

# Spatial Analysis in Polymerase Chain Reaction for Detection of *Corynebacterium diphtheriae* Post-outbreak in Samarinda 2018

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## ABSTRACT

Diphtheria is an infectious disease caused by the bacterium *Corynebacterium diphtheria*, which primarily infects the throat and upper airways, and produces a toxin affecting other organs. In severe cases, it causes myocarditis or peripheral neuropathy. In Indonesia, diphtheria has once been an epidemic but then it has decreased in cases. Although the government succeeded in eliminating diphtheria cases in 1990 through immunization programs, the disease reappeared in 2009. At the end of 2017 until the beginning of 2018, outbreaks of diphtheria had been reported in several regions in Indonesia. One of which is in East Kalimantan, Samarinda. This study was conducted one year after the 2018 outbreak to find out whether diphtheria is still present in the community and to know the recent distribution of diphtheria patients. The study was conducted by examining the patient's oral mucosal swab. Data were obtained from the Medical Record and Clinical Pathology Laboratory of AWS Hospital and Dinas Kesehatan, Samarinda. A total of 43 samples were carried out by PCR (Polymerase Chain Reaction) laboratory test, using dtx primer and spatial analysis of patients detected with diphtheria using SatScan TM Software. The examination of 43 samples showed 2 samples positive for *Corynebacterium diphtheriae*. Both of the samples were female, lived in Air Hitam and Gunung Kelua, Samarinda Ulu District. The results of the spatial analysis showed that the location of patients of diphtheria bacteria detected did not indicate clustering.

**Keywords:** PCR, *Corynebacterium diphtheriae*

## 1. INTRODUCTION

Diphtheria is an infection that affects mucous membrane upper respiratory tract or cutaneous infection. It is caused by the aerobic gram-positive bacteria, *Corynebacterium diphtheria*. Diphtheria toxins cause tissue membranes to die and accumulate in the upper throat and tonsils which causes difficulty breathing and swallowing [1]. This disease is transmitted through direct physical contact or aerosol secretions inhaled from sufferers who cough or sneeze [2]. According to WHO, there were 4,680 worldwide cases of diphtheria in 2013, in which the first rank was in Southeast Asia with 4,080 cases. In the years following, there were 7,347 cases of diphtheria in 2014 and 7097 cases in 2016. In Asia, the highest number of cases was reported in South Asia (4,016), followed by Africa (2,870) and the Western Pacific (98). Meanwhile, most cases of diphtheria in Asian countries were

recorded in India (3,380), followed by Madagascar (2,865) and Indonesia (342). In Indonesia, diphtheria was once an epidemic but then the number of cases decreased in 1990 since the Indonesian government promoted immunization programs that successfully eliminated diphtheria cases. However, diphtheria disease reappeared in 2009. According to the Ministry of Health of the Republic of Indonesia, the emergence of diphtheria outbreaks was due to the immunity gap of the population. This happens because of the accumulation of people who are susceptible to diphtheria since they do not get immunizations or complete immunizations [3].

There were 775 cases of diphtheria in 2013, then decreased in 2014 by 296 cases. In 2015, the number of diphtheria cases was reported as many as 529, then increased to 591 cases in 2016. At the end of 2017 until the beginning of 2018, a KLB

(Extraordinary Event) had been declared in Indonesia. Based on the data from the provincial health profile of East Kalimantan, no cases of diphtheria were reported in 2015. In 2016, 9 cases were reported in East Kalimantan. There had been 25 suspected cases of diphtheria from 2017 to the end of December. And in early 2018, in the Samarinda city, until the second week there were 32 suspected cases of diphtheria that continued to increase until 95 cases in the third week of January 2018 [4]. The outbreak prevention efforts were difficult to conduct since healthy people did not show symptoms but they can still transmit to others (a carrier) [3].

A study on diphtheria carriers conducted previously by Miller *et al.* (1974) found 115 diphtheria carriers [5]. To find out whether there is a diphtheria carrier, an examination can be done using PCR (Polymerase Chain Reaction) method. On PCR examination, patients who do not cause clinical manifestations but are infected with bacteria can be detected. After the carrier is known, the patient can be given treatment in order to break the chain of transmission. By detecting the carrier, its location and distribution are known [6]. The pattern of distribution of carriers of diphtheria with spatial analysis can detect the possibility of an extraordinary occurrence in the following years. We conducted this study one year after the 2018 outbreak to find out whether diphtheria is still present in the community after outbreak and to know the distribution of diphtheria patients in Samarinda in 2018.

