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Short Communication: Identification and phylogenetic analysis of *Corynebacterium diphtheriae* isolates from Jakarta, Indonesia based on partial *rpo*B gene

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Abstract. *Yasmon A, Rosana Y, Usman D, Prilandari LI, Hartono TS. 2020. Short Communication: Identification and phylogenetic analysis of Corynebacterium diphtheriae isolates from Jakarta, Indonesia based on partial rpoB gene. Biodiversitas 21: 3070-3075.* Indonesia is a developing country with the greatest potential of diphtheria, so accurate identification is needed to detect *C. diphtheriae*. The genetic relationship analysis is important to be determined so that this transmittable disease can be prevented. In this study, we reported the identification and phylogenetic analysis of *C. diphtheriae* based on partial *rpo*B gene. Twelve samples from throat swabs of diphtheria suspected patients from 2018 to 2019 were used in this study. The result of VITEK 2 ANC-Automated Testing identified that all isolates were *C. diphtheriae*. However, the identification based on partial *rpo*B gene analysis showed that only 10 out of 12 isolates were *C. diphtheriae*, while 2 other isolates were *C. argentoratense*. Of 10 *C. diphtheriae* strains/isolates, 2 strains were unique for Indonesia. Thus, it is needed to evaluate the accuracy of VITEK 2 ANC-Automated Testing and to conduct further comprehensive studies on the genetic relationships of *C. diphtheriae* strains based on multiple virulence genes, multilocus sequence typing and/or whole-genome analysis.

Keywords: *Corynebacterium diphtheriae*, *rpo*B, phylogenetic tree, Indonesian strains

INTRODUCTION

The genus *Corynebacterium* comprises more than 85 species with the characteristics of irregular- or club-shapes, non-sporulation, and aerobe (Tauch and Sandbote 2014). Of those, *Corynebacterium diphtheriae* is much more prevalent as the etiological agent of respiratory diphtheria. The diphtheria disease is an acute and communicable disease among children through contact with respiratory droplets from infected individuals (Hii et al. 2018). The diphtheria cases in particular countries varied and determined by some local factors including coverage of vaccination, education level, and socioeconomic condition (Galazka 2000). WHO reported that during 2016 there were 7.097 diphtheria cases globally in which India, Indonesia, and Madagascar countries had the highest number of diphtheria cases (Clarke 2018). In Indonesia, outbreaks of diphtheria have increased from 2010 to 2017 (MoH-RI 2017). In 2017, the outbreaks occurred in 30 provinces with 44 deaths of 954 cases (Karyanti et al. 2019).

Selective media containing tellurite is required to isolate *Corynebacterium*. Further biochemical tests are needed to differentiate *Corynebacterium* species that normally inhabiting the nasopharynx and skin (CDC 2015). One of the biochemical rapid and automatic tests is the VITEK 2 (BioMerieux) system. The assay system uses colorimetric reagent cards dedicated to *Corynebacterium* identification (Zasada and Mosiej 2018). In addition to biochemical identification, another test that can be used for bacterial identification is nucleic acid-based assays.

Several genes can be used for the identification of *Corynebacterium* species, including *rpo*B, *gyr*A, and 16S rRNA genes (Khamis et al. 2005; Gomila et al. 2012; Venezia et al. 2012; Baraúna et al. 2017). Among these genes, the *rpo*B gene is considered as a gold standard for the identification of *Corynebacterium* species (Zasada and Mosiej 2018). The *rpo*B gene is significantly more polymorphic than 16S rRNA, and the DNA fragments (434 to 452 bp) of the *rpo*B gene has a high degree of polymorphism, suggesting the replacement of 16S rRNA by partial *rpo*B genes for identification (Khamis et al. 2004; Khamis et al. 2005). Genetic relationships are important for determining the sources of infection/ outbreak, so it can be used for prevention strategies against diphtheria. Previous studies reported genetic relationships of *C. diphtheriae* strains based on more than three virulence genes (toxin, BigA, Sdr-like adhesins, spaD-type and spaH-type pilus), multilocus sequence typing and/or whole-genome analysis (du Plessis et al. 2017; Ramdhan et al. 2019). This study reported the results of a preliminary study of the VITEK 2 and partial *rpo*B gene-based identification of *Corynebacterium* species and phylogenetic analysis of *C. diphtheriae* strains isolated in Jakarta, Indonesia from 2018 to 2019.

MATERIALS AND METHODS

Ethical committee approval

This study was approved by ethical committees of the Faculty of Medicine, University of Indonesia – Cipto Mangunkusumo Hospital, and Prof. Dr. Sulianti Saroso Hospital (Approval No: 0109/UN2.F1/ETIK/2018 and 50/XXXVIII.10/VIII/2018, respectively).

