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Comparative proteomic analysis of outer membrane vesicles from *Brucella suis*, *Brucella ovis*, *Brucella canis* and *Brucella neotomae*

María del Socorro Ruiz-Palma[1,2] · Eric Daniel Avila-Calderón[1,3] · Ma. Guadalupe Aguilera-Arreola[1] ·
Ahidé López-Merino[1] · Enrico A. Ruiz[4] · María del Rosario Morales-García[5] · Edgar Oliver López-Villegas[6] ·
Zulema Gomez-Lunar[1] · Beatriz Arellano-Reynoso[7] · Araceli Contreras-Rodríguez[1]

Abstract

Gram-negative bacteria release nanovesicles, called outer membrane vesicles (OMVs), from their outer membrane. Proteomics has been used to determine their composition. OMVs contain proteins able to elicit an immune response, so they have been proposed as a model to develop acellular vaccines. In this study, OMVs of *Brucella suis*, *B. ovis*, *B. canis*, and *B. neotomae* were purified and analyzed by SDS-PAGE, transmission electron microscopy and liquid chromatography coupled to mass spectrometry to determine the pan-proteome of these vesicles. In addition, antigenic proteins were detected by western blot with anti-*Brucella* sera. The *in silico* analysis of the pan-proteome revealed many homologous proteins, such as Omp16, Omp25, Omp31, SodC, Omp2a, and BhuA. Proteins contained in the vesicles from different *Brucella* species were detected by anti-*Brucella* sera. The occurrence of previously described immunogenic proteins derived from OMVs supports the use of these vesicles as candidates to be evaluated as an acellular brucellosis vaccine.

Keywords Outer membrane vesicles · Brucellosis · Bacterial vesicles · Acellular vaccines

Introduction

Outer membrane vesicles (OMVs) were first observed in *Escherichia coli* by electron microscopy in 1966; at that time, they were designated as globules (Knox et al. 1966). Later, it became possible to identify their components in detail, such as lipopolysaccharide (LPS), phospholipids, outer membrane proteins (OMPs), periplasmic and

✉ Araceli Contreras-Rodríguez
aracelicontreras21@gmail.com; acontrerasr@ipn.mx

1 Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas (ENCB), Instituto Politécnico Nacional (IPN), Prol. Carpio y Plan de Ayala s/n, Col. Sto. Tomás, CP 11340 Mexico City, Mexico

2 División Químico Biológicas, Universidad Tecnológica de Tecámac, Km. 37.5 Carretera Federal México-Pachuca, Predio Sierra Hermosa, CP 55740 Tecámac, Estado de México, Mexico

3 Departamento de Biología Celular, Centro de Investigación y de Estudios Avanzados, Instituto Politécnico Nacional, CINVESTAV-IPN, Avenida Instituto Politécnico Nacional 2508, Col. San Pedro Zacatenco, CP 07360 Mexico City, Mexico

4 Departamento de Zoología, Escuela Nacional de Ciencias Biológicas (ENCB), Instituto Politécnico Nacional (IPN), México City, Mexico

5 Departamento de Investigación, CICATA Querétaro, Instituto Politécnico Nacional (IPN), Querétaro, Mexico

6 Departamento de Investigación, Central de Microscopía, Escuela Nacional de Ciencias Biológicas (ENCB), Instituto Politécnico Nacional (IPN), Prol. Carpio y Plan de Ayala S/N, Col. Sto. Tomás, CP 11340 Mexico City, Mexico

7 Departamento de Microbiología e Inmunología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México (UNAM), Coyoacán, 04510 Mexico City, Mexico

cytoplasmic proteins and nucleic acids (Holst et al. 2009; McConnell et al. 2011; Stevenson et al. 2018; Zhang et al. 2018). Given their composition, it has been proposed that OMVs are involved in protein transport, genetic material transference, nutrient acquisition, interkingdom communication, antibacterial activity, neutralizing phage decoy activity, virulence factor delivery, and immune response modulation (Ellis and Kuehn 2010; Veith et al. 2014; Bitto et al. 2017; Augustyniak et al. 2018; Backert et al. 2018; Maerz et al. 2018; Reyes-Robles et al. 2018). As aforementioned, OMVs transport components of the whole cell, some of them are able to elicit an immune response, and the OMVs of different bacteria have been tested as vaccines, showing promising results in the development of acellular vaccines (Kadurugamuwa and Beveridge 1998; Liu et al. 2017; Tan et al. 2018).

The genus *Brucella* is composed of ten recognized species, *Brucella melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, *B. neotomae*, *B. ceti*, *B. pinnipedialis*, *B. microti*, and *B. inopinata* (Foster et al. 2007; Godfroid et al. 2011; Scholz et al. 2008, 2010, 2016). The species, *B. papionis and B. vulpis* have been proposed recently (Whatmore et al. 2014; Scholz et al. 2016). Brucellosis induces abortion in cows and orchitis in infected males, whereas in humans, it is a febrile and systemic disease that can involve almost any organ or system of the body.

Human brucellosis is a debilitating disease characterized by undulating fever with flu-like symptoms (Seleem et al. 2010). In addition, some complications have been widely described including neurobrucellosis, sacroiliitis, spondylitis, orchitis, and endocarditis (Pappas et al. 2005). At present, there are vaccines against animal brucellosis based on live attenuated cells of *Brucella*; however, these vaccines are ineffective in humans, and the cells can be unstable and possibly revert to a virulent phenotype (Chukwu 1985; Avila-Calderon et al. 2013). Currently, the *B. abortus* RB51 and S19 strains are used to control cattle brucellosis, while *B. melitensis* Rev1 is used to vaccinate goats and sheep (Avila-Calderon et al. 2013). At present, no commercial vaccine against swine or human brucellosis is available. Some trials have been performed using *B. abortus* RB51 for swine vaccination; however, protection against *B. suis* infection has not been observed (Stoffregen et al. 2006). Due to the widespread occurrence of this disease in humans and pigs in many areas of the world, it is important to continue research to develop safer brucellosis vaccines.

A few years ago, OMVs of *B. melitensis* were tested as a vaccine in mice infected with virulent *B. melitensis*, and the results showed that the vesicles protected mice at the same level as the live vaccine strain *B. melitensis* Rev1. Moreover, among others, the proteins Omp31, Omp25, SodC, Omp16, and Omp19 were identified by proteomics in the OMVs (Avila-Calderon et al. 2012). Furthermore, OMVs from *B. abortus* 2308 and RB51 were tested as vaccines in mice

challenged with *B. abortus* 2308, and the results showed that vesicles from both strains protected mice similarly to the live vaccine strain *B. abortus* RB51. Some of the proteins identified in these vesicles are known to be *Brucella* immunogens, such as SodC, Omp2b, Omp2a, Omp10, Omp16, and Omp19 (Araiza-Villanueva et al. 2019). In addition, THP-1 cells pre-treated with OMVs from *B. abortus*-induced adherence, phagocytosis, and adhesion-molecule expression, but inhibited cytokine expression, and modulation of the host immune response (Pollak et al. 2012).

The *Brucella* species genomes share high identity (98–100%), with the more variability in genes (<95% identity) encode hypothetically surface-exposed proteins, such as OMPs (Whatmore 2009). An extensive comparison of ten *Brucella* genomes confirmed this similar core genomic structure (Whatmore 2009). Based on this high genetic similarity between *Brucella* species, it is expected that the protein cargo in purified OMVs is conserved. The orthologous proteins searching analysis of OMVs from different *Brucella* species revealed homologous proteins into the vesicles. If the antigenic properties of the proteins contained in the OMVs were determined by western blot analyses, the knowledge of such antigens could be useful for broad-range vaccine development against brucellosis. *B. melitensis*, *B. abortus* and *B. suis* represent the most pathogenic and zoonotic species spread worldwide (El-Sayed and Awad 2018). However, other species are also hazardous for humans or animals, for example, *B. ovis* is a natural rough strain (lacking O-side chain LPS) that is able to infect sheep and goats but it is not considered a zoonotic bacterium (Olsen and Palmer 2014). Although there are no reports of human cases due to *B. ovis*, its eradication from a flock is essential to avoid economical losses (Ridler and West 2011). *B. canis* is another natural rough strain that preferentially infects dogs, however, cattle have also been reported to be infected by this species (Baek et al. 2011; Cosford 2018). Due to the low virulence of *B. canis* compared to the most pathogenic strains, asymptomatic human infection is the most common presentation. However, some patients do present with *B. canis*-induced symptoms such as fever, headache, arthralgia, weakness, and constipation (Wallach et al. 2004). On the other hand, *B. neotomae* is a smooth strain that can be isolated from desert wood rats (Stoenner and Lackman 1957). It has been shown that *B. neotomae* can also cause infection in humans (Villalobos-Vindas et al. 2017). Moreover, *B. neotomae* infects the liver, lymph nodes and spleen, and induces Th1 cytokine expression in mice, similar to *B. melitensis*, *B. abortus* and *B. suis*. Therefore, *B. neotomae* also represents a potential zoonotic species (Kang et al. 2018).

