Identificación de proteínas inmunógenas de *Brucella canis* que inducen respuesta inmune humoral en humanos.

Tesis presentada a la Facultad de Ciencias Agrarias de la Universidad de Antioquia como requisito parcial para optar por el título de Doctora en Ciencias Animales

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Facultad de Ciencias Agrarias
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2014

AGRADECIMIENTOS.

A mi tutora la Dra Martha Olivera-Angel por sus enseñanzas y apoyo durante este proceso.

A los miembros del comité tutorial, doctores Cesar Segura y Carlos Muskus por sus sugerencias y críticas constructivas.

A los profesores Nélida Rodríguez, Henry Cardona y Marisol Medina y a Johanna Cardona, del posgrado en Ciencias Animales de la Facultad de Ciencias Agrarias por su colaboración durante este proceso.

A los doctores Pablo Patiño, quien en el primer año participó también en mi comité, y Fabiola Toro, quienes tuvieron la generosidad y la paciencia de compartir conmigo algunos de sus conocimientos en inmunología.

Al comité de investigaciones de la Universidad de Antioquia (CODI) y a la Dra. Tatiana Ruiz, directora del grupo de investigación Biogénesis, por su apoyo económico para la realización de este trabajo**.**

A los doctores Robinson Ramírez, Omar Triana y Juan Fernando Álzate por permitirme realizar parte de mi trabajo en sus laboratorios.

A Ana Catalina Medina y Lina Orrego por compartir conmigo sus conocimientos y asesorarme en las pruebas de 2 dimensiones.

A Camilo Álvarez y Jany Villa del grupo de inmunomodulación por su ayuda con los ensayos de isoelectroenfoque.

Al Dr. Ramesh Vemulapalli por recibirme en su laboratorio y asesorarme durante mi pasantía en la Universidad de Purdue.

A Neha Dabral, Marie Laoye y Shankar Thangamani por su amistad y generosidad durante mi estancia en la Universidad de Purdue.

A mis compañeros y amigos del Instituto Colombiano de Medicina Tropical, por su ayuda sacándome de apuros.

A mis compañeros y amigos del grupo Vericel-Biogénesis por su apoyo y colaboración en estos 4 años, en especial a July Duque por su apoyo total.

A Laura Castrillón y Juan Jacobo de la Cuesta por su ayuda, crítica y consejos.

A Viviana Hernández por su invaluable ayuda técnica.

A Ronald Peláez y al doctor Marcel Marín por sus consejos sobre las técnicas de proteómica.

Al Dr. Anthony M. Haag del Mass Spectrometry Lab, Biomolecular Resorce Facility, University of Texas Medical Branch por sus sugerencias para la realización de los análisis de masas.

A Gisela García, Jairo Alonso Mesa y los demás compañeros del Centro Nacional de Secuenciación Genómica de la UdeA, por acogerme y ayudarme con la producción de la proteínas recombinantes.

A Jesús y Adiela por su apoyo durante estos años.

A mis hermanas y sus familias por su amor y apoyo.

Infinitas gracias a todos.

DEDICATORIA.

A mis padres por su amor y apoyo total e incondicional siempre.

A mis hijos Nicolás y Sara y a mi esposo Juan Miguel, por su amor, paciencia y darme tanta felicidad, recordándome siempre que ellos son lo más importante de mi vida,

“Los amo hasta el infinito y más allá”.

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2. LISTA DE ABREVIATURAS

1D-PAGE: Electroforesis en una dimensión

2D-PAGE: Electroforesis en dos dimensiones

ADN-DNA: Acido desoxirribonulceico- Deoxyribonucleic acid

CNSG: Centro Nacional de Secuenciación Genómica

E: Especificidad

ELISA: Enzyme-linked immunosorbent assay

GuaB: Inosina 5´ fosfato deshidrogenasa

IgG: Inmunoglobulina G

IgM: Inmunoglobulina M

LC MS-MS: Liquid chromatography–mass spectrometry

MALDI TOF-TOF: Análisis de masas en tandem por Matrix-Assisted Laser Desorption/Ionization Time-of-flight.

OMP31: Outer Membrane Protein 31

PCR: Reacción en Cadena de la Polimerasa

PdhB: Piruvato deshidrogenasa **E1** subunit beta

S: Sensibilidad

TBS: Tris Buffered Saline

TBS-T: Tris Buffered Saline con Tween 20.

Tet: Regulador transcripcional de la familia TetR

Tuf: Factor de elongación Tu

VPN: Valor predictivo negativo

VPP: Valor predictivo positivo

3. RESUMEN GENERAL

En el año 2005 en Medellín, el grupo de investigación Vericel de la Universidad de Antioquia aisló *Brucella canis* de animales enfermos en la región. En el transcurso de estos 9 años se han identificado caninos y humanos positivos para la infección por serología y hemocultivo; la presencia de factores de riesgo que se asocian a la presencia de la enfermedad por prácticas inadecuadas en los criaderos y se establecieron algunas características moleculares que permitieron identificar como *Brucella canis* grupo 2 las cepas que circulan en Medellín.

Como una continuación del trabajo del grupo, durante esta tesis de doctorado se identificaron proteínas inmunógenas de *Brucella canis* (*B. canis*) útiles para el diagnóstico de la infección en humanos y otras posiblemente para caninos, utilizando análisis proteómicos de *B. canis* cepa Oliveri, aislada de un canino y cuyo genoma fue secuenciado. Se analizaron sueros de 3 grupos de personas: 1. positivas a *Brucella canis* por la prueba serológica tamiz, 2. positivas a *Brucella abortus* por rosa de bengala y 3. sueros de personas negativas para las dos pruebas serológicas.

Por MALDI TOF-TOF, 35 fragmentos fueron analizados. Se identificaron 19 proteínas citoplasmáticas; 14 fueron identificadas definitivamente y sus epítopes y antigenicidad fueron caracterizados bioinformaticamente. Se consideraron proteínas inmunoreactivas de interés las presentes solo en los inmunoblots de las muestras positivas a *B. canis.*

Para caninos se utilizó el mismo extracto proteico pero en 1DE-PAGE e inmunoblots. Se identificaron bandas inmuno reactivas de diferente peso molecular, dependiendo del estadio de la infección.

Para identificar las proteínas presentes en las bandas inmuno reactivas y de membrana, extractos totales y de membrana de la cepa Oliveri se analizaron por LC-MS/MS.

Según su antigenicidad y seleccionando proteínas que no hubieran sido reportadas previamente, se identificaron 3 proteínas citoplasmáticas inmuno reactivas para humanos y caninos: Inosina 5´ fosfato deshidrogenasa (GuaB-52 kDa), Piruvato deshidrogenasa E1 subunit beta (PdhB-49 kDa) y Factor de

elongación Tu (Tuf-42.6 kDa). El regulador transcripcional de la familia TetR (27.4 kDa) fue inmuno reactiva solo para humanos; adicionalmente, se identificó la proteína de membrana Omp31.

A continuación, de la cepa Oliveri se obtuvieron las secuencias de los genes y se realizó clonación, sub-clonación, expresión y purificación de las proteínas de interés.

Posteriormente, se evaluó la presencia de anticuerpos IgG a través de ELISA indirecta utilizando las 5 proteínas recombinantes y una mezcla 1:1 de PdhB y Tuf como antígeno, en humanos y caninos. Para esto, sueros de 385 caninos de criaderos (200 de zona urbana, 185 de zona rural) y 91 humanos (52 de zona urbana, 39 de zona rural) en contacto con estos caninos, fueron evaluadas.

Se determinó la sensibilidad (S), especificidad (E), valor predictivo positivo (VPP), valor predictivo negativo (VPN) y la concordancia de cada una de las ELISAs frente 2ME-PARP, hemocultivo y PCR.En humanos la iELISA de mejores desempeño fue la mezcla de las proteínas Pdhb y Tuf. Para caninos la iELISA no fue de utilidad pues los valores de Sensibilidad y Especificidad fueron bajos., La iELISA en conjunto con las otras pruebas diagnósticas, se constituyen en un panel para detectar esta infección en humanos, mejorando el diagnóstico y con la posibilidad de ofrecer en el corto plazo, la realización de estas pruebas en la región.

General Abstract.

Since 2005, in Medellín, Vericel research group at the University of Antioquia has studied the problem of canine brucellosis in the region. During the last 9 years, the research group has identified by serology and blood culture tests *Brucella canis*-positive canines and humans, the presence of risk factors associated with the development of this infection in kennels due to inadequate practices and established some molecular characteristics, which helped to identify the circulating *Brucella canis* in Medellin as group 2. As a continuation of the work carried by the group, during this PhD thesis, immunogenic proteins of *B. canis* useful for the diagnosis of infection in humans and canines were identified using proteomic analysis of *B. canis* str. Oliveri, isolated from a canine whose genome was sequenced in collaboration with CNSG of the University of Antioquia. Three human sera groups were analyzed: 1. *B. canis*-positive sera by serological screening test, 2. *B. abortus*-positive sera by Bengal Rose y 3. *B. canis*-negative and *B. abortus*-negative sera by both serological test.

35 fragments were analyzed by MALDI TOF-TOF, 19 cytoplasmic proteins were identified; 14 were definitively identified and their epitopes and antigenicity were characterized using bioinformatics tools. Proteins of interest were considered as immunoreactive if they were present only in immunoblots from *B. canis*-positive samples.

