JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2016. Vol. **10**(3), p. 2121-2126

Isolation and Characterization of OMPs and OMVs of *Brucella abortus* S19 and *Brucella abortus* S19Δ*per*

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https://doi.org/10.22207/JPAM.10.3.51

(Received: 12 June 2016; accepted: 20 July 2016)

Bovine brucellosis is a global zoonotic disease. Outer membrane vesicles (OMVs) and outer membrane protein fractions (OMPs) were derived from *Brucella abortus* S19 , a widely used vaccine strain and it's perosamine synthetase gene (*per)* mutant also called *Brucella abortus* S19Δ*per* using differential density ultrcentrifugation followed by their characterization through sodium dodecyl sulfate polyacrylamide gel electrophoresis and both showed immuno-reactivity with mice antisera in immunoblot. Transmission electron microscopy revealed the presence of OMVs as spherical nano-sized structures.

Keywords: *Brucella abortus* S19, brucellosis, perosamine synthetase gene, outer membrane vesicles, outer membrane proteins.

Brucellosis is a worldwide anthropozoonotic infectious disease affecting a wide range of mammals including animals, humans and marine mammals. Brucellosis causes huge total economic losses of US $ 3.4 billion in India, out of which cattle and buffalo industries accounted for 95.6% of total losses due to abortions, temporary infertility and sterility of adult animals[1]. Control of brucellosis is a way to control the zoonotic diseases in human population[2]. Vaccination is the preferred means of controlling Brucellosis, as stamping out is not the choice in Indian scenario. The WHO for Animal Health recommends the use of *B. abortus* strain 19 and *B. abortus* strain RB51 as live atttenuated vaccines for bovine brucellosis. However, these strains are not safe for pregnant animals. Although vaccination with RB51 does not interfere in routine sero-surveillance but it's resistance to rifampicin is a matter of concern which is an important antibiotic for treatment of Brucellosis[3]. Recently, a perosamine synthetase deletion (*per)* mutant of *Brucella abortus* S19, named *Brucella abortus* S19Δ*per* has been developed. S19Ä*per* exhibits intermediate rough phenotype with truncated lipopolysaccharide (LPS). The mutant strain is more safe than S19 while maintaining immunogenic properties similar to parent strain[4].

Brucella, like other Gram-negative bacteria, has ability to secrete outer membrane vesicles (OMVs) by blebbing and pinching off the segments of its outer membrane with an average diameter of 20-250nm. OMVs are composed of the constituents of outer membrane and periplasm[5,6,7,8]. OMVs bound LPS act as a natural adjuvant to immune system as well as presence of OMPs such as Omp19 and Omp16, BP26 and SOD proteins

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offer an advantage for the development of OMVs based vaccines as these immunogens induce a protective responses in their host[9].

Unlike polysaccharides, the outer membrane proteins (OMPs) are structural constitutents of bacterium which are important immunogens for induction of cell mediated immunity against intracellular pathogen[10,11]. The effectiveness of OMPs as differential diagnostic antigens and a candidate for subunit vaccine were reported in various species of animals[12,13].

The present study reports the isolation and comparison of OMVs and OMPs from *Brucella abortus* S19 and *Brucella abortus* S19Δ*per*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The *B. abortus* S19 vaccine strain was obtained from Division of Biological products (BP), IVRI. *Brucella abortus* S19Δ*per,* was developed in our laboratory as an alternate vaccine candidate.

S19 and Δ*per* were cultivated on Brucella agar (Difco) in large sized plates (150x20 mm, 50 in no.) for 3 days. Cells were harvested in phosphate-buffered saline by centrifugation at 10,000 x g to pellet bacteria. The supernatant was filtered through 0.22 micron filter (Millipore, Corp.) to obtain bacteria free filtrate. The sterility of filtrate was assessed by plating on Brucella agar followed by incubation for 72 hrs at 37ºC. During sterility test, the filtered supernatant was stored at 4ºC.

Preparations of OMVs

The OMVs were prepared according to the protocol described by Gamazo *et al*[14]., 1989, with slight modifications**.** Briefly, the sterile bacteria free supernatant was ultrafiltered through 100 KDa filter by centrifugation at 5000 x g at room temperature (Amicon Ultra-15 centrifugal filter unit, UFC910024). The ultrafilterate was then cenrifuged at 1, 94800 x g, for 2 hr at 4 ºC in Ti-70 ultra-centrifuge rotor (Beckman Coulter).The OMV pellet was washed twice and resuspended in PBS (pH 7.2-7.4), and stored at -20ºC.

