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Expression of full and fragment-B of diphtheria toxin genes in *Escherichia coli* for generating of recombinant diphtheria vaccines

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Purpose: In the present study, whole diphtheria toxin (dt) and fragment B (dtb) genes from *Corynebacterium diphtheriae* Park William were cloned into *Escherichia coli*, the purified expressed proteins were evaluated for ultimately using as a candidate vaccine.

Materials and Methods: The dt and dtb genes were isolated from bacterial strain ATCC (American Type Culture Collection) no. 13812. Plasmid pET29a+ was extracted by DNA-spin TM plasmid purification kit where genes were inserted using BamHI and HindIII-HF. Cloned pET29a+dt and pET29a+dtb plasmids were transformed into *E. coli* BL21(DE3)PlysS as expression host. The identity of the sequences was validated by blasting the sequence (BLASTn) against all the reported nucleotide sequences in the NCBI (National Center for Biotechnology Information) GenBank. Production of proteins in high yield by different types and parameters of fermentation to determine optimal conditions. Lastly, the purified concentrated rdtx and rdtb were injected to BALB/c mice and antibody titers were detected.

Results: The genetic transformation of *E. coli* DH5α and *E. coli* BL21 with the pET-29a(+) carrying the dt and dtb genes was confirmed by colony polymerase chain reaction assay and were positive to grow on Luria-Bertani/kanamycin medium. The open reading frame of dt and dtb sequences consisted of 1,600 bp and 1,000 bp, were found to be 100% identical to dt and dtb sequence of *C. diphtheriae* (accession number KX702999.1 and KX702993.1) respectively. The optimal condition for high cell density is fed-batch fermentation production to express the rdtx and rdtb at 280 and 240 Lf/mL, dissolved oxygen was about 24% and 22% and the dry cell weight of bacteria was 2.41 g/L and 2.18 g/L, respectively.

Conclusion: This study concluded with success in preparing genetically modified two strains for the production of a diphtheria vaccine, and to reach ideal production conditions to achieve the highest productivity.

Keywords: *Corynebacterium diphtheriae*, Diphtheria toxin, Gene expression, Fermentation, Immunogenicity

Introduction

The technology to produce recombinant vaccines as hepatitis B vaccine has been available for more than 30 years. VACSERA (Egyptian Company for Production of Vaccines, Sera and Drugs) is the sole manufacturer of vaccine in Egypt and one of the very few vaccine manufacturers in the African and the Middle East regions. Diphtheria vaccines are DTaP (diphtheria, tetanus, pertussis), Tdap (tetanus, diphtheria, pertussis), Td (tetanus, diphtheria), and DT (diphtheria, tetanus). These vaccines are recommended in

Egypt, is not produced locally, and is still being imported. The safer and more effective, yet recombinant diphtheria vaccine is not even being imported and is not available on the Egyptian health market, it is a novel vaccine art which is promising to be high yield, less cost and undesired side effects. The ultimate objective is to achieve the pilot scale production of recombinant diphtheria vaccine as a step towards its industrial production in Egypt, and assure its production in sufficient volume to meet projected needs.

Diphtheria is an acute bacterial disease caused by toxigenic corynebacterial strains with a high case fatality rate. The diphtheria toxin (dt) is absorbed into the circulatory system, where it can cause systemic complications such as myocarditis and neuritis when disseminated. The dt gene is carried by a family of closely associated bacteriophages (corynebacteriophages) that can incorporate into the bacterial chromosome and convert non-toxigenic, non-virulent to toxic, and virulent. A dt is produced and secreted as a single polypeptide pro-enzyme that is cleaved and reduced *in vivo* to create a toxic protein with A and B fragments. The toxin's receptor binding and translocation domains are in the B subunit, and the binding of the toxin to particular cell surface receptors is the first step in the intoxication of eukaryotic cells by dt [1].

The dt was obtained for vaccination using *Corynebacterium diphtheriae* Park-Williams number 8 or its mutant strain. Chemical alteration converts the poison into a toxoid [2]. By supplying air to the surface of an agitation tank and using a medium of beef digested with papain, surface static culture, a simple method developed by Mueller and Miller [3] in 1941, can produce approximately 200 Lf/mL of toxin in 48 hours, instead of 100 Lf/mL of toxin in 48 hours.

Diphtheria is regulated by maintaining high herd immunity by vaccination as the first line of protection. While the incidence of diphtheria decreased significantly worldwide after the introduction of the diphtheria vaccine, it continues to be a major public health problem in many countries with poor routine vaccination coverage the World Health Organization [4].

Due to comprehensive research and developments in biotechnology, recombinant protein production has become more effective in recent years. The growing need for recombinant proteins in a variety of applications, including therapeutics [5] to the fine chemicals production [6] has necessitated the enhancement of various aspects of recombinant protein production. The most common host for non-glycosylated protein expression is *Escherichia coli* [7]. The well-studied genetics, quick growth rate [8], low nutritional requirement [9], ease of achieving high cell density [10], and likelihood of genetic reprogramming or rewiring the cell are all reasons for *E. coli*'s success as an expression host [11]. The factors that affect recombinant protein development in *E. coli* can be divided into two categories: expression-level factors and process-level factors. The detection and optimization of these causes, as well as other aspects of recombinant protein production in *E. coli* [12]. Furthermore, high-throughput screening and purification of recombinant proteins [13] or advanced technologies such as genetic and metabolic engineering and multi-omics approaches have significantly enhanced recombinant protein yield [14]. Even though these advanced technologies are used to boost the efficiency of recombinant protein production, the optimization strategy remains a top priority. The optimization of recombinant protein output is usually done at the gene expression or fermentation process stages, or with a combination of factors from both levels. To achieve high levels of toxin expression, the bacteria were cultured in a medium containing inorganic phosphate, which is absent in casein, and calcium chloride was added to induce calcium-phosphate precipitates in the medium [15].

Since nutrients, gases, and trace elements (if necessary) are introduced during microbial development, fed-batch processes primarily concentrate on raising biomass concentration and thus productivity, while minimizing problems encountered in high cell density cultivations [16,17]. The recombinant product's volumetric yield is determined by both biomass concentrations and the basic cellular product yield.

For biotechnological purposes, high yield recombinant protein processing is highly desirable. Several important central elements should be considered when developing recombinant expression conditions, including the expression strain, medium form, bioprocess optimization, and mathematical modelling. The cost and reproducibility issues can be addressed by well-designed industrial scale development of one recombinant protein with optimized influential parameters and yield [18].

In this research, we concentrated on the transfer of the dt and diphtheria toxin fragment B (dtb) genes from the strain *C. diphtheriae* Park William to *E. coli* and obtained the high-level fermentative expression of the recombinant dt and dtb genes in *E. coli* BL21, accompanied by purification, immunization, and characterization. The optimization of fermentation conditions and the screening of nutrients are equally essential for

the efficient recombinant protein production by recombinant DNA plasmid. This directly affects the downstream purification and final quality and recombinant protein yield from recombinant DNA plasmid.

Materials and Methods

Gene cloning and expression

Bacterial strains, vectors, and growth conditions

C. diphtheriae Park William strain (ATCC [American Type Culture Collection] 13812) was cultured at 35°C for 48 hours with shaking at 140 rpm in Linggood medium for vaccine production and source of the dt and dtb genes [19]. *E. coli* DH5α and *E. coli* BL21were used for transformation and protein expression after cloning of the dt and dtb genes using a bacterial expression system pET29a (+) vector (Novagen, Darmstadt, Germany). The cultures were routinely grown on Luria-Bertani (LB) agar prepared as follows: tryptone 1.0 g, yeast extract 0.5 g, sodium chloride 1.0 g, distilled water up to 100 mL, and Agar 1.5 g. The pH of medium was adjusted with 1 N NaOH. The medium was autoclaved at 121°C for 20 minutes. To maintain the original and recombinant *E. coli* strains LB broth was prepared without agar and the cells were held at 4°C until needed. Each of bacterial strain was inoculated separately in 5 mL LB medium and the cultures were incubated overnight at 37°C at 150 rpm shaking. After optical density $[OD]_{600}$ reached approximately 2.5, the cultures were mixed with an equal quantity of glycerol 70% (volume per volume [v/v]), dispensed into Eppendorf tubes (1.5 mL), and stored at -20°C freezer for future use.

Isolation of genomic DNA

The BYF DNA extraction i-genomic Mini Kit was used to extract DNA from *C. diphtheriae* (iNtRON Biotechnology Inc., Seongnam, Korea). According to Sambrook and Russell [20], qualitative and quantitative estimation of extracted DNA was achieved by reading the ultraviolet (UV)-absorbance at 260 and 280 nm with a spectrophotometer (Shimadzu model UV-240) to estimate the DNA quantity and purity.

PCR amplification of the dt and dtb genes

The dt gene was determined by using specific primers designed according to Mohammadi et al. [21] and Nascimento et al. [22], based on sequences of dt gene conserved regions. However, isolation of complete dt gene was carried out by using primers flanking sequences regions, designed according to the numerous dt sequences at the GenBank database. Primers were synthesized by automated DNA synthesizer. For polymerase chain reaction (PCR) tests, Ready-To-Go PCR Beads were used.

