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Isolation, replication of Brucella OMP31 gene and transfering to an expression vector

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Abstract: Brucellosis is a chronic and debilitating disease that affects various organs and has many negative consequences in the field of health and economy in Iran. Brucella outer membrane proteins, especially OMP31, OMP28, are important in vaccine design, production of required antigens, serum diagnostics, production of safe recombinant proteins with appropriate immunogenicity, and cost-effective production. Selecting the appropriate expression and hosting system for the expression of these recombinant proteins that can be produced safely, massively and economically is one of the main priorities in the production of vaccines and antigens used. In this study, plasmid pnz8149 was extracted from *Lactococcus lactis*. Brucella OMP31 gene was then amplified into specific vectors after amplification with specific primers and digestion of nco1 and bmh1 enzymes. Using electroporation, the plasmid containing the gene was transferred to *Lactococcus lactis*. In order to select transformant lactococci, lactococci containing recombinant plasmid pNZ8149 + OMP31 were transferred to Elliker medium. Finally, the presence of recombinant plasmid pNZ8149 + OMP31 in *Lactococcus lactis* was investigated by PCR, enzymatic digestion and sequencing. The quantity and purity of pnz8149 extracted plasmid were 123.8 ng and 1.93 μg, respectively. Due to the design of specific primers for amplification of OMP31 gene of this fragment, the target of Brucella bacterium genome was successfully amplified to 723 bp. OMP31 gene insertion in pnz8149 vector was successfully performed and the presence of recombinant plasmid pNZ8149 + OMP31 in *Lactococcus lactis* was confirmed. Given that the product of the OMP31 gene is a valuable candidate for the production of vaccine, this gene can be of significant target in research associated with vaccine production for treatment of brucellosis.

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Keywords: Brucellosis, OMP31 gene, Lactococcus lactis, Vector

1 Introduction

Brucella is mainly a pathogen of animals and is transmitted to animals through feces, urine, milk and infected tissues of animals. Human infection, which occurs accidentally through contact with contaminants, especially pasteurized milk and dairy products such as cheese or occupational exposure to contaminated animals. *Brucella melitensis* and *Brucella abortus* are responsible for almost all cases of brucellosis in humans and livestock (McDermott and Arimi, 2002; Ko and Splitter, 2003; McDermott and Arimi, 2002; Rivera et al., 2002). The re-emergence of human brucellosis in many parts of the world and its use as a class B bio terrorism agent has attracted the attention of scientific authorities. Brucellosis is a fever-causing disease that occurs in thousands of infectious and non-infectious forms. Early and correct diagnosis of human brucellosis due to nonspecific clinical manifestations, slow growth in blood cultures and the complexity of serum diagnosis is very important by clinical specialists (Seleem et al., 2008; Wang et al., 2011; Thavaselvam et al., 2010). Brucellas are bacteria of pirivolan and their infectious dose is relatively low, which is usually transmitted through the skin-mucosa (Bax et al., 2007; Jacques et al., 2007; Ko and Splitter, 2003). The clinical management of brucellosis is worrying due to the high rate of failure in initial treatment and the severity of its recurrence. Due to nonspecific clinical manifestations, slow growth in blood vessels, and complexity, serum diagnosis by clinicians is very important. Therefore, since the management of this disease has failed, vaccination and immunity against this disease and also the diagnosis of this disease in livestock in order to prevent its transmission to humans through the consumption of dairy products is of great importance in controlling and preventing the disease in humans. Many efforts have been made to develop different vaccines, establish immunity, eradicate the disease, and diagnose and screen sick animals. Most of these efforts to develop vaccines, as well as the production of antigens, have also focused on the production of recombinant proteins from the outer membrane proteins of Brucella bacteria. Considering that the main cause of brucellosis in Iran is Brucella melitensis, it is possible to study the sources and linear epitopes of two strong immunogenic agents of this bacterium, including OMP28 and OMP31 outer membrane proteins, and methods of production and purification of recombinant proteins (Jacques et al., 2007). OMP, or Brucella outer membrane proteins, have been widely identified as immunogenic and protective antigens, so they can be useful antigens in the development of diagnostic methods and vaccines (Seleem et al., 2008; Wang et al., 2011). OMP has been widely identified as an indicator of immune and protective antigen (Thavaselvam et al., 2010; Bax et al., 2007). OMPs in bacteria are composed of protein, lipids, and sugars. In fact, these proteins are the same as group antigens and are purine proteins. In 1980, this protein, which is part of the major outer membrane proteins, was identified as a strong immunogenic antigen and classified according to molecular weight. The molecular weight of the first group is 94-88 KDa, the molecular weight of the second group is 33-43 KDa and the molecular weight of the third group is 25-34 KDa. Group 2 genes encode 2aOMP and 2bOMP proteins, and group 3 genes encode

31OMP and 25OMP proteins. More detailed studies show that there are five specific genes similar to 31OMP, 25OMP in Brucella melitensis (KDa 20, KDa 28 and KDa 31 proteins). The 28-kDa protein is located on chromosome 1 of the bacterium (Jacques et al., 2007; Ko and Splitter, 2003). This protein was particularly useful in measuring anti-brucellosis antibodies in infected animals and is located on chromosome I of the bacterium. The technology of recombinant proteins and monoclonal antibodies (MABs) indicates that OMPs act as weak antigens in smooth strains, *Brucella melitensis* and *Brucella abortus*, and provide little or no protection in the mouse model. Instead, group 3 proteins, especially 31OMP, are present in the form of immunogenic antigens in rams infected with *Brucella ovis* and in mice infected with *Brucella ovis*. Thus, their mutant strains are suitable candidates for vaccine production (Thavaselvam et al., 2010). Due to the prevalence of brucellosis in the world (Megersa et al., 2011; Young et al., 2005) and Iran (Ko and Splitter, 2003) and its increase in different societies (Bax et al., 2007) and also due to the widespread clinical, social and economic complications of brucellosis, the present study investigated the isolation and replication of Brucella OMP31 gene and its transfer to an expression vector and the results of this study are of particular importance in the field of vaccine design for brucellosis.