Bacterial isolation and identification

Corynebacteria samples were isolated from Eighty-nine throat swabs of diphtheria suspected patients from 2018 to 2019 at the Prof. Dr. Sulianti Saroso Hospital. The collected samples were cultivated on a selective cystine tellurite blood agar (CTBA) medium. Black colonies that grow on CTBA medium were suspected as Corynebacteria. CTBA media overgrown black colonies were transferred to Microbiology Laboratory, Faculty of Medicine-University of Indonesia- Cipto Mangunkusumo Hospital by using a cold box containing 6 ice packs. After arriving at the laboratory, a single black colony were sub-cultured on blood agar and incubated for 24 hours. After incubation, the colonies were suspended in a tube containing 0.9% NaCl to achieve turbidity comparable to 2.7 McFarland. Identification of *Corynebacterium* species was carried out using VITEK 2 ANC (anaerobic and corynebacterium card) automated microbiology systems (BioMerieux). In addition to bacterial identification, bacterial colonies were also used for bacterial genomic DNA extraction.

Extraction of bacterial genomic DNA

Ten bacterial colonies were suspended into 1x phosphate-buffered saline (PBS) and centrifuged at 14000g for 5 min. The supernatant was discarded and the pellet was extracted to obtain the pure bacterial genomic DNA. The extraction was performed using a Qiamp DNA Mini Kit (Qiagen) according to the manufacturer's instruction with the final elution of 100 µl and stored at -35ºC until used.

PCR of partial *rpo*B gene

Polymerase chain reaction (PCR) was performed by using primers (C2700F [**CGW ATG AAC ATY GGB CAG GT**] and C3130R [**TCC ATY TCR CCR AAR CGC TG**]) according to Khamis et al. (2004) as follows: 40 µl PCR Master mix Solution (i-MAX II), 0.2 µM each of primers, and 6 µl of DNA template. The thermal cycler was conducted by using Gene Amp PCR System 9700 (Applied Biosystems) with the following conditions: 94^0C for 4 min; 35 cycles of 94^0C for 30 sec, 61^0C for 30 sec, and 72^0C for 30 sec; and 72^0C for 5 min. The PCR products (434 to 452 bp) were detected on 2% agarose gel and documented by the Bio-Rad Gel Doc System.

DNA sequencing and *Corynebacterium* species identification

PCR products (434 to 452 bp) were purified using QIAquick Gel Extraction Kit (Qiagen). DNA sequencing was performed using forward and reverse primers as it is used for PCR and using BigDye® Terminator Sequencing Standard Kit v3.1 (Applied Biosystems, Foster City, CA). Results of DNA sequencing were analyzed by overlapping editing using SeqScap v2.7 (Applied Biosystems). The identification of *Corynebacterium* species was performed by comparing the sequence of isolates with those in the GenBank database by using the BLAST program.

DNA similarity and phylogenetic tree

The DNA homology and phylogenetic tree were analyzed by BioEdit 7.0.5.3 and Mega X, respectively (Hall 1999; Tamura et al. 2004). The Neighbor-Joining method was used in the phylogenetic tree analysis,. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

The sequences obtained in this study were deposited in the GenBank with the following accession no: MN956994 (S12), MN956995 (S13), MN956996 (S16), MN956997 (S22), MN956998 (S28), MN956999 (S45), MN957000 (S47), MN957001 (S68), MN957002 (S85), MN957003 (S86), MN956992 (S11), and MN956993 (S51). Strains from other countries for comparison were obtained from the GenBank with the following accession no: CP003212.1 (HC01), CP003213.1 (HC02), CP003214.1 (HC03), CP003215.1 (HC04), JRUZ01000011.1 (HC07), CP003217.1 (VA01), CP003207.1 (241), LN831026.1 (NCTC11397), CP003216.1 (PW8), CP020410.2 (FDAARGOS_197), JAQQ01000008.1 (ISS4749), NZ_JAQO01000005.1 (ISS3319), BX248355.1 (NCTC13129), AJVH01000008.1 (NCTC5011), JZUJ01000001.1 (17801), LSYP01000030.1 (3058), MIYS01000034.1 (ST291), NZ_MION01000001.1 (ST395), CP029644.1 (BQ11), CP038504.1 (TH1526), CP018331.1 (B-D-16-78), MKYG01000009.1 (Rz252), MKYM01000008.1 (Rz319), CP003206.1 (31A), CP003209.1 (BH8), CP003211.1 (CDCE 8392), CP003208.1 (INCA 402), CP003210.1 (C7), NZ_JAQN01000008.1 (ISS 4060), NZ_JAQP01000009.1 (ISS 4746), LR738855.1 (FRC0190), NZ_MSIH01000010.1 (50), NZ_MSIO01000009.1 (5010), NDHS01000017.1 (c319), NDHR01000006.1 (c324), NZ_NBNL01000006.1 (c326), LR134538.1 (NCTC 3529), and LR134537.1 (NCTC 7838).

