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Diphtheria Toxin Repressor (*dtxR*) Gene-Based Genetic Diversity of *Corynebacterium diphtheriae* Isolated in Jakarta, Indonesia, 2018–2019

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ABSTRACT

Background and Aim: In Indonesia, diphtheria cases caused by *Corynebacterium diphtheriae* are still occurring. One of the causes is probably the diphtheria toxin repressor (*dtxR*) gene, which influences toxin expression. In this study, the gene was characterized to determine the mutations that affect the DtxR protein.

Materials and Methods: The *dtxR* genes of 10 *C. diphtheria* strains isolated in Jakarta were amplified by conventional PCR. The PCR products were directed to DNA sequencing by using overlapping primers.

Results & Conclusion: No mutations showed amino acid changes. We propose that the DtxR protein is conserved among bacterial strains isolated from Jakarta.

Keywords: *Corynebacterium diphtheriae*, *dtxR*, Mutation, Phylogeny tree

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1. Introduction

Corynebacterium diphtheriae is the most common toxin-producing bacterial species that causes diphtheria **(1)** From 2016 to early 2018, the WHO reported that the number of diphtheria cases was higher as compared to 2 years earlier (15,928 to 12,309 cases) **(2)** Indonesia ranked second, with 954 cases, in 2017 **(3)** These data indicate that diphtheria is still persistent, irrespective of the vaccination program. A previous study reported that the administration of DTP3 high-dose vaccination did not result in significant differences in the protection of long-term immunity compared to low-dose vaccination **(4)** Fitriansyah also reported similar results: some diphtheria patients had a complete immunization history **(5)** Variations of bacterial virulence factors, especially the toxin, are the most likely causes of the low effectivity of the vaccine **(6)** Nakao *et al.* reported that one factor that affects the variation of bacterial virulence is the presence of mutations in the diphtheria toxin repressor (*dtxR*) gene influencing toxin expression **(7, 8)**.

The DtxR (226 AA) is an iron-dependent -repressor protein that regulates diphtheria toxin synthesis **(9)**. Previous studies reported that amino acid substitution in the DtxR protein caused decreased repressor activity **(10)**. Kolodkina *et al.* reported the missense mutation in the *dtxR* sequence on the strain of

Corynebacterium that produces a toxin at high concentrations **(11)**. Kombrova *et al.* and Nakao *et al.* (1997) also found a mutation in the *dtxR* gene of *Corynebacterium* that caused an outbreak **(7, 12)**. In Indonesia, Sunarno *et al.*, who performed partial *dtxR* characterization (200 bp) in 2014, found a variety of mutations in *dtxR* **(13)**. Moreover, in 2017, Mulyastuti *et al.* found a mutation of the *dtxR* gene in the complete *dtxR* characterization (678 bp) using four isolated samples from diphtheria patients and contacts in outbreak areas between 2013 and 2014 **(8)**. This study aims to characterize the *dtxR* gene from *C. diphtheriae* isolated from diphtheria patients in Jakarta from 2018 to 2019.

2. Materials and Methods

T Ten bacterial isolates obtained from previous studies, confirmed as *C. diphtheria* and with the ability to produce toxins **(14, 15)**, were used in this study. To amplify the *dtxR* gene, genomic bacterial DNA was extracted and purified by using a Qiamp DNA Mini Kit (Qiagen) according to the manufacturer's instruction, with a final elution of 100 µl, and stored at -35°C until used.

Polymerase chain reaction (PCR) was performed using the primers (dtxRF [CCAGCACACAACAGTCTCCA] and dtxRR [CATCTAATTTCGCCGCCTTT]). The DNA sequencing was performed using the same forward and reverse primers as used for PCR, and the obtained DNA sequences were analyzed via overlapping editing using SeqScap v. 2.7 (Applied Biosystems).

The software packages BioEdit 7.0.5.3 and Mega X were used to analyze DNA mutations and phylogenetic trees, respectively. Phylogenetic tree analysis was done using the neighbor-joining method. The maximum composite likelihood method and the units of the number of base substitutions per site were used to compute the evolutionary distances. The phylogenetic tree was generated with 1,000 bootstraps. The sequences obtained in this study were deposited at GenBank under the following accession numbers (sample IDs): MT713126 (12), MT713127 (13), MT713128 (16), MT713129 (22), MT713130 (28), MT713131 (45), MT713132 (47), MT713133 (68), MT713134 (85), and MT713135 (86).

