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

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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 10^7 bacterial cells, sus-

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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.
isolates were reported to produce too low a level of diphtheria gene was indeed expressed in vivo. Actually, several clinical diphtheriae tox gene, but it remained unclear whether the tox gene, as determined by Southern hybridization. The exact components of the PCR mixture used are shown in Fig. 1b. Cycling parameters for PCR are as follows: 95°C for 120 s, 95°C for 20 s, 55°C for 30 s, and 72°C for 60 s (30 cycles); 72°C for 600 s, and 4°C for stock. A 112-nucleotide-long PCR amplicon was obtained from the clinical specimen and from the tox gene-positive control strain, as shown in Fig. 2. Since an increased yield of amplicon was seen when the DNA template was diluted nine times, as is shown in Fig. 2, lane 7, further dilution of the DNA template prepared from a clinical specimen might give increased sensitivity for this test.

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 to be amplified with the PCR primers was 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 tox gene 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-
pective 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