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Polymorphisms of *dtxR* Gene of *Corynebacterium diphtheriae* Isolated from Diphtheria Outbreak in Indonesia

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Abstract

Background: In *Corynebacterium diphtheriae*, the *dtxR* gene plays a role in regulating diphtheria toxin synthesis. The *dtxR* gene is often used as a marker for identifying *C. diphtheriae* by the polymerase chain reaction (PCR) method because it is present in all strains of this bacterium. Mutations in the *dtxR* gene can cause the over-synthesis of diphtheria toxin and reduce PCR assays' sensitivity.
Objectives: This study aimed to describe the polymorphisms in the *dtxR* gene of *C. diphtheriae* isolated from a diphtheria outbreak in Indonesia.
Methods: Forty-eight isolates of *C. diphtheriae* were obtained from clinical samples (throat/nasopharyngeal swabs) of diphtheria cases and close contacts. The isolates were revived on a blood agar plate (BAP), bacterial colonies were harvested, and deoxyribonucleic acid (DNA) was extracted. The DNA sequencing was carried out using a Whole-genome Sequencing (WGS) approach. The data were converted and analyzed with U-gene software. The *dtxR* gene analysis was performed with *C. diphtheriae* PW8 as references.
Results: There were 59-point mutation locations in 48 isolates examined. None of these single nucleotide polymorphisms (SNPs) coded for amino acid changes. Based on the mutation pattern, seven clades/groups of the *dtxR* gene of 48 *C. diphtheriae* isolates were examined.
Conclusions: At least seven types of DNA sequences and more than 50 SNPs of the *dtxR* gene were identified in 48 *C. diphtheriae* isolates from a diphtheria outbreak in Indonesia. Although all of them are silent mutations, they must be considered in the design of PCR examination in diphtheria laboratories.

Keywords: *Corynebacterium diphtheriae*, DNA, Indonesia, Mutation

1. Background

Indonesia has experienced diphtheria outbreaks over the past few years. Based on the World Health Organization (WHO) data, Indonesia is almost always among the top five countries with the most diphtheria cases in the world (1). Meanwhile, data from the Indonesian Ministry of Health show that diphtheria cases are almost found in all provinces. In 2018, diphtheria cases reported from 28 out of 34 provinces in Indonesia, ranging from one to 385 cases. The case fatality rate (CFR) of diphtheria in Indonesia in 2018 was 2.09% nationally, less than globally (2). Several analyses have linked high diphtheria cases in Indonesia to low immunization coverage (3, 4).

Diphtheria is mainly caused by *Corynebacterium diphtheriae*, while *C. ulcerans* and *C. pseudotuberculosis* cause a small number of cases. The main virulence factor in diphtheria-causing bacteria is the diphtheria toxin. *Corynebacterium diphtheriae* contains the *dtxR* gene that plays a role in regulating diphtheria toxin synthesis (5, 6). The *dtxR* gene is often used as a marker for identifying *C. diphtheriae* by the polymerase chain reaction (PCR) method because it is present in all strains of this bacterium (7, 8). Mutations in the *dtxR* gene can cause over-synthesis of diph-theria toxin and reduce the sensitivity of PCR assays.

2. Objectives

This study describes the polymorphisms in the *dtxR* gene of *C. diphtheriae* isolated from a diphtheria outbreak in Indonesia.

3. Methods

3.1. Samples

Forty-eight isolates of *C. diphtheriae* were used as samples in this study in 2017. They were stored by the National Institute of Health Research and Development. The bacterial isolates were isolated from clinical samples (throat/nasopharyngeal swabs) of diphtheria cases and close contacts in several provinces of Indonesia (Table 1). The isolates were revived on a blood agar plate (BAP) and incubated at 37°C for 24 hours. The bacterial colonies were harvested and put into 500 μL aquadest for deoxyribonucleic acid (DNA) extraction. Table 1 shows that most of the samples came from Jakarta and Banten, accounting for about 70.8% of the total sample. In contrast, the least samples came from Aceh, Central Java, East Java, and West Kalimantan, with one isolate each. Some provinces of Indonesia have no representative isolates. Several regions, especially Eastern Indonesia, referred their diphtheria samples to BBLK Surabaya for the examination so that they were not included in the list of isolates examined.