## 2. METHODS

The study was conducted with a cross-sectional design with a spatial analytic approach by examining the patient's oral mucosal swab. Data were obtained from the Medical Record and Clinical Pathology Laboratory of AWS Hospital and Dinas Kesehatan, Samarinda. Samples had been collected in the Samarinda city area from February to April 2019. The 43 samples were obtained from the primary data collected by researchers and the secondary data from AWS Hospital and Dinas Kesehatan, Samarinda, spread across 17 Sub-Districts and 8 Districts. 16 of 43 samples were patients who had previously been diagnosed as diphtheria and 27 others were family or patient contact at the time of illness. Spatial analysis is done using SatScan TM Software version 9.4.

Metagenomic DNA was isolated using DNeasy Blood & Tissue Kits (Qiagen, Germany) following the manufacturer instructions. Metagenomic

template was extracted and checked for the quality and quantity of DNA by agarose gel electrophoresis. The PCR technique for diphtheria detection was performed according to the established protocols, using dtx primers dtxR1 (dtxR 1F GGGACTACAACGCAACAAGAA) and dtxR (1R CAACGGTTTGGCTAACTGTA) [7]. Each 25µl PCR consisted of 1x Taq Polymerase buffer, 1.5 Mm MgCl<sub>2</sub>, 4µM of each primer, and 50 ng of template. The thermocycling conditions were 35 cycles at 94°C for 60 s, 55°C for 60 s, and 72°C for 90 s, with the final extension of 72°C for 10 minutes. To verify the amplification, 15 ml of the amplified product was electrophoresed on a 1.2% agarose gel for 45 min at 120 V. The gels were stained with ethidium bromide, and the amplicons were visualized on a UV transilluminator with a band measuring 258bp of *Corynebacterium diphtheriae*.

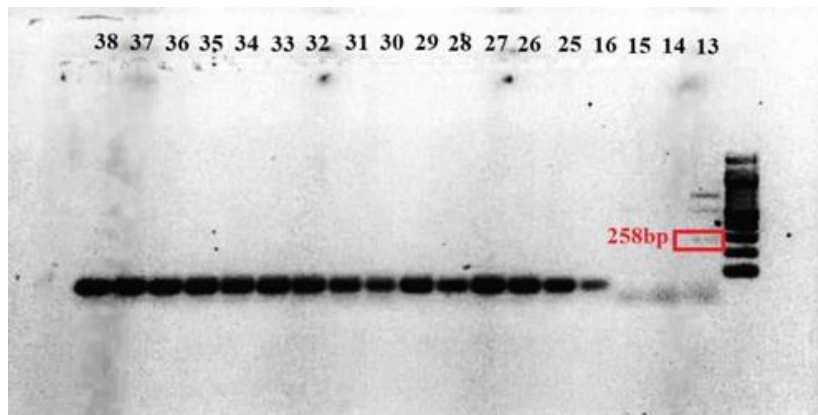
Investigations to detect toxigenic strains of *Corynebacterium diphtheriae* were constructed using Tox primers, Tox 1 (ATC CAC TTT TAG TGC GAG AAC CTT CA) and Tox 2 (GAA AAC TTT TCT TCG TAC CAC GGG ACT AA) (248; subunit A), and primer DiphT 6F (ATA CTT CCT GGT ATC GGT AGC) and DiphT 6R (CGA ATC TAC AAC AGT GTT CCA) (2297 bp; subunit B) [8]. Each 25µl PCR consisted of 1x Taq Polymerase buffer, 1.5 Mm MgCl<sub>2</sub>, 12.5 µM of (each) appropriate primer, and 50 ng of template. The thermocycling conditions were 35 cycles at or 95°C for 20 s, 55°C for 30 s, and 72°C for 1 min, ending with a final 10-min extension at 72°C. To verify the amplification, 15 ml of the amplified product was electrophoresed on a 1.2% agarose gel for 45 min at 120 V. The gels were stained with ethidium bromide, and the amplicons were visualized on a UV transilluminator.

## 3. RESULTS AND DISCUSSION

The examination of 43 samples showed 2 samples positive for *Corynebacterium diphtheriae*, namely subject number 2 and 13 (Fig. 1). The subject number 2 is a late adolescent (17-25 years) who was a patient who had been positively diagnosed with diphtheria in the past, and the subject number 13 is an adult (36 - 45 years) who was a family or household contact. Diphtheria carriers can occur in patients who were previously diagnosed with positive diphtheria. It is diphtheria. It can be caused by inadequate treatment. Diphtheria carriers can also occur in families or in house contact. Close contact that is at risk of becoming a carrier of diphtheria is someone who makes direct contact through sharing food, drinks or glasses, or stays in the same house with a case of illness within 14 days

before the onset of symptoms or onset until the case is treated. In this study, a household contact that had been infected by diphtheria bacteria had shared food and lived at home with

patients who were previously confirmed as positive cases from 14 days before the onset until the patient was treated.