For canine sera, the same protein extract was used for 1DE-PAGE and immunoblotting. Immunoreactive bands of different molecular weights, depending on the stage of infection, were identified. In order to identify the proteins present in the immunoreactive bands and the membrane proteins, total protein extracts from the Oliveri strain were analyzed by LC-MS/MS. According to their antigenicity and only selecting proteins that were not previously reported as immunoreactive, 3 cytoplasmic proteins were identified for humans and canines: Inosine 5´ phosphate deshydrogenase (GuaB-52 kDa), Piruvate deshydrogenase E1 subunit beta (PdhB-49 kDa) y Elongation factor Tu (Tuf-42.6 kDa). The transcriptional regulactor TetR (27.4 kDa) was found to be only

immunoreactive in humans. The outer membrane protein 31 (Omp31) was identified as well.

Then, the strain Oliveri gene sequences were obtained and cloning, sub-cloning, expression and purification of the proteins of interest was carried. This step was performed at two locations, Purdue University and the CNSG, in order to improve the purity of the proteins and produce the Omp31 protein.

Next, and indirect ELISA assay was performed using the recombinant proteins with the purpose of identify IgG in humans and canines; sera from 385 kennel canines (200 urban and 185 rural dogs) and 91 humans in contact with said canines (52 urban and 39 rural humans) were tested. No human had positive blood culture and 9/91 (9.9%) had positive serology. Therefore PCR was used as a comparison test with indirect ELISAs. Sensitivity (S), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV) and the agreement of each of the ELISAs were determined. A mixture of Pdhb and Tuf had the best performance in both humans and canines.

Finally, it was found that in humans, the mixture of recombinant proteins can increase the diagnostic value. For canines the iELISA wasn't useful because the sensitivity and specificity values were low. The iELISA, in conjunction with other diagnostic tests, constitute a panel for the detection of this infection in humans, improving the diagnosis and with the possibility of offering these tests in short-term in the region.

4. INTRODUCCIÓN GENERAL

La brucelosis canina es una zoonosis cuyos hospederos primarios son los caninos, en quienes causa problemas reproductivos como aborto e infertilidad, problemas óseos como disco espondilitis, problemas oculares como uveítis, entre otros (Gyuranecz et al., 2011; Hollett, 2006; Wanke, 2004; Wanke et al., 2012). En Medellín, desde 2009 se han reportado casos de infección por *Brucella canis* en caninos de criaderos (Giraldo-Echeverri et al., 2009), en caninos de albergues (Ruiz et al., 2010) y en mascotas (Agudelo-Flórez et al., 2012). En humanos, la infección se produce por contacto con secreciones provenientes de caninos infectados o por accidentes de laboratorio (Lucero et al., 2010; Wallach et al., 2004). La enfermedad puede ser asintomática y crónica, al igual que la infección producida por *B. abortus*; pueden pasar meses, e incluso años, antes de presentarse sintomatología (Krueger et al., 2014) que puede ir desde fiebre ondulante o persistente hasta casos severos como endocarditis y osteomielitis (Lucero et al., 2005b; N. Lucero et al., 2010a; Manias et al., 2013; Olivera-Angel et al., 2012). En Colombia, la bacteria fue aislada de una persona asintomática, conviviente con caninos infectados (Olivera and Di-lorenzo, 2009).

El diagnóstico de brucelosis por *B. canis* en humanos se realiza inicialmente con la Prueba de Aglutinación Rápida en Placa (2ME-PARP) con 2 Mercaptoetanol, la cual es una prueba tamiz, que detecta anticuerpos totales contra la bacteria en el suero de los pacientes, recomienda también el manual realizar una prueba de ELISA indirecta (iELISA) en suero, que detecta anticuerpos IgG o IgM (López et al., 2009). La prueba de oro es el hemocultivo, pero esta prueba presenta problemas de sensibilidad, pues su positividad depende del estadio de la infección, ya que la bacteria circula principalmente en casos agudos y poco en casos crónicos. Existen además otras pruebas

como la Reacción en Cadena de la Polimerasa (PCR), sin embargo este tipo de pruebas se están realizando solo recientemente y se utilizan principalmente para confirmar aislamientos y no para detectar ADN de la bacteria circulante en la muestra, pues se presentan problemas de especificidad (López-Goñi et al., 2008). Las pruebas serológicas como el 2ME-PARP y la IELISA, tienen problemas de sensibilidad (varían entre 40 a 90%) y especificidad (entre un 60-100%) (Baldi et al., 1997; Cassataro et al., 2004; Lucero et al., 2002, 1999; Wanke et al., 2002)lo cual se podría explicar por las dificultades al obtener antígenos específicos e inmunogénicos que reaccionen con anticuerpos IgG o IgM y que permitan realizar un diagnóstico rápido, sensible y especifico de la infección en caninos y humanos.

En nuestra ciudad, la brucelosis canina puede convertirse en un problema de salud pública, según las prevalencias encontradas en Medellín hasta hoy, debido al aumento de la población canina como animal de compañía y a la falta de regulación en la tenencia de criaderos, ya que estos se pueden convertir en reservorio de la infección para otros perros y humanos, sobre todo por el estrecho contacto de las mascotas con los niños y adultos mayores, quienes tienen un sistema inmune menos competente para enfrentar este tipo de infecciones.

Por lo anterior, la disponibilidad de un panel de pruebas diagnósticas para la detección precoz de casos humanos y caninos de brucelosis canina, podría evitar complicaciones derivadas de esta infección.

En el 2013 Castrillón y cols. reportaron un 17,3% de positividad a anticuerpos totales con la Prueba de Aglutinación Rápida en Placa con 2 beta mercaptoetanol (2ME-PARP), en humanos convivientes con caninos infectados provenientes de criaderos del área metropolitana del Valle de Aburrá (Castrillón-Salazar et al., 2013). Esto puede estar asociado probablemente al alto número de perros positivos por cultivo (23,6%) que se encontraron en este mismo estudio, lo cual evidencia la circulación de la bacteria. Además, teniendo en cuenta que no hay control para erradicación ni control de esta infección, un 10% de aumento aproximado por año del número de caninos en la ciudad de Medellín y el aumento del reporte de casos humanos en la literatura

internacional incluyendo caso de pacientes con HIV (Lawaczeck et al., 2011), demuestra la necesidad de evitar que la infección se disemine en nuestro medio y que se convierta en un problema de salud pública.

Una de las cepas aisladas de caninos en Medellín, se secuenció y se denominó *Brucella canis, cepa Oliveri* y se utilizó en este estudio para identificar proteínas inmunógenas que indujeran respuesta en humanos y caninos, posteriormente estas proteínas se produjeron en forma recombinante y se evaluaron preliminarmente en ensayos de ELISA.

Los resultados de esta investigación permitirán a corto y mediano plazo, diseñar pruebas diagnósticas para la detección precoz de casos en humanos y así evitar las complicaciones que de esta infección se pueden derivar.

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5. OBJETIVOS

5.1 Objetivo General

Identificar y caracterizar proteínas de *Brucella canis* que inducen respuesta inmune humoral en humanos y evaluar su utilidad preliminar en el diagnóstico precoz de *Brucella canis* en humanos.

5.2 Objetivos Específicos

1. Seleccionar las proteínas inmunógenas de *Brucella canis*, mediante la generación del inmunoproteoma empleando electroforesis en una y dos dimensiones y western blot con suero de caninos y humanos.

2. Identificar las proteínas reconocidas por los anticuerpos presentes en el suero de los humanos y caninos mediante espectrometría de masas y análisis bioinformáticos.

3. Clonar y expresar las proteínas con mayor reactividad frente a los sueros de humanos y caninos.

4. Evaluar la utilidad de estas proteínas inmunógenas en el diagnóstico serológico en humanos mediante ensayos de ELISA y/o Western blot.

CAPITULO I.

REVISIÓN DE LITERATURA

Se presenta como revisión de literatura el artículo titulado: **“Infección por *Brucella canis* en humanos, propuesta de un modelo teórico de infección a través de la ruta oral”**, publicado en la Revista Infectio 2013; 17(4) 193-200. Este artículo se puede consultar en el siguiente enlace, en el sitio web de la revista Infectio.
http://apps.elsevier.es/watermark/ctl_servlet?_f=10&pident_articulo=90270444&pident_usuario=0&pcontactid=&pident_revista=351&ty=57&accion=L&origen=zonadelectura&web=zl.elsevier.es&lan=es&fichero=351v17n04a90270444pdf001.pdf.

CAPITULO II

Selección de las proteínas inmunógenas de *Brucella canis*, mediante la generación del inmunoproteoma empleando electroforesis en una y dos dimensiones y western blot con suero de caninos y humanos.

En este Capítulo 2 se presenta el trabajo correspondiente al cumplimiento de los objetivos específico 1 y 2 de la tesis:

1. Seleccionar las proteínas inmunógenas de *Brucella canis*, mediante la generación del inmunoproteoma empleando electroforesis en una y dos dimensiones y western blot con suero de caninos y humanos.

2. Identificar las proteínas reconocidas por los anticuerpos presentes en el suero de los humanos y caninos mediante espectrometría de masas y análisis bioinformáticos.

Para los perros se realizó análisis de inmunoreactividad frente al mismo extracto proteico que se utilizó en 2DE-PAGE, pero usando geles de una dimensión y western blot con mezclas de sueros de 50 caninos de criaderos, posteriormente se identificaron las proteínas inmunoreactivas utilizando LC MS-MS.

No se detectaron coincidencias con las proteínas inmunogénicas en humanos, pero por ser los caninos los hospederos primarios de la infección, se evaluaron con ellos también estas proteínas.

