Heat shock transformation and expression of the plasmid containing cytolethal distending toxin of *Campylobacter fetus subsp venerealis* in *Escherichia coli*

N Herlina¹, N D Yanthi¹, R D Pratiwi¹, K S Dewi¹, F Setiyoningrum¹, D Priyoatmojo², R D P Manggung³

¹Research Center for Biotechnology, Indonesian Institute of Sciences, Indonesia, 16911, Email: nina.herlina.0212@gmail.com

²Center for Application of Technology of Isotope and Radiation, National Nuclear Agency of Indonesia, Indonesia, 12440, Email: dadangpr@batan.go.id

³Department of Animal Husbandry and Animal Health Subang, Indonesia, 41211, Email: r.dang.manggung@gmail.com

**Abstract.** The cytolethal distending toxins (cdt) is a multi-subunit toxin consisted of three subunit encoded cdtA, cdtB and cdtC. The cdt played an important role as a virulence factor of Campylobacter infection, including *C. fetus subsp venerealis*. The cdtA which responsible for binding the cdt to cell membrane, was cloned in plasmid expression and inserted into bacterial cells of *Escherichia coli* BL21(DE3). The research was conducted to evaluate the transformation using the heat shock method of a plasmid containing cdtA3 gene and the protein expression induced by various concentration of IPTG. Transformation was done using the heat shock method at 42°C for 90 second. Evaluation of the transformation was observed on the presence of E. coli BL21(DE3) colonies on Luria Bertani agar containing Ampicillin antibiotic with 100 µg/mL dosage. The recombinant protein was expressed using IPTG-induction with various concentration (0.1mM, 0.25mM, 0.5mM, 0.75mM and 1 mM). The result showed that the transformation and IPTG-induction 0.1 mM produced higher concentration of protein than other concentration applied. The protein characterization was observed with SDS PAGE and cdtA3 protein was detected on 23,4 kDa.

**Keywords:** cdtA3, Campylobacter, Escherichia coli, expression, transformation
1. Introduction
The genus Campylobacter consists of pathogens causing diseases in human and animals. Recent data from the OIE (2019) indicated the presence of Bovine Genital Campylobacteriosis (BGC) in Australia, New Zealand, Argentina, Africa, Iran, Brazil, Ireland, France, South Colombia, Uruguay, and Nigeria between January and June 2018 [1]. Among Campylobacter species, Campylobacter fetus defined as zoonotic pathogens causing diarrhea and other gastrointestinal infection in human [2]. In animals, C. fetus plays an aetiological role in reproduction diseases of livestock including infertility and abortion [3]. C. fetus is a gram negative bacteria consists of three subspecies C. fetus subspecies fetus (cft), C. fetus subspecies venerealis (cfv) and C. fetus subspecies testudinum (cft) [4, 5]. Generally, Cff colonizes the intestine of cattle, sheep and sometimes of human but may migrate to the genital tract and causing abortion [6]. Cft is a subspecies found in reptile and may infects human with variate symptoms such as fever, cough, gastritis and diarrhea [7]. In contrast, Cfv exclusively inhabits the genital tract of cattle and causes Bovine Genital Campylobacteriosis or Bovine Venereal Campylobacteriosis (BVC) [8]. The transmission of cfv and cft is coitus and because of the bacterial survival in raw and processed bull semen makes them transmissible via artificial insemination (AI) [9]. Cfv is a not detectable disease in the bull because the bull infected do not show clinical signs [10]. Transmission of cfv to the female can result several reproduction problems such as vaginitis, cervicitis, endometritis, infertility, early embryonic death and abortion [11].

Cf as the causative agent of BVC deserves special concern due to major impact of economic loses for the cattle industry [12]. Office International des Epizooties (OIE) notified BVC as a disease considered to have public health and socio-economic implications. The international trade regarding animals and animal products determine the disease as a requirement of inquiries [13].

