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Cloning, Expression and Purification of Omp16, Omp19 and Omp28 of *Brucella melitensis* S19

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To clone the Omp16, Omp19 and Omp28 genes in pET32a expression vector and purify recombinant proteins used as antigen for future serological test development. *Brucella melitensis* S19 strain was cultured and bacterial DNA was extracted. Oligonucleotide primer pair was designed based on Genbank gene sequence with *Eco*R I and *Sal* I restriction site at 5´end of the forward and reverse primers respectively. DNA amplification was performed using PrimSTAR HS DNA polymerase and the PCR product was purified by Tiangen DNA Purification Kit. Purified DNA was excised with *Eco*R I and *Sal* I from the PCR product and subsequently cloned into pET32a. Target protein expression was induced by adding IPTG to a ûnal concentration of 1 mM when the culture OD600 (optical density at 600 nm) reached 0.6. targeted proteins wereobtained using Ni-NTA agarose resin. *Brucella* Omp16, Omp19 and Omp28 genes was successfully cloned into pET32a vector. Fusion proteins were expressed and puriûed. The Omp16 Omp19 Omp28 genes were cloned into pET32a and fusion proteins were purified.

Key words: Omp16, Omp19, Brucella, DNA.

Brucella abortus is a Gram-negative, facultative intracellular *coccobacillus* which causes *Brucellosis* in humans and in cattle. In humans, *B. abortus* causes undulant fever, endocarditis, arthritis and osteomyelitis and in animals, it leads to abortion and infertility resulting in serious economic losses[1,2,3]. Humans are usually infected by *Brucella* through contacting with infected animals or their products. Therefore, prevention of human *Brucellosis* depends predominantly on the control of the disease in animals. But it has become a diagnostic puzzle due to occasional misleading, unusual presentations and non-specific symptoms. So, looking for a way to detect the infected animals is particularly important. However, many methods for *Brucellosis* test is not sensitive[4,5]. Serological tests are often a better way of diagnosis of *Brucellosis* in both humans and animals[6]. The lipopolysaccharide (LPS) in the cell wall of *Brucella* is considered as the most important antigen for immune response during infection as it elicits long lasting immune response in both vaccinated as well as infected animals. In addition, tests based on anti-LPS antibodies give false positive results due to their cross reactive nature with other gram negative bacteria[7]. In order to overcome these drawbacks of LPS antigen, studies have been carried out to identify a non-LPS group of immunogens or recombinant proteins that can be used in immunological studies and diagnosis of *Brucellosis*. Among all the recombinant proteins of *Brucella* species studied, outer membrane proteins OMPs have received major attention for their use in vaccine as well as in diagnostics. So the purified recombinant proteins are used in development of diagnostic system for

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Brucellosis. The molecular characterization of several of these OMPs has been reported over the past years. The genes Omp25, Omp31 and Omp2b, encoding the major *Brucella* Omps, respectively, have been cloned and studied[8, 9, 10,11]. Omp16 and Omp19 are lipoproteins, and expressed in all six species and known biovars of *Brucella*. It has been confirmed to be one of the key mediators of the proinflammatory response elicited by heat-killed *B. abortus*, and the monoclonal antibody against Omp16 can protect mice against a *Brucella ovis* challenge, which indicates the important biological role of Omp16 and Omp19 in *Brucella* life and the immunogenicity[12]. Omp28 is also a candidate for the serodiagnosis of *Brucellosis* and should be explored further as a potential candidate for the development of an antigen detection system for *Brucellosis* , and the efficacy of purified Omp28 in an iELISA on clinical samples from cattle, dog, sheep and goat sera was evaluated and found to be immunoreactive [13]. Recombinant proteins have role in various applications in diagnosis of infectious diseases and also as vaccine candidates. In this study ,Omp16, Omp19 and Omp28 fusion proteins of *Brucella melitensis* S19 were purified for clinical diagnosis of *Brucellosis*.