Transmission Electron Microscopy of OMVs

The OMVs isolated by ultracentrifugation were confirmed and characterized by electron microscopy. The OMV sample was fixed in 0.5% glutaraldehyde and kept for incubation for 1hr at 4°C. 12 µl of the dilutions were allowed to adsorb on carbon coated copper grid for 1 min followed by negative staining with aqueous uranyl acetate (0.5%). The micrographs were recorded at an accelerating voltage of 80 kV using a JEM-1011 DV 300W electron microscope.

Extraction of OMPs enriched fractions

The outer membrane protein fractions *B. abortus* S19 and *Brucella abortus* S19Δ*per* were prepared as per method described by Davies *et al*[15] with slight modifications.

Briefly, the pelleted organisms were resuspended in 20 ml of ice cold 20 mM Tris (pH 7.2). Organisms were inactivated by adding 0.5% formalin and the cells were washed twice with 20 mM Tris and then resuspending in 15 ml of sterile Tris and sonicated in ice at 12 µm amplitude for 30 cycles using a soniprep 150 sonicator (MSE UK Ltd.). The sonicated material was centrifuged at 5000 x g to remove the cell debris followed by ultracentrifugation of supernatant at 1, 00,000 x g for 1 hr at 4 ºc (Beckman Coulter). The pellet contained both outer and inner membrane proteins. The membrane protein preparation was then treated with 2% sodium N-laurylsarcosine (Sigma-Aldrich, USA) for selective solubilisation of inner membrane. The sarcosyl insoluble outer membrane fraction was pelleted by ultracentrifugation at 1, 00,000 x g for 1 hr at 4 ºC (Beckman Coulter).

Analysis of OMVs and OMPs by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis of OMVs and OMPs of both, *B. abortus* S19 and *Brucella abortus* S19Δ*per* was carried out as per the protocol described by Laemmli[16]. The samples were electrophoresed on 15% SDA-PAGE and stained with Coomassie brilliant blue.

Immuno-dot blot assay

The sero-reactivity of OMVs and OMPs of *B. abortus* S19 and *Brucella abortus* S19Δ*per* S19 was checked using dot blot technique. OMPs and OMVs from respective organisms was impregnated on Nitrcellulose paper and incubated with primary antibodies (anti-S19) raised in mice at 1:100 dilution followed by rabbit anti-mouse-HRPO conjugated secondary antibody (Sigma, USA) at 1:2500 at 37ºC for 1hr. The colour was developed by adding freshly prepared diaminobenzedene solution.

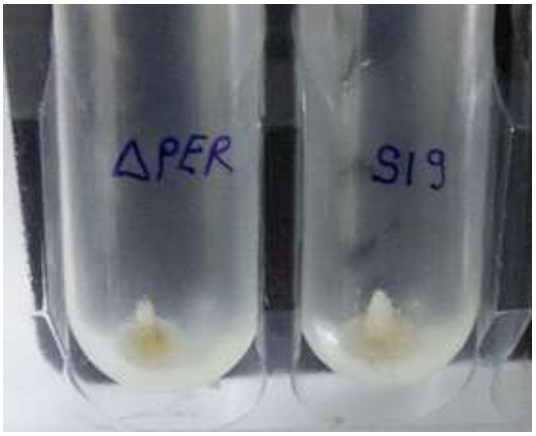

Fig. 1(a). OMVs ultra-centrifuged pellets from *Brucella abortus* S19 and *B. abortus* S19Δ*per*

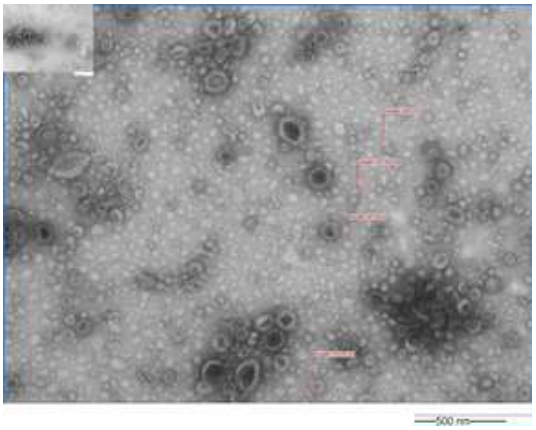

Fig. 1(b). Transmission electron microscopy of OMV of *Brucella abortus* S19Δ*per*

