Microbiology Research Journal International

30(9): 62-69, 2020; Article no.MRJI.62880
ISSN: 2456-7043
(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)

Expression of Epsilon Toxin from *Clostridium perfringens* Type D in *E. coli* Rosetta (DE3): Study on a New Vaccine Production

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Authors' contributions

This work was carried out in collaboration among all authors. Author RPL (the supervisor) designed the study, performed the statistical analysis and wrote the protocol. Author MA wrote the first draft of the manuscript. Author LAK managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/MRJI/2020/v30i930264
Editor(s):
(1) Dr. Ana Cláudia Coelho, University of Trás-os-Montes and Alto Douro, Portugal.
Reviewers:
(1) Cleide Barbieri de Souza Centro, Universitário Lusíada – Unilus Santos, Brasil.
(2) Ganapati Hegade, India.
(3) Anal Chatterjee, Barrackpore Rastraguru Surendranath College, India.
Complete Peer review History: http://www.sdiarticle4.com/review-history/62880

Original Research Article

Received 09 September 2020
Accepted 17 November 2020
Published 11 December 2020

ABSTRACT

Clostridium perfringens is a Gram-positive anaerobic bacterium which is divided based on major toxins into five types (A-E). *C. perfringens* type D causes fatal enterotoxaemia (pulpy kidney) in sheep and goats that causes heavy economic losses in domestic animals. However current enterotoxaemia vaccine has been effective in controlling of disease; Strategies for the development of effective vaccine can be achieved by the production of new generation experimental vaccines. The aim of this study was cloning and expression of epsilon toxin of *C. perfringens* type D in *E. coli* Rosetta (DE3) to improve of the immunity. Epsilon toxin gene was cloned into pJET1.2/blunt vector and pET22b (+) expression vector and finally transformed into *E. coli* Rosetta competent cells

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(DE3). The result showed that the epsilon gene of *C. perfringens* type D can be cloned and expressed in *E. coli* Rosetta™ (DE3) successfully. *E. coli* was suitable host for the expression of *C. perfringens* epsilon toxin. Clearly, future research is open for further refinement of the study.

Keywords: C. perfringens; epsilon gene; cloning; expression; pET22b; E. coli Rosetta (DE3).

1. INTRODUCTION

Clostridia are anaerobic and spore-forming bacteria which are spread in the environment and intestinal tract of human and animals. One of the most important genus of Clostridia is *C. perfringens*. *C. perfringens*, the causative agent of gastrointestinal diseases in animals and foodborne disease, gangrene and enteritis necroticans in human [1], have been studied in Iran by Rafiei and Ardehali since 1950 [2]. *C. perfringens* chromosome is a single circular with G+C content of approximately 25% and 3.6 Mbp [3]. *C. perfringens* has been classified into five types (A -E) based on major toxins [4]. *C. perfringens* type D is produced by epsilon toxin (etx), which causes fatal enterotoxaemia (pulpy kidney) in sheep and goats [5]. The toxin is secreted in the small intestine as a 33-kDa prototoxin which is activated by removal of 13 amino acids from N-terminal and 22 residues from C-terminal [6]. The accumulation of active toxin (29-kDa) increases the permeability of the animal intestinal resulted in damage of brain, lungs, and kidneys [7].

Epsilon toxin is one of the most potent toxins among bacterial toxin [8]. Vaccines have been proven to be effective against clostridial disease. Several kind of vaccine is used for immunization in animals such as bacterin-toxoids (current vaccine) toxoid and new generation vaccine .In 1992, epsilon gene of *C. perfringens* type D had been cloned, expressed in *Escherichia coli* (*E. coli*) and surveyed immunization in mice [9]. The result showed that recombinant vaccines resulted in neutralization toxin in mice [10]. In 2010, epsilon gene was also cloned and expressed in *E. coli* against pulpy kidney. The result showed that adequate immunity in ruminants [11]. *C. perfringens* is a model for genetic researches [12] PET22 as expression cloning and *E. coli* as host also has been used for cloning and engineering studies since years ago. So, the aim of this study was cloning and expression of epsilon toxin of *C. perfringens* type D in *E. coli* Rosetta (DE3) to improve of the immunity. In this study, *Nde*I and *Xho*I enzymes were used for digestion which contains cut region.