Differential diagnoses among C. fetus subspecies is essential. Unfortunately, the microbiological and molecular differentiation of cff and cfv is extremely difficult because of their complexes taxonomy, special requirements for optimal growth and phenotypic identification in the laboratory is hard to achieve. The recommended standards procedure for detection of cff and cfv are based on the 1% glycine tolerance and H2S production tests. Cff is glycine-tolerant and produce H2S, in contrast with Cf as the causative agent of BVC deserves special concern due to major impact of economic loses for the cattle industry [12]. Office International des Epizooties (OIE) notified BVC as a disease considered to have public health and socio-economic implications. The international trade regarding animals and animal products determine the disease as a requirement of inquiries [13].

Differential diagnoses among C. fetus subspecies is essential. Unfortunately, the microbiological and molecular differentiation of cff and cfv is extremely difficult because of their complexes taxonomy, special requirements for optimal growth and phenotypic identification in the laboratory is hard to achieve. The recommended standards procedure for detection of cff and cfv are based on the 1% glycine tolerance and H2S production tests. Cff is glycine-tolerant and produce H2S, in contrast with Cf as the causative agent of BVC deserves special concern due to major impact of economic loses for the cattle industry [12]. Office International des Epizooties (OIE) notified BVC as a disease considered to have public health and socio-economic implications. The international trade regarding animals and animal products determine the disease as a requirement of inquiries [13].

There are some potential virulence factors identified for diagnostic assay and/or subunit vaccine candidate. It may contains nucleoside diphosphate kinase (Ndk), type IV secretion systems (T4SS), outer membrane proteins (OMP), substrate binding proteins CjaA and CjaC, surface array proteins, sap gene, and cytolethal distending toxin (CDT) [16]. Cytolethal distending toxin (cdt) is a toxin produced by Campylobacter genus that attacks epithelial cell layer. The toxin induces continuous cellular distension, cell cycle block, DNA damage and several eukaryotic cell death [17]. This gene is important as a heterotrimeric toxin responsible for the transport of the active subunit (CdtA and CdtC) and catalytic activity (CdtB) [18].

Cdt potentially developed as a diagnostic and subunit vaccine target. The research conducted to identify the transformation of plasmid containing virulence genes of Cfv (Cdt) as candidate protein for diagnostic assay target. The expression of recombinant protein was observed through SDS PAGE analysis.

2. Materials and methods
2.1. Microbial strain and media
The E. coli BL21(DE3) was used for protein expression and plasmid pD454-SR (ATUM, Newark, California, USA) was used as plasmid vector expression. Luria Bertani medium [1% tryptone, 1% sodium chloride and 0.5% yeast extract] supplemented Ampicillin was used for protein expression.
2.2. Competent cell
Competent cell used was E. coli strain BL21(DE3).

2.3. Transformation and characterization
The plasmid pD454-SR product was transformed into E. coli BL21(DE3) using transformation and storage solution for chemical transformation (TSS) [19], then continued with heat-shock method [20]. A total of 5 μL pD454-SR was added to the 100 μL competent cells and incubated for 30 min at 0°C. Heat shock was carried out at 42°C for 90 seconds. The cells was immediately cooled on ice for 10 minutes. A total of 900 μL LBB medium then added and incubated at 37°C for 2h with agitation rate of 150 rpm. Centrifugation was performed at 12,000 g for 1 min seconds. A total of 900 μL supernatant was removed, and the remaining of 100 μL of mixture was grown in LB medium containing 100 μg/mL of ampicillin and then incubated at 37°C for 18 hours. E. coli BL21(DE3) transformants was added IPTG (isopropyl-D-thiogalactopyranoside) with various concentration start at 0.1 mM, 0.25 mM, 0.5 mM, 0.75 mM and 1 mM. The incubation was done at room temperature, 150 rpm overnight. The next day, optical density was observed at 600 nm. Furthermore, the cultures were pelleted, and cells were resuspended in loading buffer. Cells were frozen until use.

2.4. SDS PAGE characterization
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli in 15% of resolving gel and 5% of stacking gel with Coomassie Brilliant Blue staining [21].

3. Results and discussion
In this study, we tried to produce recombinant protein of cdtA3 as a diagnostic assay candidate to differentiate Campylobacter genus. The competent cell used in this research is E. coli BL21(DE3). The strain has long offered as a model organism for biotechnology research and produced recombinant protein [22]. Several advantages from using BL21(DE3) strain as a fabrication host are fast cell growth in minimal media, low acetate production when grown on high levels of glucose, low protease abundance and an amenability to high-density culture [23].