Strains from other countries for comparison were obtained from GenBank, with the following accession numbers (strains): CP003216.1 (PW8), CP020410.2 (FDAARGOS_197), CP003210.1 (C7), M80337.1(1030), M80336.1 (C7hm723), CP003209.1 (BH8), CP003211.1 (CDCE 8392), CP003208.1 (INCA 402), CP003206.1 (31A), CP003217.1 (VA01), CP003207.1 (241), CP003212.1 (HC01), CP003213.1 (HC02), CP003214.1 (HC03), CP003215.1 (HC04), LR134538.1 (NCTC 3529), LR134537.1 (NCTC 7838), LN831026.1 (NCTC11397), BX248355.1 (NCTC13129), NZ_JAQO01000005.1 (ISS3319), CP029644.1 (BQ11), KU869771.1 (M2871), KU869772.1 (M5840), CP018331.1 (B-D-16-78), CP038504.1 (TH1526), CP039522.1 (CN2000), HM231328.1 (IR74), KU869772.1 (6732), and KY817830.1**(2-15)**.

3. Results & Discussion

Based on the results, the amplicon size of the *dtxR* gene was 752 bp. This amplicon product was confirmed by electrophoresis (Figure 1).

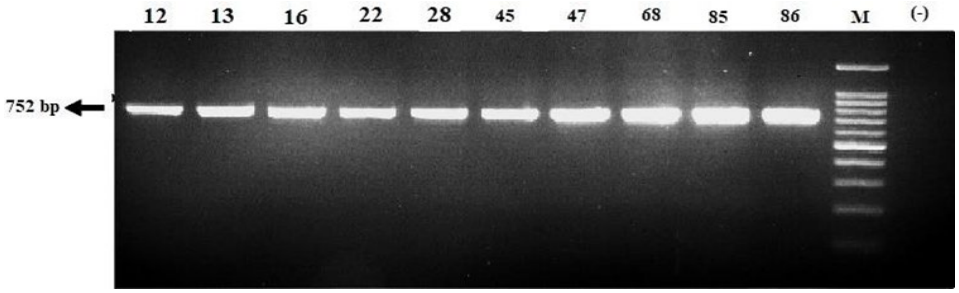

Figure 1. PCR products of the *dtxR* gene sequence (752 bp). bp: base pair; M: DNA Ladder (from bottom to top: 100–1000 bp); 12, 13, 16, 22, 28, 45, 47, 68, 85, and 86:10 isolates.

Based on the *dtxR* sequence, phylogenetic tree analysis was performed. The result showed that all strains in this study could be divided into seven clades – clades I, II, III, IV, V, VI, and VII (Figure 2), whereas 10 isolates of C. diphtheriae analyzed were divided into three clades (Clades I, II, and IV). Isolate numbers 13, 16, 22, 47, 86, and 12 belonged to the same clade and were related to strains from Brazil (HC01, CDCE8392, 241), Romania (6732ROM), India (TH1526), Australia (M5840), and Russia (215RUS). Isolate numbers 45, 68, and 85, located in Clade II, were related to strains from India (IR74), Brazil (HC02 dan 31A), the UK (ISS3319 and NCTC11397), the USA (C7 and C7hm723), and Boston (C7Bos). Isolate number 28, located in Clade IV, was associated with strains from the USA (PW8/vaccine strain), Brazil (BH8, INCA402), the UK

(NCTC3529), Australia (BQ11 dan and M2871), Malaysia (BD1678), and India (CN2000). All isolates had mutation pattern 2 (Table 1), which was the dominant mutation pattern located in the same clade (Figure 2). Isolate numbers 45, 68, and 85, located in Clade II (Figure 2), also had the same mutation pattern (Table 1).

Table 1. Mutation positions of nucleic acids of the *C. diphtheriae dtxR* gene.

