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SHORT COMMUNICATION

Genetic characterization of diphtheria *tox* B to evaluate vaccine efficacy in Indonesia

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ABSTRACT

Background and Objectives: Blocking the attachment of diphtheria toxins to host cells through the intact receptor binding site (*tox* B) was the initial mechanism of action of the diphtheria vaccine. Diphtheria outbreaks in populations with good vaccination coverage can be caused by mutations or changes in the genetic structure of the *tox* B protein. The aim of this study was to characterize the Tox B protein produced by *Corynebacterium diphtheriae* isolated from 2018 to 2019 in patients in Jakarta who had already received the diphtheria vaccine.
Materials and Methods: Of the 89 throat swab specimens of patients with a clinical diagnosis of diphtheria, 10 were positive for diphtheria and toxin. PCR was used to amplify the *tox* B DNA fragment in the 10 positive isolates. DNA sequencing was conducted with overlapping primers and the DNA sequences were analysed by using SeqScape V2.7.
Results: Of the 10 isolates, nine isolate showed a DNA mutation (G30A), but the mutation did not change the amino acid encoding arginin (silent mutation). Our findings indicate that the efficacy of the diphtheria vaccine used in Indonesia has not decreased because of mutations in the *tox* B genes not change the amino acid.
Conclusion: Overall, there are no amino acid changes in the *tox* B protein, indicating that the outbreaks are not affected by mutation in *tox* B. Another possible mechanism – overexpression of the toxin – is likely responsible for causing diphtheria in patients who have a complete history of immunization in Indonesia.

Keywords: Diphteria; Genetic characterization; Indonesia; Mutation; *Tox* B

INTRODUCTION

Corynebacterium diphtheriae is a gram-positive, aerobic, pleomorphic coccobacillus that can produce a toxin by lysogenization with a *corynebacteriophage* carrying the *tox* gene, which can increase the severity of infection (1, 2). The diphtheria toxin is a single-chain polypeptide with a molecular weight of about 58.36 kDa and 353 amino acids (3). Diphtheria toxin is an enzyme consisting of two subunits, namely the A subunit (21 kDa, encoded by the *tox* A gene) and the B subunit (39 kDa, encoded by the *tox* B gene) linked by a disulphide bond. *Tox* A is composed of a catalytic domain (C domain), which has an enzymatic function, is lethal and has an inhibitory effect on protein synthesis. *Tox* B consists of

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a translocation domain (T domain), which functions in the internalisation of toxins from the endocytosis membrane into the cytoplasm and receptor binding domains (3, 4).

The characteristic of inflammation caused by the toxin is the formation of grey pseudomembranes in the upper respiratory tract (tonsils, larynx or pharynx), which can cause difficulty breathing and swelling of the lymph nodes around the neck. Systemic dissemination can increase the severity of infection, with symptoms such as myocarditis; damage to the nerves, kidneys, and liver; and heavy bleeding leading to death (4, 5).

Diphtheria mortality rates began to decline dramatically in the 21st century after the introduction of vaccination (6). Although there are still diphtheria vaccination programmes throughout the world, including Indonesia, diphtheria cases have increased again. These cases represent a public health problem and have a high mortality rate. Based on World Health Organization (WHO) data, from 2016 to 2018 there was an increase in the number of diphtheria cases throughout the world, reaching 15,928 cases compared with the previous two years (12,309 cases). Indonesia was second ranked in the world, with 954 cases reported in 2017, a twofold increase compared with the previous year (314 cases in 2016) (7). In early 2018, 14 cases of diphtheria were reported from 11 districts/cities in four provinces (Jakarta, Banten, West Java and Lampung) (8, 9).

Supporting the Indonesian government's efforts to increase the efficacy of diphtheria vaccines, it is necessary to evaluate the role of increasing the virulence of the toxin produced by *C. diphtheriae*. Hughes et al. reported that administration of high doses of DTP3 vaccine did not make a significant difference in long-term immunity (0.1 – >1 IU/mL) protection compared with administration of low-dose vaccination (<0.01 – 0.09 IU/mL) (10). Other reports explained that there are still some patients suffered from diphtheria who have been immunized completely (11, 12). The immunization can trigger an immune response and the formation of human antibodies as protection against diphtheria which is mainly mediated by binding to the B subunit. Mutations and changes in the genetic structure of *tox* B allow the failure to recognize antibodies that cause diphtheria infection to persist even after vaccination (13-15). Mutations in the *tox* B gene can prevent the antitoxin from attaching and lead to failure of the neutralisation process, with eventual cell damage (16). It seems that complete vaccination does not guarantee protection against *C. diphtheria* infection.