Table 1. Sample Distribution by Province and Year of Isolation

Province	Year of Isolation	Quantity	Percent
1. Jakarta	2015, 2016, 2017	15	31.3
2. Banten	2010, 2014, 2015, 2016, 2017	19	39.6
3. West Java	2016, 2017	6	12.5
4. Central Java	2017	1	2.1
5. East Java	2015	1	2.1
6. Aceh	2017	1	2.1
7. West Kalimantan	2013	1	2.1
8. Central Kalimantan	2012, 2016	4	8.3
Total	2010, 2012, 2013, 2014, 2015, 2016, 2017	48	100

3.2. DNA Extraction

The DNA extraction was performed using a commercial QiaAmp kit (Qiagen) following the manufacturer's protocol. The extracted DNA was stored in 50 μL molecular-grade water. The DNA quality and quantity were checked using Qubit and gel electrophoresis to meet the MiSeq machine (Illumina) protocol (8-10).

3.3. DNA Sequencing

The DNA sequencing was carried out using a Whole-genome Sequencing (WGS) approach by a MiSeq machine (Illumina). The data were converted and analyzed with U-gene software. The *dtxR* gene analysis was performed with *C. diphtheriae* PW8 as references (8-10).

4. Results

There were 59-point mutation locations in 48 isolates (Table 2). None of these SNPs coded for amino acid changes or silent mutations. Based on the mutation pattern, there were seven clades/groups of the *dtxR* gene in 48 *C. diphtheriae* isolates from a diphtheria outbreak in Indo-nesia. Table 2 and Figure 1 show that some examined isolates demonstrated similarities to the reference sequence (*C. diphtheria* PW8). Type 7 was very prominent because there was the larg-est sequence difference compared to other isolates. Figure 1 shows the grouping of *C. diphtheriae* isolated from several parts of Indonesia based on the mutation pattern.

5. Discussion

A previous study on the variation of the *dtxR* gene was conducted by Nakao et al. on isolates from diphtheria outbreaks in Russia and its surroundings using PCR-SSCP (single-strand conformation polymorphisms) (11). The identification results showed 12 types of the *dtxR* gene. Furthermore, De Zoysa et al. did the same study on a sample from the United Kingdom. The results showed at least four variants of the *dtxR* gene from 26 isolates examined (12). In Indonesia, a previous study was reported by Mulyastuti et al. with four isolates from the Java and Kalimantan islands. The results showed three variants of the *dtxR* gene with three mutation locations (13). Furthermore, Sunarno et al. reported 10 partial sequences (162 bases) of the *dtxR* gene in isolates from the Java and Kalimantan islands. The results showed at least two variants with three mutation locations (14).

The samples examined in this study were only 48 isolates that were not proportional by province (Table 1). It is one of the limitations of this study. However, this is the first study to show a lot of sequence variations (seven types) and DNA mutations (59 SNPs) of the *dtxR* gene of *C. diphtheriae* isolated from a diphtheria outbreak in Indonesia (Table 2). This study showed that mutations occurred in approximately 9% of 683 bases in the *dtxR* gene. Most mutations were found in one isolate (53 S18), which had a similar sequence to the *C. diphtheriae* strain Dong-yang (CP074413.1) reported from China in 2021, strain CHUV2995 (LT990688.1) reported from Switzerland in 2018, and strain CMCNS703 (CP038789.1) reported from India in 2019 (NCBI Blast: Nucleotide Sequence (nih.gov)). The mutations do not cause changes in amino acids, so it is predicted that they will not affect the function of the protein formed.

Knowledge of *dtxR* mutations is essential to predict the accuracy of PCR assays for identifying diphtheria-causing bacteria. Several previous studies used the *dtxR* gene as a

Table 2. Position of Point Mutations in the *dtxR* Gene of 48 *Corynebacterium diphtheriae* Iso-lates

