tests that use as diagnostic antigen smooth LPS. Consequently, while previous studies have identified some proteins as diagnostic antigens for serological detection of brucellosis [23, 24], the use of these antigens in detection of human infection has not been explored. The main challenge in developing tests to detect *B. canis* infection is the identification of immunogenic *Brucella* canis proteins that induce an immune response in all infected animals and humans.

Therefore, multiple studies have been carried out to develop rapid and accurate methods to detect all zoonotic *Brucellae.* Notably, Enzyme-Linked Immunosorbent Assays (ELISA) [25, 26] based on the use of the lipopolysaccharides [27] and recombinant proteins [28, 29] as antigens has been evaluated. The latter is of special interest because of the potential use of species-specific proteins from different *Brucella* species. Numerous antigenic cytoplasmic and membrane proteins have been identified and proposed as candidates for this purpose in *B. abortus* and *B. melitensis* [23, 29, 30, 31, 32, 33, 34, 35, 36]. However, to the best of our knowledge, there are currently no reports of immunoproteomic characterization of antigenic proteins of *B. canis.*

Therefore, in this work we made a two phases study. In the first one, we made the identification by proteomic methods and subsequent molecular analyses using bioinformatics tools, of 14 antigenic cytoplasmic proteins of *B. canis* in humans, obtained from a Colombian bacterial isolate, *B. canis* strain Oliveri and identified 3 proteins that have not been previously reported as being immunoreactive by any author.

In the second phase, we made the expression, production, and purification of two recombinant *B. canis* proteins identified in phase 1 as immunogenic, and then we use them as antigens in indirect ELISA assays to detect human and canine brucellosis using sera samples from 91 humans and 385 canines from two geographical areas of Antioquia, Colombia.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

This study used the genome data from *B. canis* strain Oliveri isolated from a blood culture from a dog in a kennel in Medellín, Colombia, EMBL with accession numbers HG803175.1 and HG803176.1 for chromosome 1 and 2 respectively [37].

Plasmids pGEM®-T easy (Promega), pTZ57 R/T (Thermo Scientific), pRSET-A (Invitrogen) and pET-28a (Life technologies) were used. *Escherichia coli* DH5α cells (New England Labs) and *Escherichia coli* BL21 (DE3) (New England Labs) were used for transformation with the recombinant plasmids. Recombinant bacterial cells were grown routinely in LB broth (Sigma, Madison, WI, USA) and Luria Bertani (LB) agar, and if antibiotic was needed, kanamycin (Sigma, Madison, WI, USA) at 25 μg/ml or ampicillin (Sigma, Madison, WI, USA) at 100 μg/ml was added to the medium.

### 2.2. Protein extraction

To retrieve soluble proteins, a variation of the method proposed by Zhao *et al.* was used [33]. Briefly, cells from 1-liter cultures in stationary growth phase were centrifuged 15 min at 4000 x g at 4 °C, and harvested. The precipitate was washed twice with low-salt washing sample buffer (3 mM KCl, 1.5 mM KH2PO4, 68 mM NaCl, 9 mM NaH2PO4), resuspended in sonication buffer (8 M urea, 1% dithiothreitol (DTT), 4% CHAPS, and one tablet of complete protease inhibitor cocktail in 100 ml) and sonicated on ice. The solution was stored at room temperature for 1 h and then centrifuged at 12,000 x g for 1 h. The supernatant was collected and stored at -70 °C. Total protein concentration was determined using the Bradford protein assay with Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA), using bovine serum albumin (BSA) as standard.

### 2.3. 2D gel isoelectric focusing and western blotting

Regarding isoelectric focusing, aliquots of 100 μg of *B. canis* protein were used in a final volume of 125 μl of rehydration solution (8 M urea, 2% CHAPS, 40 mM DTT and 1% ampholytes); the eluted proteins were applied onto 7 cm immobilized pH nonlinear gradient (IPG) gel strips of pH 4–7 or 3–10 (Bio-Rad, Inc., Hércules, CA, USA).

IEF was performed using the Protean®IEF Cell system (Bio-Rad, Inc.) at 20 °C, to 50 microamper (μA) per strip (μA/strip), using the following parameters: Passive rehydration for 12 h at 20 °C; constant voltage of 50 V for 25 min; gradient from 50 to 500 V for 4 h; gradient from 500 to 1,000 V for 1 h; gradient from 1000 to 3000 V for 1 h; constant voltage of 3000 V up to a voltage equal to or higher than 13550 V for the pH 4–7 strips and 22000 V for the pH 3–10 strips. Analyses were performed in triplicate. The samples on the strips were reduced (10 mg/ml DTT) and alkylated (25 mg/ml iodoacetamide) in 2 ml of equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8.8 and 20% glycerol) for 20 min with shaking.

The second dimension was performed in 1.0 mm-thick and 12% polyacrylamide gels. The strips were sealed with 1% agarose (w/v) containing 0.001% bromophenol blue. For electrophoresis, a Mini Protean® Tetra Cell system (Bio-Rad, Inc., Hércules, CA, USA) was used at room temperature in standard Tris/Glycine/SDS buffer until the bromophenol blue reached the bottom of the gel. Two of the three gels were stained, the first gel was stained with Silver stain and was used to visualize the proteins, the second one used for mass spectrum analysis was stained with Oriole™ Fluorescent gel (Bio-Rad, Inc). Scanner Images of the gels were captured with ImageScannerTM III™ and analyzed on the ImageMaster™ 2D Platinum 7.0 software (GE Healthcare, Upsala, Sweden). A virtual average gel was created using three replicates.

