



Development and Application of *Dtxr* and *Tox* Genes Targeting Real-time PCR to Identify *Corynebacterium diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* Simultaneously

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Abstract

Background: *Corynebacterium diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* are known as diphtheria-causing bacteria. Although diphtheria therapy is administered based on the clinical manifestations, some cases are mild and atypical. The immediate and accurate identification of diphtheria-causing bacteria is of paramount importance to prevent the spread of the disease and provide case management as early as possible. Unfortunately, conventional methods as the gold standard are time-consuming.

Objectives: This study aimed to develop and implement a multiplex real-time PCR with the *dtxR* and *tox* genes as the target to identify three species of diphtheria-causing bacteria and screen their toxigenicity quickly and accurately.

Methods: The research sample encompassed seven reference strains, one synthetic DNA, 30 archived isolates, and 924 clinical specimens isolated from 311 diphtheria cases and 613 close contacts. The conventional methods as the gold standard and the established PCR assay were used to verify the results of multiplex real-time PCR developed in this study.

Results: The multiplex real-time PCR could identify seven reference strains, one synthetic DNA, and 30 archived isolates as accurately as the conventional methods and the established PCR. Similar to established PCR, the multiplex real-time PCR identified diphtheria-causing bacteria in 120 (38.6%) out of 311 and 12 (2%) out of 613 clinical specimens from diphtheria cases and close contacts, respectively. Meanwhile, the conventional methods identified diphtheria-causing bacteria in 79 (25.4%) out of 311 and three (0.5%) out of 613 clinical specimens.

Conclusions: The multiplex real-time PCR developed in this study can be used to identify three species of diphtheria-causing bacteria and screen their toxigenicity quickly and accurately. However, in this study, no diphtheria-causing bacteria other than *C. diphtheriae* was found in the clinical samples using the PCR or conventional methods. PCR is more sensitive than the conventional methods and can be used as an additional test in diphtheria laboratories.

Keywords: Diphtheria, *Corynebacterium diphtheriae*, *C. ulcerans*, *C. pseudotuberculosis*, Polymerase Chain Reaction

1. Background

Diphtheria is still a global health concern, especially in low- and middle-income countries. The statistics reported by the World Health Organization (WHO) indicates that countries with the highest frequency of diphtheria cases during the last five years (2015 - 2019) were India, Ethiopia, Nigeria, Madagascar, Indonesia, Yemen, Pakistan, Venezuela, and Nepal. In this regard, Indonesia is consistently among the five countries with the highest frequency of diphtheria cases each year (1). Data from the Ministry of Health of the Republic of Indonesia shows that several

regions such as East Java, West Java, Jakarta, and Banten encompassed the largest number of diphtheria cases (2). Diphtheria is a serious health problem because of its high case fatality rate (CFR) (from about 10% to > 50% in severe cases) (3). Moreover, this disease is proved to spread across regions, islands, and even countries (4).

Diphtheria is caused by *Corynebacterium diphtheriae* and two closely related species, called *C. ulcerans* and *C. pseudotuberculosis* (5). *Corynebacterium diphtheriae* is the leading cause of diphtheria and is widely isolated in developing countries. *Corynebacterium ulcerans* is more of-

ten isolated in developed countries with high immunization coverage. Meanwhile, *C. pseudotuberculosis* has rarely caused diphtheria in humans in developed and developing countries (6). The potential of these bacteria to produce diphtheria toxin makes them different from the other species of the genus *Corynebacterium*. Diphtheria toxin is a major virulence factor for diphtheria-causing bacteria and is associated with the clinical features of diphtheria disease. However, not all strains of diphtheria-causing bacteria can produce toxins (toxigenic). In nontoxigenic strains, no *tox* gene encoding toxin synthesis has been found. Nontoxigenic strains do not cause diphtheria diseases; however, their presence needs to be continuously monitored because they can cause other severe diseases such as endocarditis or bacteremia. Nontoxigenic strains can also change to toxigenic and cause diphtheria when the bacterial chromosome is inserted by a typical *Corynebacterium* carrying the *tox* gene (7, 8).