Propagation of cdt gene in E. coli BL21(DE3) results colonies grown on LB media containing ampicillin 100μg/ml while no colonies grown in LB-amp for E.coli BL21(DE3) as it shown on the figure 1. This ampicillin-resistant establishes that the Amp gene in plasmid vector has succeeded in penetrating the E. coli BL21(DE3) and expressed in the medium. Amp gene in plasmid vector induces β lactamase secretion and provokes degradation of Ampicillin so that the bacteria colonies could grow. The negative control is E. coli BL21(DE3) without plasmid and have no resistency to ampicillin and the results showed that no colonies grown.

Figure 1. Transformants grown on LB-amp (left); negative control (right).

The recombinant plasmids were isolated from growing E. coli BL21(DE3) transformants. The inducer used in our study is IPTG. Recombinant cells containing cdtA3 gene construct were induced by IPTG and after induction, the absorbance value was observed at 600 nm (Table 1). The IPTG concentration was found to be a critical phase due to protein folding [24]. The absorbance was higher
in IPTG concentration 0.1 mM about 1.5146 and decreasing slightly to 1.3400 at IPTG concentration of 1 mM. The absorbance corresponded with the results of protein expression.

IPTG as inducer is one of the most widely used expression systems in *E. coli*. IPTG can produce very high expression levels as the target protein can represent up to 50% of the total cell protein [25]. Silaban *et al.* (2018) reported that the 0.1 mM IPTG concentration is suitable for induction of hPT-2 in *E. coli* BL21(DE3) Arctic Express [26]. Their results was similar with our finding that 0.1mM IPTG was produces better protein band size. The result of IPTG concentration and the protein expression obtained in SDS PAGE (Figure 2).

**Table 1.** CdtA3 absorbance value of IPTG induced.

| IPTG concentration | Absorbance |
|--------------------|------------|
| 0.10 mM            | 1.5146     |
| 0.25 mM            | 1.4762     |
| 0.50 mM            | 1.4253     |
| 0.75 mM            | 1.4287     |
| 1 mM               | 1.3400     |

The cells induced by IPTG were centrifugated and harboring recombinant cdtA3 were lysed and resuspend with loading dye. The denaturation protein was done at 95°C for 5 minutes. The cell lysate were then analysed in 15% SDS-PAGE and stained with Coomassie Brilliant Blue-250. The protein band of 23.4 kDa was analyzed on the comparison with the Page Ruler Prestained protein ladder (Figure 2).

**Figure 2.** SDS-PAGE pattern of cdtA3 proteins after CBB staining. Lane 1-5, total proteins of cdtA3 with IPTG induction of 0.10mM (1), 0.25mM (2), 0.50mM (3), 0.75mM (4) and 1mM (5).

The cdtA3 protein plays important role for binding of the cdt holotoxin to cell membrane [27].The production of cdtA3 recombinant protein need to put high consideration of host, vector, and culture conditions (including cultivation media) [28]. Cultur condition as described above and IPTG-induction concentration has been discovered. According to our results, low concentration of IPTG (0.1 mM) produces highest protein expression level as it shown on the figure 2.

**4. Conclusion**

In summary, our study proves that transformation with heat shock methods in 42°C for 90 second was succeed and the IPTG-induction with concentration 0.1 mM provides a better protein expression.
References