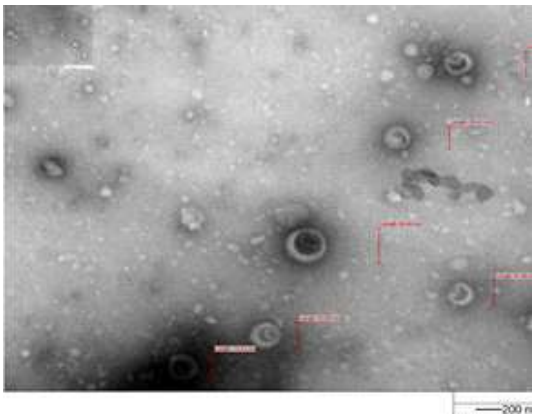

Fig. 1(c). Transmission electron microscopy of OMV of *Brucella abortus* S19

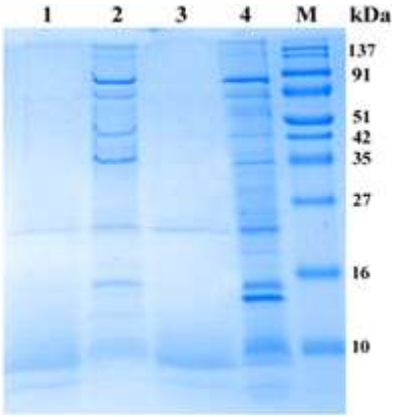

Fig .2: SDS-PAGE analysis of OMV and OMPs of *B. abortus S19 & Brucella abortus* S19Δ*per*
Lane M : Prestained marker
Lane 1 : OMV of *B.abortus* S19
Lane 2 : OMPs of *B.abortus* S19
Lane 3 : OMV of *B.abortus* S19 Ä*per*
Lane 4 : OMPs of *B.abortus* S19 Ä*per*

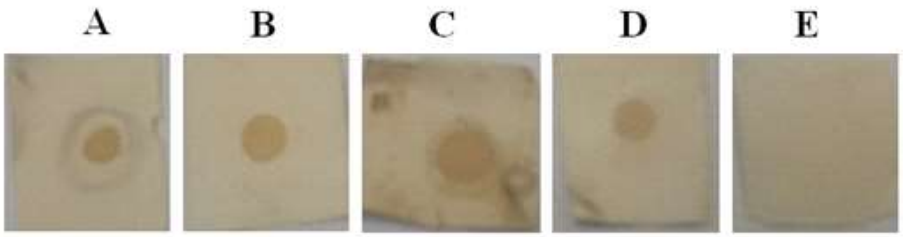

Fig. 3. Immuno-dot blot assay showing immunoreactivity
A : OMPs of *Brucella abortus* S19 showing immunoreactivity
B : OMPs of *Brucella abortus* S19Δ*per* showing immunoreactivity
C : OMVs of *Brucella abortus* S19 Δ*per* showing immunoreactivity
D : OMVs of *Brucella abortus* S19 showing immunoreactivity
E : Antigen control (PBS) showing no immunoreactivity

RESULTS

Isolation of OMVs of *B. abortus* S19 and *Brucella abortus* S19Δ*per*

The sterility of supernatant containing OMVs was checked by plating on Brucella agar plates. There was no growth of bacteria. OMVs were purified from bacterial free-culture-supernatant by differential –density-ultracentrifugation.

Transmission electron microscopy (TEM) of OMVs

The OMVs of *B. abortus* S19 and *Brucella abortus* S19Δ*per* were negatively stained with 0.5% uranyl acetate revealed the presence of multiple intact spherical vesicles with electron dense centre surrounded by a double membrane, though conventionally released few single membrane enclosed vesicles were also viewed. The size of majority of vesicles ranges between 50 nm and 200 nm Fig. 1(b) & 1(c). Shape and size of vesicles were similar in both wild type and mutant strain of S19.

SDS-PAGE analysis of OMVs and OMPs

The OMPs profiles of *B. abortus* S19 and it's perosamine synthetase mutant revealed eight bands in sarkosyl insoluble fractions. The size of OMPs ranges from 12 to 89 KDa. OMVs extracted from both strains showed a single, prominent band of 25 kDa on Coomassie brilliant blue stained gel (Fig 2). However, on silver nitrate staining, a band of lipopolysaccharide (LPS) and several minor protein bands in OMVs were observed (Fig. not shown).

Immuno-dot blot assay

Performing Immuno blot using hyperimmune serum raised in mice against *B. abortus* S19 as a source of primary antibody and rabbit anti-mouse-HRPO conjugate as secondary antibody showed similar immunoreactivity of OMVs and OMPs of both strains (Fig.**3.**).