The aim of this study was to characterize *tox* B produced by *C. diphtheriae* isolated from patients in Jakarta with a clinical diagnosis of diphtheria. This endeavour allowed us to evaluate the efficacy of the diphtheria vaccine used in Indonesia, namely whether it still has a protective effect to overcome the diphtheria problem.

MATERIALS AND METHODS

A total of 10 isolates of *C. diphtheriae* producing toxins were collected from 89 pseudomembranous lesions in the upper respiratory tract from patients with diphtheria in the Sulianti Saroso Infectious Disease Hospital, Jakarta, Indonesia, during the period of 2018–2019. Gram and Albert stains were used for microscopic examination. Culture was carried out on non-selective blood sheep agar and selective tellurite medium. The VITEK2® ANC card (BioMérieux, France) was used for identification. PCR was used to identify *tox* B gene. Toxigenicity was examined by using the Engler modification of the Elek test by the formation of precipitates due to the bond between the toxin produced by *C. diphtheriae* and the anti-toxin disc.

DNA was extracted with a Qiagen kit according to manufacturer's instructions, with a final elution volume of 50 µL. Primers of C. diphtheriae *tox* B – forward GGCGATCAGTAGGTAGCTCA and reverse GCACACGACCCCACTACCT – were used to amplify the *tox* B fragment (1026 base pairs). The PCR involved initial denaturation at 94°C for 4 minutes; 25 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds and elongation at 72°C for 1 minute; and a final extension at 72°C for 5 minutes. The PCR product was analyzed on 1.5% agarose gel, followed by DNA sequencing using overlapping primers. Overlapping editing with SeqScap v2.7 (Applied Biosystems) and bioedit was used to analyze the DNA sequencing result.

Ethical approval. The study was approved by the Ethics Committee, Faculty of Medicine, Universitas Indonesia (number ND-1175/UN2.F1/ETIK/PPM.00.02/2019).

RESULTS

Designed primer for *C. diphtheriae tox* B gene (forward 20 bp and reverse 19 bp) successfully detected amplicon product 1026 bp, same as the size of *tox* B gene *C. diphtheriae*. Forward primer of *tox* B attached to nucleotide 8 to 27 and reverse primers attached to nucleotide 1061 to 1079. Primers to detect *tox* B gene was part of the *Corynebacterium diphtheriae* genome Park William 8 (GeneBank : CP003216.1). The 1026-bp *tox* B PCR product compared to DNA Ladder 100 bp was shown in Fig. 1.

DNA mutation analysis showed that of 9 of 10 isolates had mutation in G30A, but the mutation did not change the amino acid encoding arginin (silent mutation). Sequence of DNA mutation analysis of *tox* B gene *C. diphtheriae* compared to Park William 8 as a reference strain was shown in Table 1.

DISCUSSION

Mutations in the *tox* B gene could alter the amino acid sequence, and this change could reduce the

