BIODIVERSITAS
Volume 18, Number 2, April 2017
Pages: 784-787

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d180249

Short Communication: Investigation of Diphtheria in Indonesia: *dtxR* and *tox* genes analysis of *Corynebacterium diphtheriae* collected from outbreaks

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Manuscript received: 26 March 2017. Revision accepted: 21 April 2017.

Abstract. *Mulyastuti Y, Rahayu SI, Sunarno S, Santoso S, Wasito EB. 2017. Short Communication: Investigation of Diphtheria in Indonesia:* dtxR *and* tox *genes analysis of* Corynebacterium diphtheriae *collected from outbreaks. Biodiversitas 18: 784-787.* Diphtheria outbreaks have sporadically occurred in Indonesia recently. It is suspected that toxin profile changes play an important role in vaccine efficacy and the occurrence of outbreaks. This study aimed to investigate the genetic changes of *dtxR* and *tox* genes in *Corynebacterium diphtheria* in Indonesia. Four *C. diphtheriae* toxigenic isolates circulating in current outbreak area were analyzed by comparing DNA sequences of their *dtxR* and *tox* genes to those of the PW8 vaccine strain. Among the four isolates, three point mutations were detected in *dtxR* gene while three other point mutations were detected in the *tox* gene. All six were silent mutations, suggesting that the diphtheria toxin is highly conserved at the amino acid sequence level, and indirectly indicating that the vaccine remains appropriate. Genetic variation in *dtxR* and *tox* genes of *C. diphtheriae* isolates from the recent outbreaks in Indonesia was detected.

Keywords: *Corynebacterium diphtheriae*, DNA Sequence Analysis, *dtxR* gene, *tox* gene, Indonesia

INTRODUCTION

Diphtheria vaccination has successfully lowered the incidence throughout the world, but outbreaks still occur sporadically in some countries, especially in developing countries like India, Pakistan, Indonesia, and China (WHO 2013). There was a significant increase in diphtheria cases in Indonesia, with a peak occurring in the year 2012 (1192 cases in all over Indonesia). The number decreased to 394 in 2014 (74% from East Java Province), however, it increased to 502 in 2015 (63% from East Java Province) (MoH 2016). Multiple current outbreaks in several areas in Indonesia indicate the continuous transmission of *Corynebacterium diphtheriae*. Moreover, the shift of epidemiological pattern from "children disease" to "all-age disease" is raising concerns over the changes of pathogenicity of this bacteria and the vaccine efficacy.

The pathogenicity of diphtheria results from an invasion of toxigenic bacteria at local tissue of the throat preceded by colonization and proliferation, and from protein synthesis inhibition induced by the diphtheria toxin (Murphy 1996; Burkovski 2014). Toxin as one important weapon of this pathogen has become an unsurpassed focus of research. Nontoxigenic *C. diphtheriae* generally produces milder clinical symptoms, but such strains are associated with endocarditis (Reacher et al. 2000). Diphtheria prevention which targets the diphtheria toxin is performed through vaccination, which is still based on the toxoids of the *C. diphtheriae* PW8 strain discovered in 1896 (Holmes 2000).

In diphtheria epidemic in Russia, it was found that base mutations at the *tox* operon in two of the 81 strains were not affecting the amino acid sequence of diphtheria toxin (Kolodkina et al. 2007). The structural gene encoding diphtheria toxin, *tox* gene, is carried by *corynebacteriophage* but the regulation of the expression is maintained by the regulatory elements encoded by *C. diphtheriae*, namely *dtxR*. Research in a change in *C. diphtheriae* toxin that causes diphtheria outbreaks in Indonesia has never been done so far. Analysis of the *dtxR* and *tox* genes profile is important, given that the profile changes may contribute to a reduction in the efficacy of toxoid, rendering it less useful for the prevention of diphtheria (Holmes 2000).

MATERIALS AND METHODS

Isolates and identification procedures

In this study, four *C. diphtheriae* isolates were collected from diphtheria patients and contacts in outbreak areas. Isolates studied are listed in Table 1. *C. diphtheriae* isolates were identified using conventional methods as described previously as well as using PCR assay (Efstratiou 1999, 2000; Sunarno 2015).

Toxigenicity test

The toxigenicity of *C. diphtheriae* was determined by Elek test, and the detection of *tox* gene was conducted with PCR assay (Sunarno 2015).

Polymerase Chain Reaction assay

DNA was extracted by heating a suspension consisting of one loop fresh bacterial culture in 500 µl sterile water for 10 minutes. The suspension was centrifuged at 8000 rpm. PCR for detection of *dtxR* and *tox* gene was performed in the National Institute for Health Research and Development, Jakarta, by using methods as described previously (Sunarno 2015).

