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Diphtheria Toxigenity Test Using Biomolecular and Elek test Methods

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Abstract

The main important testing for diagnosis Corynebacterium diphtheriae is a toxicity test. The Elek test is an alternative method that most widely used as a gold standard, but it requires expertise in the interpretation of results, experienced of technicians, and consumed time. The Polymerase Chain Reaction (PCR) method is also an alternative method to determine thetoxigenity, because it is faster, simpler, and easier in the interpretation of results. Regarding of Toxicity test for dihptheriae, the previous studies showed that the differences in results between the genotype-based PCR method and the phenotype-based Elek test method. This study aims to compare the results of toxicity testing using the PCR method and the Elek test method on C. diphtheriae isolates. The specimens research of C. diphtheriae are from an outbreak isolates diphtheriae with total 30 samples. The Positive and negative control of examinations are used reference strains (NCTC 3984 strains and NCTC 10356 strains). The results showed that 30 samples were toxigenic strains. There were no strains of nontoxigenic tox gene bearing (NTTB). No difference results for both PCR and Elek tests. The conclusion of those research are 100 % agreement both two PCR method and the Elek test method in determination of C. diphtheriae toxigenity.

Keywords: C. diphtheriae, Elek test, PCR, toxicity

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1. Introduction

Corynebacterium diphtheriae is a bacterium that causes diphtheria disease and is the main species of the genus *Corynebacterium* as the cause of diphtheria in humans. The disease is characterized by the presence of inflammation at the site of infection, especially in the mucous membranes of the pharynx, larynx, tonsils, nose, and skin [1-3]. Diphtheria in children became the leading cause of death before the vaccination era. In 1974, the diphtheria vaccine was included in the list of immunizations recommended by the WHO. The use of the diphtheria vaccine caused the incidence of diphtheria globally to decrease by more than 90% from 384,540 cases in 1980 to a stable state rate of about 18,000 cases. Reported since 2006 [4]. Until now diphtheria outbreaks still occur in various countries and cannot be eradicated [5]. The largest outbreak of the vaccination era occurred in the countries of the former Soviet Union in the 1990s with the victims of more than 4,000 deaths [3]. Diphtheria cases in the *South-East Asia Region* (SEAR) annually rank among the first cases of diphtheria in the world. This can be seen from the position of SEAR since 2000-2017 which always shows the highest number of diphtheria cases in the world. Indonesia is the country with the second most cases of diphtheria compared to other sear countries, namely India. The number of diphtheria cases reported in Indonesia from 2000-2017 was 7,160 cases, while India is still the country with the highest number of diphtheria cases with as many cases as possible. 79,034 cases [6]. Cases of diphtheria in 2018 spread in almost all regions in Indonesia. The number of diphtheria cases in 2018 was 1,386 cases, of which 29 cases died. The number of diphtheria cases in 2018 has almost doubled compared to 2017 with 954 cases [7]. The main virulence factor *of C. diphtheriae* is the diphtheria toxin that causes systemic complications in diphtheria. Toxins will be absorbed by the mucous membrane and then cause inflammation, destruction of the epithelium in the infected area, tissue necrosis, and the formation of pseudomembranes. Pseudomembranes are followed by the occurrence of edema of the mucosal tissue underneath so that it can cause airway obstruction. Toxins will enter the bloodstream and attack various organs, especially the heart and nerves because there are many diphtheria toxin receptors. Effects on the heart can cause myocarditis while on the nerves can lead to polyneuropathy. Death is usually caused by heart failure and respiratory distress [8, 9]. Laboratory detection of diphtheria virulence in the form of bacterial toxins is carried out through toxigenity examination. *C.diphtheriae* conventionally as *a gold standard* can be done by *in vivo* or *in vitro* examination. In *vivo* toxigenity examination consists of *a subcutaneous test* and *an intradermal test* using two *guinea pigs* as try animals.

The use of *guinea pig* as an animal is widely opposed by animal lovers, the high cost, and the risk of accidents during inoculation make it not applicative. The way *in vitro* is done through *vero cell cytotoxicity* with limits to the use of *vero cell cytotoxicity* in diagnostic laboratories that require a laboratory with special *tissue culture* facilities, The cost is expensive, takes a long time up to weeks (± 6 weeks), and technicians who have special skills in *tissue culture* engineering are needed. Another in vitro way is with the *Elek test* method [3, 10]. *The elek test* is the most widely used method for the diagnosis of diphtheria based on immunological techniques. While the examination of toxigenity genotype with *polymerase chain reaction* (PCR) method is an option because it is faster, simpler, and relatively easier in the interpretation of results [11-12]. This study aims to determine the suitability of toxigenity examination between the elek test method and bio molecular tests on specimens of extraordinary events of diphtheria.

