Iranian Journal of Microbiology
Volume 12 Number 6 (December 2020) 508-515

ORIGINAL ARTICLE

Detection of toxin-producing *Corynebacterium diphtheriae* from throat swabs of diphtheria patients using duplex real-time PCR

Yeva Rosana[1*], Luh Inta Prilandari[2], Ruhsyahadati Ajisman[2], Teguh Sarry Hartono[3], Andi Yasmon[1]

[1]Department of Microbiology, School of Medicine, Universitas Indonesia, Ciptomangunkusumo Hospital, Jakarta, Indonesia
[2]Department of Microbiology, Clinical Microbiologist Program, School of Medicine, Universitas Indonesia, Jakarta, Indonesia
[3]Department of Microbiology, Sulianti Saroso Infectious Diseases Hospital, Jakarta, Indonesia

Received: June 2020**, Accepted:** October 2020

ABSTRACT

Background and Objectives: Diphtheria is a potentially fatal disease caused by toxigenic bacterial infection, particularly from *Corynebacterium diphtheriae (C. diphtheriae)*. Isolation of *C. diphtheriae* is technically lacking in sensitivity, and Elek's test to detect toxin production has several difficulties associated with its application. Duplex real-time PCR to throat swab of suspected diphtheria patients can detect both bacteria and toxin-encoding genes simultaneously, faster, with higher sensitivity and specificity.

Materials and Methods: A total of 89 consecutive throat swabs from suspected diphtheria patients were collected from Sulianti Saroso Infectious Disease Hospital, Jakarta, during 2018 to 2019. Two pairs of primers and probes, targeting the *rpoB* gene of *C. diphtheriae* and the A-subunit of the diphtheria toxin gene, were used in this study. Parameters including annealing temperature, concentration of primers and probes, inhibitors, cross-reaction and detection limit were all optimized. Elek's toxigenicity test and clinical data were analyzed for comparison.

Results: The optimum annealing temperature was 55°C. The concentrations of *Cd* primer, *Tox* primer, *Cd* probe and *Tox* probe were 0.4, 0.6, 0.5 and 0.625 µM, respectively. DNA elution and template volumes were 50 µL and 5 µL. The detection limit was 2 CFU/mL. No cross-reaction with other microorganisms was observed. Of the 89 samples, duplex real-time PCR gave better results than the standard test, with 19 (21.3%) and 10 (11.2%) patients diagnosed with diphtheria, respectively.

Conclusion: Duplex real-time PCR increases the rate of laboratory diagnosis of diphtheria, compared to the standard method to detect potentially toxigenic *C. diphtheriae*.

Keywords: *Corynebacterium diphtheriae*; Diphtheria toxin; Real-time polymerase chain reaction

INTRODUCTION

Diphtheria is an acute, toxin-mediated bacterial infection, predominantly located in the upper respiratory tract (1). The diphtheria toxin is the major virulence factor and cause of death in diphtheria patients. There are more than 122 species of *Corynebacteria*, but only three are potentially toxigenic in human: *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* (2). The most common etiology of diphtheria is the toxigenic strain of *C. diphtheriae*, whereas *C. ulcerans* and *C. pseudotuberculosis* are zoonotic and rarely found.

Diphtheria was one of the leading causes of childhood death globally in the pre-vaccine era. After the invention of the diphtheria toxoid, the incidence of diphtheria declined, although several outbreaks still

*Corresponding author: Yeva Rosana, Ph.D, Department of Microbiology, School of Medicine, Universitas Indonesia, Cipto Mangunkusumo Hospital, Jakarta, Indonesia.
Tel: +62-3160492
Fax: +62-3100810
Email: yeva.rosana@ui.ac.id

occurred, due to the large population of non-immune adults and poor vaccination coverage in children. Asia has the highest portion of global diphtheria incidence since 2000. Among the countries in Asia, India has the largest amount of reported diphtheria cases, followed by Indonesia and Nepal (3). Diphtheria cases in Indonesia have increased, peaking in 2012, with sustained annual incidences over the following years. Diphtheria outbreak during 2017 was reported in 170 districts of 30 Indian provinces, with 954 total cases and a fatality rate of 4.61%. Three phases of outbreak response immunisation (ORI) have been established, in order to follow up on the outbreak of diphtheria. The Indonesia Ministry of Health reported 1665 diphtheria cases in 2018, with a case fatality rate of 1.74% (4). As a vaccine-preventable disease, diphtheria remains a health threat. Rapid, prompt and reliable diagnostic methods for diphtheria are needed to support clinical diagnosis.

