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Original Article

Cloning and Expression of *Com1* and *OmpH* Genes of *Coxiella burnetii* in Periplasmic Compartment of *Escherichia coli* with the Aim of Recombinant Subunit Vaccine Production

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ABSTRACT

Coxiella burnetii is an obligate and gram-negative bacteria causing query fever (Q fever) disease, despite the importance of Q fever, there is no universal vaccine against this disease. Therefore, application of the recombinant subunit vaccines which use *Com1* and *OmpH* as immunogenic proteins can be useful in this regard. To perform the current project, *Com1* and *OmpH* genes were amplified by polymerase chain reaction (PCR) method, then, the PCR products were purified by DNA precipitation technique. In order to clone, first, both genes along with the pET-22b(+) vector were digested by *NcoI* and *XhoI* enzymes and then, *Com1* and *OmpH* genes were ligated in linear vectors by T4 DNA ligase. The recombinant vectors were transformed in BL21 (DE3) strain of *Escherichia coli* and expression was induced by 1 mM Isopropyl β-D-1-thiogalactopyranoside. Expression of *Com1* and *OmpH* was investigated using 12% Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Finally, both proteins were purified by Ni-NTA columns and consequently confirmed by western blotting. The results of assessing 1% agarose gel showed that PCR amplification, DNA precipitation, and digestion of both genes were successfully performed. The results of colony PCRs and sequencing revealed that *Com1* and *OmpH* were correctly cloned in pET-22b(+) vector. Finally, the results of expression, purification, and western blotting of both proteins showed that BL21 (DE3) strain of *Escherichia coli* could be able to express *Com1* and *OmpH* proteins**.** Based on the collected data, it seems that *Escherichia coli* as an affordable and simple host can be applied to express *Com1* and *OmpH* genes. It should be mentioned that products of the present project can be examined as recombinant subunit vaccines against Q fever.

Keywords: *Coxiella burnetii*, *Com1*, *OmpH*, *E.coli*, subunit vaccine

Clonage et expression des gènes *Com1* et *OmpH* de *Coxiella burnetii* dans l'espace périplasmique d'*Escherichia coli* afin de produire des vaccins à base de sous-unités recombinants

Résumé: *Coxiella burnetii* est une bactérie obligatoire et à Gram négatif qui provoque la fièvre Q (ou coxiellose). Malgré l'importance de la fièvre Q, il n'existe pas de vaccin universel contre cette maladie. Il serait donc utile de développer des vaccins à base de sous-unités recombinants utilisant *Com1* et *OmpH* comme protéines immunogènes. Dans ce but, les gènes de Com1 et *OmpH* ont été amplifiés par PCR, puis les produits de PCR ont été purifiés par la technique de précipitation d'ADN. Pour le clonage, tout d'abord, les deux gènes ainsi que le vecteur pET-22b (+) ont été digérés par les enzymes *NcoI* et *Xhol*, puis *Com1* et *OmpH* ont été ligaturés dans des vecteurs linéaires par l'ADN ligase T4. Les vecteurs recombinants ont été transformés dans la souche BL21 (DE3) d'*Escherichia coli* et l'expression a été incitée par l'isopropyl β-D-1-thiogalactopyranoside.

L'expression de *Com1* et *OmpH* a été vérifiée par électrophorèse sur gel de polyacrylamide en présence de dodécylsulfate de sodium. Enfin, la purification des deux protéines a été effectuées par des colonnes Ni-NTA et a été ensuite confirmées par Western blot. L'évaluation des résultats sur gel d'agarose à 1% a révélé qu'une amplification PCR, la précipitation de l'ADN et la digestion des deux gènes ont été effectuées avec succès. Les résultats des PCR des colonies et le séquençage a révélé que *Com1* et *OmpH* étaient correctement clonés dans pET-22b (+). Enfin, les résultats d'expression, de purification et de Western blot des deux protéines ont montré que la souche BL21 (DE3) d'*Escherichia coli* exprimait correctement les protéines *Com1* et *OmpH*. Au vu des résultats, il semble qu'*Escherichia coli* représente une hôte abordable et simple d'application pour exprimer les gènes *Com1* et *OmpH*. Il convient de mentionner que les produits issus de ce projet peuvent potentiellement rentrer dans la composition de vaccins à base de sous-unités recombinants contre la fièvre Q.
Mots-clés: *Coxiella burnetii*, *Com1*, *OmpH*, *E. coli*, vaccin à sous-unités

INTRODUCTION

Query fever (Q fever) is known as a zoonotic disease that is observed in both developed and developing countries (Derrick, 1937). The *Coxiella.burnetii* is responsible for causing this disease and it is classified as an obligate intercellular and gram-negative bacterium (Eldin et al., 2017). This bacteria can show many dangerous biological behaviors, such as adhesion, invasion, intracellular trafficking, host-cell modulation, and detoxification (Seshadri et al., 2003).

