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A new modified medium for Simultaneous Cystinase and elek tests of bacteria causing diphtheria



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ABSTRACT

Introduction: Potentially toxigenic *Corynebacteria* (*Corynebacterium diphtheriae*, *Corynebacterium ulcerans*, and *Corynebacterium pseudotuberculosis*) can produce diphtheria toxin and stated as diphtheria causative agent. The bacteria causing diphtheria could be identified by *Cystinase* test on the Tinsdale medium, while its toxigenicity determined by Elek test on the Elek medium. This study aims to develop a new modified medium for both *Cystinase* and Elek tests simultaneously.

Methods: There were ten reference strains of bacteria used for the modified medium optimization. Moreover, 15 clinical isolates were used as samples in the modified medium testing. The result of *Cystinase* and Elek tests on the modified medium was compared with the standardized tests on the Tinsdale and Elek mediums.

Results: Twelve of 25 isolates tested on the modified medium were identified as toxigenic strain, corresponding with the result from standardized Elek test on the Elek medium. Moreover, 16 of 25 isolates tested on the modified medium were identified as positive for *Cystinase* test. The similar result was obtained using the standardized *Cystinase* test on the Tinsdale medium. This result was visible 24 hours after incubation. The modified medium was in excellent condition with the consistent result after stored in half-finished condition for 32 days at 2-8°C.

Conclusion: The modified medium developed in this study was a new good medium that could be used for *Cystinase* and toxigenicity tests simultaneously.

Keywords: diphtheria, Cystinase, Elek, medium

Cite this Article: Fitriana., Sunarno., Syarif, A.K., Karyana, M., Rosana, Y., Moehario, L.H. 2019. A new modified medium for Simultaneous Cystinase and elek tests of bacteria causing diphtheria. *Bali Medical Journal* 8(1): 334-340. DOI: [10.15562/bmj.v8i1.1231](https://doi.org/10.15562/bmj.v8i1.1231)

INTRODUCTION

The campaign and implementation of immunization program worldwide, especially diphtheria toxoid leads to the decrease of diphtheria cases globally. However, this disease has not gone and the bacteria causing diphtheria still circulate in the environment. The finding of diphtheria cases in various countries is the evidence of the existence of this disease.¹ Diphtheria outbreak in vaccination era with casualties reached thousands of people has affected Russia and surrounding area in 1990's.² Indonesia's number of cases just shifted from the second to the third position after the soaring cases in Nepal (2014) and Madagascar (2015 and 2016).³ Diphtheria cases in Indonesia has spread almost to all province from the west end (Nanggroe Aceh Darussalam) to the east end (Papua).⁴

Corynebacterium diphtheriae was identified as the causative agent of diphtheria in the 19th century.⁵ Moreover, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* were

stated as the other bacteria causing diphtheria.^{6,7} All of three species can produce diphtheria toxin if infected by certain *bacteriophage*.⁸⁻¹⁰ These bacteria also have similar biochemical reactions (positive *Cystinase* and negative *Pirazinamidase*)¹¹ and closed family relation based on 16S rRNA and *rpoB* genes analysis.¹² The biochemical reactions differentiate them from another member of Genus *Corynebacterium* and therefore, applicable to screen and identify the bacteria causing diphtheria.¹¹ On the other hand, *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* have some different clinical characteristics. Transmission of the diseases caused by *C. diphtheriae* usually man-to-man, although *C. diphtheriae* has also been isolated from the animal.^{13,14} Whereas, the diseases caused by *C. ulcerans* and *C. pseudotuberculosis* are typically transmitted through the animal (zoonotic), although transmission between humans cannot be ruled out.^{15,16}

The ability of the bacteria to produce diphtheria toxin (*toxigenicity*) could be determined by an *in*