2. MATERIALS AND METHODS

2.1 Cultivation

C. perfringens type D vaccine strain (CN409) used as the source of genomic DNA. For production of recombinant protein, a prokaryotic expression vector pET22b (+) (Novagen, Germany) was used. *E. coli* Rosetta (DE3) strain *was* applied as an expression host. *C. perfringens* type D vaccine strain was grown anaerobically in the liver extract medium using anoxomat (Mart® microbiology, Netherlands) at 37°C overnight. On the other hand *E. coli* strains were grown in LB broth medium under aerobic condition [13].

2.2 Isolation of Genomic DNA

Then bacterial cells were harvested by centrifugation and treated in TE containing 1 mg/ml lysozyme before 10% SDS and RNase-A (10 mg/ml) was added and incubated for 30 minutes at 37°C. Proteinase K (50 mg/ml) was added and incubated for 1 hour. Extraction with equivalent phenol and chloroform solution was done twice. Sodium acetate (1:10 V/V) and isopropanol (1 V/V) were added to mixture and incubated at -20°C for 20 minute at least. The DNA was then centrifuged for 10 minute at 12500 rpm in 4°C. Sediment of chromosomal DNA was washed in ethanol 70%, dried, and resuspended in TE buffer [14].

2.3 Gene Amplification

Primers were designed from nucleotide sequence of *etx* gene was retrieved in GenBank under accession number HQ179546 using oligo software. *etx* gene was amplified using forward (5′-AAT-CAT-ATG-AAA-AAA-AAT-CTT-GTA-AAA-AGT-3´) and reverse primers (5´-AAT-CTC-GAG-TTT-TAT-TCC-TGG-TGC-CTT-AAT-3´) and *Pfu* DNA polymerase (Fermentas, Germany).

PCR amplification was performed in total volume of 25 µl containing template DNA (100 ng), 1 µM for each primers (10 pmol/µl), 1.5 mM Mg^{2+},

200 µM each dNTP, 1X PCR buffer and 2 unit of *Pfu* DNA polymerase and distilled water. The following conditions were used including 95°C (5 min), followed by 30 cycles of denaturing step of 1 min at 95°C, annealing step of 1 min at 52°C and extension step of 3 min at 72°C, followed by final extension at 72°C for 15 minutes. The PCR product was analyzed by electrophoresis in 1% agarose gel containing ethidium bromide and visualized using transilluminator. The PCR product was purified using Gene JET^{TM} Gel Extraction Kit (Fermentas, Germany). Then nucleotide sequencing was carried out by SEQLAB (Sequence Laboratories Goettingen GmbH).

2.4 Cloning of *etx* Gene

pJET1.2/blunt vector was ligated with amplified *etx* gene in order to production of pJETε then transformed into *Top10 E. coli* competent cell, and screening of recombinant *E. coli Top10* clones was performed by colony PCR and antibiotic resistance (culture of suspension on LB-amp agar supplemented with ampicillin 100mg/ml) (Fermentas, Germany). The positive colonies and pET22b were digested using *NdeI* and *XhoI* enzymes (Fermentas, Germany), purified (using Gene JET^{TM} Gel Extraction Kit) and ligated with T4 DNA Ligase at 16 °C. Screening of recombinant expression vector was performed as previously described. After that, they *E. coli*/Rosetta strains were transformed using pET22ε [pET22 (+) epsilon] and screening of recombinant *E. coli*/Rosetta clones was also done by colony PCR. After purification, Sequencing was performed using Forward and Reverse primers. The results obtained by Chromas Lite and Blast software were compared with the gene sequence in GenBank.

Recombinant *E. coli* cells were grown in LB broth medium at 37°C with shaking for 0.6–0.7 OD_{600} and followed by induction with IPTG (0.5, 1 and 1.5 mM) (Fermentas, Germany) . Samples were collected every in three times. Finally, SDS-PAGE (Paya Pajouhesh, Iran) analysis was surveyed to confirm protein expression.