Except for the primer and DNA template, each bead contains all you need to run a 25-μL PCR amplification reaction. In this analysis, three separate pairs of primers were used. The first pair (dt) sequence was forward dt gene 5′-CGCGGATCCATGGGCGCTGATGATGTT-3′. Reverse dt gene 5′-CCCAAGCTTTCAGCTTTTGATTTCAAAAAATAGCG-3′. The second pair (dtb) sequence was forward dtb gene 5′-CGCGGATCCATA AATCTTGATTGGGATGTCATAA-3′. Reverse dtb gene 5′-CCCAAGCTTGCT TTTGATTTCAAAAAATAGCG-3′. Operon Technologies Company (Venlo, the Netherlands) provided all primers. The used primer (12 ng) and filtered DNA sample (40 ng) were applied to each PCR bead. Using sterile distilled water, the total volume of the amplification reaction was reached to 25 μL. The following was the amplification procedure: five minutes of denaturation at 95°C. Each of the 35 cycles is made up of the following segments: denaturation at 95°C for 1 minute; primer annealing at 60°C (dt) or 51°C (dtb) for 2 minutes; and DNA polymerization at 72°C for 2 minutes. Keep the PCR at 4°C until the end. The amplified DNA products were electroporated for around 2 hours on a 1.0% agarose gel with 1× TBE (Tris-borate-EDTA [ethylenediaminetetraacetic acid]) buffer at a constant 100 volt. The band sizes were calculated using a 100-bp ladder, and the separated bands were stained with 0.5 g/mL ethidium bromide and photographed with a gel documentation device with UV transilluminator.

Extraction of the mother and recombinant plasmids

DNA-spin TM Plasmid DNA Purification Kit (iNtRON Biotechnology Inc.) was used to extract the plasmid.

DNA purification of dt and dtb genes after electrophoresis

MEGAquick-spin TM Plus Total Fragment DNA Purification Kit (iNtRON Biotechnology Inc.) was used to purify the specific bands obtained after PCR amplification that were responsible for the dt and dtb genes.

Restriction digestion of dt and dtb genes fragments and plasmid pET 29a

Purified dt and dtb genes and isolated pET29a+ plasmid were subjected to restriction digestion reaction with BamHI and HindIII-HF restriction enzymes (New England BioLabs, Ips-

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wich, MA, USA). According to the manufacturer's instructions, 50 U of each New England BioLabs enzyme, 1× of NE Buffer 4 (recommended buffer for double digestion of BamHI and HindIII-HF), 10 μg DNA (fragments and plasmid) were used in a final volume of 100 μL and incubated at 37°C for 2 hours. After incubation, the digested fragments were purified using PCR cleanup protocol (gel extraction protocol without step of agarose gel running), and the digested plasmid was electrophoresed on 1.5% agarose gel and cut under long wave UV light, and gel slice was subjected to gel purification.

Insertion of PCR products into the cloning vector

Recovered dt and dtb genes were ligated with the linearized vector pET29a+ at BamHI and HindIII-HF recognition sites, using T4 DNA ligase (New England BioLabs). Ligation reaction was carried out in 20 μL volume with the vector insert ratio (1:1, 1:3, and 1:5) as follow: 500 U of T4 ligase, 1× ligation buffer with adenosine 5´-Triphosphate (ATP) (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT [dithiothreitol], 1 mM ATP, 25 μg/mL bovine serum albumin [BSA]), almost equal to 10 ng of the DNA (plasmid and insert), and incubated overnight at 16°C.

Bacterial transformation

Constructs of plasmid pET29a(+) was transformed into *E. coli* DH5α using Calcium-Chloride described by Sambrook and Russell [20]. The introduction of ligation mixture of pET29a(+) with dt and dtb genes to *E. coli* DH5α was done by heat shock. A ligation mixture of 10 μL was applied to 100 μL of $CaCl_2$-treated competent cells, mixed by tapping, and held on ice for 20 minutes. The mixture was then heated to 42°C for 90 seconds and then put on ice for 2–5 minutes before being added to 800 μL of pre-warmed LB medium and incubated at 37°C with slow shaking. On LB/kanamycin plates, different aliquots of these transformed competent cells were spread. After 1 hour, the plates were inverted and incubated overnight at 37°.

Screening for the positive colonies after transformation

Colony-PCR technique was used for screening the transformed positive colony that has the dt and dtb genes. PCR amplification was carried out using pET—dt and dtb genes specific primers. Colonies that at least 1 mm in diameter was picked for screening into 25 μL of PCR reaction mixture, which prepared as follows: 0.25 μL of Taq polymerase (5 U/μL), 2.5 μL of 10× Taq buffer, 0.25 μL of dNTPs (50 mM for each), 0.5 μL (10 pmol/μL) of each primer; using the following parameters: 94°C predenaturation for 5 minutes, followed 35 cycles of 40 seconds denaturation 94°C, 40 seconds annealing 57°C, 1.5-minute extension 72°C, and 10-minute final extension 72°C. Then, an aliquot of this amplification was visualized on 1% agarose gel. Sequences of pET specific primers and resulted PCR products, the first pair was forward primer (pET-F) 5´-CGTCCGGCGTAGAGGATC-3´ and the reverse primer (pET-R) 5´ ATCCGGATATAGTTCCTCCTTTC-3´. The resulting PCR products were contained of the insert gene plus 290 bp from the mother pET29a(+) plasmid.

Transformation of E. coli BL21 (DE3) pLysS and DNA sequencing

The plasmids were isolated from the positive transformed colony of *E. coli* DH5α using the DNA-spin Plasmid DNA Purification Kit (iNtRON Biotechnology Inc.), and the recombinant pET 29a(+) plasmids were transformed into expression host *E. coli* BL21 (DE3) pLysS (Novagen) using the calcium chloride transformation protocol mentioned previously with DH5α. In the same manner, the positive transformed colony was screened by colony PCR technique as above. The sequencing was done on both the DNA strands by universal pET29a(+) forward and reverse primers using ABI PRISM 3500XL DNA Sequencer (Applied Biosystems, Waltham, MA, USA).

Protein production

Optimization of conditions for high level of expression

To produce rdt and rdtb proteins, a single colony of *E. coli* BL21 (DE3) cells containing dt and dtb genes were incubated to grow overnight at 37°C with shaking 150 rpm shaker incubator in 50 mL LB medium supplemented with 50 μg/mL kanamycin. Thirty mL of overnight culture were inoculated in 3 L of modified terrific broth (TB) medium supplemented with 0.5 mg/mL kanamycin, modified TB [20]. The inoculated TB medium was incubated at 37°C with shaking at 250 rpm until the culture reach to mid logarithmic phase (OD_{600}=0.6). Then, 10 mL culture was transferred to microfuge tube for un induced control and the rest was induced overnight with 1 mM Isopropyl β-D thiogalactopyranoside (IPTG) with different fermentation condition according to Table 1.

Preparation and fractionation of cell extract

Bacterial cells were collected by centrifugation at 8,000 rpm for 20 minutes at 4°C, followed by a wash with 100 mM NaCl. The cell pellets were then re-suspended in 5 mL of lysis buffer. The pellets and buffer were mounted on ice for 30 min-

Table 1. Fermentation data sheet for rdt and rdtb

Item	Culture A	Culture B	Culture C
Agitation (rpm)	700	200–700	700
Air flow (L/hr)	5	3–5	5
pH	6.8 was adjusted by 25% NH_4OH	6.8 was adjusted by 25% NH_4OH	6.8 was adjusted by 25% NH_4OH
Duration of culture (hr)	24	27	36
Agitation/air/DO	No cascade	Agitation/air/DO cascade	No cascade
Type of fermentation	Batch fermentation	Batch fermentation	Fed-batch fermentation with 500 mL 40% glucose and 10% yeast extract with rate 2 mL/min

DO, dissolved oxygen.

utes before being sonicated in ice using an ultrasonicate set to 25 kHz with amplification of 50% in 5 times 20-second bursts. Cell debris was collected by centrifugation at 15,000 rpm for 30 minutes at 4°C. As a cell-free extract, the soluble supernatant was transferred to new tubes.

Purification using ammonium sulphate precipitation

A pilot study determines the ammonium sulphate concentration between which the bulk of the toxoid will precipitated (precipitation curve). Thereafter, dt was centrifuged at 6,700 g for 3 minutes to remove flocculates. An x volume of different percentages of saturated ammonium sulphate; 22%, 25%, 28%, 30%, 34%, and 37% was added drop wise to parallel dt, while rotating the tube on ice for 90 minutes to precipitate whole dt molecules. The precipitate was dissolved in volume of phosphate-buffered saline (PBS) by adding the PBS drop wise. Lime flocculation (LF) for each sample determined and plotted against the ammonium sulphate concentration. The whole toxin was reprecipitated using a saturated ammonium sulphate with a concentration of 20% to 40% by adding the saturated ammonium sulphate drop wise to each tube and rotating for 1–2 hours on ice. The precipitate was centrifuged at 6,700 g for 5 minutes, then, each precipitate was washed for 1 hour at 0°C with the same ammonium sulphate solution used for precipitation and centrifuged at 6,700 g for 5 minutes. After sedimentation, the precipitate in each tube was dissolved in the original volume of PBS slowly and without vortex. And dialyzed against 1,000 V of PBS at least 3–4 changes over 24–48 hours at 4°C. Purity of the dt produced by different percentages of saturated ammonium sulphate was examined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) after detoxification by 0.6% formaldehyde and dialysis. The highest overall yield with approximate purity was chosen. The protein content was assessed and whole toxin divided into suitable aliquots and kept frozen at -20°C [23].

