Biomedical Journal of Indonesia
Journal Homepage: https://bji-fk.ejournal.unsri.ac.id

Optimization of Duplex Real-time PCR to Detect *Corynebacterium ulcerans* and *C. pseudotuberculosis* from Patients with Suspected Diphtheria in Jakarta

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ARTICLE INFO

Keywords:
Duplex *Real- time PCR*
C. ulcerans
C. pseudotuberculosis

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All authors have reviewed and approved the final version of the manuscript.

https://doi.org/10.32539/BJI.v12i2.319

Received 5 January 2026;
Accepted 2 March 2026

ABSTRACT

Introduction. Diphtheria infection remains a serious public health problem in many countries with low vaccine coverage. Molecular detection plays an important role in controlling disease dissemination. Among more than 122 Corynebacterium species, only three are potentially toxigenic: *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*. Infections caused by *C. ulcerans* and *C. pseudotuberculosis* in humans usually involve close animal contact. Due to the many difficulties in identifying *C. ulcerans* and *C. pseudotuberculosis*, this study aimed to optimize Duplex Real-time PCR for rapid and precise detection. **Method.** Duplex real-time PCR was performed by using 2 pairs of primers and a probe to detect *C. ulcerans* and *C. pseudotuberculosis*. All parameters were optimized to maximize PCR amplification. Then, the primer set's specificity was tested against some microorganisms. The sensitivity test of DNA was conducted to get the detection limit. **Results**: Optimization of real-time PCR was conducted to achieve optimal amplification conditions. Annealing temperature 57 °C, Optimization of primer and probe concentration of 0.45 µM and 0.50 µM for *C. ulcerans*, Primer and probe concentrations of 0.35 µM for *C. pseudotuberculosis* yielded optimal results. The detection limit of DNA of duplex real-time PCR for *C. ulcerans* was 4.49 DNA copy number and 1.06 DNA copy number for *C. pseudotuberculosis.* **Conclusion.** Duplex *Real-time* PCR optimization result can be used as a detection of *C. ulcerans* and *C. pseudotuberculosis* for effective control of dissemination in the population. PCR results showed that all 108 clinical specimens suspect diphtheria were negative for *C. ulcerans* and *C. pseudotuberculosis*.

1. Introduction

Diphtheria infection remains a serious health problem within the country with low vaccine coverage. Most people recognize vaccination as a very effective and reasonably priced health tool.[1] World Health Organization (WHO) mentions that the vaccine to prevent diphtheria should be given as a primary series of three doses to infants and supplemented with three suitably spaced booster doses for long-lasting protection. Control over the spread in populations is possible only when early detection is done. It is important for early detection to effectively control the spread in the population. Globally, coverage of the diphtheria, tetanus, pertussis third dose (DTP3) among children aged one year increased by 13 percentage points, rising from 72% in 2000 to 85% in 2024, while Indonesia reported a coverage rate of 78% in 2024.[2]

In 2024, the World Health Organization reported that the highest numbers of diphtheria cases were recorded in Nigeria (10,595 cases), India (5,634 cases), Chad (2,599 cases), Niger (2,082 cases), Pakistan (1,053 cases), and Indonesia reported 943 cases.[3] In 2023, a total of 216 confirmed diphtheria cases were recorded across the European Union, of which 155 were attributed to *C. diphtheriae*. During the same period, 61 laboratory-confirmed cases caused by *C. ulcerans* were reported by the ECDC. *C. diphtheriae* was most frequently detected among males aged 15–24 years, in contrast to *C. ulcerans*, which was primarily found in individuals aged 45 years or above.[4] According to Torres *et al*. and Dias *et al*. study, most of the cases were in adults ≥ 65 years of age.[1,5,6]

During 2017, diphtheria outbreaks in Indonesia were reported across 170 districts/cities spanning 30 provinces, resulting in 954 confirmed cases and 44 deaths. Among these cases, the highest burden was recorded in East Java with 331 cases, followed by West Java with 167 cases. In 2018, Indonesia reported 1,665 diphtheria cases with 29 associated deaths. The largest number of cases occurred in Central Java with 385 cases, while West Java and Riau Islands each reported 225 cases. In DKI Jakarta, 18 cases were documented, including two fatalities.[7] In Indonesia, there have been no recorded cases of diphtheria brought on by *C. ulcerans* or *C. pseudotuberculosis*.[8]