2 Materials and Methods

During this experimental-laboratory study, QIAGEN Endo Free Plasmid Maxi extraction kit was prepared to extract pnz8149 plasmid from *Lactococcus lactis* and *Lactococcus lactis* NZ3900 stocks containing plasmid pNZ8149 from Pasteur Vaccine Research Institute. In this study, plasmid pNZ8149 *Lactococcus lactis* based on LacF gene was selected as a marker of selection in the food class in order to establish the ability to grow in lactose. The host strain of *Lactococcus lactis* NZ3900 has all the genes associated with lactose fermentation except the LacF gene. The NZ3900 strain is able to grow on glucose, but will also be able to grow on lactose in the presence of the LacF gene in the plasmid. A special culture medium was used to select *Lactococcus lactis* Lac + colonies.

2. 1. M17 culture medium with glucose as carbon chain (G-SGM17B)

This culture medium includes commercial culture medium M17 with: 0.5 M sucrose, 2.5% glycine, 5% glucose. Sucrose and glycine are added to M17 and sterilized for 20 minutes at 121 ° C. Sterilized glucose is added to the culture medium after cooling. To prepare 500 cc of this culture medium (G-SGM17B) according to the instructions in the M17 data sheet, the amount of M12/ 215 g with a molecular weight of 42.25 was weighed and placed in a 1000 cc bottle. According to the molecular weight of sucrose (342.2) and the required molarity (0.5), 85.55 g of sucrose was weighed and added to the bottle containing M17. Weigh 12.5 g of glycine and add 2.5% to the bottle to prepare the final concentration and reach the final volume of 400 cc using double distillation water. 25 g of glucose was added to another 200 cc bottle to make a final concentration of 5% and reached 100 cc by double distillation of water. Both bottles were sterilized at 121 ° C for 20 minutes and after cooling under the hood, they were mixed together and after sealing, they were kept at 4 °C to 8 °C for further use.

2. 2. M17 culture medium with lactose as carbon chain (L-SGM17B)

This culture medium contains commercial culture medium M17 with: 0.5 M sucrose, 2.5% glycine, 5% lactose. Sucrose and glycine are added to M17 and sterilized for 20 minutes at 121 °C. Sterilized lactose is added to the culture medium after cooling. To prepare 500 cc of this culture medium (L-SGM17B), according to the instructions in the M17 data sheet, the amount of g21.125 M17 with a molecular weight of 42.25 was weighed and placed in a bottle with a volume of 1000 cc. According to the molecular weight of sucrose (342.2) and the required molarity (0.5), the amount of sucrose g85.55 was weighed and added to the bottle containing M17. Weigh 12.5 g of glycine and add 2.5% to the bottle for the final concentration. Was double distilled using water to reach a final volume of 400 cc. 25 g of lactose was added to another 200cc bottle to make a final concentration of 5% and reached 100 cc by double distillation of water. Both bottles were sterilized at 121 °C for 20 minutes and after cooling under the hood, they were mixed together and after sealing, they were kept at 4 °C to 8 °C for later use.

2. 3. Washing solution

This solution contains 0.5 M sucrose and 10% glycerol.

2. 4. Suspension solution

This solution contains 0.5 M sucrose, 10% glycerol and 0.05 M EDTA.

2. 5. Prepare a recovery solution

This solution contains G-SGM17B culture medium plus 20 mM MgCl2 plus 2 mM CaCl2.

2. 6. Elliker culture medium

Elliker is a special culture medium that can be used to select *lactococcus lactis* Lac + colonies. In this rich culture medium all cells can grow, both lactose positive and lactose negative, but when lactose is added as the only carbon source, the cells that undergo lactose fermentation are colonized. They produce yellow spots. This culture medium includes: 20 grams per liter of tryptone, g5 per liter of yeast extract, 4 grams per liter of sodium chloride, 1.5 grams per liter of sodium acetate, 0.5 grams per liter of lactose-positive ascorbic acid and 15 grams per liter of solid culture medium or 1.5% agar was added. The medium should be sterilized at 151 °C for 15 minutes. After sterilization, 0.5% lactose and 0.004% purple bromocrocellulose are added to it and after cooling, it is divided into plates for culture medium. Half of the yeast extract concentration can be used to create less background color in negative colonies (Figure 1).

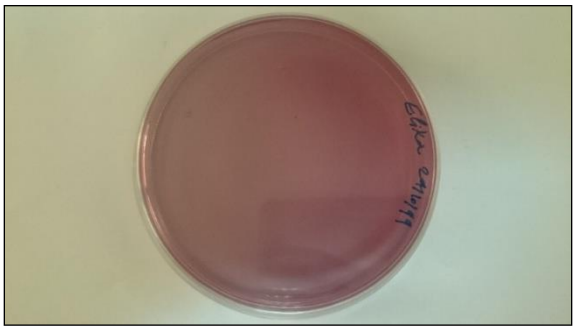

Figure 1: Elliker culture medium prepared according to Elliker culture medium instructions.

