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Construction of Eukaryotic Expression Vector of Main *B. Melitensis* S19 Outer Membrane Proteins

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To evaluate *in vitro* the mechanism of function of *Brucella* major outer membrane proteins and the interaction with cells. The *Brucella* outer membrane proteins Omp10, Omp16, Omp19, Omp25, Omp28, Omp31, Omp2a genes were cloned to pMD18-T simple carrier; Liked them to the carrier pefgp - n1 for eukaryotic expression; Examined the carrier on HEK 293 cell and detected the transfection efficiency. Cloned *Brucella* main outer membrane proteins and constructed their eukaryotic expression vectors, further examined the transfection efficiency on HEK 293 cells. Built carriers have happened expression in HEK 293 cells. This study provided the necessary materials for later research on relationships between cells and *Brucella* main outer membrane proteins.

Key words: B. Melitensis S19,outer membrane proteins, Carriers construction, Eukaryotic expression.

Brucella, the etiologic agent, is a gram negative, facultative intracellular bacterium classified based upon host preference where goat, swine, and cattle are preferred by the most common agents implicated in human *Brucellosis*, *Brucella melitensis*, *Brucella suis*, and *Brucella abortus*, respectively[1-5] and existed in rural tropical communities in most parts of the world, including the Middle East, Africa, Latin America, Central Asia and several Mediterranean countries[6-7]. *Brucella* are highly infectious and can be readily transmitted in aerosolized form[8-9]. Successful infection of the host by *Brucella* reflects the ability of the bacterium to establish itself in an intracellular environment favorable for its replication. *Brucella* survive and replicate predominantly in macrophages, where outer membrane proteins (Omps) plays a critical role[10].The outer membrane of *Brucella* is mainly composed of major outer membrane proteins (OMPs). Interest in the *Brucella* major outer membrane proteins (OMPs) stems initially from their potential as protective antigens. This notion started in the early 1980s but interest in the major OMPs has since then evolved. The *Brucella* major OMPs were initially identified by in the SDS-I cell wall fraction shown to be protective in a mouse model[11-14]. The major outer membrane proteins (OMPs) of *Brucella* were characterised as potential immunogenic and protective antigens and also they display diversity and specific markers have been identified for *Brucella* species, biovars, and strains, including the recent marine mammal *Brucella* isolates for which new species names have been proposed. Although several virulence factors are known, the basis for virulence in

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Brucella infections remains poorly understood. Early studies on virulence factors were directed at the structural features of the outer membrane. The outer membrane contains two recognized virulence factors: lipopolysaccharide (LPS) and outer membrane proteins. LPS was recognized for its virulence role when naturally occurring isolates lacking LPS showed reduced survival. However, the role of outer membrane proteins in survival and virulence remains not very clear. Here, we cloned the main brucella outer membrane protein gene, and connects them to eukaryotic expression vector pefgp-n1. This report identifies *Brucella* genes that encode proteins that may associated with stress, as well as global bacterial regulatory mechanisms, induced following early intracellular infection. Transfection of HEK 293 cell for study of *Brucella* outer membrane proteins interaction with cells lay a foundation.

MATERIALS AND METHODS

Bacterial strains, plasmids

Brucella melitensis S19 was provided by the China CDC, pefgp-n1 expression vector and HEK 293 cells were maintained in key laboratry of animal biotechnology, *DH5*α competent cells were bought from Beijing Tiangen biological technology, pMD18-T simple carriers were bought from TaKaRa biological technology.

Culture of bacteria and genomic DNA extraction

Brucella melitensis S19 were routinely cultured in TSA, when these bacteria were grown in solid medium, the above medium was supplemented with 1.5%(w/v) agar. *Bacterial* DNA from cultures of *Brucella melitensis S19* grown over night was extracted using a Tiangen DNA extraction kit.

Primer design and PCR amplification

According to the Genbank published Omp10, Omp16, Omp19, Omp25, Omp28,Omp31 and Omp2a genes Sequence, we designed specific PCR primers for the full-length amplification of selected genes with the corresponding enzyme restriction site (Omp10, Omp16, Omp19, Omp25 and Omp28 with *EcoR I* and *Sal I* restriction site, Omp31 and Omp2a with *BamH I* and *Xho I*) at 5´end of the forward and reverse primers respectively (lowercase are protection base and enzyme site). Primers sequence are as follows:

