DNA Sequence Analysis of \textit{dtxR} Gene (Partial) of \textit{Corynebacterium diphtheriae} Causing Diphtheria in Jawa and Kalimantan Islands, Indonesia

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Abstract

\textsc{Background:} \textit{dtxR} gene is a global regulator that can be used as a marker for detection of \textit{Corynebacterium diphtheriae} (\textit{C. diphtheriae}) and it is also a representative tool for mapping purpose (molecular typing) of this bacteria. The aim of this study was to analyze the DNA sequences of partial \textit{dtxR} gene of \textit{C. diphtheriae} causing diphtheria in some region of Indonesia. DNA sequence analysis was used to verify the accuracy of the in-house multiplex polymerase chain reaction (PCR) method that used for detection of \textit{C. diphtheriae} in the clinical specimen as well as a preliminary study to determine the strain diversity of \textit{C. diphtheriae} circulating in Indonesia.

\textsc{Methods:} Ten PCR products targeting the \textit{dtxR} gene that have been detected as positive \textit{C. diphtheriae} previously by in-house multiplex PCR used as samples in this study. The DNA sequencing carried out by Sanger method and the sequence data was analyzed by Bioedit software offline and basic local alignment sequence typing (BLAST) online.

\textsc{Results:} All of DNA sequence analyzed in this study were similar or identical to the \textit{dtxR} gene sequence data of \textit{C. diphtheriae} registered in GenBank. Within the 162 nucleotides (base 150-311) of \textit{dtxR} gene that analyzed, at least 2 clonals were found among 10 samples. Substitutions of 2 nucleotides (base 225 and 273) was detected, both were silent mutation.

\textsc{Conclusion:} Ten partial DNA sequences of \textit{dtxR} genes in this study verify the accuracy of in-house multiplex PCR which used to identify the bacteria causing diphtheria in the clinical specimen. The DNA sequences also represent the existing diversity of the bacteria causing diphtheria circulating in Indonesia.

\textsc{Keywords:} \textit{dtxR}, \textit{C. diphtheriae}, diphtheria, Indonesia

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Introduction

Diphtheria is a vaccine preventable disease. The use of diphtheria toxoid vaccine globally reduces the prevalence of diphtheria cases significantly.(1,2) However, the largest diphtheria outbreak since the introduction of vaccination was observed in Russia and the Newly Independent States of the former Soviet Union on the 1990s with at least 150,000 morbidities and more than 4,000 mortalities. This outbreak makes a great influence worldwide.(3,4) World Health Organization (WHO) data showed that diphtheria is still a global health problem, especially in developing countries, including Indonesia. In fact, in the last few year Indonesia was ranked 2\textsuperscript{nd} and 3\textsuperscript{rd} cases of diphtheria globally after India and Nepal.(5) Currently, the spread of diphtheria cases have reached almost all provinces in Indonesia, from Aceh to Papua province with a quite high case fatality rate (CFR).(6)

