PlpE Epitopes of Pasteurella multocida Fusion Protein as Novel Subunit Vaccine Candidates

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Original Article

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Abstract

Background: *Pasteurella multocida* is the causative agent of many diseases. Antimicrobial treatment disadvantages highlight the need to find other possible ways such as prophylaxis to manage infections. Current vaccines against this agent include inactivated bacteria, live-attenuated bacteria, and nonpathogenic bacteria, which have disadvantages such as lack of immunogenicity, reactogenicity, or reversion to virulence wild bacteria. Using bioinformatical approaches, potentially immunogenic and protective epitopes identified and merged to design the best epitope fusion form in case of immunogenicity as a vaccine candidate. **Materials and Methods:** In this study, the fusion protein (*PlpE1 + 2 + 3*) and full *PlpE* genes (*PlpE-Total*) were cloned in pET28a in BL21 (DE3) firstly and later in pBAD/gIII A and expressed in Top10 *Escherichia* coli. Overlap polymerase chain reaction (PCR) using different primers for 5' and 3' end of each segment produced fusion segment 1 + 2 and (1 + 2) +3 fragments and was used for cloning. **Results:** Cloning of both *PlpE1 + 2 + 3* and *PlpE-Total* into the pET28a vector and their transform into the BL21 (DE3) *E. coli* host was successful, as the presence of the cassettes was proved by digestion and colony PCR, however, their expression faced some challenges independent of expression inducer (isopropyl β-d-1-thiogalactopyranoside) concentration. **Conclusion:** Changing the vector to pBAD/gIII A and consequently changing the host to Top10 *E. coli* have resulted in sufficient expression, which shows that Top10 *E. coli* may be a good substitute for such cases. Furthermore, it is concluded that adding 8M urea results in sufficient purification, which hypothesizes that denature purification is better for such cases than native one. Purified proteins headed for further analysis as vaccine candidates.

Keywords: *Fusion PlpE, Pasteurella Multocida, vaccine candidate*

Introduction

Pasteurella multocida is a Gram-negative, nonmotile, nonspore-forming, and facultative aerobic/anaerobic coccobacillus that is the etiological agent of a wide range of animal diseases such as pneumonia in cattle and sheep, atrophic rhinitis in swine, hemorrhagic septicemia in buffalo and cattle, and fowl cholera in chicken.[1] P. multocida-related infections also suffer economic incomes due to its damage to sheep, goats, rabbits, poultry, and other livestock industries. besides high mortality rates, *P. multocida* inhibit gain weight of poultry and causes a shortage in production which suffer farm holders lifestyle. This bacterium is responsible for 30% of total cattle deaths around the world and losses of one billion dollars annually in this industry in North America alone.[3] Besides these findings, it is responsible for considerable economic losses of pigs, poultry, and cattles. It has also been isolated both from healthy animals as a member of the oropharyngeal flora of calves and does not cause a serious disease but, in stress conditions, it is one of the bacterial pathogens associated with disease.[4] *P. multocida* strains are classified into five capsular serogroups (A, B, D, E, and F) and 16 somatic lipopolysaccharide serotypes numbered from 1 to 16.[5]

Vaccination of livestock plays a vital role in improving the health and welfare of livestock and preventing animal to human transmission, which is one of the main goals of public health strategies. The present vaccine candidates against this agent consist of live-attenuated vaccines, which have several disadvantages such as induction of short-term or ineffective

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immunity[6,7] and also possibility of reversion to virulence in cases the attenuation mechanism is not defined.[8] and Killed vaccines which their effectiveness varies from inefficiency to short-term stimulation of immunity.[9,10]

Considering the limitations of current vaccines, there is a clear need to develop vaccines for containing no risk to cause a disease, decreasing the side effects causing an acute rather than chronic infection, induction of immunity after exposure, least antigenic variation, and no attack to the cells of the immune system, not having an environmental or animal reservoir. One such approach is the development of new subunit vaccines, which would have some advantages, such as easier production and avoiding the possibility of reversion to virulence.[11]

Hatfaludi *et al*., in 2012, assessed 71 candidate proteins identified by reverse vaccinology; this study concluded that the surface of conserved outer membrane PlpE protein, while not required for virulence, was exposed and could elicit a protective immune response when delivered to chickens in denatured form. Accordingly, it was assumed that linear epitopes are sufficient to induce immunity.[12] Our goal was to identify epitope regions of PlpE protein common to all serotypes and construct a polytope, which offers the possibility of a vaccine that will induce protection against all *P. multocida* serotypes where all available vaccines protect the vaccinated animals only against serotype from which vaccine was produced.