### 2.4. Immunoblots

The third gel was used for immunoblots, this process was made for triplicate; proteins were transferred to nitrocellulose membranes using Towbin buffer (0,025 M Tris, 0.192 M glycine) with 20% ethanol at 100 V for 1 h at 4 °C using the Mini Trans-Blot® module (Bio-Rad, Inc.) [38]. The presence of spots in the membranes was verified using Ponceau stain. The membranes were then washed and blocked with TBS (Tris Buffered

saline pH 7.4; Tris Base and NaCl) plus 5% non-fat milk at 4 °C overnight. The next day, the membranes were washed three times for 5 min with TBS-T (Tris buffer phosphate 1X, pH 7.4 containing 0.5% Tween-20). Immunoblots were incubated in triplicate with each one of the following groups of serum samples, which were collected in previous studies and kept at -20 °C until use: Group 1: Mixture of nine *B. canis*-positive human sera by the 2-mercaptoethanol rapid slide agglutination test (2ME-RSAT); Group 2: Mixture of ten *B. abortus*-positive but *B. canis*-negative human sera by performing the Bengal Rose Test and 2ME-RSAT, respectively; Group 3: Mixture of twenty *B. canis* and *B. abortus*-negative sera by performing the Bengal Rose Test and 2ME-RSAT, respectively, as negative control.

All the participants in this study signed an informed consent form. The ethics committee of the Universidad de Antioquia (Colombia) approved this study, Act No. 11-15-365, June 2011.

Serum samples were mixed and used at 1:500 dilutions in TBS plus 5% non-fat milk; based on previous standardization, using immunoblot assays from 1D-Gel electrophoresis (data not shown). Membranes were incubated with diluted sera, and placed on a shaker for 1 h at 37 °C, washed three times with TBS-T for 5 min, incubated with a previously standardized 1:10000 dilution of anti-human IgG (A3188) or IgM (A3437) alkaline phosphatase goat antibody (Sigma-Aldrich St Louis, MO, USA) in TBS plus 5% milk at 37 °C for 1 h (previously standardized using 1D immunoblot assays; data not shown). Membranes were washed as described above to reveal immunoreactive spots using a BCIP/NBT solution (Amresco, Solon, Ohio USA).

Spots were selected if they met the following criteria: 1) being positive in at least 2 out of three gels treated with sera of *B. canis*-infected humans but not in those treated with *B. abortus*-infected or negative control sera; 2) not having been previously reported as immunoreactive by others authors; and 3) that the signal was intense in the immunoblot. Selected spots were excised from the polyacrylamide gel, sliced into small pieces and sent in sterile distilled water to the Mass Spectrometry Lab at the Biomolecular Resource Facility of the University of Texas Medical Branch (Galveston, USA), where they were processed as follows: each spot was incubated with trypsin (10 μg/ml in 25 mM ammonium bicarbonate, pH 8.0, Promega Corp.) at 37 °C for 6 h. Afterwards, 1 μL of the digested sample was placed on a MALDI plate and allowed to dry. One μL of the matrix compound (acid alpha-cyano-4-hidroxycinamic, Aldrich Chemical Co.) was then added to the sample and allowed to dry.

### 2.5. Mass analysis and protein identification

Subsequently, MALDI TOF MS analysis was carried out using the Applied Biosystems 5800 Proteomics Analyzer for peptide mass fingerprinting and MS/MS analysis. After MALDI analysis, MALDI MS/MS analysis was performed for the 10 most abundant ions of each sample. To identify proteins specifically belonging to the *B. canis,* strain Oliveri, a bioinformatics analysis from raw data was performed. For this purpose, the Mascot against the whole NCBI-nr and SwissProt protein databases was used, and for visualization, the Scaffold (Proteome Software, Inc. Portland, OR, USA) software was used.

The likelihood of protein match was determined using the expected values and the Mascot protein scores. Mascot search parameter values were established as 2 for missed cleavage of variable. MOWSE (Molecular Weight SEarch) scores greater than 83 were considered significant ($P < 0.05$).

Once the proteins were identified in *B. canis* strain Oliveri, they were characterized using multiple bioinformatic tools in order to determine patterns that could influence antibody production. Protein sequences were located and downloaded of the *B. canis* strain Oliveri genome (EMBL accession numbers HG803175.1 and HG803176.1) for further analyses.

### 2.6. Physicochemical properties calculation

To confirm the physicochemical parameters, such as molecular weight and isoelectric point, the ExPASy ProtParam tool was used, submitting the amino acid sequence of each protein identified. (http://web.expasy.org/protparam/) [39].

### 2.7. Subcellular localization prediction

Analysis of protein subcellular localization was initially made using PSORTb v3.0, (http://www.psort.org/psortb/) [40]. However, due to inconclusive results, and with the aim of confirming the obtained results by PSORTb, a second subcellular localization analysis was applied to protein sequences using the CELLO subcellular localization predictor v.2.5 (http://cello.life.nctu.edu.tw/) [41].

### 2.8. Multiple alignment analysis

To determine the level of phylogenetic conservation of the proteins, multiple alignments and phylogenetic trees were constructed. Using the EBI WU-BLAST (http://www.ebi.ac.uk/Tools/sss/wublast/) [42] against the UniProt Knowledgebase, homologues of each protein were obtained from the reference strains of each of the *Brucella* genus species (*B. abortus* bv.1 str 9–941, *B. melitensis* bv.1 str 16M, *B. suis* 1330, *B. ovis* ATCC 25840, *B. canis* ATCC 23365, *B. canis* HSK A52141, *B. ceti* B1/94, *B. pinnipedialis* B2/94, *B. neotomae* 5K33, *Brucella inopinata* BO1 and *B. microti* CCM 4995). BLASTP (http://blast.ncbi.nlm.nih.gov/) [43] was used to retrieve non-*Brucella* genus protein homologues, with an established threshold of $10^{-20}$. The sequences obtained were aligned with CLUSLATW [44], provided by Unipro UGENE (http://ugene.unipro.ru/) [45]. The p-distance between each of the immunoreactive *B. canis* strain Oliveri proteins and its homologues in the reference strains mentioned above was calculated using MEGA6 [46].