2. 7. Cell predisposition (*Lactococcus lactis*)

In order to prepare susceptible cells for the transfer of recombinant plasmids, Lactococcus lactis NZ3900 from the Biology Bank of Razi Vaccine and Serum Research Institute, Arak Central Region Branch, was used according to the following method. This process was performed based on the method of the German Institute of Molecular Biotechnology GmbH (2015).

First day: 5 ml of G-SGM17B culture medium was cultured using Lactococcus lactis NZ3900 stocks in freeze-80 °C of Razi Vaccine and Serum Research Institute and kept overnight at 30 °C without aeration.

Second day: 50 ml of G-SGM17B culture medium was cultured in a ratio of 1 to 100 from the previous culture medium (day one) and kept at 30c temperature overnight without aeration.

Third day: To the 50 ml of fully grown crop on the second day, 400 ml of the new G-SGM17B medium was added and stored in a greenhouse at 30 °C until the optical density reached 0.2 to 0.3. The bacterial mass was then precipitated using a centrifuge at 6000 g at 4 °C. The precipitated cells were washed with 400 cc of washing solution and re-precipitated using a centrifuge at 6000 rpm. The precipitated cells were re-dissolved using the suspension solution, kept on ice for 15 minutes, and pelleted again using a centrifuge. The washing operation was repeated using 100 ml of washing solution and the cells were re-precipitated using centrifugation. Finally, the resulting plates were dissolved again in 4 ml of washing solution and stored in 1.5 ml stock vials for later use at -80 °C.

2. 8. Preparation and extraction of plasmid pNZ8149

Lactococcus lactis NZ3900 stocks containing plasmid pNZ8149 were obtained from the Pasteur Vaccine Research Institute. In order to prepare plasmid pNZ8149 for the preparation of recombinant plasmid 2 falcons containing 5ml of G-SGM17B culture medium was contaminated with a strain prepared from Pasteur Institute and kept at 30 °C overnight. A fresh culture with a ratio of 1 to 100 was prepared from the previous culture with 200 ml of new culture medium. When the optical density of the culture medium reached 0.2 to 0.3, the bacterial mass was precipitated using a centrifuge and the resulting cells were used to extract the plasmid as a sample. Plasmid extraction was performed using QIAGEN Endo Free Plasmid Maxi extraction kit with catalog number 12362 according to the kit manufacturer's instructions.

2. 9. Preparation of PCR products of OMP31 gene

Design and manufacture of primers:

The primers were designed and ordered for synthesis according to the sequence of Brucella bacterium and OMP31 gene and the addition of cleavage sites for NcoI (go) and XbaI (return) using primer 6 software. Table 1 shows the primers sequences:

Table 1. The primers sequences.

Forward Primer:
CTGCGGTTTTAATGCCTAAAGTA eneG
CCATGG: oCN1
Anchor: TGA
Forward Primer Sequence:
5′-TACCATGGATATGAAATCCGTAATTTTGGCGTC-3′
TM: 74/7 Melting Point
Reverse Primer:
Xba1: TCTAGA
Tag: GTA GTA GTA GTA GTG GTA
Reverse Primer Sequence:
5′-CATTCTAGA GTA GTA GTA GTA GTG GTAGAA CTT GTA GTT CAG ACC GA-3′
TM: 71/9 ºC Melting Point

2. 10. Polymerase chain reaction (PCR):

The polymerase chain reaction was performed using specific primers designed to amplify the OMP31 gene with the following reaction conditions and volume (Tables 2 and 3).

Table 2. Polymerase chain reaction conditions for OMP31 gene amplification

Step	Number of Cycles	Time (Minutes)	Temperature
Denaturation	-	20	95
Annealing	×35	1	95
		1	59
		1	72
Extension/Elongation		10	72
		5	4

Table 3. Volume of polymerase chain reaction to amplify OMP31 gene.

Materials	Volume(µl)
Master Mix contains single polymerase enzyme	25
Nuclease-free water	22
Forward primer	1
Reverse primer	1
Sample (Brucella bacteria cultured from culture)	1
Total volume	50

2. 11. Enzymatic digestion of plasmids and polymerase chain reaction products

Due to the presence of the NcoI and XbaI enzymes in the genomic map of the circular plasmid pNZ8149 and in order to create a linear plasmid with a sticky end, an enzymatic digestion reaction was performed for the plasmids extracted using NcoI (ThremoScientific, # ER057) and XbaI (Thremo Scientific # ER0681) was performed with the following conditions and volume of reactions (Table 4). After the digestion reaction, the quantity and quality of the digested (linear) plasmid were evaluated using a nanodrop device and gel electrophoresis for use in subsequent reactions.

Table 4. Digestive reaction volume for plasmid digestion using two enzymes NcoI and XbaI.

Materials	Volume(µl)
NcoI Digestive Enzyme (Thremo Scientific, # ER0571 (long Action, 1-6h)	2/5
Digestive enzyme XbaI (XbaI, Thremo Scientific # ER0681 (long Action, 1-16)	2/5
Tango Buffer (Tango Buffer, Thremo Scientific)	25
Nuclease-free water	25
Sample (plasmid pNZ8149) extracted from step 3-13	5
Total	60

Also, to create an OMP31 gene fragment with a sticky end on both sides (′5 and ′3) that can bind to both sides of the plasmid (′5 and ′5); an enzymatic digestion reaction was also performed for the produced polymerase chain products. NcoI and XbaI enzymes with the conditions and volume of reactions were performed as described in the table below (Table 5). After the digestion reaction, the quantity and quality of the digested PCR products were evaluated using a nanodrop device and gel electrophoresis for use in subsequent reactions.