DNA sequencing and data analysis

Results of the sequencing were analyzed by Bioedit software version 7.2.5. The results of DNA sequences of *tox* and *dtxR* genes were then compared with the complete genome sequence of *C. diphtheriae* PW8 strain using BlastN online program. This complete genome sequence has been published previously with GenBank accession No. CP003216.1. The phylogenetic tree was analyzed using the MEGA software version 6.06 by inserting the nucleotide base sequence data of *dtxR* gene.

This study has been approved by Ethical Committee of Faculty of Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia.

RESULTS AND DISCUSSION

All four isolates exhibited amplification product corresponding to the *dtxR* gene, which is the species-specific marker for *C. diphtheriae.* The amplification product of 100 bp corresponding to fragment A and 539 bp corresponding to fragment B of the diphtheria toxin were also obtained. Based on the data obtained, all four sequences showed 99% similarity with the *dtxR* gene from the PW8 strain of *C. diphtheriae*.

Analysis of *dtxR* gene sequencing results showed mutations located in open reading frame (ORF). The difference between all isolates and the PW8 strain was caused by a transition of GGC→GGT at position 273 (both encode Glycine). Moreover, isolates L3 and L4 also had transitions of GTT→GTC at position 225 (both encode Valine) and CTC→CTA at position 639 (both encode Leucine).

Analysis of *tox* gene sequencing results showed three nucleotide changes. Isolates L1, L2, L3, and L4 all had transitions of TTG→CTG at position 415 (both encode Leucine) and AGG→AGA at position 705 (both encode Arginine). Isolates L3 and L4 both had a transition of GAT→GAC at position 84 (both encode Aspartic acid). All mutations in *dtxR* and *tox* genes do not produce amino acid substitutions. We compared our finding with the previous report from Trost et al since there is no previous genotypic data about diphtheria isolate in Indonesia. Mutations in *dtxR* gene are shown in Table 2, while *tox* gene mutations are shown in Table 3.

Phylogenetic analysis based on the *dtxR* gene predicted a close relationship between isolates L3 and L4 with HCO1, CDCE 8392 and 241 strains (Figure 1). The analysis also predicted a close relationship between isolates L1 and L2 with C7, HCO2 and 31A strains.

Discussion

DNA sequence analysis of *dtxR* and *tox* genes in this study indicates that all isolates and the PW8 strain used for diphtheria toxoid production had identical amino acid sequences. However, the variation in *dtxR* and *tox* genes in four isolates within this study clearly demonstrates the necessity for further studies with both larger sample size and wider scope, to facilitate the development of strategies and surveillance policies to prevent further outbreaks. Further epidemiological research based molecular typing (i.e. Ribotyping or MLST) of strains and toxins circulating in Indonesia is necessary to reveal routes of transmission and adaptation mechanism of *C. diphtheriae*.

A seroprevalence study by Hughes *et al.* in Indonesian children concluded that high DTP3 vaccination coverage does not provide long-term immunity when compared with low vaccination coverage (Hughes et al. 2015). Earlier research led to speculation that natural variations in *dtxR* and *tox g*enes may be associated with the bacterial ability to produce a toxin (Perera and Corbel 1990). Any significant change in the amino acid sequence of fragment B may lead to failure in recognition by the diphtheria toxoid-induced antibody. On the other hand, production of diphtheria toxoid through inactivation process by chemical modification implies that there are variations in both characteristic and quality of toxoid. Toxoid tends to be less immunogenic unless given in large quantities or double dose (Metz 2005; Baxter 2007). Failure to recognize the toxin and a decrease in vaccine quality may reduce protection level conferred by vaccination, as does inadequate dosage. These factors may increase the risk of an outbreak.

Table 1. Origin of *C. diphtheriae* isolates in this study

Sample No.	Patient/Contact	Gender/Age	Immunization history	Origin	Year
L1	Contact	M/12 y	Unknown	Java	2012
L2	Patient	M/5 y	Unvaccinated	Java	2014
L3	Contact	F/11 y	Unknown	Kalimantan	2014
L4	Patient	M/15 y	Unknown	Kalimantan	2013

Table 2. Mutation in *dtxR* gene in this study compared to previous report (Trost 2012)

Strain	Origin	Position of nucleotide		
		225	273	639
PW8	Isolated from a diphtheria patient in New York, 1896, widely used toxoid vaccine producer; *tox*$^+$	GTT	GGC	CTC
L1	Isolated from a diphtheria contact in Java; 2012, *tox*$^+$	GTT	GGC → GGT	CTC
L2	Isolated from a diphtheria patient in Java; 2014, *tox*$^+$	GTT	GGC → GGT	CTC
L3	Isolated from a diphtheria contact in Kalimantan, 2014, *tox*$^+$	GTT→GTC	GGC → GGT	CTC→CTA
L4	Isolated from a diphtheria patient in Kalimantan, 2013, *tox*$^+$	GTT→GTC	GGC → GGT	CTC→CTA
C7(β)$^{tox+}$	Derivate of the avirulent isolate C7, 1954, widely used laboratory strain, *tox*$^+$	GTT	GGC → GGT	CTC→CTA
HCO1	Isolates from a blood sample from a patient with fatal endocarditis in Rio de Janeiro, 1993	GTT→GTC	GGC → GGT	CTC→CTA
CDC-E8392	Isolated from a diphtheria patient, originally from CDC, *tox*$^+$	GTT→GTC	GGC → GGT	CTC→CTA
241	Isolated from a diphtheria patient in Rio de Janeiro, 1981; *tox*$^-$	GTT→GTC	GGC → GGT	CTC→CTA
31A	Isolated from a diphtheria patient (vaccinated adult) in Rio de Janeiro; 1978; *tox*$^+$	GTT	GGC → GGT	CTC