Assay of lime flocculation

The Ramon method was used to determine the LF value of the toxin solution. One LF unit is known as the amount of toxin that reacts with one unit of anti-toxin using this method [24].

Immunization of mice

The animal facility of (VACSERA) provided females 8 weeks old BALB/c mice and weighing 15–18 g, who were kept under aseptic conditions at 25°C, 12 hours of light per day, sterilized pelleted food, and sterile water. The BALB/c mice were divided into five classes, each with six mice. For the first injection, CFA was emulsified in equal volumes with rdt, rdtb, and ST dtx (as positive controls), formalin dtx, and saline (as negative controls). For the second injection, ICF (immune-mediated cancer field) was emulsified in equal volumes with rdt, rdtb, and ST dtx (as positive controls), formalin dtx, and saline (as negative control), the creamy white emulsion was made by vertexing a 1:1 adjuvant and antigens mixture vigorously. The third injection, on the other hand, was given without any adjuvant. The time between three injections was 2 weeks [25]. Seven days after each injection, post-immunization sera were collected. All three injections were given at a dose of 10 μg/100 μL to each party. The total dose was 200 μL intraperitoneally injected [26]. Blood was taken from the tails of BALB/c mice. Sera were collected by centrifugation at 3,000 rpm at room temperature for 15 minutes and stored at -70°C.

Detection of rdt and rdtb specific antibody by ELISA

The anti-immunoglobulin G (IgG) antibodies that have been adsorbed to the surface of polystyrene microtiter wells react with the IgG present in the samples in this assay [27]. Anti-IgG antibodies conjugated with horseradish peroxidase are applied after washing to remove unbound proteins. These en-

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zyme-labeled antibodies bind to the previously bound IgG and form complexes. The enzyme bound to the immunosorbent is measured using a chromogenic substrate, 3,3´,5,5´-tetramethylbenzidine (TMB), after another washing phase (TMB). The amount of bound enzyme varies directly with the amount of IgG in the sample being tested; hence, the absorbance at 450 nm is a measure of the amount of IgG in the test sample. The amount of IgG in the test sample can be extrapolated from the standard curve generated from the norms, and sample dilution can be taken into account, 100 μL of Standard 0 (0.0 ng/mL) in duplicate was added to enzyme-linked immunosorbent assay (ELISA) plate, standard 1 (9.38 ng/mL), standard 2 (18.75 ng/mL), standard 3 (37.50 ng/mL), standard 4 (75 ng/mL), standard 5 (150 ng/mL), standard 6 (300 ng/mL), and standard 7 (600 ng/mL); 100 μL of rdt, rdtb, and ST dtx (as positive control) and Formalin dtx and saline (as negative control) sample into pre-designated wells. The contents of the wells were aspirated and washed 4 times after the microtiter plate was incubated at room temperature for 60 minutes. Every well received 100 μL of diluted enzyme antibody conjugate, which was incubated at room temperature for 30 minutes while the plate was kept covered in the dark and level, and then washed 4 times. Each well received 100 μL of TMB substrate solution, which was incubated in the dark at room temperature for 10 minutes before receiving 100 μL of stop solution. The absorbance of the contents of each well was measured at 450 nm.

Statistical analysis

Differences in the antibody level for groups of each type of inbred mice strain were compared. Humeral and cellular immune response were compared between the inbred mice groups by using (independent samples T-test). Average means and standard deviation were also determined by using Microsoft Excel software (Microsoft Corp., Redmond, WA, USA). All p-value <0.01 were considered statistically significant.

Ethics statement

The animal studies were performed after receiving approval of the Institutional Animal Care and Use Committee in Cairo University (IACUC approval no., 62, 05/2016).

Results

There is significant interest in cloning of the dt and dtb genes to approach of commercial recombinant diphtheria vaccine production. At the beginning of this study, DNA was extracted and then the PCR was used to amplify the genes under study, then the cloning of these genes on a suitable genetic vector. At the end, this vector was introduced into the *E. coli*

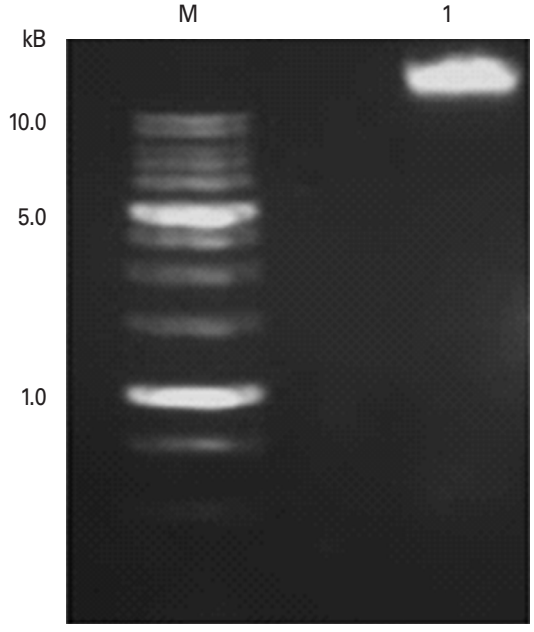

Fig. 1. Extraction and detection of the complete bacterial genomic DNA of *Corynebacterium diphtheriae* (lane 1). Lane M, DNA size marker (Tiangen Biotech Co. Ltd., Beijing, China).

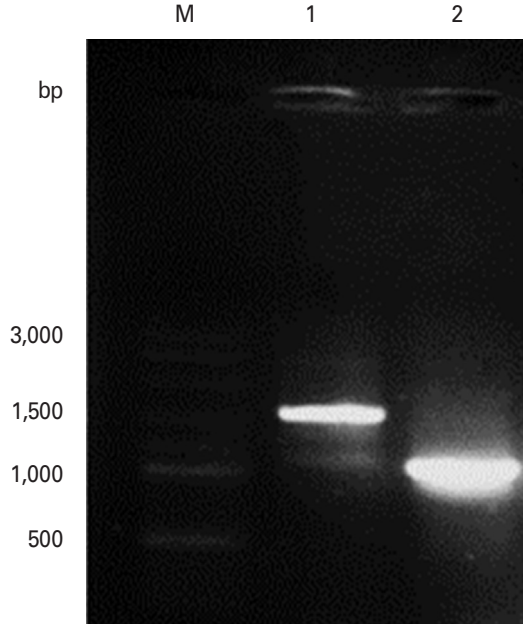

Fig. 2. *Corynebacterium diphtheriae*, diphtheria toxin (dt) gene polymerase chain reaction amplified profile on an agarose gel (lane 1), diphtheria toxin fragment B gene (dtb) (lane 2). Lane M, DNA size marker (Vivantis # NL 1407; Vivantis, Shah Alam, Malaysia).

bacteria to the study of gene expression, detection of protein products for these genes, and their production at the semi-industrial level. Immunological evaluation and production of antibodies to these protein products of these genes were also performed in mice.

Gene cloning

Amplification and detection of dt and dtb genes after genomic DNA extraction

The bacterial genomic DNA from *C. diphtheriae* strain was extracted and separated by agarose gel electrophoresis as shown in Fig. 1. The obtained results indicated that two genomic DNA samples of *C. diphtheriae* strain lane 1 gave one band upper 10 kB without any smear DNA. So, the above result exhibited that the genomic DNA was highly purified and un-fragmented. The dt and dtb genes were detected and amplified by direct PCR. Fig. 2 showed that the obtained PCR products representing the dt and dtb genes were approximately 1,600 bp and 1,000 bp, respectively.

Cloning of purified pET29a(+) vector and dt and dtb genes

DNA-spin TM Plasmid DNA Purification Kit (iNtRON Biotechnology Inc.) was used to isolate and purify plasmid vector from *E. coli* containing pET29a(+), and the purified pET29a(+) plasmid vector was used to clone the dt and dtb genes obtained from *C. diphtheriae* to construct the recombinant vector that was used. To prepare the linear vector, a double restriction digestion reaction using restriction enzymes BamHI and Hind III-HF was performed on the pET29a(+) plasmid vector, and then the linear vector was purified using a PCR cleanup kit. The purified dt and dtb genes were also subjected to a double restriction digestion reaction using the restriction enzymes BamHI and Hind III-HF (New England BioLabs). After restriction digestion and purification of the dt and dtb genes with a PCR cleanup kit, the dt and dtb genes were ligated with plasmid pET29a(+) using T4 DNA ligase (New England BioLabs). The ligated recombinant vectors were then transformed into competent *E. coli* DH5α cells, and the *E. coli* DH5α carrying the recombinant vectors with dt and dtb inserts was immune to kanamycin at 50 g/mL. The recombinant vectors containing dt and dtb genes and the purified linear vector after extraction from *E. coli* DH5α competent cells was loaded to agarose gel to examine its molecular weight.