In this study, OMVs were purified from *B. suis*, *B. ovis*, *B. canis* and *B. neotomae*, and the proteins contained in the OMVs were determined by mass spectrometry. The analysis of the pan-proteome of *Brucella* vesicles allowed

classifying the OMVs cargo into clusters of orthologous proteins. In addition, the presence of proteins contained in the OMVs recognized by anti-*Brucella* antibodies were determined by western blot.

Materials and methods

Bacterial strains and growth conditions

Brucella suis ATCC 23444 (1330), *B. ovis* ATCC 25840 (63/290), *B. canis* ATCC 23365 (RM6/66) and *B. neotomae* ATCC 23459 (5K33) were used in this study. All strains were grown on trypticase soy agar (BD Bacto™) plates supplemented with yeast extract (BD Bacto™) (0.5%) (TSA-YE).

OMV isolation and purification from culture medium

OMV purification was performed according to the protocol described by Avila-Calderon et al. (2012). Briefly, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* were cultured in bulk on TSA-YE plates by incubating for 48 h at 37 °C. Cultures were harvested with a rubber policeman and suspended in 25 mL of sterile 0.1 M phosphate-buffered saline (PBS). The cells were pelleted by centrifugation at $10,000 \times g$ for 30 min at 4 °C, and the supernatant was filtered through a 0.22 µm pore filter (Millipore Corporation) to remove the remaining bacteria. A sterility test was performed on the supernatant by culturing an aliquot on a TSA-YE plate, followed by incubation for 7 days at 37 °C. The OMVs were obtained by ultracentrifuging the sterile supernatant at $100,000 \times g$ for 2 h at 4 °C. The pellet was washed twice with 25 mL of sterile PBS. Finally, vesicles were suspended in 1 mL of sterile PBS. For each strain, the total protein concentration was determined using a PIERCE-BCA Kit (Thermo Fisher Scientific Incorporated) following the manufacturer's recommendations. OMVs were purified with a density gradient using OptiPrep (Sigma-Aldrich, Incorporated) according to the protocol of Fernandez-Moreira et al. (2006). Briefly, OptiPrep was diluted with sterile PBS to final concentrations of 10, 15, 20, 25 and 30%. Then, 2.6 mL of the OptiPrep solution was layered sequentially, from high to low density in an ultracentrifuge tube. OMVs were loaded at the bottom of the tube. Tubes were centrifuged at $100,000 \times g$ for 16 h at 4 °C. The OMVs appeared as an opalescent band in the density gradient. Then, the OMVs were collected, washed twice with sterile PBS at $100,000 \times g$ for 2 h at 4 °C, and finally, suspended in 500 µL of PBS. The OMV samples were stored in 0.5 mL aliquots at − 20 °C until use.

Observation of OMVs by electron microscopy

Twenty microliters of purified OMVs from *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* (approximately 25 µg of protein) were placed onto copper grids coated with formvar and dried using filter paper. Phosphotungstic acid (1%) was added, and grids were allowed to dry for 10 h at room temperature, and then they were observed with a JEOL model JEM 10–10 transmission electron microscope. Micrographs were taken with ATM image capture engine V.5.4.2 software at different magnifications. To determine differences in the size and number of purified vesicles produced by each *Brucella* species, the OMV diameters were measured and the number of vesicles was counted from ten fields. To avoid differences in vesicle counting, the same protein concentration was placed onto the copper grids. One-way ANOVA with Tukey's post hoc test was used for statistical analysis (95% confidence interval). GraphPad Prism V.5.01 was used for the statistical analysis.

To observe the vesicles released from *Brucella* species, each strain was grown on TSA-YE plates overnight and then covered with molten soft agar. Once the agar solidified, it was cut into small cubes (2 mm). All preparations were stained with osmium tetraoxide (OsO_4). Images were obtained using the aforementioned transmission electron microscope at the Microscopy Facility of ENCB-IPN, Mexico City, Mexico.

Denaturing polyacrylamide gel electrophoresis

SDS-PAGE was performed in 15% acrylamide slab gels using the method described by Laemmli (1970). The gels were stained with a Bio-Rad[R] Silver Stain Kit. The molecular sizes of the purified OMV proteins were determined by comparing their electrophoretic mobility with that of a wide range of molecular mass markers (Page Ruler™ Prestained protein ladder, Thermo Fisher Scientific) using ImageJ V.1.49.

Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS)

After obtaining OMV proteins by SDS-PAGE, each gel was cut into four sections in duplicate. Each section was reduced with 50 mM dithiothreitol, alkylated with iodoacetamide and finally "*in gel*" digested with trypsin. The peptides were desalted using a Zip Tip[R] (Millipore Corp) and then concentrated in a Speed-Vac SPD 1010 Thermo Electron.

All gel sections were dissolved in 50% acetonitrile containing 1% acetic acid. Then, they were placed directly into a Finnigan LCQ iron trap mass spectrometer. LC–MS/MS analysis was performed with a Pico Frit needle/ RP C18 column (New Objective, Woburn, MA, USA) using a fast

gradient system with 5–60% solution B (100% acetonitrile with 1% acetic acid) over 45 min. The electrospray ionization source voltage was set at 1.8 kV, and the capillary temperature was set at 130 °C. Collision-induced dissociation (CID) was performed using 25 V of collision energy and 35–45% (arbitrary units) normalized collision energy, and the scan had the wide band activated. All spectra were obtained in the positive-ion mode. Data acquisition and deconvolution were carried out using X-calibur software on a Windows XP PC system at the Proteomic Facility of the Instituto Nacional de Biotecnología-UNAM, Cuernavaca, Mexico. The MS/MS spectra from enzymatically generated peptides were analyzed by Sequest software from Finnigan (Palo Alto, CA, USA) and the MASCOT software package search engine from Matrix Science Ltd (Boston, MA, USA) that interprets mass spectral data into protein identities.

Determination of the OMV pan-proteome

The peptide sequences obtained by LC–MS/MS from OMVs from *Brucella* species were analyzed using BLASTP to determine the identity of the proteins (NCBI (https://www.ncbi.nlm.nih.gov) and UniProt (https://www.uniprot.org/uniprot). Evpedia (http://student4.postech.ac.kr/evpedia2) and OrthoVenn (http://www.bioinfogenome.net/OrthoVenn) online software were used to analyze the gene ontology terms enrichment (Wang et al. 2015). To predict the subcellular location of each protein, PSORTb V.3.0 from the ExPASy Bioinformatics Resource Portal (http://www.psort.org/psortb/index.html) and ProtCompB from the Softberry database (http://linux1.softberry.com/berry.phtml?topic=pcompb&group=programs&subgroup=proloc) were used. In addition, the MyHits database (https://myhits.isb-sib.ch/) was used to determine the motif sequence on each protein.

Antisera preparation

The immunization protocol was performed with whole inactivated smooth and rough *Brucella* strains to corroborate cross-reactivity between the antibodies against *Brucella* OMV antigens. *B. abortus* 2308 or *B. canis* RM 6/66 were cultured on TSA-YE plates for 36 h at 37 °C. Cultures were centrifuged and then the cells were used to obtain a bacterial suspension adjusted to OD at 600 nm of 0.8. A 10 mL aliquot from the suspension was centrifuged and the pellet was washed twice with PBS. The pellet was resuspended in 10 mL of PBS with 10% aluminum hydroxide. CFUs/mL were determined by plating on TSA-YE plates. The bacterial suspension was inactivated, and a sterility test was performed by culturing an aliquot of the bacterial suspension onto a TSA-YE plate and incubating for 36 h at 37 °C. Two-month-old New Zealand rabbits (1.5 and 2 kg) were immunized subcutaneously with 1 mL of either *B. abortus* or

B. canis in an aluminum hydroxide (10%) suspension. Two boosts were performed 15 and 30 days after the first immunization. Finally, the rabbits were euthanized and the serum was separated from the clotted blood and stored at − 20 °C until use.

