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

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The \textit{tox} gene of \textit{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 \textit{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 \textit{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 \textit{tox} gene of \textit{Corynebacterium diphtheriae} in the clinical sample, although no vaccination history was known in this case.

Several cases in which the \textit{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 \textit{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 \textit{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 \textit{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-

FIG. 1. Primers and PCR mixture. The report of Ratti et al. (4) was referred for the DNA sequence of the \textit{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 tox gene was indeed expressed in vivo. Actually, several clinical specimens and from the 112-nucleotide-long PCR amplicon was obtained from the clinical specimen might give increased sensitivity for PCR analysis. A 57-nucleotide-long internal sequence of this test.

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 9 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.

In this study, we succeeded in detecting a part of the C. diphtheriae tox gene, using DNA templates diluted with distilled water. Lines: 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 9 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.

FIG. 3. Internal sequence of the tox gene, detected by sequencing analysis. Line A shows the internal sequence of the tox gene amplified by PCR using primers 1 and 2. Line B shows the nucleotide sequence of the PCR amplicon determined by the dye terminator method using primer 1. n, undefined nucleotide residue.
perspective 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