Diphtheria toxin may cause an acute serious infection in humans.[9] There are more than 122 species of *Corynebacterium* in the list, but only three species are potentially toxigenic, including *C. diphtheriae*, *C. pseudotuberculosis,* and *C. ulcerans*. These pathogens are related to various infectious diseases in animal and human hosts.[10] Zoonotic diphtheria linked to *C. ulcerans* and *C. pseudotuberculosis* in humans is typically related to animal exposure. No confirmed cases of direct interpersonal spread involving *C. ulcerans* or *C. pseudotuberculosis* have been reported. The incubation period typically lasts up to seven days, although longer durations may occur. Risk factors for *C. ulcerans* and *C. pseudotuberculosis* include contact with dogs, cats, cows, horses, goats, sheep, camels, and pigs.[10,11]

Toxic strains of *C. ulcerans* causing respiratory diphtheria-like illnesses are being increasingly reported from developing countries with large populations of livestock and/or deficient child immunization programs.[10,12] Asymptomatic rural workers may harbor *C. ulcerans* in the nasopharynx without showing clinical signs of infection.[10] *C. ulcerans* is also capable of producing diphtheria toxin (DT), as well as phospholipase D (*pld*). The clinical effects of DT commonly include recurrent epistaxis during infection, cutaneous lesions resembling classical diphtheria, tracheobronchial involvement with pseudomembrane formation, and hemorrhagic manifestations. The detached pseudomembrane from its current site can create an obstruction of the airways and might increase mortality. The pld gene expression is linked to health problems in the lower respiratory system. It is often related to symptoms like pneumonia and the formation of granulomatous tumors in the lungs.[5,10] The rapid identification was mostly developed for *C. diphtheriae* detection.

Identification of Corynebacterium often does not reach the species level of *C. ulcerans* and *C. pseudotuberculosis* due to several practical limitations; routine laboratory diagnostic procedures mainly target *C. diphtheriae* as the primary etiological agent of diphtheria. Conventional phenotypic and biochemical methods have limited ability to accurately differentiate closely related Corynebacterium species, while advanced techniques such as molecular assays or sequencing are not always available because of cost and resource constraints. In addition, the low prevalence of *C. ulcerans* and *C. pseudotuberculosis* results in a lower priority for their routine identification and surveillance. A study on the detection of *C. ulcerans* and *C. pseudotuberculosis* has previously been conducted by Sunarno *et al*. However, the study did not utilize direct clinical specimens from patients and relied on conventional PCR methods.[8] Therefore, the development of a duplex real-time PCR assay to detect *C. ulcerans* and *C. pseudotuberculosis* is considered essential.

Routine laboratory testing often faces significant challenges in accurately identifying *C. ulcerans* and *C. pseudotuberculosis*; real-time PCR assays can be used as rapid diagnostic tools for detection. The advantages of this method are to reduce the risk of transmission from clinical specimens to laboratory personnel, to increase the possibility of detection in all laboratories without having to receive throat swabs, and to increase the potential for detection in positive microscopic slides that failed to grow in culture. The objectives of this study were to design and develop a duplex real-time PCR capable of detecting the *rpoB* gene of *C. ulcerans* and the *pld* gene of *C. pseudotuberculosis.*

2. Methods

Clinical Specimen

There were 108 throat swab specimens studied. Specimens from patients suspected of toxigenic diphtheria with clinical symptoms are sore throat, fever, and pseudomembrane in the tonsil, pharynx, or nasal cavity, were collected from Prof. Dr. Sulianti Saroso Infectious Diseases Hospital and sent to the Clinical Microbiology Laboratory Universitas Indonesia-Cipto Mangunkusumo Hospital Jakarta, from December 2017 to August 2019. The throat swab was sent by amies transport medium. Ethical clearance for this study was granted by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (Ref. No. 0109/UN2.F1/ETIK/2018).

Sample Preparation and DNA Extraction

A total of 108 specimens were centrifuged at 12,000 rpm for 5 minutes to obtain a pellet, while the supernatant was carefully removed. Following the manufacturer's protocol, DNA extraction from the pellet was then carried out using the QIAamp DNA Mini Kit (Qiagen), with a final elution volume of 50 µL.

Positive Control

A synthetic positive control was obtained using gBlocks® gene fragments, consisting of a 330 bp fragment corresponding to *C. ulcerans* and a 140 bp fragment corresponding to *C. pseudotuberculosis*.