Antigenicity of the orthologous proteins in the *Brucella* OMVs

From the 30 clusters of the 4 *Brucella* species, the prediction of antigenicity was performed *in silico*. The analysis focused on the proteins with superficial subcellular location in the outer membrane identified through the alignment of orthologous proteins to obtain a consensus sequence. The antigenicity of the proteins with subcellular location of outer membrane found in the OMVs was predicted by alignments to orthologous proteins to obtain a consensus sequence using UniProt UGENE V.1.30. The antigenicity of the consensus protein was analyzed with VaxiJen V.2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) using the default threshold value (Doytchinova and Flower 2007). In addition, B and T cell epitope predictions were performed for each protein using the BCPREDS server (http://ailab.ist.psu.edu/bcpred/predict.html), and the predictions were performed with a specificity of 80% and an epitope length of 20 amino acids (El-Manzalawy et al. 2008). In addition, MHCpred V.2.0 (http://www.ddg-pharmfac.net/mhcpred/MHCPred/) was used to predict the T cell epitopes based on binding affinities to the MHC-I and MHC-II molecules (Guan et al. 2003). The server was adjusted to predict epitopes with a binding affinity greater than 15 for DRB1*0101, the most common allele in the human population (Vishnu et al. 2017). The B and T cell epitope density in a given protein was calculated by dividing the number of predicted epitopes by the length of the protein. The cumulative score was calculated by adding the score obtained from the VaxiJen server and the B and T cell epitope density values (Hisham and Ashhab 2018).

Detection of antigenic proteins in OMVs derived from B. suis, B. ovis, B. canis and B. neotomae

In this study, the antigenicity and cross-reactivity of the proteins contained in OMVs from two smooth *Brucella* strains (*B. suis* and *B. neotomae*) and two rough strains (*B. ovis* and *B. canis*) were analyzed.

Briefly, 30 µg of OMVs from *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* were loaded onto a 15% SDS-PAGE gel and run at 90 V for 2 h. A wide range of molecular mass markers was included (PageRuler™ Prestained protein ladder, Thermo Fisher Scientific). The proteins were transferred to PVDF membranes (Immobilon-P Millipore^R) in a semi-dry chamber for 30 min at 20 V. The PVDF membranes

were washed with TBS-T (20 mM Tris–Cl, 150 mM NaCl, 0.05% Tween 20, pH 7.5) for 5 min. The membranes were blocked with 5% low-fat dry milk in TBS-T for 2 h at room temperature. Subsequently, membranes were washed three times with 10 mL of TBS-T. After that, membranes were incubated for 2 h at room temperature with rabbit anti-*B. abortus* 2308 and anti-*B. canis* 23365 sera diluted 1:5,000 in TBS-T. Membranes were washed three times with TBS-T and incubated for 1 h at room temperature with a secondary antibody (anti-rabbit IgG, the whole molecule) coupled to peroxidase (Sigma-Aldrich) diluted 1:5,000 with TBS-T. Then, the membranes were washed three times and treated with an Immobilon Western Kit (Millipore[R]). The molecular mass of the proteins was calculated with a Gel Doc system (Bio-Rad) and Image Lab™ software (Bio-Rad).

Results

OMVs of *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* showed a spherical shape and bilayer lipid membrane by electron microscopy (Figs. 1 and 2). In addition, the release of OMVs from the surface of whole cells was observed in all *Brucella* species tested in this work. In particular, cells of *B. suis* (1330) released the smallest vesicles, with an average diameter of 30 nm (measured from ten fields) (Fig. 1). Purified vesicles from *B. suis* were slightly larger, with an average diameter of 47.05 nm (Fig. 2). *B. ovis* vesicles were observed surrounding the cells, with an average size of 84.71 nm (Fig. 1), and purified OMVs had a similar average diameter of 83.88 nm (Fig. 2). The micrographs showed vesicles with an average size of 84.55 nm surrounding *B. canis* cells, while the average size of purified OMVs from this species was 69.40 nm (Figs. 1 and 2). The OMVs from *B. neotomae* observed in thin sections from whole cells had an average diameter of 58.55 nm, and purified vesicles had an average diameter of 69.36 nm (Figs. 1 and 2).

The protein profiles of the different *Brucella* OMVs observed by SDS-PAGE were very similar (Fig. 3). In all species, OMVs displayed two main bands of 20 and 23 kDa (Fig. 3). *B. suis* OMVs clearly exhibited more protein bands from 10 to 127 kDa. OMVs from *B. canis* and *B. neotomae* shared very similar protein profiles from 21 to 72 kDa, with the exception of one band present at 11 kDa in OMVs from *B. canis* (Fig. 3).

The proteins contained in OMVs from *Brucella* species were identified by LC–MS/MS. The hits obtained from the mass spectrometry analysis were used for protein identity searching with BLASTP from the NCBI, using the corresponding *Brucella* genome. A query result was considered significant only if the overall score was > 25 and if at least two tryptic peptides, as well as their fragment ions, matched the protein. LC–MS/MS analysis revealed 333,

230, 135 and 375 hits (identified proteins) for *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* OMVs, respectively. These hits were analyzed with BLASTP from the NCBI database and the UniProt BLAST tool using the respective genomes. The numbers of hits that were unambiguously identified in the genomes and both duplicates were 264, 214, 131 and 352 for *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* OMVs, respectively; these protein sequences were used for further analysis (Supplementary Tables 1, 2, 3 and 4). In Supplementary Tables 1, 2, 3 and 4, molecular weight (Mw), isoelectric point (pI), locus, Clusters of Orthologous Groups (COG) and protein motif are shown, among other additional information about the identified proteins. In addition, subcellular localization analysis revealed a similar subcellular distribution among cargo proteins found in *Brucella* OMVs (Fig. 4a). The cytoplasmic proteins were the most abundant, followed by membrane and periplasmic proteins at a similar ratio, and the proteins with an extracellular location were the least abundant. Although the proportion of cytoplasmic proteins was the highest, this kind of protein has been considered a normal component of OMVs, and density gradient purification did not preclude the presence of cytoplasmic proteins in proteomic analysis (Cahill et al. 2015).

To determine the putative function of the proteins identified in the OMVs, their peptide sequences were analyzed according to COG annotations. After COG identification, it was possible to determine the OMV pan-proteome (Fig. 4b). The identified proteins were grouped into 157, 147, 101 and 212 clusters of orthologous proteins for OMVs purified from *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae*, respectively. Only 30 clusters (117 orthologous proteins) were shared between the OMVs of the four *Brucella* species (Table 1) (Fig. 4b). The summary of the molecular functions indicated that the ion binding (GO:0043167) cluster was the most shared among all four *Brucella* OMVs, followed by those for nucleic acid binding (GO:0003676) and transporter activity (GO:0005215) (Fig. 5). Other orthologous proteins and GO terms shared between all four *Brucella* species are listed in Table 2. Remarkably, some proteins involved in *Brucella* virulence, such as Omp16, Omp31, Omp25, SodC, and BhuA, identified in the OMVs proteome of all *Brucella* species (core proteome). Notably, *B. suis*, *B. ovis* and *B. neotomae* OMVs shared the highest number of clusters (49) for the main functional classifications: ion binding (GO:0043167), hydrolase activity (GO:001687), nucleotide binding (GO:0000166) and binding (GO:0005488) (Fig. 5b). These clusters include proteins such as invasion protein B homologue BruAb10366, BamD, Omp19, Omp10, and TolB. *B. suis* and *B. neotomae* OMVs had the second highest number of shared clusters (39), with functions associated with ion binding (GO:0043167), nucleic acid binding (GO:0003676) and transferase activity (GO:0016740)