Table 5. Digestive reaction volume for digestion of PCR products using NcoI and XbaI enzymes.

Materials	Volume (µl)
NcoI Digestive Enzyme (Thremo Scientific, # ER0571 (long Action, 1-6h)	2
Digestive enzyme XbaI (XbaI, Thremo Scientific # ER0681 (long Action, 1-16)	2
Tango Buffer (Tango Buffer, Thremo Scientific)	6
Nuclease-free water	38
Samples (PCR products) produced	3
Total	51

2. 12. Purification of polymerase chain reaction products and linear plasmids

The products of the gel plasmid digestion reaction were electrophoresed and the resulting 2500 linear plasmid bands were removed from the gel and purified using a purification kit. The polymerase chain reaction products were also purified using a kit according to the kit instructions for use in subsequent reactions. The quantity and quality of purified plasmids and PCR products were evaluated before further use using nanodrop and gel electrophoresis.

2. 13. Binding reaction of OMP31 gene and linear plasmid

The OMP31 gene was produced, digested and purified in the previous steps to linear plasmid pNZ8149 digested using NcoI and XbaI enzymes and purified to form a recombinant cyclic plasmid at the site of plasmid cloning site using T4 (Thermo scientific) enzyme. Reaction conditions, volume of materials used in the reaction and their concentration are as follows (Table 6).

Table 6. Conditions and volume of the binding reaction of OMP31 to the linear plasmid pNZ8149.

Material	concentration	volume
Linear Plasmid - Dena Linear Vector	113ng/µ	1
Insert DNA fragment (purified OMP31 gene fragment)	45ng/µ	11
T4 enzyme (Thermo Scientific Cat No: # EL0011)	5Weiss U/µ	1/5
Tango Buffer	10X	2
Nuclease-free water	-	3/5
Total volume	-	20

After mixing the ingredients, it was kept at 22 °C for one hour and then kept in the refrigerator (4 °C – 8 °C) overnight.

2. 14. Electroporation

In order to transfer the recombinant vectors to susceptible cells, 40 µl of the prepared susceptible cells was poured into a cool electroporation jar (refrigerated before use) and 1 µl (50-100 ng) of the recombinant vectors pNZ8149, Was added to it. Bio-Rad Genepulser device with the following settings was used to create pores in cells prone to recombinant vectors:

Voltage: 2000 volts - Electrical potential: 25 microfarads - Resistance: 2000 ohms and 4.5 to 5 seconds shock. After shock 1, it was transferred to 1.5 ml micro tubes and 1 ml of the recovery solution was added to it and kept in an incubator at 30 °C for 1.5 hours. After incubation time, 10, 100 and 1000 macro liters of electroporated medium were cultured in culture medium prepared from M17 with lactose (L-SGM17B).

2. 15. Selection of recombinant lactococci

Elliker culture medium was used to select recombinant lactococci (lactococci containing recombinant plasmid pNZ8149 + OMP31). As mentioned, Elliker is a special culture medium that can be used to select *lactococcus lactis* colonies Lac^+. In this rich culture medium, all cells can grow, both lactose-positive and lactose-negative, but when lactose is added as the sole carbon source, the cells that undergo lactose fermentation are colonized. They produce yellow spots. In order to select and separate the recombinant bacteria from the culture medium and the bacteria grown in the previous section, 100 macro liters were cultured in each plate of Elliker-agar medium and kept in an incubator overnight at 30 °C. Colored cultures were prepared from yellow colonies on cultured plates.

2. 16. Confirmatory tests to confirm cloning results

Cultures prepared from recombinant clones were precipitated using a centrifuge and the resulting plates were used as a sample for confirmatory tests. Also, a new culture with a ratio of 1 to 100 was prepared from the previous culture with 200 ml of new culture medium. When the optical density of the culture medium reached 0.2 to 0.3, the bacterial mass was precipitated using a centrifuge and the resulting cells were used to extract the plasmid as a sample. Plasmid extraction was performed using QIAGEN Endo Free Plasmid Maxi extraction kit with catalog number 12362 according to the kit manufacturer's instructions.

Finally, the following confirmatory tests were performed to confirm the presence of recombinant plasmid and also to confirm cloning:

Digestion reaction of recombinant plasmid with NcoI-Digestion reaction of recombinant plasmid, and XbaI-Digestion reaction of plasmid-Recombinant with NcoI and XbaI - Polymerase chain reaction of recombinant plasmids was done using specific primers OMP31.

In order to analyze the data, confirmatory tests were performed to ensure the correct cloning, which indicates the correct placement of the gene in the vector. Vector sequencing after recombination also confirmed the results of the initial tests.

3 Results and Discussions

3. 1. Plasmid extraction

The results of evaluations performed on the extracted plasmid showed that *Lactococcus lactis* strains containing plasmid pNZ8149 were prepared from Pasteur Institute and after culturing using bacterial deposition and according to the instructions of the extraction kit, the plasmid was successfully extracted. And the presence of plasmid pNZ8149 was confirmed. The quantity and quality of the extracted plasmid evaluated using the nanodrop device was reported to be

123.8 ng / µl and purity of 1.93, respectively (Figure 2).