**Figure 1.** Lane 1 and 2 showed a band measuring 258 bp using the dtx primer

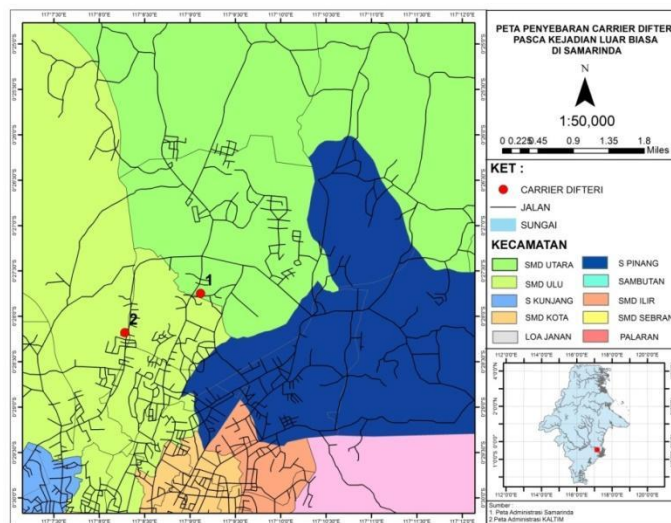
A study on carriers conducted in Dansai District, Loei Province, Thailand in 2012 found carriers at all ages. The study also found carriers in patients with a complete history of immunization and incomplete immunization history [9]. In this study, both samples that showed diphtheria bacteria had a complete history of immunization. This is similar to a study of diphtheria carriers that have been carried out in Surabaya City, Indonesia in 2011 to 2015 that found carriers in patients with a complete history of immunization. The study used a case control design where no significant differences were found between diphtheria carrier patients with complete immunization history and patients with incomplete immunization history [10]. According to Husada *et al.* 2015, the immunization status obtained was only based on interviews without checking the immunization documentary evidence. This explained why in some studies the carrier of diphtheria immunization status had no relationship or significant impact. The Centers for Disease Control and Prevention (CDC) states that

immunized individuals can still be carriers or carriers of diphtheria bacteria. People who have received immunization can still be a *Corynebacterium diphtheria* reservoir; bacteria can still infect but do not cause symptoms [11].

The results of the examination found that two samples that showed the presence of diphtheria bacteria were female. Research on diphtheria during the 1990 outbreak in Russia also showed that the majority of the patients were female [11]. Other studies regarding diphtheria carriers carried out by Husada *et al.* (2015) also showed higher results in female patients [10]. Although there is no literature that can explain the significant difference in risk regarding sex in the incidence of diphtheria, some studies show that the infection is found to be higher in women. It is presumed that the level of adequacy of protein intake and the insufficient energy of women can be one of the causes of decreased immunity so that women are more susceptible to infectious diseases (Table 1).

**Table 1.** Distribution of research samples based on regional distribution

District	Number of locations (n)	Percentage (%)
Kecamatan Samarinda Ulu	6	27.27
Kecamatan Sungai Pinang	5	22.73
Kecamatan Palaran	3	13.63
Kecamatan Sambutan	1	4.55
Kecamatan Samarinda Kota	2	9.09
Kecamatan Samarinda Ilir	1	4.55
Kecamatan Sungai Kunjang	2	9.09
Kecamatan Samarinda Sebrang	2	9.09
<b>Total</b>	<b>22</b>	<b>100</b>