3. RESULTS

The result of electrophoresis showed that the length of PCR product was 984 bp (Fig. 1).

Amplified and purified *etx* gene (using Gene JET^{TM} Gel Extraction Kit) was ligated into digested vector pJET1.2/blunt and transformed *Top10 E. coli* successfully. The colony PCR and antibiotic resistance result confirmed the presence of the inserted fragment (Figs. 2, 3).

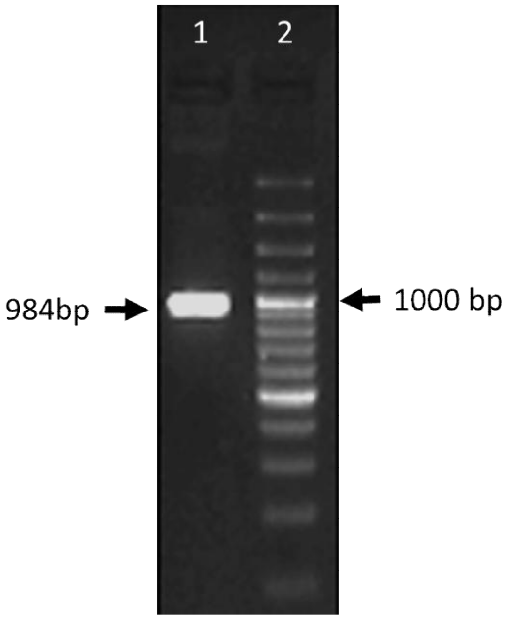

Fig. 1. Electrophoresis of the polymerase chain reaction product on agarose gel (1%)
Lane 1, PCR product epsilon toxin C. perfringens type D, lane 2, 100 bp DNA size marker

The result of restriction enzyme analysis of the plasmid DNA from the positive clones also confirmed the presence of the insert (Fig. 4).

After successful subcloning of the pET22ε, *E. coli* Rosetta competence cells were transformed in this vector. The colony PCR result also confirmed its. Sequencing result revealed that sequence of inserted gene was consistent accession number HQ179546 (99%). The result of SDS-PAGE showed that the optimum expression of recombinant protein was obtained with 0.5 mM IPTG and incubation time 3 h (Fig. 5).

4. DISCUSSION

C. perfringens causes severe economic losses in livestock and poultry industries, and wild life. Some *Clostridium* is the cause of disease in animals due to the release of exotoxins (e. g. alpha (*cpa*), beta (*cpb*), epsilon (*etx*), and iota (*ia*) [15]. This toxin causes enterotoxaemia in sheep and goat [16]. In 1954, the first strain of *C. perfringens* type D was isolated from intestinal contents of enterotoxaemia of sheep and goats

[17]. Further research has shown widespread infection for years all over the country. Vaccination seems to be an effective way to control clostridial diseases [18]. *C. perfringens* is a suitable model for cloning and engineering studies [12] for production of new generation vaccine. So, the aim of this study was cloning and expression of epsilon toxin of *C. perfringens* type D *E. coli* Rosetta (DE3) in order that improves of the immunity.

In this research the cloning vector was used pJET1.2/ blunt which is a 2974 bp linear plasmid. This vector has advantages the other vectors because these vectors with the use of Pfu enzyme which has proof reading activity, ligation can be corrected completed. This vector contains a lethal gene at the distance of 372-371 bp which will be destroyed cells [19] but after insertion of foreign fragment into cloning site, the ability of propagate will be survived. So, there is no need to observe the blue and white colonies. This vector has an extensive Multiple Cloning Site which has T7 promoter and an ampicillin resistance gene. During ligation, DNA is inserted at the end of the vector, which contains the 5-phosphoryl group, to form a recombinant circular plasmid of 3958 bp. After transformation, only transformed bacteria will grow in the LB agar plus ampicillin medium. After this step, the colonies were examined. The result of colony PCR showed 984 bp fragments which confirmed the previous result.