Materials and Methods

Genome extraction

The *P. multocida* serotype A1, X73 received from the Razi Vaccine and Serum Research Institute of Iran (Karaj, Iran). The genome was extracted by high pure polymerase chain reaction (PCR) Template Preparation Kit (Roche, Germany) and stored at −20°C.

SOEing polymerase chain reaction

PlpE gene of *P. multocida* A1 serotype was extracted from NCBI. It was amplified by PCR method using designed forward and reverse primers 5'-CCATGGGCATGAA-ACA AATCGTTTTAAA-3' and 5'-CTCGAGTTGTGCTTGGTG ACTTTTTTC-3', respectively, that was already known for this serotype in NCBI.[13]

The primers were constructed by Sinaclone (Tehran, Iran). The amplified *PlpE* gene was then sequenced by Bioneer (Daejeon, Korea). The location of the DNA coding sequences of the three selected peptides (*PlpE 1 + 2 + 3)* was found on the sequenced *PlpE* gene, and consequently, their primers were designed, as shown in Table 1.

The sequence of *PlpE-Total* was also used as a template for amplification of the three selected epitope regions for constructing fusion form. Three different segments amplified separately using designed primers, as shown in Table 1. The fragments 1 and 2 were overlapped by SOEing PCR to construct a fusion form (1 + 2) without a linker due to separated epitopes. Then, the latter DNA fragment was mixed with fragment 3 and fused using SOEing PCR program. The final product of overlap PCR is segment 1 + 2 + 3 as a fused form called *PlpE1 + 2 + 3*.

For segments 1, 2, and 3, initial denaturation was at 94°C for 5 min, and denaturation and annealing and extension were at 94°C for 45 s, 55°C for 45 s, and 72°C for 30 s, respectively, and repeated for 30 cycles. The final extension was performed at 72°C for 7 min. For segments 1 + 2 and (1 + 2) +3, PCR program is as above but needs pre-PCR stage. Primers designed considering overlap segments and restriction sites for *Nco*I and *Xho*I. CCATGG sequence is a restriction site for *Nco*I and CTCGAG sequence is restriction sites for *XhoI*.

To merge segment 1 to segment 2 and also segment 1+2 to segment 3, a extra cycle called pre-PCR performed as 94C for 3 minutess followed by 40C for 5 minutes and finally 72C for 1 min. Then, it was paused to add the related primers followed by PCR cycle, as discussed above.

Cloning

The mixture was incubated at a temperature appropriate for that restriction enzyme (37°C) for 3–5 h. PCR products of *PlpE-Total*, *PlpE1 + 2 + 3,* and pET-28a(+) and pBAD/gIII A vectors were digested using *NcoI* and *XhoI* restriction enzymes. Then, the ligation of digested PCR fragments and vectors was done.

Briefly, 10 μL of 2X ligase buffer, 50 ng of vectors, 100 ng of PCR product, and 5 Weiss units of T4 DNA ligase were mixed and H_2O was added to total volume of 20 μL. Ligation was carried out as overnight incubation at 4°C. When the vectors were used, vector and insert DNA were mixed in 1:3 molar ratio, 5 Weiss units of T4 DNA Ligase (Fisher Scientific, USA) and 2 μL of 10X ligase buffer were added, and the mixture was incubated 16 h at 4°C.

Competent BL21 (DE3) (Novagen, Germany) and Top10 (Novagen, Germany) *Escherichia coli* cells were prepared. Briefly, a single fresh *E. coli* colony was inoculated in LB medium until the OD600 reached 0.5–0.6 to obtain a middle of logarithmic phase culture. Then, the culture was incubated on ice and centrifuged at 3500 rpm for 5 min. The supernatant was decanted and the pellet was resuspended in ice-cold buffer. The cells were spun down at 3500 rpm for 5 min at 4°C. Finally, the supernatant was decanted and the pellet was resuspended gently. 100 μL of aliquots was stored at −80°C.