In general, particularly in livestock, ruminants are known as primary reservoirs of Q fever. As a result, this disease can be transferred through urine, stool, milk, and birth products of pollutant ruminants (Parker et al., 2006). It has been shown in ruminants that Q fever can cause abortion, stillbirth, premature delivery, and delivery of weak offspring. Whereas, in humans, it can damage vital organs (e.g., brain, lung, liver, and heart). Consequently, this disease is introduced as a huge threat to human health and animal husbandry industry; therefore, fighting with Q fever is dispensable (Anderson et al., 2013). Although indispensable and inactive vaccines of Q fever are extensively applied in different areas of the world, it has been disclosed that conventional methods owing to their limitations (efficiency, availability, and side effects) are not able to control this disease. Therefore, identification and employment of a potent strategy must be put on agenda (Soler and Houdebine, 2007). It seems that recombinant vaccines (e.g., subunit vaccines) that use recombinant DNA technology can be regarded as a strong alternative to overcome the limitation of conventional vaccines (Soler and Houdebine, 2007). Designing recombinant subunit vaccines depends on immunodominant or immunogenic proteins which belong to outer membrane proteins (OMPs). According to immunoproteomic analysis, there are 169 immunodominant proteins in *C. burnetii* which can be appropriate candidates to design a subunit vaccine against Q fever. It has been demonstrated that among 169 proteins, CUB1910 (Com1), CBU0612 (OmpH), CBU1718 (GroEL), CBU0236 (Tuf-2), and CBU009 (YbgF) are the most immunodominant proteins (Glazunova et al., 2005; Xiong et al., 2012). According to the literature review, it seems that *Com1* and *OmpH* are more reliable than other proteins to apply. Therefore, in the present study, these proteins were chosen to be expressed in *Escherichia coli (E. coli)*. It must be mentioned that *Com1* is introduced as an outer membrane-associated seroreactive protein of *C. burnetii* (Hendrix et al., 1990). Whereas, *OmpH* is introduced as a putative outer membrane chaperone protein required to release translocated proteins from the plasma membrane (Xiong et al., 2012).

MATERIAL AND METHODS

Collection and frameshift investigation of nucleotide sequences. In order to conduct the current study, nucleotide sequences of *Com1* (Accession No: Z11828) and *OmpH* (Accession No: NC_002971) genes were collected from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih .gov/). In addition, CLC workbench software was used to investigate the frameshifts of both genes. Furthermore, signal sequences of genes were identified and separated by SignalP 4.1 online server (http:// www.cbs.dtu.dk/services/SignalP/).

Amplification of *Com1* and *OmpH* genes using polymerase chain reaction. At first, genomic DNA of *C. burnetii* was extracted from killed bacteria by DNA extraction kit (Thermo, USA). The quality and quantity of extracted DNA were analyzed by 1% agarose gel and Nanodrop, respectively. To design forward and revers primers of both genes, Primer Premier software (version 5) was employed (Table 1). Moreover, in order to digest and ligate genes in multiple cloning sites of pET-22b(+) vector (Novagen, USA), 5' end of both forward primers contained restriction site of *NcoI*, whereas 5' end of both revers primers comprised *XhoI* restriction site (underlined regions). Mixture of both polymerase chain reaction (PCR) reaction included 1µl of qualified genomic DNA, 0.5µl of each primer (20 pmol), 2.5 µl of 10 X PCR buffer, 1 µl of Mgcl2 (10mM), 2.5 µl of dNTPs mixture (2 mM), and 0.2 u of *Pfu* (Vivantis, Malaysia) enzyme. Thermocycler programs of both PCRs contained 1 initial denaturation cycle of 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 60°C for 30 s and 72 °C for 2 min, and 1 final extension cycle of 72 °C for 10 min.

Cloning of *Com1* and *OmpH* genes. To clone *Com1* and *OmpH* genes in the pET-22b(+) vector, a direct cloning strategy was considered. In this case, to increase the efficiency of digestion, first, the PCR products of both genes were purified by ethanol DNA precipitation. Thereafter, *Com1* and *OmpH* fragments were digested by *NcoI* (New England Biolab, USA) and *XhoI* (New England Biolab, USA) restriction enzymes, as well as the pET-22b(+) vector was linearized by these enzymes. After digestion, digested *Com1* and *OmpH* fragments were inserted in the linear vector by T4 DNA ligase (Vivantis, Malaysia) enzyme. Ligation was conducted based on three inserts of one vector ratio. Then, the ligation products were transformed into DH5α strain of *E. coli* using a heat-shock method. Finally, produced colonies which were screened based on antibiotic resistance were applied to confirm ligation with the help of colony-PCR, sequencing by T7 promotor, and T7 terminator primers (Table 1).