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Received: 2018-07-04
Accepted: 2018-08-23
Published: 2019-04-01

vitro test. The usual *in vitro* test to identify *toxigenicity* of bacteria causing diphtheria is Elek test that found and published in 1949.¹⁷ A number of modifications was done to get more optimal results.¹⁸⁻²⁰ On the other hand, Tinsdale medium has developed for *Cystinase test* to identify bacteria causing diphtheria. This medium could be used to differentiate bacteria causing diphtheria (*C. diphtheriae*, *C. ulcerans*, *C. pseudotuberculosis*) from the other member of the genus *Corynebacterium* by brownish halo formation around bacterial colonies.²¹ A number of the Tinsdale medium modifications was also carried out by some investigator.^{22,23}

The *toxigenicity test* is crucial for diphtheria case management and also monitoring of toxigenic strain spreading.^{24,25} In this case, Elek test is the method that recommended and used widely.²⁶ On the other hand, several studies showed benefits and advantages of the Tinsdale medium to identify the bacteria causing diphtheria.^{27,28} In this study, we have developed a new modified medium that could be used for *Cystinase* and Elek tests simultaneously. Ethical approval for this study was obtained from the Health Research Ethics Committee, Faculty of Medicine, Universitas Indonesia-Cipto Mangunkusumo Hospital (Approval number 367/H2.F1/ETIK/V/2014).

MATERIALS AND METHODS

Sample

Some reference strains of bacteria were used to optimize the modified medium, including *C. diphtheriae* NCTC 10356, *C. diphtheriae* NCTC 10648, *C. diphtheriae* NCTC 3984 and *C. ulcerans* NCTC 12077, the other member of genus *Corynebacterium* (*Corynebacterium striatum* NCTC 764, *Corynebacterium minutissimum* ATCC 23346, and *Corynebacterium pseudodiphthericum* ATCC 10700) and the other bacteria (*Klebsiella pneumoniae* ATCC BAA-1144, *Staphylococcus aureus* ATCC 12493, and *Streptococcus pneumoniae* ATCC 10015). Moreover, 15 clinical isolates from the patients and the closed contacts were used as samples in the modified medium testing. These isolates have identified as *C. diphtheriae*, *C. pseudodiphthericum* and *Corynebacterium imitans* by standardized conventional methods.²⁹

Elek test on The Elek Medium

Bacterial toxigenicity was determined by the standardized Elek test on the Elek medium as described previously.²⁰ A half milliliter of Newborn Calf Serum (Sigma) mixture with 2.5 ml Elek medium base at 50-55 °C and poured into the 4.5 cm petri dish, trimmed, and waited until it became solid. Diphtheria antitoxin disc was put right in the

center of this plate. Three control strains, including two toxigenic *C. diphtheriae* (NCTC 10648 and NCTC 3984), one non-toxigenic *C. diphtheriae* (NCTC 10356) and the examined samples were inoculated around the antitoxin disc. The plate was incubated at 37°C for 24-48 hours. Precipitation line formed between the antitoxin disc and the inoculation place was observed on the 24th and 48th hour of incubation.

Cystinase test on The Tinsdale Medium

The standardized *Cystinase* test was conducted on the Tinsdale medium as described previously with a few modifications.²¹ Tinsdale agar base which contains 20 g Protease peptone, 15 g agar, 5 g NaCl, 5 g Yeast extract and 0.24 L-Cysteine was diluted in 1000 ml distilled water, dissolved and sterilized in an autoclave. Tinsdale supplement contains 0.43 g Natrium thiosulfate (Na₂S₂O₃) and 0.35 g Kalium tellurite (K₂TeO₃) was diluted in a little amount of distilled water and put in 100 ml serum. The Tinsdale agar base (50-55°C) was mixed with The Tinsdale supplement with 10:1 ratio, then poured into a petri dish and waited until it became solid. *C. diphtheriae* isolates (positive control), *C. striatum* isolate (negative control) and the examined samples were streaked on this medium and incubated at 37°C for 24-48 hours. The brownish halo formed around bacterial colonies was observed on the 24th and 48th hour of incubation.

