

RESEARCH ARTICLE

Antibiotic Susceptibility Pattern of *Corynebacterium diphtheriae* Isolated from Outbreaks in Indonesia 2010-2015

Kambang Sariadji*, Sunarno, Nelly Puspandari, Masri Sembiring

Center for Biomedical and Basic Technology of Health, National Institute of Health Research and Development, Jl. Percetakan Negara 29, Jakarta, Indonesia

*Corresponding author. E-mail: kambang_sar@yahoo.com

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Abstract

BACKGROUND: Diphtheria cases are treated with both anti-diphtheria serum (ADS) and antibiotics. Penicillin and erythromycin are the primary choices for any *Corynebacterium diphtheriae* (*C. diphtheriae*) infection. Antibiotic susceptibility pattern of *C. diphtheriae* has not been reported in Indonesia since 1982. The improper use of antibiotics, as well as, the consumption of antibiotics without prescription, will increase the resistance of *C. diphtheriae*. This study aims to determine the susceptibility pattern and the effectiveness of *C. diphtheriae* to several antibiotics.

METHODS: A total of 57 *C. diphtheriae* isolates were recultured by using Blood Agar (BA) and Cystine Tellurite Blood Agar (CTBA). Afterward, these isolates were identified by using API Coryne. The antibiotic susceptibility pattern was determined by using Kirby Bauer Method based on CLSI M45-A2.

Introduction

Diphtheria is a disease caused by *Corynebacterium diphtheriae* (*C. diphtheriae*). The primary virulence factor of *C. diphtheriae* is an endotoxin that is released by the bacteria once inside the human host. The target organs of this bacterium are tonsils, pharynx, larynx, nasal, mucous membranes and skin. The clinical symptoms of diphtheria are sore throat, membrane lesions in the throat with a tender cervical lymph nodes enlargement. Toxin production and colonization of *C. diphtheriae* should be considered

RESULTS: These studies revealed that as the primary choice of diphtheria cases, 10.5% of *C. diphtheriae* isolates were resistant to Penicillin and 5.3% were resistant to erythromycin. Seven isolates (12%) showed resistance to more than one antibiotics, such as tetracycline and clindamycin, vancomycin and clindamycin, penicillin and tetracycline, also penicillin and vancomycin. Moreover, 4 isolates (7%) were resistant to 3-5 antibiotics. All isolates were susceptible to moxifloxacin and linezolid.

CONCLUSION: Some *C. diphtheriae* isolates were resistant to penicillin and erythromycin, which are known as the primary choice for the treatment of *C. diphtheriae* infection. Even though, all isolates susceptible to moxifloxacin and linezolid.

KEYWORDS: *Corynebacterium diphtheriae*, resistance, antimicrobial

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in diphtheria infection. Diphtheria, Pertussis, Tetanus (DPT) immunization program has been implemented in some developing countries. It has been proven to reduce the incidence of diphtheria in the last three decades. Low coverage of DPT immunization will increase the risk of *C. diphtheriae* infection.(1-3)

Currently, the recommended treatments of diphtheria are anti-diphtheria serum (ADS) and antibiotics, which are given simultaneously. The antitoxin will neutralize the unbound toxin that circulating in the blood and cell/tissue, while the antibiotic eliminate the bacteria. Consequently, it prevents toxin production and breaks the transmission

in communities. Penicillin and erythromycin are recommended antibiotics to treat diphtheria by the World Health Organization (WHO). However, it has been reported that there are *C. diphtheriae* which resistant to penicillin, oxacillin, erythromycin, rifampicin, tetracycline and clindamycin.(4-6) Another problem that has been identified in the treatment of diphtheria is the increase of multidrug resistance *C. diphtheriae*. Therefore, it is necessary to monitor the antimicrobial susceptibility of *C. diphtheriae* continuously.

In 2008, 14.8% isolates of *C. diphtheriae* in Brazil showed resistance to penicillin G, even erythromycin and azithromycin were still effective, but the susceptibility to erythromycin had decreased.(6) Meanwhile, in Canada around 2011, multidrug resistant *C. diphtheriae* were isolated from a skin wound. These isolates were resistant to clindamycin and erythromycin.(7) A study in Indonesia showed that *C. diphtheriae* is resistant to tetracycline since 1982. During that time, 86% of 133 *C. diphtheriae* isolated from diphtheria patients were resistant to tetracycline.(8)

The gold standard of antimicrobial susceptibility test for *C. diphtheriae* is broth microdilution method. However, this method is quite challenging and has some limitations, such as time-consuming, a high risk of contamination and more complicated rather than disk diffusion method. Disk diffusion is another alternative method to determine the antimicrobial susceptibility of *C. diphtheriae*. This method is simpler, easier and cheaper. The reason for choosing *Staphylococcus aureus* and *Streptococcus spp.* as a susceptibility breakpoints for *C. diphtheriae* is because both of them are gram-positive bacteria.

