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

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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.
In this study, we succeeded in detecting a part of the \textit{C. diphtheriae} tox gene, but it remained unclear whether the tox gene was indeed expressed in vivo. Actually, several clinical diphtheriae tox gene, but it remained unclear whether the 112-nucleotide-long PCR amplicon was obtained from the clinical specimen might give increased sensitivity for the diagnosis of diphtheria and because of the absence of selection criteria for making retrovirus toxin (5). This strongly suggests that localized transmission of \textit{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 \textit{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 \textit{C. diphtheriae} in their clinical laboratories. It is therefore important to establish methods for making retro-

![FIG. 2. Result of PCR analysis of the \textit{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 \textit{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 \textit{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.]

\begin{figure}
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\includegraphics[width=\textwidth]{figure2.png}
\caption{FIG. 2. Result of PCR analysis of the \textit{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 \textit{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 \textit{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.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{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.}
\end{figure}
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


