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

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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...
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 decay 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 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 × 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 × 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 × 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 × 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 (OsO4). 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-RadR 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 TipR (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 solution 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 Finngan (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/evpedia) 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.

**Antiserum 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/mhcppred/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) 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®). 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 transferase activity (GO:0016740) (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. 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).
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
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 Boigeegrain 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, Avila-Calderon 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...
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...
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 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:00042597; 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: 0000255HAMAP-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:0046,872; 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:0005215; F: transporter activity |
| Cluster 26 | 4                 | Outer membrane protein assembly factor BamA [ECO: 0000255HAMAP-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: 0000255HAMAP-Rule: MF_00600} | GO:0005737; C: cytoplasm; GO:0005524; F: ATP binding; GO:0042026; P: protein refolding |
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 (Cloeck-aert 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.
sein A2 is involved in Fe³⁺ ion (ferric iron) import (Roop et al. 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/βα-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 Fe³⁺ ion (ferric iron) import (Roop et al. 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 (Dalio 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.

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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.

References

Anderson ES, Paulley JT, Martinson DA, Gaines JM, Steele KH, Roop RM 2nd (2011) The iron-responsive regulator Irr is required for wild-type expression of the gene encoding the heme transporter BhuA in Brucella abortus 2308. J Bacteriol 193:5359–5364. https://doi.org/10.1128/jb.00372-11

Araiza-Villanueva M, Avila-Calderon ED, Flores-Romo L et al (2019) Proteomic analysis of membrane blebs of Brucella abortus 2308 and RB51 and their evaluation as an acellular vaccine. Front Microbiol 10:2714. https://doi.org/10.3389/fmicb.2019.02714

Augustyniak D, Seredynski R, McClean S, Roszkowski J, Rosznio-wski B, Smith DL, Druis-Kawa Z, Mackiewicz P (2018) Virulence factors of Moraxella catarrhalis outer membrane vesicles are major targets for cross-reactive antibodies and have adapted during evolution. Sci Rep 8:4955. https://doi.org/10.1038/s41598-018-23029-7

Avila-Calderon ED, Lopez-Merino A, Jain N, Peralta H, Lopez-Villegas EO, Sriranganathan N, Boyle SM, Witonsky S, Contreras-Rodriguez A (2012) Characterization of outer membrane vesicles from Brucella melitensis and protection induced in mice. Clin Dev Immunol 2012:352493. https://doi.org/10.1155/2012/352493

Avila-Calderon ED, Lopez-Merino A, Sriranganathan N, Boyle SM, Contreras-Rodriguez A (2013) A history of the development of Brucella vaccines. Biomed Res Int 2013:743509. https://doi.org/10.1155/2013/743509

Backert S, Bernegger S, Skoroko-Glonek J, Wessler S (2018) Extracellular HtrA serine proteases: an emerging new strategy in bacterial pathogenesis. Cell Microbiol 20:e12845. https://doi.org/10.1111/cmi.12845

Baek BK, Park MY, Islam MA, Khatun MM, Lee SI, Boyle SM (2011) The first detection of Brucella canis in cattle in the Republic of Korea. Zoonoses Public Health 59:77–82. https://doi.org/10.1111/j.1863-2378.2011.01429.x
Delpino MV, Cassataro J, Fossati CA, Goldbaum FA, Baldi PC (2018) An update on research and clinical aspects of Brucella canis. In: Cloeckaert A, Weynants V, Godfroid J, Verger JM, Grayon M, Zygoiridou M, eds. Archives of Microbiology (2021) 203:1611–1626.

El-Manzalawy Y, Dobbs D, Honavar V (2008) Predicting linear protective antigens against pathogenic Brucella spp. through combining support vector machines and immune epitope prediction. J Bacteriol 190:1557–1566. https://doi.org/10.1128/JB.01382-05

Gamazo C, Moriyon I (1987) Release of outer membrane fragments by exponentially growing Brucella melitensis cells. Infect Immun 55:609–615. https://doi.org/10.1128/IAI.55.3.609-615.1987