Elek and Cystinase tests on The Modified Medium

The medium consists of Tinsdale_Elek (T-E) medium base (Proteose peptone, starch, NaCl, and other compounds), T-E supplement (Na₂S₂O₃, K₂TeO₃ and other compounds), and Newborn Calf Serum (NBCS).³⁰ The T-E medium base was diluted in distilled water, dissolved, poured 2.5 ml into the tubes and sterilized in the autoclave at 121°C for 15 minutes. Two and a half milliliter of the T-E medium base (50-55°C) was mixed with 0.025 ml T-E supplement and 0.5 ml NBCS, poured into the 4.5 cm petri dish and waited until solid. The further procedures were performed similar to the modified Elek test, as described before.²⁰ The negative control for Elek test was non-toxigenic *C. diphtheriae*, while the negative control for *Cystinase* test was *Corynebacterium striatum*. Precipitation line between each bacterial inoculation and the antitoxin disc as well as the brownish halo around the bacterial inoculations were observed on the 24 and 48 hours after incubation.

The result of Elek and *Cystinase* tests on the modified medium compared with the standardized Elek test on the Elek medium and the standardized

Cystinase test on the Tinsdale medium. The stability of the modified medium stored in the 2-8°C temperature assessed serially on day-1, day-2, day-4, day-8, day-16, and day-32.

RESULTS

Elek test on the Elek Medium

Standardized Elek test on the Elek medium (Fig. 1) showed that precipitation line appeared on 2

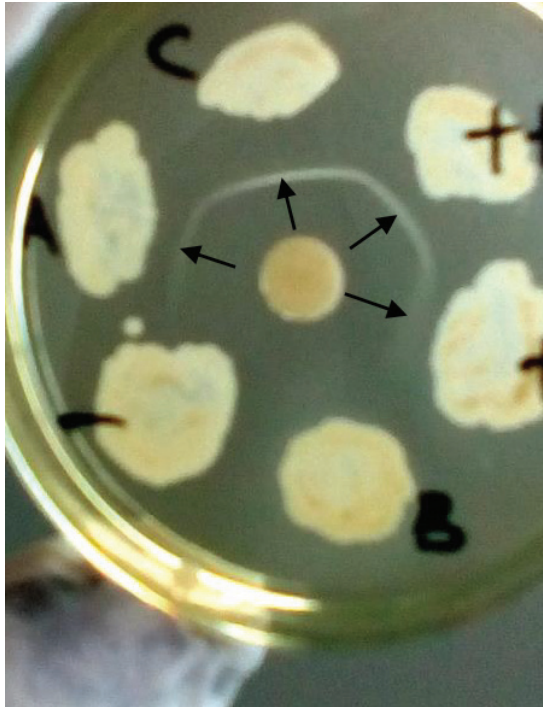


Figure 1. Standardized Elek test on the Elek medium: precipitation line showed by the arrow

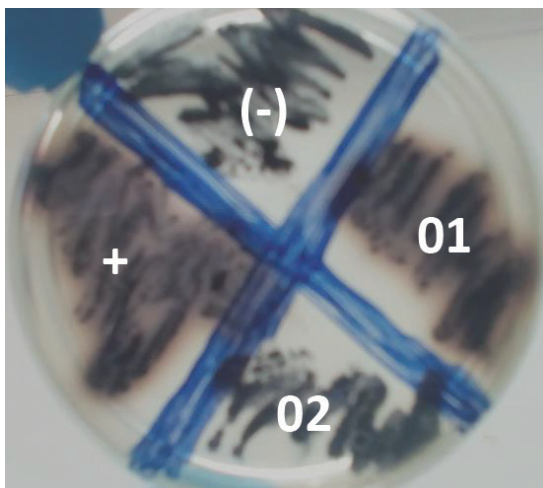


Figure 2. Standardized *Cystinase* test on the Tinsdale medium: positive results marked by a brownish halo around suspected colonies (right and left sides)

positive controls (toxigenic *C. diphtheriae* NCTC 10648 (++) and NCTC 3984 (+)), the sample A, and sample C, while no precipitation line observed on the negative control (non-toxigenic *C. diphtheriae* NCTC 10356 (-)) and sample B. These results showed that sample A and C were the toxigenic strains, while sample B was the non-toxigenic strain.