Diagnosis and treatment of diphtheria are based on clinical signs and symptoms. Microscopic examination previously showed non-specific diphtheroid morphology, and could not differentiate between toxigenic and non-toxigenic *Corynebacterium* sp. and other flora in the upper respiratory tract. Culturing followed by toxigenicity testing is considered the gold standard for testing of laboratory-confirmed cases, but is time-consuming, requires expertise and is only done in referral laboratories. Polymerase chain reaction (PCR) testing is a fast, sensitive and specific method. De Zoysa et al. (2016) developed a quadriplex real-time PCR assay for detection of *C. diphtheriae, C. ulcerans, C. pseudotuberculosis* and diphtheria toxin genes (2). As *C. diphtheriae* is the most frequent cause of diphtheria, detection of this species can support patient diagnosis and direct them to seek prompt treatment and control of infection. Therefore, this study focused on optimising duplex real-time PCR to detect both the bacteria and diphtheria toxin-carrying gene of diphtheria. Optimised duplex real-time PCR was performed using clinical samples of suspected diphtheria patients.

MATERIALS AND METHODS

Clinical specimens and patients. Specimens were collected over the period from October 2018 to June 2019, after the implementation of ORI in Jakarta. Throat specimens were taken from the base of the pseudomembrane lesion, using Amies swabs from suspected respiratory diphtheria patients admitted to Sulianti Saroso Infectious Disease Hospital, Jakarta. Informed consent was obtained in all cases. The study was approved by the Ethics Committee, Faculty of Medicine, Universitas Indonesia, number 0109/UN2.F1/ETIK/2018 and Sulianti Saroso Infectious Disease Hospital, Jakarta, number 50/XXXVIII.10/VIII/2018.

The first throat swabs collected at the time of admission were examined using standard methods in Sulianti Saroso Infectious Disease Hospital, Jakarta, to support diagnosis. Swabs were stored in cryotubes containing sterile phosphate buffered saline (PBS) at 2 to 8°C, until further processing for PCR preparation. Secondary data, including age, gender, clinical symptoms, signs, vaccination history and treatment during hospitalisation and outcome were analysed based on medical records.

Culturing and toxigenity testing. Throat swabs were cultured in non-selective sheep blood agar and selective tellurite medium. Both media were incubated at 37°C, and observed for suspected colonies in 24 to 48 hours. Suspected black colonies in tellurite medium were stained with Gram/Albert's stain, and identified using VITEK 2 ANC cards. *C. diphtheriae* isolates were sent for biotyping and toxigenicity testing to the National Referral Laboratory at Balai Besar Laboratorium Kesehatan (BBLK) Surabaya, Indonesia. Biochemical reactions were used to determine the biotype of the isolate. The toxigenicity test performed was Engler's modified Elek's test (5).

Specimens preparation and DNA extraction. Cryotubes containing swabs were vortexed for 2 min, and then 500 µL of the solution was transferred into an Eppendorf tube and centrifuged at 12,000 rpm for 5 min. The pellet was separated from the supernatant and stored at 80°C until extraction. DNA extraction was performed using QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. After extraction, the DNA was stored at -20°C prior to PCR testing. Sterile PBS was used as a negative control for each extraction batch, in order to detect cross-contamination.

Duplex real-time PCR. This study used two pairs of primers and specific probes targeting the RNA polymerase b-subunit-encoding gene *(rpoB) C. diph-*

theriae and the A-subunit diphtheria toxin gene *(Tox)*, as previously reported by De Zoysa et al. (2), with modified flourophores and quenchers. The primer's nucleotide sequences for the *rpoB* gene target were (dip_rpobF-CGTTCGCAAAGATTACGGAACCA), (dip_rpobR-CACTCAGGCGTACCA ATCAAC), while the nucleotide probe sequence was (CdipHP-FAM-AAGTTCCGGGGC TTCTCGATAT-TCA-BHQ~1). The primer's nucleotide sequences for the *Tox* gene target were (toxAF-CTTTTCTTC-GTACCACGGGACTAA), (toxAR-CTATA-AAACCCTTTCCA ATCATCCTC), while the nucleotide probe sequence was (diptoxHP-TAM-RA-AAGGTATACAAA AGCCAAAATCTGGTA-CACAAGG-BHQ~1). Amplicons fragment sizes for *rpoB* and *toxA* genes were 97 bp and 117 bp, respectively.