Gamazo C, Winter AJ, Moriyon I, Riezu-Boj JJ, Blasco JM, Diaz R (1989) Comparative analyses of proteins extracted by hot saline or released spontaneously into outer membrane blebs from field strains of Brucella ovis and Brucella melitensis. Infect Immun 57:1419–1426. https://doi.org/10.1128/IAI.57.5.1419-1426.1989

Gee JM, Kovach ME, Grippie VK, Hagtus S, Walker JV, Elzer PH, Roop RM 2nd (2004) Role of catalase in the virulence of Brucella melitensis in pregnant goats. Vet Microbiol 102:111–115. https://doi.org/10.1016/j.vetmic.2004.05.009

Godfroid J, Scholz HC, Barbier T et al (2011) Brucellosis at the animal/eco-system/human interface at the beginning of the 21st century. Prev Vet Med 102:118–131. https://doi.org/10.1016/j.prevetmed.2011.04.007

Gonzalez D, Grillo MJ, De Miguel JM et al (2008) Brucellosis vaccines: assessment of Brucella melitensis lipopolysaccharide rough mutants defective in core and O-poly saccharide synthesis and export. PLoS ONE 3:e2760. https://doi.org/10.1371/journal.pone.0002760

Guan P, Doytchinova IA, Zygoouri C, Flower DR (2003) MHCPred: a server for quantitative prediction of peptide-MHC binding. Nucleic Acids Res 31:3621–3624. https://doi.org/10.1093/nar/gcf001

Haurat MF, Aduse-Opoku J, Rangaranjan M, Dorobantu L, Gray MR, Curtis MA, Feldman MF (2011) Selective sorting of cargo proteins into bacterial membrane vesicles. J Biol Chem 286:1269–1276. https://doi.org/10.1074/jbc.M110.185744

Hisham Y, Ashhab Y (2018) Identification of cross-protective potential antigens against pathogenic Brucella spp. through combining pan-genome analysis with reverse vaccinology. J Immunol Res 2018:1474517. https://doi.org/10.1155/2018/1474517

Holst J, Martin D, Arnold R, Huergo CC, Oster P, O’Hallahan J, Rosenqvist E (2009) Properties and clinical performance of vaccines containing outer membrane vesicles from Neisseria meningitidis. Vaccine 27(Suppl 2):B3–B12. https://doi.org/10.1016/j.vaccine.2009.04.071

Jain-Gupta N, Waldrop SG, Tenpenny SG, Boyle SM, Srinanganathan N (2019) Rough Brucella neotomae provides protection against Brucella suis challenge in mice. Vet Microbiol 239:1084–1087. https://doi.org/10.1016/j.vetmic.2019.108447

Kadurugamuwa JL, Beveridge TJ (1998) Delivery of the non-membrane-permeative antibiotic gentamicin into mammalian cells by using Shigella flexneri membrane vesicles. Antimicrob Agents Chemother 42:1476–1483. https://doi.org/10.1128/AAC.42.6.1476

Kang YS, Brown DA, Kirby JE (2018) Brucella neotomae recapitulates attributes of zoonotic human disease in a murine infection model. Infect Immun 87:1–12. https://doi.org/10.1128/IAI.00255-18

Knox KW, Vesk M, Work E (1966) Relation between excreted lipopolysaccharide complexes and surface structures of a lysine-limited culture of Escherichia coli. J Bacteriol 92:1206–1217. https://doi.org/10.1128/JB.92.4.1206-1217.1966

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685. https://doi.org/10.1038/227680a0

Lam JS, Taylor VL, Islam ST, Hao Y, Kocincova D (2011) Genetic and functional diversity of Pseudomonas aeruginosa lipopolysaccharide. Front Microbiol 2:118. https://doi.org/10.3389/fmicb.2011.00118

Liu Q, Yi J, Liang K, Zhang X, Liu Q (2017) Salmonella Choleraesuis outer membrane vesicles: proteomics and immunogenicity. J Basic Microbiol 57:852–861. https://doi.org/10.1002/jobm.201700153

Luo X, Zhang X, Wu X, Yang X, Han C, Wang Z, Du Q, Zhao X, Liu SL, Tong D, Huang Y (2018) Brucella downregulates tumor...
necrosis factor-alpha to promote intracellular survival via Omp25 regulation of different microRNAs in porcine and murine macrophages. Front Immunol 8:2013. https://doi.org/10.3389/fimmu.2017.02013