Diphtheria is caused by a group of bacteria known as potentially toxigenic \textit{Corynebacteria}, consisting of \textit{Corynebacterium diphtheriae} (\textit{C. diphtheriae}).
The DtxR protein encoded by regulator (chromosome known as the diphtheria toxin repressor/gene is regulated/repressed by a set of genes in the bacterial chromosome mostly located. The binding of DtxR -
forming hydrogen binding.(19) some study predicts its role as the dimerization stabilizer by and the contribution of this domain remains unclear although and ancillary region that also involves several amino acid binding of the metal ion occurred at 2 different site, primary that involves several amino acid residues. Otherwise, the
2 dimerization at the almost opposite palindromic region shown the potency of typing) of bacteria causing diphtheria. Several studies has as a genetic marker for detection and mapping (molecular
tox-PO (diphtheria toxin production both in vitro and in vivo.(12,13) The dtxR gene is also referred as a global regulator or metalloregulator as its function in regulating several genes involved in iron uptake and transport, including Siderophore or Corynebactin.(14,15)
The DtxR is a 25 kDa protein that consists of 226 amino acid residues. DtxR proteins are divided into three domains, helix-turn-helix (residues 1-73), metal binding site (residues 74-140), and Sre homology 3 (SH-3) (residues 147-226). The region between N-terminal (helix-turn-helix and metal binding site) and C-terminal, residues 125-139 consist of tether region rich in amino acid Proline.(16) N-terminal is the region which binds to the tox-PO gene and the metal ion mostly located. The binding of DtxR - tox-PO occurred by 2 dimerization at the almost opposite palindromic region that involves several amino acid residues. Otherwise, the
binding of the metal ion occurred at 2 different site, primary and ancillary region that also involves several amino acid residues. (17,18) SH-3 domain is located at the C-terminal and the contribution of this domain remains unclear although some study predicts its role as the dimerization stabilizer by forming hydrogen binding.(19)
The dtxR is a multifunction gene, hence it can be used as a genetic marker for detection and mapping (molecular
typing) of bacteria causing diphtheria. Several studies has shown the potency of dtxr gene as a marker of C. diphtheriae and also for C. ulcerans and C. pseudotuberculosis.(20,21) In addition, dtxr gene also used for typing of C. diphtheriae causing diphtheria outbreak in Russia and Ukraine based on the mutation analysis by polymerase chain reaction, single-strand conformation polymorphism (PCR SSCP) and direct sequencing.(22,23) This study aimed to analyze the DNA sequences of 10 C. diphtheriae dtxr partial genes that have detected by in-house multiplex PCR method in the clinical specimen from diphtheria cases and close contacts in some regions of Indonesia. In this study, we use sequence analysis to verify the accuracy of in-house multiplex PCR method to identify the bacteria causing diphtheria, as well as a preliminary study to determine the strains diversity of C. diphtheriae circulating in Indonesia.

Methods

Samples
This study focused on analyzing DNA sequence of partial dtxr gene. The samples were 10 PCR product consist of C. diphtheriae dtxr gene fragment. The origin of samples were clinical specimen (throat swab) from suspected diphtheria cases and close contact that sent to the Research Laboratory of Infectious Diseases Prof. dr. Sri Oemijati for the bacteria causing diphtheria detection and identification. The clinical specimens were collected and sent to the laboratory in 2013-2014 period. Amies transport medium in 2-8°C temperature was used for the bacterial viability maintenance in the specimen transportation.

Identification of the bacteria causing diphtheria was performed by conventional methods and in-house multiplex PCR assay with 5 pairs of PCR primer targeting dtxr and tox gene as we described previously.(21) The bacterial DNA for in-house multiplex PCR samples was extracted from clinical specimen by using QiaAmp commercial kit (Qiagen, California, USA) according to the instructions of the manufacturer with a few modification.(24) Another PCR by monoplex technic was performed on the C. diphtheriae positive samples only. The second PCR assay was performed using the same method with in-house multiplex PCR assay except the primer pair used were only one primer pair corresponding to dtxr gene (dtxR1_F and dtxR1_R). Ten products of second PCR were then used as a sample for DNA sequencing analysis of dtxr gene in this study.

DNA Sequencing and Data Analysis
DNA sequencing was conducted by Sanger method. Sap Exo-IT kits were used to purify the genomic DNA. The PCR master mix contained 1 μL template DNA (PCR product), 1.6 μM dtxr primer (dtxR1_F and dtxR1_R), 0.5 μL premix BigDye Terminator v.3.1, 3μL sequencing buffer v.3.1 and ddH₂O 4.5 μL. The sequencing was performed by using 3500 Series Genetic Analyzers (Applied Biosystems, California, USA) with following conditions: 96°C for 1 minute followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 seconds.
The data obtained from the sequencing process was analyzed using Bioedit software offline and BLAST software online based on reverse complement sequence alignment (forward and reverse) and graphs chromatography. DNA sequences of \textit{dtxR} gene were compared with reference strain (PW8) data and also with the previously published sequences registered in GenBank (www.ncbi.nlm.nih.gov/genbank/).

## Results

### Samples

The characteristics of the sample showed that the samples were collected from different areas with 2 variations of bacteria (Table 1). All samples (product of second PCR) were separated by gel electrophoresis with gel red staining (Biotium, California, USA) and analyzed by Geldoc XR plus (Biorad, California, USA) (Figure 1). All samples were expected to have amplification product with 162 bp band corresponding to primer pair targeting \textit{dtxR} gene (dtxR1_F and dtxR1_R) and without non-specific bands.