RESULTS AND DISCUSSION

Based on the partial *rpo*B gene sequence, all twelve isolates showed DNA fragments with the same sizes between 400 and 500 bp (Figure 1). PCR products of collected *Corynebacterium* isolates could be varied (434-452 bp) (Khamis et al. 2004); however, the difference of 18 bp in the PCR products can not be visualized on agarose gel (Figure 1). The varied DNA fragments are due to *rpo*B polymorphism among species (Khamis et al. 2004). Because of the polymorphism, several studies have used the partial *rpo*B sequences and showed that the partial *rpo*B has the superior ability for differentiating *Corynebacterium* species and useful for taxonomic

classification (Khamis et al. 2005; Gomila et al. 2012; Venezia et al. 2012; Baraúna et al. 2017).

Species identification using VITEK 2 ANC-Automated Testing showed that all isolates were *C. diphtheriae*. However, identification based on the partial *rpo*B sequence-based *Corynebacterium* species has different results, i.e. only 10 isolates (S12, S13, S16, S22, S28, S45, S47, S68, S85, and S86) out of 12 isolates were *C. diphtheriae*, while 2 other isolates (S11 and S51) were *C. argentoratense* (Figure 2).

C. argentoratense was first reported in 1995 and differs from *C. diphtheriae* (Riegel et al. 1995). The phenotypic characteristics of *C. argentoratense* were the presence of pyraziminidase and alkaline phosphatase, but absent of nitrate reductase, and cannot ferment maltose (Riegel et al. 1995). *C. argentoratense* is one of the human skin microbiota and its role as pathogenic bacteria in humans is still not fully understood (Bomholt et al. 2013; Fernández-Natal et al. 2016). Some studies have reported the isolation of *C.argentoratense* from clinical specimens such as upper and lower respiratory tracts, ear, blood, catheter, conjunctiva, and biofilm (Riegel et al. 1996; Bernard et al. 2002; Babay and Kambal 2004; Martins et al. 2009; Haas et al. 2011).

Misidentification by VITEK 2-Automated Testing has been previously reported. A multicenter study reported that identification by VITEK 2 using clinical isolates showed 4.9% low discrimination, 4.6% incorrect identification, and 0.3% unidentified isolates, and the results of *Corynebacterium* identification showed misidentification of *C. urealyticum* strain from 51 *Corynebacterium* species (not included *C. argentoratense*) (Rennie et al. 2008). Another VITEK MS- Automated Testing also showed misidentifications of two *C. afermentans* isolates as *Lactobacillus* species (Navas et al. 2014). Moreover, *C. ureicelerivorans* and *C. minutissimum* were also misidentified as *C. mucifaciens* and *C. aurimucosum*, respectively (Navas et al. 2014). To the best of our knowledge, there is no report about misidentifications of 2 *C. argentoratense* isolates as *C. diphtheriae* by VITEK 2 ANC-Automated Testing. Thus, we suggest the need for specific evaluation for the identification of *Corynebacterium* species in diphtheria diseases.

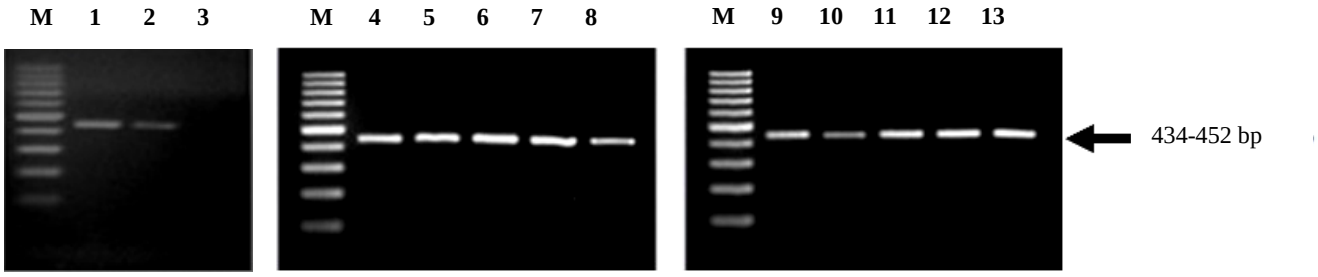

Figure 1. Results of PCR products of the partial *rpoB* gene sequences (434-452 bp). bp: base pair. 3: negative control. 1,2,4-13: 12 collected isolates, showed a positive result on selective tellurite media and VITEK 2 ANC-Automated Testing. M: DNA Ladder (from bottom to top: 100-1000 bp).