The obtained results indicated that the mother pET29a(+) plasmid lane 1 gave one band upper 10 kB but the recombinant vectors containing dt and dtb genes gave two bands (Fig. 3). It was found that the level of the bands of the recombinant vectors containing dt and dtb genes (lanes 2 and 3) were higher than the mother plasmid (lane 1), and the bands were found in recombinant vector containing dtb gene (lane 3) was higher than recombinant vector containing dtb gene (lane 2), due to the large dt gene size in comparison with dtb gene.

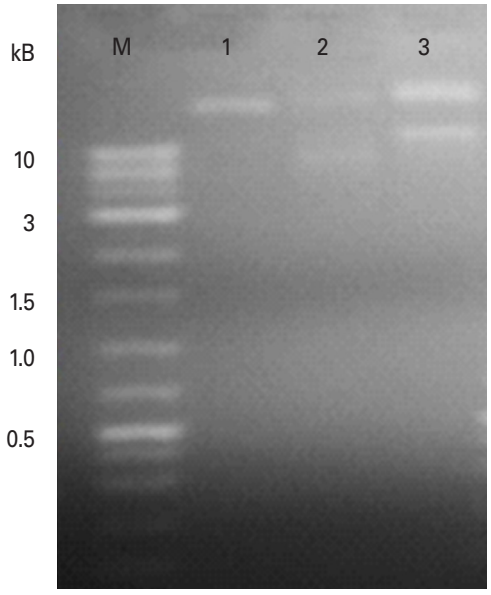

Fig. 3. Agarose gel electrophoresis for of pET 29a(+) plasmid (lane 1), pET29a-diphtheria toxin fragment B (dtb) (lane 2), and pET29a-diphtheria toxin (dt) (lane 3) constructs obtained after transformation of *Escherichia coli* DH5α strain beside of DNA size marker (lane M) (iNtRON Biotechnology Inc., Seongnam, Korea).

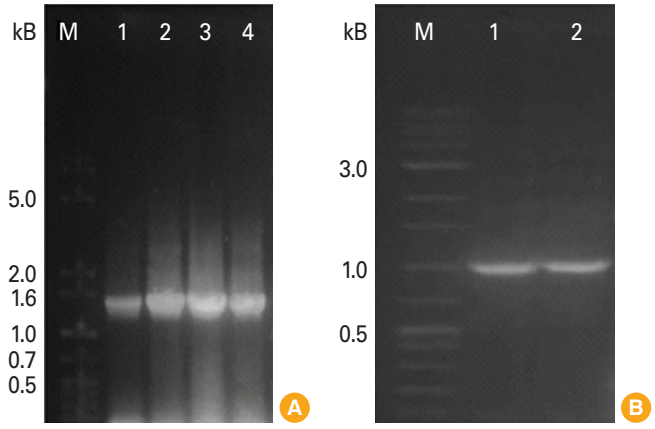

Fig. 4. (A) Colony-polymerase chain reaction (PCR) for pET29a-diphtheria toxin (dt) construct in DH5α strain (lanes 1 and 2). Lane M, DNA size marker (Tiangen Biotech Co. Ltd., Beijing, China). (B) Colony-PCR for pET29a-diphtheria toxin fragment B (dtb) construct in DH5α strain (lanes 1 and 2). Lane M, DNA size marker (iNtRON Biotechnology Inc., Seongnam, Korea).

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Confirmation of the successful cloning in E. coli DH5α

After the DNA was extracted from *E. coli* DH5α colonies containing recombinant pET29a-dt and pET29a-dtb plasmids were confirmed by colony-PCR technique. The purified construct was tested using a PCR reaction to amplify the fragment containing the dt and dtb genes' cloning sites against a DNA ladder. Fig. 4 displays the PCR results of two recombinant *E. coli* DH5α strains carrying pET29a-dt and two recombinant *E. coli* DH5α strains carrying pET29a-dtb. After the gel was examined using UV transilluminator it showed that the dt gene amplified by PCR showed a strong band on 1,600 bp as shown in Fig. 4A while that dtb demonstrated positive amplification at molecular size 1,000 bp as shown in Fig. 4B. The obtained results suggested that the desired genes are presented in genetically engineered strains of *E. coli* DH5α. Also, the genes were the ideal molecular weight size, as previous studies reported.

Propagation of pET29a-dt and pET29a-dtb in E. coli BL21

Recombinant pET29a(+) plasmid isolated from *E. coli* DH5α and harboring dt and dtb genes were transformed into *E. coli* BL21 competent cells then spread over LB/kanamycin media plate. One aliquot of *E. coli* BL21 competent cells transformed with pET29a-dt was spread over LB/kanamycin media plate and an aliquot of normal *E. coli* BL21 competent cells aliquot spread over LB/kanamycin media plate as a negative control. Media plates were incubated overnight. The obtained results exhibited that no colony in LB/kanamycin media plate spread with the *E. coli* BL21 competent cells, because the *E. coli* BL21 competent cells did not harbor pET29a(+) plasmid carrying the kanamycin resistant gene. While the *E. coli* BL21 competent cells transformed with pET29a-dt and pET29a-dtb gave many colonies in LB medium containing 50 μg/mL of kanamycin. So, the *E. coli* BL21 competent cells transformed with pET29a-dt and pET29a-dtb acquired kanamycin resistance.

Confirmation of successful cloning in E. coli BL21

The DNA was extracted from the *E. coli* BL21 colonies which containing recombinant pET29a-dt and pET29a-dtb plasmids by colony-PCR technique. The purified construct was tested using a PCR reaction to amplify the fragment containing the dt and dtb genes' cloning sites against a DNA ladder. The PCR analysis of two recombinant *E. coli* BL21 strains which containing pET29a-dtb and four recombinant *E. coli* BL21 strains which containing pET29a-dt was shown in Fig. 5. After the gel was examined using a UV transilluminator it showed that the dt fragment amplified by PCR showed a strong band on approximately 1,600 bp while that dtb demonstrated positive amplification at a molecular size of approximately 1,000 bp. The obtained results suggested that the desired genes are presented in genetically engineered strains of *E. coli* BL21. Also, the genes were the ideal molecular weight size, as previous studies had decided.

Amplification the dt and dtb genes using specific primers of pET29a(+) plasmid

Colony-PCR was performed for bacterial colonies grown on

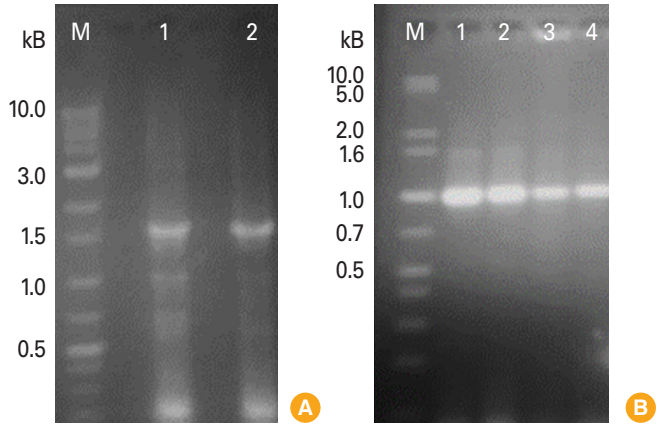


Fig. 5. (A) Colony-polymerase chain reaction (PCR) for pET29a-diphtheria toxin (dt) construct in BL21 strain (lanes 1 and 2). Lane M, DNA size marker (iNtRON Biotechnology Inc., Seongnam, Korea). (B) Colony-PCR for pET29a-diphtheria toxin fragment B (dtb) construct in BL21 strain (lanes 1 and 2). Lane M, DNA size marker (Tiangen Biotech Co. Ltd., Beijing, China).