Base Positions	PW8 [a]	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6	Type 7
39	T	.	.	.	.	.	.	C
54	G	.	.	.	A	.	.	.
66	A	.	.	.	.	.	T	.
72	C	.	.	.	.	.	.	T
75	T	.	.	.	.	.	.	C
93	T	.	.	.	.	.	.	C
126	C	.	.	T	T	.	.	.
180	C	.	.	.	.	.	.	T
195	A	.	.	.	.	.	.	G
198	G	.	.	.	.	.	.	A
204	C	.	.	.	.	.	.	T
207	C	.	.	.	.	.	.	T
210	T	.	.	.	.	.	.	G
225	T	.	.	.	.	C	.	C
234	A	.	.	.	.	.	.	G
246	T	.	.	.	.	.	.	A
270	T	.	.	.	.	.	.	C
273	C	.	T	.	.	T	.	.
309	C	.	.	.	.	.	.	A
318	C	.	.	.	.	.	.	T
321	T	.	.	.	.	.	.	C
339	A	.	.	.	.	.	.	G
357	A	.	.	.	.	.	.	G
358	T	.	.	.	.	.	.	C
372	T	.	.	.	.	.	.	C
373	C	.	.	.	.	.	.	A
393	A	.	.	.	.	.	.	G
402	T	.	.	.	.	.	.	G
405	C	.	.	.	.	.	.	T
414	C	.	.	.	.	.	.	A
423	C	.	.	.	.	.	.	T
429	T	.	.	.	.	.	.	C
456	T	.	.	.	.	.	.	C
459	T	.	.	.	.	.	.	C
462	C	.	.	.	.	.	.	T
471	C	.	.	.	.	.	.	A
474	C	.	.	.	.	.	T	.
493	A	.	.	.	.	.	.	G
504	T	.	.	.	.	.	A	.
507	C	.	.	.	.	.	T	T
516	T	.	.	.	.	.	C	C
521	T	.	.	.	.	.	.	C
534	G	.	.	.	.	.	.	A
537	T	.	.	.	.	.	.	C
540	A	.	.	.	.	.	.	G
552	T	.	.	.	.	.	.	C
558	C	.	.	.	.	.	T	T
564	T	.	.	.	.	.	A	.
567	T	.	.	.	.	.	.	C
579	C	.	.	.	.	.	T	.
585	T	.	.	.	.	.	.	C
600	C	.	.	.	.	.	.	G
603	C	.	.	.	.	.	.	T
613	A	.	.	.	.	.	.	C
639	C	.	.	.	.	A	T	G
640	C	.	.	.	.	.	A	A
652	G	.	.	.	.	.	.	A
654	T	.	.	.	.	.	C	.
663	T	.	.	.	.	.	.	C