Fig. 1 Electron microscopy micrographs of OMVs from *B. suis*, *B. ovis*, *B. canis* and *B. neotomae*. Agar-embedded whole bacteria were processed for thin sectioning and negatively stained with OsO_4. OMVs were released from the bacterial surface (arrowheads). Bar = 100 nm
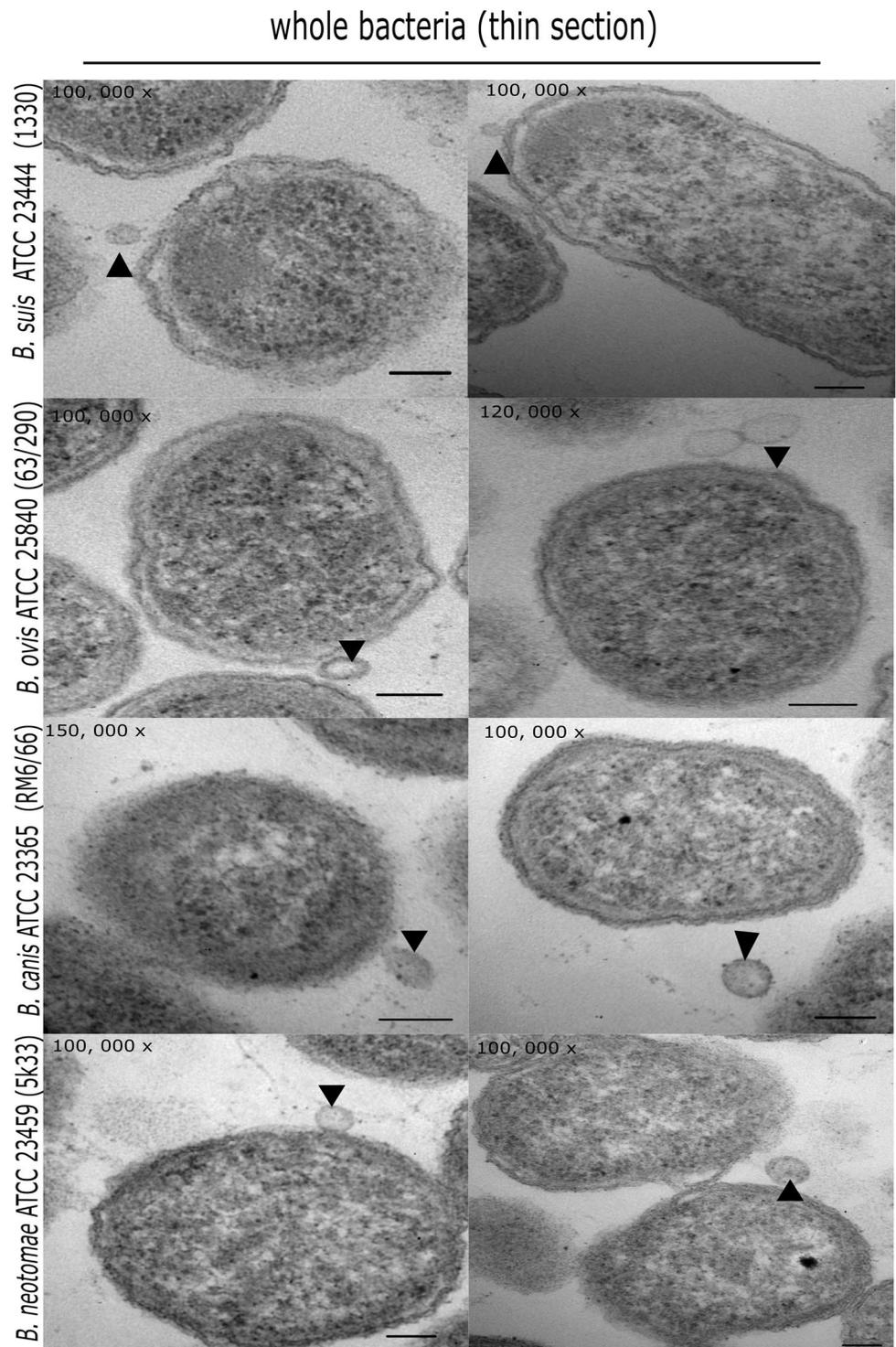
(Fig. 5c); Omp25, Omp31, ActR and other virulence proteins were grouped in these clusters.

Other proteins identified only in the OMVs of individual *Brucella* species, referred to as singletons, were found as follows: 101 in *B. suis*, 65 in *B. ovis*, 30 in *B. canis*, and 127 in *B. neotomae*. These proteins were not classified into orthologous clusters (Table 1), and they are listed in the Supplementary Tables 1, 2, 3 and 4. Among these singletons, the most common functional classifications were catalytic activity (GO:0003824), binding (GO:0005488), nucleotide binding (GO:0000166), small molecule binding (GO:0036094) and organic cyclic compound binding (GO:0097159).
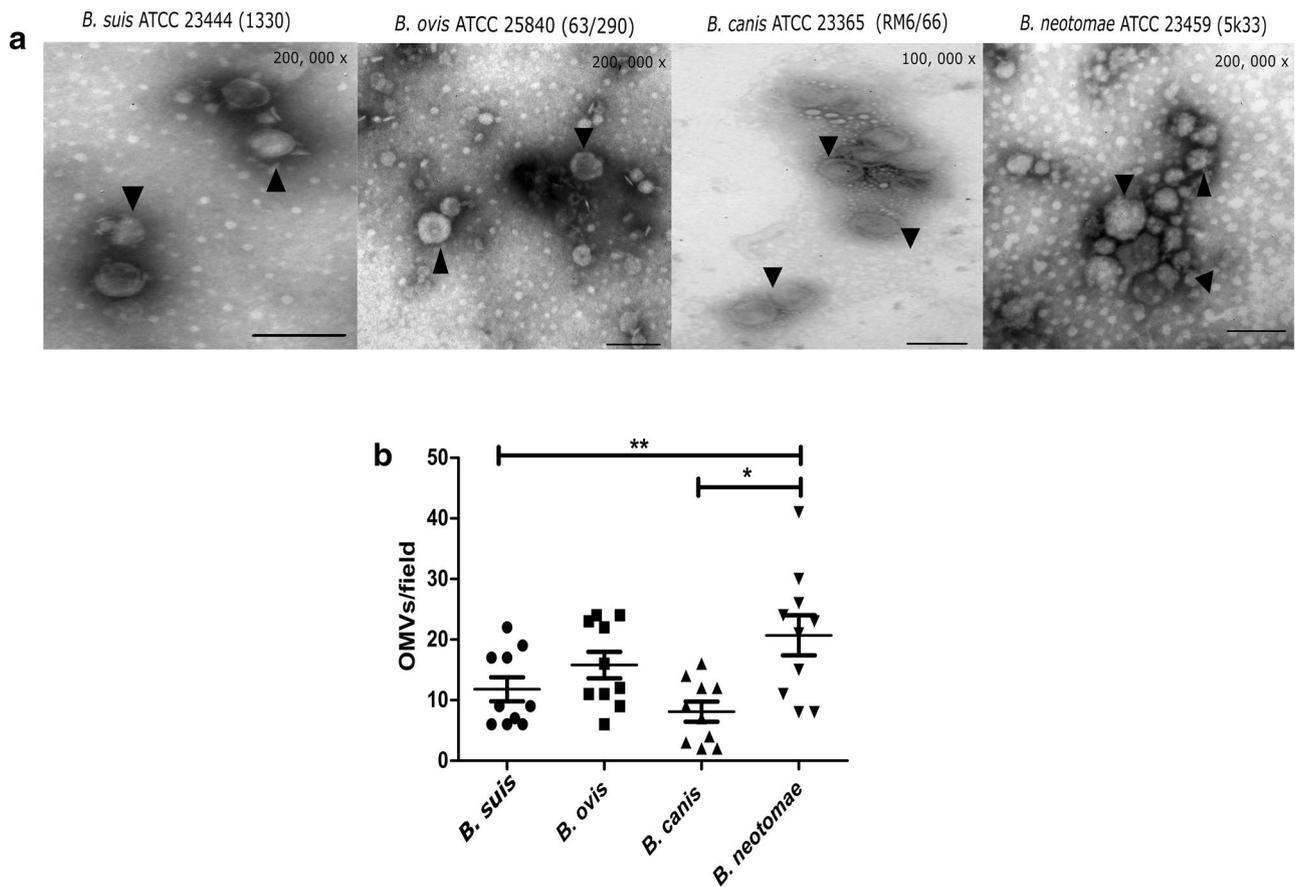
Fig. 2 Purified OMVs from *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* observed by electronic microscopy. **a** OMVs stained with phosphotungstic acid showed vesicles with a lipid bilayer membrane (arrowheads). **b** Graph representing the number of vesicles counted from ten fields for each strain (one-way ANOVA, 95% confidence interval). A significant difference was observed. $*P < 0.05$, $**P < 0.01$. Bar = 100 nm

