Retrospective Diagnosis of Diphtheria by Detection of the 
*Corynebacterium diphtheriae* tox Gene in a Formaldehyde-Fixed 
Throat Swab Using PCR and Sequencing Analysis

TAKAKO KOMIYA,1 NAOHIRO SHIBATA,1 MASAFUMI ITO,2 MOTOHIDE TAKAHASHI,1 
AND YOSHICHIKA ARAKAWA1*

Department of Bacterial and Blood Products, National Institute of Infectious Diseases, Tokyo,1 and Department of 
Pathology, Nagoya University Hospital, Nagoya,2 Japan

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The *tox* gene of *Corynebacterium diphtheriae* was detected in a formaldehyde-fixed throat swab taken from a 
68-year-old man who was reported to have died of suffocation due to a pharyngeal tumor. DNA templates 
prepared from bacterial cells fixed with 10% formaldehyde were subjected to a PCR analysis with *tox*-gene 
specific PCR primers. The resultant 112-nucleotide-long PCR product was sequenced using a dye terminator 
method, and an expected 57-nucleotide-long internal sequence of the *tox* gene was detected. This method is 
applicable for retrospective diagnosis in diphtheria cases in which only a formaldehyde-fixed clinical sample 
is available.

The number of diphtheria cases in Japan has fallen dramatically with the introduction of effective vaccines, such as diphtheria-pertussis-tetanus or diphtheria-tetanus. Over the past 10 years, there have been fewer than five diphtheria cases reported annually and only two deaths registered at the Ministry of Health and Welfare, Japan. Accordingly, the number of clinicians who have encountered cases of diphtheria has been decreasing, and it is becoming very difficult to make an accurate diagnosis of diphtheria in first-line medical settings. It is therefore assumed that many sporadic diphtheria cases have been overlooked or misdiagnosed, e.g., as sudden death of unknown cause. We herein report a practical method that enabled us to make a retrospective diagnosis of diphtheria by PCR and sequencing analysis using formaldehyde-fixed clinical specimens.

A throat swab specimen extracted from a 68-year-old man who was reported to have died of suffocation due to a pharyngeal tumor was suspended in a 10% formaldehyde solution and stocked for 53 days. A pathologist later suspected that this death might have been a case of diphtheria, because many gram-positive rods were visualized in the fixed throat tissue by Gram staining (data not shown). So he asked us to search for the presence of the *tox* gene of *Corynebacterium diphtheriae* in the clinical sample, although no vaccination history was known in this case.

Several cases in which the diphtheria *tox* gene was detected in cultured bacterial cells by PCR have been documented (2, 3); thus, we first used a conventional PCR method as described in these reports. However, no *tox* gene was detected in the formaldehyde-fixed sample. It was speculated that fixation of bacteria with a 10% formaldehyde solution for 53 days produced excessive alkylation of bacterial DNA, which might have blocked PCR amplification, as was reported for a previous attempt to detect virus genome DNA in formaldehyde-fixed tissue samples (1). Since there are no reports on the detection of the *tox* gene in formaldehyde-fixed clinical specimens, we designed two sets of original PCR primers by GENETYX 
MAC System version 5.0.0 (Software Development Company Ltd., Tokyo, Japan), referring to the total *tox* gene sequence reported previously (4), and a primer set, 5′-AAGTGACGTA TCCAGG-3′ and 5′-CCGACTTGCTCCAT-3′, gave better results for amplification of a 112-nucleotide-long fragment (Fig. 1a). A DNA template was prepared from the formaldehyde-fixed sample as follows. Approximately 10⁷ bacterial cells, sus-

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* Corresponding author. Mailing address: Department of Bacterial and Blood Products, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771, ext. 500. Fax: 81-42-561-7173. E-mail: yarakawa@nih.go.jp.