Las muestras de humanos y caninos utilizadas en la realización de esta tesis fueron recolectadas durante la investigación: Factores asociados con la seropositividad a *Brucella canis* en criaderos caninos de dos regiones de Antioquia, Colombia. Castrillón-Salazar, L.; Giraldo-Echeverri, C. A.; Sánchez-Jiménez, M.M.; Olivera-Angel M. Cadernos de Saúde Pública 2013; 29:1955-1973.

Articulo sometido a Journal of Proteome Research.

IDENTIFICATION OF *Brucella canis* IMMUNOREACTIVE CYTOPLASMIC PROTEINS IN HUMANS.

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ABSTRACT

Brucella canis is a pathogenic bacterium that can produce infections in dogs and humans. There is no information regarding immunogenic proteins that can induce immune response in humans. We identified by 2D-GEL and immunoblot, immunoreactive proteins for humans using serum samples positive to *Brucella canis* infection. These spots were analyzed using MALDI-TOF-TOF and predicted proteins were identified. Of the 35 protein spots analyzed, 14 proteins were identified and subsequently characterized, using bioinformatics tools. These protein set could be used in the development of sensitive and specific tests for the diagnosis of canine brucellosis in humans.

INTRODUCTION

Brucellosis, one of the most widespread zoonosis in the world (Pappas, 2010), is caused by several species of Gram-negative coccobacilli that belong to the genus *Brucella* (Galińska and Zagórski, 2013). These facultative intracellular pathogens (von Bargen et al., 2012) can infect a wide range of mammals; however, their host preference and pathogenicity may vary (Godfroid et al., 2005). The most studied zoonotic *Brucella* species are *Brucella melitensis, B. abortus* and *B. suis* (Atluri et al., 2011). Nonetheless, other species are of zoonotic concern as well, such as *Brucella canis*, whose main reservoir is

canines. Canine brucellosis can be transmitted in a venereal or an oral fashion by contact with infected secretions (N. Lucero et al., 2010a). Its symptoms, that are not as severe as other *Brucella* infections, include embryonic mortality, abortions in bitches, neonatal morbidity and mortality, epididymitis and prostatitis in dogs, and infertility in both sexes. In humans, its symptoms are nonspecific and may vary from flu-like symptoms to endocarditis and septicemia (Makloski, 2011). In addition, some cases are under-reported; this may be due to lack of specific symptoms and of accurate diagnosis techniques (Pappas et al., 2005).

Therefore, multiple studies have been carried out to develop rapid and accurate methods to detect all zoonotic *Brucellae.* Notably Enzyme-Linked Immunosorbent Assays (ELISA) (Al Dahouk and Nöckler, 2011) based on the use of the lipopolysaccharides (Tiwari et al., 2013; Xin et al., 2013) and recombinant proteins (Seco-Mediavilla et al., 2003; Tan et al., 2012) as antigens has been evaluated. The latter is of special interest because of the potential use of species-specific proteins from different *Brucella*. Numerous antigenic cytoplasmic and membrane proteins have been identified and proposed as candidates for this purpose in *B. abortus* and *B. melitensis* (Abbady et al., 2012; Al Dahouk et al., 2006; Contreras-Rodriguez et al., 2006, 2003; Ko et al., 2012; Pakzad et al., 2009; Seco-Mediavilla et al., 2003; Yang et al., 2011; Zhao et al., 2011), although, to the best of our knowledge, there are no reports of immunoproteomic characterization of antigenic proteins contained in *B. canis*.

Here we report 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 including 5 proteins that have not been previously reported as immunoreactive by any author.

MATERIALS AND METHODS

Bacterial Strain: A field strain isolated from an infected dog (*B. canis* strain Oliveri; EMBL accession numbers HG803175.1 and HG803176.1) from a kennel in Medellín, Colombia, was used to perform the proteomic procedures.

Biochemical and molecular tests were used to confirm the identification of the bacteria as *B. canis* (Ortiz et al., 2012).

Proteins extractions: To retrieve the soluble proteins, a variation of the method proposed by Zhao *et al.* was used (Zhao et al., 2011). 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 KH_2PO_4, 68 mM NaCl, 9 mM NaH_2PO_4), 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. The total protein concentration was determined using the Bradford protein staining assay with Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA), using bovine serum albumin (BSA) as standard.

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).

The parameters used were the following: 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 replicas.

Immunoblots: The third gel was used for immunoblots; the proteins were transferred to nitrocellulose membranes using Towbin buffer (0,025 M Tris, 0.192 M glycine) with 20% methanol at 100 V for 1 h at 4°C using the Mini Trans-Blot® module (Bio-Rad, Inc.) (Towbin et al., 1979). The presence of spots in the membranes was verified using Ponceau stain, and 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. These samples 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.

The serum samples were mixed and used at dilutions 1:500 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 shaking 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 above to reveal immunoreactive spots using a BCIP/NBT solution (Amresco, Solon, Ohio USA).

Spots were selected if they complied with the criteria of 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, or if not immunoreactive, and 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 in 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. Afterward, 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 applied in the sample and allowed to dry.

Mass analysis and protein identification: Subsequently MALDI-TOF/TOF 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 in the 10 most abundant ions of each sample. To identify the proteins specifically belonging to the *B. canis,* strain Oliveri, a bioinformatics analysis from raw data was performed. For this purpose, the Mascot against the NCBI-nr and SwissProt protein databases was used, and for visualization, the software Scaffold (Proteome Software, Inc. Portland, OR, USA).

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, characterization using multiple bioinformatics tools were conducted in order to determine patterns which could influence antibody production. The protein sequences were located and downloaded of the *B. canis* strain Oliveri genome (EMBL accession numbers HG803175.1 and HG803176.1) for further analyses.

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/) (Gasteiger E., Hoogland C., Gattiker A., Duvaud S., Wilkins M.R., Appel R.D., 2005).

Subcellular localization prediction: Analysis of the subcellular localization of the protein was originally made using PSORTb v3.0, (http://www.psort.org/psortb/) (Yu et al., 2010). However, due to inconclusive results and with the aim of confirming the obtained results by PSORTb, a second subcellular localization analysis was applied to the protein sequences using the CELLO subcellular localization predictor v.2.5 (http://cello.life.nctu.edu.tw/) (Yu et al., 2004).

N- and O-glycosylation prediction and signal peptide detection

For the prediction of potential N- and O-glycosylation sites, GlycoPP v1.0, a webserver for glycosite prediction in prokaryotes, was used (http://www.imtech.res.in/raghava/glycopp/) (Chauhan et al., 2012). For both analyses, predictions were based on average surface accessibility. Because most glycosylated proteins in prokaryotes are membrane bound or extracytoplasmic, further analyses were carried out to complement the results of both the subcellular localization prediction and the glycosylation analysis. The presence and location of signal peptides were predicted using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011), with default values.

Analysis of protein domain composition: For the analysis of domain composition of the antigenic proteins, InterProScan

(http://www.ebi.ac.uk/Tools/pfa/iprscan/) was used, with its applications: BlastProDom, FprintScan, HMMPIR, HMMPfam, HMMSmart, HMMTigr, ProfileScan, HAMAP, PatternScan, SuperFamily, SignalPHMM, TMHMM, HMMPanther and Gene3D (Quevillon et al., 2005; Zdobnov and Apweiler, 2001).

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/) (Altschul, 1997) 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/) (Altschul et al., 1990) was used to retrieve non-*Brucella* genus protein homologues, with an established threshold of 10^{-20}. The sequences obtained were aligned with CLUSLATW (Thompson et al., 1994), provided by Unipro UGENE (http://ugene.unipro.ru/) (Okonechnikov et al., 2012). 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 (Tamura et al., 2013).

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 (Larsen et al., 2006) and confirmed with the immunogenicity prediction software using the Kolaskar & Tongaonkar Antigenicity algorithm (Kolaskar and Tongaonkar, 1990). Antigenicity was also determined with SCRATCH Protein Preditor Software using the COBEpro and ANTIGENpro algorithms (http://scratch.proteomics.ics.uci.edu/) (Cheng et al., 2005). Potential conformational epítope 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) (Kelley and

Sternberg, 2009) and the prediction based on the structure epitopes using the software Ellipro (http://tools.immuneepitope.org/tools/ElliPro/iedb_input) (Ponomarenko et al., 2008).

RESULTS

Proteomic analysis by the 2D-PAGE yielded 19 spots of immunogenic proteins. Those were highly expressed or reactive to *B. canis*-positive human sera but not to *B. abortus* or negative controls. 14 from the 19 spots analyzed, were successfully identified including their functions, 6 of them had not previously been reported as immunoreactive for other *Brucella* species: cold shock protein, S-adenosylmethionine synthetase, 2-oxoisovalerate dehydrogenase E1 component subunit alpha, triosephosphate isomerase, fructose-1,6-bisphosphate aldolase, inosine-5'-monophosphate dehydrogenase and ribosomal subunit interface protein.

The other five, showed low MOWSE scores (<83) and were not included in the final analysis; these proteins were acetyltransferase, DnaK, 50S ribosomal protein L7/L12, LysR family transcriptional regulator and TetR family transcriptional regulator.

Proteins identified as immunogenic were predicted as cytoplasmic-based in the subcellular localization servers and none had a predicted N- or O-glycosylation site or signal peptides.

Protein function in most of the identified proteins belongs to two categories; 31% are involved in energy metabolism and 21% in protein synthesis. The remaining 48% are 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 are present in all species from genus *Brucella* and are highly conserved, with identities of 95% or more. In addition, a homolog of each protein was found in closely related taxa, such as *Ochrobactrum*, with identities of up to 87%.