Table 1. Positive Controls of Corynebacterium ulcerans and Corynebacterium pseudotuberculosis

DNA synthetic	Length	Amount Delivered	GC Content	Molecular Weight
C.ulcerans	330	500 ng	47.88%	203775.7
C.pseudotuberculosis	140	250 ng	53.57%	86386.8

Table 2. Primer and Probe *C. ulcerans* and *C. pseudotuberculosis*

Gene Target	Product	Oligonucleotide Sequence	bp
Primers *C. ulcerans*	Forward	TTCGCATGGCTCATTGGCAC	20
	Reverse	TCCAGGATGTCTTCCAGTCC	20
Probe *C. ulcerans* HEX	Probe	CCAGCAGGAGGAGCTGGGTGAA	20
Primers *C. pseudotuberculosis*	Forward	CTACAGCAAATCGGCCAGTCT	21
	Reverse	CGTCATCCACGCCTTGAGT	19
Probe *C. pseudotuberculosis* FAM	Probe	TGCGATTGCCCACCGCGTTTTA	21

Primer Design

The *C. ulcerans* primer and probes used in the study are based on the research of De Zoysa *et al.* to detect the *rpoB* gene, and the *C. pseudotuberculosis* primer and probes used were based on the research of Spier *et al.* to detect the *pld* gene.[1,13] DNA sequences were analyzed through online alignment using the Basic Local Alignment Search Tool (BLAST).

Real-Time PCR

Real-time PCR reaction mixture (20 µL): 1x reaction, 10 µL SensiFAST™ Real-Time PCR Kits, 0.45 µM each of primers and 0.50 µM probe *C. ulcerans*, 0.35 µM each of primers and probe *C. pseudotuberculosis*, 1 µl of DNA synthetic *C. ulcerans,* and 1 µl of DNA synthetic *C. pseudotuberculosis* for template. Reaction condition: initial denaturation at 95 °C for 3 min was followed by 45 amplification cycles at 95 °C for 15 s and a combined annealing/extension step at 56-66°C for 45 s. Real-time PCR amplification was performed using a Bio-Rad instrument according to the manufacturer's guidelines. Optimization of PCR parameters, such as annealing temperature, primer and probe concentrations, reaction times, and cycle numbers, was completed before testing clinical samples.

Specificity and sensitivity of real-time PCR

The specificity of the real-time PCR assay was assessed using DNA from various pathogens that may potentially result in false-positive reactions, including: *Mycobacterium tuberculosis*, *Herpes Simplex Viruses*, *Candida albicans, Streptococcus pneumonia, Epstein-Barr Virus* (EBV), *Klebsiella pneumonia, Streptococcus pyogenes*, *Cytomegalovirus*, *Helicobacter pylori, Staphylococcus aureus*, *Escherichia coli*, *Moraxella catarrhalis*, *Pneumocystis jirovecii*, *Neisseria meningitides*, *Neisseria gonorrhoeae*, *Legionella pneumophila*, *Chlamydia pneumonia*, and *Mycoplasma pneumonia*. Serial dilutions of synthetic DNA from *C. ulcerans* and *C. pseudotuberculosis* were used to evaluate the sensitivity of the real-time PCR and to define its limit of detection.

3. Results

Primer Design

In this study, we designed two sets of primers for *C. ulcerans* and *C. pseudotuberculosis*. Forward and reverse primers of *C. ulcerans* consist of 20-base arrangements and produce an amplicon with a size of 98 base pairs. The *rpoB* gene of *C. ulcerans* has a length of 3537 bp. Forward primers of *C. ulcerans* attach to 178 to 197 bases, and reverse primers attach to 256 to 275 bases, which attach to the *rpoB* gene, which is part of the *C.ulcerans* genome, Japan isolation: Hokkaido (GeneBank: AB828262.1) (Figure 1).

The forward primer of *C. pseudotuberculosis* consists of 21 bp, and the reverse primer consists of 19 bp and produces an amplicon with a size of 66 base pairs. The Phospholipase D (*pld*) gene has a length of 924 bp. Forward primer of *C. pseudotuberculosis* attaches 98 to 118 bases, and reverse primers attach 145 to 163 bases. Primer attached to the *rpoB* gene, which is part of the *C. pseudotuberculosis* base of the Brazil isolate (GeneBank: CP038431.1) (Figure 2).