In Indonesia and other developing countries, antibiotics are available without a prescription. Consequently, the emergence of multidrug resistant *C. diphtheriae* become another problem in controlling diphtheria. The aim of this study is to determine the pattern of resistance and the effectiveness of some antibiotics against *C. diphtheriae*. This study presents the resistance pattern of mono and multidrug resistance *C. diphtheriae* to antibiotics that frequently used in diphtheria treatment in Indonesia.

Methods

Bacterial Isolates

Fifty-seven *C. diphtheriae* isolates were isolated from diphtheria outbreaks investigation in Indonesia during 2010-2015. All isolates were obtained from nasopharynx and throat swab of diphtheria patients in several provinces.

Twenty-four isolates are from Banten, 14 isolates are from West Kalimantan, 1 isolate is from West Java, 10 isolates are from Jakarta, 1 isolate is from Central Kalimantan and 7 isolates are from East Java. All isolates were stored in Trypticase Soy Broth (TSB) with 20% glycerol, at -80°C. Laboratory examination was conducted at the Bacteriology Laboratory, Center for Biomedical and Basic Technology of Health, Ministry of Health Indonesia in January-December 2015.

Re-culture and Antimicrobial Susceptibility Testing of *C. diphtheriae*

All isolates were recultured on Blood Agar (BA) and Cystine Tellurite Blood Agar (CTBA) medium. The isolates were reidentified by using API Coryne®. The susceptibility test was performed by disk diffusion method. Mueller Hinton agar medium containing 5% sheep blood was used in this method.(8-13) The antibiotic susceptibility test was performed by using disc diffusion method, based on Clinical and Laboratory Standards Institute (CLSI) M45. Medium that used in antibiotics susceptibility test was Mueller Hinton agar medium containing 5% sheep blood.(8-13)

Antibiotics used in this antibiotic susceptibility testing were erythromycin (15 µg), rifampin (5 µg), linezolid (30 µg), clindamycin (2 µg), moxifloxacin (5 µg), gentamicin (10 µg), Trimethoprim-sulfamethoxazole, (15 µg), vancomycin (30 µg), tetracycline (30 µg) and benzylpenicillin (10 µg). There was no breakpoint criteria for *C. diphtheriae*, therefore in this study, the breakpoint referred to *Streptococcus spp.* for antibiotic other than penicillin, and *Staphylococcus spp.* for breakpoints of penicillin(6,7) We used *Streptococcus pneumoniae* ATCC 49619 as internal quality control for *C. diphtheriae* antibacterial susceptibility testing.

Statistical Analysis and Ethical Approval

Data analysis of antimicrobial resistance pattern and profile was performed by using WHONET software version 5.6. The Ethical approval was obtained from Ethical Committee of National Institutes of Health Research and Development, Ministry of Health Indonesia, with the clearance number: LB.02.01/5.2/KE.315/2015.

Results

Biochemical identification of 57 reculture isolates on CTBA and BA was shown in Table 1. Meanwhile, the antimicrobial susceptibility pattern of 57 *C. diphtheriae* isolates by using disc diffusion method was shown in Table 2.

Table 1. Biochemical identification confirmation.

Number of Samples	Characteristics Colony After 24 -48 Hours		Microscopic (Albert Stain's)	Biochemical Test
	CTBA	BA		
57	Black colonies	White colonies	The metachromatic granules of <i>Diphtheria bacilli</i>	1*

Biochemical test 1*: nitrate reduction (+), pyrazinamidase (-), pyrrolidonyl arylamidase (-), alkaline phosphatase (-), beta glucuronidase (-), beta galactosidase (-), alpha glucosidase (-/+), N-acetyl-B glucosaminidase (-), esculin (-), urease (-), gelatine (-), glucose (+), ribose (-), xylose (-), mannitol (-), maltose (+), lactose (-), sucrose (-), glycogen (+), catalase (+), hemolysis (+/-).

Note: (+): positive reaction
(-): negative reaction

Table 2. Susceptibility of *C. diphtheriae* to tested antibiotics.

Antibiotic	Dose	n	%R	%I	%S
PEN	10 µg	57	11	5	84
MFX	5 µg	57	0	0	100
GEN	10 µg	57	1.8	3.5	94.7
VAN	30 µg	57	8.8	5.3	86
TCY	30 µg	57	84.2	7	8.8
CLI	2 µg	57	5.3	5.3	89.5
LNZ	30 µg	57	0	0	100
RIF	5 µg	57	1.8	0	98.2
SXT	1,25/23,75	57	22.8	0	77.2
ERY	15 µg	57	5.3	3.5	91.2

%R: Resistance Percentage, % I : Intermediate percentage, %S: Susceptible percentage, PEN: Penicillin G, MFX: Moxifloxacin, GEN: Gentamicin, VAN: Vancomycin, TCY: Tetracycline, CLI: Clindamycin, LNZ: Linezolid, RIF: Rifampicin, SXT: Trimethoprim/Sulfamethoxazole, ERY: Erythromycin.