### DNA Sequencing and Data Analysis

DNA sequence variation was shown among 10 samples and the DNA profile of \textit{dtxR} gene in all samples similar with reference strain (PW8) (Table 2, 3). There were two point mutation within 162 bp amplified fragment of \textit{dtxR} genes (base 150-311) among 10 samples. In addition, twelve different mutations were also detected in another sequence registered on GenBank.

## Discussion

The samples used in this study were originally collected from three provinces in Indonesia that located in two islands, Jawa and Kalimantan, as shown in Table 1. These samples only grouped by island territory because the population mobility that influences the disease's transmission is relatively more common among the regions on one island. Ministry of Health data showed that several diphtheria cases found in Kalimantan during 2014. The highest cases found in West Kalimantan, followed by South Kalimantan and East Kalimantan, meanwhile, no diphtheria case found in Central Kalimantan. Based on the data, diphtheria cases in Java Island dominated by East Java, followed by Banten, West Java, Jakarta, and Central Java in the same year. No diphtheria case found in Jogjakarta. (6) Since Indonesia consist of 34 provinces, these three provinces do not represent Indonesia, but at least those data can be used as a preliminary description of the bacteria that cause diphtheria in Indonesia.

### Table 1. Characteristic of samples by origin and bacterial toxigenicity.

| Origin of Sample (Island) | Toxigenic | Non-Toxigenic | Number |
|---------------------------|-----------|--------------|--------|
| Kalimantan                | 6         | 1            | 7      |
| Jawa                      | 1         | 2*           | 3      |
| **Total**                 | **7**     | **3**        | **10** |

*1 person is residing temporarily

![Figure 1](image-url) The result of second PCR: 100bp DNA ladder (line1), sample 1-10 (line 2-11), negative control ddH$_2$O (line 12), positive control (line 13).
Table 2. DNA sequences profile of partial dtxR genes (base 150 – 311) of 10 C. diphtheriae sample in this study.

| Strain | 150 | 160 | 170 | 180 | 190 | 200 | 210 | 220 |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|
| PW 8   | CGATGGACTT GTCGTTGTCG CCTCAGACCG CAGTCTACAA ATGACACCGA CAGGCCAC TTTAGCGACT GCACTTATGC |
| sample-1 | C   |     |     |     |     |     |     |     |
| sample-2 | C   |     |     |     |     |     |     |     |
| sample-3 | C   |     |     |     |     |     |     |     |
| sample-4 | C   |     |     |     |     |     |     |     |
| sample-5 | C   |     |     |     |     |     |     |     |
| sample-6 | C   |     |     |     |     |     |     |     |
| sample-7 |     |     |     |     |     |     |     |     |
| sample-8 |     |     |     |     |     |     |     |     |
| sample-9 |     |     |     |     |     |     |     |     |
| sample-10 |     |     |     |     |     |     |     |     |

| Mutation Position |
|-------------------|
| Samples are grouped by bacterial toxigenicity, toxigenic and nontoxigenic. In this study, we did not obtain the non-toxigenic tox gene-bearing (NTTB) strains, which is defined as genotypically tox positive but do not express the protein.(25,26) The bacterial toxigenicity determine bacterial pathogenicity and pathology of disease. Toxigenic strain produces diphtheria toxin that responsible for systemic clinical symptom of disease.(27) C. diphtheriae toxigenic are major diphtheria causative agent in developing countries, included Indonesia. (21)

| Strain | 230 | 240 | 250 | 260 | 270 | 280 | 290 | 300 | 310 |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| PW 8   | GTAACATCG CTTAGCTGAG CGCCTCTTA CCGATATCAT TGCCCTAGAT ATCAATAAG TTCACGATGA AGGCTGCGC | TG |
| sample-1 | T   |     |     |     |     |     |     |     |     |
| sample-2 | T   |     |     |     |     |     |     |     |     |
| sample-3 | T   |     |     |     |     |     |     |     |     |
| sample-4 | T   |     |     |     |     |     |     |     |     |
| sample-5 | T   |     |     |     |     |     |     |     |     |
| sample-6 | T   |     |     |     |     |     |     |     |     |
| sample-7 | T   |     |     |     |     |     |     |     |     |
| sample-8 | T   |     |     |     |     |     |     |     |     |
| sample-9 | T   |     |     |     |     |     |     |     |     |
| sample-10 | T   |     |     |     |     |     |     |     |     |

