The Journal of Animal & Plant Sciences, 29(5): 2019, Page: 1254-1261
ISSN: 1018-7081

CONSTRUCTION OF RECOMBINANT *LACTOBACILLUS CASEI* EXPRESSING *AEROMONAS VERONII* OMPF PROTEIN GENE

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ABSTRACT

Aeromonas veronii infection is significant common worldwide exists in aquatic environment, closely related to haemorrhagic septicaemia, severe skin and muscles tissues ulcers with high mortality. Therefore an effective and safe technique to eradicate *Aeromonas veronii* infection is urgently required. In this study we built-up straightforward system to construct the live recombinant lactobacillus oral OmpF vaccine candidate against newly isolated *A. veronii* strain TH0426. One protein of interest OmpF (1038bp) was expressed in secreting prokaryotic shuttle expressing plasmid pPG612.1. We identified strong expressions of novel recombinant plasmid by using extraction of plasmid, digestion by enzymes, PCR, immunoblot and sequence analysis. The recombinant plasmid pPG612.1-OmpF was transformed onto the *Lactobacillus casei* CC16 by electroporation, and developed the recombinant strain on *L.casei*-pPG612/OmpF. The results show OmpF gene had successfully inserted onto the expression recombinant vectors and achieved recombinant *Lactobacillus* systems.

Keywords: *Aeromonas veronii*, OmpF, gene cloning, prokaryotic expression, recombinant *Lactobacillus casei*

INTRODUCTION

Aeromonas veronii infections are the most common immerging bacterial disease causes heavy losses in aquaculture, fresh water fish farming as well as ornamental fish farming industry (Aijun Lu *et al*. (2016). *A. veronii* clinical manifestations of infection includes heamorrhagicsepticaemia, severe skin and muscles ulcers, necrosis and sudden elevation of high mortality (Jingjing *et al*., (2016); Webb *et al*., (2015) & Bhuyan *et al*., 2018). It also causes severe infection in human, dairy animals Austin DA. (1999). It is therefore considered as a foremost economic barrier between fish farming and profitable production of fish industry (Webb *et al*., 2015).

A. veronii virulence factors includes enterotoxins, adhesis, lipase, aerolysin, invasins, phospholipase and extracellular serine protease, temperature-sensitive protease, aerolysin, collagenase, elastase, cytotoxin, enterotoxins, glycerophospholipidslipo polysaccharide and bundle-forming pilus (Senderovich *et al.* 2012; Yi *et al.,* 2013) ; Beaz-Hidalgo R and Figeuras*,* 2013).

The frequent application of bactericidal additives are result to the least amount of success rate, it develop resistance gene to many group with inescapable side effects in aquaculture, vaccination is still questionable but it can be consider as most safe, essential and effective tool to avoid a range of diseases. Many bacteria are now seems resistances against several of antibiotics, because of excessive use during infectious diseases (Thompson and Adams 2004). The production and development of ideal vaccine is not only influenced by a variety of immunological factors, such as type perfect antigen but also by competent composition and delivery factors.

The gram-negative microorganisms outer membrane proteins (Omp) serve important part in the virulence, due to their positioned on the outermost layer, this make easy to contact to cells of the host, and ideal to involve in stimulation of several defense factors of immunity (Ebanks *et al*., 2005).

Outer membrane protein possesses high attentions, because it is now identified as ideal antigen for recombinant vaccines, it has absolute abilities to develop immune responses of host and use for drug remedy (Khushiramani 2008). From recent few years Gram-positive, noninvasive, food-grade bacterium *Lactobacillus* strains are use as vector to transport immunogenic recombinant proteins.