Expression and purification of *Com1* and *OmpH* proteins. To express *Com1* and *OmpH* proteins, after confirmation of ligation, recombinant pET-22b(+) vectors were transformed into competent BL21 (DE3) strain of *E. coli*. At the next step, for each protein, a transformed colony was used for incubation in 10 ml of LB ampicillin medium at 37 °C overnight, in order to refresh, 1 ml of incubated medium was added to 99 ml of fresh LB ampicillin medium at 37 °C for 2 h to gain optical density=0.6. The experiment was continued by adding 1 mM isopropyl β-D-thiogalactoside (Sigma-

Table 1. Sequences of primers employed in the present study

Genes	Primer sequences(5'-3')	PCR product size
OmpH	F: ccatggcgcaaacggttgggcttgtcgatatgcgtcag	
OmpH	R:ctcgagtttcaatgctgatacaacgtttgaagtaatatcc	452 bp
Com1	F: ccatggcggccccctctcaattcagtttttctcctcaac	
Com1	R: ctcgagcttttctacccggtcgatttctttttgaagg	720 bp
T7 Promotor	F: taatacgactcactataggg	
T7 terminator	R: gctagttattgctcagcgg	*

Underlined regions belonged to restriction sites of *NcoI* and *XhoI*.
*Colony PCR product size of *OmpH* and *Com1*genes were 754 bp and 1022bp, respectively.
PCR: Polymerase chain reaction

Aldrich, USA) (IPTG) and incubation at 37 °C for 5 h. To find the best time of expression, sampling was performed 0, 1, 2, 3, 4, and 5 h after IPTG induction. Induction products were used for protein extraction; in order to perform this action, at first, each sample was harvested by centrifugation at 5,000 rpm for 15 min and the pellets were re-suspended in enough volume of TSE (Tris-HCL 200 mM pH=8.0, Sacarose 500 mM, and Ethylenediaminetetraacetic acid 1 mM) buffer, and then incubated on ice for 1 h. Finally, the samples were pelleted by refrigerated centrifuge at 12,000 rpm for 20 min and their supernatant was collected to load on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Purification of expressed proteins was conducted by Ni-NTA columns. In this case, at first, samples were dialyzed at 4 °C overnight and after that columns were equilibrated and washed by cold phosphate buffer saline. Thereafter, the dialyzed samples containing proteins were loaded on columns and by passing the samples, the columns were washed by 20 mM imidazole at 25 °C. Finally, the proteins were eluted from the columns by 4 ml of 25 mM imidazole.

Confirmation and concentration measurement of purified proteins. In order to confirm purified proteins, western blotting was applied. First, purified proteins were visualized on 12% SDS-PAGE and proteins were transferred from gels to polyvinylidene difluoride (PVDF) membranes (Roche Diagnostic, Germany) using semi-dry apparatus. The PVDF membranes were blocked by 5% skim milk in Tris Buffered Saline Tween 20 buffer (TBST) for 2 h. Thereafter, the membranes were washed by TBST buffer and embedded in primary antibody (Thermo, USA) (anti-His-tag) solution diluted with a blocking buffer (1:100) for 2 h. The membranes were washed by a TBST buffer and incubated with a secondary antibody (Thermo, USA) (1:200 diluted Blocking) for 2 h. At last, after washing with TBST buffer, 15 ml of Tetramethylbenzidine (Sigma-Aldrich, USA) was added to each PVDF membrane. Moreover, the Bradford assay was applied to measure protein concentrations. To this end, a standard curve was created by serial dilution of bovine serum albumin (Sigma-Aldrich, USA) (BSA).

RESULTS

Amplification of *Com1* and *OmpH* genes. To investigate the amplification of *Com1* and *OmpH* genes, 1% agarose gels were run. Findings of the present study revealed that the *Com1* gene with 720 bp in length (Figure 1A), as well as *OmpH* with 452 bp in length (Figure 1B), were successfully amplified. A high fidelity enzyme, namely *Pfu* was used to prevent mutation in the current study. The results of sequencing using T7 primers showed that the PCR of both genes was correctly performed.