Antigenicity of the OMV proteins

Previous reports demonstrated that *B. melitensis* OMVs induced protection in mice challenged with virulent *Brucella*; therefore, a search for antigenic proteins in the OMVs was performed in this work (Avila-Calderon et al. 2012; Araiza-Villanueva et al. 2019). The antigenicity of the orthologous proteins was analyzed and prediction of B and T cell epitopes. In Table 3, some orthologous proteins are listed, as well as their antigenicity score, B and T cell epitope density and cumulative score. As mentioned, some orthologous proteins found in the OMVs, such as Omp16, Omp25, Omp31, SodC, BhuA and catalase, have been well characterized as virulence factors, and some of them have been previously used as subunit vaccines (Avila-Calderon et al. 2013; Araiza-Villanueva et al. 2019). Therefore, some uncharacterized orthologous outer membrane proteins were analyzed; the localization of proteins in the surface of the cells could improve the probability of interacting with host cells and induced an immune response. The analysis showed that putative lipoprotein YiaD had the highest cumulative score, while the orthologous periplasmic oligopeptide-binding protein had the lowest score. All orthologous proteins tested were antigenic and possessed B and T cell epitopes (Table 3).

Detection of antigenic proteins in OMVs derived from *B. suis*, *B. ovis*, *B. canis* and *B. neotomae*

To detect antigenic proteins in OMVs purified from *B. suis*, *B. ovis*, *B. canis* and *B. neotomae*, western blotting was performed using anti-*Brucella* antibodies. The results showed a band of approximately 23 kDa in all OMVs from the four *Brucella* strains tested (Fig. 6a). Moreover, a protein of approximately 55 kDa that was recognized by anti-*B. abortus* 2308 serum was detected in OMVs from *B. suis*, *B. canis* and *B. neotomae.* However, another 60 kDa band was more evident in *B. canis* and *B. neotomae* OMVs than in *B. suis* OMVs (Fig. 6a, lanes 3 and 4). Two proteins of approximately 15 and 21 kDa were detected by *anti-B. canis* 23365
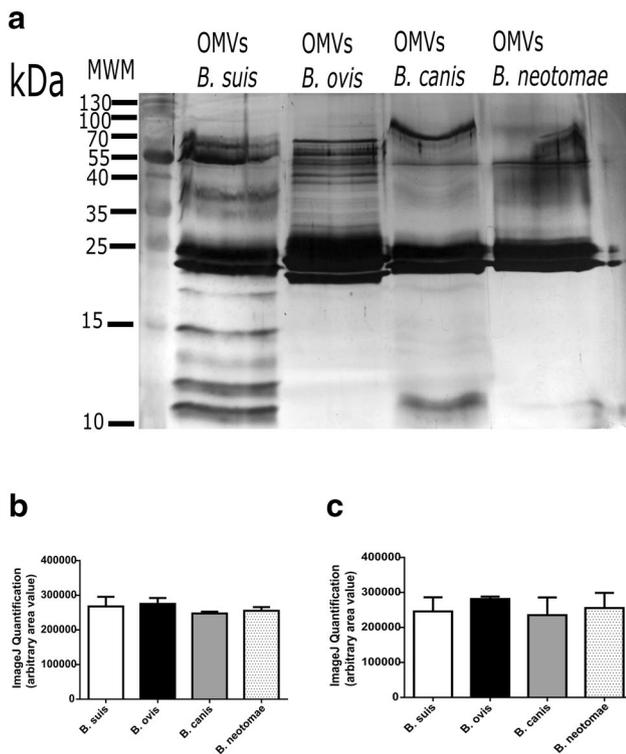
Fig. 3 SDS-PAGE protein profile of OMVs purified from *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae*. OMVs were purified by differential centrifugation and loaded onto a 15% acrylamide gel for electrophoresis. MWM, molecular weight marker. Lane 1: protein profile of OMVs purified from *B. suis* ATCC 23444. Lane 2: protein profile of OMVs purified from *B. ovis* ATCC 25840. Lane 3: protein profile of OMVs purified from *B. canis* ATCC 23365. Lane 4: protein profile of OMVs purified from *B. neotomae* ATCC 23459. One hundred micrograms of OMV proteins were loaded into each well

serum in OMVs from the four *Brucella* strains (Fig. 6b). In addition, proteins of approximately 25 and 30 kDa were observed using *anti-B. canis* 23365 serum, mainly in the OMVs from *B. canis* and *B. neotomae* (Fig. 6b, lanes 3 and 4).

Discussion

The first study concerning *Brucella* vesicles was performed by Gamazo and Moriyon (1987), who observed that *B. melitensis* strain 16M (smooth) and the mutant B115 (rough) released membranous material containing lipopolysaccharide, proteins, and phospholipids. Later, Gamazo et al. (1989) observed vesicles in isolates of *B. ovis*, while Boigegrain et al. (2004) identified Omp31 and Omp25 in the vesicles of *B. suis* 1330 using monoclonal antibodies. These early studies were restricted to describing the morphology of the vesicles and the qualitative composition determined by SDS-PAGE or through specific antibodies. More recently,
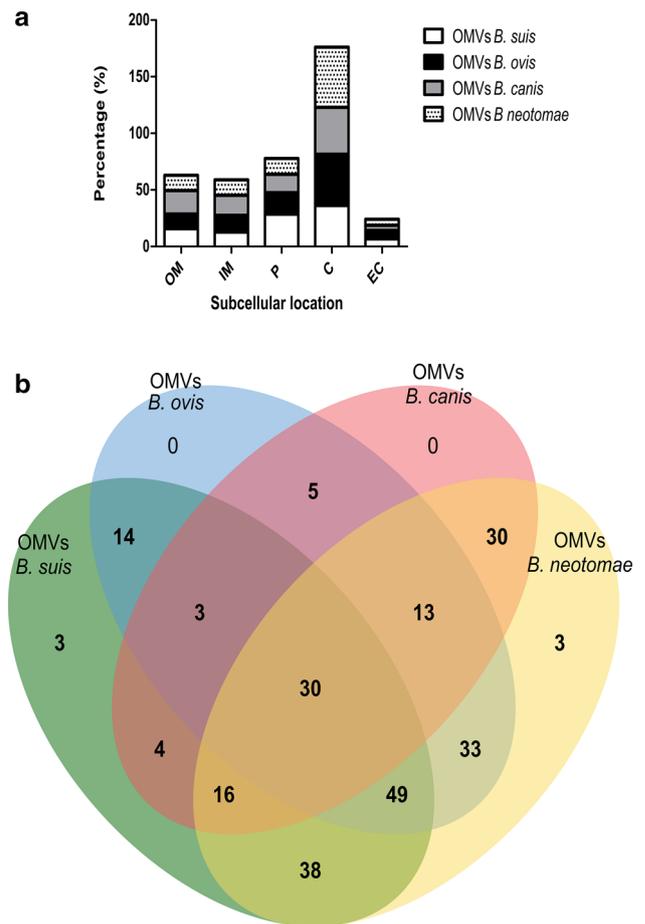

Fig. 4 *In silico* analysis of proteins from *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* OMVs. **a** Subcellular locations of OMV proteins based on PSORT3b and the Softberry database. **b** Venn diagram showing the pan-proteome of the OMVs from *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae*. Outer membrane (OM); inner membrane (IM); periplasmic (P); cytoplasmic (C); extracellular (EC)

Table 1 Clusters of orthologous proteins and singletons from OMVs of *Brucella* species

OMVs from species	Proteins	Clusters	Singletons
B. suis	264	157	101
B. ovis	214	147	65
B. canis	131	101	30
B. neotomae	352	212	127