Table 3. Mutation in *tox* gene in this study compared to previous report (Trost 2012)

Strain	Origin	Nucleotide position		
		84	415	705
PW8	Isolated from a diphtheria patient in New York, 1896, widely used toxoid vaccine producer; *tox*$^+$	GAT	TTG	AGG
L1	Isolated from a diphtheria contact in Java; 2012, *tox*$^+$	GAT	TTG→CTG	AGG→AGA
L2	Isolated from a diphtheria patient in Java; 2014, *tox*$^+$	GAT	TTG→CTG	AGG→AGA
L3	Isolated from a diphtheria contact in Kalimantan, 2014, *tox*$^+$	GAT→GAC	TTG→CTG	AGG→AGA
L4	Isolated from a diphtheria patient in Kalimantan, 2013, *tox*$^+$	GAT→GAC	TTG→CTG	AGG→AGA
31A	Isolated from a diphtheria patient (vaccinated adult) in Rio de Janeiro; 1978; *tox*$^+$	GAT	TTG→CTG	AGG→AGA

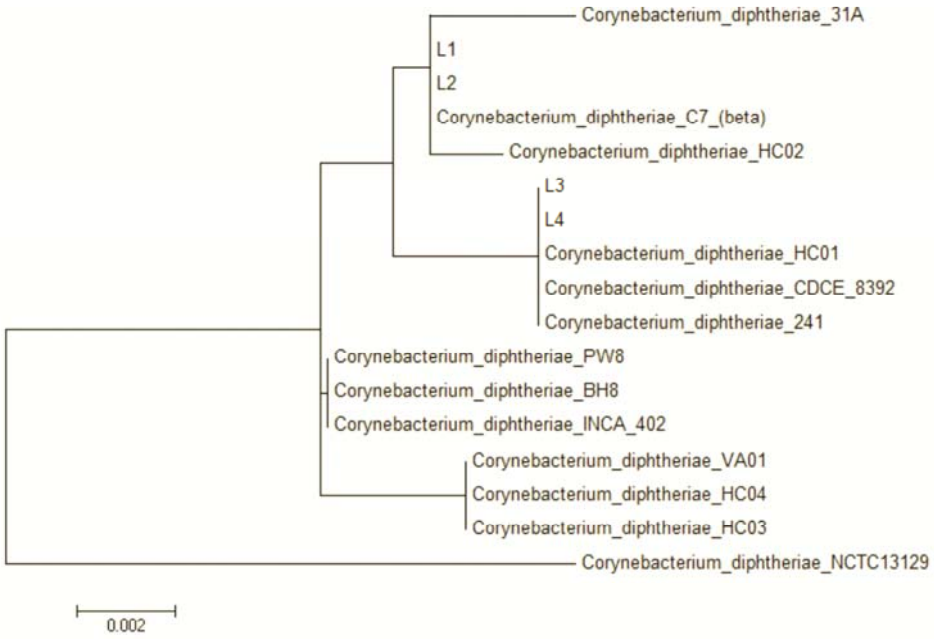

Figure 1. Phylogenetic analysis based on dtxR gene

This study is the first report of genetic variation in diphtheria isolates in Indonesia. Mutations in *dtxR* and *tox* genes of *C. diphtheriae* toxigenic strain were detected in isolates from several regions of Indonesia. All mutations did not lead to a change in the amino acid sequence of the toxin, leaving it identical to the amino acid sequence of *C. diphtheriae* PW8 strain which has been used in vaccine development. Therefore, it is concluded that the currently used vaccine is still appropriate for diphtheria prevention in Indonesia. The result is similar to the finding of Nakao et al. (1996), which showed that the decrease in vaccine efficacy was not the primary cause of the large diphtheria outbreak in Russia.

These results should be an important source of information for policy makers in Indonesia, especially concerning quality assurance of vaccine production, also its distribution and administration. It is important to enhance the coverage and effectiveness of diphtheria vaccination since it is currently still the most effective prevention mechanism against diphtheria outbreak. Moreover, it is important to increase awareness and ensure that good surveillance and laboratory diagnostics are in place.

ACKNOWLEDGEMENTS

This research was funded by *Riset Pembinaan Iptek Kedokteran* from Ministry of Health and Education Grants from Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia (DPP SPP FKUB).

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