2. Materials and Methods

The research sample is a *C. diphtheriae* isolate stored because of the 2019 Extraordinary Events (KLB) investigation totaling 30 samples. The isolate is replanted (recultured) in *the Blood Agar* (BA) medium and incubated at a temperature of 37 °C for 24 hours. Growing bacterial colonies are used for genotype toxigenity examination by PCR and phenotype methods with *the Elek test* method.

2.1. Procedure Elek test

Columbia media *so that* [Oxoid] 15 mL in a threaded cap tube is sterilized using an *autoclave* at 121 °C for 15 minutes, let stand to a temperature of 50 °C, added 3 mL *Newborn Calf Serum* (NBCS) [Thermo Scientific] slowly, and poured into a petri dish measuring 60 x 15 mm as much as 5 mL. *The* antitoxin disk containing 1,000 IU of Serum Anti Diphtheria (ADS) [Biopharmaceuticals] is placed in the middle of the petri dish; the bacterial colony is inoculated at the edges at a distance of 1 cm from the place of the antitoxin disk, and incubated at a temperature of 37 °C for 48 hours.

2.2. Procedure PCR

The DNA extraction process is carried out using *the Quick-DNATM Miniprep Plus Kit* [Zymo Research]. The DNA of the *extracted template* is stored in a *freezer* with a temperature of -20 °C if not directly worked on. The DNA amplification process in the PCR process is carried out using *a master mix* of *MyTaqTM HS Red Mix* [Bioline] with a *primary* set of *Integrated DNA Technologies* (IDT) DT1 *forward* (5'-GT TTGCGTCAATCTTAATAGGG-3') nucleotide position 15-36 and *primary* DT2 *reverse* (5'-ACCTTGGTGTGATCTACTGTTT-3') nucleotide position 1622-1643 to detect *C. toxphtheriae* with *tox* gene targets. Amplification with the *primer* produces DNA fragments measuring 1,600 *bp*. The final volume of the master mix made is 25 µL consisting of 12.5 µL *2x MyTaq HS Red Mix*, 1 µL *primer* DT1 *forward* 10 µM , 1 µL *primer* DT2 *reverse* 10 µM, 5.5 µL *molecular water* [Sigma], and 5 µL DNA *template*. The PCR stage consists of the *ion pre-denaturate* stage at 95 °C for 5 minutes and then followed by 35 amplification cycles consisting of the denaturation stage . for 1 minute at 94 0C, *annealing* stage for 1 minute temperature 55 °C, and *extension* stage for 1 minute at 72 °C. The final stage of the amplification process is *the final stage of extension* at 72 °C for 10 minutes. Furthermore, the results of DNA or amplicon amplification are carried out electrophobresis process using mupid-exu electrophoresis machine [Advance] and 2% agarosa gel [Invitrogen] which is stained with red gel [Biotium] in the TAE 1X [Thermo Scientific] *buffer* at a voltage of 70 volts for 2 hours. Electrophoresis results are then visualized using *Doc XR Plus Gel* [Biorad].

3. Results and discussion

The results of the toxigenity examination conducted on 30 samples using the PCR and elek test methods were presented in the form of table 1. The results of the toxigenity examination using both methods showed 30 samples (100%) positive results.

3.1. Elek Body

An overview of the results of the toxigenity examination with the *Elek test* method can be seen in figure 1. Samples number 1-4 form a white precipitation line between the antitoxin disc (center) and the bacterial inoculation site as seen in positive control (+). This indicates that the samples are toxygenic strains. Meanwhile, in negative control (-) there is no visible line of precipitation. A total of 30 samples that have been examined by *the Elek test* method showed positive results of precipitation is a toxgenic *strain*.

3.2. PCR

An overview of the results of the toxigenity examination with the PCR method can be seen in figure 2. In sample numbers 1-5, there is a DNA band measuring 1,600 *bp* as seen in positive control (+). This indicates the presence of *the tox* gene in the sample and it is concluded that the samples are toxigenic strains. Meanwhile, in negative control (-) there is no visible DNA band. A total of 30 samples that have been examined by pcr method are known to be toxygenic strains. The results showed that the toxigenity examination of 30 samples of *C. diphtheriae* isolates using the PCR method was 100% in accordance with the results of the toxigenity examination with the *Elek test* method. The results of this study are in accordance with the study conducted by Mikhailovich *et al.* In Russia between1990-1994, it showed a 100% correlation between conventional PCR methods in detecting subunits A and B of *the tox* gene with *the Elek test* method [13]. The results of this examination also showed all samples of *Corynebacterium diphtheriae* were toxygenic both genotif and phenolic. There are no isolate samples that are either negative or both of them are negative. Toxigenity testing is very important to do because not all C. *diphtheriae* bacterial isolates produce toxins [14]. Examination of the toxicity of

