Tropical Biomedicine 35(3): 775–780 (2018)

Evaluation of Conventional PCR for Detection of Toxigenic *Corynebacterium diphtheriae* Strains in Malaysia

Liow, Y.L., Rohaidah, H., Fairuz, A., Hazwani, H., Nurul Ain, D. and Norazah, A.*
Bacteriology Unit, Infectious Diseases Research Centre, Institute for Medical Research, 50588 Kuala Lumpur, Malaysia
*Corresponding author e-mail: norazah@imr.gov.my
Received 4 November 2017; received in revised form 27 February 2018; accepted 27 February 2018

Abstract. Diphtheria is an infectious but vaccine preventable disease caused by *Corynebacterium diphtheriae* and humans are the only reservoir. While toxigenic strains most frequently cause pharyngeal diphtheria, non-toxigenic strains commonly cause cutaneous infections. In 2016, there was a sudden increase in cases of *C. diphtheriae* reported in Malaysia. The toxigenic strains are currently determined using Elek's test and are carried out only in the reference laboratory. With the sudden increase in diphtheria cases in Malaysia, it is important for local laboratories in state hospitals to be able to perform a rapid, reliable diagnostic test for the detection of the exotoxin. In this study, we aimed to evaluate the application of conventional PCR method to detect toxigenic strains of *C. diphtheriae* compared to the Elek's test. Forty-eight *C. diphtheriae* strains were subjected to PCR detection of toxin gene A and B subunits, and also Elek's test. The A and B subunits of the toxin gene were detected in all *C. diphtheriae* strains except for one strain which was isolated from a foot ulcer. Elek's test was also positive for all the PCR positive strains. This study showed 100% co-relation between the results of PCR and Elek's test assay. The conventional PCR can be used at the state laboratories for rapid detection of toxin genes in toxigenic *C. diphtheriae* cultures, thus early treatment can be given to the patients while waiting for Elek's test results.

INTRODUCTION

Corynebacterium diphtheriae, also known as the 'Klebs-Löffler' bacillus, is the etiologic agent of diphtheria (Murphy, 1996). It is a Gram-positive, aerobic, non-motile, toxin-producing, rod-shaped bacteria belonging to the order Actinomycetales, which are typically found in the soil (Disease Control Division MOH Malaysia, 2017). The vital virulence factor of *C. diphtheriae* is the diphtheria exotoxin, which consists of two major functional fragments: A and B carried by the lysogenic specific Beta phage. The non-toxic B fragment attaches and penetrates the host cell while the highly toxic fragment A detaches, and inhibits protein synthesis leading to cell death (Holmes, 2000).

Diphtheria remains an endemic disease in large part of the world (Adler, Mahony and Friedman, 2013; Bhagat *et al.*, 2015) and to a very small extent in Eastern Europe (Wagner *et al.*, 2012) despite the introduction and the success of mass herd immunization in many countries (Clarke, 2017). Diphtheria infection outbreak was first reported in Malaysia in the 1980s and the number of diphtheria cases reported to the WHO (World Health Organization) was 131 and since the first report, a surveillance on the cases was started by the Ministry of Health (MOH) Malaysia and followed up by WHO (World Health Organisation (WHO), 2017). According to the health facts in 2014 by the Disease Control Division under the MOH, there was a diminishing trend where the incidence rate and mortality rate for vaccine preventable disease of diphtheria in 2013 have decrease to 0.01(per 100,000 populations). However, in 2016, there was a

sudden surge in numbers of diphtheria cases reported in Malaysia by WHO, a total of 31 diphtheria cases compared to 2015, 2014 and 2013 which are reported to have 4, 2, and 4 cases, respectively (Planning Divisions, 2016; World Health Organisation (WHO), 2017). Furthermore, the disease came into focus when a non-vaccinated girl in Malacca, Malaysia died of systemic diphtheria in June 2016, followed by more reported cases in the same year (The Straits Times, 2017). This is worrying as diphtheria could potentially re-emerge as a serious public health threat even though Malaysia has been reported to have been well under control for decades (World Health Organisation (WHO), 2017).