Cystinase test on The Tinsdale Medium

Standardized *Cystinase* test on the Tinsdale medium (Figure 2) could be interpreted easily. Positive results for *Cystinase* test on positive control (*C. diphtheriae*) and sample 01 were marked by a brownish halo around bacterial colonies. The result indicated that sample 01 was highly suspected as the bacteria causing diphtheria. The brownish halo was not visible on the negative control (*C. striatum*) and sample 02.

Elek and *Cystinase* tests on The Modified Medium

Simultaneous Elek and *Cystinase* tests on the modified medium (Figure 3) were interpreted based on precipitation line and brownish halo formation.

Both of precipitation line and brownish halo were visible on sample 01 as well as two positive controls (toxigenic *C. diphtheriae* NCTC 10648 (++) and NCTC 3984 (+)). Therefore, the sample 01 was most likely identified as bacteria causing diphtheria (positive *Cystinase* test) and toxigenic (positive Elek test). Meanwhile, the brownish halo

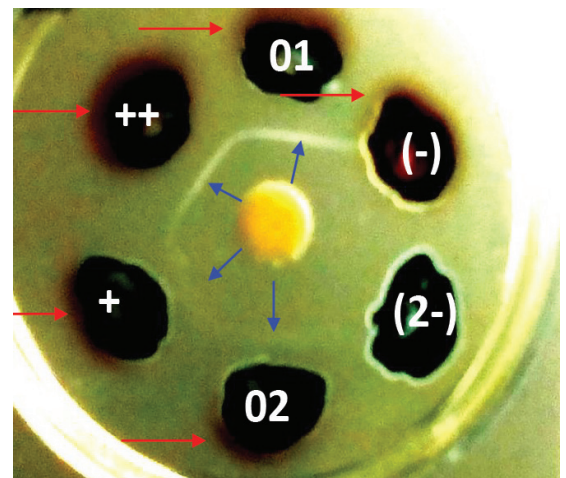


Figure 3. Simultaneous Elek and *Cystinase* tests on the modified medium: positive for *Cystinase* test marked by brownish halo formation (red arrow) around bacterial inoculation, while positive for Elek test marked by precipitation line (blue arrow) between the inoculation place and antitoxin disc

Table 1. The accuracy of simultaneous *Cystinase* and Elek tests on the modified medium compared with standardized tests on the Elek and Tinsdale mediums