DISCUSSION.

This study presents six novel cytoplasmic proteins not previously reported as immunoreactive for *Brucella* (Identification 1-6; Table 1), but some of them have been reported as immunoreactive in other bacteria: inosine-5'-monophosphate dehydrogenase in *Burkholderia multivorans* and *Burkholderia cenocepacia,* (Shinoy et al., 2013) and S-adenosylmethionine synthetase in *Bordetella pertussis* (Altindiş et al., 2009)*,* both genera are proteobacteria as is *Brucella.*

Although the use of immunoproteomics analysis for protein identification with diagnostic interest has increased in the last decade (DelVecchio et al., 2002b; Eschenbrenner et al., 2006; Khan et al., 2006; Strain et al., 2002) there are no reports in human and dogs for *B. canis*. Literature reports analysis in *B. abortus* (Al Dahouk et al., 2006)*, B. melitensis* (Shinoy et al., 2013)(Zhao et al., 2011) and *B. suis genomes* (Al Dahouk t al., 2013)(Al Dahouk et al., 2009) in different hosts such as camels (Abbady et al., 2012), humans (Connolly et al., 2006), bovines (Pajuaba et al., 2012) and goats (Yang, 2012; Yang et al., 2011; Zhao et al., 2011), analyzing *Brucella abortus* interesting the 5 proteins not included in final analysis, they were established as immunoreactive for *B. abortus* by other authors, namely enolase, 50S ribosomal protein L7/L12, acetyltransferase, pyruvate dehydrogenase subunit Beta and cysteine synthase A, reported by Conolly *et al*. (Connolly et al., 2006).

Zhao *et al*. studying *B. melitensis,* reported glyceraldehyde-3-phosphate dehydrogenase, elongation factor Ts, molecular chaperone DnaK and 50S ribosomal protein (Zhao et al., 2011). Molecular chaperone DnaK and the 50S ribosomal protein L7/L12 were also reported by Al DahouK (Al Dahouk et al., 2006). Yang *et al*. (Yang et al., 2011) 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.*

All the immunoreactive proteins are not necessarily cytoplasmic, but are more easily identifiable with the 2DE-PAGE method than membrane proteins.

Regarding the location of the proteins, the 2DE-PAGE method is useful for the identification of cytoplasmic proteins and this is why we report no membrane protein; for this purpose, LC MS-MS (Liquid chromatography Mass spectrometry analysis) technique is more useful.

Concerning function, our results demonstrate that 31% of the proteins are involved in energy metabolism and 21% in protein synthesis, and that they are different from those of Sandalakis et al. (Sandalakis et al., 2012) who reported in *B. abortus* that 16% of the proteins are involved in energy metabolism and 14% in protein synthesis, when the proteome was analyzed for antibiotic resistance.

In the genus *Brucella,* there are not substantial genomic differences, but there are few polymorphisms or genomic variations, instead (Wattam et al., 2012) that produce variations in the proteins sequences, that may confer host specificity and differences in the pathogenicity, as the *virB* virulence operon (de la Cuesta-Zuluaga et al., 2013; Paredes-Cervantes et al., 2011).

The *B. canis* immunogenic proteins identified in the present study could be useful in the short term for the development of sensitive and specific diagnostic tools for the detection of *B. canis* in humans, which could complement the already existing tests (López et al., 2009; Lucero et al., 2005a; Marzetti et al., 2013). Several tests for the detection of *B. canis* based on the M-strain have been developed and currently used, like the one made by Lucero et al., 2002 who used a saline extract and Wanke et al. (Wanke et al., 2002) who used a heat saline extract; Daltro di Oliveira et al. (de Oliveira et al., 2011) used heat soluble bacterial extracts and sonicated extracts of a *B. canis* wild strain, similarly to Barrouin–Melo et al. (Barrouin-Melo et al., 2007); all of these studies report sensitivity and specificity over 90% in canines; other authors such as Baldi et al. (Baldi et al., 1997, 1994) and Cassataro et al. (Cassataro et al., 2004) used *B. abortus* and *B. melitensis* to produce recombinant proteins and obtained variable results in sensitivity and specificity.

In our particular case, the use of *B. canis* strain Oliveri is an advantage, because it could be used for the development of diagnostic tests directly from a strain circulating in our region.

The following step would be according to the theoretical antigenicity of each protein, to select several of them to produce recombinantly for ELISA or Western blotting with human sera, and determine their diagnostic utility with the aim of improving the diagnosis of this disease.

Conflict of interest

The authors declare no financial or commercial conflicts of interest.

Acknowledgments and financial support

We are grateful to Dr. José Robinson Ramirez and the team of his Grupo de Inmunomodulación, Universidad de Antioquia, for their contribution with the IEF.
We are grateful to Dr. Anthony Haag and his team of Mass Spectrometry Lab, Biomolecular Resource Facility, University of Texas Medical Branch – Galveston, USA for the services provided in the identification of proteins by MALDI-TOF/TOF.
This work was supported by Universidad de Antioquia and sustainability strategy 2013-2014 to Biogénesis group, COLCIENCIAS through a contractual research grant for PhD students to MM Sánchez-Jiménez and the Comité de Investigaciones de La Universidad de Antioquia-CODI research 20127003.

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CAPITULO III

Clonación, expresión y purificación de las proteínas inmunogénicas para humanos.

En este Capítulo 3 se presenta el trabajo correspondiente al cumplimiento del tercer objetivo específico de la tesis:

3. Clonar y expresar cuatro de las proteínas con mayor reconocimiento de los sueros

Para cumplir este objetivo 3 se tuvo la posibilidad de secuenciar el genoma de *Brucella canis* cepa Oliveri en el Centro Nacional de Secuenciación Genómica de la Universidad de Antioquia, (http://www.udea.edu.co/portal/page/portal/bActualidad/Principal_UdeA/UdeANoticias/Ciencia1/Descubrieron%20los%20genes%20de%20la%20bacteria%20Brucella%20canis),(http://www.genome.jp/kegg-bin/show_organism?org=bol), http://www.udea.edu.co/portal/page/portal/bibliotecaAlmaMater/secciones/investigacion/2013/F36B1736867053FDE04018C83D1F331C), a partir de este genoma se descargaron las secuencias de los genes que codifican las proteínas identificadas previamente y sus respectivas secuencias para proceder a realizar los procesos de clonación, expresión y purificación de las proteínas evaluadas por ELISA.

Comparison of *Brucella canis* genomes isolated from different countries shows multiple variable regions.

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Abstract

Brucella canis is a pathogenic bacterium for dogs and their zoonotic potential has been increasing in recent years. In this study, we report the sequencing, annotation and analysis of the complete genome of *Brucella canis* strain Oliveri isolated from a dog in a breeding kennel in Medellín, Colombia, South America.

Whole genome shotgun sequencing was carried out using the ROCHE 454 GS FLX Titanium technology at the National Center for Genomic Sequencing-CNSG in Medellin, Colombia. The assembly procedure was performed using Newbler v2.6. In the genome annotation process, each contig was analyzed independently using as reference *B. suis* ATCC 1330 chromosomes.

The annotation was then manually curated with the ARTEMIS software.

The GC content in the genome was 57%. A total of 2,132 and 1,158 CDS (Coding DNA Sequences) features were annotated in chromosomes 1 and 2 in *B. canis* str. Oliveri, respectively.

The genome showed a perfect synteny with other *B. canis* and *B. suis* strains whose genomes are complete annotated in GenBank.

This new genome could be useful for the development of diagnostic tools and for vaccines search as well, in order to reduce the health impact of this infection in both, dogs and humans. The sequence was deposited in EMBL-EBI with accession numbers HG803175.1 and HG803176.1 for chromosome 1 and 2, respectively.

Keywords: Genome; Sequencing; *Brucella canis*.

Introduction

Brucella is a genus of Gram-negative intracellular coccobacilli that belongs to the Proteobacteria phylum, comprised of ten well-characterized species. This bacterium can infect a wide range of animal hosts, including humans. Due to the relevance of the genus in public health and the need for basic evolutionary studies, a great amount of research is being performed including genome sequencing and analysis of reference strains.

Reports of genomic analyses have included *B. melitensis* (DelVecchio et al., 2002), *B. suis* (Paulsen et al., 2002; Tae et al., 2012, 2011), *B. abortus* (Halling et al., 2005), *B*. *microti* (Audic et al., 2009), *B. ovis* (Tsolis et al., 2009), *B. canis* (Wattam et al., 2009) and *B. pinnipedialis* (Audic et al., 2011). Genomic data seem to indicate that the genus should be comprised of a single species with multiple strains, namely, *B. melitensis* (Chain et al., 2005)

B. canis is a veterinary pathogen that affects the reproductive tract of dogs and can be isolated from blood and other body fluids or tissues of infected animals (Corrente et al., 2010; Gyuranecz et al., 2011; Wanke, 2004).

This bacterium can be transmitted to humans exposed to infected dog secretions, or bacterial laboratory cultures (Wallach et al., 2004), inducing many symptoms from mild flu-like to severe complications (Krueger et al., 2014; Lucero et al., 2005a; N. Lucero et al., 2010b; Manias et al., 2013; Marzetti et al., 2013; Olivera-Angel et al., 2012).

The phylogenomic analysis of *B. canis* has shown that this agent is closely related to *B. suis* and that the former originated from the latter around 22,000 years ago (Foster et al., 2009). Despite the recent publishing notes regarding *B. canis* genome sequencing, no available publication has focused on its analysis (Gao et al., 2012; Kim et al., 2012; Wang et al., 2012).