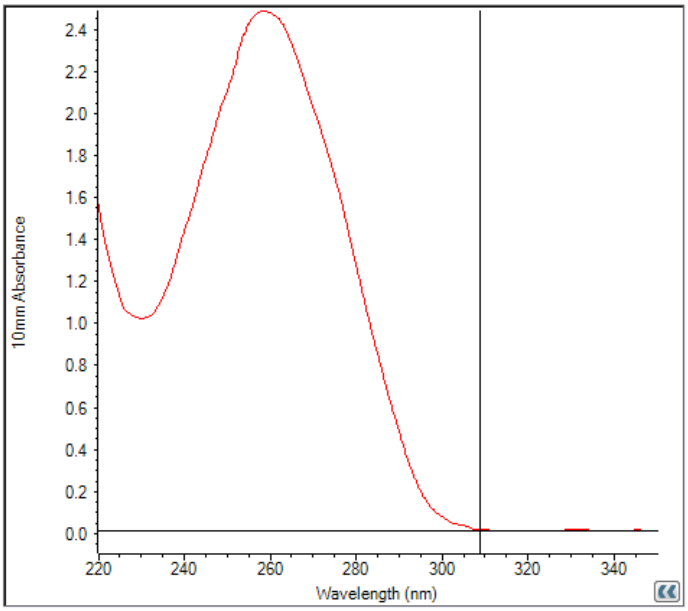

Figure 2: Quantitative and qualitative evaluation of the extracted plasmid pNZ8149.

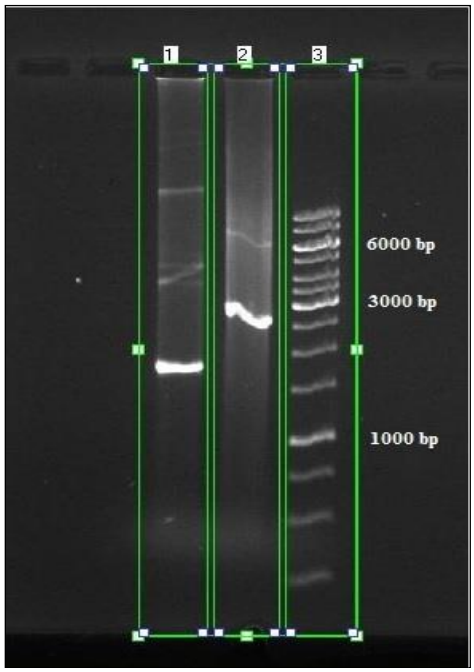

Figure 3: Results of extracted plasmid gel electrophoresis. [The first column of the undigested plasmid is pNZ8149, which has three super coils, annular and linear plasmid bands. Second column of digested plasmid pNZ8149 using NcoI and XbaI enzymes. Third column of 1 kb DNA marker of Fermatas brand, concentration 1 ng /µl (1.5 macro liters)].

3. 2. Production of gene PCR products from OMP31

The results of designing specific primers for amplification of OMP31 gene of this target fragment of Brucella bacterium genome during the mentioned conditions and with determined

volumes showed that the quantity and quality of the amplified fragment was suitable for use in later stages and was successfully amplified. This product has a concentration of 487.2 ng/µl and its purity (absorption ratio at 260 wavelength compared to absorption ratio at 28 wavelength) was reported to be 1.49 (Figure 3).

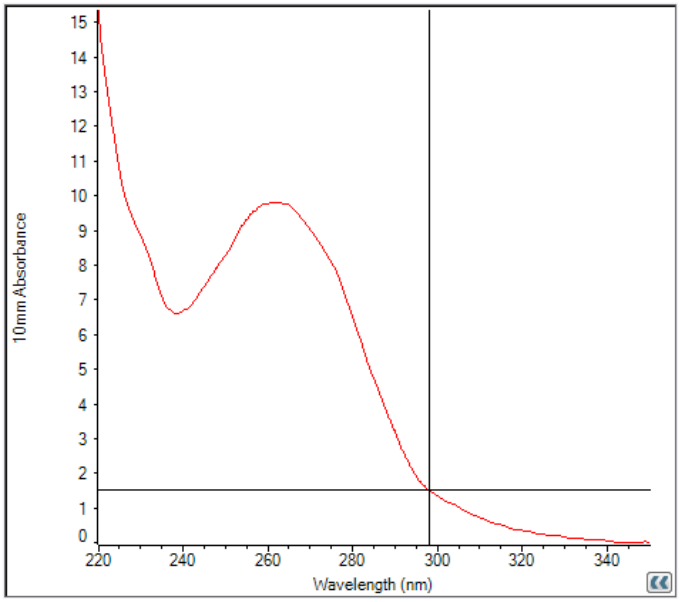

Figure 4: Quantitative and qualitative evaluation of OMP31 gene PCR products.

According to the fact that the PCR product size of the OMP31 gene should be 723 bp, the fragment was amplified correctly (Figure 5).

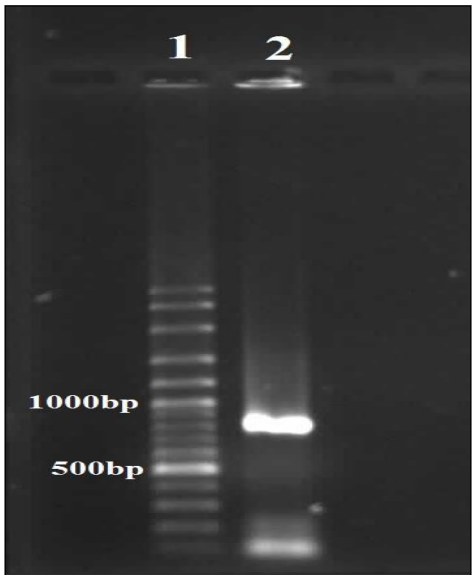

Figure 5: Gel electrophoresis image of PCR products of OMP31 gene. [Column 1: Vivantis bp100 DNA marker concentration 1 ng /µl (1.5 macro liters). Column 2: OMP31 gene PCR products (2 macro liters)].