Fifteen *C. diphtheriae* isolates (26.3%) with no resistance are susceptible to all antibiotics tested and 42 isolates are resistant to at least one antibiotic. The profile showed some isolates resistance to several antibiotics (multi-resistant) (Table 3).

Discussion

The purpose of antibiotic treatment for diphtheria cases is to eliminate bacteria from the infection site, consequently it will stop toxins production and the transmission of the disease in the community.(3) The antibiotic susceptibility testing is used to guide the antibiotics used in diphtheria cases. The appropriate use of antibiotic in diphtheria cases will inhibit the toxin production and prevent transmission from the patient or asymptomatic carrier to others.

Table 3. Profile of *C. diphtheriae* antibiotics resistance.

Resistance Profile	Number of Isolate	% Isolate
NR	15	26
CLI	2	3.5
TCY	25	43.9
PEN	1	1.8
RIF	1	1.8
SXT	2	3.5
ERY	1	1.8
TCY, CLI	1	1.8
VAN, CLI	1	1.8
VAN, TCY	3	5.3
PEN, VAN	1	1.8
PEN, TCY, CLI	1	1.8
PEN, GEN, VAN, TCY, CLI	3	5.3
Total	57	100%

NR: No resistance, CLI: Clindamycin, TCY: Tetracycline, PEN: Penicillin G, VAN: Vancomycin, GEN: Gentamicin, RIF: Rifampicin, SXT: Trimethoprim/Sulfamethoxazole, ERY: Erythromycin.

Table 2 shows that all 57 isolates are sensitive to moxifloxacin and linezolid. There are 84.2% *C. diphtheriae* isolates that resistant to tetracycline, 22.8% resistant trimethoprim-ssulfamethoxazole, 10.5% resistant to penicillin, 8.8% resistant to vancomycin, 5.3% resistant to erythromycin and 5.3% resistant to clindamycin.

The profile of antibiotic susceptibility result of *C. diphtheriae* is shown on Table 3, there are some isolates that resistance to two or more antibiotics, commonly called as multidrug resistant bacterium. Irrational use of antibiotics due to misdiagnosis and misuse of antibacterial agent, will cause the occurrence of multidrug resistance.

The literature of multi-drug resistant for *C. diphtheriae* are rarely found. Nevertheless, some study reported about multi-drug resistant *C. diphtheriae* isolates. There were 20% of isolates in Vietnam resistant to some antibiotics by microdilution broth and disc diffusion.(7) A study conducted in Brazil showed that 97% of *C. diphtheriae* strains are resistant to 4-7 antimicrobials, including mupirocin, penicillin and/or ampicillin, oxacillin, ceftazidime, aztreonam, tetracycline and/or lincomycin, clindamycin, erythromycin. Those studies used disc diffusion and E-test methods.(6) On the contrary, the susceptibility data collected from an outbreak in Russian Federation in the early 1990s showed that 2.4% isolates had monoresistance to trimethoprim and rifampin but no isolates as multidrug resistant was found. It also reported from a study in Polish, whereas there was a multidrug resistance *C. diphtheriae* case in Canada which

was resistant to clindamycin, erythromycin, tetracycline and trimethoprim-sulfamethoxazole.(7,17-20)

In our study, there were 6 isolates (10.5%) that resistance to two antibiotics, such as tetracycline and clindamycin, vancomycin and clindamycin, vancomycin and tetracycline, also penicillin and vancomycin. Then 4 isolates (7%) resistance to 3-5 antibiotics. All isolates susceptible to moxifloxacin and linezolid and 15 out of 57 *C. diphtheriae* isolates (26.3%) are susceptible to all antimicrobials tested.

A study conducted in Cipto Mangunkusumo Hospital, Jakarta in 1982 reported that 33 out of 188 samples are identified as *C. diphtheriae* subtype mitis.(8) All isolates were tested by disc diffusion and E-test, simultaneously. It showed that the agreement value between disc diffusion and E-test method were 94.9%.(6)

Tetracycline resistance *C. diphtheriae* is uncommon in some countries, but some study in Europe countries revealed *C. diphtheriae* that resistant to tetracycline, after treatment of intravenous tetracycline. Tetracycline resistance *C. diphtheriae* was also reported in 1982 in Cipto Mangunkusumo Hospital, Jakarta. In that study, 133 samples (86%) showed resistance to tetracycline.(10) Moreover, the study reported all the isolates susceptible to penicillin and erythromycin. It study have similarity with our research, especially in tetracycline resistance. On the contrary, the research of susceptibility test in Brazil reported that 12.8% out of 47 isolates of *C. diphtheriae* was resistant to tetracycline, by using minimum inhibitory concentration (MIC) method.