Table 1. PCR primers for outer membrane proteins

Genes and primers	Primer sequence[a]	Fragment length
Omp10	O10A:ccggaattcATGAAACGCTTCCGCA O10B:acgcgtcgacGCCGGCGTTGC	378bp
Omp16	O16A:ccggaattcATGCGCCGTATCCAGTCGATTG O16B:acgcgtcgacCCGTCCGGCCCCGTTGA	504bp
Omp19	O19A:ccggaattcATGGGAATTTCAAAAGCAAGTCTG O19B:acgcgtcgacGCGCGACAGCGTCAC	531bp
Omp25	O25A:ccgaattcATGCGCACTCTTAAGTCTCTC O25B:acgcgtcgacGAACTTGTAGCCGATGC	639bp
Omp28	O28A:ccggaattcATGAACACTCGTGCTAGC O28B:acgcgtcgacCTTGATTTCAAAAACGAC	750bp
Omp31	O31A:gcgagctcATGAAATCCGTAATTTTGGCGT O31B:cccaagcttGAACTTGTAGTTCAGACCGAAG	720bp
Omp2a	O2A1:gcgagctcATGAACATCAAGAGCCTTCTCCTTG O2A2:cccaagcttGAACGAGCGCTGGAAGCGAACG	1035bp

[a]Restriction sites and protection base are underlined.
The amplification was performed based on the following conditions: 5 min at 98°C; 30 cycles of 60 s at 94°C, 2 min at 55°C, 60 s at 72°C, then10 min at 72°C for further extension. After reaction, the PCR products were detected with 1% agarose gel electrophoresis and observations.

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Plasmid construction

By a small amount of amplification *Brucella*, bacterial genome kit(Tiangen Bio,Inc) was used to extract its genome. After high-fidelity enzyme amplification, we obtained PCR production of *Brucella* major outer membrane protein gene. Then PCR production were also extended by rTaq enzyme(TaKaRa Bio,Inc) and purpose fragments end with A base were obtained. After connected with pMD18-T simple carrier (TaKaRa Bio,Inc) for the night, recombinant plasmid transformed, coated board, picked cloning, shaked bacteria, extracted plasmid, enzyme digested identification, sent to sequencing company, then recombinant plasmid were transformed *DH5*α to extract of plasmid again. After enzyme digestion plasmid products and pefgp-n1 vector from the digested product were detected with 1% agarose gel electrophoresis and observations, DNA band was sliced under long wave UV and recovered by Tiangen Purification KitPurified plasmid by the corresponding enzyme restriction site (Omp10, Omp16, Omp19, Omp25 and Omp28 with *EcoR I* and *Sal I* restriction site, Omp31 and Omp2a with *BamH I* and *XhoI*). They were ligated with *T4 DNA ligase* (TaKaRa Bio,Inc) and we named pefgp-Omp10, pefgp-Omp16, pefgp-Omp19, pefgp-Omp25, pefgp-Omp28, pefgp-Omp31, pefgp-Omp2a, respectively. The ligation products were transformed into the *DH5*α competent cells and dispersed onto LB agar plates containing 100ng/mL ampicillin. By restriction enzyme identification, PCR and sequence analysis, The recombinant plasmid was then transformed into the *DH5*α competent cells for recycling a large number of plasmid.

Transient transfection

The Connected purpose fragment pefgp - n1 expression vector were built in this study. HEK 293 cells were grown in 6-well culture plates to about 60% to 70% confluence. The cells were then transfected with pefgp-Omp10, pefgp-Omp16, pefgp-Omp19, pefgp-Omp25, pefgp-Omp28, pefgp-Omp31, pefgp-Omp2a and vector control DNA using a lipofectin reagent (Invitrogen). After 24 hours infection, cells transfection efficiency were detected.

Blood counting chamber detection cell transfection efficiency

HEK 293 cells were grown in 6-well culture plates and treated with DMEM (high glucose, Gibco) supplemented with 10% FBS (Gibco). Cells were transfected for 24 h, and harvested and washed with PBS and subjected to blood counting chamber to detected green fluorescent protein cell percentage in total number of about 200 cells each of the sample.

RESULTS

Genomic DNA extraction and cloning main brucella outer membrane proteins gene into pMD18-T simple vector

Brucella melitensis gene was extracted using a Tiangen DNA extraction kit and analyzed on 1% (w/v) agarose gel (Figure 1). *Brucella melitensis* Omp10, Omp16, Omp19, Omp25, Omp28, Omp31 and Omp2a genes were amplified from genomic DNA of *B. melitensis S19* strain. The PCR products analyzed on 1% (w/v) agarose gel displayed a target fragment with the correct size

