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Original Article

Molecualr Cloning of the capsular antigen F1 of *Yersinia pestis* in pBAD/gIII plasmid

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Abstract

Yersinia pestis which is the causative agent of pneumonic plague and distributed in all continents has led to many deaths during the history. Because of its high mortality rate, it must be diagnosed and treated at the earliest time post infection and therefore, rapid diagnostic tests are required. In the present study, we cloned the coding sequence of F1 capsular antigen of the bacteria in the pBAD/gIII plasmid for later expression and purification of the protein to produce poly and monoclonal antibodies against this antigen, and subsequently to develop rapid and efficient diagnostics tools for *Y. pestis* infections.

Keywords: *Yersinia. pestis*; Caf1; Capsular antigen F1; pBAD

INTRODUCTION

Yersinia pestis was first isolated by Alexander Yersin in 1894 in Hong Kong, after the spread of infection from mainland China (1). The bacterium is a Gram negative, non-motile, facultative anaerobic rod that exhibits bipolar staining. It is a zoonotic pathogen and its infectious dose is extremely low, estimated between 1 and 10 organisms, which makes it one of the most virulent bacteria identified (2). Clinically, it appears in three different forms including bubonic, septicemic and pneumonic plague (3). Bubonic and pneumonic plague infections are associated with high mortality rate (2,4). If remains untreated, the mortality rate of bubonic plague is about 50-90%, and untreated meningitis, pneumonia, or septi-cemia is fatal in most cases. (1). The primary pulmonary plague, although rare, has the mortality rate of 100% if untreated and more than 50% with antimicrobial treatment (5). Therefore, development of efficient, rapid and convenient methods for detection of bacterial agent at the earliest time of the infection is necessary (6). In addition, because plague is a fulminating disease and the clinical diagnosis is unspecific, the treatment should not be delayed by waiting for bacteriological confirmation or antibody seroconversion which can take more than one week (7). *Y. pestis* produces at 37 °C a specific F1 antigen which forms a large gel-like capsule (caf1), readily soluble in the culture media and the F1- negative phenotype is rarely encountered. Previous preliminary studies have evidenced F1 antigen in animal tissues and serum of one fourth of culture-positive patients (8). Therefore, various detection methods depend-ing on the detection of F1 antigen or anti-F1 antibodies have been developed to date (9-13). The aim of the present study was to obtain the coding sequence of the F1 antigen and its cloning in a suitable expression vector for its expression and subsequent production of polyclonal and monoclonal antibodies against F1 antigen.

MATERIALS AND METHODS

Bacterial strains, plasmid and growth condition

Cloning procedure was performed in Top *Escherichia. coli* strain (Invitrogen, USA).

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Competent cells were prepared by calcium chloride method as described earlier (14-15) and the bacteria were propagated and cultured in Luria-Bertani (LB) (HiMedia, India) medium at 37 °C. Whole cell DNA extract of *Y. pestis* was obtained from Pasteur Institute of Iran (Tehran, Iran).

For T/A cloning, pTZ57R/T plasmid (Fig. 1A; Thermo-scientific, USA) was used. The expression vector pBAD/gIII A (Fig. 1B) was purchased from Invitrogen . In order to maintain the stability of the plasmids, ampicillin (Sigma, Germany) was added to the culture medium at a final concentration of 100 µg/ml.

Primer designing and polymerase chain reaction amplification of the caf1 gene

In order to amplify the caf1 coding sequence, specific primers were designed according to the caf1 gene sequence retrieved from Gene bank (Accession number: NC_006323.1). Table 1 represents the nucleotide sequences and features of the designed primers.

The primers were prepared when received and used for polymerase chain reaction (PCR) amplification of the gene. PCR was performed with High fidelity PCR enzyme mix (Thermoscientific, USA) and the PCR condition for amplification of the caf1 included a primary denaturation step of 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 45 s at 55 °C for annealing, and 45 s at 72 °C for elongation. The conditions also included a final elongation step of 10 min at 72 °C. PCR products were analyzed using 1% agarose gel elcrophoresis.