MATERIALS AND METHODS

Bacterial strains, plasmids

Brucella melitensis S19 was provided by the China CDC, pET32a expression vector was maintained in key laboratry of animal biotechnology, *BL21 (DE3)* and *DH5á* competent cells were bought from *Beijing Tiangen* 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 Omp16, Omp19 and Omp28 genes Sequence, we designed specific PCR primers with *Eco*R I and *Sal* I restriction site at 5´end of the forward and reverse primers respectively (lowercase are protection base and enzyme site). Primers sequence are as follows:

The amplification was performed based on the following conditions: 5 min at 98 !; 30 cycles of 60 s at 94 !, 2 min at 55 !, 60 s at 72 !, then10 min at 72 ! for further extension. After reaction, the PCR products were detected with 1% agarose gel electrophoresis.

Cloning of Omp16, Omp19 and Omp28 genes into pET32a vector

DNA band was sliced under long wave UV and recovered by *Tiangen* Purification Kit. Purified DNA and pET32a vector from the digested product by *Eco*R I and *Sal* I (TaKaRa Bio, Inc.) were ligated with T4 DNA ligase (TaKaRa Bio,Inc). The ligation product was transformed into the DH5á competent cells and dispersed onto LB agar plates containing 100 ng/mL ampicillin. By restriction enzyme identification, PCR and sequence analysis, The recombinant plasmid was then transformed into the expression host *E. coli* BL21 (DE3) competent cells and dispersed onto LB agar plates containing 100 ng/mL ampicillin. After 16-18 h incubation at 37 !, colonies on the agar plate that contained recombinant plasmids were detected. For confirmation, Recombinant plasmid was extracted by *Tiangen* Plasmid Extraction Kit and digested by *Eco*R I and *Sal* I restriction enzymes (TaKaRa Bio, Inc.).

Expression and purification of Omp16, Omp19 and Omp28 fusion protein

The exponential-phase culture of the confirmed Omp16, Omp19 and Omp28 transformed colonies were induced by adding IPTG to a ûnal concentration of 1 mM when the culture OD600 (optical density at 600 nm) reached 0.6 and checked for expression after 6 h. Induced cells, as well as uninduced cells exposed to the same conditions, were lysed in lysis buffer and analysed by 12% SDS-PAGE.

In order to ascertain the location of the expressed fusion protein, 1 mM PMSF was added to inhibit the proteolytic degradation of protein, the bacterial cell suspension was sonicated for 10 min with a pulse interval of 8 s. After confirmation of the solubility, Cleared lysate was then mixed with Ni-NTA agarose resin for 1 h to allow the 6xHis tagged protein to bind with Ni^{+2} in the column. Unbound proteins were washed from column using wash buffer (8 M urea, 100 mM

NaH_2PO_4, 10 mM Tris-Cl, pH 6.3) additionally containing 1% triton X-100 and 10% glycerol. 10 mM imidazole was also included in lysis and washed buffer to prevent the nonspecific binding of contaminating proteins to the Ni-NTA resin. Finally 6xHis tagged Omp16, Omp19 and Omp28 proteins were eluted from column using elution buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-Cl, pH 4.5) and checked in SDS-PAGE [14].

RESULTS

Genomic DNA extraction and Cloning Omp16, Omp19 and Omp28 into pET32a vector

Brucella melitensis Omp16, Omp19 and Omp28 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 pertaining to the amplification of the related DNA (Figure 1). Recombinant plasmid Omp16, Omp19 and Omp28 genes ligated pET32a vector was digested by *Eco*R‘!and *Sal*‘!restriction enzymes. Right recombinant plasmid was named pET32a-Omp16, pET32a-Omp19 and pET32a-Omp28, respectively (Figure 2).

Expression of recombinant Omp16, Omp19 and Omp28 fusion proteins

The cloned recombinant plasmid in the pET32a expression system were led to the expression of the protein of Omp16 Omp19 and Omp28 at 37 ! after induction with IPTG induced 6h. The amount of protein expressed is shown in Figure 3.