Mutation Types	DNA Mutation Positions			Amino Acid Changes	Isolate IDs
	255	273	639		
1	T	C	C	No	PW8, 28
2	C	T	A	No	12, 13, 16, 22, 47, 86
3	-	T	-	No	45, 68, 85

PW8: referral strain; C: cytosine; T: thymine; A: adenine

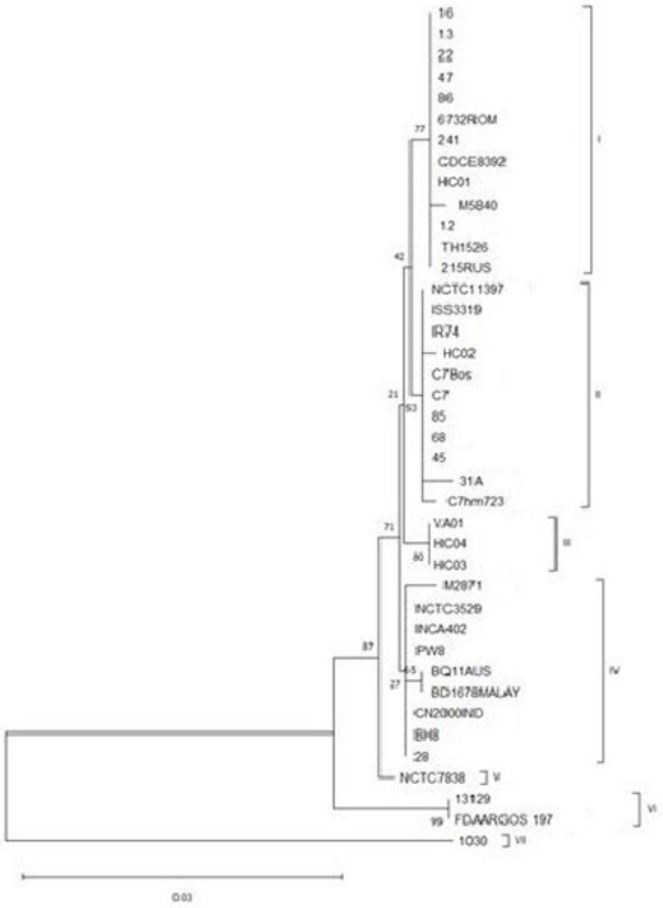

Figure 2. A phylogenetic tree based on the *dtxR* gene (681 bp) of *C. diphtheria* strains. The strains analyzed in this study were 12, 13, 16, 22, 28, 45, 47, 68, 85, and 86, and the other symbols represent the reference strains taken from GenBank data.

All isolates analyzed in this study shared similarities with strains from other countries. Nakao and Sunarno reported that the sequence of *dtxR* could show the diversity of *C. diphtheria* strains **(7, 13)**. Instead of phylogenetic analysis, the gold standards to show the phylogenetic relationship among strains are 16 sRNA and *rpoB* gene analysis **(16)**. Moreover, to obtain valid and comprehensive conclusions on the genetic relationships of *C. diphtheria*, characterization based on several virulence genes, multilocus sequence typing, and/or whole-genome analysis are required **(17)**. Therefore, in this study, the phylogenetic analysis only predicts and shows the diversity of strains rather than describing the true phylogenetic relationship.

Mutations in the *dtxR* gene could affect not only the production of DtxR but also the toxin expression **(18)**. However, in this study, the mutation in the *dtxR*

sequence was silent, and there was no change in the amino acids sequence (Table 1). Therefore, mutations that occurred in the *dtxR* gene did not affect the DtxR function as a repressor of toxin synthesis. This result is in agreement with the finding of Muliastuti and Sunarno, who detected no amino acid substitution of DtxR protein in isolates from the outbreak in Indonesia **(8, 14)**. Based on that, it can be proposed that the *dtxR* sequences found in Jakarta are preserved. Although these results differ from those found in other countries, it is possible that the geographic area influences the occurrence of mutations. However, this assumption needs to be confirmed by further research.

5. Conclusion

No mutations showed amino acid changes. We propose that the dtxR protein is conserved among bacterial strains isolated from Jakarta.

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None.

Conflict of Interest

The authors declare no conflicts of interest.

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