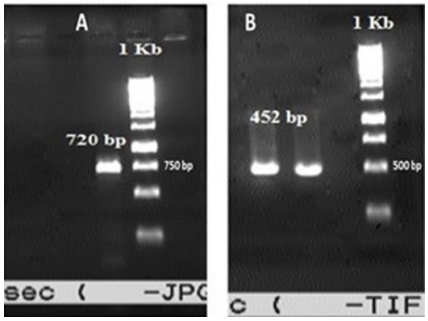

Figure 1. The PCR results of *Com1* and *OmpH* genes on 1% agarose gel. A: The amplification of Com1 with 720 bp in length; B: OmpH amplification with 452 bp in length. For both Figures, 1kb DNA ladder (Vivantis, Malaysia) was used.

Cloning of *Com1* and *OmpH* genes. As noted, the DNA precipitation was performed to increase the efficiency of digestion. Visualization of the precipitated PCR product on 1% agarose gel showed that intended bands of *Com1* and *OmpH* were successfully recovered and all PCR contaminations (e.g., primer dimers) were completely eliminated. The result of pET-22b(+) (as vector) digestion using *NcoI* and *XhoI* was visualized by electrophoresis on agarose gel, which demonstrated that the vector was well linearized. Colony-PCR and sequencing were performed in order to confirm ligation. The results of *Com1* and *OmpH* colony-PCR

and sequencing using T7 primers showed that both genes were correctly cloned into the pET-22b(+) vector.

As noted, *Com1* and *OmpH* proteins were purified by Ni-NTA columns (Qiagen, Germany). The results of purification revealed that *Com1* with a molecular mass

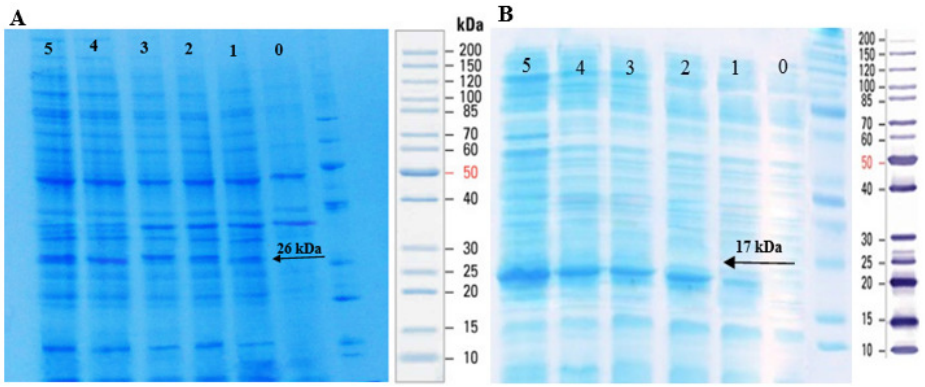

Figure 2. The results of time-point study of *Com1* (A) and *OmpH* (B) expression on SDS-PAGEs (12%) which were stained with Coomassie Blue. In both Figures, 0-5 columns related to 0-5 h after adding 1mM IPTG at 37 ºC, respectively.

Expression, purification, and confirmation of Com1 and OmpH proteins. In order to induce the expression of both proteins, 1 mM IPTG was added and sampling was performed 0, 1, 2, 3, 4 and 5 h after induction. The expression assessment of Com1(figure 2A) and OmpH(figure 2B) proteins on 12% SDS-PAGE showed that the maximum expression of both proteins occurred 5 h after induction.

of 26 kDa and also, *OmpH* with a molecular mass of 17 kDa were clearly purified (Figure 3).The manifestation of both proteins on PVDF membranes by western blotting demonstrated that expression and purification were successful (Figure 4). Finally, the results of Bradford assay indicated that concentration of *Com1* was 0.6 g/L, whereas the concentration of *OmpH* was 0.9 g/L.

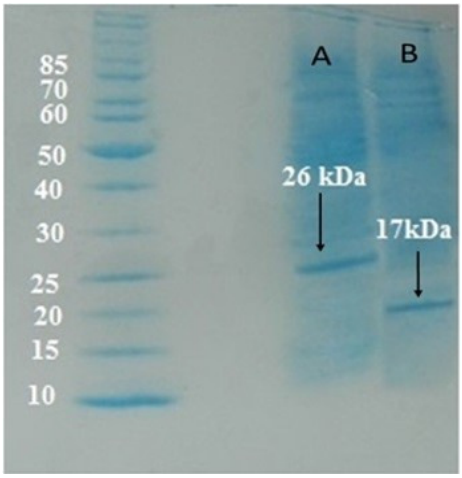

Figure 3. The results of protein purification using Ni-NTA columns on the SDS-PAGE gel (12%) which was stained with Coomassie Blue. Lane A: *Com1* with a molecular size of 26 kDa. Lane B: *OmpH* with a molecular size of 17 kDa.