The optimisation of parameters in the PCR, including annealing temperature, primer concentration, probe concentration, and inhibitors was performed prior to applying for clinical samples. Optimisation was performed using *C. diphtheriae* American Type Culture Collection (ATCC) 13812 isolate as the positive control. DNA for the positive control was extracted using QIAamp DNA minikit (Qiagen) and purification was performed using QIAamp DNA purification kit (Qiagen). Positive and negative controls were used in each reaction of PCR. The negative control used was nuclease-free water (NFW). To avoid cross-contamination, different rooms and areas were used for PCR reagent mixing, DNA extraction, templating and amplification. Filter tips were used for all pipetting steps. The optimum condition was decided based on the value of Cycle threshold (Ct) and the intensity of fluorescence signal. Amplification was done using an IQ5 iCycler Multicolor Detection System (Biorad).

Specificity and sensitivity of PCR. Duplex real-time PCR specificity was tested in DNA of microorganisms known to potentially cause false positive results, including upper respiratory tract flora, such as *Streptococcus pneumoniae* (ATCC 6303), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 25922), *Corynebacterium argentorantense* (clinical isolate), *Moraxella catarrhalis* (clinical specimen), *Neisseria meningitidis* (clinical specimen) and pathogens which symptom can mimic diphtheria were *Streptococcus pyogenes* (ATCC 19615), *Neisseria gonorrhoeae* (ATCC 43069), *Ebstein-Barr virus* (clinical specimen), *Herpes Simplex virus* (clinical specimen), and *Candida albicans* (ATCC 10231). The sensitivity of duplex real-time PCR was tested using a serial dilution of 0.5 McFarland (1.5×10^8 CFU/mL) of the positive control.

RESULTS

Patient characteristics. Eighty-nine patients were enrolled in this study, and throat swabs were collected from each patient at the time of admission. The range of patient ages was between 1 to 70 years, with a median age of 13 years, and consisted of 41 males and 48 females. The presence of pseudomembrane and throat pain was observed in all patients (Table 1).

Culturing and toxigenity testing. Out of 89 cultured specimens identified by VITEK2 ANC cards, 12 isolates of *C. diphtheriae* (Cd) were revealed. Two of 12 isolates showed negative results on biochemical tests using pyramidase, cystinase, nitrate, urea, and fermentation of glucose, maltose, starch and sucrose. Positive tests for toxigenic *C. diphtheriae* were observed in 10 specimens (11.2%), consisting of *C. diphtheriae var mitis* (70%) and *C. diphtheriae var gravis* (30%) (Fig. 1).

Optimisation of duplex real-time PCR. Optimisation of PCR conditions was carried out. The optimum annealing temperature was 55°C. The optimum elution volume and DNA template volumes were 50 and 5 µL, respectively. A duplex sigmoid curve was formed for positive results in PCR, showing the detec-

Table 1. Characteristics of the patients included in this study

Characteristic		Total patients (N = 89)
Gender (n (%))	Male	41 (46.2)
	Female	48 (53.7)
Age (years (%))	Median (range)	13 (1-70)
	Child (<18)	56 (62.9)
	Adult (≥18)	33 (37.1)
Clinical symptom in admission (n (%))	Pseudomembrane	89 (100)
	Throat pain when swallowing	89 (100)
	Fever	86 (96.6)
	Snoring	33 (37.1)
	Bullneck	22 (24.7)

tion of potentially toxigenic *C. diphtheriae* (Fig. 2).

Specificity and sensitivity of duplex real-time PCR. A panel of microorganisms including upper respiratory tract flora and pathogens which symptom can mimic diphtheria were tested, in order to determine the specificity of duplex real-time PCR. There was no cross-reaction of PCR assay with any of the DNA tested. Specificity of primers was also analysed by primer Basic Local Alignment Search Tool (BLAST) program. The detection limit of duplex real-time PCR was 2 CFU/mL.