Brucella genomes lack plasmidic DNA and contain two chromosomes of approximately 2.1 and 1.2 Mbp in length. Both carry ribosomal gene clusters and approximately 3200 protein-coding genes have been described in each species. In general, *Brucella* genomes are highly conserved, with less than 6% nucleotide sequence variation, attributed to the recent origin of the genus (Halling et al., 2005). Methodologies for sequencing *Brucella* genomes have evolved from the Sanger capillary technique (Halling et al., 2005), to the 454 WGS (Whole Genome Sequencing) methodology combined with Sanger sequencing to fill the gaps (Crasta et al., 2008), up to date, when *Brucella* genome studies of different strains have been done using the Illumina platform (Ding et al., 2011).

In the present article we report the full genome sequence of a Colombian isolate of *B. canis* str. Oliveri (access numbers HG803175.1 and HG803176.1) previously reported as Group 2 (Ortiz et al., 2012), using the 454 FLX titanium technologies with the whole genome shotgun strategy. After read assembly, 34 contigs were obtained with an average coverage of 28X. Chromosome 2 was finished using PCR and Sanger sequencing; chromosome 1 was partially degapped using the same strategy. Comparative analysis with *Brucella* reference genomes showed several indel events, some being *B. canis*-specific and others specific of the Colombian strain.

Material and Methods

Bacterial Culture and DNA extraction: The *B. canis* strain from a blood culture in tryptic soy broth (Becton Dickinson, Franklin Lakes, NJ, USA) was isolated in tryptic soy agar (Becton Dickinson, Franklin Lakes, NJ, USA), from a dog in a kennel in Medellín, Colombia. The strain was confirmed as *B. canis* using a biochemical test such as urease production and molecular tests (Ortiz et al., 2012). One colony was then inoculated in tryptic soy broth and incubated for 2 days at 37°C; this liquid culture was used for DNA extraction.

For the genomic DNA extraction, a column-based method was used following the 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).

Whole Genome Shotgun Sequencing and Assembly: Whole genome shotgun sequencing was carried out using the ROCHE 454 GS FLX TITANIUM technology at the National Center for Genomic Sequencing-CNSG, Universidad de Antioquia, Medellin, Colombia, following all the standard protocols. One fourth of PTP (picotiter plate) was used and 289,912 reads were obtained, representing 93,261,046 raw bases. Read dataset quality was analyzed using FASTQC software, to quickly obtains some summary statistics to check the quality of the run (Andrews, 2009). The assembly procedure was performed using Newbler v2.6 with default options for *de novo* genome assembly. Contig scaffolding was carried out using the ABACAS algorithm (Assefa et al., 2009) with *Brucella sius* 1330 chromosomes, as reference.
Chromosome 2 and 1 were completed using PCR, cloning and capillary sequencing for both strands. Gap flanking regions were used to design the primers.

Genome annotation: For the genome annotation process, each chromosome was analyzed independently. The RATT algorithm was used for automatic annotation using as reference the *B. suis* ATCC 1330 genome with Genbank accession numbers NC_004310.3 and NC_004311.2. Each chromosome was then manually curated using the ARTEMIS software. Some of the features had to be edited since the RATT tool incorrectly identified many non-ATG starting codons or skipped several genes.

Genome Comparison: The program MAUVE v2.3.1 served for entire genome alignment and comparative analysis of Indels and SNPs between *B. canis* str. Oliveri, *B. canis* ATCC 23365*, B. canis* HSKA 52141 and *B. suis* 1330. Output tables were filtered and edited using custom PERL and Python scripts. FASTA package was used for global comparisons of genes and predicted protein sequences.

Results

Genome assembly and annotation

Whole genome shotgun in one quarter PTP of 454 FLX Titanium produced a total of 289,912 reads with a GC content of 57%, an average length of 321 and a Phred quality score of 31. Total read bases summed 93,326,476 with no ambiguous nucleotides. Genome assembly with Newbler v2.6 produced 34 contigs with an average coverage of 28X, only one contig presented an aberrant read depth of 1.9X. This contig was also very small with only 141

bases, it might represent an aberrant product of the assembler and therefore was excluded from further analyses. The length of the 33 remaining contigs ranged from 552 to 439,538 bases, the N50 genome assembly value was of 294,016 bases with 99.91% of the bases in the assembly with a Q40 quality value. Repetitive elements within the genome suffer compression during the assembly process, resulting in contigs with a higher read depth, often multiples, of the average contig depth. Contigs 20, 22, 28 and 30 clearly presented such phenomena. BLAST comparisons of such contigs showed them to be ribosomal, IS3 (chr2) and IS711 (portion) sequences, repeated two or more times in the genome.

The following step involved contig reordering based on its position within the chromosome. For this purpose genome alignment with the close reference strain, *B. suis* 1330, was carried out using the MUMMER genome aligner. Once the contigs had been assigned and positioned, the ABACAS automatic algorithm reordered and constructed pseudochromosome of both molecules. Contigs 28, 29 and 32 were excluded by ABACAS in the pseudochromosome.

For further confirmation of the contig scaffolding with the reference chromosomes, PCR amplification and capillary sequencing was carried out for both chromosomes. Chromosome 2 of *B. canis* str. Oliveri was fully validated and neither ambiguous bases nor gaps remained. In chromosome 1, comparative analysis with other *B. canis* genomes, showed that gaps between contigs were related to ribosomal gene clusters, to part of the IS711 insertion sequence, to the translation elongation factor Tu gene and to some repeated sequences at the ends of neighboring contigs. These gaps were partially removed and those belonging to rDNA, repetitive motifs, *tuf1* and *tuf2* and part of the IS711 element were not filled. Twenty gaps remained in this chromosome. The final sizes of the chromosome I and II were 2,111,919 and 1,206,736 bp, respectively.

Genome annotation was carried out using the automated annotation transfer tools of the Sanger institute, RATT (Otto et al., 2011); the *B. suis* 1330 genome was used as reference. The complete chromosome manually curated allowed the annotation of features that were lost by the RATT script or wrongly transferred. Finally, a total of 2,132 and 1,158 CDS features were annotated in chromosomes 1 and 2 of *B. canis* str. Oliveri, respectively built using circos (Krzywinski et al., 2009)

B. canis str. Oliveri genome structure

The genome showed perfect synteny with other *B. canis* and *B. suis* strains whose genomes are completely annotated in GeneBank. The total length of the genome is 3,318,655 bp. A total of 5 insertion elements, including IS711, were identified in chromosome 1 and 2 in chromosome 2. These are identical to the ones previously reported in *B. canis* ATCC 23365. *B. canis* species-specific deletions and insertions were detected in both chromosomes, which ranged from 1 to 357 bp, most of them involving coding regions. Each chromosome in *B. canis* str. Oliveri presented two specific sequence deletions. In chromosome 1, deletions involved 218 and 2 bases affecting the tRNAGLU gene and one intergenic region, respectively. In chromosome 2, deletions were of 1 and 7 bases in intragenic regions.

Protein comparison

Predicted peptides of *B. canis* str. Oliveri were aligned (global alignment) with its respective ortholog in *B. canis* HSK A52141, *B. canis* ATCC 23365 and *B. suis* 1330. This analysis showed that proteins sharing 100% identity were 82% with *B. canis* ATCC 23365, 63% with *B. canis* HSKA 52141 and 71% when compared to *B. suis* 1330 (Table 5). The lower protein identity with the HSK A52141 is noteworthy. When compared to other *B. canis* strains and *B. suis,* inspection of the annotated CDS features in the HSK A52141 genome showed several differences at the start codon of several genes; this could explain the protein identity differences observed between the two isolates.

We considered as very divergent proteins those that had an amino acid identity below 60% with its respective orthologs between *B. canis* str. Oliveri and the other reference strains compared. In this subclass, we observed 7% of divergence when compared to *B. canis* ATCC23365, 14% against *B. canis* HSK A52141 and 2.25% against *B. suis* 1330.

Single nucleotide polymorphisms (SNPs)

SNPs between *B. canis* str. Oliveri and the other three reference genomes studied were calculated. For chromosome 1; 90, 108 and 1408 SNPs were found compared with *B. canis* ATCC 23365, *B. canis* HSK A52141 and *B. suis* 1330, respectively. In chromosome 2, in the same order, 82, 81 and 918 SNPs were detected. This data correlates with the percentage of proteins that showed 100% identity, indicating that *B. canis* str. Oliveri is closer to *B. canis* ATCC 23365. Forty-eight SNPs were unique to the Colombian *B. canis* str. Oliveri.

Discussion

Bacterial genome sequencing has opened a new era in the analysis of pathogenic bacteria. In the western hemisphere *B. canis* str. Oliveri is the first field strain whose genome is sequenced and fully annotated; sequence information from *B*. *canis* ATCC 23365 of the United States and *B. canis* HSK A52141 from South Korea, had not been published at the time of the start of this investigation.

Regarding genome structure, *B. canis* str. Oliveri has a GC-content of 57%, similar to that of both Chinese isolates: the dog strain 118 (57.27%) (Gao et al., 2012) and the human strain BCB018 (Wang et al., 2012).

Regarding size, when these three strains were compared, str. Oliveri showed a larger genome size; 3,318,655 versus 3,234,827 bp for strain 118 and 3,247,324 bp for strain BCB018.

As was described in earlier investigations, within the *Brucella* genome, it is common to find deletion events, more frequently than insertions (Chain et al., 2005; Foster et al., 2008; Gao et al., 2012; Kim et al., 2012; Wang et al., 2012). Apart from the indels common to all the *B. canis* genomes examined, there were specific mutations of the Colombian isolate. This map of variations could be used as candidate for molecular epidemiology studies.