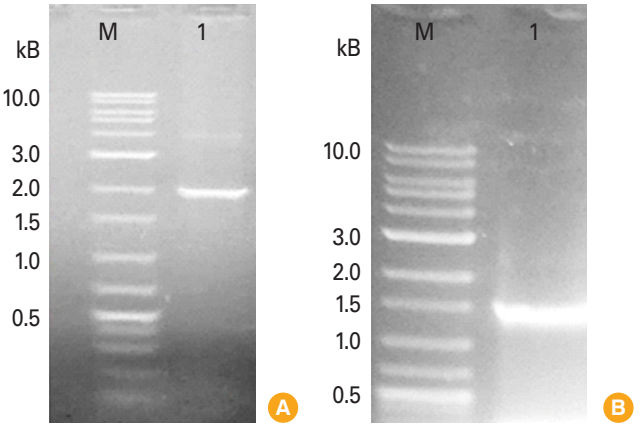
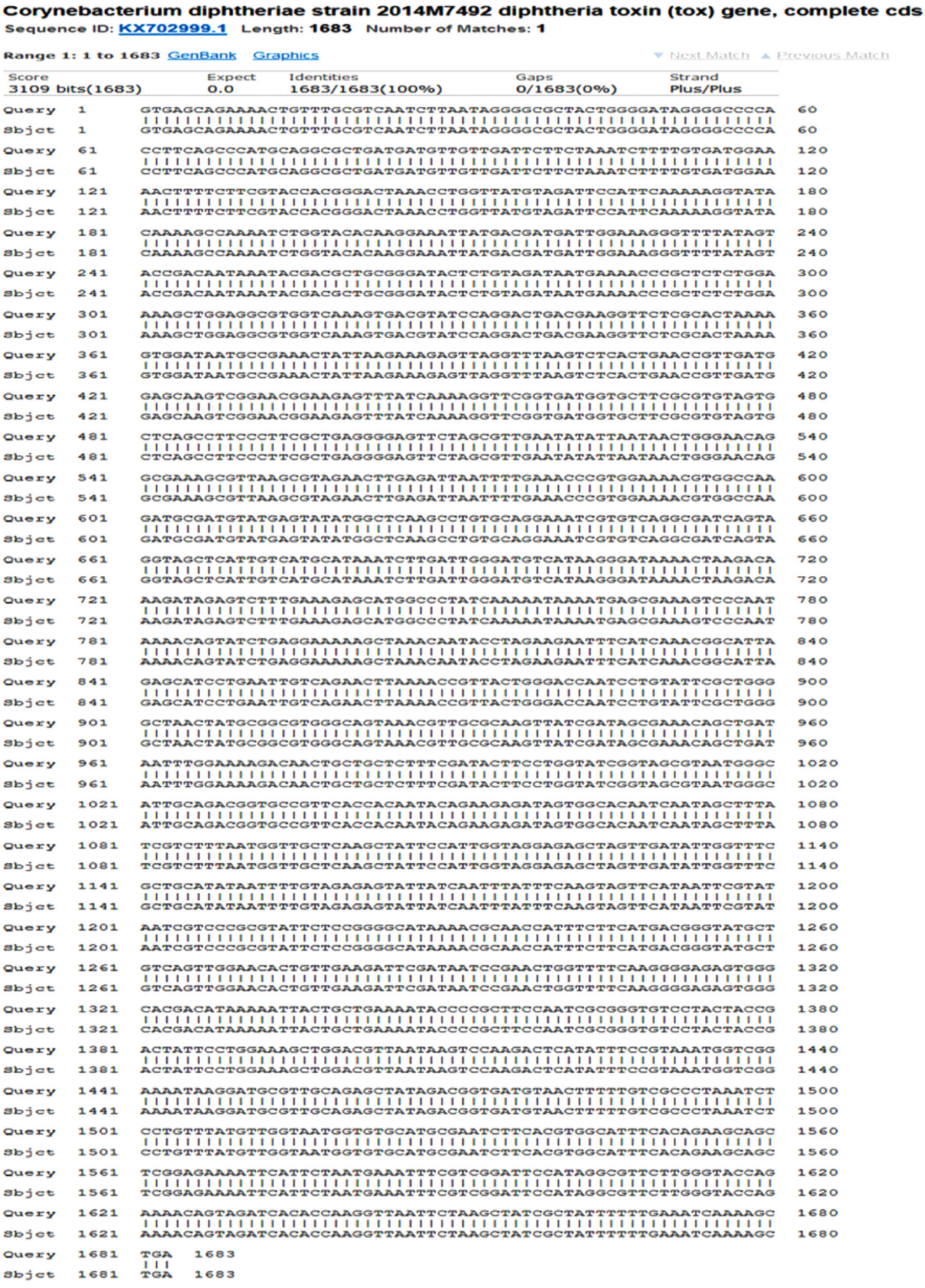


Fig. 6. (A) Polymerase chain reaction (PCR) analysis of pET29a-diphtheria toxin (dt) construct extracted from BL21 strain with plasmid specific primers (lane 1). Lane M, DNA size marker. (B) PCR product for pET29a-diphtheria toxin fragment B (dtb) construct in BL21 strain with plasmid specific primers (lane 1). Lane M, DNA size marker (iNtRON Biotechnology Inc., Seongnam, Korea).



Corynebacterium diphtheriae strain 2014M7492 diphtheria toxin (tox) gene, complete cds

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Range 1: 1 to 1683 GenBank Graphics Next Match Previous Match

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Query  1681  TGA  1683
Sbjct  1681  TGA  1683
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Fig. 7. The NCBI (National Center for Biotechnology Information) Blast of diphtheria toxin (dt) sequence in relation to *Corynebacterium diphtheriae* (accession number KX702999.1).

 https://doi.org/10.7774/cevr.2022.11.1.12

antibiotic (kanamycin) containing medium and the plasmid specific primers to detect the right position of the genes on the plasmid. The colony of pET29a-dt was found positive amplification at 1,973 bp with plasmid specific primers (Fig. 6A). Likewise, colony was recorded in plate pET29a-dtb since they demonstrated positive amplification at molecular size 1,337 bp with the plasmid specific primers (Fig. 6B). These obtained fragments can be used to perform DNA sequencing analysis so that the sequence of the complete gene can be obtained without any deficiency.

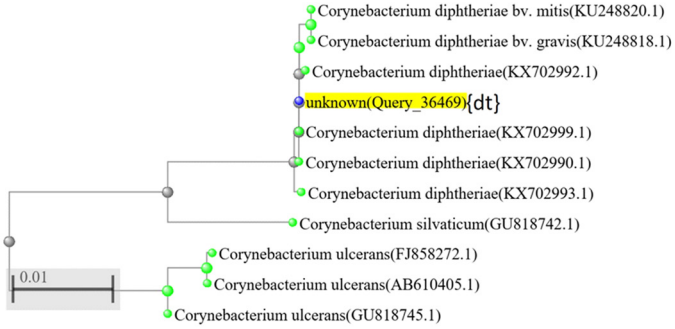


Fig. 8. The NCBI (National Center for Biotechnology Information) Neighbor Joining of diphtheria toxin (dt) in comparison with other sequences.

DNA sequencing of dt and dtb genes using specific primers of pET29a(+) plasmid

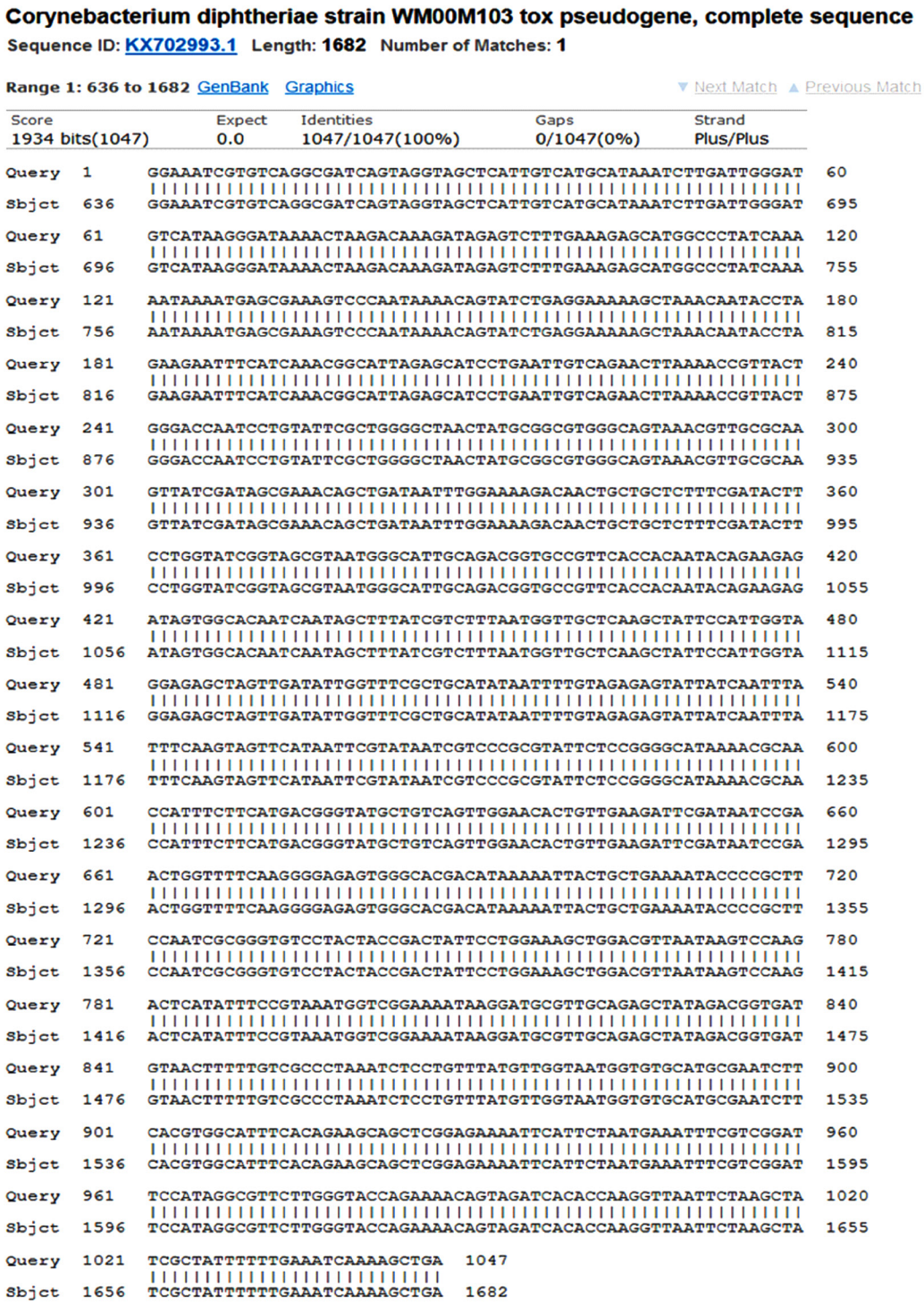
DNA-spin Plasmid DNA Purification kit (iNtRON Biotechnology Inc.) was used to isolate and purify the recombinant plasmids (pET29a-dt and pET29a-dtb). The ABI PRISM 3500XL DNA Sequencer was used to sequence both DNA strands using universal pET29a(+) forward and reverse primers (Applied Biosystems). The dt sequence (saved in GenBank under accession number MW833977) is shown in Fig. 7 and the open reading frame consisted of 1,683 bp. Sequences obtained were analyzed for variability or homogeneity through National Center for Biotechnology Information (NCBI) Blast and the phylogenetic tree was drown as shown in Fig. 8. The DNA sequence of dt was found to be 100% identical to the sequence of *C. diphtheriae* strain 2014M7492 dt (tox) gene (accession number KX702999.1).