[1] Balzan C, Rosangela EZ, Leticia TG, Agueda PCV 2020 Ciência Rural 50(3):1-14.
[2] Lúcio EC, Mércia RB, Rinaldo AM, Rita CCM, José WP 2019 Braz J Microbiol.
[3] Truyers I, Luke T, Wilson D, Sargison N 2014 BMC Vet Res10:280.
[4] Mcgoldrick A, Chanter J, Gale S, Parr J, Toszeghy M, Line K 2013 J Microbiol Methods 94(3):199-204.
[5] Fitzgerald C, Tu ZC, Patrick M, Stiles T, Lawson AJ, Santovenia M, Gilbert MJ, van Bergen M, Joyce K, Pruckler J 2014 Int J of Systematic and Evolutionary Microbiology 64:2944–2948.
[6] Wagenaar JA, Van Bergen MA, Blaser MJ, Tauxe RV, Newell DG, van Putten JP 2014 Clin Infect Dis 58(11):1579-86.
[7] Patrick ME, Gilbert MJ, Blaser MJ, Tauxe RV, Wagenaar JA, Fitzgerald C 2013 Emerg Infect Dis19(10): 1678-1680.
[8] Stynen, A.P 2011 J. Bacteriol 193, 5871–5872.
[9] Modolo JR, Lopes CAM, Genari T 2000 Arg Bras Vet Meg Zootec 52(2): 96-97.
[10] Given MD 2018 Animal 12:165–171.
[11] BonDurant RH 2005 Vet Clinics of North America-Food Anim 21:383–408.
[12] Yen-Hung L, Wataru Y, Yu-Tsung H, Chuns-Hsing L, Wang-Hui S, Po-Ren H 2019 J Microbiol 52:122-131.
[13] McMillen L, Fordyce G, Doogan VJ, Lew AE 2006 J Clin Microbiol 44:938-945.
[14] Leece JG 1958 J Bacteriol 76:312-316.
[15] Fouts DE, Mongodin EF, Mandrell RE, Miller WG, Rasko DA, Ravel J, Brinkac LM, DeBoy RT, Parker CT, Daugherty SC 2005 PLoS Biol 3(1):15.
[16] Thi A, Siomar C, Soares, Anderson R, Santos, Luís C, Guimarães, Eudes Barbosa, Sintia S, Almeida, Vinicius AC, Abreu, Adriana R, Carneiro, Rommel TJ, Ramos, Syeda M, Bakhtiar, Syed S, Hassan, David W, Ussery, Stephen On, Artur S, Maria PS, Andrey PL, Anderson M, Vasco A 2012 Gene 145 45(9):5285
[17] Martinez I, Estibaliz M, Estibaliz C, Cecilia G, Rodrigo Al, Aurora FA 2006 Int J Med Microbiol 296:45-48.
[18] Pons BJ, Vignard J, Mirey G 2018 Toxins 11(10):595.
[19] Chung CT, Niemela SL and Miller RH. 1989 Proc. Natl. Acad. Sci. 86(7):2172-5.
[20] Froger A, Hall JE 2007 J Vis Exp 253.
[21] Laemmli 1970 Nature 227(5259):680-5.
[22] Kim S, Haeyoung J, Eun-Youn K, Jihyun FK, Sang YL and Sung HY 2017 Nucleic Acid Res 45(9):5285–5293.
[23] Yoon,SH., Jeong H, Kwon SK and Kim JF 2009 Genomics, biological features, and biotechnological applications of Escherichia coli B: ‘Is B for better?!’. In: Lee,SY (ed). Systems Biology and Biotechnology of Escherichia coli. Springer, Berlin, pp. 1–17.
[24] Rizkiya PR, Silaban S, Hasan K, Kamara DS, SubROTO T, Soemitro S and Maksum IP 2015 Procedia Chemistry 17:118-124.
[25] Rosano GL, Ceccarelli EA 2014 Front. Microbiol 5:172.
[26] Silaban S, Gaffar S, Simorangkir M, Maksum IP and Subroto T 2019 IOP Conf. Series: Earth and Environmental Science 217.
[27] Asakura M, Samosornsuk W, Taguchi M, Kobayashi K, Misawa N, Kusumoto M, Nishimura K, Matsuhisa A, Yamasaki S 2007 Microbiol Pathogenesis 42:174-183.
[28] Hayad, Seyed MG, Farahani, Najmeh, Golichenari, Behrouz, Sahebkar, Amirhossein 2018 24(6):718-725.

Acknowledgements

This research was supported by PRN LPDP Ministry of Finance and Ministry of Research and Technology Flagship Program during 2020-2021 fiscal years. Contract No: 59/E1/PRN/2020.