Lactobacillus strains have excellent possessions which makes these microorganism prominent attractive candidates as safe oral vaccines antigens delivery carriers. It induces a non specificimmuno adjuvant effects and has capabilities to stay exist and colonize in the epithelial mucosal cells, respiratory tract, intestinal tract, and urogenital tract to induce a strong mucosal immune behavior (Xinpeng *et al.,* 2016), and induce the non-specific immunoadjuvant effects (Alander *et al*., 1999, Lee *et al*; 2004). *Aeromonas veronii* novel strain TH0426 was selected in this study, it was previously isolated from "*Pelteobagrusfulvidraco*" "Zhejiang Institute of Freshwater Fisheries" Zhongguan village, China, its entire genome sequence and information was documented in our previous work (Kang *et al*., 2016).

In this study OmpF of *A veronii* TH0426 is used as antigen and cloned onto structural polypeptide the external milieu cell surface using pPG612.1 plasmid, *Lactobacillus casei CC16* was employed as vector for an antigen-delivery. The outcome of this research we successfully constructed a recombinant *lactobacillus* recombinant plasmid that expresses *A veronii* TH0426 OmpF gene and can be employ vaccine candidate against pathogenic *A. veronii*.

MATERIALS AND METHODS

Materials: The DNA genome extraction was performed by TIANGEN" Genomic DNA Kit (Biotech Beijing), for separation of the plasmid and (Pure Plasmid Mini kit CW BIO) was used. Primers synthesis and DNA sequencing were carried out by Shanghai Sangon Biotechnology Company China. Protein molecular weight (MW) markers were obtained from (CW BIO). BamHI and XhoI TaKaRa Biotechnology Co., Ltd. (Dalian, China) and were used following to the manufacturers' instructions, sodium dodecyl sulphate-polyacrylamide gel (SDS) preparation kit was purchased from (Sigma-Aldrich). 5xSDS-PAGE sample Loading Buffer (nzy tech Lisboa. Portugal). Coomassie brilliant blue G-250 was used (BIO-RAD) for immunoblot process. Mouse anti-*Carassiusauratus* polyclonal antibodies (pAb) was developed before this study start in the molecular laboratory of Jilin Agriculture University China, and stored -80 Celsius (˚C) in our laboratory. Standard super optimal broth (SOC medium) purchased commercially (SIGMA-ALDRICH). Chloramphenicol (Cm) (Sigma, USA) was utilized at final concentration of 10 µg/ml. Luria–Bertani (LB) and Mann Rogosa Sharpe (MRS) agar and broth routinely prepared in our lab. Analysis of sequences was acted by using NCBI servers (www. ncbi. nlm. nih. gov/BLAST), and by Primer Premier Program.

Bacterial Strains and growth conditions: The newly isolated *Aeromonas veronii* strain TH0426 used in present study is deposited in MCCC (Marine Culture Col-lection of China) as MCCC 1K02718. GenBank Accession No: CP012504 (Kang *et al*., 2016). It was preserved in Luria Bertani Broth (LB) with 20% glycerol at -80˚C. After thawing, the bacteria was cultured on LB agar plate, and kept for incubation at 37ºC for 16 hours aerobically. Once checking the purity, single colony was cultured in Luria broth and kept in shaker at 37ºC for 24 hours. DNA extraction was achieved following the instructions of supplied manual of "TIANGEN" deoxyribonucleic acid (DNA) extraction kit for DNA template, pMD18-T vector procured from TaKaRa Bio-Engineering Co., Ltd.; Taq, E. coli DH5α was procured by Sangon Biotech Co., Ltd. (Shanghai, China). *E. coli* cells of strain MC1061 were commercially purchased by (MoBiTec GmbH, Germany), the secreting prokaryotic shuttle expressing plasmid pPG612.1 was obtained from molecular laboratory Jilin Agriculture University. *Lactobacillus casei* CC16 used in this study was isolated previously from the gut of Carp fish and maintained in glycerol at -80˚C (FS 16S ribosomal RNA gene NCBI GenBank Accession No. KC404975.1).