Although the diagnosis and treatment of diphtheria are based on clinical manifestation, some cases are mild and atypical. Laboratory tests are needed to rule out a differential diagnosis (9, 10). The fast and accurate laboratory tests help prevent the spread of diphtheria-causing bacteria and provide case management as early as possible. However, the gold standard for the laboratory examination of diphtheria using conventional methods has several limitations. The conventional methods were completed in 3 - 5 days, and the results were affected by sample transportation and history of antibiotic administration. This is a major problem for countries such as Indonesia. In this case, PCR can be an alternative, which is fast and relatively less affected by sample delivery problems and history of antibiotic use (10). Diphtheria laboratory examination using the PCR method has long been introduced as a screening method to examine toxigenicity. Some PCR assays focus on detecting toxigenic strains, while others include the identification of nontoxigenic strains (11, 12). However, PCR usually cannot be used to distinguish between toxigenic and non-toxigenic *tox* gene bearing (NTTB) strains. We previously succeeded in developing PCR to predict the NTTB strains; however, it is still limited to two types, namely base-25 deletion or base-55 deletion NTTB (13).

2. Objectives

The study aimed to develop and implement a multiplex real-time PCR to identify three species of diphtheria-causing bacteria and screen their toxigenicity quickly and accurately using the *dtxR* and *tox* genes as the target.

3. Methods

3.1. PCR Primers and Probes

The multiplex real-time PCR in this study used two target genes: the *dtxR* gene for species identification and

the *tox* gene for bacterial toxigenicity screening (Table 1). Only two pairs of the PCR primers were used according to the target genes. Meanwhile, the used probe consisted of one probe for the *tox* gene and three other probes for the *dtxR* gene of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* with a competitive system. Accordingly, the probe was designed to distinguish three species of diphtheria-causing bacteria regarding the differentiation of *dtxR* gene sequences. The PCR primers and probes were designed semi-manually by aligning the sequences of *tox* and *dtxR* genes of three species of diphtheria-causing bacteria. Sequence alignment was carried out using BioEdit on the variations of *dtxR* and *tox* genes of three diphtheria-causing bacteria. A pair of the PCR primers and a probe targeting the *tox* gene were designed in the conserved and specific regions for all three species and the pairs of PCR primers targeting the *dtxR* gene. Some parameters, including G-C content, Tm, run, and repeat, were obtained with PerlPrimer software. The specificity of the PCR primer and probe was analyzed with online Primer-BLAST. The delta G (free energy) value of the self-dimer or cross-dimer at the 3' end was not < -7 kcal/mol (14).

3.2. Samples

The research sample encompassed seven reference strains of *Corynebacterium* spp, a synthetic DNA, 30 archived isolates, and 924 clinical specimens. The reference strains consisted of three isolates of toxigenic *C. diphtheriae* (ATCC 13812, NCTC 10648, and NCTC 3984), one isolate of non-toxigenic *C. diphtheriae* (NCTC 10356), one isolate of non-toxigenic *C. ulcerans* (NCTC 12077), one isolate of *C. striatum* (NCTC 764), and one isolate of *C. minutissimum* (ATCC 23348). A synthetic double-stranded DNA (gBlock, Integrated DNA Technologies, Inc.) identical to the *dtxR* gene of *C. pseudotuberculosis* 316 was used to substitute the *C. pseudotuberculosis* isolate not available in this study.

The archive isolates were as follows: 10 isolates of toxigenic *C. diphtheriae*, one isolate of non-toxigenic *C. diphtheriae*, and 19 isolates of non-diphtheria-causing bacteria (namely *Neisseria meningitidis*, *N. gonorrhoeae*, *Streptococcus pneumoniae*, *S. agalactiae*, *Staphylococcus aureus*, *S. epidermidis*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Enterobacter sakazakii*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Salmonella typhimurium*, *Haemophilus influenzae b*, *Shigella flexneri*, *Aeromonas hydrophila*, *Vibrio cholerae*, *Escherichia coli*, *Clostridium tetani*, and *Candida albicans*). The *C. diphtheriae* and *C. ulcerans* isolates and synthetic DNA were used as a positive control, while the others were used as a negative control in the PCR optimization method. The clinical specimen in this study were throat swabs sent to the laboratory for diphtheria examination. In this study, 924 throat swabs were collected from 311 diphtheria clinical cases and 613 close contacts. The clinical

cases were diagnosed by a physician when the signs and symptoms matched the diphtheria criteria described in the National Diphtheria Surveillance Guideline.

3.3. Conventional Methods

The reference strains and the archived isolates were revived and re-identified by microscopic and biochemical examination and Elek tests. On the other hand, the clinical specimens were identified using conventional methods according to the WHO guidelines for diphtheria laboratory (15). The diphtheria-causing bacteria were identified using API Coryne® (bioMérieux), while the bacterial toxigenicity was determined using modified Elek tests (16). After the clinical specimens were examined using the conventional methods, the samples were transferred to 0.5 mL Aquadest in the microtube for the DNA extraction.