Moreover, the dtb sequence is shown in Fig. 9 and the open reading frame consisted of 1,047 bp. Sequences obtained were

Table 2. The growth and production of dtx in batch fermentation and rdtx and rdtb in batch and fed batch fermentations

Culture type	Bacterial purity	Volume of 25% NH_4OH (mL)	Agitation	Final dissolved oxygen (%)	Dry cell mass (g/L)	Optical density at 600 nm	Time	LF value (Lf/mL)	Protein nitrogen content (mg/mL)
Dtx: batch fermentation	++	100	200	18	3.12	2.1	At the end of cultivation	170	0.6
							After ultrafiltration	1,500	5.7
							After purification	1,100	4.2
rdtx A: batch fermentation	++	80	700	16	2.7	1.8	At the end of cultivation	200	-
							After ultrafiltration	1,800	-
							After purification		-
rdtx B: batch fermentation with cascade	++	68	200–700	30	2.95	2.07	At the end of cultivation	230	-
							After ultrafiltration	2,150	-
							After purification		-
rdtx C: fed batch fermentation	++	125	700	24	3.65	2.41	At the end of cultivation	280	1.12
							After ultrafiltration	2,700	9.5
							After purification	1,900	7.0
rdtb A: batch fermentation	++	75	200	14	2.4	1.73	At the end of cultivation	170	-
							After ultrafiltration	1,400	-
							After purification		-
rdtb B: batch fermentation with cascade	++	90	200–700	27	2.8	1.85	At the end of cultivation	200	-
							After ultrafiltration	1,600	-
							After purification		-
rdtb C: fed batch fermentation	++	115	700	22	3.06	2.18	At the end of cultivation	240	0.87
							After ultrafiltration	2,100	7.5
							After purification	1,300	4.8

LF, lime flocculation.



Corynebacterium diphtheriae strain WM00M103 tox pseudogene, complete sequence

Sequence ID: KX702993.1 Length: 1682 Number of Matches: 1

Range 1: 636 to 1682 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
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Fig. 9. The NCBI (National Center for Biotechnology Information) Blast of diphtheria toxin fragment B (dtb) sequence in relation to *Corynebacterium diphtheriae* (accession number KX702993.1).

analyzed for variability or homogeneity through NCBI Blast and the phylogenetic tree was drown as shown in Fig. 10. The DNA sequence of dtb was found to be 100% identical to the sequence of *C. diphtheriae* strain WM00M103 tox pseudogene (accession number KX702993.1).

Protein production

Expression and production of rdt and rdtb protein using pilot scale

Fig. 11 depicts a standard schematic fermentation process in a 5 L fermenter at a pilot scale (New Brunswick) to generate

 https://doi.org/10.7774/cevr.2022.11.1.12

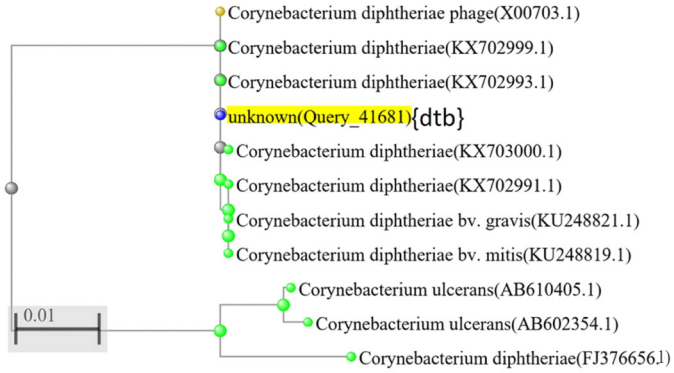

Fig. 10. The NCBI (National Center for Biotechnology Information) Neighbor Joining of diphtheria toxin fragment B (dtb) in comparison with other sequences.

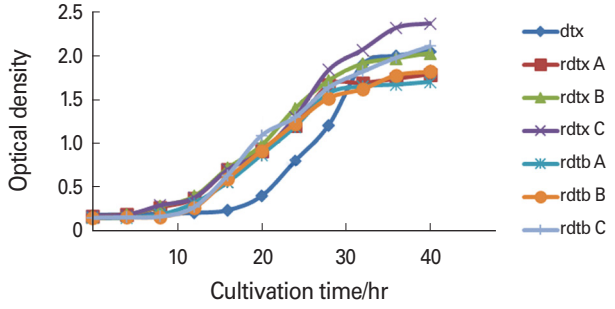

Fig. 11. Growth kinetics of *Corynebacterium diphtheria* and *Escherichia coli* BL21 in the three different fermentation condition.

dtx, rdtx, and rdtb protein using adjusted TB medium. The OD (cell dry weight) of the dtx culture increased after 16 hours of growth, while the expression of the recombinant protein increased after 12 hours of fermentation. The real growth rate began to stabilize at 36 and 40 hours for rdtx and rdtb, respectively, when the fermentation phase was completed. During the fermentation process, the levels of rdtx and rdtb expression as well as protein production increased, peaked, and then declined. The best operating conditions for producing rdtx and rdtb protein on a pilot scale were investigated and compared to conventional bacterial fermentation. Determine the best method of fermentation and the best growing conditions for producing more protein and biomass. The highest recombinant protein production was obtained in culture C, which was a batch culture followed by a fed batch using glucose and yeast extract as an additional carbon source to achieve high cell densities. During cultivation at 600 OD illustrated in Fig. 11, biomass was measured. The highest LF values concerned for batch C fermentation production of rdt and rdtb were 280 and 240, respectively, and higher than dtx LF value which was

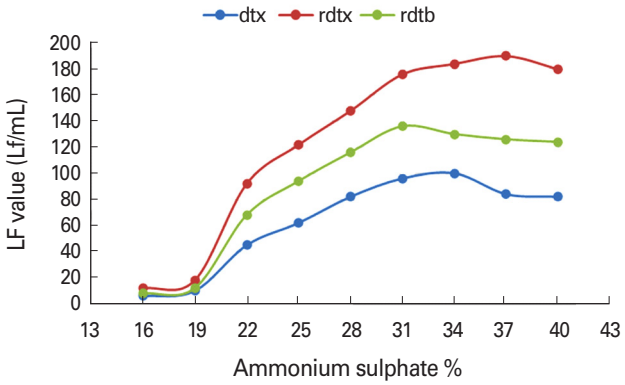

Fig. 12. Lime flocculation (LF) value of dtx, rdtx, and rdtb after ammonium precipitation.

170 that proved batch C fermentation conditions were the optimum to produce highest immunogenic yield protein. At the end of each culture of fermentation, the cultures were harvested. One L centrifuged and pellets of dt culture and batches A, B, and C of rdtx and rdtb cultures were dried at 60°C for 48 hours. The dry cell mass of dt culture was 3.1 g/L and the volume of supernatant was 2,900 mL; the dry cell mass of rdt batch A was 2.7 g/L and the volume of supernatant was around 2,930 mL; the dry cell mass of rdt batch B was 2.95 g/L and the volume of supernatant was 2,875 mL; and the dry cell mass of rdt batch C was 3.65 g/L Batch A, B, and C had rdtb dry mass of 2.4, 2.8, and 3 g/L, respectively, and the supernatant were 2,920, 2,870, and 2,900, the results were shown in Table 2. Different volumes of 25% NaOH were used in each culture to maintain pH 6.8 till end of culture. Agitation varies from 200 to 700 rpm and final dissolved oxygen differ in cultures according to cascade with air supply and agitation as shown in Table 2.