For transformation, briefly, 100 μl aliquot of competent *E. coli* was mixed gently with gel-extracted, cleaned up ligation products. The mixture was incubated on ice and heat shocked at 42°C and then incubated on ice. Luria-Bertani (LB) was added to the mixture and incubated at 37°C for 80 min by gentle agitation (100 rpm). The cells were centrifuged

Table 1: Primer specifications used for overlap polymerase chain reaction

Primer	Description	Sequence	Amplicon Size (bp)
PlpE-FA	Segment 1	TATACCATGGCTGGTGGTGGCGGTAGCGC	260
PlpE-RBA		ATTATATTGAGAAGTAAGATTTTC	
PlpE-FAB	Segment 2	GAAAATCTTACTTCTCAATATAATG	220
PlpE-RCB		CGTACCCTCGATCTGGTTTG	
PlpE-FBC	Segment 3	ATCAAACCAGATCGAGGGTACGAT	200
PlpE-RC		TAAACTCGAGTTCAGCTTTTCCTACACCAA	
PlpE-FA	Segment 1+2	TATACCATGGCTGGTGGTGGCGGTAGCGC	460
PlpE-RCB		CGTACCCTCGATCTGGTTTG	
PlpE-FA	(Segment 1+2) + (segment 3)	TATACCATGGCTGGTGGTGGCGGTAGCGC	640
PlpE-RC		TAAACTCGAGTTCAGCTTTTCCTACACCAA	

Primers designed considering overlap segments and restriction sites for NcoI and XhoI. CCATGG is restriction sites for NcoI and CTCGAG is restriction sites for XhoI

and resuspended in 100 μL of LB. Transformed cells were plated on selective medium containing appropriate antibiotic (100 μg/mL ampicillin or 30 μg/mL kanamycin).

Expression and purification

For the production of His-tagged fusion proteins, recombinant BL21 (DE3) *E. coli* were grown in Luria Broth containing isopropyl β-d-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1, 0.4, and 1 mM for the induction of gene expression under the control of phage T7 promoter (Kothari *et al*., 2006), and kanamycin was added to a final concentration of 30 μg/ml as a selection marker.

Recombinant *E. coli* Top10 were grown in Luria Broth containing serial dilution (0.2%, 0.02%, 0.002%, 0.0002%, and 0.00002%) of L-Arabinose (Merck, Germany) for the induction of gene expression. Ampicillin to a final concentration of 100 μg/ml was a selection marker.

Induced cultures were centrifuged and the pellets were resuspended in binding buffer (Ayalew *et al*., 2008) containing 8M urea. The cells were freeze-thawed 20 times, for 20 s, 10 s interval and then sonicated 20 times on ice, 20 s interval and the lysates were centrifuged and supernatants were saved. Ni-NTA (Qiagen) columns were used for the purification of fusion proteins on the bases of nickel affinity of histidine-tagged proteins. The purity of elutes was monitored on glycine-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Western blotting is used to confirm the target proteins.

Results

Amplification of *PlpE-Total* and construction of the chimeric gene

Sequence evaluation

The sequencing result of amplified *PlpE* confirmed that *P. multocida* strain X-73 lipoprotein E (*PlpE*) gene (Genbank: EF219452.1) has been used.

Overlap PCR using different primers for 5' and 3' end of each segment produced fusion segment 1 + 2 and (1 + 2) +3 fragments and was used for cloning, as shown in Figure 1. The final sequence of overlap fragments was 640 bp but not 680 bp.

pET-28a(+) and pBAD/gIII A vectors including gene of interest were transformed into the prepared BL21 (DE3) and Top10 competent *E. coli,* respectively. PCR performed to select 5 colonies which uptaken genes of interests, digestion (*NcoI* and *XhoI*) and colony.

Expression and purification

For gene expression in *E. coli*, the gene constructs and recombinant plasmids were introduced to the BL21 (DE3) and Top10 *E. coli,* and recombination was verified by restriction enzyme digestion, colony PCR, and sequencing. For the production of His-tagged fusion proteins, recombinant BL21 (DE3) *E. coli* were grown in Luria Broth containing IPTG to a final concentration of 0.5 mM for the induction of gene expression under the control of phage T7 promoter (Kothari *et al*., 2006) as supplier recommendation. Recombinant Top10 *E. coli* were grown in LB containing 0.002 mM L-arabinose which was the best concentration for both *PlpE-Total* and *PlpE1 + 2 + 3*. Cloning into pET28a vector in BL21 (DE3) *E. coli* was successful but not its expression. The presence of the cassettes in the pBAD/gIII A vector in Top10 *E. coli* was confirmed and also its expression for both *PlpE-Total* and *PlpE1 + 2 + 3* had an adequate amount of proteins as shown in figure 2.

Discussion

Our goal was to identify polytope regions of PlpE protein common to all serotypes and construct a polytope, which offers the possibility of a vaccine that will induce protection against all *P. multocida* serotypes.