There are several diagnostic methods to detect toxigenic *C. diphtheriae* strains (Androulla Efstratiou *et al.*, 2000) that could assist clinicians in achieving early diagnosis and timely intervention with diphtheria antitoxin (Bisgard *et al.*, 2000) but not all are widely available in Malaysia. By introducing accessible rapid reliable tool in Malaysia, local state hospital laboratories need not rely mainly on reference laboratory for results. In this present study, we aimed to evaluate the application of conventional PCR (Pallen *et al.*, 1994; Nakao and Popovic, 1997) method as a rapid and reliable tool for detection of toxigenic *C. diphtheriae* strains, compared to the Elek's test (Androulla Efstratiou *et al.*, 2000) that detects toxin expression.

MATERIALS AND METHODS

Bacterial strains and clinical specimens

A total of 32 clinical isolates of *C. diphtheriae* were randomly selected from patients with suspected respiratory or cutaneous diphtheria from year 2016-2017. The isolates were received from microbiology laboratories of hospitals in Malaysia for confirmation of identification and specialised testing. Upon arrival at the reference laboratory of Institute for Medical Research (IMR) Kuala Lumpur, Malaysia, the cultures were promptly inoculated onto blood agar; after overnight incubation at 37°C, the cultures were subjected to standard microbiological laboratory procedures (Androulla Efstratiou *et al.*, 2000) and identification using API Coryne (BioMèrieux, France). Also included were 16 *C. diphtheriae* cultures which are in the IMR culture collection and one reference strain (NCTC 3984; toxin gene positive) for analysis. The strains were collected from the source as detailed in Table 1. They were maintained in sterile beads added with rabbit serum in tryptic soy broth 15% glycerine solution at -70°C until needed. Prior to use, the strains were streaked onto a sterile blood agar plate (tryptic soy agar II with 5% cow blood) and were incubated at 37°C overnight.

Modified Elek toxigenicity test

All isolates were simultaneously assayed by the modified Elek's test, prepared as described previously (Kathryn H. Engler, 1997). Using a 3ml of medium (2.5ml of Elek's base and 0.5ml of Newborn Bovine Serum) in a 4.5cm diameter petri disk, the 10IU/disc of antitoxin (20µL of a 500-IU/ml stock solution) was place in the middle of the petri dish. Heavy inoculum of a maximum of five isolates and one control strain per petri dish were streaked at a distance of 9 mm from the edge of the anti-toxin disk. *C. diphtheriae* NCTC 3984 was used as positive control. A minimum incubation of 24 hours was required before the reading of the precipitin line.

Extraction of DNA

DNA extractions were carried out using Promega Maxwell® Cultured Cells DNA Kit. Two to five colonies were picked from the fresh cultured plates and transferred to well #1 of each cartridge and thoroughly mixed with the lysis buffer. Thereafter, the cartridge preparations were as described in the manufacturer's protocol.

PCR

All isolates were subjected to PCR amplification of toxin gene A and B subunits. Two sets of primers (*Tox* 1: ATCCACTTTTAGT GCGAGAACCTTCGTCA and *Tox* 2: GAAAA CTTTTCTTCGTACCACGGGACTAA, Dipht 6F: ATACTTCCTGGTATCGGTAGC, Dipht 6R:

CGAATCTTCAACAGTGTTCCA) targeting the diphtheria toxin (DT) gene subunits A and B were used (Nakao and Popovic, 1997). The PCR reaction mixture consisted of 1µL of DNA, 10.5µL of sterile Milli-Q water, 0.5µL of each appropriate primer, and 12.5µL of ready-to-use Master Mix (MyTag HS Mix, BIO-25046, Bioline, USA) were all added into a 0.2ml thin walled PCR tube and mixed well. The PCR cycles were as follows; initial denaturation at 95ºC for 1 minutes, 35 cycles of denaturation at 95ºC for 15 seconds, annealing at 58ºC for 15 seconds, and extension at 72ºC for 15 seconds before cooling down and stored at 4ºC. *C. diphtheriae* NCTC 3984strain was used as positive control. The PCR products were then visualised by agarose gel electrophoresis.

RESULTS & DISCUSSION

All 49 types of *C. diphtheriae* strains (including the reference strain) were positive for both A and B subunits of the diphtheria toxin gene. These strains were also positive by Elek's test (Table 1), Only one isolate which is C523, where the *C. diphtheriae* was grown from a foot ulcer, was negative for toxin gene by PCR and also negative by Elek's test. This is in concordance with the study conducted in Russia between 1990-1994 (Mikhailovich *et al.*, 1995), where our study also showed a 100% correlation between the standard conventional PCR that detects the A and B subunits of diphtheria toxin (DT) gene and Elek's test.