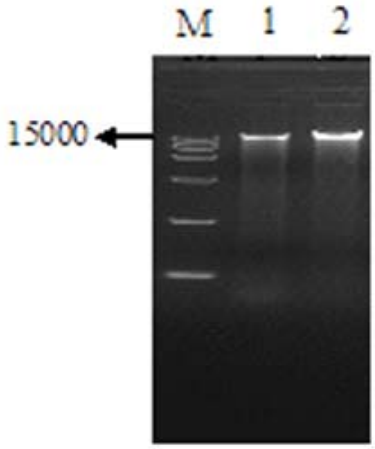
Table 2. Blood counting chamber detected cell transfection efficiency

Samples	Total number of cells	Fluorescent cells number	Transfection efficiency
Untreated cells	196	0	0%
pefgp-Omp10	219	71	32.42%
pefgp-Omp16	210	62	29.52%
pefgp-Omp19	180	45	25.00%
pefgp-Omp25	220	67	30.45%
pefgp-Omp28	197	53	26.90%
pefgp-Omp31	215	60	27.91%
pefgp-Omp2a	213	60	28.17%
pegfp-n1	199	53	25.15%
Negative control	179	0	0%

pertaining to the amplification of the related DNA (Fig. 2). Recombinant plasmid pMD18-T simple vector was digested by *EcoR I*, *Sal I* and *BamH I*, *Xho I* restriction enzymes and send to Sangon Biotech, Shanghai for determination of the sequence, the recombinant plasmid was successfully linked and named pMD18-Omp10, pMD18-Omp16, pMD18-Omp19, pMD18-Omp25, pMD18-Omp28, pMD18-Omp31, pMD18-Omp2a, respectively (Fig. 3).

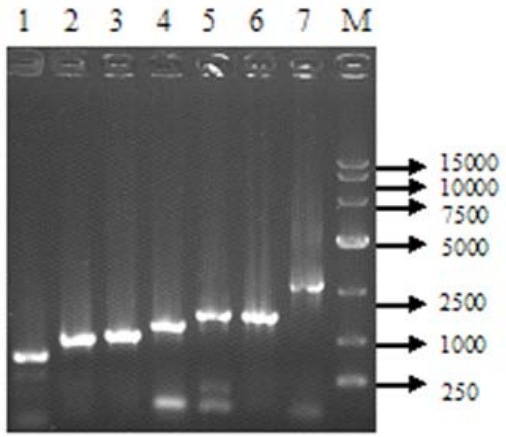
Main brucella outer membrane proteins genes ligated pefgp-n1 vector

The cloned recombinant plasmid and pefgp-n1 vector in the *DH5a* was extracted and digested by *EcoR I*, *Sal I*and *BamH I*, *Xho I* restriction enzymes, then ligated into pefgp-n1. The



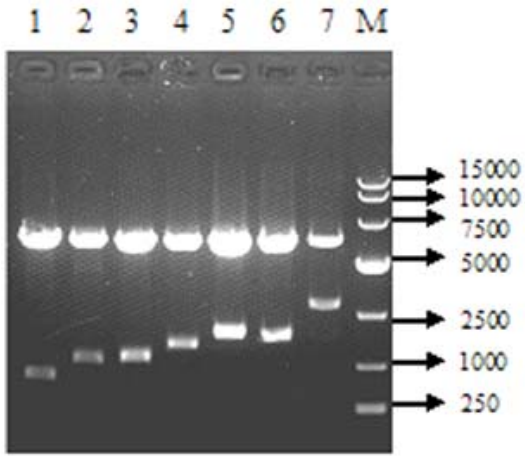
M: Standard DNA molecules; 1: Genome; 2: Genome

Fig. 1 . Extraction of brucella genome



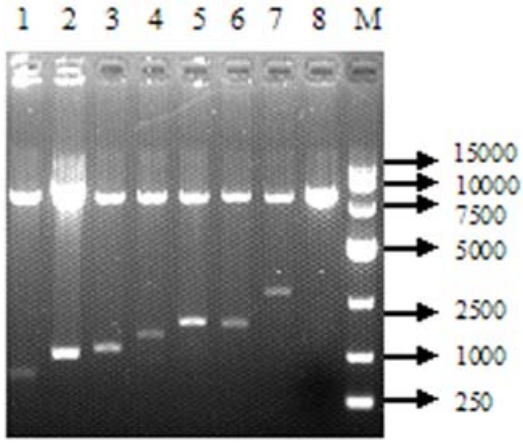
1: Omp10 (381 bp); 2: Omp16 (507 bp); 3: Omp19 (534 bp); 4: Omp25 (642 bp); 5: Omp28 (753 bp); 6: Omp31 (723 bp); 7: Omp2a (1023 bp); M: DL15000DNAMarker