3.4. Multiplex Real-time PCR

DNA was extracted using the commercial QIAamp DNA Mini Kit (Qiagen) (17). The DNA in 50 µL molecular water was stored at -20°C for the DNA template in the PCR examination. The multiplex real-time PCR process was run in the CFX 96 thermal cycler (Biorad). The PCR mix consisted of 10 µL SensiFAST™ Probe No-ROX (Bioline), 0.5 µL each PCR primer, 0.5 µL each probe, 1 µL ddH₂O, and 5 µL DNA template. The PCR conditions followed an initial denaturation phase at 95°C for 3 min, with 35 cycles of a denaturation phase at 95°C for 10 s and annealing and extension phases at 60°C for 30 s. The PCR results were interpreted according to the sigmoid curve and Ct value of each fluorophore. The Cy5 fluorophore was used as a marker for *C. diphtheriae*, while Hex and FAM fluorophores were used as markers for *C. pseudotuberculosis* and *C. ulcerans*, respectively. For bacterial toxigenicity markers, a Texas red fluorophore was used. When a sigmoid curve appeared with a Ct value < 36 on the Cy5 and Texas red fluorophores, it was concluded as toxigenic *C. diphtheriae*. When only Cy5 appeared, it was concluded as nontoxigenic *C. diphtheriae*. The same applied to the Hex fluorophore for *C. pseudotuberculosis* and the FAM fluorophore for *C. ulcerans*. If only the Texas red fluorophore is present, the test should be repeated.

3.5. Established Conventional PCR and Data Analysis

The established conventional PCR assay was carried out to ensure the accuracy of the multiplex real-time PCR assay. The procedure of the conventional PCR assay was according to the protocol described in the previous study (13). The multiplex real-time PCR results were considered accurate when the results were similar to the established conventional PCR results, even though the conventional methods showed different results.

4. Results

4.1. PCR Primers and Probes

The developed multiplex real-time PCR in this study used two pairs of PCR primers and four Taqman probes (Table 1). Primer-dimer binding risks were decreased by minimizing the number of the PCR primers. The sequences analysis showed acceptable criteria for the primer and probe, including the presence of conserved and specific sequences with 40 - 60% GC content (Data is not presented here).

4.2. Conventional Methods

The re-identification of isolates showed that the results matched the data of the isolates (Table 2). The identification of the clinical specimens using conventional methods showed that the diphtheria-causing bacteria (*C. diphtheriae*) were isolated from 79 (25.4%) out of 311 and three (0.5%) out of 613 clinical samples isolated from diphtheria cases and close contacts, respectively. Eighty of the 82 isolates were identified as toxigenic using the modified Elek test; however, two isolates were non-toxigenic.

4.3. PCR Assay

Seven reference strains and one synthetic DNA were used to optimize the PCR reaction (Figure 1). For three isolates of toxigenic *C. diphtheriae* (ATCC 13812, NCTC 10648, and NCTC 3984), the sigmoid curve for both fluorophores (Cy5 dan Texas red) appeared. In contrast, for one isolate of non-toxigenic *C. diphtheriae* (NCTC 10356), one isolate of non-toxigenic *C. ulcerans* (NCTC 12077), and one DNA synthetic of *C. pseudotuberculosis*, there was only one sigmoid curve of fluorophore (Cy5 for *C. diphtheriae*, FAM for *C. ulcerans*, and Hex for *C. pseudotuberculosis*). On the other hand, no sigmoid curve of fluorophore appeared for the two isolates of non-diphtheria-causing bacteria (NCTC 764 and ATCC 23348). Thirty archived isolates were accurately identified using a multiplex real-time PCR as there were 10 isolates toxigenic *C. diphtheriae*, one isolate non-toxigenic *C. diphtheriae*, and 19 isolates negative for diphtheria-causing bacteria (Table 2). The results also showed that 130 toxigenic *C. diphtheriae* and two non-toxigenic *C. diphtheriae* were identified in 120 (38.6%) out of 311 clinical specimens from cases and 12 (2%) out of 613 clinical specimens from close contacts. No *C. ulcerans* and *C. pseudotuberculosis* were found in the clinical specimen in this study. The findings were similar to the established PCR results.