### 2.9. Antigenic peptides and site prediction

Two methods were employed to evaluate antigenicity. The sequences of the proteins of interest were screened for predicted lineal B cell epitopes using the BepiPred algorithm software [47], and confirmed with the immunogenicity prediction software SCRATCH Protein Preditor Software using the COBEpro and ANTIGENpro algorithms (http://scratch.proteomics.ics.uci.edu/) [48]. Potential conformational epitopes were also evaluated, modeling by homology the three-dimensional structures of the proteins, using Phyre v2.0 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id = index) [49] and the prediction based on the structure epitopes using the software Ellipro (http://tools.immuneepitope.org/tools/ElliPro/iedb_input) [50].

### 2.10. Samples

Serum samples from 385 dogs from 20 kennels, and 91 humans in contact with these dogs were obtained and were used to evaluate the performance of two purified recombinant proteins as diagnostic antigens. The samples were classified into urban area (positive kennels) and rural area (negative kennels) serum samples, as described previously [17, 51, 52].

The University of Antioquia Ethics Committee approved this study (Act No. 11-15-365, June 2011 and Act No. 77, June 2012), in adherence to the Declaration of Helsinki Principles. Institutional Review Board approval was obtained, and all participants or their legal guardians provided signed informed consent before entering the study.

### 2.11. Diagnostic tests

All serum samples obtained from dogs were previously evaluated by 2ME-RSAT, blood culture, and PCR; human serum samples were previously evaluated by 2ME-RSAT and blood culture [17, 52].

### 2.12. Cloning, expression and purification of two recombinant proteins

From LB agar, one colony of *B. canis* strain Oliveri was inoculated into LB broth, grown overnight, and incubated for 24 h at 37 °C. This liquid culture was used for genomic DNA extraction using a column-based method following manufacturer's instructions (QIAGEN, DNeasy Blood & Tissue Kit, CAT# 69504). DNA concentration was measured using UV light absorption at 260 nm and Picogreen fluorescence (INVITROGEN, Quant-iT™ PicoGreen® dsDNA Assay Kit, CAT# 69504).

For human samples, we had previously identified 14 immunogenic cytoplasmic proteins of *Brucella canis* strain Oliveri by 2DE-PAGE, mass spectrometry analysis, and bioinformatics. According to their antigenicity score, and the lack of reports identifying them as immunogenic for humans in other *Brucella* sp., two proteins were selected and produced by recombinant methods. These purified proteins were subsequently used as antigens in iELISA. The two proteins were: Pyruvate dehydrogenase E1 subunit beta (PdhB) (49 kDa) and elongation factor Tu (Tuf) (42.6 kDa). The primers used for cloning are listed in Table 1.

The complete process of producing the recombinant proteins is described below:

From the *B. canis* Oliveri DNA, genomic regions that encoded the proteins of interest were PCR amplified and ligated into pTZ57 R/T or pGEM-4T easy. These recombinant plasmids were used to transform *E. coli* DH5α cells. The sequence integrity of the amplicons was verified. The ligated region was subsequently excised and subcloned in pET-28a or pRSET-A which were further used to transform *E. coli* BL21 (DE3) cells.

One colony of each recombinant *E. coli* was seeded in 200 ml of LB broth with Kanamycin at 25 μg/ml or Ampicillin at 100 μg/ml, depending on the expression vector used. Exponential-phase culture was induced using 1 mM IPTG (Isopropyl-β-D-1-Thiogalactopyranoside) at 37 °C, and protein expression was analyzed at 2, 4, and 6 h post-induction. Time-specific protein expression was analyzed by electrophoresis using a 12.5% polyacrylamide gel.

Six hours post-induction, bacterial cultures were used for further experiments. The bacterial pellets were dissolved in a buffer (50mM $NaH_2PO_4$, 300 mM NaCl and 10 mM Imididazol) to conserve the native structure of proteins, sonicated for 35 min with pulses of 1 min with 10 s intervals. To assess protein solubility, bacterial suspensions were centrifuged (8500 g for 35 min) at 4 °C and supernatants and cell pellets were analyzed by electrophoresis using a 12.5% polyacrylamide gel.

Once the presence of proteins in the supernatants was verified, the proteins were purified using a Biologic Duoflow Pathfinder 20 chromatography system (BioRad, Hercules, CA, USA) and BioScale mini Profinity IMAC Cartridge of 1 ml with histidine (His-Tag) binding affinity (BioRad, Hercules, CA, USA). The purity of the purified proteins was confirmed by Coomassie blue staining of the polyacrylamide gel after electrophoresis. Protein concentration was estimated by Bradford proteins assay using Bovine Serum Albumin as a standard. The proteins were stored at 4 °C and their purity evaluated using Agilent 2100 Bioanalyzer (Agilent techonologies Inc. Santa Clara, CA, USA). The reactivity of these proteins was further tested by iELISA.

### 2.13. Indirect microplate iELISA for IgG antibody detection

iELISA test for detection of antigen-specific IgG was standardized using the purified recombinant proteins. First, a pilot study was conducted using 0.1, 0.5 and 1 μg of recombinant protein per well to determine the optimal concentration at which to coat the wells. This pilot iELISA was carried out using positive and negative samples.

To test human and canine sera for antigen-specific antibodies, proteins were diluted to an optimal concentration in coating buffer (4.42 g of $Na_2CO_3$ and 5.04 g of $NaHCO_3$ in a liter of distilled water, pH 9.6), and used to coat wells of a 96-well MICROLON 600 (high binding) plate (Greiner Bio-One, Monore, NC, USA). Briefly, each well was coated with 100 μl of the diluted protein (one of the two proteins or a combination of PdhB and Tuf at equal concentrations), and the plate was incubated at 4 °C overnight. Plates were washed four times with TBS-T (Tris Buffered Saline with Tween 20) for 5 min. Plates were blocked with 200 μl of blocking buffer (TBS with 5% non-fat milk) and incubated at 37 °C for 1 h and then washed again as previously mentioned. Finally, 50 μl of diluted human and canine serum samples (1:500 in blocking buffer), were added to each well. Each sample was tested in triplicate.