Table 1. Results of toxigenity examination using PCR and Elektest methods

		Elek test		
		Positive	**Negative**	**Sum**
PCR	**Positive**	30	-	30
	Negative	-	-	-
	Sum	30	0	30

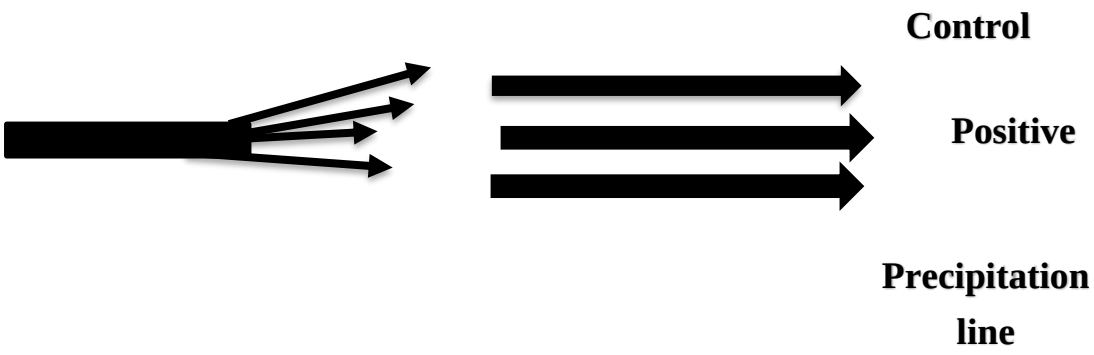

Figure 1. Example of the results of the toxigenity examination with *the Elek test* method