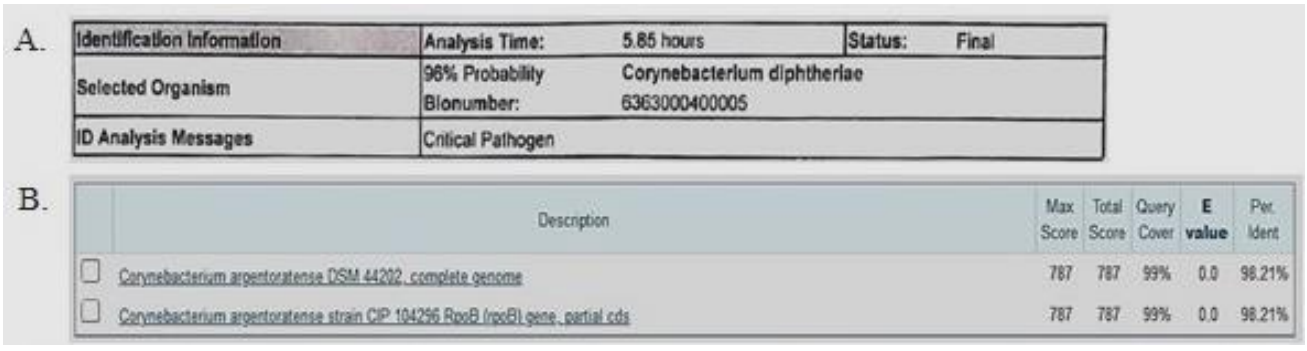

Figure 2. Result of isolate identification based on VITEK 2 ANC-Automated Testing as *Corynebacterium diphtheriae* (A), and by BLAST program with the filter of 90-100% identity and query coverage identified as *C. argentoratense* (B)