For human serum samples, positive control consisted of a mix of three human serum in equal quantity of ul of each samples, that were previously positive by 2ME-RSAT to *Brucella canis*. LPS depletion was not performed, since *Brucella canis* does not have the LPS O antigen, so the interference of this structure with the performance of the immunoblot is minimal. For specificity control, a mix of three human samples that were found to be positive by Rose Bengal Test (RBT) to *Brucella abortus* was used, and as negative control, a mix of three human serum samples negative by 2ME-RSAT and RBT was used. All control samples were run in duplicate.

For canine serum samples, a mix of three samples (obtained from kennels previously identified as being *B. canis* positive) positive by 2ME-RSAT, blood culture, and PCR was used as a positive control; and a mix of three samples (obtained from kennels without reported clinical cases of canine brucellosis) negative for *Brucella canis* by 2ME-RSAT, blood culture, and PCR was used as a negative control. All control samples were run in duplicate.

Plates were incubated at 37 °C for 1 h with constant shaking and subsequently washed four times. After appropriate dilution, 50 μl of one of the following secondary antibodies was added to each well: alkaline phosphatase labeled anti-human IgG (g-chain specific) (Sigma-Aldrich, Madison, WI, USA) or alkaline phosphatase labeled anti-dog IgG (whole molecule). Plates were incubated at 37 °C for 1 h and washed. For color development, 100 μl of TMB substrate solution 3,3′,5,5′-tetramethylbenzidine (0,4 g/L) and peroxide (0,02% $H_20_2$) solution (Thermo Scientific Meridian Rd, Rockford, IL, USA) was added to dog serum samples. The reaction was stopped by the addition of 100 μl of 0.185 M sulfuric acid, and absorbance was read at 450 nm in an EPOC™ ELISA reader (BioTek, Winooski, VT, USA).

For human serum samples, p- Nitrophenyl phosphate (Amresco, Solon, OH, USA) was used as substrate and the reaction was stopped by3N NaOH; absorbance was read at 405 nm.

### 2.14. Statistical analysis

Data were stored in an excel worksheet (Microsoft office, 2007), and analyzed using SPSS 19 software (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp). For each of the diagnostic tests, the percentage of positive and negative samples was calculated. Cutoff values for each of the tested recombinant proteins were

**Table 1. List of primers used for cloning genes.** These primers were designed based on the sequence of *Brucella canis* Strain Oliveri, to amplify the specific genes to produce recombinant proteins.

| Protein name | Primer name | Primer sequence (5′ to 3′) | Sequence length CDS Amplicon Size (nt) | Sequence length CDS (aa) | Calculated MW (kDa) |
|---|---|---|---|---|---|
| Piyruvate dehydrogenase (pdhB) | BR1128_pdhb_NdeI_Fw | CCCCATATGATGCCCATAGAAATTCTCATGC | 1386 | 483 | 51 |
| | BR1128_pdhb_XhoI_Rv | GGGCTCGAGTTAAGCGGTATAGGTAATGGCTTTC | | | |
| Elongation Factor EF_Tu1 (Tuf_1) | BR1235_tuf1_BamHI_Fw | CCCGGATCCATGGCAAAGAGTAAGTTTGAACGTACG | 1176 | 426 | 46 |
| | BR1235_tuf1_XhoI_Rv | GGGCTCGAGTTACTCGATGATCGACGAGACG | | | |

determined by Receiver Operating Characteristic (ROC) curves. In addition, sensitivity, specificity, positive and negative predictive values, likelihood ratio for positive and negative results, and agreement between tests according to calculated Kappa coefficient were also evaluated.

## 3. Results

### 3.1. Proteomic analysis by 2D-PAGE yielded 19 spots of immunogenic proteins

Spots that were highly expressed or reactive to human sera positive for *B. canis* but not for *B. abortus* or negative controls were selected (Figure 1). Of the 19 spots analyzed, the identity and function of 14 of them were successfully identified (Table 2). Of those, 7 had not been previously reported as being immunoreactive for other *Brucella* species: 1) cold shock protein, 2) S-adenosylmethionine synthetase, 3) 2-oxoisovalerate dehydrogenase E1 component subunit alpha, 4) triosephosphate isomerase, 5) fructose-1,6-bisphosphate aldolase, 6) inosine-5′-monophosphate dehydrogenase, and 7) ribosomal subunit interface protein. The remaining five (not included in Table 2), showed low MOWSE scores (<83) and were not included in the final analysis; these proteins were 1) acetyltransferase, 2) DnaK, 3) 50S ribosomal protein L7/L12, 4) LysR family transcriptional regulator, and 5) TetR family transcriptional regulator.

### 3.2. Protein cellular location

Protein cellular location of those identified as immunogenic, were predicted as cytoplasmic-based in the subcellular localization servers.

Function in almost half (52%) of the identified proteins, could be classified into two categories; 31% was involved in energy metabolism, and 21% in protein synthesis. The remaining 48% were distributed in other categories, namely, central intermediary metabolism, regulatory functions, amino acid biosynthesis, cellular processes, protein fate, purines, pyrimidines, nucleosides and nucleotides, and transcription.

Regarding genome structure, all proteins were present in all genus *Brucella* species and were highly conserved, with identities of 95% or more (Data non-show). In addition, a homolog of each protein was found in closely related taxa, such as *Ochrobactrum*, with identities of up to 87%.

### 3.3. Cloning, expression and purification of two recombinant proteins

Two proteins: PdhB and TuF were chosen according to their immunogenicity to produce them recombinantly.

Purity of each of the two proteins was greater than 95%. Final concentration of the proteins was adjusted to 1.5 mg/ml, and were stored at -20 °C until use. Best performance in iELISA was observed when ELISA plates were coated with 0.1 μg of protein per well.