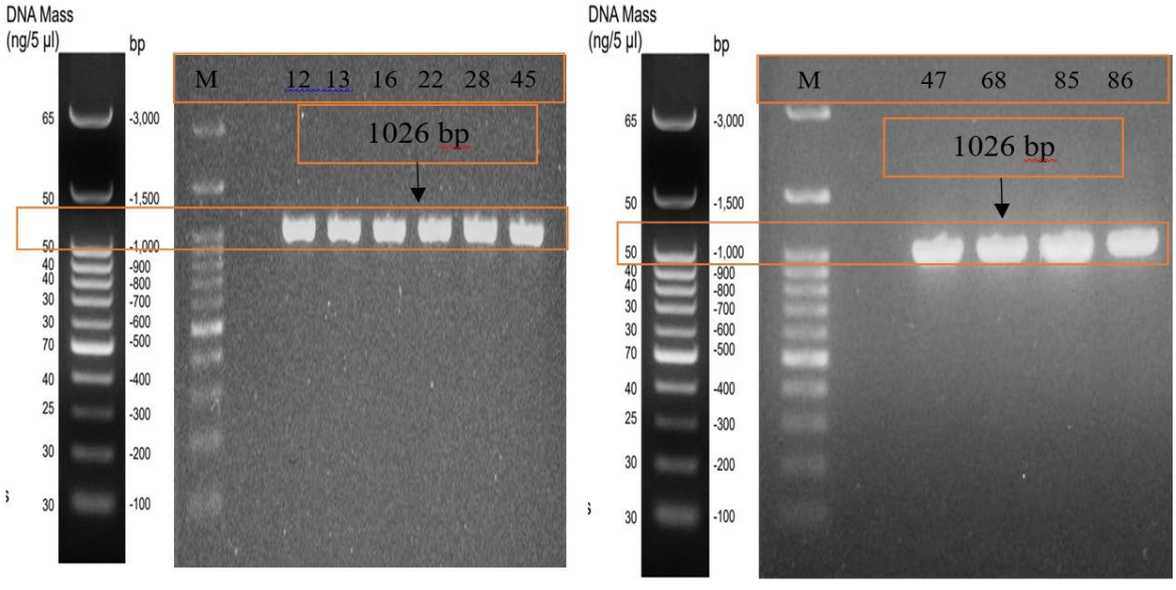

Fig. 1. Amplicon product of *tox* B gene fragment (1026 bp) on gel electrophoresis.
M: DNA ladder (100 – 1000 bp), 10 isolates: 12, 13, 16, 22, 28, 45, 47, 68, 85, 86.

Table 1. Sequence DNA mutation of *tox* B gene *C. diphtheriae* in Jakarta, Indonesia

Isolate	Position DNA mutation			Change of Amino Acid	Mutation Type
	28	29	30		
PW8	A	G	G	Arg	Reference strain
12	A	G	A	Arg →Arg	Silent Mutation
13	A	G	A	Arg →Arg	Silent Mutation
16	A	G	A	Arg →Arg	Silent Mutation
22	A	G	A	Arg →Arg	Silent Mutation
28	A	G	G	Arg	No Mutation
45	A	G	A	Arg →Arg	Silent Mutation
47	A	G	A	Arg →Arg	Silent Mutation
68	A	G	A	Arg →Arg	Silent Mutation
85	A	G	A	Arg →Arg	Silent Mutation
86	A	G	A	Arg →Arg	Silent Mutation

*Nucleotide substitution: G30A (9 isolate). A=Adenine, G= Guanine. Arg = Arginine

effective of diphtheria vaccines. However, in this study the mutation in the *tox* B sequence was silent mutation– it did not change the amino acid sequence. Therefore, this mutations did not affect on the Tox B function as a binding and translocation toxin into cells.

Our findings indicate that the efficacy of the diphtheria vaccine used in Indonesia has not decreased because of mutations in the *tox* B genes encoding the toxin. Other factors are probably responsible for causing diphtheria in patients with a complete history of diphtheria vaccination: variation in bacterial virulence or overexpression of the toxin due to the presence of mutations in toxin promoter-operators (*tox* PO) (17, 18).

Tox PO is a segment of DNA located on the upstream of the genes that regulate expression of diphtheria toxins. Consensus -35 region *Tox* PO is similar to the location of Σ70 *E. coli* which starts at position 74 of the structural toxin gene and there are 2 possible tox promoter with consensus-10 region starting at position-54 and-48 from *tox* gene structural (2, 19, 20). Variances of bacterial virulence that have been reported are mutations in the toxin promoter-operator region (*tox* PO) that affect toxin expression (19). The research of Kolodkina et al. show that *C. diphtheriae* promoter/operator confirm that an increased level of toxin production by strains is determined by the mutation located in the 9-bp palindrome, which overlaps the –10 sequence of the promoter and the operator region (16).