Sample	Results in 24 hours			Results in 48 hours		
	Elek Medium	Tinsdale Medium	Modified Medium**	Elek Medium	Tinsdale Medium	Modified Medium**
<i>C. diphtheriae</i> NCTC 10648	+	+	+/+	+	+	+/+
<i>C. diphtheriae</i> NCTC 3984	+	+	+/+	+	+	+/+
<i>C. diphtheriae</i> NCTC 10356	-	+	-/+	-	+	-/+
<i>C. ulcerans</i> NCTC 12077	-	+	-/+	-	+	-/+
<i>C. striatum</i> NCTC 764	-	-	-/-	-	-	-/-
<i>C. minutissimum</i> ATCC 23346	-	-	-/-	-	-	-/-
<i>C. pseudodiphthericum</i> ATCC 10700	-	-	-/-	-	-	-/-
<i>K. pneumoniae</i> ATCC BAA-1144	-	-	-/-	-	+	-/+
<i>S. aureus</i> ATCC 12493	-	-	-/-	-	-	-/-
<i>S. pneumoniae</i> ATCC 10015	-	-	-/-	-	-	-/-
<i>C. diphtheriae</i> *	+	+	+/+	+	+	+/+
<i>C. diphtheriae</i> *	+	+	+/+	+	+	+/+
<i>C. diphtheriae</i> *	-	+	-/+	-	+	-/+
<i>C. diphtheriae</i> *	+	+	+/+	+	+	+/+
<i>C. diphtheriae</i> *	+	+	+/+	+	+	+/+
<i>C. diphtheriae</i> *	+	+	+/+	+	+	+/+
<i>C. diphtheriae</i> *	+	+	+/+	+	+	+/+
<i>C. diphtheriae</i> *	+	+	+/+	+	+	+/+
<i>C. diphtheriae</i> *	-	+	-/+	-	+	-/+
<i>C. diphtheriae</i> *	+	+	+/+	+	+	+/+
<i>C. diphtheriae</i> *	+	+	+/+	+	+	+/+
<i>C. diphtheriae</i> *	+	+	+/+	+	+	+/+
<i>C. diphtheriae</i> *	+	+	+/+	+	+	+/+
<i>C. diphtheriae</i> *	+	+	+/+	+	+	+/+
<i>C. pseudodiphthericum</i> *	-	-	-/-	-	-	-/-
<i>C. pseudodiphthericum</i> *	-	-	-/-	-	-	-/-
<i>C. imitans</i> *	-	-	-/-	-	-	-/-

* clinical isolate

** Elek test / *Cystinase* test**Table 2.** The stability of the modified medium based on the storage duration

Sample	Results (<i>Cystinase</i> test / Elek test)					
	1 day	2 days	4 days	8 days	16 days	32 days
Sample A	+/+	+/+	+/+	+/+	+/+	+/+
Sample B	-/+	-/+	-/+	-/+	-/+	-/+
Sample C	-/-	-/-	-/-	-/-	-/-	-/-

without the precipitation line was visible on sample 02 as well as negative control for Elek test (non-toxigenic *C. diphtheriae* NCTC 10356 (-)). Thus, the sample 02 was highly suspected as bacteria causing diphtheria (positive *Cystinase* test), but non-toxigenic (negative Elek test). Furthermore, there was no precipitation line (negative Elek test) nor halo around bacterial inoculation (negative *Cystinase* test) found on the negative control for *Cystinase* test (*C. striatum* (2-)), *C. striatum* is not the bacteria causing diphtheria that unable produce diphtheria toxin.^{31,32}

The accuracy of the simultaneous Elek and *Cystinase* tests on the modified medium compared with the separated standardized Elek and *Cystinase* tests on the Elek and Tinsdale mediums is described in Table 1.

The simultaneous Elek and *Cystinase* tests on the modified medium were concordant with standardized Elek test on the Elek Medium and standardized *Cystinase* test on the Tinsdale medium. *C. diphtheriae* NCTC 10648, *C. diphtheriae* NCTC 3984 and ten clinical isolates were interpreted as the toxigenic strain of bacteria causing diphtheria, while *C. diphtheriae* NCTC 10356, *C. ulcerans* NCTC 12077, and two clinical isolates were interpreted as the non-toxigenic strain of bacteria causing diphtheria based on these tests. The other samples were interpreted as a non-diphtheria causative agent, except for *K. pneumoniae* ATCC BAA-1144. In this study, *K. pneumoniae* ATCC BAA-1144 could produce atypical halo on the modified medium as well as Tinsdale medium 48 hours after incubation.

The modified medium stored in half-finished mode (without T-E supplement and Newborn Calf Serum). This method was done to accelerate medium production, considering theoretically, the Tinsdale medium will only be stable for four days of storage.²⁹ Our result shows the medium stability could be maintained for more than a month at 2-8 °C storage (Table 2).