### 3.4. Indirect microplate iELISA for IgG antibody detection

For the evaluation of the two recombinant proteins as diagnostic antigens in iELISA assay, cutoffs were determined using ROC curves. The area under the curve and the cutoff values for each of the two iELISAs are presented in Table 3.

For human serum samples, iELISA results were compared with PCR results since only 9.9% of the samples were positive by 2ME-RSAT, and none by blood culture (Table 4). The best iELISA performance was shown when a combination of PdhB and Tuf proteins was used as antigen with a Sensitivity (S): 98%, Specificity (Sp): 73%, Negative Predictive Value (NPV): 97% and Positive Predictive Value (PPV): 76%. Kappa coefficient was 0.696; suggesting a good agreement between tests. The number of observed agreements between tests was 76/91 (83.52%). PdhB and Tuf presented S: 91% and 86%, respectively; Sp: 77 and 71%, respectively; NPV: 90 and 85%, respectively; PPV: 78 and 73%, respectively. Kappa coefficient for PdhB was 0.672, which is considered to reflect a good agreement between tests. The number of observed agreements between tests was 71 (78.02%). Kappa coefficient for Tuf was 0.563, which is considered to reflect a moderate concordance between tests. The number of observed agreements between the tests was 73 (80.22%) (Table 5).

Results for iELISA tests for 9 human serum samples that were positive by 2ME-RSAT are explained in Table 6.

For canine serum samples, iELISA results were compared with 2ME-RSAT, blood culture and PCR results (Table 7). Using a mix of PdhB plus Tuf, or using Tuf only, iELISA demonstrated the best performance with S: 75%, Sp:64%, PPV: 40%; NPV: 89%, and had fair agreement with PCR results. Using PdhB as an antigen, the number of observed agreements between the tests was 237 (61.56%) whereas for a mix of Tuf and PdhB, the number of observed agreements between tests was 258 (67.01%).

## 4. Discussion

In this study, we used proteomic methods and subsequent molecular and bioinformatics analyses to identify antigenic cytoplasmic proteins of *B. canis*, obtained from a Colombian bacterial isolate *B. canis* strain Oliveri, in human sera. We identified six novel cytoplasmic proteins that had not been previously reported as being immunoreactive for *Brucella* (Identification 1–6; Table 2). Nonetheless, some of them have been

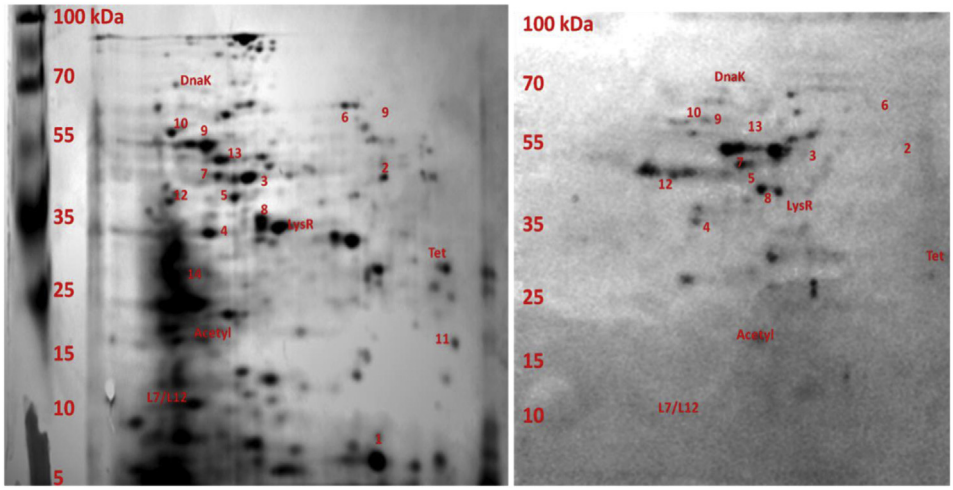


**Figure 1.** 12% 2DE-PAGE and immunoblot with serum of group 1. Red numbers are the 14 proteins identified, listed in Table 2.

**Table 2.** Immunoreactive soluble proteins from *B. canis* strain Oliveri as determined by 2DE and MALDI-TOF/TOF.

| $P_{ID}$ | Protein | Gene name | Mowse score | Protein length aa | PI* | MW (Da) Obt/Theo | Seq. Cov** | Uniq pep*** | GenBank Accesion in *Brucella canis* strain Oliveri | Antigenicity | Function |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Cold shock protein cspa | *cspa* | 327 | 69 | 6.53 | 7362/7432 | 80,3 | 3 | CDL76877.1 | 0,772 | Cellular processes |
| 2 | S-adenosylmethionine synthetase | *metK* | 595 | 421 | 8.15 | 43250/45642 | 20,8 | 7 | CDL77531.1 | 0,743 | Central intermediary metabolism |
| 3 | 2 oxoisovalerate dehydrogenase E1 component subunit alpha | *oxo* | 240 | 410 | 6.28 | 41717/45790 | 5,6 | 4 | CDL78065.1 | 0,594 | Energy metabolism |
| 4 | Triosephosphate isomerase | *tpiA*$^{-1}$ | 429 | 254 | 5.63 | 24922/26485 | 10,6 | 3 | CDL76533.1 | 0,246 | |
| 5 | Fructose-1,6-bisphosphate aldolase | *fbaA* | 438 | 354 | 5.93 | 30654/38777 | 19,6 | 4 | CDL78397.1 | 0,408 | |
| 6 | Inosine-5′-monophosphate dehydrogenase | *guaB* | 454 | 497 | 7.22 | 52843/52280 | 7,8 | 4 | CDL77898.1 | 0,212 | Purines, pyrimidines, nucleosides and nucleotides |
| 7 | Cysteine synthase A | *cysA* | 136 | 341 | 5.41 | 32898/36728 | 22 | 6 | CDL76448.1 | 0.340 | Aminoacid biosynthesis |
| 8 | Glyceraldehyde-3-phosphate dehydrogenase | *gap* | 124 | 335 | 6.26 | 35757/36236 | 19.4 | 4 | CDL77103.1 | 0.356 | Energy metabolism |
| 9 | Phosphopyruvate hydratase (enolase) | *eno* | 1040 | 425 | 5.03 | 46227/45261 | 25.8 | 10 | CDL76527.1 | 0.462 | |
| 10 | Pyruvate dehydrogenase subunit Beta-pdhB | *pdhB* | 340 | 461 | 4.7 | 45521/48998 | 9.7 | 3 | CDL76523.1 | 0.590 | |
| 11 | 50S ribosomal protein L10 | *rpiJ* | 203 | 172 | 9.6 | 18026/17913 | 5.4 | 3 | CDL76639.1 | 0.667 | Protein synthesis |
| 12 | Elongation factor Ts | *tsf* | 148 | 305 | 5.02 | 32789/31491 | 15.8 | 7 | CDL76556.1 | 0.789 | |
| 13 | Elongation factor Tu | *tuf1* | 755 | 391 | 5.27 | 42383/42605 | 28.8 | 8 | CDL78401.1 | 0.565 | |
| 14 | Ribosomal subunit interface protein | *yfiA* | 511 | 197 | 5.4 | 21082/21570 | 37.2 | 4 | CDL75581.1 | 0.567 | Purines, pyrimidines, nucleosides and nucleotides |