Purification of Omp16, Omp19 and Omp28 fusion proteins

The soluble and insoluble fraction of recombinant proteins were detected (Figure 4) and both existed , but the insoluble fraction were more than insoluble in all of them.The recombinant protein from the cell lysate was purified under native conditions with 500 mM of imidazole in elution buffer, as shown in (Figure 5)

DISCUSSION

Brucella melitensis is a worldwide disease, unless controlled, it may cause serious reproductive losses and present a risk in human and animals, so *Brucella* tests has an important role in the diagnosis of *Brucellosis*. Main antigenic structure which is imported in the diagnosis of *Brucella melitensis*. The smooth lipopolysaccharide structure of the cell surface is the main antigenic structure. *Brucella*, which is a gram negative bacterium, has a lipopolysaccharide structure (LPS)in the outer membrane in S colony phase and has a surface that is in contact with the outer surface. This smooth lipopolysaccharide structure plays a very important role in agglutination tests. So classical serological techniques rely mainly on the detection of antibodies to LPS, however the false-positive reactions because of cross-reactivity with LPS from other bacteria[7] and other drawbacks of anti-LPS antibodies have generated an increasing interest in the detection of antibodies to alternative antigens, mainly outer membrane proteins (Omps) and cytoplasmic proteins[15]. Previous research indicated that outer membrane proteins have very strong immunogenicity, Omp16 and Omp19 exist in all the *Brucella* outer membrane proteins[16]. And they can cause cellular immunity and immune protection[17,18,19]. Omp28 was also extracellular

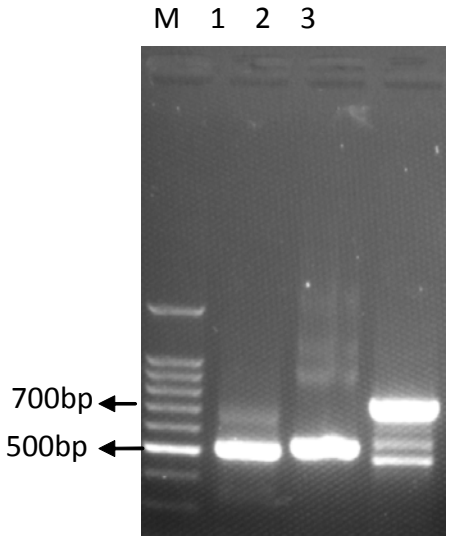
Table 1. PCR primers for outer membrane proteins

Genes and primers	Primer sequence[a]	Fragment length
Omp16A	ccggaattcATGCGCCGTATCCAGTCGATTG	507
Omp16	BacgcgtcgACTTACCGTCCGGCCCCGTTGA	
Omp19A	ccggaattcATGGGAATTTCAAAAGCAAGTCT	534
Omp19B	GacgcgtcgACTCAGCGCGACAGCGTCA	
Omp28A	ccggaattcATGAACACTCGTGCTAGC	53
Omp28B7	acgcgtcgCTTACTTGATTTCAAAAACGAC	

[a]Restriction sites and protection base are underlined

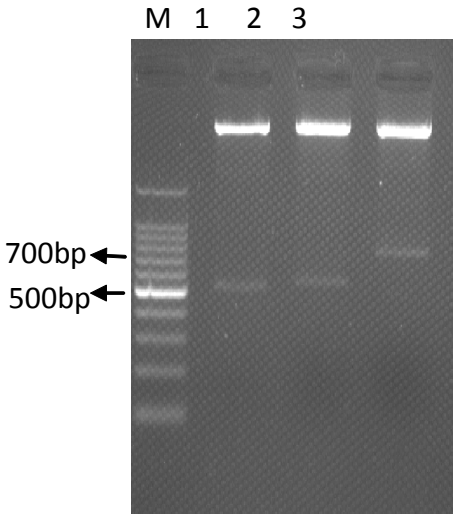
soluble proteins, relative to other outer membrane proteins, it has the advantages of easy to test[20]. In this study, a prokaryotic expression vector for *Brucella* Omp16, Omp19 and Omp28 were constructed. The fussion protein vectors pET32a-Omp16, pET32a-Omp19 and pET32a-Omp28 were expressed by IPTG induction and examined by SDS-PAGE, then purified by Ni-NTA agarose resin.