Amplification of membrane protein F of *A veronii* TH0426: Specific primers were intended by Primers Premier 5.0 on the source of putative ompF sequence of *A. veronii* strain TH0426 (Uniport Accession No A0A0E2LBJ1_9GAMM). Amplification of ompF gene was carried out using *A. veronii* TH0426 genomic DNA, and gene specific forward (5′GGATCCCAAGACAACACCTGGTATG 3' containing a Bam HI site 3′CTCGAGGCTGACACCTTCGACTTTC 5' (reverse) containing an XhoI site (underlined).

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The PCR was performed in 25 µl reaction volume containing template DNA 1µl, each primer 1µl, dNTP (Promega) 2 µl, 10 x Ex Taq buffer (TaKaRa) 2.5 µl, Taq DNA polymerase (QIAGEN) 0.25 µl, ddH2O 17.25 µl. Each reaction was consist of 30 cycles using the following conditions; initial denaturing temperature 94°C/5 min; denaturing for 94°C during 1 min; annealing temp 60°C/1 min, 58.5°C/1 min elongation at 72°C/1min, the final extension was set at 72°C/10 min. Amplified PCR product was sprint in to 1% agarose gel for electrophoresis and scrutinized with GelDoc (Herolab GmbH Germany). The identified fragments were isolated from superfluous gel by using sterilized scalpel carefully to avoid extra part of gel. Purification of OmpF gene fragment has been done by following the direction of manual supplied by AXYGEN DNA gel extraction kit and analyzed using a gel documentation system.

Cloning, transformation and sequencing of OmpF gene: The construction of recombinant plasmid was carried out as described earlier by Yigang and Yijing (2007) with some modifications. The coding region of gene OmpF was effectively amplified from genomic DNA by primers targeted to the coding region. Double digestion of the PCR amplicons has been performed directly by system: purified PCR product10 µl, 10 x buffer (K) (Promega) 5.0, BamHI 2.0µl and XhoI 2.0µl

and ddH_2O 31 µl in ThermoQ (ER-CHB-T1) dry bath for 6 h at 37°C. The digested PCR amplified product was ligated into commercial pMD-18T vector. Reaction mixture was prepared for (10µl), pMD18T-vector 2.0 µl, SolutionI 5.0 µl (Takara Biotech), digested PCR product 1.0µl, ddH_20 2.0µl, and was managed at 16°C for 8 hours. The digested fragments and the vector were further profiled on agarose gel electrophoresis, further it was purified to identify obtained aim gene fragment. The ligated OmpF purified fragment was transformed onto the competent cells *E. coli* DH5α by gradually mixing and placed in ice for 40 min, further it was followed by heated in water bath at 42°C for 90 seconds and immediately placed in ice for 3 min. The cells were developed in LB broth and kept in shaking incubator on 37°C for 3 hours, after incubation the cells are centrifuged at 4500 rpm for 15 min at 4 °C.

The obtained transformed recombinants cells were propagated on LB agar supplemented with Ampicilline (200µg/mL) further it was incubated at 37°C for 12 hours. After incubation white uniformed colonies are suspected possess the recombinant plasmid. Single pale colonies were separately cultured in LB liquid medium with addition of Ampicilline (200µg/mL). After getting growth, recombinant plasmid extraction was carried out by supplied manual of plasmid extraction kit, whereas it was further confirmed by PCR analysis.

The positive fresh clones were also profiled to electrophoresis and were confirmed through sequencing by Sangon Biotech Company.

The identity of Outer membrane protein F (OmpF) nucleotide and amino acid sequences were scrutinized with National Center for Biotechnology Information (NCBI) Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Secretory expression Plasmids, DNA manipulation and connection with OmpF gene: The cloning and nucleic acid manipulation of secretory expression plasmid pPG612.1 was achieved by Plasmid extraction, digestion with restricted enzymes and purification as described before. The target bands were examined by electrophoresis. Furthermore extraction of plasmid and connection of purified pPG612.1 with pMD18-T-OmpF plasmid has been conducted in same manner as discussed previously. PCR has been performed for confirmation of recombinant plasmid.