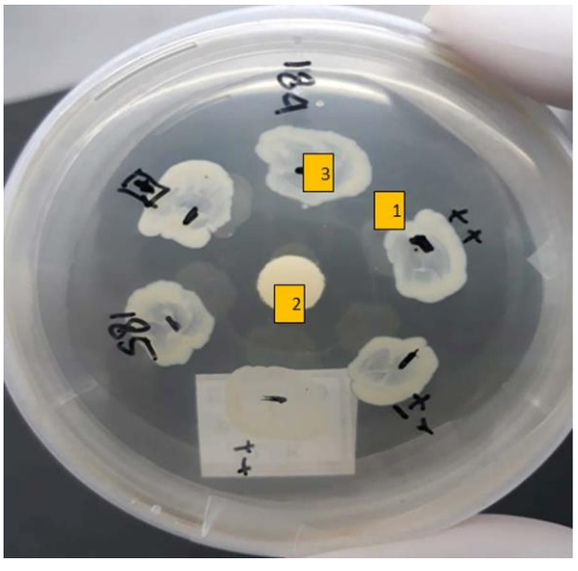

Fig. 1. Elek toxigenicity test. Immunoprecipitation line formed between Diphtheria anti toxin disc (centre) and *C. diphtheriae* isolate (numbered), indicating Diphtheria toxin production. Positive control (*C. diphtheriae var. gravis* NCTC 10648) and negative control (*C. diphtheriae var. belfanti* NCTC 10356) were used as comparison.

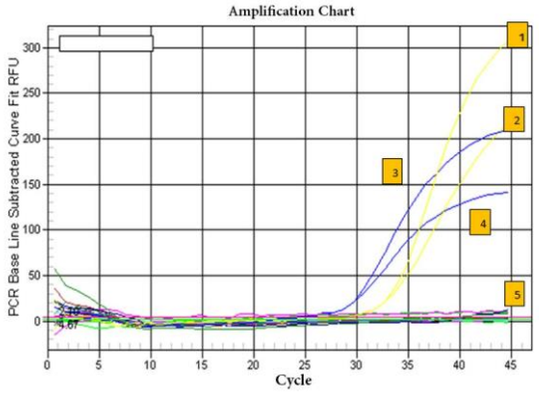

Fig. 2. Example of duplex real time PCR result, positive result indicated by duplex sigmoid curve. Yellow sigmoid curve (upper curve): *C. diphtheriae* positive control; yellow sigmoid curve (lower curve): *Tox* positive control; blue sigmoid curve (upper curve): positive *C. diphtheriae* on sample; blue sigmoid curve (lower curve): positive *Tox* on sample; flat multicolor curve: negative control and negative result on other samples.

Duplex real-time PCR on clinical samples. Optimised duplex real-time PCR was performed on all 89 samples. A duplex sigmoid curve indicated detection of *C. diphtheriae* and *Tox* gene, i.e., a positive result. The detection of both bacterium and diphtheria toxin gene indicated the presence of potentially toxigenic *C. diphtheriae*. Nineteen of 89 specimens showed a positive result in duplex real-time PCR (21.3%).

Comparison of culturing/toxigenity testing and duplex real-time PCR. There were differences in the number of positive results by culturing/toxigenity testing (10/89) and duplex real-time PCR (19/89). In this study, duplex real-time PCR method increased the rate of positive detection from 11.2% to 21.3% (Table 2).

Table 2. Comparison of standard method and duplex real time PCR result

	Culture/toxigenity test (n=89)	**Duplex real-time PCR (n=89)**
Positive result	10 (11,2%)	19 (21,3%)
Negative result	79 (88,8%)	70 (70,7%)

The 10 patients with positive results from culturing/toxigenity testing were children and adults, in equal proportion. All patients had no history of mobilisation, locally and abroad, nor any contact with confirmed diphtheria cases. Most patients did not receive complete primary diphtheria immunisation during childhood. Only 10% of patients had complete outbreak response immunisation. Most patients did not undergo antibiotic therapy prior to hospital admission. Two of 10 patients (20%) had complications including severe airway obstruction and carditis, and later died.

The 19 patients with positive results from duplex real-time PCR mostly consisted of children. Ten of 19 patients (52.6%) also showed positive results from culturing/toxigenity testing. Most patients did not receive complete primary diphtheria immunisation,

and did not receive ORI in most cases. Four out of 19 patients (21.1%) had complications including severe airway obstruction, and later died. Characteristics of patients with positive results are shown in Table 3.