Ammonium precipitation curve of dtx, rdtx and rdtb after purification

According to pilot test, dtx, rdtx, and rdtb were precipitated between ammonium sulphate concentrations 22%–34%, 22%–37%, and 22%–31%, respectively. LF values of dtx, rdtx batch C, and rdtb batch C after purification and concentration were 1,500, 2,700, and 2,100 Lf/mL (Fig. 12).

Protein content determination and LF value

The antigenic purity of each batch of toxin depends entirely on the LF and the protein nitrogen content of the toxin. The antigenic purity of the recombinant dt and dtb were estimated and found to be in a suitable range to the obtained from *C.*

diphtheriae. The total protein content of dtx, rdtx, and rdtb produced from different cultures were estimated by Lowery method using BSA as standard (Table 3) and it was observed that the rdtx produced from culture C (1.12 mg/mL) contain double the protein level of the dtx (0.6 mg/mL) while rdtb produced in culture C was 0.87 mg/mL. Concentration using ultrafiltration increase protein nitrogen content about 10 times, while during purification using ammonium sulphate precipitation protein nitrogen content reduced about 25% as illustrated at Table 4.

Evaluation of dtx, rdtx, and rdtb purification

The titer in terms of protein content (100 μg/100 μL) correlates with the intensity of the dtx protein band (58 kDa) observed on SDS-PAGE. Individual protein molecules lost their identity and formed wide molecular complexes within broad molecular mass limits after being exposed to formaldehyde, the detoxifying agent; the toxoid displayed diffused banding in accordance with this (lane 5). *C. diphtheriae* Park William strain was cultured on Linggood media (production media) (lane 2) evaluation of concentration and purification with ammonium sulphate precipitation of dtx presented on lane 1 and rdtx in lane 3 and 4 (Fig. 13). While the rdtb-clarified from lysate of *E. coli* cells was purified with ammonium sulphate precipitation and rdtb-concentrated in was submitted to analysis by SDS-PAGE silver stain (Fig. 14).

Table 3. Lime flocculation value of dtx, rdtx, and rdtb batches

Dt type		Lime flocculation value (Lf/mL)				
	Batch culture	Primary culture	18-hr culture	At the end of culture	After ultra filtration	After purification
Dtx		20	55	170	1,500	1,100
Rdtx	Batch A	22	25	200	1,800	-
	Batch B	22	26	230	2,150	-
	Batch C	22	28	280	2,700	1,900
Rdtb	Batch A	18	23	170	1,400	-
	Batch B	18	26	200	1,600	-
	Batch C	18	28	240	2,100	1,300

Dt, diphtheria toxin.

Table 4. Comparison of protein nitrogen content for production, purification, and concentration of dtx, rdtx, and rdtb

Dt type	Protein content		
	At the end of culture (μg/mL)	After ultrafiltration (mg/mL)	After purification (mg/mL)
Dtx	600	5.7	4.2
rdtx Batch C	1,120	9.5	7.0
rdtb Batch C	870	7.5	4.8

Dt, diphtheria toxin.

Immunogenicity

In the first, second, and third injections, the findings showed that vaccinated groups had significantly higher IgG titers than control groups. For both immunized types, there were no sub-

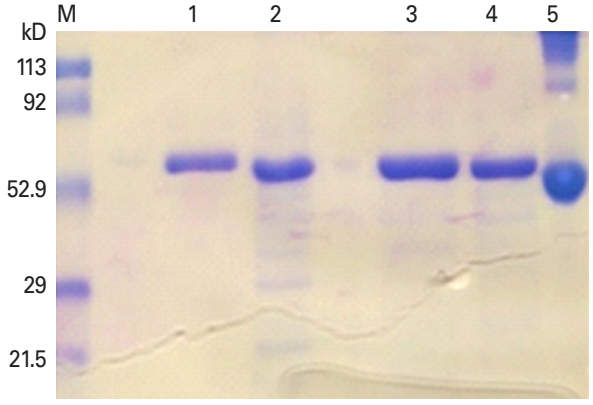

Fig. 13. Evaluation of the production and purification of dtx and rdtx using ammonium sulphate precipitation. The dtx demonstrating band of similar molecular weight 58 kDa (lane 1), crude dtx in Linggood medium (lane 2), purified and concentrated rdtx (lane 3 and 4) as compared with dtxd standard Toxoid shows diffused band (lane 5) and (lane M) molecular weight marker (Bio-Rad Cat. No. 1610305; Bio-Rad, Hercules, CA, USA).

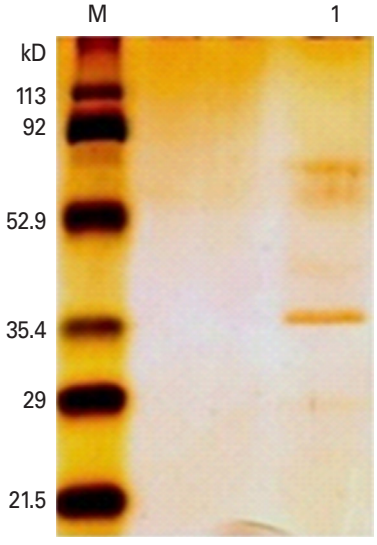

Fig. 14. Silver-stained SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis, 12%) of the purified concentrated rdtb. Lane M, molecular weight marker (Bio-Rad Cat. No. 1610305; Bio-Rad, Hercules, CA, USA); lane 1, purified concentrated rdtb at 37 kD.

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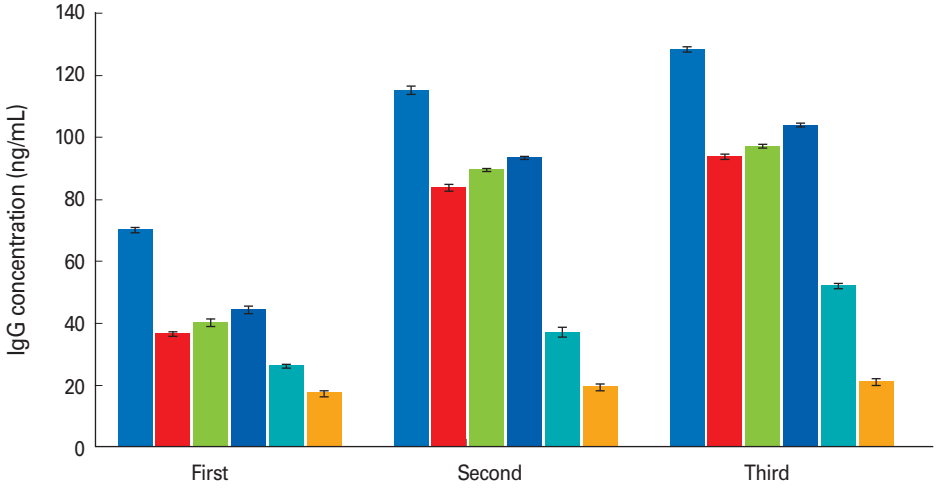

Fig. 15. Effect of boosting on the resulting immunoglobulin G (IgG)-antibody titer. BALB/c mice were immunized intraperitoneal with 200 μL with phosphate-buffered saline (negative control), rdtb (10 μg/100 μL), rdtx (10 μg/100 μL), St-dtx (10 μg/100 μL) (positive control), F dtx (10 μg/100 μL), and DT (10 μg/100 μL) on day 0, 14, 28 and bled on 7, 21, 35. Total IgG titers were determined by the enzyme-linked immunosorbent assay and are expressed as mean±standard deviation.

stantial variations in titers between the second and third injections, although there were significant differences between the first and second or third injections. ELISA was used to determine the basic immune response in terms of total IgG. Between the vaccinated and control groups, there was a significant difference in titer ($p<0.01$). Fig. 15 shows that total IgG for rdtx is higher than rdtb and ST dtx, but with a substantial difference from control and less than DT commercial vaccine, with no significant difference from F dtx.

Discussion

This research was carried out to characterize some of the genes responsible from *C. diphtheriae* Park William strain to develop of the recombinant diphtheria vaccines. The genomic DNA was initially isolated from *C. diphtheriae*. The isolated DNA quantity was defined to be adequate and pure to carry out subsequent studies on isolated DNA samples based on agarose gel electrophoresis and spectrophotometer experiments. In addition, direct PCR detected and amplified the dt and dtb genes. The molecular weight of PCR products representing both the dt and dtb genes was approximately 1,600 bp and 1,000 bp, respectively. These findings were consistent with several previous studies by Mohammadi et al. [21] and Nascimento et al. [22] in which the same molecular weights of the dt and dtb genes were confirmed.