Preparation of *E.coli* MC1061were performed as described earlier by Sambrook*et al*., (2001), the competent MC1061 cells were culture on L.B plate supplemented by 34 mg/ml Cm and incubated overnight at 37°C. The single white pale colonies were collected and further cultured in 10 mL LB broth with addition of 20 µg/ml Cm, and allowed to grow at 37^oC (300 rpm), until to get $OD_{600}$for 3 hours.

The cell culture was dispensed onto centrifuged tube (Nunc 200mL), and kept in ice for 20 minutes, further it was centrifuged at 4000 rpm for 15 min at 4^oC. Subsequent resuspensions had been performed in the same tube. The cells were dispensed in 30 mL of chilled 0.1M Calcium Chloride ($CaCl_2$) and carefully mixed with pipette. The suspended cells were then transfer into 50 mL falcon tubes, and held on ice for 40 minutes, further it was centrifuged in same settings.

The cells were aseptically shifted in another sterilized tube containing 8 mL of pre-chilled 0.1M CaCl2 supplemented with glycerol (15%), it was slowly mixed by pipette, approximately 140 µL dispensed into the sterilized tubes and again placed on ice. The cells were chilled in liquid nitrogen for 10 min. E. coli MC1061 100µL of cells was dispensed into a pre-chilled 15 mL Falcon tubes. Recombinant pPG612.1-OmpF 5µL was added onto the cells and swirl the tube to evenly homogenize the DNA and cells. The recombinant pPG612.1-OmpF cells lysates and control tubes was maintained on ice for 30 minutes, warm therapy has been fixed by placing cells on water bath at 42 ºC for 90 seconds. Instantly took out and exposed the cells to ice for 2 minutes. The cells were propagated with Super Optimal Broth (SOC) medium (900 µL) and kept for vigorous shaking at 37 ºC for 1hour. The cells were dense by centrifugation for 15 minutes with the same system and again resuspended in 200µL of sterilized SOC medium. The harvested cells were propagated on L.B plates for positive and negative control, containing Cm 20 µg/ml. The obtain cells was incubated at 37 ºC for 18 hours.

The positive clones of recombinant plasmid were further cultured in 50 ml of LB broth containing Cm (Sigma) 20 µg/ml, and incubated for 18 hours. The extraction of has been followed by the instructions provided in manual CW BIO Mini plasmid kit. The aspect of correct constriction of pPG612.1-OmpF and its successful transformation was authenticated by sequencing through Sangon Biotech Company. *L.casei* CC16 was grown in MRS broth by adding glucose 0.5% (w/v) anaerobically, the obtained growth was further cultured in MRS liquid and incubated for 3 hours; OD was 600 = 0.6-0.8, the cells were then centrifuged with low temperature 4ºC at 4000× rpm. Frequent twice washing of the cells done with pre-chilled 50 mL Ethylenediamine Pyrocatechol buffer (EPWB) buffer and at once with ice-cold 50 mL EPB solution. The cells were homogenized with 500µl ice-cold electroporation buffer.

Transformation of *L. casei* CC16 with recombinant pPG612.1-OmpF: The recombinant plasmid pPG612.1-OmpF was assorted with *Lactobacillus casei* CC16 cells for electroporation; the cells temperature was maintained below at -0 ºC for 5 min. The aggregated cells were aseptically transited onto 2 mm sterilized pre-cooled

Micro Pulser cuvette (Bio-Rad). Electroporation was performed by adjusting voltage at 2.5 kV and 5 milliseconds for pulse time and kept for incubation for 37 ºC anaerobically. After 2h of incubation the cells were propagated equally by glass spreader on MRS agar plates containing 10µg /mL Cm and incubated at 37 ºC anaerobic condition for 24 h. The solitary colonies were chosen from each plate and inoculated in MRS liquid medium supplemented with 10µg /mLCm, further incubated for 12 hours at 37 ºC without shaking. Recombinant plasmid was identified by PCR and double digestion analysis

Expression of recombinant *L casei*-pPG612.1-OmpF: For expression evaluation, recombinant *L casei*-pPG612.1/OmpF was cultured on MRS broth with adding Cm (10 µg/ml) and glucose (2 %) at 37 °C overnight.