Maerz JK, Steimle A, Lange A, Bender A, Fehrenbacher B, Frick JS (2018) Outer membrane vesicles blebbing contributes to B. vulgatus mpk-mediated immune response silencing. Gut Microbes 9:1–12. https://doi.org/10.1080/19499076.2017.1344810

Martin-Martin AI, Caro-Hernandez P, Sancho P, Tejedor C, Cloeckaert A, Fernandez-Lago L, Vizzacino N (2009) Analysis of the occurrence and distribution of the Omp25/Omp31 family of surface proteins in the six classical Brucella species. Vet Microbiol 137:74–82. https://doi.org/10.1016/j.vetmic.2008.12.003

McConnell MJ, Rumbo C, Bou G, Pachon J (2011) Outer membrane vesicles as an acellular vaccine against Acinetobacter baumannii. Vaccine 29:5705–5710. https://doi.org/10.1016/j.vaccine.2011.06.001

Murphy K, Park AJ, Hao Y, Brewer D, Lam JS, Khursigara CM (2014) Influence of O polysaccharides on biofilm development and outer membrane vesicle biogenesis in Pseudomonas aeruginosa PA01. J Bacteriol 196:1306–1317. https://doi.org/10.1128/jb.01463-13

Nieves W, Heang J, Asakrah S, Zu Bentrup KH, Roy CJ, Morici LA (2010) Immunospecific responses to bacterial elongation factor Tu during Burkholderia infection and immunization. PLoS ONE 5:e14361. https://doi.org/10.1371/journal.pone.0014361

Olsen SC, Palmer MV (2014) Advancement of knowledge of Brucella over the past 50 years. Vet Pathol 51:1076–1089. https://doi.org/10.1177/0300985814540545

Pappas G, Akritidis N, Bosiljkovski M, Tsianos E (2005) Brucellosis. N Engl J Med 352:2325–2336. https://doi.org/10.1056/NEJMr050570

Pasquevich KA, Garcia Samartino C, Coria LM et al (2010) The protein moiety of Brucella abortus outer membrane protein 16 is a new bacterial pathogen-associated molecular pattern that activates dendritic cells, induces a Th1 immune response, and is a promising self-adjuvanting vaccine against systemic and oral acquired brucellosis. J Immunol 184:5200–5212. https://doi.org/10.4049/jimmunol.0902209

Pollak CN, Delpino MV, Fossati CA, Baldi PC (2012) Outer membrane vesicles from Brucella abortus promote bacterial internalization by human monocytes and modulate their innate immune response. PLoS ONE 7:e50214. https://doi.org/10.1371/journal.pone.0050214

Reyes-Robles T, Dillard RS, Cairns LS, Silva-Valenzuela CA, Houseman M, Ali A, Wright ER, Camilli A (2018) Vibrio cholerae outer membrane vesicles blebbing contributes to Brucella abortus strain RB51. Am J Vet Res 76:1802–1808. https://doi.org/10.2460/ajvr.67.10.1802

Tan K, Li R, Huang X, Liu Q (2018) Outer membrane vesicles: current status and future direction of these novel vaccine adjuvants. Front Microbiol 9:783. https://doi.org/10.3389/fmicb.2018.00783

Tsolis RM, Sheshadi R, Santos RL et al (2009) Genome degradation in Brucella ovis corresponds with narrowing of its host range and tissue tropism. PLoS ONE 4:e5519. https://doi.org/10.1371/journal.pone.0005519

Veith PD, Chen YY, Gorasia DG, Chen D, Glew MD, O’Brien-Simpson NM, Cecil JD, Holden JA, Reynolds EC (2014) Porphyromonas gingivalis outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors. J Proteome Res 13:2420–2432. https://doi.org/10.1021/pr501227e

Verdiguier-Fernandez L, Oropeza-Navarro R, Basurto-Alcantara FJ, Castaneda-Ramirez A, Verdugo-Rodriguez A (2017) Omp31 plays an important role on outer membrane properties and intracellular survival of Brucella melitensis in murine macrophages and HeLa cells. Arch Microbiol 199:971–978. https://doi.org/10.1007/s00203-017-1360-7

