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

Yeva Rosana¹, Luh Inta Prilandari², Ruhsyahadati Ajisman³, Teguh Sarry Hartono³, Andi Yasmon¹

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

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**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
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. C. ulcerans, and C. pseudotuberculosis are 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 toxicity 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 (Qia-gen), 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. diphe-
theriae and the A-subunit diphtheria toxin gene (Tox), as previously reported by De Zoyza et al. (2), with modified fluorophores and quenchers. The primer’s nucleotide sequences for the rpoB gene target were (dip_rpoB-CGTTCGCAAGATTACGGAACCA), (dip_rpoBR-CACTCGGGTACCA ATCAAC), while the nucleotide probe sequence was (CdipHP-FAM-AAGTTCGGGGCC TCTCGATATCA-BHQ-1). The primer’s nucleotide sequences for the Tox gene target were (toxAB-CTTTTCTTCGACCGAGACTAA), (toxAR-CTATATTAAACCTTTCGACATCCT), while the nucleotide probe sequence was (diptoxHP-TAMRA-AAGTATACAAA AGCCAAAATCTGGTACACAAGG-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 argentrantense (clinical isolate), Moraxella catarrhalis (clinical isolate), Neisseria meningitidis (clinical isolate) 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 × 10^4 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 toxigenicity 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 pyridoxidase, 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 admission (n (%)) | Pseudomembrane 89 (100) |
|                               | Throat pain when swallowing 89 (100) |
|                               | Fever 86 (96.6)        |
|                               | Snoring 33 (37.1)      |
|                               | Bullneck 22 (24.7)     |

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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.

**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).

|                        | 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%)                 |

Fig. 1. Elek toxigenicity test. Immunoprecipitation line formed between Diphtheria anti toxin disc (centre) and *C. diphtheriae* isolate (numbered), indicating Diphtheria tox- in production. Positive control (*C. diphtheriae var. gravis NCTC 10648*) and negative control (*C. diphtheriae var bel-fanti 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.

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 quadruplex 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/toxigenicity 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 mits            | Positive | Positive   | Positive C. diphtheriae/Tox | Died    |
| 2   | 7           | No                   | No                             | Positive                  | mits                     | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 3   | 9           | Complete             | Complete                       | Positive                   | mits                     | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 4   | 11          | No                   | No                             | Positive                   | mits                     | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 5   | 17          | No                   | No                             | Positive                   | mits                     | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 6   | 19          | No                   | No                             | Positive                   | mits                     | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 7   | 28          | Complete             | No                             | Positive                   | mits                     | Positive | Positive   | Positive C. diphtheriae/Tox | Died    |
| 8   | 43          | Not clear            | Not clear                      | Positive                   | mits                     | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 9   | 46          | Not clear            | Not clear                      | Positive                   | mits                     | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 10  | 60          | Not clear            | No                             | Positive                   | mits                     | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 11  | 2           | Incomplete           | Not clear                      | Positive                   | mits                     | Positive | Positive   | Positive C. diphtheriae/Tox | Died    |
| 12  | 4           | Complete             | No                             | Positive                   | Negative                 | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 13  | 6           | Incomplete           | Incomplete                     | Positive                   | Negative                 | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 14  | 8           | Incomplete           | No                             | Negative                   | Positive                 | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 15  | 9           | Incomplete           | No                             | Negative                   | Positive                 | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 16  | 13          | Complete             | No                             | Negative                   | Positive                 | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 17  | 19          | No                   | No                             | Negative                   | Positive                 | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 18  | 42          | No                   | No                             | Negative                   | Positive                 | Positive | Positive   | Positive C. diphtheriae/Tox | Survived|
| 19  | 43          | Not clear            | No                             | Negative                   | Positive                 | Positive | Positive   | 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 discordant 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 Zoya (2008) reported toxigenic C. diphtheriae var mitis were present in most cases (21). Benamrouche et al. (2016) reported biotype mi-
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 toxigenicity-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 specimens, 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. diptheriae. Duplex real-time PCR increase laboratory diagnosis of diphtheria compared to culture and toxigenicity method.

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