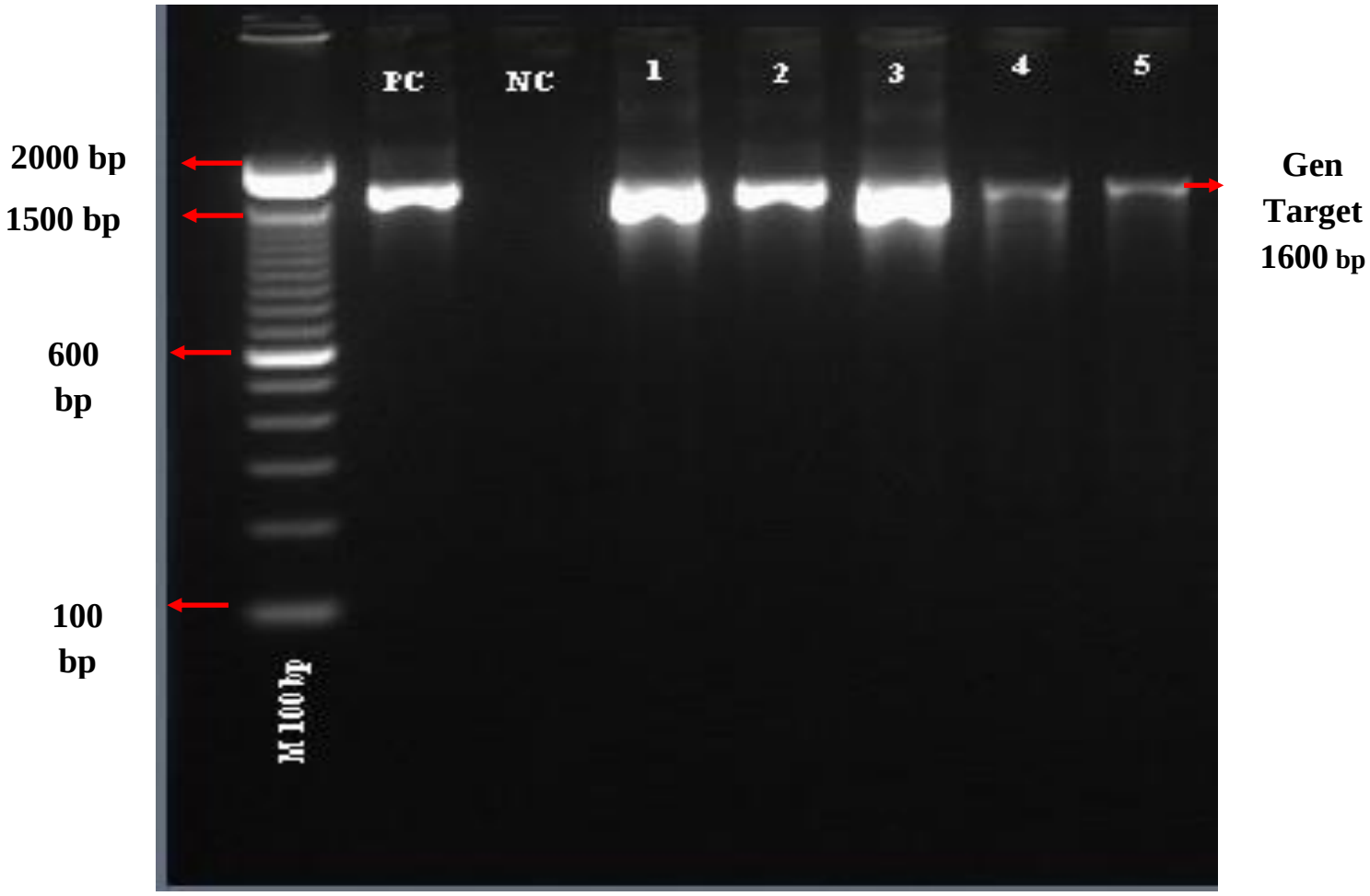

Figure 2. Example of the results of the toxigenity examination by PCR method, Description: M100: Marker 100 bp , PC : Positive control, NC: Negative Control, sample 1,2,3,4,5: target gene examination 1600 bp.

C. diphtheriae is important for investigating cases and treatment. Toxygenic *strains* require management as soon as possible with ADS and antibiotics [15-16]. Early diagnosis and treatment with ADS and antibiotics can prevent complications and death. Delayed treatment will cause the toxin to spread throughout the body and bind to toxin receptors on the surface of cells, especially nerve cells and the heart. Toxins that have bonded to the cell surface cannot be neutralized with ADS so that the cell will experience damage and death. Therefore, the decision to provide ADS is based on a clinical diagnosis and there is no need to wait for laboratory confirmation [17]. In the examination of toxigenity with The *Elek test* method (figure 1) as an alternative *to gold standard* showed 30 positive samples with the formation of precipitation lines. The formation of this precipitation is a reaction bond between antitoxin (ADS) as an antibody and diphtheria toxin as an antigen released by bacteria in the culture media. If bacteria produce diphtheria toxin, there will be antigen-antibody bonds that form a precipitation line between antitoxins and bacterial inoculations. However, *the Elek test* is particularly susceptible to misinterpreting interpretations of results because some *strains* produce very weak precipitation lines, requiring experienced technicians because will affect the sensitivity of the results of the examination, it takes 2-3 days, non-pure antitoxins can produce nonspecific precipitation lines that cause. False positive results, clarity and accuracy of the test depend on media constituents, antitoxin concentration, and the use of the right type of control.11. However, the concern of the *Elek test* cannot distinguish nttb strains from non to genic strains. *Nttb* strain is a nontosygenic *strain* that has the *tox* gene but is not expressed so that it does not produce toxins phenotypically [17, 18]. At the examination of the toxigenity method PCR (figure 2) showed 30 samples formed a target gene band at 1600 bp. PCR methods are an option because they are faster, simpler, and relatively easier in interpreting results. The PCR method is based on the detection of bacterial DNA, both living and dead bacteria, in contrast to conventional methods that are based on isolating live bacteria from a sample 12. However, conventional PCR has limitations, namely the indistinguishability of NTTB strains from toxygenic strains.

This can cause differences in the interpretation of examination results, namely positive toxgenic PCR but non to specgenic conventionally so that clinically it can have implications for Therapy, especially in the administration of ADS and the selection of antibiotics 12. Therefore, if the PCR result is positive toxgenic, *Elek test* should be done to detect the expression of toxin [19-20]. PCR toxicity screening is still insufficient to identify *pathogenic strains of C. diphtheriae* and confirm toxin production by organisms. Pcr method can be used as a quick and reliable diagnosis tool for the benefit of screening for C. *diphtheriae* toxicity to reduce the spread of disease and lower the disease. Death toll. This supports the use of PCR methods for the determination of C. *diphtheriae* toxigenity without waiting for the results of the *Elek test*.

4. Conclusions

The results of the study in the toxigenity test of 30 samples of *Corynebacterium diphtheriae* isolate showed 100% conformity of the results both with the PCR method that detects genotifally and the elektest method that detects Phenotifally. Pcr method is a fast and reliable alternative method, especially for the benefit of screening C. *diphtheriae* toxicity examination that can be applied in the laboratory. However, the certainty of the toxigenity *of C.diphtheriae* phenotype with the *Elek test* method is still recommended to be done.

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