In other hands, nontoxigenic strain usually relates to diphtheria with mild symptom, such as pharyngitis. Even though, nontoxigenic diphtheria strain cannot be neglected. Nontoxigenic diphtheria may cause severe disease, for instance, endocarditis and bacteremia.(28-30) This strain also can transform to toxigenic strain. One of sample that analyzed in this study is dtxR gene of nontoxigenic diphtheria strain, obtained from clinical specimen of hospitalized patient with mild diphtheria symptom. Samples were not classified by subtype (gravis, mitis, intermedius...
| Strain                  | Mutation Position |
|------------------------|-------------------|
| 180 195 198 204 207 210 225 234 246 252 273 309 |                  |
| PW 8 (consensus)       | C A G C C T T A T C C C |
| sample-1               | . . . . . . . . . . . . T |
| sample-2               | . . . . . . . . . . . . T |
| sample-3               | . . . . . . . . . . . . T |
| sample-4               | . . . . . . . . . . . . T |
| sample-5               | . . . . . . . . . . . . T |
| sample-6               | . . . . . . . . . . . . T |
| sample-7               | . . . . . . . . . . . . T |
| sample-8               | . . . . . . . . . . . . T |
| sample-9               | . . . . . . . . . . . . T |
| sample-10              | . . . . . . . . . . . . T |
| C.d.96/10              | C . . . . . . . . . . . . T |
| 16194                  | C . . . . . . . . . . . . T |
| FRC0004                | C . . . . . . . . . . . . T |
| 11852                  | C . . . . . . . . . . . . T |
| 39091                  | C . . . . . . . . . . . . T |
| 7275                   | C . . . . . . . . . . . . T |
| IR74                   | C . . . . . . . . . . . . T |
| 6732                   | C . . . . . . . . . . . . T |
| CORDTXRAB:173-853      | C . . . . . . . . . . . . T |
| 9337                   | C . . . . . . . . . . . . T |
| CD95/407               | C . . . . . . . . . . . . T |
| C.d.96/552             | C . . . . . . . . . . . . T |
| CD95/211               | C . . . . . . . . . . . . T |
| IR125                  | C . . . . . . . . . . . . T |
| CD95/305               | C . . . . . . . . . . . . T |
| 2005-3187              | C . . . . . . . . . . . . T |
| 25298                  | G A T G C G A . . . . . A |
| 39077                  | G A T G C G A . . . . . A |
| CORDTXRAC:208-888      | G A T G C G A . . . . . A |
| HC02                   | . . . . . . . . . . . . T |
| HC01                   | . . . . . . . . . . . . T |
| CDCE 8392              | . . . . . . . . . . . . T |
| 241                    | . . . . . . . . . . . . T |
| 681-1361               | . . . . . . . . . . . . T |
| 31A                    | . . . . . . . . . . . . T |
| NCTC 13129             | . . . . . . . . . . . . T |
| BH8                    | . . . . . . . . . . . . T |
| INCA 402               | . . . . . . . . . . . . T |
| VA01                   | . . . . . . . . . . . . T |
| HC04                   | . . . . . . . . . . . . T |
| HC03                   | . . . . . . . . . . . . T |
| C7                     | . . . . . . . . . . . . T |
| CORDTXRAA:690-1370     | . . . . . . . . . . . . T |
and belfanti) because some samples only genotypically detected by PCR (undetected by conventional methods) hence it is impossible to identify the subtype.