The differences found may be explained by the bacterial adaptation to hosts and environments that produce genetic changes and therefore loss of genomic material unnecessary in the pathogenesis process, or produce genetic polymorphisms, as has been reported by other authors (Atluri et al., 2011; Gao et al., 2012; Kim et al., 2011)

Genome sequencing of several *B. canis* strains around the world, and also from different hosts, such as dogs and humans, it is very important to establish which characteristics are conserved or different between the strains. Perhaps, depending on their environment and host, the pathogenic mechanisms and co-evolution processes could have generated small differences in the genetic material of the bacterium. These changes could be useful in the future to determine what generates the differences in virulence and host specificity.

The genome sequence of *B. canis* str. Oliveri can be used as the starting point in the development of specific diagnostic tools for early detection of infection in dogs and humans,

as well as in the development of vaccines, all of which could help avoid the epidemiological, public health and economic complications caused by the disease.

Conclusions

We report here the complete and annotated genome sequence of *B. canis* str. Oliveri, isolated from a dog in Medellín, Colombia. It shows unique genomic characteristics that indicate that within a species, there are differences in genome structure associated to its geographical origin. This genome could also be useful in the development of diagnostic tools and vaccines, in order to reduce health complications of this infection in dogs and humans.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by Universidad de Antioquia sustainability strategy 2013-2014, COLCIENCIAS through a contractual research grant for PhD students to MM Sánchez-Jiménez and Convenio Especial de Cooperación No. 767 from Colciencias-Colombia and Vicerrectoría de Investigación, Universidad de Antioquia.

We also thank July Duque, Veronica Bermudez and Juan Vélez for their technical assistance and biologist Juan Jacobo de la Cuesta for his help in the translation of this paper.

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CAPITULO IV.

Evaluación mediante ensayos de ELISA de la utilidad de las proteínas recombinantes en el diagnóstico serológico en humanos y caninos.

En este Capítulo 4 se presenta el trabajo correspondiente al cumplimiento del cuarto objetivo específico de la tesis:

4. Evaluar la utilidad de estas proteínas inmunógenas en el diagnóstico serológico en humanos mediante ensayos de ELISA y/o Western blot.

Para la evaluación de las pruebas de ELISA indirecta utilizando como antígenos las proteínas recombinantes producidas, en el caso de los humanos se debían evaluar frente a una prueba que pudiera servir como punto de comparación. Debido a que en los humanos no se tuvieron hemocultivos positivos y solo 9/91 (9,9%) fueron serológicamente positivos con 2ME-PARP, se utilizó una prueba de PCR en la que se amplifico un fragmento de 450 pares de bases que se había estudiado previamente en una prueba que se aplicó a caninos.

En el artículo "**Application of a polymerase chain reaction test for the detection of *Brucella canis* from clinical samples of canines and humans",** publicado en la Revista Colombiana de Ciencias Pecuarias (Rev Colomb Cienc Pecu 2014; 27:3-11), http://rccp.udea.edu.co/index.php/ojs/article/view/910/1032 se observa la Sensibilidad, Especificidad, Valor Predictivo Positivo, Valor Predictivo negativo y concordancia entre las pruebas y demuestra la utilidad de esta PCR como punto de comparación para las muestras de humanos.

Para los caninos las ELISAS se compararon utilizando serología, hemocultivos y la PCR mencionada anteriormente.

En el manuscrito: **"Development and Evaluation of Indirect Enzyme-Linked Immunosorbent assays using recombinant proteins to diagnosis infection by *Brucella canis* in humans and dogs"** se muestra la metodología de producción de las proteínas recombinantes y la evaluación de la utilidad de estas proteínas mediante pruebas de ELISA indirecta.

Development and Evaluation of Indirect Enzyme-Linked Immunosorbent assays using recombinant proteins to diagnose infection by *Brucella canis* in humans and dogs.

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Abstract

The diagnosis of *Brucella canis* infection in humans and dogs is challenging in Colombia due to the unavailability of highly specific and sensitive diagnostic assays for the detection of infection. The objective of this work was to develop and evaluate Indirect Enzyme-Linked Immunosorbent assays using recombinant proteins for the diagnosis of *Brucella canis* in humans and dogs. Four cytoplasmic proteins of *B. canis* strain Oliveri: Inosine 5´ phosphate dehydrogenase (52 kDa), Pyruvate dehydrogenase E1 subunit beta **(**49 kDa), Elongation factor Tu (42.6 kDa), TetR-family transcriptional regulator (27.4 kDa) and one membrane protein OMP31, were previously identified as immunogenic in humans and dogs using western blot analysis and identifying the proteins with mass spectrometry analysis. The genes encoding these proteins were PCR-amplified using the genomic DNA of *B. canis* strain Oliveri that was isolated from a dog in Medellin, Colombia, as a template. Genes were initially cloned in the pGEM-T easy vector or pTZ57R/T vector and *Escherichia coli* DH5α cells were transformed with the recombinant plasmids. Following the verification of the sequence integrity of the amplicons, the genes were excised and subcloned in pRSET-A or pET-28a expression vectors. *Escherichia coli* BL21 cells were transformed with the resulting constructs. Protein expression was induced using IPTG and the proteins were subsequently purified. The utility of these purified proteins as diagnostic antigens for the detection of IgG antibodies in sera obtained from humans and dogs was assessed using indirect ELISA. PdhB and Tuf proteins showed potential as target antigens for the identification of *B. canis* infection in humans but not in dogs.

The use of recombinant antigens for the development of iELISA assays to detect *B. canis*-specific antibodies in human serum could be a potential tool to improve the infection diagnosis.

Keywords: zoonoses, ELISA test, *B. canis* str. Oliveri

1. Introduction

Canine brucellosis is a zoonotic disease that affects canines resulting in several symptoms including abortions, infertility, discospondylitis and uveitis, among others (Hollett, 2006; Wanke, 2004; Wanke et al., 2012). Since 2009, several cases of *Brucella canis* infection in kennel canines (Giraldo-Echeverri et al., 2009), shelter dogs (Ruiz et al., 2010) and pets (Agudelo-Flórez et al., 2012) have been reported in Medellin, Colombia. In humans, infection occurs through contact with contaminated secretions from infected dogs or as result of laboratory accidents (Castrillón-Salazar et al., 2013)(Lucero et al., 2010; Wallach et al., 2004). Similar to the infection produced by *B. abortus*, the disease in humans can be asymptomatic (Olivera and Di-lorenzo, 2009) or chronic; it may take months, even years, before symptoms appear (Krueger et al., 2014). The symptoms can range from undulating or persistent fever to severe cases such as endocarditis and osteomyelitis (Lucero et al., 2010, 2005b; Manias et al., 2013). In Colombia, the bacterium was first isolated from an asymptomatic person cohabiting with infected dogs (Olivera and Di-lorenzo, 2009).

B. canis infection in humans is initially diagnosed using 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 (Lucero et al., 2005a), is recommended as a confirmatory test. The gold standard, however, is the blood culture, but this test has sensitivity issues as the bacteria can mainly be isolated from acute cases of infection where it is found readily circulating in the host (Lucero et al., 2005a). There are other recently developed tests, such as Polymerase Chain Reaction (PCR) (Sánchez-Jiménez et al., 2013), which is often used to confirm specie of isolates. Serological tests such as 2ME-RSAT and iELISA have problems of sensitivity (ranging from 40 to 90%) and specificity (between 60-100%) (Baldi et al., 1994; Cassataro et al., 2004; Delpino et al., 2004; Lucero et al., 2005a; Wanke et al., 2002) which might be explained by the difficulties in obtaining specific immunogenic antigens that react with IgG or IgM antibodies in serum of infected host and that would allow for a rapid, sensitive and specific diagnosis of *B. canis* infection in dogs and humans.

The development of diagnostic tests for the detection of *B. canis* infection is problematic as the humoral immunity induced by *B. canis* in humans is poorly characterized. This type of immunity has mainly been studied in *B. melitensis* and *B. abortus* infections (Bowden et al., 1995), (Baldi et al., 1996), but the conclusions of these studies cannot be extrapolated to *B. canis* infection as *B. canis* is a rough species, while the other two above mentioned species are phenotypically smooth.

Diagnostic tests that detect infection with smooth *Brucella* species mainly utilize smooth LPS. Infection with rough species cannot be detected by tests that involve smooth LPS as a diagnostic probe. Consequently, some previous studies have identified protein antigens for diagnosis of brucellosis (Sascha et al, 2006; Texeiras et al, 1997); without proved useful in human diagnosis.

The main difficulty in developing tests to detect infection with *B. canis* is to identify potential immunogenic proteins that induce an immune response in the infected human and that can serve as diagnostic tools for the development of rapid, sensitive and specific assays.

This work reports the purification and production of five recombinant *B. canis* proteins, which were previously identified as immunogenic in humans and their assessment in indirect ELISA assays using sera samples from 91 humans and 385 canines from two geographical areas of Antioquia, Colombia. The results of this research will allow the design of novel strategies for early detection of human brucellosis cases.

2. Material and Methods

2.1 Bacterial strains

This study used the genome information from *Brucella canis* strain Oliveri isolate of 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).