Villalobos-Vindas JM, Amuy E, Barquero-Calvo E, Rojas N, Chacón-Díaz C, Chaves-Olarte E, Guzman-Verri C, Moreno E (2017) Brucellosis caused by the wood rat pathogen Brucella neotomae: two case reports. J Med Case Rep 11:352. https://doi.org/10.1186/s13567-014-0072-0

Villanueva J, Hargrave DM, Holmgren J, O’Sullivan W, Sandvig K (2009) Secreted proteins and designing novel multitope peptide vaccines against Brucella melitensis. J Proteome Res 13:2420–2432. https://doi.org/10.4049/jimmunol.0900099

Seleem MN, Boyle SM, Sriranganathan N (2010) Brucellosis: a re-emerging zoonosis. Vet Microbiol 140:392–398. https://doi.org/10.1016/j.vetmic.2009.06.021

Soler-Llorens P, Gil-Ramirez Y, Zabalza-Barangua A et al (2014) Mutants in the lipopolysaccharide of Brucella ovis are attenuated and protect against B. ovis infection in mice. Vet Res 45:72. https://doi.org/10.1186/s13567-014-0072-0

Song T, Mika F, Lindmark B, Liu Z, Schild S, Bishop A, Zhu J, Camilli A, Johansson A, Vogel J, Wai SN (2008) A new Vibrio cholerae sRNA modulates colonization and affects release of outer membrane vesicles. Mol Microbiol 70:100–111. https://doi.org/10.1111/j.1365-2958.2008.06392.x

Stevenson TC, Cywes-Bentley C, Moeller TD, Weyant KB, Putnam D, Chang YF, Jones BD, Pier GB, DeLisa MP (2018) Immunization with outer membrane vesicles displaying conserved surface polysaccharide antigen elicits broadly antimicrobial antibodies. Proc Natl Acad Sci U S A 115:E3110–E3115. https://doi.org/10.1073/pnas.1718341115

Stoesser TG, Lackman DB (1957) A preliminary report on a Brucella isolated from the desert wood rat, Neotoma lepida Thomas. J Am Vet Med Assoc 130:411–412

Stoffregen WC, Olsen SC, Bricker BJ (2006) Parenteral vaccination of domestic pigs with Brucella abortus strain RB51. Am J Vet Res 67:1802–1808. https://doi.org/10.2460/ajvr.67.10.1802

Tu during Burkholderia infection and immunization. PLoS ONE 25231 20000 47

Wattam AR, Foster JT, Mane SP et al (2014) Comparative phylogenomics and evolution of the Brucella reveal a path to virulence. J Bacteriol 196:920–930. https://doi.org/10.1128/jb.01091-13
Whatmore AM (2009) Current understanding of the genetic diversity of \textit{Brucella}, an expanding genus of zoonotic pathogens. Infect Genet Evol 9:1168–1184. https://doi.org/10.1016/j.meegid.2009.07.001

Whatmore AM, Davison N, Cloeckaert A et al (2014) \textit{Brucella papionis} sp. nov., isolated from baboons (\textit{Papio} spp.). Int J Syst Evol Microbiol 64:4120–4128. https://doi.org/10.1099/ijs.0.065482-0

Zhang K, Wang H, Guo F, Yuan L, Zhang W, Wang Y, Chen C (2016) OMP31 of \textit{Brucella melitensis} 16M impairs the apoptosis of macrophages triggered by TNF-alpha. Exp Ther Med 12:2783–2789. https://doi.org/10.3892/etm.2016.3655

Zhang X, Yang F, Zou J, Wu W, Jing H, Gou Q, Li H, Gu J, Zou Q, Zhang J (2018) Immunization with \textit{Pseudomonas aeruginosa} outer membrane vesicles stimulates protective immunity in mice. Vaccine 36:1047–1054. https://doi.org/10.1016/j.vaccine.2018.01.034

Zygmunt MS, Blasco JM, Letesson JJ, Cloeckaert A, Moriyon I (2009) DNA polymorphism analysis of \textit{Brucella} lipopolysaccharide genes reveals marked differences in O-polysaccharide biosynthetic genes between smooth and rough \textit{Brucella} species and novel species-specific markers. BMC Microbiol 9:92. https://doi.org/10.1186/1471-2180-9-92

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