Cloning and subcloning of the caf1 gene

In order to clone the amplified fragment, it was gel purified using GeneJet Gel Extraction kit (Thermoscientific, USA) according to the manufacturer's instruction. Then, the purified fragment was ligated to the pTZ57R plasmid using T4 DNA ligase (Thermoscientific, USA) according to the manufacturer's instruction. The ligation mixture was incubated at 22 °C for 10 min, and then transformed to competent Top10 *E. coli* cells.

Screening of the recombinant clones was performed on LB-Agar medium containing 100 µg ampicillin/ml. Some of the obtained bacterial colonies were cultivated overnight and followed by plasmid purification using Gene Jet Plasmid Miniprep kit (Thermoscientific, USA).

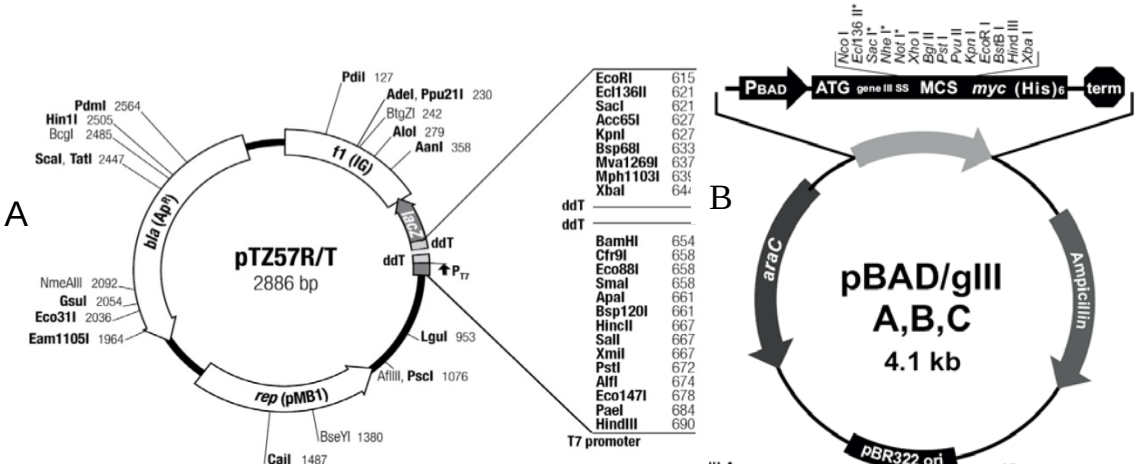

Fig. 1. A; Schematic representation of the pTZ57R/T, B; Schematic representation of the pBAD/gIII A plasmids (obtained from the manufacturers brochure).

Table 1. Nucleotide sequence and features of the designed primers.

Primer Name	Sequence	Feature
CAFPBFR	CATG**CCATGG**CAATGAAAAAAATCAGTTCCG	*Nco*I, Start codon
CAFPBRV	CCC**AAGCTT**CCTTGGTTAGATACGGTTACGG	*Hind*III

Then, the fidelity of the cloning was verified with restriction endonuclease digestion of the extracted plasmids with FastDigest™ *Nco*I and *Hind*III enzymes (Thermoscientific, USA). Finally the fidelity of the cloned sequences was verified by DNA sequencing.

Following authentication of the cloned sequence, sub-cloning of the caf1 gene to the pBAD/gIII A plasmid was performed. In this regard, both recombinant pTZ57R plasmid and the pBAD vector were digested with *NcoI* and *Hind*III restriction endonucleases followed by gel purification, and then the fragments were ligated using T4 DNA ligase and used to transform competent Top10 *E. coli* strain. Finally, the authenticity of the obtained recombinant pBAD plasmids was verified by restriction endonuclease digestion.