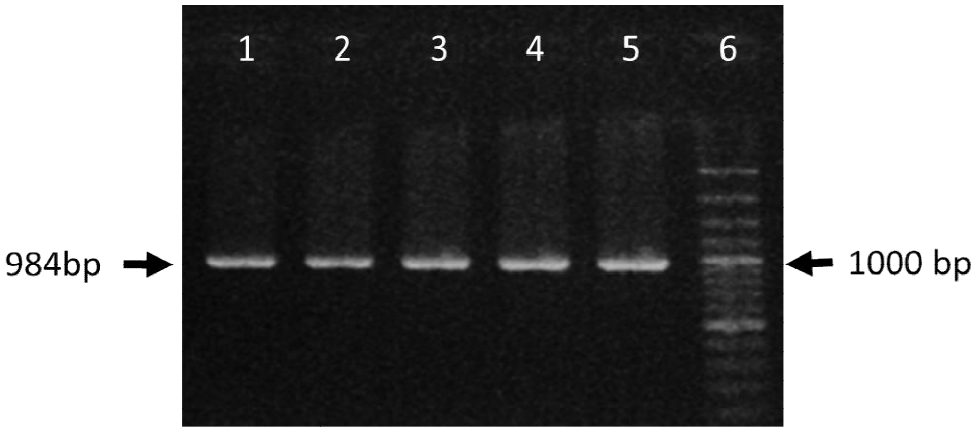

Fig. 2. Positive colonies screened using colony PCR

Lanes 1-5, PCR product epsilon toxin C. perfringens type D, lane 6, 100 bp plus DNA size marker

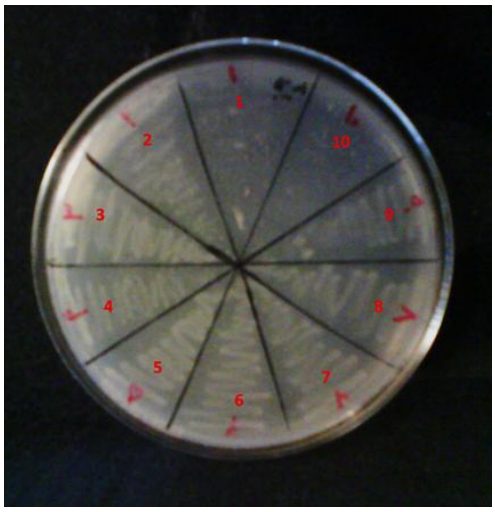

Fig. 3. Positive colonies screened with antibiotic resistance (LB agar supplemented with ampicillin 100 mg/ml)

Numbers 2-9 resistant to ampicillin and numbers 1, 10 sensitive to ampicillin

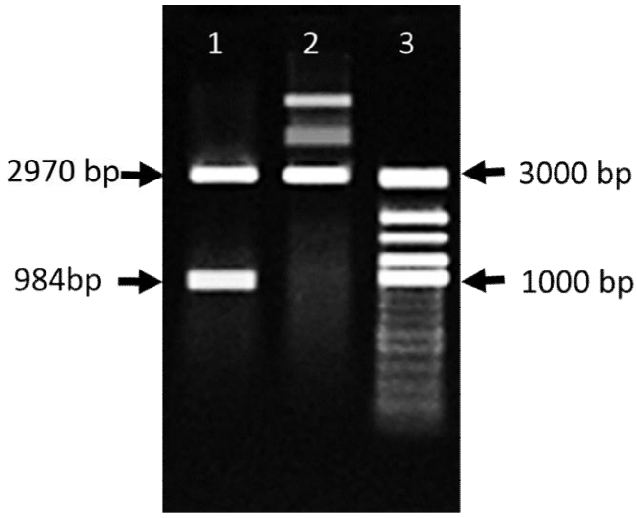

Fig. 4. Digestion of pJET /ε recombinant plasmid using *NdeI* and *XhoI*
Lane 1: pJET /ε cut, lane 2: pJET /ε uncut, lane 3:100bp plus DNA size marker