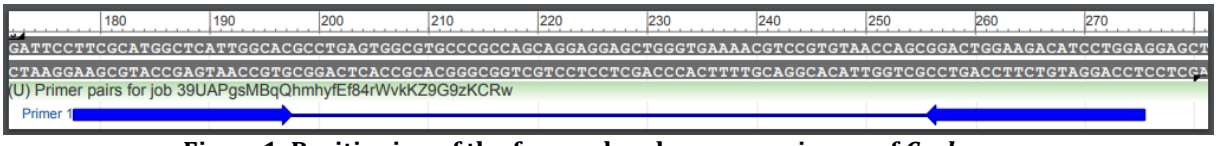

Figure1. Positioning of the forward and reverse primers of *C. ulcerans*

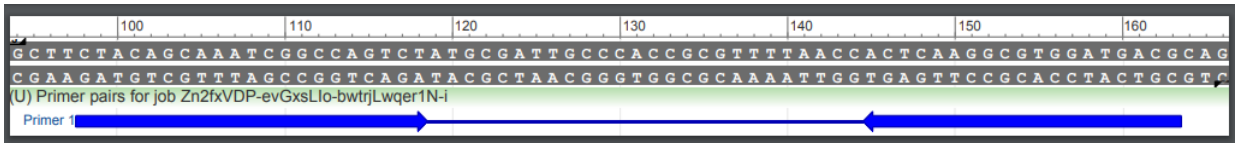

Figure 2. Positioning of the forward and reverse primers of *C. pseudotuberculosis*

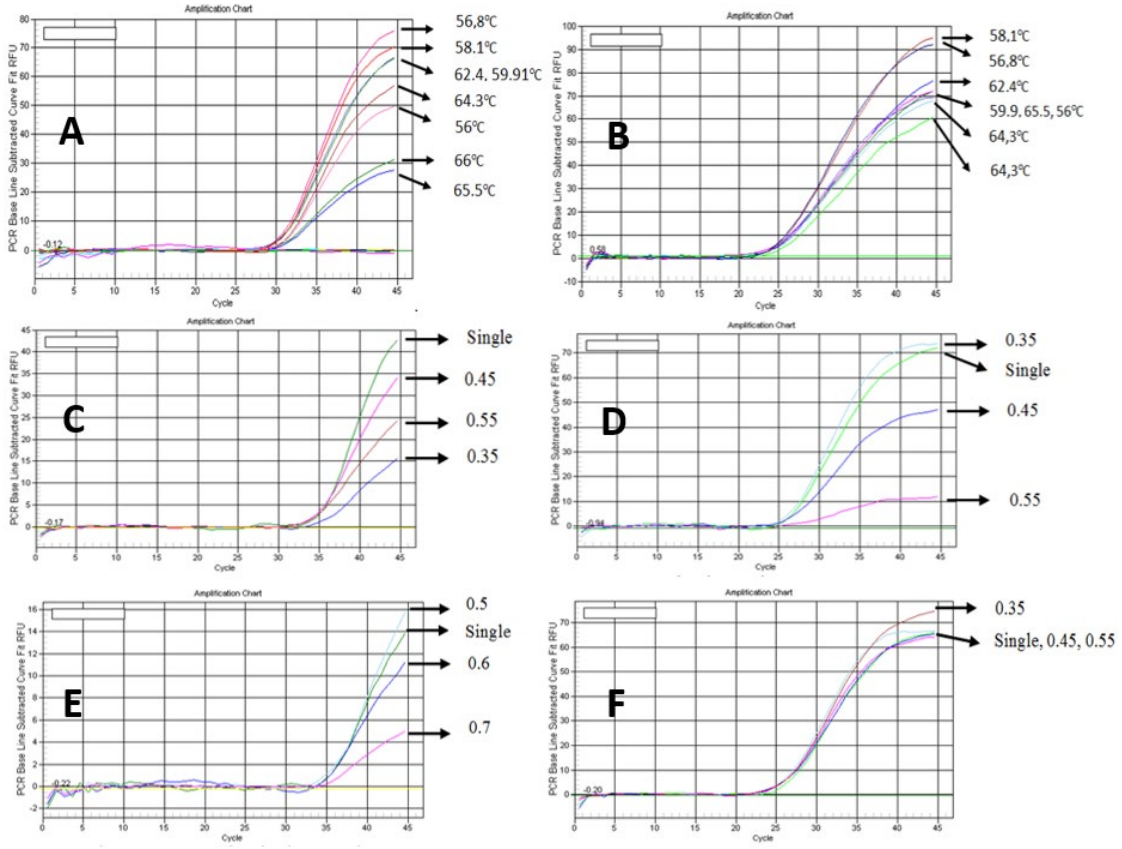

Figure 3. Optimization results for duplex real-time PCR conditions. A = results of optimization of annealing temperature of *C. ulcerans*, B = results of optimization of annealing temperature of *C. pseudotuberculosis*, C = results of optimization of primer concentrations of *C. ulcerans*, D = results of optimization of primer concentrations of *C. pseudotuberculosis*, E = results of optimization of *C. ulcerans* probe concentration, F = Results of optimization of *C. pseudotuberculosis* probe concentration