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REFERENCES

1. Zasada AA. *Cornybacterium diphtheriae* infections currently and in the past. *Przegl Epidemiol* 2015; 69: 439–444, 569-574.
2. Zajdowicz SLW, Holmes RK (2016). Phage Conversion and the Role of Bacteriophage and Host Functions in Regulation of Diphtheria Toxin Production by Corynebacterium diphtheriae. In: The Mechanistic Benefits of Microbial Symbionts, Advances in Environmental Microbiology. Springer International Publishing, Switzerland. pp. 15-45.
3. De Zoysa A, Efstratiou A, Mann G, Harrison TG, Fry NK. Development, validation and implementation of a quadriplex real-time PCR assay for identification of potentially toxigenic corynebacteria. *J Med Microbiol* 2016; 65: 1521-1527.
4. Riedel S, Morse SA, Mietzner TA, Miller S, Hobden JA, Detrick B, et al (2019). *Corynebacterium diphtheriae* (Aerobic Non Spore Forming Gram Positive Bacilli: *Corynebacterium, Listeria, Erysipelothrix*). In: Medical Microbiology. Ed, Jawetz, Melnick, Adelbergs. McGraw Hill Professional, 28th ed. USA, pp.196-199.
5. Talaro KP, Chess B (2008). Foundation in Microbiology. McGraw-Hill Companies. New York, USA.
6. Beh P, Byard R W. Diphtheria and lethal upper airway obstruction. *Forensic Sci Med Pathol* 2015; 11: 133-135.
7. World Health Organization. Diphtheria vaccine: WHO position paper, August 2017 – Recommendations. *Vaccine* 2018; 36: 199-201.
8. Tosepu R, Gunawan J, Effendy DS, Ahmad LOAI, Farzan A. The outbreak of diphtheria in Indonesia. *Pan Afr Med J* 2018; 31: 249.
9. Husada D, Primayani D, Marbun K, Kartina L, Puspitasari D, Tirthaningsih N, et al. Risk factors of diphtheria carriers in Indonesian children. *Southeast Asian J Trop Med Public Health* 2018; 49: 660-669.
10. Hughes GJ, Mikhail AFW, Husada D, Irawan E, Kafatos G, Bracebridge S, et al. Seroprevalence and determinants of immunity to diphtheria for children living in two districts of contrasting incidence in East Java, Indonesia. *Pediatr Infect Dis J* 2015; 34: 1152-1156.
11. Swart EM, Van Gageldonk PGM, De Melker HE, Van Der Klis FR, Berbers GAM, Mollema L. Long-term protection against diphtheria in the Netherlands after 50 years of vaccination: results from a seroepidemiological study. *PLoS One* 2016; 11(2): e0148605.
12. Kitamura N, Le TTT, Le LT, Nguyen LD, Dao AT, Hoang TT, et al. Diphtheria Outbreaks in Schools in Central Highland Districts, Vietnam, 2015–2018. *Emerg Infect Dis* 2020; 26: 596-600.
13. Abulmagd S, Emara M, Aziz S, El- Domany R. Evaluation and characterisation of A and B fragments of *Corynebacterium diphtheriae* toxin towards recombinant diphtheria vaccine. *Indian J Med Microbiol* 2013; 31: 3-9.
14. Le TV, Nguyen VTT, Nguyen QH, Nguyen TTT, Duong TTN, Ly TTT, et al. The evaluation of anti-diphtheria toxoid antibodies in healthy population in Kon Tum, Vietnam: a population-based study. *IJID*

Reg 2022; 3: 171-176.
15. Abdul A, Lichtman A, Pillai S (2012). Cellular and molecular immunology. Elsevier Health Sciences, 7th ed. Philadelphia, USA.
16. Kolodkina VL, Titov LP, Sharapa TN, Drozhzhina ON. Point mutations in tox promoter/operator and diphtheria toxin repressor (*dtxR*) gene associated with the level of toxin production by *Corynebacterium diphtheriae* strains isolated in Belarus. *Mol Gen Mikrobiol Virusol* 2007; (1): 22-29.
17. Doyle CJ, Mazins A, Graham RMA, Fang N-X, Smith HV, Jennison AV. Sequence analysis of toxin gene–bearing *Corynebacterium diphtheriae* strains, Australia. *Emerg Infect Dis* 2017; 23: 105-107.
18. Holmes RK. Biology and Molecular Epidemiology of Diphtheria Toxin and the tox Gene. *J Infect Dis* 2000; 181 Suppl 1: S156-167.
19. Mokrousov I. Corynebacterium diphtheriae: genome diversity, population structure and genotyping perspectives. *Infect Genet Evol* 2009; 9: 1-15.
20. Kunkle CA, Schmitt MP. Analysis of a DtxR-regulated iron transport and siderophore biosynthesis gene cluster in *Corynebacterium diphtheriae*. *J Bacteriol* 2005; 187: 422-433.