[a] Reference

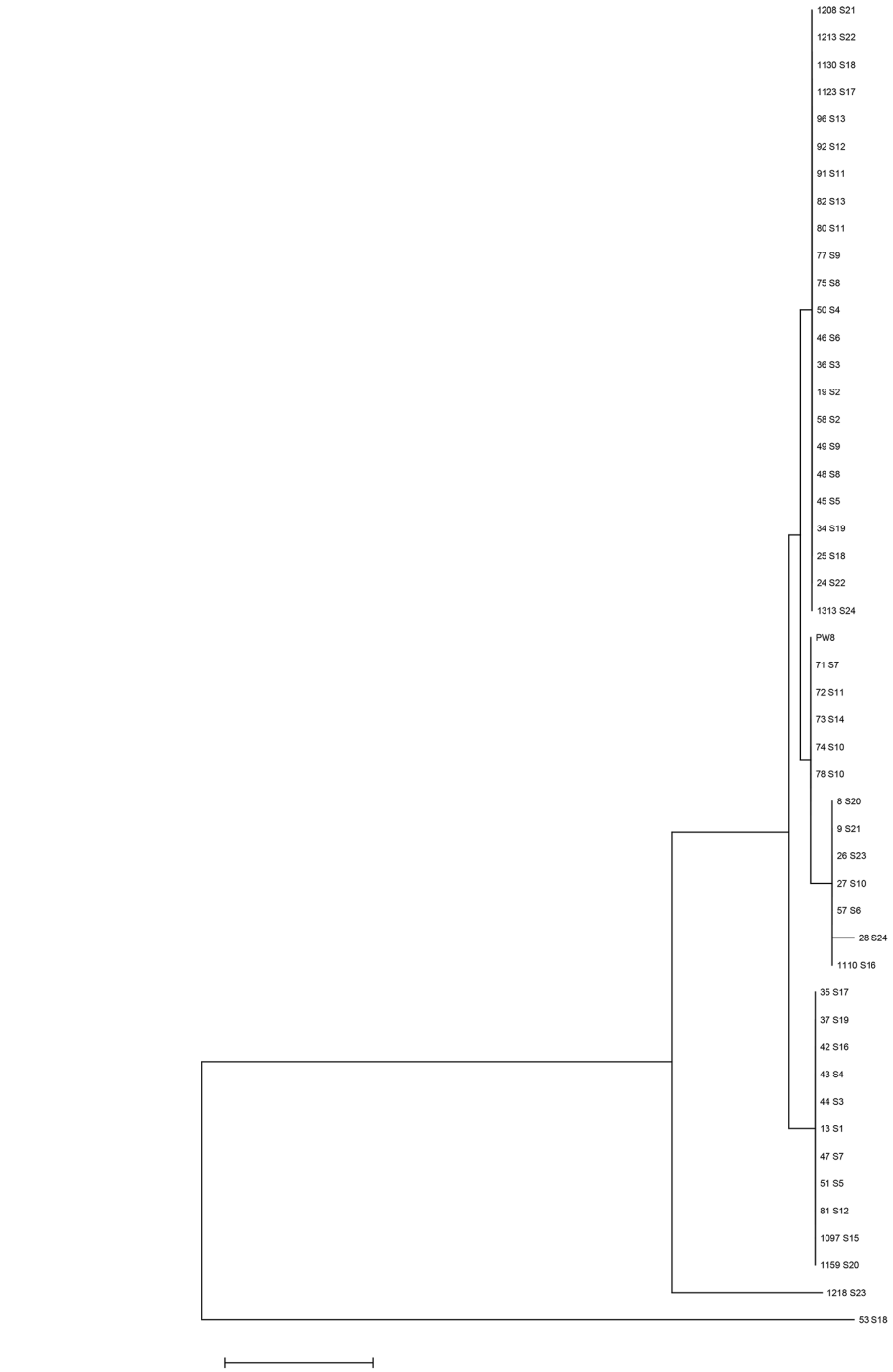

Figure 1. Phylogenetic description based on the mutation pattern of the *dtxR* gene

marker for diphtheria-causing bacteria. Initially, Pimenta et al. developed a PCR assay to detect *C. diphtheriae* with the *dtxR* gene as a marker. The results showed that the *dtxR* gene could be used to identify toxigenic and non-toxigenic *C. diphtheriae* (7). Furthermore, De Zoysa et al., Pimenta et al., and Torres et al. also developed a PCR targeting the *dtxR* gene as a marker of *C. diphtheriae* (15-17). The *dtxR* gene is also known as a marker for *C. ulcerans* and *C. pseudotuberculosis*.

The *dtxR* gene is predicted better than other genes. Sunarno et al. showed that the *dtxR* gene was more conserved than the *pld* gene and differentiated between species more than the *16s rRNA* gene. Therefore, the *dtxR* gene was used as a target for PCR examination to simultaneously identify three bacterial species causing diphtheria (8, 18). In addition, the *dtxR* gene functions as a regulator of diphtheria toxin synthesis. Mutations in certain regions have been shown to cause uncontrolled diphtheria toxin synthesis. In this case, the *dtxR* gene function is closely related to the availability of Fe in the environment where bacteria grow (19, 20). A literature search showed that the mutations found in this study did not affect the effectiveness of the established PCR examination because they were not located at the attachment site of the PCR primer or its probe. How-ever, this is a warning in the PCR examination for diphtheria.

5.1. Conclusions

At least seven types of DNA sequences and more than 50 Single Nucleotide Polymorphisms (SNPs) of the *dtxR* gene were identified in 48 *C. diphtheriae* isolates from a diphtheria outbreak in Indonesia. Although all of them are silent mutations, they must be considered in the design of the PCR examination in diphtheria laboratories.

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Footnotes

Authors' Contribution: FF, RDS, SS, and NP contributed to the conception and design of the study, acquisition of data and interpretation, and drafting and writing of the manuscript; NA, YH, SN, FM, YR, and AR contributed to the data collection and laboratory work; KK, KS, IS, DF, and TF contributed to the data analysis and revision of the manuscript.

Conflict of Interests: None.

Data Reproducibility: It was not declared by the authors.

Ethical Approval: This *in vitro* study did not use human and animal cells as samples. The ethical clearance was exempted as stated by the Ethics Committee for Health Research, National Institute of Health Re-search and Development No. LB.02.01./2/KE.216/2017.

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Informed Consent: This study did not use humans as subjects, and no informed consent was needed

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