Eight out of a total of 32 clinical isolates of *C. diphtheriae* from patients with suspected respiratory or cutaneous diphtheria from year 2016-2017 were adults, age ranging 18-81 years old. Even though diphtheria is uncommon in most parts of the world, many serological studies in developed countries showed an increasing susceptibility to diphtheria especially in the adult population (Völzke *et al.*, 2006). This may be due to adults do not compulsorily need to receive booster for diphtheria toxoid vaccination after being vaccinated as a child or the awareness of it is not there due to poor information disseminated amongst clinicians and the public. In Malaysia, sporadic cases still occur from time to time, most probably related to incomplete or unvaccinated individuals. The current diphtheria re-emergence in Malaysia emphasizes the need for rapid and reliable method such as the application of PCR, which can distinguish toxigenic from non-toxigenic isolates. This rapid detection will definitely help in the prompt treatment with diphtheria anti-toxin which are not easily accessible except in major hospitals.

In standard laboratories procedures, suspected colonies are tested for toxin production using the Elek's test, which takes 24–48 hours before any positive reaction can be observed. The preparation of Elek's media and the procedure is time consuming and sometimes need to be repeated because of plate contamination or inconclusive results. Also, Elek's test is prone to misinterpretation especially in microbiological laboratories that rarely performed this. PCR amplification and visualization of PCR product would only take approximately 4 hours. In some rare cases, the presence of toxin gene in the isolates of *C. diphtheriae* does not necessarily express a biologically active protein (Zakikhany, Neal and Efstratiou, 2014). However, from our study, there is 100% correlation between PCR positive and Elek's test. This supports that the use of PCR for determination of toxin-producing *C. diphtheria*e.

PCR method is only available in universities and private laboratories in Malaysia and in other state hospitals in Malaysia. So, by having PCR assay easily available and routinely performed in most microbiological laboratories Malaysia, physicians can be informed much earlier regarding the toxin gene positivity in the isolated *C. diphtheria*e without waiting for the results of the Elek's test. Hence, it is important for laboratories in Malaysia to be able perform conventional PCR without having to rely mainly on Elek's test offered in reference laboratory for results.

Toxigenic strains frequently cause pharyngeal diphtheria and sometimes cutaneous disease but the non-toxigenic strains commonly cause cutaneous disease