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FIG. 1. Primers and PCR mixture. The report of Ratti et al. (4) was referred to for the DNA sequence of the *C. diphtheriae tox* gene (a). The PCR primers used were designed using the GENETYX System (Software Development Co., Ltd.). Cyclic parameters for PCR are given in the text, and the composition of the PCR mixture (b) was determined by the protocol provided by Boehringer Mannheim Ltd.

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2400
The nucleotide sequence was determined using the dye terminator cycle sequencing FS ready reaction kit (Applied Biosystems, Foster City, Calif.) and the ABI Prism 377 XL DNA sequencing system (Applied Biosystems), with primer 1 used for PCR analysis. A 57-nucleotide-long internal sequence of the tox gene was amplified with the PCR primers detected (Fig. 3).

In this study, we succeeded in detecting a part of the C. diphtheriae tox gene, but it remained unclear whether the tox gene was indeed expressed in vivo. Actually, several clinical isolates were reported to produce too low a level of diphtheria toxin for detection, despite carrying the tox gene. But it was strongly suspected that this was a real diphtheria case because of clinical symptoms and pathological observations, as described above.

It is becoming more and more difficult to make accurate diagnoses of diphtheria, especially in those countries where the numbers of diphtheria cases have been dramatically reduced through successful national immunization programs conducted by the national health authorities. In Japan, only two diphtheria death cases, including the one presented herein, have been reported over the past 10 years, and only a few clinicians tend to consider diphtheria in differential diagnoses, even in cases of acute bacterial respiratory infections involving pharyngeal edema or pseudomembrane. We assume that not a few sporadic diphtheria cases could be overlooked in Japan annually. This speculation is supported by the finding obtained by the national surveillance on serum levels of antibody to diphtheria toxin that most Japanese, even those above 45 years old who have not been immunized with diphtheria-tetanus or diphtheria-pertussis-tetanus, have a high level of antibody to diphtheria toxin (5). This strongly suggests that localized transmission of C. diphtheriae and undetected or asymptomatic sporadic diphtheria cases still occur in Japan, causing the elevation of levels of antibody to diphtheria toxin as a booster effect. In cases of sporadic and asymptomatic diphtheria, isolation and identification of C. diphtheriae from clinical samples are very difficult, because only a few clinicians have experience in diagnosing diphtheria and because of the absence of selection media for C. diphtheriae in their clinical laboratories. It is therefore important to establish methods for making retro-

![FIG. 2. Result of PCR analysis of the C. diphtheriae tox gene, using DNA templates diluted with distilled water. Lanes: 1, formaldehyde-fixed clinical specimen diluted once; 2, formaldehyde-fixed clinical specimen diluted 3 times; 3, formaldehyde-fixed clinical specimen diluted 9 times; 4, formaldehyde-fixed clinical specimen diluted 27 times; 5, formaldehyde-fixed C. diphtheriae PW8 (a tox gene-positive strain) diluted once; 6, formaldehyde-fixed PW8 diluted 3 times; 7, formaldehyde-fixed PW8 diluted 9 times; 8, formaldehyde-fixed C. diphtheriae NT05 (a tox gene-negative strain) diluted once; 9, formaldehyde-fixed NT05 diluted 3 times; 10, formaldehyde-fixed NT05 diluted 9 times; 11, non-formaldehyde-fixed PW8 diluted once; 12, non-formaldehyde-fixed PW8 diluted 3 times; 13, non-formaldehyde-fixed PW8 diluted 9 times; 14, non-formaldehyde-fixed NT05 diluted once; 15, non-formaldehyde-fixed NT05 diluted 3 times; 16, non-formaldehyde-fixed TN05 diluted 9 times; M, DNA marker.](Image 312x101 to 550x170)
spective diagnoses of diphtheria using formaldehyde-fixed clinical samples, especially in cases of death from an unknown cause. Thus, the method described in this study would seem useful and practical in those cases in which only formaldehyde-fixed clinical specimens are available for diagnosis.

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REFERENCES