In this work, the important stage was to load the dt and dtb genes into the plasmid vector pET29a(+) and then make a genetic transformation of *E. coli* and determine the occurrence of the genetic transformation with the recombinant vector. In two phases, the genetic transformation process was carried out. First, the transformation of *E. coli* DH5α, for the purpose of plasmid multiplication, was performed and then the genetic transformation of *E. coli* BL21 with the objective of genetic expression of the dt and dtb genes under study, was carried out. In an experiments after the genetic transformation of *E. coli* DH5α and *E. coli* BL21, it was confirmed that the pET29a(+) carrying the dt and dtb genes, detected by colony PCR technique. At the LB/kanamycin media, the transformed *E. coli* DH5α and *E. coli* BL21 were positive for growth. These findings have shown that the progress of the genetic transformation and gene transfer experiences under research has been observed and is also compatible with several previous studies, such as Mohammadi et al. [21], Nascimento et al. [22], and Romaniuk et al. [28].

In addition, the amplification of the dt and dtb genes using specific pET29a(+) plasmid primers explained that positive amplification was observed in the pET29a- dtb colonies at 1,337 bp with plasmid-specific primers and colonies were reported in the pET29a-dt plate as they showed positive amplification with the plasmid-specific primers at molecular size 1,973 bp. This means that, as previous studies have determined, the genes were at the optimal size plus 290 bases of the mother plasmid vector and the obtained fragments can be used without any deficiency to perform DNA sequencing analysis to sequence the complete used genes. The sequencing was

also carried out by universal pET29a(+) forward and reverse primers using ABI PRISM 3500XL DNA Sequencer on both DNA strands (Applied Biosystems). The dtb sequence's open reading frame consisted of 1,047 bp and was found to be 100% like the tox pseudogene of *C. diphtheriae* strain WM00M103 (accession number KX702993.1). The open reading frame of the dt sequence consisted of 1,683 bp and was found to be 100% dt (tox) gene of *C. diphtheriae* strain 2014M7492 (accession number KX702999.1). The previous findings are in line with the results of previous research [21,22,29]. Despite the various methods and strains used, the purpose of this research is consistent with previous studies.

On the other hand, *C. diphtheriae* strain used for production of dtx was PW8 CN 2000 which contains *tox* ω^+ Phage in agreement with Wahby et al. [30]. LF value is the first international reference reagent used for evaluation of culture and production of dtx as described by Preneta-Blanc et al. [31], it was about 200 Lf/mL at the end of culture as expected and in concordance with Sundaran et al. [32]. The purification of crude dtx (culture filtrate) was performed by stepwise ammonium sulphate precipitation at concentration ranging from 25% to 34% according to Stefan et al. [33]. Different acrylamide gel concentrations were used for detection of the largest MW range for the examined protein samples, one migrating band appeared at MW of 58 kDa (the expected MW of dtx molecule) and compared with standard dtx. Since dtx contains some sensitive sites that react very easily with formaldehyde, even at low concentrations, formaldehyde treatment transforms the wild toxin into a non-toxic immunogenic toxoid. The electrophoretic study of dtx purification and detoxification in relation to standard toxoid showed three significant differences that result from the reaction with formaldehyde. First, the toxin bands shifted; second, the ratio of nicked toxoid shape as two 21.0 (fragment-A) and 37.3 kDa (fragment-B) fragments to seemingly intact toxoid (58 kDa) changed; and finally, the protein bands became more diffuse. Cross-linking within the toxin and/or between amino acids present in the toxoid and the toxin has been attributed for this effect by Metz et al. [34].

A standard medium (TB) along with other additional components was selected and optimized at the mini pilot scale fermentation of rdtx and rdtb, according to Sundaran et al. [32] and Tan et al. [35]. The effects of fermentation form and cascade on cell growth and biomass development in recombinant *E. coli* were investigated using batch and fed batch fermentations in modified TB medium. High density biomass and protein level expression were compared in three different 3 L bioreactors to determine the most favorable kinetic parameters and optimal growth conditions for higher quantity protein and biomass production. The total protein concentration in the collected 1 mL cell pellets from the overall cultures was calculated using the Lowery method, and protein expression was analyzed using a 15% SDS-PAGE gel. The results show that culture C produces a lot of total protein and biomass, while cultures B and A produce less. After 12 hours of batch culture, adding yeast extract and glucose resulted in higher protein content (per gram of cell mass), and then fed batch techniques were used to achieve high cell densities [36]. Fed-batch systems produced a high cell density and increased recombinant protein production by 4–8 folds [10,37]. The relationship between growth rates in time and change in biomass can easily be noticed as the exponential growth period between 12 and 22 hours is the most remarkable. Culture was induced after 1 mM IPTG final concentration for induction of the T7 promoter-mediated gene expression and to generate rdtx and rdtb. During fermentation, it was also discovered that as the bacterial culture expanded, partial pressure of oxygen (pO_2) saturation decreased, owing to the culture's increased O_2 uptake. The stirrer speed was kept in cascade mode to maintain a higher pO_2 concentration, with continuous aeration using filter air and feed addition. The pO_2 controller is triggered by any change in pO_2 combined with a pH change. According to Packiam et al. [18], a total of 500 g of glucose was used during fermentation. Since dtx protein is a hydrophobic protein, it was possible to prevent aggregation by using strains like the BL21(DE3)-derived. Protein aggregation can be avoided by using a slow expression rate, which can be accomplished by using certain growth conditions: low temperature start culture with 35, then reduce to 28 after adding low concentration inducer 1 mM IPTG, short induction period of 24–36 hours. Using protease deficient BL21(DE3) strains, identifying and replacing unique protease sites, co-expression of protease inhibitors, or secretion of the target protein to the periplasm or external medium, protein degradation can be prevented. Cells were broken and subsequent procedures were carried out at ice-cold temperatures in the presence of protease inhibitors. Lowering the temperature also helps to avoid unwanted disulfide bonds [38].

In addition, when tested the hypothesis that whether rdtx or rdtb could replace the toxoid produced by formaldehyde treatment in the vaccination against diphtheria infection, the vaccination of BALB/c mice by rdtx and rdtb was applied and

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the resulting immune response was estimated in terms of antibody response (total IgG). This view was supported by the observation of previous studies indicated that both rdtx and rdtb are immunogenic. Moreover, it has been suggested that the immunological reactions of rdtb fragment of dtx may account for the presence of some multiple antigenic determinants that are unlikely to be present in the toxoid as described by Usuwanthim et al. [39]. Our results have demonstrated that mice that have been immunized by rdtx, rdtb, formaldehyde treated dtx, standard toxoid, and DPT (diphtheria-pertussis-tetanus) (as positive control), induced specific IgG responses. The antibody titers of the various vaccinated groups and the positive control groups did not vary significantly, but the vaccinated groups of mice exhibited significantly higher total IgG titers than the negative control groups. Total IgG was produced in the immunized groups following the first immunization and the titers have been raised significantly after the second immunization. dtx induced mixed TH1/TH2 response agrees with McNeela et al. [40]. There was no correlation as expected and is in concordance with McNeela et al. [40].

The utilization of rdtx and rdtb with Freund's adjuvant instead of the toxoid may help in minimizing the problem that arises with the currently used vaccine. For example, vaccination of dtxd in alum generally cannot stimulate mucosal immune response which limits their ability to infect the mucosal tissues such as the respiratory tract and resulting in the emerging limitation of the current vaccine schedule against diphtheria and this agrees with Clements and Griffiths [41]. Also, the expression of this part of dt (like rdtb) was successfully achieved in *E. coli* to overcome of the serious disadvantages of the traditional anti-diphtheria vaccines. Thus, exploring new antigen substances with sufficient efficacy for stimulating protective responses to dt as descrbed by Bazaral et al. [42]. Furthermore, using an appropriate non-toxic antigen for injection to susceptible animals to produce neutralizing antibodies for passive immunization eliminates the need for full length dtx injection to horses and this resolves the ethical issues. Although vaccination of natural fragment-B alone has proven difficult due to its high instability, Cabiaux et al. [43] found that it is usually accompanied by rapid degradation. However, the immune response in mice after application of recombinant dtx or rdtb was highly stable after vaccination, and the obtained findings regarding immune response in mice after application of rdtx and rdtb comply with Nascimento et al. [22].

In conclusion, this research has shown that PCR techniques are efficient molecular methods for amplifying the dt and dtb genes responsible for generating recombinant diphtheria vaccines and purifying, cloning, sequencing, and expressing these targeted genes in *E. coli* BL21. By determining the DNA sequences of the genes under study, they are completely identical to the genes preserved in the gene bank. The proteins were produced in abundant quantities by controlling production conditions and were at the exact molecular weights. When isolating and purifying these recombinant vaccines for use in immunization studies, it was discovered that there was no substantial difference in antibody titers between the vaccinated groups and the positive control groups, but the vaccinated groups of mice developed significantly higher total IgG titers than the negative control groups. From the above, we see that the possibility of producing new and advanced recombinant vaccines is possible and more efficient than the old methods of preparing such vaccines.

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