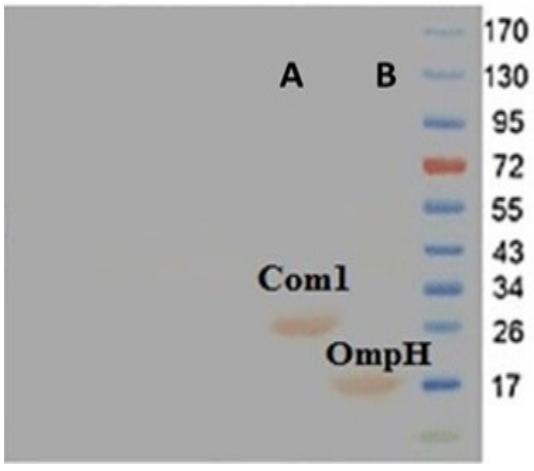

Figure 4. The results of western blotting using an anti- His antibody. First lane: Protein marker (Thermo, USA). Lane A: *Com1* with a molecular size of 26 kDa. Lane B: *OmpH* with a molecular size of 17 kDa.

DISCUSSION

The Q fever as a zoonotic disease can provoke extreme damages to human health and animal husbandry industry; therefore, prevention of this disease is important (Derrick, 1937). In general, vaccination against pathogenic organisms is performed by attenuated, inactivated, and subunit vaccines (Lo, 1987). To produce attenuated vaccines, living organisms are applied; therefore, it seems that attenuated organisms may achieve their pathogenicity during application and become pathogenic for their hosts (Hansson and Sta, 2000). Although inactivated vaccines used nonviable organisms and they do not have problems with attenuated vaccines, their production is too onerous and expensive. Hence, to overcome problems of live and inactivated vaccines, subunit vaccines using immunogenic proteins of the organism were introduced. Despite the advantage of these vaccines, separation of immunogenic proteins from organisms is known as an enormous challenge to produce subunit vaccines (Lo, 1987). Nowadays, applying recombinant DNA technology has been able to make a huge revolution in the production of subunit vaccines (Kristensen et al., 1996). In fact, this technology can produce immunogenic recombinant proteins without problems of traditional methods (Hansson and Sta, 2000). The present study was designed to clone and express *Com1* and *OmpH* genes (as the most immunogenic antigens) in the prokaryotic system with the aim of recombinant subunit vaccine against Q fever. In general, the type of recombinant protein has a vital role in selection of expression system (Forouharmehr et al., 2018). For instance, proteins which do not require post-translational modification can be expressed in prokaryote, such as *E. coli*. In view of cost-efficiency and convenience of prokaryotic systems, researchers who work in this area prefer to optimize this system for expression of simple proteins (Forouharmehr et al., 2018). Given that, *Com1* and *OmpH* are simple proteins which were expressed in *E. coli*. In prokaryotic systems, proteins can be expressed in the cytoplasm or periplasmic space of bacteria. It has been concluded that expression of recombinant proteins in periplasmic space is much better than cytoplasm because periplasm is clearer than cytoplasm; therefore, purification of recombinant proteins is easier (Jahandar and Forouharmehr, 2019). In view of this matter, pET-22b(+) was applied in the current study. This plasmid contains a potent signal peptide named PelB that can direct recombinant protein to the periplasm. According to the results of expression, purification, confirmation, and measurement of *Com1* and *OmpH* proteins, it can be claimed that *E. coli* as host and pET-22b(+) as plasmid could express both proteins with high yield and low contamination. In a previous study, it has been shown that applying *E. coli* along with pET-22b(+) could express human serum albumin with a high concentration (Ghafari et al., 2013). In addition, more recently, it has been reported that *E.coli* expression system using the pET-22b(+) as expression vector, including r3epitope linked to IL2 gene was efficient enough to express protein by the 0.3 g/l concentration (R2=0.989) for 5 h at 37 °C (Nazifi et al., 2018).

Nowadays, recombinant DNA technology is widely used in most areas of biology. In general, the expression of recombinant immunogenic proteins with the aim of subunit vaccines production is known as one of the most important applications of this technology. As mentioned, the present study was performed to express recombinant *Com1* and *OmpH* as the most immunogenic proteins of *C. burnetii* in *E. coli*. Based on the obtained results, both proteins could be successfully expressed in *E. coli* as a prokaryotic system. Therefore, it seems that *E. coli* can be applied to express *Com1* and *OmpH* genes as an affordable and simple host.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

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