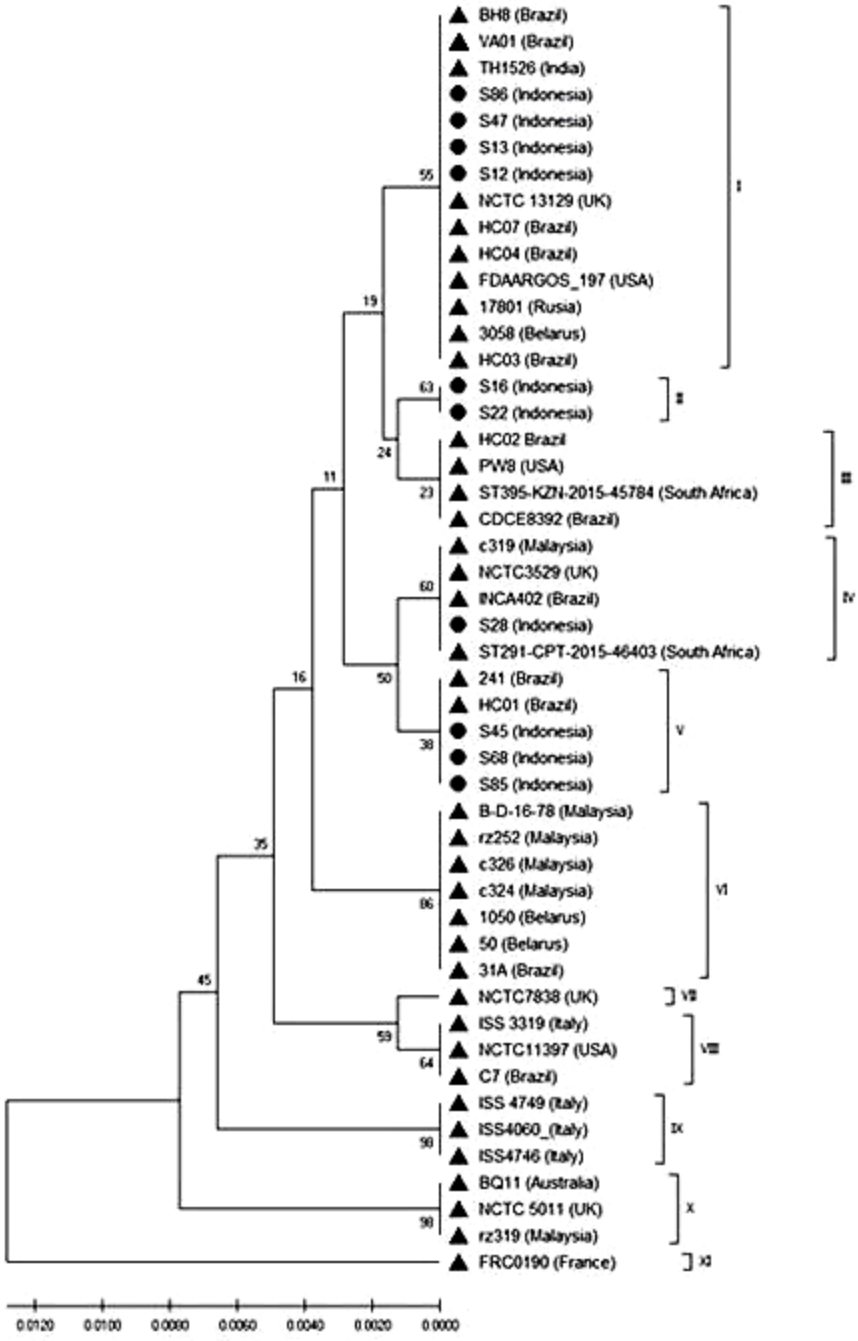

Figure 3. Phylogenetic tree based on partial *rpo*B gene of *Corynebacterium diphtheriae.* Indonesian strains are indicated by black circles. Comparison strains from other countries are indicated by black triangles. Bacterial strains are named following strain codes and countries where bacteria isolated. I-IX: Clades

The phylogenetic tree classified 48 strains involved in this study into eleven clades (Figure 3) with 96.5-100% similarity. Strains within each clade have a 100% DNA sequence similarity. The DNA similarity between strains in clades I and II, I and IV, I and V, II and IV, II and V, and IV and V was 99.5, 99.2, 99.5, 99.2, 99.5, and 99.7%. Ten strains of *C.dphtheriae* originating from Indonesia in this study were clustered into 4 clades (clade I, II, IV, and V). Four Indonesia strains (S12, S13, S47, and S86) in clade I were closely related to strains from Brazil (VA01, HC03, HC04, BH8, and HC07), India (TH1526), United Kingdom (NCTC 13129), United States (FDAARGOS 197), Belarus (3058), and Russia (17801). Clade II consisted of two Indonesia strains (S16 and S22). In clade IV, one Indonesia strain (S28) was closely related to South African strain (ST291-CPT-2015-46403), Malaysia (c319), United Kingdom (NCTC 3529) and Brazil (INCA 402). In clade V, three Indonesia strains (S45, S68, and S85) were closely related to two Brazil strains (HC01 and 241). Overall, 8 strains of *C. diphtheriae* originating from Indonesia within

clade I, IV, and V are closely related to other strains from other countries, and 2 strains (S16 and S22) were unique for Indonesia. The strain coded NCTC 13129 is a strain that caused a large outbreak in the former Soviet Union (Cerdeno-Tarraga et al. 2003) and closely related to four Indonesia strains (S12, S13, S47, and S86).

Based on the patient's residence, four Indonesia strains in clade I were isolated from patients living in South Jakarta (S86), Bekasi (S47), East Jakarta (S13), and Bogor (S12). Three strains in clade V were isolated from patients living in East Jakarta (S45), Bekasi (S68), and West Jakarta (S85). Strains in clade II (S16 and S22) and clade IV (S28) were isolated from patients living in Central Jakarta; therefore these strains are unique for Central Jakarta. Seven strains in clade I and V were dispersed in 3 cities (Jakarta, Bekasi, and Bogor), indicating the possibility of strains circulating in 3 cities. However, we do not have data relating to the patient's travel history. Diphtheria disease will remain a health problem due to the movement of people (Sadoh and Oladokun 2012; Seth-Smith and Egli 2019). Thus, the information on the travel history of infected patients is important to predict the potential transmissions within one country or among countries, so this disease could be prevented in the future.

Phylogenetic analysis in this study was based on a partial *rpo*B gene, so it can only be used to predict the genetic relationships. Further study is needed to obtain valid and comprehensive conclusions on the genetic relationships of *C. diphtheria*, based on several virulence genes, multilocus sequence typing, and/or whole-genome analysis (du Plessis et al. 2017; Ramdhan et al. 2019). The study is important for understanding the emergence of outbreaks (Seth-Smith and Egli 2019). For now, next-generation sequencing (NGS) technologies can also result in comprehensive data of pathogens, including virulent factors, drug resistance, toxin, and other genomic sequences contributing to diphtheria outbreaks (Trost et al. 2012; Sangal et al. 2017).

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