Avila-Calderón et al. (2012) determined the composition of OMVs purified from *B. melitensis* by proteomics, reporting 29 proteins in the vesicles, some of them related to immunological protection. The most recent study describes the presence of SodC, Omp2b, Omp2a, Omp10, Omp16, and Omp19, among other proteins, in the vesicles of *B. abortus* 2308 and the rough mutant RB51 (Araiza-Villanueva
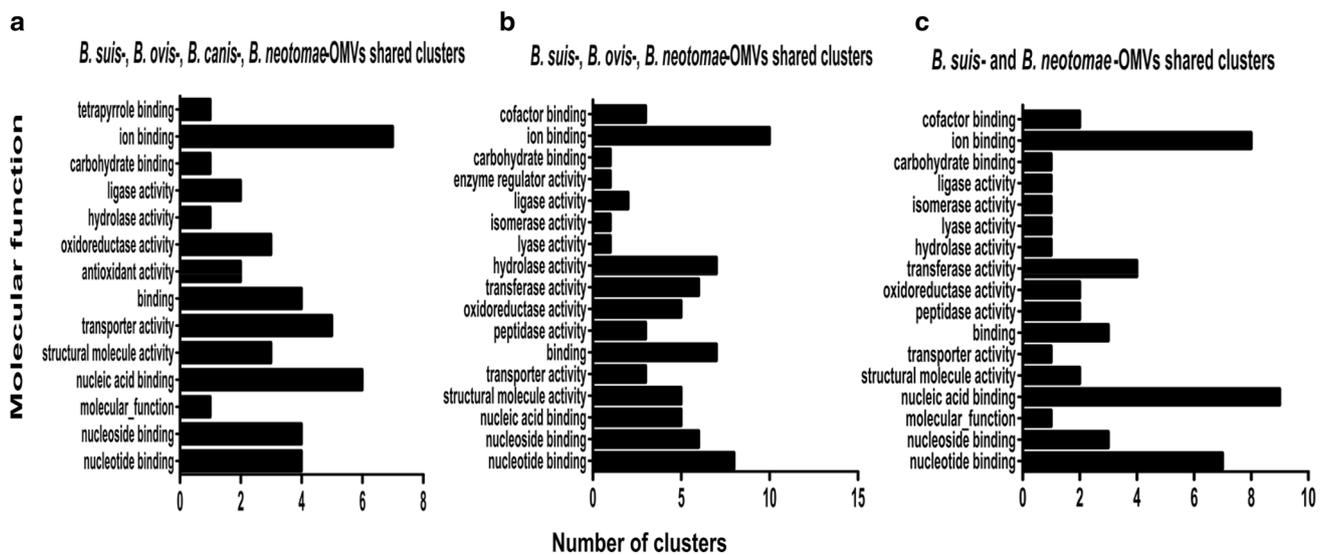
Fig. 5 Functional classification of the *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* OMV proteins. The analysis of proteins was performed by gene ontology classification. **a** *B. suis*, *B. ovis*, *B. canis*, and *B. neoto-* *mae* OMV shared clusters; **b** *B. suis*, *B. ovis*, and *B. neotomae* OMV shared clusters; **c** *B. suis* and *B. neotomae* OMV shared clusters

et al. 2019). Here, through a proteomic analysis, the proteins in the OMVs of other *Brucella* species not previously described were obtained and the pan-proteome of these vesicles was determined.

Although vesicles from *B. suis* and *B. ovis* were previously reported, their protein composition had not been described in detail. In the cases of *B. canis* and *B. neotomae*, this study is the first report of OMVs in these species.

Regarding the number of OMVs released by different strains, we must note that *B. neotomae* released more vesicles than *B. canis* and *B. suis*. Furthermore, differences in the sizes and protein profile of the vesicles were recorded. Of the four *Brucella* species studied in this work, *B. ovis* and *B. canis* are natural rough strains lacking the O-side chain of LPS. Specifically, the genome of *B. ovis* has a 15 kb deletion and therefore lacks the *wboA* and *wboB* genes that are essential for the production of smooth LPS. In addition, the presence of point mutations in the genes of the *wbk* operon involved in O-side chain synthesis has been reported in the genome. In the case of the genome of *B. canis*, a deletion of 351 bp affects the *wbkF* and *wbkD* genes in the *wbk* operon, which are also involved in the synthesis of *Brucella* LPS (Tsolis et al. 2009; Zygmunt et al. 2009). It has been demonstrated that the lack of the LPS O-side chain alters cargo proteins and the release of OMVs. For instance, a *Klebsiella pneumoniae wbb-O* mutant that lacks the O-side chain has an altered OMV protein composition (Cahill et al. 2015). Moreover, the O-side chain influences the size of the OMVs released from *Pseudomonas aeruginosa*, producing two forms of O-side chain antigen: the common polysaccharide antigen (CPA, short with a neutral charge) and the O-specific antigen (OSA, negatively charged and highly immunogenic) (Lam et al. 2011). The OMVs from a *P. aeruginosa* OSA mutant strain were smaller than the OMVs isolated from the wild-type strain, and the OMVs isolated from a CPA mutant strain were larger than the OMVs from the OSA mutant strain but smaller than the OMVs from the wild-type strain (Murphy et al. 2014). Based on these findings, it was expected that the lack of the O-side chain in rough *Brucella* species may result in differences in the protein profile and size of OMVs between the smooth and rough *Brucella* species tested.

Proteomic analysis revealed differences in the cargo proteins of *Brucella* OMVs; fewer proteins were found in OMVs purified from *B. ovis* (214 proteins) and *B. canis* (131 proteins) than in the OMVs from the smooth *B. suis* (264 proteins) and *B. neotomae* (352 proteins) strains. As mentioned above, the lack of the O-side chain influences cargo protein sorting into OMVs and their diverse functions. The mechanism to select cargo proteins for packing into OMVs has been proposed to be selective, and it is not dependent on the protein abundance in the bacterial cell but is related to LPS structural integrity (Bonnington and Kuehn 2014). Experiments performed in *Porphyromonas gingivalis* demonstrated that the lack of an O-side chain does not affect OMV release; instead, it affects protein sorting into OMVs (Haurat et al. 2011). Despite the variability in the number of proteins found in the OMVs, a large number of orthologous protein clusters were shared between the *Brucella* species. The distribution of the subcellular locations of the identified proteins in the *Brucella* species vesicles tested in this work support the hypothesis

Table 2 Orthologous protein clustering and functional classification from *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* OMVs

ID	Number of proteins	Swiss-Prot hit	GO annotation
Cluster 3	6	Periplasmic oligopeptide-binding protein	GO:0042597; C: periplasmic space; GO:0005215; F: transporter activity; GO:001533; P: peptide transport; GO:0015031; P: protein transport
Cluster 6	4	Outer membrane lipoprotein Omp16	GO:0009279; C: cell outer membrane; GO:0016021; C:integral component of membrane
Cluster 7	4	Porin omp2b	GO:0009279; C: cell outer membrane; GO:0046930; C: pore complex; GO:0015288; F: porin activity; GO:0006811; P: ion transport
Cluster 12	4	Superoxide dismutase [Cu–Zn]	GO:0042597; C: periplasmic space; GO:0046872; F: metal ion binding; GO:0004784; F: superoxide dismutase activity
Cluster 13	4	25 kDa outer membrane immunogenic protein	GO:0009279; C: cell outer membrane; GO:0016021; C: integral component of membrane
Cluster 14	4	Probable lipoprotein YiaD	GO:0009279; C:cell outer membrane; GO:0016021; C: integral component of membrane; GO:0005886; C:plasma membrane
Cluster 15	4	Elongation factor Tu{ECO: 0000255|HAMAP-Rule: MF_00118}	GO:0005737; C:cytoplasm; GO:0005525; F:GTP binding; GO:0003924; F:GTPase activity; GO:0003746; F:translation elongation factor activity
Cluster 19	4	Catalase	GO:0042597; C:periplasmic space; GO:0004096; F:catalase activity; GO:0020037; F:haem binding; GO:0,046,872; F:metal ion binding; GO:0042744; P:hydrogen peroxide catabolic process
Cluster 20	4	31 kDa outer membrane immunogenic protein	GO:0009279; C:cell outer membrane; GO:0046930; C:pore complex; GO:0015288; F:porin activity; GO:0006811; P:ion transport
Cluster 22	4	Haem transporter BhuA	GO:0009279; C:cell outer membrane; GO:0016021; C:integral component of membrane; GO:0004872; F:receptor activity; GO:0005215; F:transporter activity
Cluster 26	4	Outer membrane protein assembly factor BamA {ECO: 0000255|HAMAP-Rule: MF_01430}	GO:0009279; C:cell outer membrane; GO:0016021; C:integral component of membrane; GO:0043165; P:gram-negative-bacterium-type cell outer membrane assembly; GO:0051205; P:protein insertion into membrane
Cluster 29	4	Iron uptake protein A2	GO:0016020; C:membrane; GO:0030288; C:outer membrane-bounded periplasmic space; GO:0009579; C:thylakoid; GO:0046872; F:metal ion binding; GO:0006811; P:ion transport; GO:0055072; P:iron ion homeostasis
Cluster 30	4	25 kDa outer membrane immunogenic protein	GO:0009279; C:cell outer membrane; GO:0016021; C:integral component of membrane
Cluster 31	4	60 kDa chaperonin groL {ECO: 0000255|HAMAP-Rule: MF_00600}	GO:0005737; C:cytoplasm; GO:0005524; F:ATP binding; GO:0042026; P:protein refolding