DNA sequencing is widely used in molecular techniques, to define a genetic marker for organism detection and identification, including mapping purpose.(31) One of the objectives of DNA sequences analysis in this study is to verify the in-house multiplex PCR results that used for bacterial identification (C. diphtheriae, C. ulcerans and C. pseudotuberculosis) from clinical specimen that performed before.(21) The in-house multiplex PCR assay has some advantages compare with established methods. This is a new method and result confirmation by DNA sequencing carried out to ensure no mismatch in the amplification process. DNA sequence analysis using BLAST program and BioEdit software show that all samples have a similar or identical DNA sequence of C. diphtheriae PW8 (consensus sequence) and several strains registered on GenBank. The in-house multiplex PCR have been applied for the bacteria causing diphtheria identification in the Research Laboratory of Infectious Diseases Prof. dr. Sri Oemijati routinely.

Beside for PCR result confirmation, DNA sequence of partial dtxR gene are used for initial description of the existing bacteria causing diphtheria diversity in Indonesia. Table 2 and 3 showed that there are at least two clones of bacteria among 10 samples analyzed. Two samples with Cytosine (C) \(\rightarrow\) Thymine (T) substitution at 273 base position are originally from Jawa Island. Otherwise, 8 samples with T \(\rightarrow\) C substitution at 225 base position and C \(\rightarrow\) T substitution at 273 base position are from Kalimantan Island (7 samples) and from Jawa Island (1 sample), who reside temporarily. Two types of the DNA sequence are similar to the data that already registered on GenBank. Seven samples from Kalimantan Island (sample 1, 2, 3, 4, 6, 8 and 9) and 1 sample from Jawa Island (sample 10) are similar to data from strain 6732, 9337, CD95/407, Cd96/552, CD95/211, CD95/305, HC01 and CDCE 8392 and 241. Meanwhile, 2 samples from Jawa Island (samples 5 and 7) are similar to data from strain CORDTXRAB: 173-853, IR125, HC02, 681-1361, 31A, C7 and CORDTXRAA: 690-1370. From these facts, if the same analysis applied to the sample used in this study, we assume that there would be more variation of DNA sequences.

DNA is the blueprint of amino acid sequences in protein, the DNA sequence of dtxR gene can be used to predict the amino acid sequences of DtxR protein. The amino acid sequence can be used to determine bacterial virulence indirectly, mainly related to the repressor functions of dtxR in diphtheria toxin synthesis.(32-34) Prediction of the amino acid translated from DNA sequence in this study cover 53 amino acid residues (51 to 103) from start codon (ATG) of dtxR genes. Fifty-three amino acids (residue 51–103) that made up of 162 bases analyzed in this study (starting from the start codon) were DGLVYVASTR SLQMTPTGRT LATAVMRKHR LAERLLTDII GLDINKVHDE ACR. Some of the amino acid position in the range of 51 to 103 that need more concern and proven may affect the repressor function including amino acid residues 50, 72, 77, 79, 80, 83, 84, 88, 98, 100, 102 and 104.(17,34,35)

Most of the amino acid residues roles in metal ion binding in the metal binding site domain. Amino acid residue 102 (Cysteine) has a central role in metal ion binding. Substitution in these positions, except with Aspartic Acid, will inactive the repressor and toxin production would not affect by the environment Fe level.(36) Cysteine in that position is the only Cysteine that assembles DtxR proteins. DNA mutation in 10 observed sample and also in Genbank data are the silent mutation (no amino acid substitutions) which is predicted will not change the dtxR function, although further study is still needed for confirmation.(37)

It should be underlined that the result obtained in this study is only preliminary data about the bacterial causing diphtheria variety and the disease’s transmission, and also about amino acid that arrange DtxR protein structure. Further analysis using more reliable genotyping method, such as multilocus sequences typing (MLST) and ribotyping should be performed for more sample.(38-40)

**Conclusion**

DNA sequences of partial dtxR genes in this study verify the accuracy of in-house multiplex PCR used to identify the bacteria causing diphtheria. In addition, these results illustrate the genetic diversity of C. diphtheriae strains circulating in Jawa and Kalimantan Islands, Indonesia. This study also ensures that there are no amino acid substitutions encoded by dtxR gene related to the base nucleotide
mutations observed. The Limitations of this study is the limited number of samples has not been able to describe the reality of genetic diversity of strains circulating in Indonesia diphtheria.

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