3. 3. Enzymatic digestion and purification of plasmid pNZ8149 and PCR products of OMP31

gene

Plasmid pNZ8149 has the cleavage site of NcoI and XbaI enzymes at its cloning site. The primers were also designed to amplify the OMP31 gene by adding the NcoI and XbaI cleavage sites on both sides of the fragment. Therefore, in order to insert the pure fragment of OMP31 gene into the plasmid and perform the binding reaction and generate the recombinant plasmid, the plasmid and PCR products need to be purified and of good quality. Therefore, the produced PCR products as well as the extracted plasmid were fermented using NcoI and XbaI enzymes and digested with the mentioned conditions and volumes. These products were then purified using a kit (specifications) and the results of the quantity and quality of plasmid and purified PCR products were mentioned (Table 7 and Figure 6).

Table 7. Quantitative and qualitative evaluation results of digested and purified products of PCR and plasmid pNZ8149.

Product	Quantity (concentration)	Purity (Wavelength absorption ratio 260/ Wave absorption ratio 280)
Purified plasmid	123/8	1/93
Purified PCR products	45	2

Figure 6: Results of gel electrophoresis of purified pNZ8149 linear plasmid products and PCR products of OMP31 gene. [First column: Fermatas brand kb1DNA marker concentration 1 ng /µl (1.5 macro liters). Column 2: Linearly purified plasmid pNZ8149 after enzymatic digestion reaction (2 macro liters). Column 3: Purified product Digestion of PCR products of OMP31 gene (2 macro liters)].

3. 4. Binding reaction of OMP31 gene fragment and linear plasmid pNZ8149

The results of the quantity and quality of the purified products as well as the instructions of the T4 enzyme used (Thermo Scientific) were combined with the mentioned conditions and volumes. Also, 2 ng/µl of the gel reaction was electrophoresed and the results of gel electrophoresis of the binding reaction product were shown in the following figure (Figure 7).

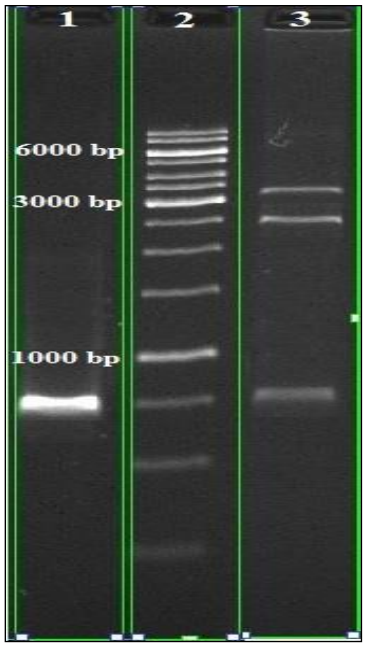

Figure 7: Results of gel electrophoresis of binding reaction products and PCR after recombinant bond binding reaction. [Column 1: PCR product of recombinant plasmid after ligation reaction (2 µl). Column 2: Fermatas brand kb1 DNA marker concentration 1 ng /(l (1.5 macro liters). Column 3: Bonding reaction product (2 marker liters)].

According to the results of gel electrophoresis of the third column, the result of 2l2 electrophoresis is the bonding products in three sizes: bp723, bp2550 and bp3273, which are related to: PCR product of OMP31 gene, linear plasmid pNZ8149 and recombinant plasmid (bp2550 + bp723) From the connection reaction. Also, the formation of a third band in the size of about 300 bp3 indicates and confirms the correct performance of the binding reaction, which, of course, must be done by using the polymerase chain reaction of the product from the said band on the gel, as well as sequencing PCR products and other tests. To be finally approved.

3. 5. Additional tests to confirm the recombination of plasmid pNZ8149

In order to confirm the bonding reaction of the resulting band on the electrophoresis gel, which was 3300 in size, it was separated using a razor blade and placed in a microtubule. After purifying the product from the gel, the polymerase chain reaction was performed using specific primers of the OMP31 gene, the results of which were displayed in a single column after gel electrophoresis. The formation of a favorable band in the size of 723 bp indicates the presence of a pattern in the harvested product. Also, a sample of purified products was sent to Knowledge-based Company for sequencing.

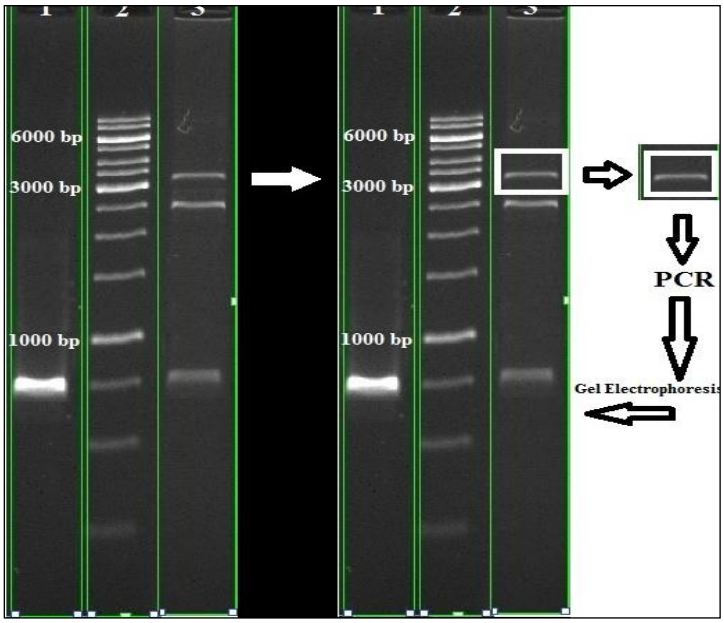

Figure 8: PCR of product harvested from gel electrophoresis of bonding reaction products. [Column 1: PCR product of recombinant plasmid after ligation reaction (2 µl). Column 2: Fermatas brand kb1 DNA marker concentration 1 ng/µl (1.5 macro liters). Column 3: Bonding reaction product (2 marker liters)].