Optimization of duplex real-time PCR

Optimization of the duplex real-time PCR reaction was performed to obtain optimal amplification results. Annealing temperature optimization demonstrated that the optimum temperature for amplification of *C. ulcerans* was 56.8 °C, while for *C. pseudotuberculosis* it was 58.1°C and 56.8°C, both yielding comparable Ct values. However, because the assay was conducted using duplex real-time PCR, a single annealing temperature was required for simultaneous amplification. Therefore, an annealing temperature of 57°C was selected, as it provided optimal performance for both targets.

Based on optimization experiments, primer concentrations of 0.45 µM for *C. ulcerans* and 0.35 µM for *C. pseudotuberculosis* were optimum in a 20 µL reaction volume. Under the same reaction conditions, probe concentrations of 0.50 µM for *C. ulcerans* and 0.35 µM for *C. pseudotuberculosis* yielded optimum performance.

Specificity and sensitivity of duplex real-time PCR

To evaluate assay specificity, the duplex real-time PCR was challenged with DNA from a wide selection of microorganisms, including: *Mycobacterium tuberculosis, Herpes Simplex Viruses, Rubella, Influenza A, Influenza B, Streptococcus pneumonia, Epstein-Barr Virus (EBV), Candida albicans, Streptococcus pyogenes, Cytomegalovirus, Helicobacter pylori, Staphylococcus aureus, Klebsiella pneumonia, Escherichia coli, Moraxella catarrhalis, Pneumocystis jirovecii, Neisseria meningitides, Neisseria gonorrhoeae, Legionella pneumophila, Chlamydia pneumonia, and Mycoplasma pneumonia.* Specificity testing revealed the absence of cross-reactivity with non-target microorganisms, while the analytical sensitivity of the duplex real-time PCR reached 4.49 DNA copies for *C. ulcerans* and 1.06 DNA copies for *C. pseudotuberculosis.*

Detection of *C. ulcerans* and *C. pseudotuberculosis* in Clinical specimens

PCR results revealed that *C. ulcerans* and *C. pseudotuberculosis* were both negative in 108 clinical samples from suspected diphtheria cases.

4. Discussion

C. ulcerans and C. pseudotuberculosis can be a health problem for suspected respiratory diphtheria in many developed countries. Until now, there have been no reports of *C. ulcerans* and *C. pseudotuberculosis* identified from suspected diphtheria patients in Indonesia. Many cases with clinical diphtheria were detected by positive microscopic results, but rarely had positive culture. These difficulties in confirmation using culture may be due to inadequate specimen transportation,

overuse of antibiotics, and overdiagnosis.

In this study, *C. ulcerans* was assessed for the presence of the *rpoB* gene, and *C. pseudotuberculosis* for the presence of the *pld* gene by duplex real-time PCR, as stated in several studies by Zoysa *et al*., Torres *et al.*, Mancini *et al.*, Schuhegger *et al*., and Spier *et al*. They have evaluated the primers and probe used in this study for clinical samples (throat), in that the primers and probe were highly specific for *C. ulcerans* and *C. pseudotuberculosis*. In this study, the positive control using standardized *C. ulcerans* and *C. pseudotuberculosis* DNA syntetic was worked properly; our designed primer was able to detect *C. ulcerans* and *C. pseudotuberculosis*.[1,5,13,14,15]

Based on annealing temperature optimization, amplification was optimal at 57 °C. Primer concentration optimization further indicated that final concentrations of 0.45 µM for *C. ulcerans* and 0.35 µM for *C. pseudotuberculosis* yielded optimum results in a total reaction volume of 20 µL. Research conducted by Spier *et al* states that the primary concentration used to detect the *pld* gene *of C. pseudotuberculosis* is 400 nM or 0.4 µM in each primer. So there is not too much difference between the primary concentrations used in this study and those conducted by Spier *et al*. While the research conducted by De Zoysa *et al*. uses primary concentrations to detect *C. diphtheriae* and *C. ulcerans* based on the *rpoB* gene, which is 0.5 mM in each primer. The primary concentration used by De Zoysa *et al* has a large difference in concentration compared to the primary concentration used in this study.[1,13]