Fig. 2. PCR amplification fragment



1:pMD18-Omp10; 2:pMD18-Omp16; 3:pMD18-Omp19; 4:pMD18-Omp25; 5:pMD18-Omp28; 6:pMD18-Omp31; 7: pMD18-Omp2a; M: DL15000DNAMarker

Fig. 3 . Identification of recombinant plasmid pMD18-T simple vector with double enzyme digestion



1: pefgp -Omp10; 2: pefgp 8-Omp16; 3: pefgp -Omp19; 4: pefgp -Omp25; 5: pefgp 8-Omp28; 6: pefgp -Omp31; 7: pefgp -Omp2a; 8: pefgp-n1; M: DL15000DNAMarker

Fig. 4. Identification of recombinant plasmid pefgp-n1vector with double enzyme

ligated products were then used to transform *DH5α*. Extraction of plasmid after enzyme digestion identification were shown on 1% (w/v) agarose gel, as shown (Fig. 4). Eukaryotic expression vector were successfully built and named pefgp-Omp10, pefgp-Omp16, pefgp-Omp19, pefgp-Omp25, pefgp-Omp28, pefgp-Omp31, pefgp-Omp2a, respectively .

Results of Cell transfection efficiency

When HEK 293 cells were grown in 6-well culture plates to about 60% to 70% confluence, they were transfected. After 24 h, cells were