of a conserved or compensatory sorting mechanism to select vesicle protein content independent of the presence of complete LPS. In this regard, Murphy et al. (2014) observed a greater number of periplasmic proteins and a smaller number of OMPs in the *P. aeruginosa* OSA mutant strain (containing negatively charged LPS), while in the CPA mutant strain (displaying a neutrally charged LPS), a lower number of periplasmic proteins and a greater number of OMPs were found (Murphy et al. 2014).

The *Brucella* LPS structure differs from the LPS of enterobacteria, and these differences could impact OMV biogenesis and protein composition. For example, the negative charge in *Brucella* LPS and enterobacterial LPS is at the core (as is the case for *K. pneumoniae* and *E. coli*). However, the negative charge in enterobacterial LPS resides in the phosphate groups, whereas in *Brucella*, a positively charged core oligosaccharide branch not linked to the O-antigen balances the negative internal LPS charges (Frirdich et al. 2005;

Table 3 Antigenicity and B and T cell epitope density in the orthologous proteins identified in the *Brucella* OMVs

Cluster	Accession*	Swiss-Prot hit*	Subcellular location	Antigenicity (score)	B cell epitope density	T cell epitope density	Cumulative score
Cluster 3	P06202	Periplasmic oligopeptide-binding protein	P	Probable ANTIGEN (0.4956)	0.011	0.190	0.6966
Cluster 7	Q45078	Porin omp2b	OM	Probable ANTIGEN (0.6617)	0.016	0.12	0.7977
Cluster 10	P55561	Uncharacterized outer membrane protein y4mB	OM	Probable ANTIGEN (0.6200)	0–008	0.189	0.8170
Cluster 14	P37665	Probable lipoprotein YiaD	OM	Probable ANTIGEN (0.8707)	0.018	0.322	1.2107
Cluster 18	N/A**	Hypothetical protein	IM	Probable ANTIGEN (0.7554)	0.032	0.064	0.8514
Cluster 24	N/A**	Hypothetical protein	P	Probable ANTIGEN (0.6027)	0.014	0.131	0.7477
Cluster 25	N/A**	Hypothetical protein	S	Probable ANTIGEN (0.7222)	0.023	0.108	0.8532
Cluster 26	B5FJ24	Outer membrane protein assembly factor BamA	OM	Probable ANTIGEN (0.5882)	0.019	0.163	0.7702
Cluster 27	N/A**	Hypothetical protein	P	Probable ANTIGEN (0.6028)	0.022	0.126	0.7508
Cluster 28	N/A**	Hypothetical protein	OM	Probable ANTIGEN (0.5819)	0.014	0.209	0.8049
Cluster 29	Q55835	Iron uptake protein A2	OM	Probable ANTIGEN (0.5478)	0.011	0.150	0.7088

*Protein identity assigned to the cluster group by the OrthoVenn database

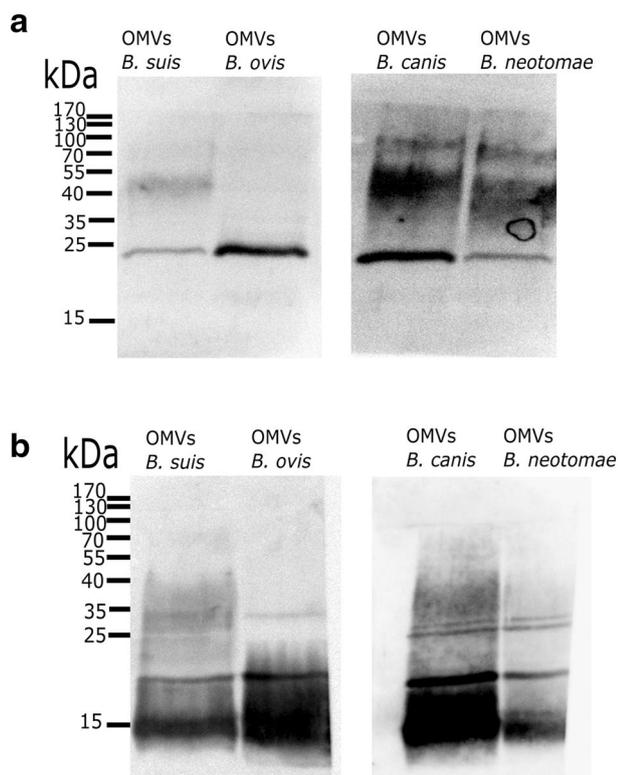**Uncharacterized protein with an unassigned (N/A) Swiss-Prot hit

Fig. 6 Western blot analysis of purified OMVs derived from *B. suis*, *B. ovis*, *B. canis* and *B. neotomae*. **a** Antigenic proteins of OMVs from *B. suis* (lane 1), *B. ovis* (lane 2), *B. canis* (lane 3) and *B. neotomae* (lane 4) detected by a rabbit anti-*B. abortus* 2308 serum. **b** Antigenic proteins of OMVs from *B. suis* (lane 1), *B. ovis* (lane 2), *B. canis* (lane 3) and *B. neotomae* (lane 4) detected by a rabbit anti-*B. canis* 23365 serum

Soler-Llorens et al. 2014; Fontana et al. 2016). Perhaps the lack of O-side chains in rough strains imbalances the charge at the *Brucella* surface, affecting both the number and the types of proteins found in the OMVs. A smaller number of proteins were identified in the OMVs from the rough strains compared to the smooth strains. However, the results showed no differences in the number of vesicles or subcellular locations of the identified proteins in the *B. ovis*, *B. canis* and *B. suis* OMVs. Likely, LPS does not affect the number of OMVs released but does affect the number of proteins packaged into the vesicles. The *Brucella wadC* gene encodes a glycosyltransferase necessary for synthesis of a core oligosaccharide branch. Further experiments with the *Brucella wadC* mutant are needed to analyse whether an imbalanced charge at the *Brucella* surface affects vesiculation. There is little information about the differences in the LPS of smooth *Brucella* species; however, there are reports concerning differences in the proportion of the A and M epitopes in the O-side chain of *Brucella* species. For instance, *B. neotomae* expresses fewer A and M epitopes than *B. suis* (Cloeckaert et al. 1998). This smaller proportion of epitopes in the O-side chain may contribute to the differences in vesiculation between the *B. neotomae* and *B. suis* strains observed in this work.

In OMVs from the *Brucella* strains tested, 30 orthologous clusters with a total of 117 proteins were shared. From these clusters, the GO enrichment showed three main classifications: GO:0009279 cell outer membrane (cellular component), GO:0016021 integral component of membrane (cellular component) and GO:0006811 ion transport (biological process). A large number of shared clusters among the *B.*

suis, *B. ovis*, and *B. neotomae* OMVs were observed (49); 38 clusters were shared between *B. suis* and *B. neotomae* OMVs, and 33 were shared between *B. ovis* and *B. neotomae* OMVs. The genomes of *Brucella* species share a high identity (98–100%), and a large number of variable genes (< 95% identity) are hypothetically surface proteins, such as OMPs (Whatmore 2009). Regarding the *Brucella* species tested, *B. suis* is phylogenetically most closely related to *B. canis*, whereas *B. ovis* and *B. neotomae* are in separate clades (Wattam et al. 2014). Thus, we cannot discount that the genetic background reflects protein sorting in *Brucella* OMVs.