In our previous work, based on bioinformatics studies, three different conserved segments of PlpE fulfilled the designed criteria. Briefly, the selection of epitope region via IEDB tools, possessing high antigenicity via

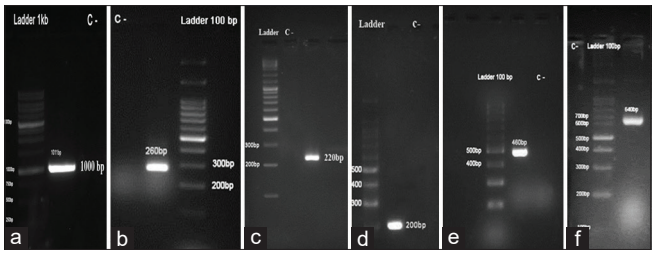

Figure 1: Overlap polymerase chain reaction for segment amplification. (a) Amplification result of *PlpE* gene of *Pasteurella multocida*, 1011 bp, (b) amplification result of segment 1, 260 bp, (c) amplification result of segment 2, 220 bp, (d) amplification result of segment 3, 200 bp, (e) amplification result of segment 1 + 2, 460 bp, (f) amplification result of segment 1 + 2 + 3, 640 bp. C−: Control negative, C+: Control positive

immunomedicine group, is including different serotypes' coverage via homology modeling, amelioration of half-life via ProtParam, and antibody accessibility. Conserved region 1 comprising amino acids (aa) 23–104 of PlpE, conserved region 2 from aa 135 to 202, and conserved region 3 from aa 262 to 321 are hypothesized as the most promising segments of PlpE to confer protection against *P. multocida*. The candidate fusion protein for cloning and expression was fragment 1 + fragment 2 + fragment 3.

In this study, the construction of both expression cassettes via pET28a vector within BL21 (DE3) *E. coli* was successful, however, the expression resulted no protein after evaluating 20 colonies on glycine-SDS-acrylamide gel despite successful cloning. Surprisingly, changing the vector to pBAD/gIII A within Top10 *E. coli* has resulted in sufficient expression, which hypothesizes that Top10 *E. coli* may be a good substitute for CodonPlus BL21 (DE3) *E. coli*.

Conclusion

We also came to this conclusion that 8M urea within lysis buffer results in sufficient purification, which hypothesizes that denature purification is better for such cases than native one 3 times in yield despite the gIII in vector which makes the target protein secretory.

Hatfaludi *et al*., in 2012, used 10 mM of 2ME in addition of 8M urea to insoluble protein including PlpE for purification and showed that denature purification is suitable for PlpE protein despite its expression vector or host[12] which supports our hypothesis also.

Further studies are underway to evaluate *in vivo* immunogenicity of these cloned and expressed subunit candidate vaccines against infection by *P. multocida*.

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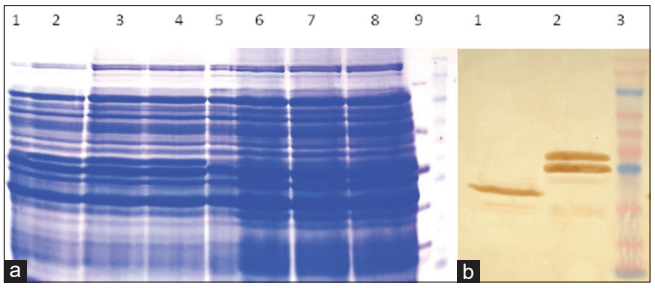

Figure 2: Expression of recombinant Top10 *Escherichia coli* cells. (a) Glycine-sodium dodecyl sulfate 12% acrylamide gel, (b) Western blot. (a1) Top10 *Escherichia coli* carrying pBAD vector. (a2-a4): Induced recombinant Top10 *Escherichia coli* carrying pBAD vector and *PlpE-Total* (42 and 45 kD). (a5) Not induced Top10 *Escherichia coli* carrying pBAD vector. (a6-a8) Induced recombinant Top10 *Escherichia coli* carrying pBAD vector and *PlpE1 + 2 + 3* (33 kD). (b1) Induced recombinant Top10 *Escherichia coli* carrying pBAD vector and *PlpE1 + 2 + 3*. (b2) Induced recombinant Top10 *Escherichia coli* carrying pBAD vector and *PlpE-Total*. (a9 and b3) Prestained protein marker

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Conflicts of interest

There are no conflicts of interest.

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