The obtained culture of *L casei*-pPG612.1/OmpF centrifuged at 4000 rpm for 15 min at 4°C to acquire the new recombinant cells. The cells are washed three times with sterile phosphate-buffer saline (PBS, pH 7.4) washed cells were boiled at 100°C for 10 min and centrifuged at 4000× g for 10 min, whereas purified cell lysates of *A veronii* TH0426 has also been prepared in same method for analysis.

For immunoblot analysis *L casei*-pPG612.1/OmpF cell were centrifuged at 2300 × g for 10 min, approximately 15 µl of sample was loaded onto the wells of SDS-PAGE gel. The samples were run with molecular weight marker (CW BIO) for accurate analysis.

The gels were run between 20 and 45 min at 80 and 120 V for resolving and stacking gel respectively. The treated gel was stained for 12 hours by coomassie brilliant blue G-250. Finally gels were scanned obtained bands was determined using TotalLab v2002.03 software program (Nonlinear Dynamics Ltd).

Western blot analysis: To appraise the expressions of *L casei*-pPG612.1/OmpF, the cell lysates (40 µl) was assorted with 10 ml 5 x SDS sample loading buffer, and boiled at 100 ºC for 10 minutes. The obtained stewed cells of *L casei*-pPG612.1/OmpF were centrifuged with the system of 12,000 rpm for 10 minutes. The collected supernatant samples with same amount were dispensed onto the SDS-12% polyacrylamide gel for SDS electrophoresis process.

After completion of electrophoresis, the gel was saturated in transfer buffer 12 min to reduce the quantity of tris and glycine. PVDF filter transfer membrane was kept on shaking platform and dipped in 100% ethanol for 60 min. The authenticity of the induced proteins was verified by Western blot analysis with primary antibodies minute before using for further process. The gel was tightly close sandwiched between the several blotting papers and immune-Blot PVDF membrane (Bio-Rad), this sandwich was fixed into the blotting apparatus (BIO-RAD), electro-eluted run at 200 milliamperes (mA) for 1 hour in transfer buffer. Skimmed milk 5% (w/v) diluted in Tris Buffered Saline with Tween (TBST) was used to block the membrane. The membrane was further incubated and vigorously shakes with polyclonal antibodies (pAb) mice anti *A. veronii* antibody 1:50 diluted with Tris-buffered saline solution for 2 h at 25 ºC. The membrane was allowed to wash gently three times with TBS. Finally membrane was incubated with the second antibody (1:2000) HRP Goat-anti-mouse IgG (Bios, Beijing, China) for 1hour, then it was washed with 1 × TBST. The obtained blots were finally visualized by chemiluminecence detection with Western ECL substrate (Thermo Scientific) and Amersham Imager 600 (GE Healthcare, UK.)

RESULTS

A 1038 base pair (bp) fragment was amplified by PCR method using two specific primers (Fig.1 A). As it is shown in (Fig.1B) the gene fragment was obtained from the recombinant plasmids pMD18-T-OmpF and was digested by enzymes BamHI and XhoI. The fragments found about 1038 bp. It was further profiled by PCR and amplified gene fragment found 1038 bp. The length was consistent with the estimated size. This result confirmed that OmpF gene fragment effectively embedded onto the cloning vector pMD18-T (Fig 1C). The sequencing result also demonstrated the gene was 1038 bp, in line with what have expected in advance.

Cloning and transcription of recombinant plasmids pPG612.1 Omp F: The OmpF gene is successfully double digested with restriction enzymes, the length of plasmid was approximately found 2600bp, and about 1038bp gene fragments were obtained from enzymes digestion. PCR amplification obtained around 1038bp gene fragment. So pMD18-T-OmpF gene fragment was inserted into selected *Lactobacillus* expression vector pPG612.1 (Fig. 2).