5. Discussion

This study developed a multiplex real-time PCR to identify diphtheria-causing bacteria, similar to previous studies by de Zoysa et al. and Badell et al. (18, 19). However, the *dtxR* gene was used in this study, while the aforementioned

Table 1. PCR Primers and Probes in Present Study

PCR; Primers & Probes	Sequence (5' - 3')	Gene	Target
DIP_UL_PSE_F	CCTACAGTTAGCCAAACMGTTGC	<i>dtxR</i>	<i>Corynebacterium diphtheriae</i> , <i>C. ulcerans</i> , <i>C. pseudotuberculosis</i>
DIP_UL_PSE_R	CGGCAGGCTTCATCGTGMAC	<i>dtxR</i>	<i>C. diphtheriae</i> , <i>C. ulcerans</i> , <i>C. pseudotuberculosis</i>
DIPHATHER	Cy5-ACTTGTCGTTGTCGCCTCaGACC-BHQ2	<i>dtxR</i>	<i>C. diphtheriae</i>
ULCER	FAM-TAGTCGCaTCcGACCGAGC-BHQ1	<i>dtxR</i>	<i>C. ulcerans</i>
PSEUDOT	Hex-GTAGTTGCgTcTGACCGTAGTCTTCAA-BHQ1	<i>dtxR</i>	<i>C. pseudotuberculosis</i>
TOX_B_F	CAGTTGGAACACTGTTGAAGATTCTGAT	<i>tox</i>	<i>C. diphtheriae</i> , <i>C. ulcerans</i> , <i>C. pseudotuberculosis</i> (toxigenic)
TOX_B_R	CGACCATTTACGGAATATGAGTCTTGGAC	<i>tox</i>	<i>C. diphtheriae</i> , <i>C. ulcerans</i> , <i>C. pseudotuberculosis</i> (toxigenic)
TOX_B	Texas Red-AACGTCCAGCTTCCAGGAATAGTCG-BHQ2	<i>tox</i>	<i>C. diphtheriae</i> , <i>C. ulcerans</i> , <i>C. pseudotuberculosis</i> (toxigenic)

Table 2. Comparison of Samples Identification Using Multiplex Real-time PCR Established Conventional PCR, and Conventional Methods

Variables	Multiplex Real-time PCR	Established Conventional PCR	Conventional Methods
Reference isolates/synthetic DNA			
Toxigenic <i>Corynebacterium diphtheriae</i>	3	3	3
Non-toxigenic <i>C. diphtheriae</i>	1	1	1
<i>C. ulcerans</i>	1	1	1
<i>C. pseudotuberculosis</i> ^a	1	1	NA
Negatif for diphtheria-causing bacteria	2	2	2
Total	8	8	7
Archived isolates			
Toxigenic <i>C. diphtheriae</i>	10	10	10
Non-toxigenic <i>C. diphtheriae</i>	1	1	1
<i>C. ulcerans</i> / <i>C. pseudotuberculosis</i>	0	0	0
Negatif for diphtheria-causing bacteria	19	19	19
Total	30	30	30
Clinical samples			
Toxigenic <i>C. diphtheriae</i>	130	130	70
Non-toxigenic <i>C. diphtheriae</i>	2	2	2
<i>C. ulcerans</i> / <i>C. pseudotuberculosis</i>	0	0	0
Negatif for diphtheria-causing bacteria	792	792	852
Total	924	924	924

Abbreviation: NA, not applicable.

^a Synthetic DNA.

researchers used the *rpoB* gene. Previously, we showed that the *dtxR* gene had some advantages over *16S rRNA* and *pld* genes in identifying *C. ulcerans* and *C. pseudotuberculosis* (20). Moreover, we detected and identified three species of diphtheria-causing bacteria, while they only identified two (*C. diphtheriae* and *C. ulcerans*/*C. pseudotuberculosis*).

Furthermore, we used a pair of the PCR primers to identify three species; however, they used more pairs. Using only a pair of PCR primers would decrease the risk of primer-dimer binding and reduce costs imposed by the

PCR primer synthesis. We used the *tox* gene as a target to identify toxigenicity, similar to their study. Unlike our previous study (13), we did not identify the NTTB strains to reduce the cost in this study because of the high cost of the PCR probe. Furthermore, previous studies documented that NTTB strains were never observed in diphtheria samples in Indonesia (4, 10, 13). The NTTB strain originated in Indonesia was isolated from the non-diphtheria sample (21).