Based on probe concentration optimization, final concentrations of 0.50 µM for *C. ulcerans* and 0.35 µM for *C. pseudotuberculosis* were optimum in a 20 µL reaction volume. Research conducted by De Zoysa *et al* states that the concentration of the probe is used to detect *C. diphtheriae* and *C. ulcerans* based on the *rpoB* gene, each with a concentration of 0.2 mM. So that the concentration of the probe used has a very wide range, between the one used by De Zoysa *et al.,* compared to the probe concentration used in this study. While the research conducted by Spier *et al.,* the concentration of the probe used to detect the *pld* gene *C. pseudotuberculosis* had a probe concentration of 80 nM or 0.08 µM. Research conducted by Spier *et al* has a concentration of probes that is closer to the concentration conducted in this study when compared with research conducted by De Zoysa *et al*.[1,13]

All clinical specimens were analyzed using duplex real-time PCR, and all suspected samples were negative for *C. ulcerans* and *C. pseudotuberculosis*. This finding may be explained by the low prevalence of these organisms, as reported in previous studies by De Zoysa *et al*., Torres *et al*., and Ott *et al.* In addition, the samples in this study were collected in Jakarta, an urban setting where human interaction with companion animals and livestock is relatively limited. In contrast, studies with larger sample sizes and specimens obtained from rural areas with extensive animal husbandry may show a higher prevalence of *C. ulcerans* and *C. pseudotuberculosis*, thereby increasing the likelihood of their detection.[1,5,9]

The second possibility for the negative duplex real-time PCR resulted in *rpoB* and *pld* genes might not be enough to be used as an identification target. Pilot study of Sunarno *et al*. reported the *dtxR* and *pld* genes exhibited high specificity for their respective targets, *C. ulcerans* and *C. pseudotuberculosis*, making them suitable molecular markers for the PCR assay.[8] Sing et al. also reported successful detection of *C. ulcerans* using a combination of *rpoB* and 16S rDNA genes.[16] Some researchers also reported successful *C. ulcerans* identification targeting *rpoB* and tox genes De Zoysa *et al*., Torres *et al*., Mancini *et al*.[1,5,14] We assume the development of other genes and more than 1 gene target as PCR primer is still needed for better sensitivity and specificity of rapid *C. ulcerans* and *C. pseudotuberculosis* detection.

Overall, diphtheria caused by *C. ulcerans* and *C. pseudotuberculosis* follows a zoonotic transmission pattern, originating from animal sources, so most infected individuals are those living near livestock farms or those in direct contact with livestock products. The analyzed samples of suspected diphtheria patients came from urban areas of Jakarta and its surroundings, most of whom had no contact with livestock farms. This contrasts with the pattern of *C. diphtheriae* transmission, which occurs person to person and is dominated by respiratory spread through coughing, sneezing, or direct contact with an infected patient. Transmission of *C. ulcerans* and *C. pseudotuberculosis* occurs through contact with domestic animals, including dogs, cats, sheep, horses, cattle, goats, and pigs.[12,17,18]

The main strength of this study is that it represents the first report on the detection of *C. ulcerans* and *C. pseudotuberculosis* using duplex real-time PCR in Indonesia. The optimized duplex real-time PCR method developed in this study can be applied in hospitals or clinical laboratories to support advanced diagnostic services for diphtheria and diphtheria-like infections. However, this study has several limitations, including the limited number of samples analyzed and the use of only a single primer set for the detection of *C. ulcerans* and *C. pseudotuberculosis*. It is expected that this study will serve as a foundation and open opportunities for further research on *C. ulcerans* and *C. pseudotuberculosis* in Indonesia.

5. Conclusion

Duplex real-time PCR to detect *C. ulcerans* and *C. pseudotuberculosis* from a patient with suspected diphtheria still has potential as a diagnostic tool, but further research is still needed. Human clinical isolates of toxigenic *C. ulcerans* and *C. pseudotuberculosis* are extremely rare and could be under-reported. Advanced study of many clinical

specimens suspected of diphtheria is fundamental to prove this assay.

6. Author Contribution

D.U. experimented and wrote the manuscript with support from Y.R and A.Y. Y.R. and A.Y. helped supervise the project.

7. Acknowledgements

Thank you to all those who have helped with this research. Especially to the supervisors for their guidance, advice, and feedback to make this journal writing well accomplished. Deep appreciation is extended to the Laboratory of Clinical Microbiology, Faculty of Medicine, Universitas Indonesia-Cipto Mangunkusumo Hospital, for support and collaboration.

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