Vectors used were pGEM®-T easy (Promega), pTZ57R/T (Thermo Scientific), pRSET-A (Invitrogen) and pET-28a (Life technologies). *Escherichia coli* DH5α cells (New England Labs) and *Escherichia coli* BL21 (DE3) (New England Labs) were used for transformation of the recombinant plasmids. The recombinant bacterial cells were grown routinely in LB broth (Sigma, Madison, WI, USA) and Luria Bertani (LB) agar, in presence of Kanamycin (Sigma,

Madison, WI, USA) at 25 µg/ml or Ampicillin (Sigma, Madison, WI, USA) at 100 µg/ml if needed. *E. coli* was grown in Luria Bertani (LB) agar (Sigma, Madison, WI, USA) and LB broth (Sigma, Madison, WI, USA); if the media required antibiotic to select bacteria with the plasmid and construct inserted, Ampicillin or Kanamycin, (Sigma, Madison, WI, USA), was added.

2.2 Samples.

Sera of 385 dogs from 20 kennels and 91 humans in contact with these dogs was obtained from a serum bank and was used to evaluate the performance of the five purified recombinant proteins as diagnostic antigens. Classification of the stored serum samples was carried out according to previous studies into urban area (positive kennels) and rural area (negative kennels) serum samples (Castrillón-Salazar et al., 2013; de la Cuesta-Zuluaga et al., 2013; Sánchez-Jiménez et al., 2013).

2.3 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. (Castrillón-Salazar et al., 2013; Sánchez-Jiménez et al., 2013).

2.4 Polymerase chain reaction for human samples.

A previously reported multiplex Polymerase chain reaction (PCR) (López-Goñi et al., 2008), with good sensitivity and specificity, was used for dog samples (Sánchez-Jiménez et al., 2013). For human samples, 450 bp primers (BMEI0535 Fw: GCGCATTCTTCGGTTATGAA and BMEI0536 Rv: CGCAGGCGAAAACAGCTATAA; Immunodominant antigen, gene *bp26)* (López-Goñi et al., 2008) were used for PCR. Briefly, DNA was extracted from human blood samples using the DNeasy blood and tissue kit (QIAGEN Inc., Valencia, CA, USA) following manufacturer's instructions. Each DNA sample was diluted 1:100 and used immediately or stored at -20 ºC until further use (Sánchez-Jiménez et al., 2013).

2.5 Cloning, expression and purification of 5 recombinant proteins.

From LB agar, one colony of *B. canis* strain Oliveri was inoculated in LB broth, grown overnight and incubated for 24 hours at 37°C; this liquid culture was used for genomic DNA extraction using a column-based method following the 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 humans, a previous work (data not published) identified 14 immunogenic cytoplasmic proteins of *Brucella canis* strain Oliveri by 2DE-PAGE, mass spectrometry analysis and bioinformatics tools. According to their antigenicity score and the absence of reports identifying them as immunogenic in other *Brucella* sp. for humans, four proteins were selected and produced by recombinant methods. These purified proteins were subsequently used as antigens in iELISA. The four proteins include: Inosine 5´ phosphate dehydrogenase (52 kDa), Pyruvate dehydrogenase E1 subunit beta **(**49 kDa), Elongation factor Tu (42.6 kDa) and TetR-family transcriptional regulator (27.4 kDa). Outer Membrane Protein 31 (OMP31), which has species-specific sequence differences, has been reported as immunogenic in other species of *Brucella* genus (Thavaselvam et al., 2010; Tiwari et al., 2013) including *Brucella canis* in a murine model (Clausse et al., 2013). The primers used for cloning are listed in Table 1.

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

Genetic regions that encoded the proteins of interest were PCR amplified (Figure 1) and ligated into pTZ57R/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 concentration, depending on the expression vector used. Exponential-phase culture was induced using 1 mM IPTG (Isopropyl-β-D-1-Thiogalactopyranoside) at 37° C, and the protein expression was analyzed at two, four and six hours post-induction. Membrane Omp31 expression was induced overnight at room temperature to obtain a soluble fraction. Time-specific protein expression was analyzed by electrophoresis using a 12.5% polyacrylamide gel.

6 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 minutes with pulses of 1 minute

with 10 sec intervals. For assessing the protein solubility, the bacterial suspensions were centrifuged (8500 g for 35 minutes) at 4°C and the 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 (Figure 2 and 3). Protein concentration was estimated by Bradford method using Bovine Serum Albumin as a standard. The proteins were stored at 4°C and their purity was evaluated using Agilent 2100 Bioanalyzer (Agilent techonologies Inc. Santa Clara, CA, USA). The reactivity of the proteins was further tested by iELISA.

2.6 Indirect microplate iELISA for IgG antibody detection.

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

To test the sera from humans and dogs for antigen-specific antibodies, the proteins were diluted to an optimal concentration in a 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 were coated on 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 five 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 minutes. Plates were blocked with 200 µl of blocking buffer (TBS with 5% non-fat milk) and incubated at 37°C for 1 hour and then washed again as mentioned previously. Finally, 50 µl of human and dog serum samples, diluted 1:500 in blocking buffer, were added to each well. Each sample was tested in triplicates.

In humans, positive control was a mix of three human samples that were found to be positive by 2ME-RSAT to *Brucella canis*. 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 samples found to be negative by 2ME-RSAT and RBT was used. C*ontrols were all run in duplicates.*

In dogs, a mix of three samples (obtained from kennels previously identified as being *B. canis* positive) found to be 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) found to be negative for *Brucella canis* by 2ME-RSAT, blood culture and PCR was used as a negative control. C*ontrols were all run in duplicates.*

Plates were incubated at 37°C for 1 hour with constant shaking and subsequently washed four times. 50 µl of one of the following secondary antibodies was added to the wells after appropriate dilution: alkaline phosphatase labelled anti-human IgG (g-chain specific) (Sigma-Aldrich, Madison, WI, USA) or alkaline phosphatase labelled anti-dog IgG (whole molecule). Plates were reincubated at 37°C for 1 hour and washed. For color development, 100 µl of TMB substrate solution (3,3',5,5'-tetramethylbenzidine (0,4g/L) and peroxide (0,02% H_20_2) solution (Thermo Scientific Meridian Rd, Rockford, IL, USA) was added for 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.

2.7 Statistical analysis.

All the results were registered in an excel file (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 one of the diagnostic tests, the percentage of positive and negative samples was estimated. Cutoff values for each one of the tested recombinant proteins were determined by Receiver Operating Characteristic (ROC) curves. In addition, sensitivity, specificity, positive and negative predictive values, likelihood ratio for positive and negative results and concordance between tests according Kappa were also evaluated.

3. RESULTS

PCR results

By PCR, 43/91 (47.3%) human serum samples were positive. All the positive human serum samples were obtained from urban areas. For dogs, 93/385 (24.2%) serum samples were positive. All the positive dog serum samples were also obtained from urban areas (Table 2).

Cloning, expression and purification of five recombinant proteins.

Purity of the five proteins was upper 95%. Final concentration of the proteins was 1.5 mg/ml and they 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.

Indirect microplate iELISA for IgG antibody detection.

For evaluation of the five recombinant proteins as diagnostic antigens in iELISA assay, cutoffs were determined using ROC curves. Roc curve in humans is shown in Figure 3 and in canines in Figure 4; area under the curve and the cutoff values for each of the five iELISAs are presented in Table 3.

For human serum samples, iELISA results were compared with PCR results because only 9.9% of the samples were positive by 2ME-RSAT and none were positive 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; hence, there was a good concordance between tests. Number of observed agreements between the 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 concordance between tests. Number of observed agreements between the tests was 71 (78.02%). Kappa coefficient for Tuf was 0.563 which is considered to reflect a moderate concordance between tests. Number of observed agreements between the tests was 73 (80.22%).

For Tet, GuaB and Omp31 proteins, S: was 77, 67 and 53 %, respectively; Sp: 58, 58 and 52%, respectively; NPV: 74, 67, 56%, respectively and PPV: 62, 59 and 50%, respectively. Kappa coefficient for Tet and GuaB reflected a fair concordance between tests. For Tet

protein, the number of observed agreements between the tests was 61 (67.03%). The number of observed agreements between the tests for GuaB was 57 (62.64%) and for Omp31 were 48 (52.75%). Coefficient Kappa for Omp31 indicated that there was a poor concordance between tests.

Results for iELISA tests for 9 human serum samples that were positive by 2ME-RSAT are explained in Table 5.

For canine serum samples, iELISA results were compared with 2ME-RSAT, blood culture and PCR results (Table S1). When proteins Tet, GuaB and Omp31 were used as antigens, iELISA results showed poor concordance to 2ME-RSAT, blood culture and PCR results. Using a mix of PdhB plus Tuf or using Tuf only, iELISA demonstrated best performance with S: 75% , Sp:64%, PPV: 40%; NPV: 89% and had a fair concordance to 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 the tests was 258 (67.01%).

When results from PCR and blood culture were compared, a sensitivity of 92% and a specificity of 86% was found and there was a moderate concordance between tests. When PCR and 2ME-RSAT were compared, S: 78% and Sp: 86% was found; comparison of results from 2ME-RSAT and blood culture showed S: 67% and Sp: 93%. All these results show low levels of sensitivity and specificity.

Regarding results for dog serum samples, 110/385 (28.6%) were negative by 2ME-RSAT, blood culture, PCR, and by iELISA for PdhB-Tuf, PdhB and Tuf proteins. 68/385 (17.7%) were positive only for PdhB and negative by other tests. 63/385 (16.4%) were positive for PdhB-tuf and Tuf by iELISA but negative by other tests. 36/385 (9.4%) were negative by 2ME-RSAT, blood culture and PCR but positive by iELISA for PdhB-Tuf, PdhB and Tuf proteins. 19/385 (4.93%) were positive by 2ME-RSAT, blood culture, PCR, and by iELISA for PdhB-Tuf, PdhB and Tuf proteins.