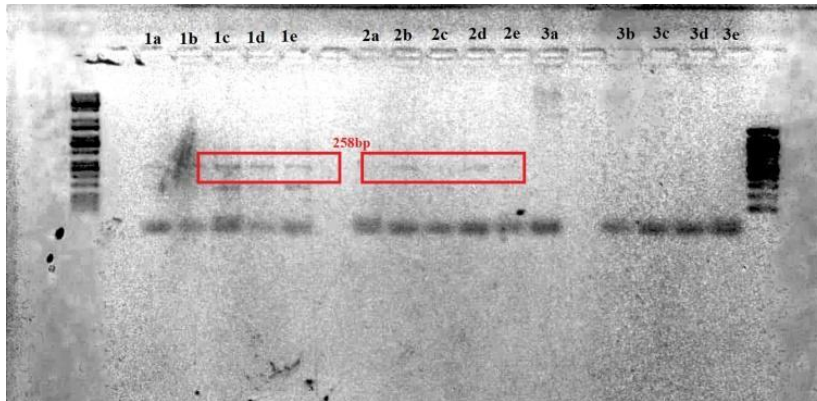


**Figure 2.** Distribution of diphtheria patient locations in 17 sub-districts and 8 sub-districts

The study found that there were 2 subjects (without clinical manifestations of diphtheria) that were infected by diphtheria bacteria based on the results of laboratory tests using the PCR technique. The two subjects were found in Kelua Mountain and Air Hitam Village, both of which were located in one sub-district, namely Samarinda Ulu District. Samarinda Ulu Subdistrict is the most populated sub-district in Samarinda City. In 2015, there were 135,814 inhabitants of Samarinda Ulu Subdistrict, with a density of 6,982 people per 12 square km. 6 locations of patients were diagnosed with diphtheria from a total of 22 locations (Table 1). This is in line with a study conducted when a diphtheria outbreak occurred in Russia in 1990, where cases of diphtheria were found mostly in areas with high population density [10]. This is also one of the factors that play a role in the case of diphtheria being a carrier patient. The spread of the carrier occurred in two villages in Samarinda City, after analysis using SatScan TM Software version 9.4 showed no pattern of spread or clustering

formed. The carrier case shows 4.7% of all subjects examined (Figure 2).

Other investigations were carried out to detect subunit A and subunit B of *Corynebacterium Diphtheria* toxigenic strains. The investigations were carried out using the primary set of Tox 1 Tox 2 and Dipht 6F and Dipht 6R with the target band size of subunit A 248 bp and subunit B 297 bp [7]. The results of using dtx primers in one sample were positive; however, the results were not positive when using primary tox and dipht. It indicated that after extraordinary events, no bacteria were found with toxigenic strains (Fig. 3). Toxin production is regulated by a set of genes called the tox / dtx gene. Dtx genes are carried by bacteriophages which integrate into non-toxigenic and non-virulent strains of kratom to become virulent and highly genetically modified. The expression of the dtx gene in producing toxins is regulated by the dtxR gene catalysed by iron.



**Figure 3.** Lane 13 shows a 258bp band using primary dtx

#### 4. CONCLUSIONS

The examination of 43 samples showed 2 samples positive for *Corynebacterium diphtheriae*. Both samples were female who lived in Air Hitam and Gunung Kelua, Samarinda Ulu District. The results of the spatial analysis showed that the location of patients of diphtheria bacteria detected did not indicate clustering.

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#### REFERENCES

- [1]. WHO 2017 Diphtheria Data in Vaccines and Disease
- [2]. Setiati S, Alwi I, Sudoyo AW, Stiyohadi B and Syam AF 2014 Buku ajar ilmu penyakit dalam jilid I *InternaPublishing* Jakarta 1132-53
- [3]. Kementrian Kesehatan RI 2016 Data dan Informasi Profil Kesehatan Indonesia
- [4]. Dinas Kesehatan Kota Samarinda 2015 Profil Kesehatan Kota Samarinda
- [5]. Miller L W, Bickham S, Jones W L, Heather C D and Morris R H 1974 Diphtheria Carriers and the Effect of Erythromycin Therapy *Antimicrobial Agents and Chemotherapy* 6(22) 166-169
- [6]. Nailul I and Soenarnatalina 2015 Analysis of Spatial Data of Diphtheria Disease in East Java Province during the year 2010 and 2011 *Bulletin of Health System Research* Vol 18 No 2 DOI: 10.22435/hsr.v18i2.4353.211-219
- [7]. Nakao H and Popovic T 1997 Development of a direct PCR assay for detection of the diphtheria toxin gene *Journal of Clinical Microbiology* p. 1651-1655
- [8]. Nakao H, Pruckler J M, Mazurova I K, Narvskaja O V, Glushkevich T, Marijevski VF, Kravetz A N, Fields B S, Wachsmuth I K and Popovic T 1996 Heterogeneity of diphtheria toxin gene, tox, and its regulatory element, dtxR, in *Corynebacterium diphtheriae* strains causing epidemic diphtheria in Russia and the Ukraine. *J Clin Microbiol* 34 1711-6
- [9]. Pailin P, Sittisak S, Pimrat K, Junti K, Jivapaisarnpong T, Paveenkittiporn W and Pittayawonganon C 2015 Epidemiological and Serological Study of Re-emerging Diphtheria in Dansai District, Loei Province, Thailand, June to October 2012 *Outbreak Surveillance and Investigation Reports* 8(1) p 13--21
- [10]. Husada, D, Primayani D, Marbun K, Kartina L, Puspitasari D and Thirtaningsih N W 2018 Risk Factors of Diphtheriae Carriers in Indonesia Children *South Asian J Trop Med Public Health* 49(4) 669-660
- [11]. CDC 2015 Epidemiology and Prevention of Vaccine-Preventable Disease *Diphtheriae* 107-118