RESULTS

PCR amplification and gel purification of the caf1 gene

Agarose gel electrophoresis of the PCR products confirmed the amplification of the caf1 gene. A band of about 500 bp observed on 1% agarose gel confirms the amplification of the caf1 fragment.

Then the amplified fragment was gel purified and the quality of the purified fragment was verified by agarose gel electrophoresis (Fig. 2).

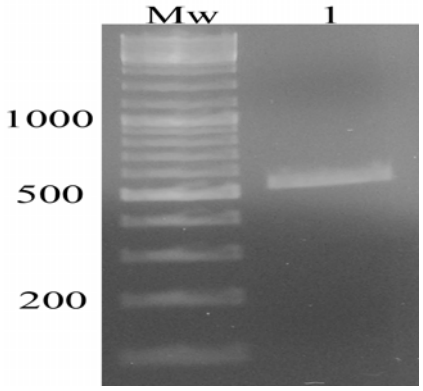

Fig. 2. Gel electrophoresis of PCR product. The amplification of the caf1 fragment was confirmed by the presence of a band about 500 bp (lane 1).

Cloning of the caf1 gene to the pTZ57R plasmid

Following ligation reaction and transformation of the competent Top10 *E. coli* cells, the ampicillin resistant colonies were cultivated overnight in LB medium containing 100 µg/ml ampicillin and then used for plasmid preparation. Agarose gel electrophoresis of the prepared recombinant plasmids indicated the possibility of cloning of the fragment when compared to non-recombinant empty pTZ57R plasmid (Fig. 3A). Afterwards, to further evaluate the cloning, the recombinant plasmids were digested with *Nco*I and *Hind*III restriction endonucleases. As it is illustrated in Fig. 3B , agarose gel electrophoresis of the digestion products confirmed the cloning by revealing two bands of about 2800 and 500 bps corresponding to the linearized pTZ57R plasmid and caf1 fragment, respectively.

The fidelity of cloning was also verified by DNA sequencing. Comparison of the sequencing results with the caf1 sequences deposited in Gene bank database was performed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) which confirmed the authenticity of the cloned sequence.

Sub-cloning of the caf1 gene to the pBAD/gIII plasmid

pBAD/gIII plasmid was used as an expression vector. Following verification of the cloned sequence by DNA sequencing, it was double digested with *Nco*I and *Hind*III restriction endonucleases and ligated to the similarly digested ends of the pBAD/gIII A plasmid. Following transformation of the competent Top10 *E. coli* cells with the ligation mixture and selection of the recombinant clones on LB-agar medium containing ampicillin, some colonies were cultivated overnight and subjected to plasmid preparation. Restriction endonuclease digestion of the prepared plasmids confirmed the cloning by revealing two bands of about 4000 and 500 bp corresponding to the linearized pBAD/gIII A plasmid and caf1 gene, respectively (Fig. 4).

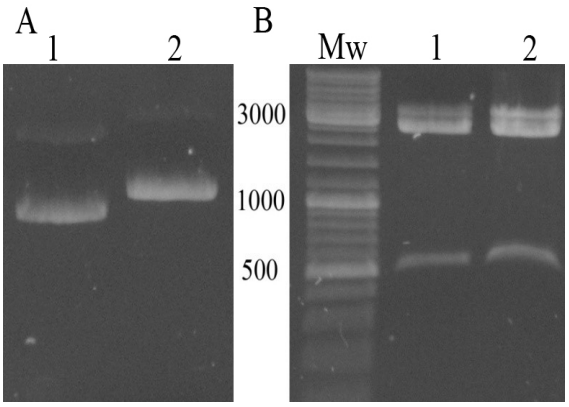

Fig. 3. A; comparison of the recombinant pTZ57R plasmid with non-recombinant plasmid confirmed the cloning by size difference: 1; Non-recombinant pTZ57R plasmid, 2; Recombiant pTZ57R plasmid, B; Evaluation of the cloning in the pTZ57R plasmid by restriction endonuclease digestion with *Nco*I and *Hind*III enzymes. As it is shown in lanes 1 and 2, digestion resulted in the release of two 500 and 2800 bp fragments corresponding to the caf1 and linearized pTZ57R plasmid, respectively. Mw; molecular weight marker.