In conclusion, expression vector for *Brucella melitensis* outer membrane proteins of pET32a-Omp16, pET32a-Omp19 and pET32a-Omp28 was successfully constructed. The target



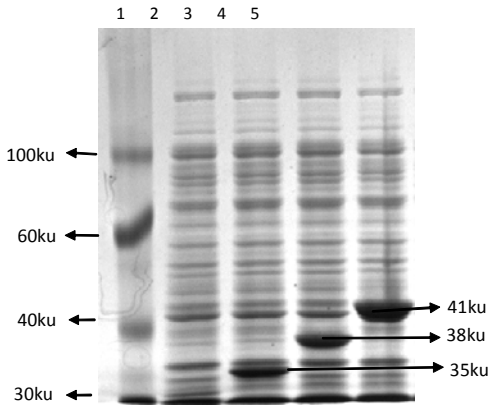
Lane M: DL5000 DNA ladder; 1-3 Omp16, Omp19 and Omp28 DNA amplified fragments.

Fig. 1. The gel electrophoresis of Omp16, Omp19 and Omp28 by PCR with genome DNA extracted from *Brucella melitensis*



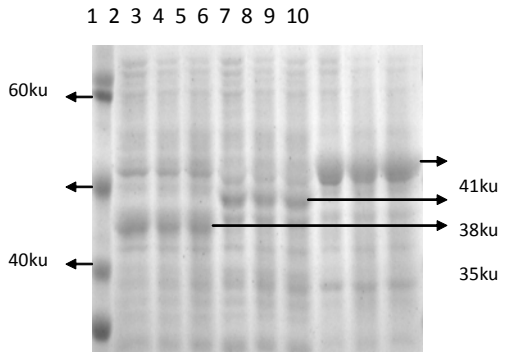
Lane 1-3: identiûcation of pET-Omp16, Omp19 and Omp28 by double digestion with *Eco*R'!and *Sal*'!, two bands with the length of 507/534/753 and 5.9 kb were generated; lane M: marker DL5000 DNA ladder.

Fig. 2. Identiûcation of prokaryotic expression vector of Omp16, Omp19 and Omp28



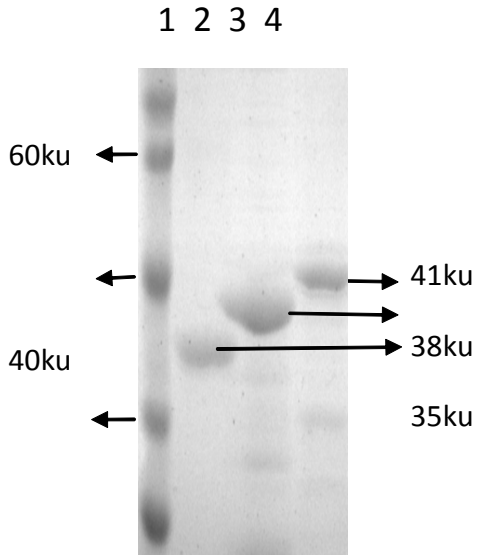
Lanes 1: Prestained protein marker; Lanes 2-5: Negative control, Omp16, Omp19 and Omp28 fusion protein induced expression with 1 mM IPTG.

Fig. 3. SDS–PAGE analysis of expression of Omp16, Omp19 and Omp28 in pET32a vector (in *E. coli* BL21).



Lane 1: Prestained proteins marker; Lane 2: Mycoprotein of Omp16; Lane 3: The soluble fraction of Omp16; Lane 4: Insoluble fraction of Omp16; lane5:mycoprotein of omp19; lane6: The soluble part of Omp16; Lane 7: Insoluble fraction of omp19; Lane 8:Mycoprotein of Omp28; Lane 9: The soluble fraction of Omp28; Lane 10: Insoluble fraction of Omp28.

Fig. 4. SDS–PAGE analysis the soluble and insoluble fraction of recombinant proteins



Lane 1: Prestained proteins marker; Lane 2: The purified Omp16; Lane3: The purified Omp19; Lane3: The purified Omp28.

Fig. 5. SDS-PAGE analysis of puriûcation of Omp19 and Omp28 in pET32a vector with elution buffer under native conditions

proteins were strongly expressed, and highly purified proteins were obtained. This study laid a foundation for further research of animal examination, immunogenicity and protection of *Brucellosis.*

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