Transformation and identification of constructed recombinant *L.casei* CC16 pPG612.1-OmpF: The recombinant plasmid pPG612.1-OmpF was successfully transformed into *L.casei*CC16. The obtained results shows the effectively construction of recombinant *Lac: casei*CC16 bacteria. The aim gene fragment 1038bp of *A. veronii* Omp F was obtained from PCR identification, further it was double digested by restricted enzymes BamHI and XhoI, in result about 1038bp of the target gene fragment and 2800bp of the expression vector fragment were obtained (Fig 3 A), Finally it was confirmed by PCR and sequences analysis (Fig. 3 B) this results indicates that the recombinant *Lac: casei* CC16 bacteria expressing the OmpF N terminus gene had been obtained in result of pPG612.1-OmpF/ *Lac: casei* CC16 constriction.

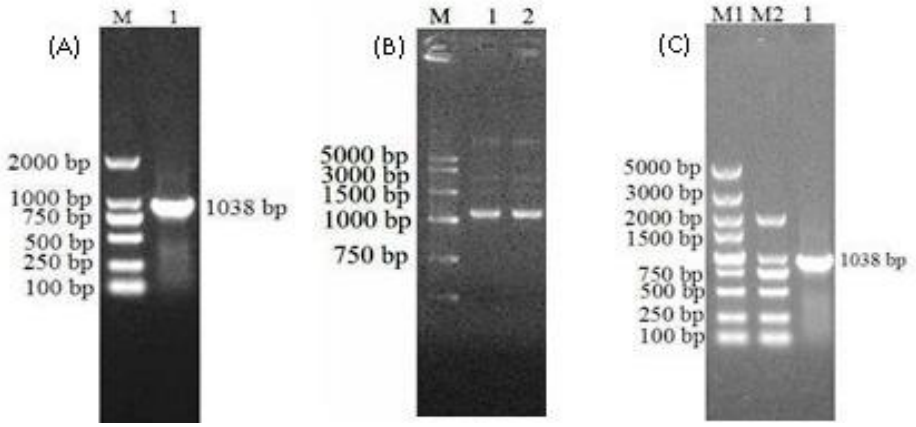

Fig1. (A): Identification of aim gene OmpF fragment after purification, M: DL2000, Lane 1 purified OmpF. (B): Identification of recombinant plasmid by PCR pMD-18T-OmpF. M: DL5000. Lane1, 2 pMD-18T-OmpF, (C): Identification of pMD-18T-OmpF by PCR, M1; DL5000, M2: DL2000, Lane 1 purified OmpF.

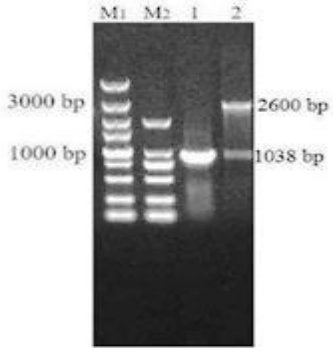

Fig. 2. M1 : DL5000; M2 : DL2000, lane 1: PCR product of plasmid pPG612.1-OmpF by PCR. Lane2: recombinant plasmid pPG612.1-OmpF digested with restriction enzymes BamHI and XhoI

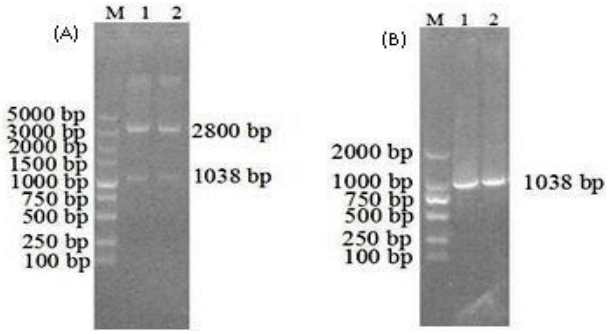

Fig 3.(A): Digestion of recombinant plasmid pPG612.1-OmpF after electroporated into *L.casei*CC16 M : DL5000 DNA Marker; 1-2: Digestion of recombinant plasmid; (B): PCR of recombinant plasmid pPG612.1-OmpF after electroporated into *L.casei* CC16.M DL2000 DNA Marker; 1-2: PCR product of recombinant plasmid.