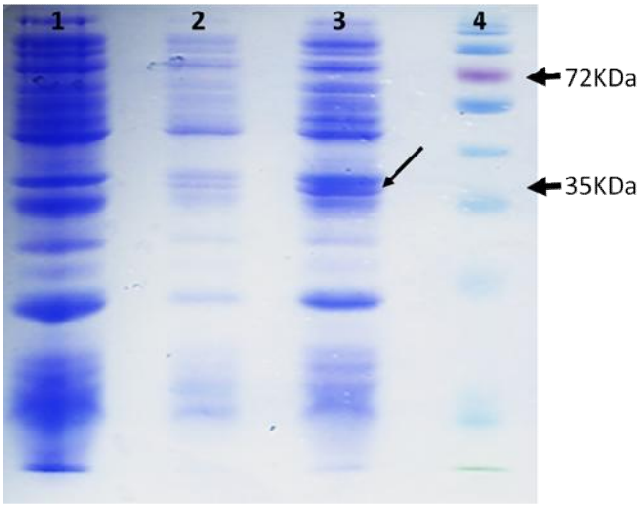

Fig. 5. SDS-PAGE analysis of the recombinant protein with coomassie blue staining
Lane 1, Negative control (E. coli Rosetta "DE3" strain without pET22b+), Lane 2-3 Recombinant E. coli strain after 1 h induction, Recombinant E. coli strain after 3 h induction, Lane 4, Protein molecular size marker (Page Ruler™)

In this study, *Nde*I and *Xho*I enzymes were used for digestion which contains cut region. *Xho*I and *Nde*I enzymes at the end of 5′ and 3´ of the gene has restriction site, respectively. The enzyme-digested and undigested plasmids were loaded on the agarose gel. The result showed that several clear bands more than 3 kb and one band more than 0.9 kb were observed in the undigested plasmid but only one sharp band approximately 2 kb (2974 bp), and epsilon toxin band (about 984 bp) were observed in the digested plasmid. These findings suggest that the epsilon toxin gene was inserted into the recombinant vector successfully.

After successful cloning of the pET22ε, *E. coli* Rosetta/ε competence cells were transformed in this vector. The colony PCR result confirmed that epsilon-toxin was transformed into the *E. coli*/ Rosetta/ε bacteria. Sequencing result also were consistent with the sequences reported in GenBank under accession number HQ179546 (99%).

Some recombinant vaccines have been prepared and evaluated in animals [20,21]. Souza et al. cloned the epsilon toxin into vector pET-11a and expressed. The result of study was successfully for animal immunization [10]. Nagahama et al. have perviously cloned the *cpa* gene and evaluated. The result was protective in mice [22]. Tang et al. cloned and sequenced enterotoxin (*cpe*) from *C. perfringens* type C [23]. Furthermore, recombinant vaccines against *cpe* from *C. perfringens* type A strain did not show any side effect on mice and guinea pigs [24]. Experimental recombinant vaccine Iota and TpeL have been expressed in *E. coli* [25,26]. Moreover, it has been reported that recombinant NetB vaccine is effective against enteritis necrotic in birds [27]. In other hand, recombinant multivalent vaccines also have been prepared and compared to the conventional vaccines [28, 29,30]. In this research the optimum expression of recombinant protein was obtained with 0.5 mM IPTG and incubation time between 2 and 4 h which is consistent with previously study [31]. Our study showed that the epsilon gene of *C. perfringens* type D can be cloned and expressed in *E. coli* Rosetta successfully.

5. CONCLUSION

In Conclusions the results obtained showed that this can be a suitable candidate as a recombinant vaccine for prevention of clostridial diseases. It is purposed more research on recombinant vaccine development be done in the future. It is hoped that in the future with further researches, the recombinant vaccine may replace the current vaccine.

ACKNOWLEDGEMENT

The authors wishes to express their warm thanks to all of the scientific members and staffs of anaerobic vaccine production department of Razi Vaccine and Serum Research Institute.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/62880