None of the negative or specificity human and dog serum controls was positive by iELISA.

4. DISCUSSION

In this work, we have identified five recombinant proteins that can be used as potential diagnostic antigens to improve the 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 five proteins used as antigens, best results were found using PdhB-Tuf mix (S 98% and Sp 73%) and PdhB (S 91% and Sp 77%) for human serum samples. The results of iELISA and PCR had good Kappa coefficient (0.696 and 0.672 respectively) indicating that they had a good concordance. The high specificity of these two iELISAs that use PdhB-Tuf and PdhB as antigens could be explained because a comparison was made with the results of PCR, which is a direct test to detect DNA and not antibodies.

There are a few iELISA assays to detect the infection in humans that have been described previously (Krueger et al., 2014; Lucero et al., 2010, 2005a, 2002, 1999; Oliveira et al., 2010). These iELISAs demonstrate variable sensitivities and specificities (70-100%) due to the infection develops low quantity of antibodies, consequently is difficult to use it in diagnosis; so far, there are no new developments to detect specific bacterial components. Until now, the disease is under-diagnosed due to a general lack of the serological testing techniques which leads to faulty conception about the disease prevalence (Lucero et al., 2005a).

PdhB protein is a novel 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 (Sun et al., 2014) and *Mycoplasma capricolum* sub sp. *Capripneumoniae* in goats (Zhao et al., 2013). 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 (Ke et al., 2000). These genes are involved in protein synthesis and antibiotic resistance mechanisms in *Brucella* (Halling and Jensen, 2006). In *Brucella canis* strain Oliveri, two *tuf* genes were found upon sequencing of the genome (data not published). *tuf* has been used as a phylogenetic marker in *Streptococcus* (Picard et al., 2004), *Enterococcus, Lactobacillus,*

Bifidobacterium lactis (Li et al., 2012) *and Yersinia* (Ventura et al., 2003) and has been reported to be immunogenic protein of *Burkholderia pseudomallei* in mouse (Nieves et al., 2010) and humans (Shinoy et al., 2013), but its immunogenic potential in *Brucella* species has not been verified.

The other two cytoplasmic proteins, GuaB (S: 67%, Sp 58%) and Tet (S: 77% Sp 58%), were tested as antigens in iELISA but they failed to provide good results for the detection of antigen-specific antibodies in human serum samples. The bacteria adapt to changes in their environment by regulating gene transcription and TetR family transcriptional regulators constitute one of the largest groups of bacterial transcription factors (Ahn et al., 2012); this might explain the low specificity obtained when this protein is used as a diagnostic antigen.

Regarding membrane protein, Omp31 has been previously found to be of limited value for the diagnosis of brucellosis in humans, sheep and dogs (Cassataro et al., 2004) by iELISA. Tiwari et al. (Tiwari et al., 2013) reported a S 62.2% and Sp 94.7% when Omp31 was used as an antigen in iELISA for detection of *Brucella* in human serum samples. In our analysis, we found an S 53% and Sp 52% on using Omp31 in iELISA for detection of human brucellosis. Omp31 showed promising results when used as an experimental vaccine against *Brucella canis* in murine model (Clausse et al., 2013) .

Using the recombinant proteins as antigens for detection of canine brucellosis cases did not deliver encouraging results. Even though using a mix of Tuf and PdB-Tuf as antigens showed promise 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 the detection of *B. canis* in canines. This is very low when compared to the results obtained on using total extracted protein (Wanke et al., 2002) as antigen, where the other authors report a S and Sp 100%.

Baldi et al. (Baldi et al., 1997, 1994) used a *Brucella abortus* cytoplasmic fraction free of LPS as antigen and obtained S 93.3% and Sp 98%.

Wanke et al. (Wanke et al., 2002) demonstrated a sensitivity of 85-100% and a specificity of 94-96.7% for different antigens used in iELISA for the detection of in dogs. The type of antigens used, cytosolic or membrane antigens of *Brucella* sp. as opposed to recombinant proteins of a wild strain, could account for the difference in the S and Sp between our tests.

De Oliveira et al. (de Oliveira et al., 2011) 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 in dogs and Barrouin-Melo et al (Barrouin-Melo et al., 2007) reported S 95% and Sp 91% on using total extracted protein as an antigen.

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

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 (Wanke et al., 2002). 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. Also, serum samples of dogs receiving antibiotic treatment might show negative agglutination but be positive by iELISA (Wanke et al., 2002).

5. CONCLUSIONS

In humans, recombinant proteins PdhB and Tuf showed promise 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 a high specificity and sensitivity. However, they can still serve as potentially useful diagnostic antigens in iELISA which can be performed as an adjunct complementary diagnostic test in combination with other tests including 2ME-RSAT, blood culture and PCR, for the diagnosis of *B. canis* infection in dogs.

iELISA assay will probably deliver better results when a mix of all the proteins is used as an antigen 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 nonfat milk is used to dilute the serum samples and the dilution factor is reduced.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by Universidad de Antioquia sustainability strategy 2013-2014 and Comité de Investigaciones UdeA-CODI research 20127003; COLCIENCIAS through a contractual research grant for PhD students to MM Sánchez-Jiménez; Convenio Especial de Cooperación No. 767 from Colciencias-Colombia and Vicerrectoría de Investigación, Universidad de Antioquia; and Department of Comparative Pathobiology, College of Veterinary Medicine, Purdue University, Indiana, United States.

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CONCLUSIONES GENERALES

1. Se secuenció el genoma de una cepa nativa de *Brucella canis* (*B. canis* cepa Olivera) aislada de un canino de la ciudad de Medellín.

2. Se generó el inmunoproteoma de *B. canis* cepa Oliveri frente a sueros de humanos y caninos y se identificaron 5 proteínas con potencial inmunogénico para humanos.

3. Se produjeron en forma recombinante las proteínas citoplasmáticas PdhB, Tuf, Tet, GuaB y la proteína de membrana Omp31.

4. La prueba de PCR probada en humanos y caninos es útil para la identificación de DNA circulante de la bacteria en sangre total.

5. En forma individual las proteínas PdhB y Tuf mostraron buenos resultados de S, E, VPP y VPN, cuando se realizó ELISA mezclando estas dos proteínas, estos valores se incrementaron, por lo cual la ELISA indirecta se debe hacer en el futuro con esta mezcla de proteínas.

6. Se completó un panel de pruebas diagnósticas que incluyen: serología por 2ME-PARP, Hemocultivo, PCR y ELISAi con las proteínas recombinantes, que es útil en la detección de la infección por *Brucella canis* en humanos.

RECOMENDACIONES

1. Revisar las proteínas inmunogénicas identificadas pero no producidas en forma recombinante y mirar cuales de ellas con buenos resultados de antigenicidad, se podrían usar para desarrollar nuevas pruebas diagnósticas e inclusive vacunas.

2. Generar una proteína quimera con los epítopes más antigénicos de las proteínas PdhB y Tuf para incrementar la especificidad de la prueba o para vacunas.

3. Realizar modificaciones a la metodología de la pruebas de ELISA indirectas para mejorar la sensibilidad y especificad de las pruebas: 1. Mezclar todas las proteínas y usarlas como antígeno en un mismo pozo; 2. Aumentar la concentración del antígeno para sensibilizar los platos de 0,1 a 0,5 µg; 3. Disminuir la astringencia de la prueba rebajando de 5% a 1% el uso de leche descremada para diluir las muestras y los anticuerpos

4. Realizar las pruebas con anticuerpos polivalentes.

5. Realizar pruebas de aplicación clínica de la ELISA desarrollada en este trabajo en población general de humanos para determinar su desempeño como prueba diagnóstica.

ANEXOS

Anexo 1. Reglamentos de revistas en las que se publicaron o se sometieron artículos derivados de esta tesis.

Revista Infectio:
http://revistainfectio.org/site/portals/0/ojs/index.php/infectio/article/viewFile/602/569

Revista Colombiana de Ciencias Pecuarias:
http://rccp.udea.edu.co/index.php/ojs/about/submissions#authorGuidelines

Veterinary Microbiology
http://www.elsevier.com/wps/find/journaldescription.cws_home/503320?generatepdf=true

Revista Veterinaria y Zootecnia.
http://vetzootec.ucaldas.edu.co/downloads/normas%20editoriales%20VZ_espanol.pdf

Journal of proteomics
http://www.elsevier.com/journals/journal-of-proteomics/1874-3919/guide-for-authors

Anexo 1: Otros artículos derivados del trabajo en la línea de brucelosis canina en los cuales participe durante el doctorado.

1. Ortiz LF, Muskus C, Sánchez MM, Olivera M. Identification of *Brucella canis* group 2 in colombian kennels. RCCP 2012; 25: 615-619

2. De la Cuesta-Zuluaga JJ, Sánchez-Jiménez MM, Martínez-Garro J, Olivera-Angel M. Identification of the *virB* operon genes encoding the type IV secretion system, in Colombian *Brucella canis* isolates. Vet Microbiol 2013; 163:196-9

3. Castrillón-Salazar Laura, Giraldo-Echeverri Carlos Andrés, Sánchez-Jiménez Miryan Margot, Olivera-Angel Martha. Factores asociados con la seropositividad a *Brucella canis* en criaderos caninos de dos regiones de Antioquia, Colombia. Cad. Saúde Pública. 2013; 29: 1955-73.