Immunoblot Analysis: Overnight cultures of *L casei*-pPG612/OmpF grown in MRS broth supplemented with glucose. The cell lysates were analyzed by immuno-blot analysis. Staining by coomassie blue gel showed the clear appearance of a~45 kD fusion protein in the cell lysates of *L casei*-pPG612/OmpF induced by glucose (Fig. 4A). Western blot analysis showed that an immunoreactive band was detected as identical about 45 kD as observed in the SDS–PAGE (Fig. 4B). The results show that MW of the *L casei*-pPG612-OmpF was identical to the predicted value of purified Omps of *A. veronii* TH0426 previously identified; moreover glucose promoter from *L casei*CC16 can be used to competently induce the expression of *A. veronii* protein that was heterologous.

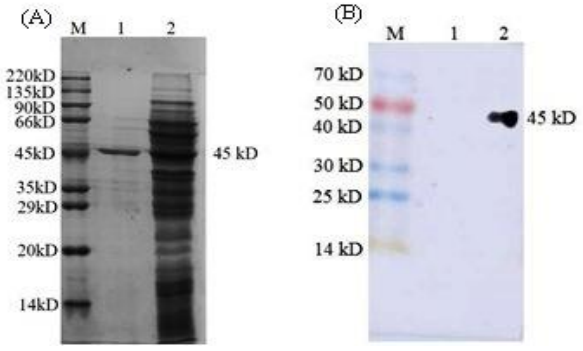

Fig.4 (A): A) SDS-PAGE Lane 1,2 Coomassie blue gel staining indicates the expression of KD ~45 MW fusion protein in cell lysates of *L casei*-pPG612/OmpFinduced by glucose, an immunoreactive band was observed. Lane3.4 Lane 3,4 an abundant for protein with same size (B).Western blot analysis of *L casei*-pPG612/OmpFM. Marker, lane 1, negative control lane 2, Expression in recombinant strain found identical 45 kD as seen in the SDS–PAGE.

M2 Western blot molecular mass markers expressed in kD.

DISCUSSION

Aeromonas veronii is now confirmed as to cause massive outbreaks in fish with heavy losses and high mortalities (Dong *et al.,* 2017). Several attempts are in progress for the development of fully protective vaccines against pathogen *A. veronii* still remains challenge. The gram negative bacteria outer membrane proteins seems as outstanding targets for the development subunit vaccines (Achouak *et al*., 2001)

In previous study OmpF had expressed high endpoint titers in *A. hydrophila*, in same work recombinant OmpF reported has ability of antigen for vaccine candidate. (Yadav *et al*., 2014). Thus perceptive the importance of OmpF as a putative adhesion and its potential was selected for suitable candidates for advanced recombinant vaccines against *A. veronii*.

Lactobacillus casei at the present has great attention and considered as an antigen delivery vector for recombinant mucosal vaccines (Dong *et al*., 2013, Bhuyan *et al*., 2018). Recombinant *Lactobacillus casei* strains have an excellent capability to survive in intestinal tract when delivered for oral immunization (Kong *et al*., 2019). Thus for the production of recombinant vaccine *Lactobacillus casei* CC16 was utilized in present study as a carrier for antigen delivery. Initially the OmpF gene was successfully amplified using specific primers incorporating precise recognition sites for the two restriction enzymes BamHI and XhoI. PCR amplification showed the size of amplified OmpF gene was 1038 bps (Fig.1A). The recombinant plasmid pMD18-T amplified through PCR and showed that OmpF gene was effectively cloned to the N-terminal and C-terminal region (Fig 1B and C).