3. 6. Selection of recombinant colonies after electroporation

Elliker culture medium was used to select recombinant lactococci (lactococci containing recombinant plasmid pNZ8149 + OMP31). Also, selection and isolation of recombinant bacteria from culture medium and grown bacteria 100 µ were cultured in each plate of Elliker-Agar medium and kept in an incubator overnight at 30 °C. The growth results of bacteria grown in Elliker-Agar culture medium are shown in the figure below (Figure 9).

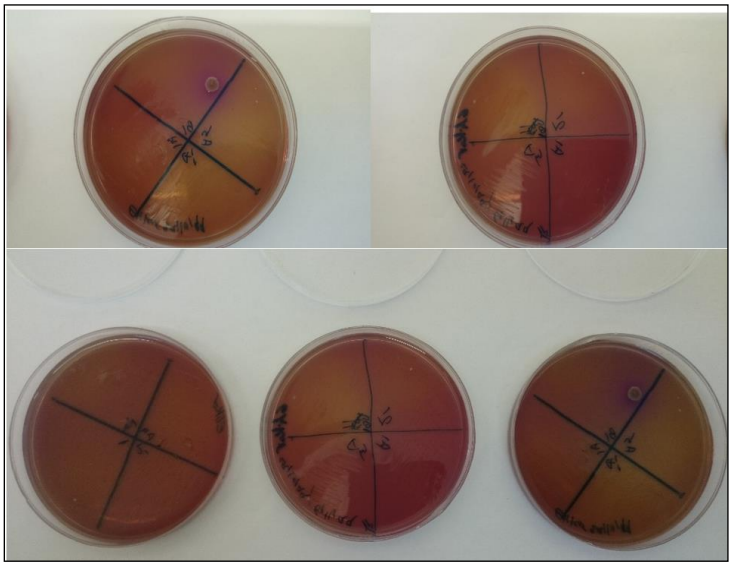

Figure 9: Elliker Agar culture medium after contamination with bacteria electrophoresed with recombinant plasmid.

Depending on the shape in parts of the culture medium where bacteria grew with the

recombinant plasmid; the culture medium is yellow. Colonies grown in these areas were re-cultured in L-SGM17 medium and after growth; they were stocked with glycerol and DMSO and stored at -80 °C for further studies of gene expression.

The results of this study indicate that the quantity and quality of plasmid pnz8149 extracted using nanodrop device were evaluated at 123.8 ng/ μl and purity of 1.93. Also, for amplification of OMP31 gene by designing specific primers, due to specific conditions and volume with a length of 723 bp, its amplification was successful. Finally, OMP31 gene insertion in pnz8149 vector was performed correctly and its cloning accuracy through PCR, enzymatic digestion and sequencing confirmed the presence of recombinant plasmid pNZ8149 + OMP31 in Lactococcus lactis.

Studies have shown that brucellosis is a chronic and debilitating disease that affects various organs and has many negative consequences in the field of health and economy in Iran (Spickler, 2018) and also creating sustainable safety and screening in livestock are the main strategies to control and prevent this disease (Bax et al., 2007). Outer membrane proteins, especially the two proteins OMP28 and OMP31, are important in vaccine design as well as in the production of antigens required for serum diagnosis. It is important to produce safe recombinant proteins that have good immunogenicity and can also be produced economically. Choosing the right expression system as well as the right host to express these recombinant proteins that can be produced safely, massively and economically is one of the next priorities in the production of vaccines and antigens used. In this regard, the present study shows that the nisin-controlled gene expression system developed by the NIZO Food Research Institute is an expression system that is easy to implement and has many benefits. This system leads to high expression of homologous and heterologous genes in studies of gene function and is suitable for obtaining a large amount of the product of a gene in the human food class as well as the feed class used in livestock. It is also used in metabolic engineering studies. Other advantages include the expression of membrane proteins in eukaryotes and prokaryotes (Kunji et al., 2003). The study was performed considering the importance of brucellosis and the vital role of Brucella melitensis bacterial membrane protein in immunization in livestock and eradication and eventual eradication of the disease as well as the unique benefits of the controllable expression system of nisin cloning gene encoding OMP31 protein. The vector pNZ8149 was targeted by Lactococcus lactis strain NZ3900 and the expression of this protein was targeted using the nisin expression system (Cassataro et al., 2007). Also, this study was designed and performed only in the first part, i.e. replication, cloning and placement of genes in plasmids. As detailed, the OMP31 gene fragment was designed by specific primers for amplification, enzymatic digestion, and purification, and its binding reaction was performed with pNZ8149 plasmids, which were linearized by enzymatic digestion at the NcoI and XbaI cleavage sites. Confirmation tests were performed to ensure that the cloning was performed correctly, indicating the correct placement of the gene in the vector. Vector sequencing after recombination also confirmed the results of the initial tests (Figure 10).