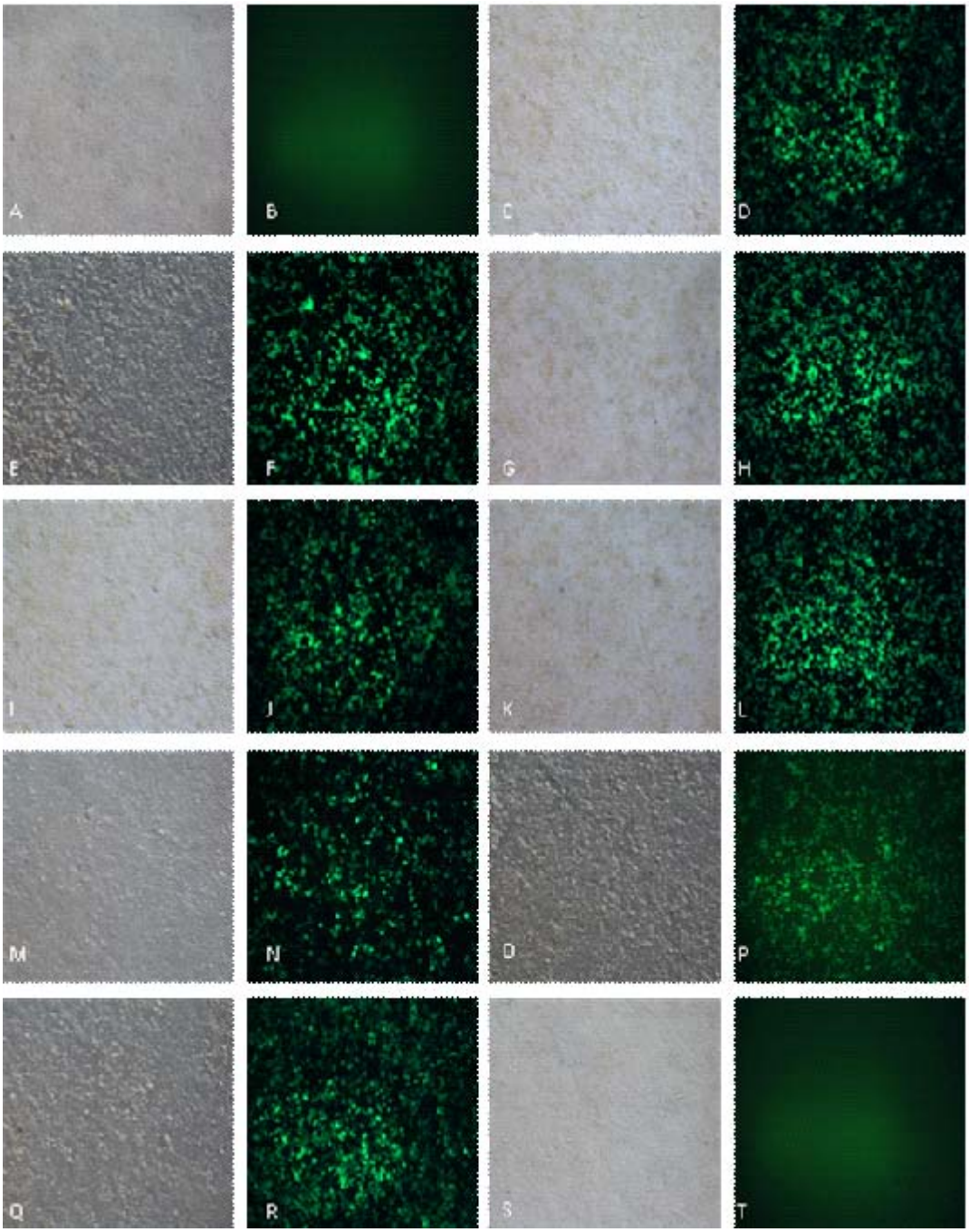


Fig. 5. The transient transfection results

observed through fluorescent inverted microscope, fusion protein of green fluorescence were successfully observed (Fig. 5). Then cells were harvested and washed with PBS, and calculated cell transfection efficiency by blood counting chamber, As shown (Table 2), transfection efficiency of pefgp-Omp10, pefgp-Omp16, pefgp-Omp19, pefgp-Omp25, pefgp-Omp28, pefgp-Omp31, pefgp-Omp2a and pegfp-n1 were about 30%.

The green fluoreseence expression of outer membrane proteins (OMPs) after transfeetion (100×): A, C, E, G, I, K, M, O, Q and S are HEK 293 cells after transfeetion in light (100×); B, D, F, H, J, L, N, P, R and T are the green fluoreseence expression of untreated cells, pefgp-Omp10, pefgp-Omp16, pefgp-Omp19, pefgp-Omp25, pefgp-Omp28, pefgp-Omp31, pefgp-Omp2a, pegfp-n1 and negative control respectively after transfeetion in Exciting light (100×).

DISCUSSION

The ability to efficiently infect through mucosal surfaces and persist in the host, its highly infectious nature and the insidious, systemic, chronic, debilitating aspects of the disease are features that make *Brucella* a viable threat to public health while earning it recognition by the CDC as a select agent with potential use in a bioterrorist act[13]. *Brucella melitensis* is a world wide disease, unless controlled, it may cause serious reproductive losses and present a risk in human and animals, so understanding of *Brucella* system disease mechanism is very important for prevention and control of brucellosis[14]. Outer membrane proteins (OMPs) are important immunogens in most of the gram negative bacteria. Investigations on *Brucella* OMPs were initiated in 1980 by sequential extraction methods using different enzymatic and detergent treatments and the characterization of these proteins was carried out on the basis of molecular masses[15-16]. Knowing the inherent stimulatory properties of the lipid moiety of bacterial lipoproteins, in recent years, scientific studies have shown that *Brucella* outer membrane proteins that causes the body's provoking immune response against *Brucella* has been investigated in cattle and murine model by immunoblot analysis and humoral as well as cell mediated immune response studies[17-19]. Previous studies on OMPs of different *Brucella abortus* strains have shown 3 main groups including group-I proteins having molecular masses between 88-94 kDa, group-II between 36 and 38kDa and group-III between 31 and 34 kDa and 25-27 kDa[20]. Although most of OMPs seen in our study also fall in the same groups, there were two additional distinct protein groups not reported by these workers. These included higher molecular mass proteins (107.1 and 151.3 kDa) and low molecular weight proteins (<20 kDa). Low molecular weight proteins in Brucella species have also been reported by Tibor[15]. Differences could be attributed to variations in bacterial strains and the method of OMPs extraction[16]. In order to study the relationship between the main outer membrane protein of brucella and cells, In this study, that the cloned experiment the brucella outer membrane protein outer membrane protein Omp10, Omp16, Omp19, Omp25, Omp28, Omp31, Omp2a genes, and connected it to the carrier pMD18-T simple and the pefgp-n1 expression vectors were done. Outer membrane proteins of eukaryotic expression vector were constructed. In order to verify the availability of carriers, the cells were then transfected with pefgp-Omp10, pefgp-Omp16, pefgp-Omp19, pefgp-Omp25, pefgp-Omp28, pefgp-Omp31, pefgp-Omp2a and vectors control DNA using a lipofectin reagent. After 24 hours infection, cells transfection efficiency were detected. As the results showed, built carriers have happened expression in HEK 293 cells. These results for animal immune experiments, the disease mechanism of cellular level and brucella outer membrane protein and virulence of brucella relationships provided the necessary materials.

In conclusion, main *brucella* outer membrane proteins genes were cloned; The green fluorescent protein fusion expression vector were built; In order to verify the availability of carrier, transienting transfection cell experiment were done in HEK 293 cell and cell transient transfection efficiency were dected by blood counting chamber.

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