The pMD18-T-OmpF was successfully connected into expression plasmid pPG612.1,

This plasmid has absolute abilities to express recombinant Omp(Kong *et al*.,2019), because it is based on the promoter of glucose operon, the result shows it takes major part on the expression of recombinant OmpF, it also posses the structures of ssUsp secretion signal sequence for cloning (Yigang *et al*., 2008).

Electrophoresis result reveals that the OmpF gene was successfully inserted in pPG612.1 plasmid and size of recombinant plasmid fragment was 1038 bps (Fig.2). MC1061 was selected due to its heat shock transformation capabilities. This recombinant positive strain is appropriate for cloning, amplification and expression of plasmid DNA of varied Gram-positive bacteria for expression in *Lactobacillus casei* (Villaverde and Carrio, 2003). The recombinant OmpF was shown successfully expressed in MC1061. In this study we found glucose has ability as carbon source which can facilitate in induction as well as for analysis of expression of OmpF protein (Chen *et al.,* 2014).

The recombinant plasmid pPG612.1-OmpF was assorted with *L.casei* CC16 cells for electroporation. Newly recombinant plasmid cloning verification was done by PCR, re-digestion and DNA sequencing. The data shows the target gene of recombinant OmpF 1038bp was effectively inserted into *L. casei* CC16 (Fig 3 A-B).

Thus the findings of present study suggest that the recombinant *Lac:casei* CC16 bacteria expressing the OmpF N terminus gene had been obtained in result production of Lc-pPG612.1-OmpF.

The newly constructed product shows OmpF protein expression evaluated by immunoblot analysis, the product of the Lc-pPG612.1-OmpF was concentrated in the pellet, representing that the expressed recombinant OmpF protein expression was observed in the form of inclusion bodies. The result found the expressed protein is successfully able to bind pAb and HRP antibodies and strong immunoreactive bands of single~ 45 kD were observed in similar positions as observed in Immunoblot analysis. (Fig 4 A-B). This research shows the expression of the Lc-pPG612.1-OmpF that was in agreement with the reported earlier (Yadav *et al.,* 2014). In our previous study it has been observed that genetically recombinant Lactobacillus strains Lc-pPG1-OmpAI and Lc-pPG2-OmpAI are capable to provide protection in common carp when orally immunized against *A. veronii* (Zhang 2018).

Thus the development of new recombinant secreted *Lactobacillus casei* oral live vector CC16 co-expressing *A. veronii* TH0426 OmpF protein which can be useful in development of new oral live recombinant vaccines against *Aeromonas veronii*.

Conclusion: In conclusion the construction of using live recombinant *lactobacillus casei* CC16 with secreting prokaryotic shuttle plasmid pPG612.1 expressing *Aeromonas veronii* OmpF protein gene has been successfully carried out. We have identified the target protein strong expressions in recombinant *L.casei* by SDS-PAGE western blotting and the fragment was found 45kD same size. The resultant recombinant plasmid Lc-pPG612-OmpF can be used for the oral immunization of fish against *Aeromonas veronii.*

Significance and Impact of the Study: In this study successfully we developed recombinant live *Lactobacillus casei for* oral immunization. It provides encouraging platform for the improvement of vaccines against *A. veronii* infection. Humans, animals and fish are equally affected from this highly pathogenic bacteria *A. veronii*. Vaccine development for this pathogenic strain is tried for the first time so; the literature, information's, recent status of disease, and the system developed for this vaccine could protect all the hosts. Serious diseases like diarrhea and muscle ulcer in humans and different animals while numerous adverse health conditions with high mortality rate in fish and other aquaculture are due to its infection. The recombinant vaccine developed in

this research could be beneficial on wide level. Moreover, the techniques used in the prosecution of this research project and data obtained all are of prime importance in relevant future studies.

Acknowledgements: This work was supported by the National Natural Science Foundation of China (Project No. 31201927) and the Key Scientific and Technological Project of Jilin Provincial Science & Technology Department (Project No. 20150204065NY).

Conflict of interests: All authors declare that they have no conflicts of interests.

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