Table 1. Data of isolates tested with PCR method and Elek's test

Culture collection no.	Date collection	Gender	Age (years)	Source of Isolate	Location	PCR toxic gene		ELEK's Test
						ToxA	ToxB	
C122	1987	female	15	Pseudomembrane Tissue	Johor	+	+	+
C123	1987	female	7	Throat swab	Terengganu	+	+	+
C20	1981	n/a	n/a	Culture Collection	IMR	+	+	+
C21	1981	n/a	n/a	Culture Collection	IMR	+	+	+
C488	2008	male	7	Culture Collection	Pahang	+	+	+
C517	2010	male	5	Throat swab	Negeri Sembilan	+	+	+
RZ252	2016	female	7	Nasopharyngeal	Malacca	+	+	+
RZ319	2016	male	6	Throat Swab	Kedah	+	+	+
RZ356	2016	male	3	Throat Swab	Sabah	+	+	+
RZ358	2016	male	5	Throat Swab	Sabah	+	+	+
RZ373	2016	female	41	Throat Swab	Negeri Sembilan	+	+	+
RZ378	2016	male	n/a	Throat Swab	Kedah	+	+	+
RZ379	2016	male	n/a	Throat Swab	Kedah	+	+	+
C110	1986	female	n/a	Nasal Discharge	Pahang	+	+	+
C13	2005	n/a	n/a	NCTC 3984	NCTC 3984	+	+	+
C325	2003	female	6	Throat Swab	Terengganu	+	+	+
C111	1986	male	2	Nasal Swab	Selangor	+	+	+
C113	1986	male	4	Throat Swab	Kedah	+	+	+
C198	1991	n/a	n/a	culture collection	Johor	+	+	+
C319	2001	n/a	11	Throat Swab	Kelantan	+	+	+
C324	2002	female	n/a	Skin lesion	Terengganu	+	+	+
C326	2005	female	8	Throat swab	Terengganu	+	+	+
RZ553	2016	female	20	Throat Swab	Kedah	+	+	+
RZ597	2016	male	3	Throat Swab	Selangor	+	+	+
RZ600	2016	female	4	Throat Swab	Perak	+	+	+
RZ632	2016	female	7	Throat Swab	Sabah	+	+	+
RZ656	2016	female	4	Throat Swab	Sabah	+	+	+
RZ658	2016	male	20	Throat Swab	Kedah	+	+	+
RZ659	2016	female	21	Throat Swab	Kedah	+	+	+
RZ693	2016	male	29	Throat Swab	Selangor	+	+	+
RZ523	2016	male	81	right foot ulcer	Sarawak	-	-	-
C25	2005	n/a	n/a	culture collection	IMR	+	+	+
C322	2002	male	38	Throat Swab	Pahang	+	+	+
RZ 11/17	2017	female	11	Throat Swab	Selangor	+	+	+
RZ41/17	2017	female	1	Throat Swab	Selangor	+	+	+
RZ70/17	2017	male	1	Throat Swab	Selangor	+	+	+
RZ71/17	2017	male	9	Throat Swab	Kedah	+	+	+
RZ72/17	2017	male	1	Throat Swab	Selangor	+	+	+
RZ80/17	2017	male	7	Throat Swab	Selangor	+	+	+
RZ87/17	2017	female	38	Throat Swab	Selangor	+	+	+
RZ114/17	2017	female	11	Throat Swab	Terengganu	+	+	+
RZ121/17	2017	male	13	Throat Swab	Malacca	+	+	+
RZ122/17	2017	female	18	Throat Swab	Terengganu	+	+	+
RZ138/17	2017	female	2	Throat Swab	Selangor	+	+	+
RZ151/17	2017	female	2	Throat Swab	Selangor	+	+	+
RZ156/17	2017	male	10	Throat Swab	Selangor	+	+	+
RZ175/17	2017	female	4	Throat Swab	Sabah	+	+	+
RZ176/17	2017	female	4	Throat Swab	Sabah	+	+	+
RZ206/17	2017	male	3	Throat Swab	Terengganu	+	+	+

+: positive; –: negative; n/a: not available.

(Bishai and Murphy, 2015). In this study, one isolate C 523, which was from a foot ulcer, was diphtheria toxin gene negative while one skin lesion C324 was toxin gene positive. The toxigenic C324 strain from a skin lesion was in IMR culture collection since 2002. However, there was no additional information recorded. The strain C 523 from Sarawak was collected from a leg ulcer of a patient with chronic ulcer which also grew methicillin-resistant *Staphylococcus aureus* (MRSA) (Sia, Liow and Ahmad, 2017) . To the best of our knowledge, this is also the first case of cutaneous diphtheria reported in Malaysia.

CONCLUSION

Based on this study, conventional PCR is reliable in rapidly detecting *toxin* gene and it may serve as a rapid identification of toxigenic *C. diphtheriae strains*. It is important that all laboratories of state hospitals have the capacity to conduct PCR assay for the urgent identification of toxigenic isolates of *C. diphtheriae* strains especially in the times of re-emergence of this disease.

Acknowledgement. We thank the Director General of Ministry of Health Malaysia for permission to publish this manuscript.

Funding:
This work was supported by the National Institutes of Health, Malaysia [JPP-IMR 16-056]; [NMRR-16-1421-32070]

Conflict Interest:
All authors declare that there is no conflict of interest in this study.

REFERENCE

Adler, N.R., Mahony, A. & Friedman, N.D. (2013). 'Diphtheria: Forgotten, but not gone', *Internal Medicine Journal* **43**(2): pp. 206-210. doi: 10.1111/imj.12049.

Androulla Efstratiou, K.H.E. (2000). 'Current Approaches to the Laboratory Diagnosis of Diphteria', *The Journal of Infectious Diseases. Public Health Laboratory Service, London* **181**(Suppl 1): pp. 138-45. doi: 10.1086/315552.

Bhagat, S. (2015). 'Persistence of Corynebacterium diphtheriae in Delhi & National Capital Region (NCR)', *Indian Journal of Medical Research* **142**(October), pp. 459–461. doi: 10.4103/0971-5916.169212.

Bisgard, K.M. (2000). 'Diphtheria Toxoid Vaccine Effectiveness: A CaseControl Study in Russia', *The Journal of Infectious Diseases* **181**(s1), pp. S184–S187. doi: 10.1086/315562.