The proteins shared among the OMVs from all four *Brucella species*, Omp16, Omp25, Omp31, SodC, and BhuA, are able to elicit an immunological response and are involved in *Brucella* pathogenesis. In particular, BhuA is required by *B. abortus* to maintain chronic brucellosis infection in a mouse model (Anderson et al. 2011). Moreover, it was reported that for *B. abortus*, the protein Omp16 activates dendritic cells and induces an immune response, while Omp25 is essential for *Brucella* to enter and survive inside murine macrophages (Martin-Martin et al. 2009; Pasquevich et al. 2010). Recently, it was demonstrated that *B. suis* Omp25 suppresses signaling and production of TNFα, a critical cytokine for eradication of *B. suis* infection (Luo et al. 2018). Furthermore, the protein Omp31 is essential for internalization of *B. melitensis* 16M and impairs apoptosis in murine macrophages, leading to bacterial persistence (Zhang et al. 2016; Verdiguel-Fernandez et al. 2017). In addition, Omp31 is involved in membrane stability; in particular, a *B. melitensis* Omp31 mutant was more susceptible to polymyxin B and sodium deoxycholate than the wild-type strain (Verdiguel-Fernandez et al. 2017). The proteins Omp16, Omp25, and Omp31 have an OmpA-like motif, and this domain has a β/α/β/α-β (2) structure typical of the Tol/Pal protein system. It has been demonstrated that OmpA stabilizes linkages between the outer membrane and peptidoglycan, and OmpA is thought to be a critical regulator of OMV biogenesis (Schwechheimer et al. 2013). Downregulation of OmpA expression increased OMV production in *Vibrio cholerae* (Song et al. 2008), and the proteins Omp16, Omp25, and Omp31 could be involved in OMV biogenesis. In addition, these three proteins have been previously studied because they are able to induce protection against *Brucella* in vivo, and their recombinant proteins have been proposed as potential subunit brucellosis vaccines (Avila-Calderon et al. 2013).

BhuA, Omp31 and the iron uptake protein A2 were found in clusters shared by OMVs of all *Brucella* species tested. BhuA serves as a TonB-dependent haem transporter in *B. abortus* 2308, while the iron uptake protein A2 is involved in Fe^{3+} ion (ferric iron) import (Roop 2012). Omp31 from *B. suis*, *B. melitensis* and *B. ovis* also has been described as a haemin-binding protein (Delpino

et al. 2006). The haem group represents an important iron source for *Brucella* during their intracellular lifestyle. OMVs released inside host cells may serve as vehicles for iron acquisition during haem trafficking. Harsh environments or stress, such as passing through the host, have been described to increase vesiculation and improve bacterial survival (Ellis and Kuehn 2010).

Another common orthologous protein found in the OMVs from all species tested was catalase; however, this enzyme is not essential for *B. melitensis* goat infection, and it has an antioxidant function. A *B. melitensis kat* mutant (a catalase mutant) displayed hypersensitivity to hydrogen peroxide (Gee et al. 2004). Thus, catalase carried in *Brucella* vesicles could contribute to avoidance of macrophage antimicrobial mechanisms, such as the oxidative burst. The elongation factor EF-Tu has been reported as a membrane-associated protein identified in OMVs from *Burkholderia pseudomallei* and *Acinetobacter baumannii*. EF-Tu associated with *A. baumannii* OMVs has been associated with cell attachment; EF-Tu bound to fibronectin in western blot-based binding assays (Dallo et al. 2012). EF-Tu induced specific IgG and IgA antibodies in immunized mice and IFN-γ in mouse splenocytes. Moreover, EF-Tu immunization reduced lung bacterial loads in mice challenged with *Burkholderia thailandensis* (Nieves et al. 2010). As mentioned above, *Brucella* EF-Tu (tufA) was identified in the OMVs of all *Brucella* species tested in this work, suggesting that it may be involved in the induction of the immune response.

The western blot results showed that there are some antigenic proteins in the OMVs from *B. suis*, *B. ovis*, *B. canis* and *B. neotomae*. In this sense, Gamazo et al. (1989) reported the electrophoretic profiles of OMVs obtained from several field strains of *B. ovis* and *B. melitensis*. At that time, they classified these protein profiles into four groups according to their molecular mass: group A (25.0–29.0 kDa), group B (21.5–22.5 kDa), group C (18.0–19.5 kDa) and group D (13–15.5 kDa). Based on this classification, the 23 kDa immunogenic protein observed in the OMVs of the *Brucella* species tested here could be classified in group B (Gamazo et al. 1989).

Western blots showed that more OMV proteins were detected by *anti-B. canis* 23365 serum compared to the anti-*B. abortus* 2308 serum. These observations could be explained by the lack of the LPS O-side chain on the whole cells of the rough *B. canis* strain. OMPs are more exposed on rough *Brucella* strains compared to smooth *Brucella* strains (Gonzalez et al. 2008). Based on the close phylogenetic relationships of the members of the *Brucella* genus, it was expected that the vesicles of the species tested here would contain similar protein cargo. Through western blotting using antibodies against rough and smooth *Brucella* strains, we demonstrated that similar antigenic proteins are present in the OMVs from rough and smooth strains.

By means of bioinformatics analysis, it was possible to identify a great number of orthologous proteins in the OMVs from the four *Brucella* species tested here. In previous studies of OMV proteins, orthologous proteins such as Omp31, Omp25, SodC, and Omp19 were identified in the OMVs of *B. melitensis* 16M (smooth strain), *B. melitensis* VTRM1 (rough mutant), *B. abortus* 2308 (smooth strain), and *B. abortus* RB51 (rough vaccine strain).

These orthologous proteins, among others, were also identified in the OMVs of *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* analyzed in this study (Avila-Calderon et al. 2012; Araiza-Villanueva et al. 2019). In fact, sera from mice immunized with vesicles from *B. abortus* 2308 and RB51 recognized proteins from 10 to 70 kDa in the purified *Brucella* vesicles. Most likely, some of these proteins are the aforementioned orthologous proteins (Araiza-Villanueva et al. 2019).

The heterologous protection conferred by *Brucella* vaccines has been explored previously. For instance, the *B. neotomae* rough mutant strain provided protection against *B. suis* 1330 infection in a mouse model (Jain-Gupta et al. 2019). Furthermore, immunization with OMVs from *B. melitensis* or *B. abortus*-induced protection in a mouse model similar to that induced by the commercial *B. melitensis* Rev1 or *B. abortus* RB51 vaccines (Avila-Calderon et al. 2012; Araiza-Villanueva et al. 2019).

Some antigenic and protective immunogens were found in the OMVs from the *Brucella* species tested in this work. Specifically, SodC, Omp25, Omp16, and Omp31 proteins, which were previously shown to be protective against brucellosis, could contribute to making OMVs good candidates for developing acellular vaccines (Avila-Calderon et al. 2013). Like rough *Brucella* strains, OMVs purified from rough *Brucella* strains could be used as vaccines, with the advantage that OMVs are not infectious like whole *Brucella* cells. On the other hand, because some antigenic proteins, such as Omp25, were found as well, the OMVs could also be used to detect antibodies against *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* for diagnostic purposes.

Conclusions

The results of this research revealed new insights into OMVs content from *Brucella* species not previously described and the putative roles of cellular components, such as the LPS O-side chain and OmpA-like proteins. In addition, the presence of iron-binding proteins in OMVs may be involved in nutrient uptake in harsh conditions, which is especially useful for the intracellular lifestyle of *Brucella* species. The orthologous proteins previously identified as immunogenic, as well as the protection-inducing proteins found in the OMVs of these *Brucella* species

make these nanostructures very attractive for the development of an acellular vaccine that could induce immune cross-protection.

Author contributions Conceptualization and design of the experiments: MSRP, EDAC, and ACR. Performed research: MSRP, EDAC, ACR, and MGAA. Formal analysis of the data: MSRP, EOLV, EDAC, ZGL, BAR, and ACR. Resources: MGAA, EAR, BAR, and ACR. Writing of the original draft: MSRP, EDAC, MGAA, MRMG, and ACR. Writing, review, and editing the manuscript: MSRP, EDAC, ALM, EAR, and ACR. All the authors approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interests None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper. The authors declare that they have no competing interests in relation to this work.

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