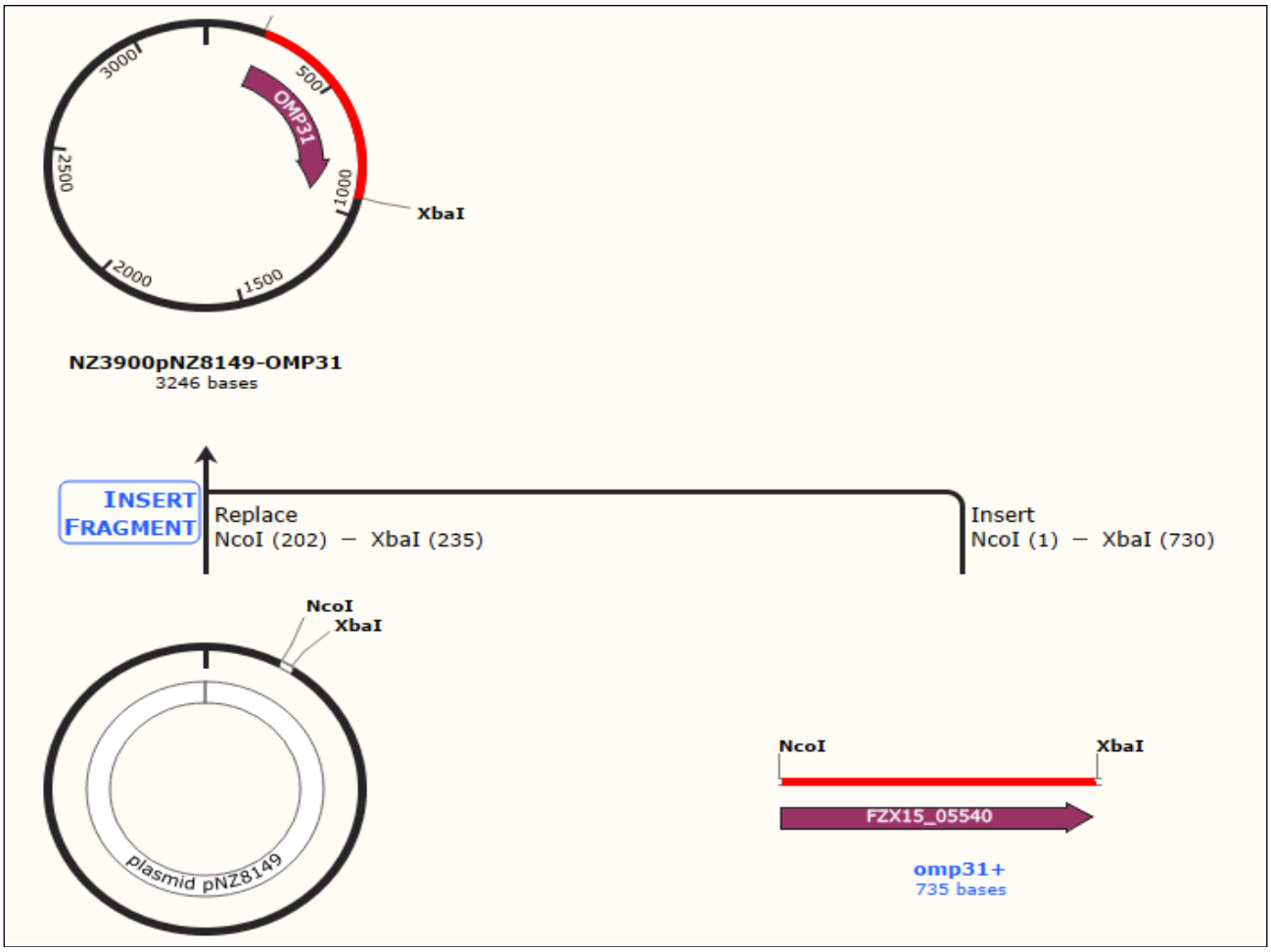

Figure 10: Graphic summary of the design and implementation of OMP31 gene cloning in plasmid pNZ8149.

Also, the recombinant vector was introduced to *Lactococcus lactis* NZ3900 using electroporation and the recombinant strain was kept at -80 °C for further study. This recombinant strain is a food and nutrient class that has the potential to express OMP31 protein into the intercellular space by secretory sound due to its nisin expression system (Figure 11).

Figure 11: OMP31 gene sequence inside pNZ8149 vector and part of vector sequence on both sides of gene.

Although the expression function of OMP31 protein, its amount and purity need to be tested in further studies to reveal its mechanism. This study was performed on cloning the gene encoding OMP31 protein in pNZ8149 vector and inserting it into *Lactococcus lactis* NZ3900. The safety of recombinant proteins that have good immunogenicity and can also be produced economically has limitations. The researchers suggest that considering the outer membrane proteins, especially the two proteins OMP28, OMP31 in vaccine design and also the production of antigens required in serum diagnostics by isolating and amplifying the Brucella OMP31 gene and transferring it to an expression vector in the continuation of this study. Selection of an

appropriate expression system as well as a suitable host for the expression of these recombinant proteins that can be produced safely, massively and economically; One of the next priorities is the production of vaccines and antigens used.

4 Conclusion

Overall, the findings of this study showed that due to the importance of brucellosis and the essential role of the membrane protein of *Brucella melitensis* in immunization in livestock and eradication and eventual eradication of the disease, as well as the unique benefits of a controllable expression system Cloning of the gene encoding OMP31 protein in vector pNZ8149 was targeted by *Lactococcus lactis* strain NZ3900 and expression of this protein was targeted using nisin expression system. In this study, only the first part of gene replication, cloning and placement in plasmid was designed and performed. The results of this research can be used in the fields of health, immunization, commercial vaccines and economics.

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Conflict of interests

The authors have no conflicts of interests to declare.

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