The mechanism of tetracycline-resistant due to different cytoplasmic membrane generates and prevents binding of tetracycline to the 30s ribosomal subunit, then the protein synthesis of the cytoplasmic membrane can continue. Another tetracycline-resistant mechanism is efflux pump due to mutation of *tetAB* genes, this mechanism enables the bacteria pump the antibiotic out of the cell. This mechanism prevents tetracycline accumulate within the bacteria in toxic level so the bacterial protein synthesis will not be inhibited.(14,15)

In this study, 10.5% *C. diphtheriae* isolates resistant to penicillin and 5.3% resistant to erythromycin. The previous study in 1982 with the same method showed that *C. diphtheriae* still susceptible to penicillin and erythromycin. Another study in French and Poland showed that 42 *C. diphtheriae* isolates were susceptible to erythromycin and 6 of them have the decreased susceptibility to penicillin. Essentially, the *in vitro* activity of erythromycin is better than penicillin.(16)

Some studies reveal that some *C. diphtheriae* isolates are resistant to some drugs, such as penicillin G, oxacillin and other antibiotics which are used for the treatment of diphtheria, for instance rifampin, tetracycline and clindamycin. The presence of β -lactams resistance against *C. diphtheriae* should be concerned, especially in the administration of penicillin for patients who have endocarditis because of *C. diphtheriae* infections. It could increase the risk of treatment failure.(6) A study in Brazilian reported that a patient with endocarditis bacterialis caused by multidrugs resistance *C. diphtheriae* infection was showing a treatment failure. Based on the study, clinician must reconsider the use of resistant to β -lactams antibiotic if the clinical condition of patient with systemic infection does not improve after being treated with β -lactams antibiotics.(6)

According to CLSI document M45-A2 guideline, the microdilution broth method is recommended in *C. diphtheriae* susceptibility testing. We already conducted our research with this method by using an automated reading incubation system (ARIS) (Thermo Scientific, Massachusetts, USA) to determine identification and antimicrobial susceptibility testing of *C. diphtheriae*, nevertheless there are several limitations of the sensititre ARIS. First, it only detects genus level of corynebacterium species. Second, the breakpoints of determining of the antibiotic concentration have small range values. Therefore, when examining *Streptococcus pneumoniae* ATCC 49619 as a validity test, the results are out of the range. Third, the sensititre plate with lysed horse blood medium for *C. diphtheriae* antimicrobial susceptibility testing is not available in Indonesia. We already optimatized an *in house* plate by adding lysed horse blood manually, but the result was not satisfying. We also tried antimicrobial susceptibility testing by using strip diffusion agar, such as E-test® (Biomeureux, Marcy-l'Étoile, France) and Ezy® test (Himedia, Mumbai, India), but those products are out of stock for several months in Indonesia. Therefore, we use disc diffusion method as an alternative, because it is easier and cheaper compare to others method and is available in Indonesia.

C. diphtheriae have several resistance mechanisms which are related to mutations in particular genes. *C. diphtheriae* resistant erythromycin mechanism relates to a mutation in ErmX genes which is expressed in methyl transferase enzymes. Resistance to tetracycline relates to *teM* and *tetAB* genes mutation. *C. diphtheriae* that resistant to penicillin occurred as a result of mutation in genes encoded penicillin-binding protein or due to bacterial

genes required penicillin-binding protein. Penicillin resistant *C. diphtheriae* may also occur due to the limited of bacteria transport system in the outer membrane which prevents penicillin reach cytoplasmic membrane where the penicillin-binding protein located. Vancomycin resistance occurs due to the enzyme in bacteria resistant cells. The mechanism of resistance identified in the Tn1546-based antibiotic resistance was shown to involve alteration of this dipeptide residue from D-ala-D-ala to d-alanyl-d-lactate (D-ala-D-lac), a dipeptide with substantially lower affinity for the antibiotic.(14,15)

Conclusion

Based on this study, some of *C.diphtheriae* are resistant to several antibiotics that used as primary choice of diphtheria. *C.diphtheriae* is resistant to penicillin (10.5%) and erythromycin (5.3%). It is also identified that there are 6 isolates (10,5%) that are resistant to two other antibiotics such as tetracycline and clindamycin, vancomycin and clindamycin, vancomycin and tetracycline, also penicillin and vancomycin. Then, 4 isolates (7%) are found to be resistance to 3-5 antimicrobial. Only moxifloxacin and linezolid show 100% susceptible and 14 out of 57 *C.diphtheriae* isolates (25%) are susceptible to all antibiotics tested. Those results could be used as a recommendation for diphtheria treatment guidelines in Indonesia.

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