* Isoelectric point.
** Sequence coverage.
*** Unique peptides.

**Table 3.** Cutoff values and area under curve for the different recombinant proteins used as antigens in iELISA for the detection of human and canine brucellosis.

| | Proteins iELISA | Area under curve | Standar error. | Confidence interval 95% | | Cutoff |
|---|---|---|---|---|---|---|
| | | | | Lower limit | Upper Limit | |
| Human samples | PdhBTuf | 0,859 | 0.043 | 0,776 | 0,942 | 0,210 |
| | PdhB | 0,878 | 0,038 | 0,803 | 0,953 | 0,135 |
| | Tuf | 0,712 | 0,054 | 0,606 | 0,817 | 0,091 |
| Canine samples | PdhBTuf | 0,702 | 0,040 | 0,624 | 0,781 | 0,200 |
| | PdhB | 0,702 | 0,040 | 0,624 | 0,781 | 0,195 |
| | Tuf | 0,695 | 0,033 | 0,631 | 0,759 | 0,190 |

**Table 4. Detection of brucellosis by 2ME-RSAT, blood culture, and PCR assays in human and canine sera**. Only 9 (9.9%) humans were positive by 2ME-RSAT. None of the human samples was positive by blood culture. 15.3% of dogs were positive by 2ME-RSAT, 13.5% by blood culture and 24.2% by PCR.

| | Diagnostic Test | | | | | |
|---|---|---|---|---|---|---|
| | 2ME-RSAT | | Blood culture | | PCR | |
| Samples | Negative (%) | Positive (%) | Negative (%) | Positive (%) | Negative (%) | Positive (%) |
| Humans (n = 91) | 82 (90.1) | 9 (9.9) | 91 (100) | 0 (0) | 48 (52.7%) | 43 (47,3) |
| Dogs (n = 385) | 326 (84,7) | 59 (15,3) | 333 (86,5) | 52 (13,5) | 292 (75,8) | 93 (24,2) |

**Table 5.** Results of Sensitivity (S), specificity (Sp), PPV, NPV, Likelihood ratio positive, Likelihood ratio negative, Kappa index and concordance to the five iELISAs evaluated in humans.

| iELISA vs PCR | S | 95% IC | E | 95% IC | VPP | 95% IC | VPN | 95% IC | LR+ | LR- | Kappa/Concordance | Number of observed agreements |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PdBtuf | 98 | 93–100 | 73 | 60–85 | 76 | 65–88 | 97 | 92–100 | 3.61 | 0.03 | 0,696 Good | 76 (83.52%) |
| PdhB | 91 | 82–99 | 77 | 65–89 | 78 | 67–89 | 90 | 81–99 | 3.96 | 0.117 | 0.672 Good | 71 (78.02%) |
| Tuf | 86 | 76–96 | 71 | 58–84 | 73 | 60–85 | 85 | 74–96 | 2.97 | 0.197 | 0.563 Moderate | 73 (80.22%) |

**Table 6.** Results by iELISA tests for the 9 human samples positive by 2ME-RSAT.

| Number of samples | 2ME-RSAT | PCR | iELISa Tests (PdhB, Tuf, PdhBTuf) |
|---|---|---|---|
| 3 | Positive | Positive | Positive all proteins |
| 2 | Positive | Positive | Positive PdhB.<br>Negative: PdhBTuf and Tuf |
| 1 | Positive | Positive | Positive: PdhB, Tuf, PdhBTuf. |
| 1 | Positive | Positive | Positive: PdhB, Tuf, PdhBTuf. |
| 1 | Positive | Positive | Positive: PdhB, Tuf, PdhBTuf. |
| 1 | Positive | Negative | Positive: PdhB and PdhBTuf.<br>Negative: Tuf |

**Table 7.** Results of Sensitivity (S), specificity (Sp), PPV, NPV, Likelihood ratio positive, Likelihood ratio negative, Kappa index and concordance to the five iELISAs evaluated in dogs.