The sensitivity of multiplex real-time PCR developed in

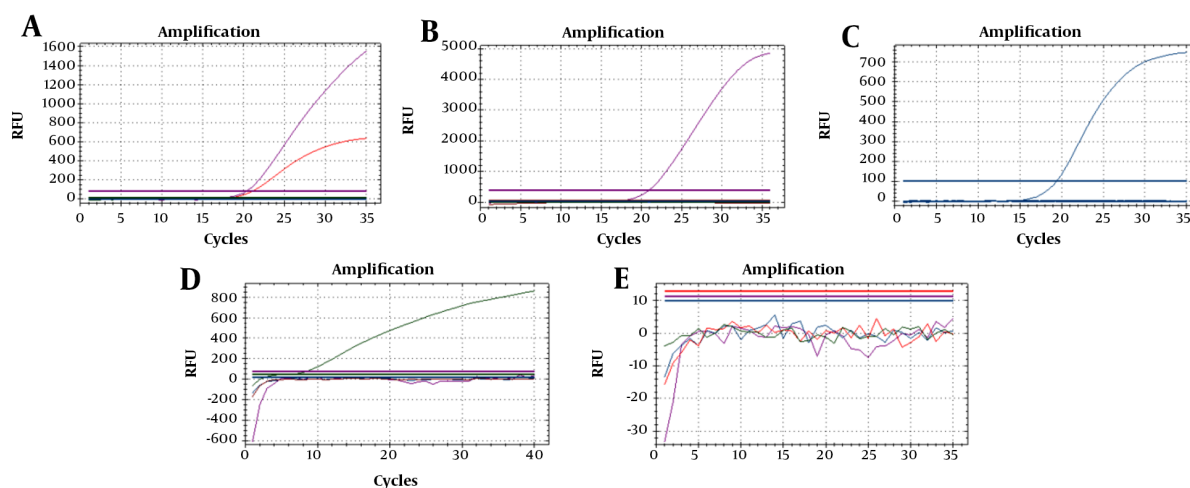


Figure 1. PCR optimization results. A, Toxigenic *Corynebacterium diphtheriae* (Cy5 and Texas Red); B, Non-toxicogenic *C. diphtheriae* (Cy5); C, Non-toxicogenic *C. ulcerans* (FAM); D, Non-toxicogenic *C. pseudotuberculosis* (DNA Synthetic) (Hex); E, Non-diphtheria-causing bacteria.

this study was quite acceptable. Regarding the limit of detection (LOD), the sensitivity was predicted by a 10-fold dilution of 0.5 Mc Farland bacteria cells up to 10^{-6} (approximately 30 CFU/200 uL bacteria cell or 30 CFU/50 uL DNA template). This means this method can detect target bacteria < 10 CFU/reaction. The sensitivity of PCR is also predicted based on its performance in identifying target bacteria in the clinical specimen. In this case, PCR was approximately 1.5 - 4 folds more sensitive than the conventional methods (38.6%: 25.4% for cases and 2%: 0.5% for close contacts). Previous studies showed that PCR was approximately two folds more sensitive than conventional PCR (11, 13). On the other hand, the efficiency of PCR in this study was in an acceptable range (90 - 110%) for four targets (data not shown). The PCR efficiency was estimated by $E = 10^{[-1/\text{slope}]} - 1$, as described previously (22).

The specificity of the PCR developed in this study was also quite acceptable. The PCR could correctly identify *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* for reference strains and synthetic DNA. Unfortunately, we found no bacteria for the archived isolates or the clinical specimens using conventional methods and PCR. The differentiation of the results between PCR and conventional methods for the clinical specimens was not predicted due to specificity weakness or mismatching. It is supported by the similarity of results between multiplex real-time PCR and established PCR assay using different PCR primers (13). Similar to a previous study (13), the identification of diphtheria-causing bacteria in the clinical samples indicated that the causative agents of diphtheria in Indonesia were dominated by toxigenic *C. diphtheriae*. This is different from the findings for developed countries where non-toxicogenic *C. diphtheriae* and *C. ulcerans* are widely isolated

(23, 24). However, we still believe that *C. ulcerans* and *C. pseudotuberculosis* can cause health problems, and this issue needs to be addressed in the future in Indonesia.

5.1. Conclusions

The developed multiplex real-time PCR in this study can identify three species of diphtheria-causing bacteria and screen their toxigenicity fastly and accurately. However, in this study, no diphtheria-causing bacteria other than *C. diphtheriae* was found in the clinical samples using PCR or conventional methods. PCR is more sensitive than conventional methods and can be used as an additional test in diphtheria laboratories.

Footnotes

Authors' Contribution: Study concept and design, S. S.; Acquisition of data, Y. H., N. A., S. N. S., and A. R.; Analysis and interpretation of data, S. S. and N. P.; Original draft preparation, N. P. and F. M.; Drafting the manuscript and literature searching, D. F. and T. F.; Manuscript editing and literature searching, R. D. S.; Editing, and visualization, R. N. and L. A. L.; Final manuscript checking and review, F. E.

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