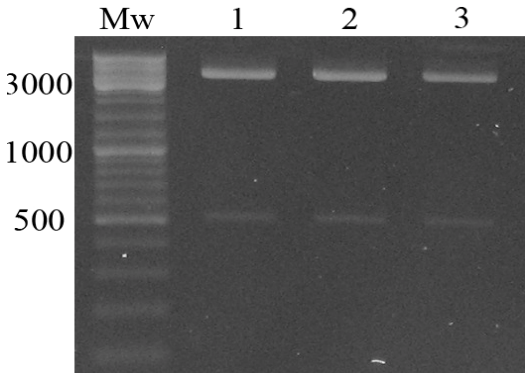

Fig. 4. Restriction endonuclease digestion of the recombinant pBAD/gIII A plasmids. Gel electrophoresis of the digestion mixtures confirmed the sub-cloning by revealing two bands of about 4000 and 500 bp (1-3) corresponding to the linearized plasmid and caf1 fragment, respectively. Mw; molecular weight marker.

DISCUSSION

The aim of the present study was to amplify and clone the coding sequence of *Y. pestis* caf1 gene into the pBAD/gIII expression plasmid. *Y. pestis* is the causative agent of the plague, a highly contagious disease, which in addition to its flea-borne transmission, is also transmissible directly from human to human in pneumonic plague (16).

Because of the rapid spread and progression of the human-to-human airborne infection (short incubation period up to 2–3 days), and very high mortality rates (approaching 100% if untreated) (17), rapid and concise detection of the disease is of highly importance. Conventional methods for the detection of *Y. pestis* depending on the biochemical and bacteriological experiments are labor intensive, time consuming and require special expertise (1).

PCR based methods have been also developed, however, they require advanced laboratory instruments which may not be available in every laboratory (18-21). One of the simplest and fastest methods for the detection of *Y. pestis* is based on the immunoassay techniques including ELISA. These methods have been developed on the basis of *Y. pestis* capsular antigen F1 (1). The *Y. pestis* caf 1 is a plasmid (pFra)-encoded proteinaceous capsule which is synthesized in the large quantities by the pathogen (22). Since this is a highly immunogenic protein, it has been used for the development of various immunoassay methods depending on the direct detection of the F1 antigen or the anti F1 antibodies (9,10).

Hence, in order to produce specific poly and monoclonal antibodies against the F1 antigen, this protein was procured. In order to produce recombinant F1 protein, its coding sequence was amplified which was then cloned in the pTZ57R/T plasmid and followed by verification of the cloning with restriction endonuclease digestion and DNA sequencing. Then, the cloned fragment was cloned into the pBAD/gIII plasmid. pBAD/gIII A plasmid is an expression vector which harbors the araBAD promoter that provides tight, dose-dependent regulation of heterologous gene expression (23).

It also contains the gIII secretion signal which permits secretion of recombinant protein into the periplasmic space (24). In addition, the araC gene which encodes the regulatory protein araC for tight regulation of the PBAD promoter has been cloned in this vector (25). Overall, this features made the pBAD/gIII A plasmid an efficient plasmid for

the high yield production of recombinant proteins in addition to their efficient secretion to the periplasmic space. Due to a very low amount of bacterial proteins present in the periplasmic space, purification of recombinant proteins would be performed easier than the cytoplasmic expressed recombinant proteins (24).

CONCLUSION

Here we amplified and cloned the *Y. pestis* capsular protein F1 antigen in the pBAD/gIII A plasmid. This plasmid could be used in later studies for high yield production of the protein and its subsequent application for production of poly and monoclonal antibodies to develop immunoassay based detection kits for the Y. pestis bacteria and plague disease.

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