DISCUSSION

Clinical symptoms and signs of diphtheria are related to the diphtheria toxin which causes complications in several organs; mainly myocard, nerves and kidneys. There are three species of *Corynebacterium* which can produce the diphtheria toxin; *C. diphtheriae, C. ulcerans* and *C. pseudotuberculosis.* Of the three toxigenic species, *C. diphtheriae* is the most frequent cause, while infection of *C. ulcerans* and *C. pseudotuberculosis* are zoonotic and rarely found.

In this study, *C. diphtheriae*, as the main cause, and diphtheria toxin gene, as the major virulence factor, are the targets of the real-time PCR assay. The main purpose of the assay is for rapid and prompt treatment, and infection control. Infection caused by *C. diphtheriae* originates from human-to-human transmitted disease, and requires an infection control strategy during treatment. All parameters in the real-time PCR assay including annealing temperature, primers concentration, probes concentration and inhibitors, were optimised to give an optimal result. There is no single protocol or single set of conditions that is optimal for all real-time PCR assays; therefore, each new real-time PCR assay requires optimisation, in order to overcome potential issues (6, 7).

This study used two pairs of primers, targeting *rpoB* gene of *C. diphtheriae* and *Tox,* representative of the A-subunit diphtheria toxin gene. DeZoysa et al. (2016) developed primer-targeting *C. diphtheriae* and toxins in a quadriplex PCR assay, and the primers were a reference point for this study. Target genes previously reported to detect *C. diphtheriae* were *dtxR, 16S rRNA* and *rpoB* (8-10). Khamis et al. (2005) compared *rpoB* and *16S rRNA* gene sequencing techniques for molecular identification of *C. diphtheriae* isolate, and reported that *rpoB* gene was both simpler and more efficient for identification in clinical practice (11).

The PCR method, using *Tox* as a gene target, was previously used, albeit with a difference in amplicon fragment length. Heuser et al. (1993) developed a primer with an amplicon fragment length of 910 bp (12). Pallen et al. (1994) developed a primer with

Table 3. Patients characteristic based on culture/toxigenity test and duplex real time PCR

No.	Age (years)	Primary immunisation	Outbreak response immunisation	Antibiotic prior admission	Culture *(C. diphtheriae)*	Biotype	Elek's test	Duplex real-time PCR test		Outcome
1	3	No	No	Not clear	Positive	mitis	Positive	Positive	*C. diphtheriae/Tox*	Died
2	7	No	No	No	Positive	mitis	Positive	Positive	*C. diphtheriae/Tox*	Survived
3	9	Complete	Complete	Yes	Positive	gravis	Positive	Positive	*C. diphtheriae/Tox*	Survived
4	11	No	No	No	Positive	gravis	Positive	Positive	*C. diphtheriae/Tox*	Survived
5	17	No	No	No	Positive	mitis	Positive	Positive	*C. diphtheriae/Tox*	Survived
6	19	No	No	No	Positive	gravis	Positive	Positive	*C. diphtheriae/Tox*	Survived
7	28	Complete	No	No	Positive	mitis	Positive	Positive	*C. diphtheriae/Tox*	Died
8	43	Not clear	Not clear	Not clear	Positive	mitis	Positive	Positive	*C. diphtheriae/Tox*	Survived
9	46	Not clear	Not clear	No	Positive	mitis	Positive	Positive	*C. diphtheriae/Tox*	Survived
10	60	Not clear	No	No	Positive	mitis	Positive	Positive	*C. diphtheriae/Tox*	Survived
11	2	Incomplete	Not clear	Yes	Negative			Positive	*C. diphtheriae/Tox*	Died
12	4	Complete	No	No	Negative			Positive	*C. diphtheriae/Tox*	Survived
13	6	Incomplete	Incomplete	No	Negative			Positive	*C. diphtheriae/Tox*	Survived
14	8	Incomplete	No	Yes	Negative			Positive	*C. diphtheriae/Tox*	Died
15	9	Incomplete	No	Yes	Negative			Positive	*C. diphtheriae/Tox*	Survived
16	13	Complete	No	No	Negative			Positive	*C. diphtheriae/Tox*	Survived
17	19	No	No	No	Negative			Positive	*C. diphtheriae/Tox*	Survived
18	42	No	No	Yes	Negative			Positive	*C. diphtheriae/Tox*	Survived
19	43	Not clear	No	Yes	Negative			Positive	*C. diphtheriae/Tox*	Survived

an amplicon fragment length of 249 bp, later followed by Mikhailovich et al. (1995) and Nakao et al. (1997) (13-15). This method was recommended by WHO, and has become widely used (16). DeZoysa et al. (2016) developed a 117 bp primer, which was a reference point for this study (2). Shorter amplicon fragment lengths may generate more efficient PCR reactions (17).