Bishai, W.R. & Murphy, J.R. (2015). 'Diphtheria and Other Corynebacterial Infections', in *Harrison's Principles of Internal Medicine, 19e*. Available at: http://accessmedicine.mhmedical.com/content.aspx?aid=1120797994.

Clarke, K. (2017). *Review of the Epidemiology of Diphtheria- 2000-2016*. Available at: http://www.who.int/immunization/sage/meetings/2017/april/1_Final_report_Clarke_april3.pdf (Accessed: 27 June 2017).

Disease Control Division MOH Malaysia. (2017). 'Case Definitions for Infectious Diseases', *Ministry of Health Malaysia*, 3rd Edition(January), p. 124.

Holmes, R.K. (2000). 'Biology and molecular epidemiology of diphtheria toxin and the tox gene.', *The Journal of infectious diseases*. American Society for Microbiology, Washington, DC, 181(Supplement 1), pp. S156-67. doi: 10.1086/315554.

Kathryn, H. & Engler, T.G. (1997). 'A modified Elek Test for Detection of Toxigenic Corynebactera in the Diagnostic Laboratory', *Journal Of Clinical Microbiology* **35**(February 1997), pp. 495-498.

Mikhailovich, V.M. (1995). 'Application of PCR for detection of toxigenic Corynebacterium diphtheriae strains isolated during the Russian diphtheria epidemic, 1990 through 1994', *Journal of Clinical Microbiology*, pp. 3061–3063. Available

at: http://pubmedcentralcanada.ca/pmcc/articles/PMC228639/pdf/333061.pdf (Accessed: 4 August 2017).

Murphy, J.R. (1996). 'Corynebacterium Diphtheriae', *Medical Microbiology*. University of Texas Medical Branch at Galveston, pp. 107–118. doi: 10.1093/nar/gkg874.

Nakao, H. & Popovic, T. (1997). 'Development of a direct PCR assay for detection of the diphtheria toxin gene', *J. Clin. Microbiol.*, **35**(7): pp. 1651-1655. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC229815/pdf/351651.pdf (Accessed: 4 August 2017).

Pallen, M.J. (1994). 'Polymerase chain reaction for screening clinical isolates of corynebacteria for the production of diphtheria toxin.', *Journal of Clinical Pathology* **47**(4): pp. 353-6. doi: 10.1136/jcp.47.4.353.

Planning Divisions, H.I.C.M. (2016). *Health Facts (reference data of 2015)*. Available at: http://www.moh.gov.my/images/gallery/publications/KKM HEALTH FACTS 2016.pdf (Accessed: 31 August 2017).

Sia, C.G., Liow, Y.L. & Ahmad, N. (2017). 'A Case Report: Corynebacterium diphtheriae Cutaneous Diphtheria with Co-Infection of MRSA in Malaysia', in *The Annual Scientific Meeting on Emerging Infectious Threat (ASMEIT) 2017*. Malaysian Society of Infectious Disease and Chemotherapy.

The Straits Times (2017). *Outrage over diphtheria deaths in Malaysia. The Straits Times*. Available at: http://www.straitstimes.com/asia/outrage-over-diphtheria-deaths-in-malaysia (Accessed: 21 November 2017).

Völzke, H. (2006). 'Susceptibility to diphtheria in adults: Prevalence and relationship to gender and social variables', *Clinical Microbiology and Infection*. doi: 10.1111/j.1469-0691.2006.01477.x.

Wagner, K.S. (2012). 'Diphtheria in the postepidemic period, Europe, 2000-2009', *Emerging Infectious Diseases*, 18(2), pp. 217–225. doi: 10.3201/eid1802.110987.

World Health Organisation (WHO) (2017). *WHO (World Health Organization) vaccine-preventable diseases: monitoring system global summary. Diphtheria reported cases in Malaysia, World Health Organisation*. Available at: http://apps.who.int/immunization_monitoring/globalsummary/timeseries/tsincidencediphtheria (Accessed: 27 October 2017).

Zakikhany, K., Neal, S. & Efstratiou, A. (2014). 'Emergence and molecular characterisation of non-toxigenic tox gene-bearing corynebacterium diphtheriae biovar mitis in the United Kingdom, 2003-2012', *Eurosurveillance* **19**(22): pp. 1-8. doi: 20819 [pii].