| iELISA vs PCR | S | 95% CI | E | 95% CI | PPV | 95% CI | NPV | 95% CI | LR+ | LR- | Kappa/Concordance | Number of observed agreements positive or negative. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pdBtuf | **75** | 66–84 | 64 | 59–70 | 40 | 33–48 | 89 | 85–93 | 2.08 | 0.391 | 0.306 fair | 237 (61.56%) |
| tuf | **75** | 66–84 | 64 | 59–70 | 40 | 33–48 | 89 | 85–93 | 2.08 | 0.391 | 0.306 fair | 258 (67.01%) |
| pdhB | 65 | 55–74 | 61 | 55–66 | 34 | 27–41 | 84 | 79–89 | 1.67 | 0.574 | 0.193 poor | 258 (67.01%) |
| iELISA vs 2ME-RSAT | | | | | | | | | | | | |
| pdhB | 73 | 62–84 | 60 | 54–65 | 25 | 18–31 | 92 | 89–96 | 1.83 | 0.450 | 0.179 poor | 237 (61.56%) |
| tuf | 24 | 18–30 | 92 | 88–96 | 71 | 60–83 | 60 | 54–65 | 3.00 | 0.826 | 0.171 poor | 236 (61.30%)<br>205.3 (53.33%) |
| pdBtuf | 24 | 18–30 | 92 | 88–96 | 71 | 60–83 | 60 | 54–65 | 3.00 | 0.826 | 0.171 poor | 236 (61.30%) |
| iELISA vs Blood culture | | | | | | | | | | | | |
| pdhB | 72 | 59–83 | 59 | 53–64 | 21 | 15–27 | 93 | 89–96 | 1.76 | 0.475 | 0.149 poor | 232 (60.26%) |
| tuf | 75 | 63–87 | 59 | 54–65 | 22 | 16–29 | 94 | 91–97 | 1.83 | 0.424 | 0.173 poor | 237 (61.56%) |
| pdBtuf | 75 | 63–87 | 59 | 54–65 | 22 | 16–29 | 94 | 91–97 | 1.83 | 0.424 | 0.173 poor | 237 (61.56%) |
| PCR vs 2ME-RSAT | 78 | 67–89 | 86 | 82–89 | 49 | 39–60 | 96 | 93–98 | 5.57 | 0.256 | 0.514 Moderate | 325 (84.42%) |
| 2ME-RSAT vs Blood culture | 67 | 55–80 | 93 | 90–96 | 59 | 47–72 | 95 | 92–97 | 9.57 | 0.355 | 0.569 Moderate | 344 (89.35%) |
| PCR vs Blood culture | 92 | 85–100 | 86 | 83–90 | 52 | 41–62 | 99 | 97–100 | 6.57 | 0.093 | 0.591Moderate | 336 (87.27%) |

reported as immunoreactive in other bacteria: inosine-5′-monophosphate dehydrogenase in *Burkholderia multivorans* and *Burkholderia cenocepacia,* [53] and S-adenosylmethionine synthetase in *Bordetella pertussis* [54], both genera are proteobacteria, as is *Brucella.*

Although the use of immunoproteomics for identification of proteins of diagnostic interest has increased in the last decade [55, 56, 57, 58], there are currently no reports in either human or dogs for *B. canis*. Previous studies have reported immunoreactive proteins of *B. abortus* [23], *B. melitensis* [33, 53] and *B. suis* [59, 60], recognized by sera of different hosts, such as camels [30], humans [61], bovines [62] and goats [32, 33, 63]. Regarding *Brucella abortus*, contrary to other studies, the 5 proteins showing low MOWSE scores, and thus not included in our final analysis (enolase, 50S ribosomal protein L7/L12, acetyltransferase, pyruvate dehydrogenase subunit Beta, and cysteine synthase A), had been previously identified as immunoreactive for *B. abortus* by Conolly *et al.* [61].

In the context of *B. melitensis*, Zhao *et al.* reported glyceraldehyde-3-phosphate dehydrogenase, elongation factor Ts, molecular chaperone DnaK, and 50S ribosomal protein [33] as being immunoreactive. Molecular chaperone DnaK, and the 50S ribosomal protein L7/L12 were also reported by Al DahouK [23]. In addition, Yang *et al.* [32] identified elongation factor Tu of *B. melitensis,* using animal and human sera. It is noteworthy that acetyltransferase, molecular chaperone DnaK, and 50S ribosomal protein L7/L12 did not have a significant MOWSE score in our analysis; however, their presence indicates that these three proteins are immunogenic in *B. melitensis*, *B. abortus* and *B. canis.*

Regarding protein subcellular localization, cytoplasmic proteins can be easily identified by 2D-PAGE method, while liquid chromatography mass spectrometry analysis (LC MS-MS) is more appropriate to identify membrane proteins. In this study, we have focused on the identification of cytoplasmic immunoreactive proteins by performing 2D-PAGE, and therefore the identification of potential immunoreactive membrane proteins is not reported.

Concerning function, our results showed that the majority of identified proteins (31%) were involved in energy metabolism, and 21% in protein synthesis, being different from those reported by Sandalakis et al. [64], who reported in *B. abortus* that 16% of the immunoreactive proteins were involved in energy metabolism, and 14% in protein synthesis, when the proteome was analyzed for antibiotic resistance.

While in the genus *Brucella* there are no substantial genomic differences, there are instead few polymorphisms or genomic variations [65] that produce variations in protein sequences, which may confer host specificity and differences in pathogenicity, such as the *virB* virulence operon [51, 66].

The *B. canis* immunogenic proteins identified in the present study could be useful in the short term to develop sensitive and specific diagnostic tools for the detection of *B. canis* in human sera, which could complement the already existing tests [16, 67, 68]. Several tests for the detection of *B. canis* based on the M-strain have been developed and are currently used in the clinical setting, like those made by Lucero et al., 2002 who used a saline extract; Wanke et al. [18] who used a heat saline extract; Daltro di Oliveira et al. [69] used heat soluble bacterial extracts and sonicated extracts of a *B. canis* wild strain, similarly to Barrouin–Melo et al. [70]. All of these studies report sensitivity and specificity greater than 90% in canines. However, variable results in sensitivity and specificity were observed by other authors, such as Baldi et al. [20, 71] and Cassataro et al. [21], which used *B. abortus* and *B. melitensis* to produce recombinant proteins.

In our particular case, the use of *B. canis* strain Oliveri is an advantage, as it could be used to develop diagnostic tests directly from a strain circulating in our region. The following step would be to select several of

these proteins, according to the theoretical antigenicity, and produce them recombinantly in order to be used in assays, such as ELISA or Western blotting, to detect such proteins in human sera. Confirmation of diagnostic use of these assays will ultimately provide an improved test for timely diagnosis of this disease.