In this study, it was found that the optimum primer concentrations were 0.4 µM for *rpoB* and 0.6 µM for *Tox*. DeZoysa et al. (2016) previously used a concentration of 0.5 mM for all four primers (2). The differences arise from the PCR machine used, as well as from the different methods, enzymes, total reaction volumes and PCR conditions of different assays. This advantage of probe-based, real-time PCR is its sensitivity and specificity. The probe reported here is likely more specific than SYBR® green for real-time PCR. The optimum probe concentrations were identified as 0.5 µM for *rpoB* and 0.625 µM for *Tox*.

Optimised duplex real-time PCR was performed for all patients, and showed that the proportion of children suffering from diphtheria (62.9%) was higher than for adults (37.1%). A large proportion of non-immune adults may potentially be causing an epidemic, especially if there are also susceptible children (18). Based on gender, the proportion of females (53.9%) was slightly higher than males (46.1%). Females tend to exhibit less seroprotection than males, likely explained by gender-specific post-vaccination immune responses (19).

The use of automated VITEK 2 ANC cards identified 12 *C. diphtheriae* isolates. Identification and biotyping by biochemical testing (pyrazinamidase, nitrate, urea, cystinase, fermentation of glucose, maltose, starch and sucrose) found 10 of 12 isolates were *C. diphtheriae*. There were two disconcordant results between both methods. According to WHO, a presumptive identification screening test of toxigenic *C. diphtheriae* is essential for laboratory diagnostics (20). Efrastratiou et al. (1996) recommended a screening test of pyrazinamidase and cystinase, in order to avoid misidentification (16).

Biotyping of *C. diphtheriae* isolates showed 70% proportion of *C. diphtheriae var mitis* and 30% proportion of *C. diphtheriae var gravis.* A previous report on *C. diphtheriae* isolates from Africa, Asia and Middle East by De Zoysa (2008) reported toxigenic *C. diphtheriae var mitis* were present in most cases (21). Benamrouche et al. (2016) reported biotype mitis found among most of 157 *C. diphtheriae* isolates in Algeria (22). Biotyping differentiation is limited to epidemiologic investigation purposes (16).

Optimised duplex real-time PCR showed an increase in positive results (21.3%), compared to culturing and toxigenity testing (11.2%). Mothershed et al. (2012) reported that real-time PCR was able to detect the *tox* gene in clinical specimens containing only a few DNA types, approximately two to three copies of the target gene (23). Antibiotic use prior to sampling may also influence the culturing results. In this study, many patients with PCR-confirmed cases who showed negative results in culturing and toxigenity testing had histories of antibiotic treatment prior to sampling (55.6%). Antibiotics may kill the viable bacteria, affecting the culture result, while the PCR assay is less affected by antibiotics.

Mortality occurred in two out of 10 (20%) patients that were positive in both culturing and in duplex real-time PCR. There were two fatal cases in patients with positive PCR results who were negative according to culturing. The causes of mortality in this study were severe airway obstruction and carditis. Truelove et al. (2019) reported airways obstruction causing 60-65% mortality in diphtheria patients, while toxic cardiomyopathy was responsible for 20-25% of mortality. All fatal cases already showed diphtheria anti-toxin at time of admission. Diphtheria anti-toxin decreased mortality in 76% of patients, but anti-toxin only neutralised circulating toxins, and its effectiveness was correlated with timely administration (24).

Diphtheria is a vaccine-preventable disease. Diphtheria vaccination during childhood consists of three doses of primary vaccinations, followed by a booster vaccination; one dose under two years of age, and three doses at school age. After three doses of primary vaccination, most children will receive a protective immunity level. Boosters keep the immunity level high, as immunity wanes overtime (25). In Indonesia, primary diphtheria immunisation began in 1976 and school-age boosters started in 1984, while boosters administered at under two years of age started in 2014 (26). WHO also recommends regular adult booster vaccinations over their lifetime, but the implementation is different between countries (27). Due to diphtheria outbreak in several areas of Indonesia, three phases of ORI targeting the most susceptible persons (<19 years) were held, in order to build herd immunity. Most of the suspected cases and laboratory-confirmed cases did not have any his-

tory of primary immunisation, nor ORI, and had no immunity against diphtheria toxin as a result.