DISCUSSION

Like other Gram-negative bacteria, the outer membrane of *Brucella*, grown in liquid or solid media, spontaneously release OMVs. The physical presence of OMVs, isolated by differential density ultracentrifugation of bacteria free filterate of *B. abortus* S19 and *Brucella abortus* S19Ä*per,* were confirmed by transmission electron microscopy. Spherical shaped intact OMVs with a diameter of 50-200nm (avg. diameter 100nm (approx.) were seen as observed previously[14,17]. No membrane debris, observed by electron microscopy using negative staining confirmed the purity of OMVs from both strains.

The spontaneous release of OMVs from bacterial surface and gentle isolation procedures should minimize cytoplasm leakage and prevent the contamination of cell debris that follow cell disruption[18].

The visualization of OMVs ensured that the protein concentrations were due to actual intact OMVs having different electrophoretic migration pattern as compare to their respective OMPs. However, the denatured electrophoretic protein profiles of OMPs and OMVs, from both strain, showed no discernible differences (Fig. 2). When SDS-PAGE gel was treated silver nitrate for LPS staining, there was an observation of LPS band along with few minor bands of OMPs indicated that OMVs isolated from both strains contained both LPS and OMPs as their constituents.

Lipopolysaccharide (LPS), an activator of innate immunity through the Toll-like receptor 4(TLR-4) pathway, is constitutively produced in Gram-negative bacteria. The concentration of LPS in OMVs doesn't depend upon the rough or smooth phenotype of bacteria[19].

Brucella abortus S19 and *B.abortus* S19Δ*per* OMVs exhibited a major band at approx., 25 KDa that would likely be an OMP. This corroborates earlier findings of Cassataro *et al*[20] and Delpino *et al*[21] and Martin-Martin *et al*[22].

Gamazo and Moriyon[17] also observed a band of 25 KDa in the OMVs of both smooth *B. melitensis* 16M and a rough strain *B. melitensis* B115. Outer membrane proteins are the principal components of *Brucella* OMVs as has been reported previously from other Gram-negative bacteria[23,24]. Boigegrain *et al*[25] identified Omp25 from *B. suis* using monoclonal antibodies, whereas mass spectrometry was used by Lamontagne *et al*[26] to identify the Omp25 (Omp3a) in OMVs from *B. abortus*.

OMPs were extracted successfully from sonicated bacteria of using 2% sodium N-laurylsarcosine which selectively solubilise only inner membrane.Outer membranes are totally

resistant to solubilisation by Sarcosine[27]. However, other ionic and neutral detergents such as sodium-dodecyl-sulfate(SDS) and Triton-x-100 solubilise both outer and inner memebrane. Sodium N-laurylsarcosine with a concentration ranging from 0.5% to 2 % results good yield of OMPs without enzymatic treatment which may alter the structure of the OMPs.

Winter *et al*[28], reported that OMPs of different *B.abortus* strains have shown three major groups. Group -I proteins having molecular mass between 88-94 KDa, group-II between 36-38 KDa and group-III between 25-27KDa to 31-34 KDa.

Tibor *et al*[29], reported low molecular weight proteins in *Brucella* species as we also observed 12 KDa band in OMPs electrophorectic profile. Differences in the molecular weight of OMPs may be attributed to variations in bacterial strains and the method of OMP extraction. We found both OMVs and OMPs immunoreactive by immune- blot dot using hyper immune atisera against *B.abortus* S19 raised in mice. Presence of LPS and OMPs, able to activate innate immunity through TLR 4 and TLR 2 dependent pathways respectively, in OMVs could prove them a better acellular vaccine candidate. It will be worthwhile to mention here that OMVs isolated using traditional methods have been used as vaccine candidate against *N. meningitidis Vibrio cholerae*, *Bordetella pertussis*, and other Gram-negative pathogens[30,31]. The OMVs based vaccines can be delivered by different routes and also have been found protective[31,32,33]. OMVs harbour immunogenic molecules including OMPs. OMVs of *Brucella* would act as acellular sub-unit vaccine candidate and therefore, we are at present actively involved in establishing the protective immune response of OMVs, equipped with OMPs, against brucellosis.

ACKNOWLEDGEMENTS

We are thankful to the Director, IVRI for providing facilities. We are also thankful to Dr. V.K. Baranwal, Principal Scientist and Incharge (Plant Virology Unit) and Dr. Jasweer Singh, Division of Plant Pathology, IARI, New Delhi for providing TEM facilities. Funding by DBT is highly appreciated and acknowledged.

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