DISCUSSION

The bacteria causing diphtheria (*C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*) are the primary targets to be detected in diphtheria diagnosis and surveillance.^{6,24,33,34} In this case, Elek test is a method used to examine bacterial toxigenicity globally. Bacterial toxigenicity was determined by the insertion of the *tox* gene which is carried by *phage* to bacterial chromosome.³⁵ Bacteria that are not inserted by this *Corynephage* cannot produce diphtheria toxin. Moreover, failure of gene expression might also happen if some deletion occurred in certain positions in

the *tox* gene sequence so that the toxin cannot be produced.³⁶ Diphtheria toxin is a major virulence factor of the bacteria causing diphtheria, but non-toxigenic strain should not be ignored.³⁷ Elek test principle is immunoprecipitation. The antigen (diphtheria toxin) produced by the bacteria will be bonded with the antibody (antitoxin), forming a precipitation line which can be seen with the naked eye.¹⁷ If the inoculated bacteria do not produce a toxin, the precipitation line will not appear (Fig. 1). The thickness of the medium, inoculation distance with the antitoxin disc, antitoxin level and source of Proteose peptone influence the speed and successfulness of the test.^{17,20,29}

Standardized *Cystinase* test is done on the Tinsdale medium.^{21,29} *Cystinase* test was positive if the brownish halo around the bacterial colonies appears (Fig. 2) which caused by the interaction between Kalium tellurite (K_2TeO_3) and Hydrogen sulfide (H_2S) produced by bacteria from L-Cystine and Sodium thiosulfate.²² *Cystinase* test is beneficial for identification of the bacteria causing diphtheria.²⁸ This test is very specific because of only diphtheria-causing bacteria are (*C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*) from the genus *Corynebacterium* that is positive for *Cystinase* test. However, *Cystinase* test has several limitations. The storage time after plating is short (around four days) to keep medium stability is one of them. Therefore, many clinical laboratories might not provide stock for routine examination.²⁶

Some materials of the Elek medium are similar with the Tinsdale medium composition, such as Proteose peptone, NaCl, agar, and serum.³⁸ In this study, we have developed a modified medium, which combines the Elek and Tinsdale mediums in a new one. This medium is suitable for Elek and *Cystinase* tests simultaneously (Fig. 3). Table 1 showed that result of *Cystinase* and Elek tests on the modified medium was similar to standardized tests on the Tinsdale and Elek Mediums. *Cystinase* test on both Tinsdale medium or modified medium exhibited, *K. pneumoniae* could produce atypical halo formation within 48 hours of incubation. Therefore, the identification of the bacteria causing diphtheria by *Cystinase* test needs a microscopic examination to differentiate genus *Corynebacterium* from the others. We highly recommend interpreting the *Cystinase* test in 24 hours. *K. pneumoniae* is not a member of the genus *Corynebacterium* and it is not included in bacteria causing diphtheria. It is a Gram-negative bacteria that rule on the antimicrobial resistant spreading worldwide.^{39,40}

The simultaneous Elek and *Cystinase* tests on the modified medium could reduce cost and time for laboratory examination. The modification was also

done to solve Tinsdale short age storage problem that only lasts for four days by making a half-finished medium. The condition of the half-finished modified medium in the temperature of 2-8°C was stable for more than one month (Table 2).

CONCLUSION

Therefore, the modified medium developed in this study is suitable for Elek and *Cystinase* tests simultaneously. Elek and *Cystinase* tests are essential to identify the bacteria causing diphtheria, but further tests are required for laboratory confirmation, including a complete biochemical analysis to determine the species and biotype.²⁹

ACKNOWLEDGMENT

This study funded by Hibah UI 2015. We are thanking The Head of Clinical Microbiology, Faculty of Medicine, Universitas Indonesia and the staffs, The Head of The Center for Biomedic and Basic Technology of Health, The Coordinator of Prof. dr. Sri Oemijati Infectious Disease Research Laboratory, Person in charge of the Bacteriology Laboratory and all of the staffs who helped and supported this study.

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