The PCR assay has significantly contributed to the development of a molecular approach to laboratory diagnosis of diphtheria, rapidly giving toxigenity-presumptive results (16). A valid negative presumptive result would exclude diagnosis, affecting further treatment. However, the molecular method could not give information about toxin production related to gene expression (28). The success of the culture method is influenced by the quality of specimen, transport, media and reagent used. Viable microorganisms were not always found in clinical specimens or were below the culture limit of detection. Antibiotic use prior to sampling may also influence the culture result. In cases where culture method cannot confirm diagnosis, the role of PCR, the more sensitive method, becomes important. Rapid and valid laboratory diagnostics will increase prompt treatments, and result in better outcome in patients.

CONCLUSION

Duplex real time PCR has been optimised to detect potentially toxigenic *C. diphtheriae*. Duplex real-time PCR increase laboratory diagnosis of diphtheria compared to culture and toxigenity method.

ACKNOWLEDGEMENTS

This study was funded by International-indexed Publication Grant of Universitas Indonesia 2018 (PITTA). We would like to thank our colleagues, Lasma Susi Simanjuntak, the team from Department of Microbiology School of Medicine Universitas Indonesia, and Sulianti Saroso Infectious Diseases Hospital Jakarta for technical assistance and the collaborative cooperation in sample collection.

REFERENCES

1. Leber A (2016). Clinical microbiology procedures handbook. 4th ed. Washington: ASM Press. pp: 390-399.
2. De Zoysa A, Efstratiou A, Mann G, Harrison TG, Fry NK. Development, validation and implementation of a quadruplex real-time PCR assay for identification of potentially toxigenic *Corynebacteria*. *J Med Microbiol* 2016; 65:1521-1527.
3. Clarke, Kristie. Review of the epidemiology of diphtheria 2000-2016. US Centre Disease Control Prevention. 2016. https://www.who.int/immunization/sage/meetings/2017/april/1_Final_report_Clarke_april3.pdf
4. Kementerian Kesehatan Republik Indonesia. Data dan informasi profil kesehatan Indonesia 2018. https://pusdatin.kemkes.go.id/resources/download/pusdatin/profil-kesehatan-indonesia/Data-dan-Informasi_Profil-Kesehatan-Indonesia-2018
5. Engler KH, Glushkevich T, Mazurova IK, George RC, Efstratiou A. A modified elek test for detection of toxigenic *Corynebacteria* in the diagnostic laboratory. *J Clin Microbiol* 1997;35:495-498.
6. Grunenwald H (2003). Optimization of polymerase chain reaction. In: Methods in molecular biology vol 226; PCR protocols. 2nd edition. Humana Press Inc. pp: 89-100.
7. Innis M, Gelfand D. Optimization of PCRs (1990). In: PCR Protocol, a guide to methods and applications. San Diego, CA: Academic Press, Inc. pp: 3-12.
8. De Zoysa A, Efstratiou A, Hawkey PM. Molecular characterization of diphtheria toxin repressor *(dtxR)* genes present in nontoxigenic *Corynebacterium diphtheriae* strains isolated in the United Kingdom. *J Clin Microbiol* 2005;43:223-228.
9. Pimenta FP, Matias GAM, Pereira GA, Camello TCF, Alves GB, Rosa ACP, et al. A PCR for *dtxR* gene: Application to diagnosis of non-toxigenic and toxigenic *Corynebacterium diphtheriae. Mol Cell Probes* 2008;22:189-192.
10. Sunarno, Mulyastuti Y, Puspandari N, Sariadji K. DNA sequence analysis of *dtxR* gene (partial) of *Corynebacterium diphtheriae* causing diphtheria in Jawa and Kalimantan Islands, Indonesia. *Indones Biomed J* 2017;9:91.
11. Khamis A, Raoult D, La Scola B. Comparison between *rpoB* and 16S rRNA gene sequencing for molecular identification of 168 clinical isolates of *Corynebacterium. J Clin Microbiol* 2005;43:1934-1936.
12. Hauser D, Popoff MR, Kiredjian M, Boquet P, Bimet F. Polymerase chain reaction assay for diagnosis of potentially toxinogenic *Corynebacterium diphtheriae* strains: Correlation with ADP-ribosylation activity assay. *J Clin Microbiol* 1993;31:2720-2723.
13. Pallen MJ, Hay AJ, Puckey LH, Efstratiou A. Polymerase chain reaction for screening clinical isolates of *Corynebacteria* for the production of diphtheria toxin. *J Clin Pathol* 1994;47:353-356.
14. Mikhailovich VM, Melnikov VG, Mazurova IK, Wachsmuth IK, Wenger JD, Wharton M, et al. Application of PCR for detection of toxigenic *Corynebac-*