In this work, we have identified two recombinant proteins that can be potentially used an as diagnostic antigens in order to improve diagnosis of human brucellosis caused by *B. canis*. However, we have also demonstrated that their utility for the diagnosis of canine brucellosis is limited.

Of the two proteins used as antigens, the best results for human serum samples were obtained using PdhB-Tuf mix (S 98% and Sp 73%), and PdhB (S 91% and Sp 77%). The results of iELISA and PCR had a good Kappa coefficient (0.696 and 0.672 respectively), indicating that they had good agreement. The high specificity of these two iELISAs that use PdhB-Tuf and PdhB as antigens is supported by the comparison with the PCR results, which is a direct test to detect DNA and not antibodies.

Previously, a few iELISA assays to detect human brucellosis have been described, however they exhibited variable sensitivities and specificities (70–100%). Because this infection elicits a response with low antibody production, it is consequently difficult to use antibodies to diagnose this disease [16].

PdhB protein is a protein that has been reported to be immunogenic in *Brucella* spp. It has been found to be an immunogenic protein of *Mycoplasma bovis* in cattle [72], and *Mycoplasma capricolum* sub sp. *Capripneumoniae* in goats [73]. The PdhB protein of *Mycoplasma* sp. might have some similar epítopes to PdhB protein of *Brucella canis*.

The elongation factor Tu, encoded by *tuf* genes, is a GTP binding protein that plays a central role in protein synthesis. Depending on the bacterial species, one to three *tuf* genes are generally present per genome, and they can be horizontally transferred between species [74]. These genes are involved in protein synthesis and antibiotic resistance mechanisms in *Brucella* [75]. In *Brucella canis* strain Oliveri, two *tuf* genes were found upon genome sequencing (Sanchez-Jiménez et al., 2015). *tuf* has been used as a phylogenetic marker in *Streptococcus* [76], *Enterococcus, Lactobacillus, Bifidobacterium lactis* [77], *and Yersinia* [78]. Furthermore, it has been reported to be an immunogenic protein of *Burkholderia pseudomallei* in mouse [79] and humans [53], but its immunogenic potential in *Brucella* species has not yet been verified.

Using recombinant proteins as antigens for detection of cases of canine brucellosis did not deliver encouraging results. Even though using a mix of Tuf and PdB-Tuf as antigens showed potential for detection of human brucellosis, the iELISA assay based on the same proteins was found to have a S 75% and Sp 64% when used for detection of *B. canis* in canines. This is very low when compared to the results obtained when using total extracted protein [18] as antigen, where other authors report a S and Sp 100%.

Wanke et al. [18] demonstrated a sensitivity of 85–100% and a specificity of 94–96.7% for different antigens used in iELISA for the detection of brucellosis in dogs. The type of antigens used, cytosolic or membrane antigens of *Brucella* sp. as opposed to recombinant proteins of a wild-type strain, could account for the difference in the S and Sp between our tests.

De Oliveira et al. [69] used heat soluble bacterial extract from a wild *Brucella canis* strain as an antigen, and reported S 91% and Sp 100% in iELISA for the detection of brucellosis in dogs, and Barrouin-Melo et al [70] reported S 95% and Sp 91% using total extracted protein as an antigen.

A good diagnostic method to detect infection in dogs is highly desired, as it is needed to detect *Brucella* infection in early-infected and asymptomatic dogs that are in contact with *Brucella* positive dogs. iELISA may have a better sensitivity to detect positive cases than 2ME-RSAT, since agglutination tests are observer-dependent and require a higher levels of antibodies for correct detection than iELISA, which is analyzed by a machine and can detect lower levels of antibodies. It is thus crucial to identify other potential diagnostic probes for iELISA that can accurately identify seropositive animals.

Regarding iELISA results with recombinant proteins, complete antigens might not serve as good diagnostic probes because, even though they include entire bacterial components, they might give rise to specificity problems as there would be many antigens common to other bacterial genus [18]. Also, false positives reported in our work can be explained by comparing iELISA results with 2ME-RSAT results, as iELISA would detect antigen-specific antibodies but agglutination might be negative as a complete antigen is used. In addition, serum samples of dogs receiving antibiotic treatment might show negative agglutination but be positive by iELISA [18].

## 5. Conclusions

Altogether, our results show that for detection of human brucellosis, recombinant proteins PdhB and Tuf showed potential as diagnostic antigens for the detection of *B. canis* infection by iELISA. Using both, PdhB and Tuf proteins, improves the efficiency of the iELISA test.

None of the tested recombinant proteins were able to detect canine brucellosis with high specificity and sensitivity. However, they could still serve as potentially useful diagnostic antigens in iELISA performed as complementary test to other tests including 2ME-RSAT, blood culture, and PCR, for diagnosis of *B. canis* infection in dogs.

iELISA assay will probably deliver better results when as an antigen a mix of all the proteins is used in a single test, with concentrations increasing from 0.1 to 0.5 μg of protein per well, and also when a lower concentration of non fat milk is used to dilute the serum samples, and the dilution factor is reduced.

The diagnosis of *Brucella canis* infection is challenging due to the unavailability of highly specific and sensitive diagnostic assays.

The identification of *Brucella canis* immunoreactive cytoplasmic proteins made in this paper opens the possibility to use these proteins as antigens to develop diagnostics test.

The results of this research will allow the design of novel strategies for early detection of human and animal brucellosis cases.

## Declarations

### *Author contribution statement*

M. Jiménez: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

J. de la Cuesta-Zuluaga, G. Garcia-Montoya and N. Dabral: Performed the experiments; Analyzed and interpreted the data.

J. Alzate and R. Vemulapalli: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

M. Olivera-Angel: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### *Competing interest statement*

The authors declare no conflict of interest.

## Additional information

No additional information is available for this paper.

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