terium diphtheriae strains isolated during the Russian diphtheria epidemic, 1990 through 1994. *J Clin Microbiol* 1995;33:3061-3063.

15. Nakao H, Pruckler JM, Mazurova IK, Narvskaia OV, Glushkevich T, Marijevski VF, et al. Heterogeneity of diphtheria toxin gene, tox, and its regulatory element, *dtxR*, in *Corynebacterium diphtheriae* strains causing epidemic diphtheria in Russia and Ukraine. *J Clin Microbiol* 1996;34:1711-1716.
16. Efstratiou A, Engler KH, Mazurova IK, Glushkevich T, Vuopio-Varkila J, Popovic T. Current approaches to the laboratory diagnosis of Diphtheria. *J Infect Dis* 2000; 181 Suppl 1:S138-145.
17. Debode F, Marien A, Janssen É, Berben G, Bragard C. The influence of amplicon length on real-time PCR results. *Biotechnol Agron Soc Environ* 2017;21:3-11.
18. Galazka A. The changing epidemiology of Diphtheria in the vaccine Era. *J Infect Dis* 2000;181 Suppl 1:S2-9.
19. Völzke H, Kloker KM, Kramer A, Guertler L, Dören M, Baumeister SE, et al. Susceptibility to diphtheria in adults: prevalence and relationship to gender and social variables. *Clin Microbiol Infect* 2006;12:961-967.
20. Efstratiou A, George RC. Screening tests for the presumptive identification of *Corynebacterium diphtheriae* in a diagnostic laboratory. *J Clin Microbiol* 1996; 34:3251.
21. De Zoysa A, Hawkey P, Charlett A, Efstratiou A. Comparison of four molecular typing methods for characterization of *Corynebacterium diphtheriae* and determination of transcontinental spread of *C. diphtheriae* based on BstEII rRNA gene profiles. *J Clin Microbiol* 2008;46:3626-3635.
22. Benamrouche N, Hasnaoui S, Badell E, Guettou B, Lazri M, Guiso N, et al. Microbiological and molecular characterization of *Corynebacterium diphtheriae* isolated in Algeria between 1992 and 2015. *Clin Microbiol Infect* 2016;22:1005.e1-1005.e7.
23. Mothershed EA, Cassiday PK, Pierson K, Mayer LW, Popovic T. Development of a real-time fluorescence PCR assay for rapid detection of the Diphtheria toxin gene. *J Clin Microbiol* 2002;40:4713-4719.
24. Truelove SA, Keegan LT, Moss WJ, Chaisson LH, Macher E, Azman AS, et al. Clinical and epidemiological aspects of diphtheria: a systematic review and pooled analysis. *Clin Infect Dis* 2020;71:89-97.
25. World Health Organization. Diphtheria vaccine: review of evidence on vaccine effectiveness and immunogenicity to assess the duration of protection ≥10 years after the last booster dose. 2017. https://www.who.int/immunization/sage/meetings/2017/april/2_Review_Diphtheria_results_April2017_final_clean.pdf
26. Anggraeni N, Dian PY, Umar AN, Mazanova D, Handini S, Widodo NH, et al. Pedoman Pencegahan dan Pengendalian Difteri. 2017. 1-51. https://sehatnegeriku.kemkes.go.id/wp-content/uploads/2018/01/buku-pedoman-pencegahan-dan-penanggulangan-difteri.pdf
27. Weinberger B. Adult vaccination against tetanus and diphtheria: the European perspective. *Clin Exp Immunol* 2017;187:93-99.
28. Efstratiou A, Engler KH, Dawes CS, Sesardic D. Comparison of phenotypic and genotypic methods for detection of diphtheria toxin among isolates of pathogenic Corynebacteria. *J Clin Microbiol* 1998;36:3173-3177.