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Development of a Direct PCR Assay for Detection of the Diphtheria Toxin Gene

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PCR has proved to be a reliable tool for the detection of the diphtheria toxin gene, *tox*, and its use has allowed for the rapid differentiation between toxigenic and nontoxigenic strains. In this study, this PCR was further developed, evaluated, and standardized to detect this gene directly from clinical specimens. Optimal conditions for collection, transport, and storage of the clinical specimens and isolation and purification of DNA from the clinical specimens were defined. With two sets of primers that detect the A and B subunits of the diphtheria toxin gene, sensitivity levels of 50 and 500 CFU/PCR mixture, respectively, were achieved. This PCR was evaluated with 162 clinical samples collected from patients with diphtheria and other upper respiratory tract infections, as well as from healthy individuals.

The largest diphtheria epidemic in the developed world began in Russia in 1990. By 1995 it had spread to all 15 newly independent states of the former Soviet Union. At least 20 imported cases were also reported in neighboring central and western European countries and in two U.S. citizens (3). This is occurring at a time when, in most of the world, the incidence of diphtheria has declined as a result of widespread immunization with diphtheria toxoid, and toxigenic *Corynebacterium diphtheriae* strains are only rarely identified. However, recent studies suggest that more than 50% of the adult population in the developed world, including the United States, lacks protective levels of diphtheria toxin antibodies (8, 9, 16). Thus, diphtheria could potentially reemerge as a public health problem even in countries where it has been well controlled for decades, as has happened in the former Soviet Union. Because prognosis improves with early treatment with diphtheria antitoxin and the disease may not be recognized clinically in countries where the disease is not epidemic, rapid methods that can detect toxigenic *C. diphtheria* strains within hours of collection of the clinical specimen are needed.

PCR has proved to be a reliable tool for the detection of the diphtheria toxin gene, *tox*; its use has allowed for the rapid differentiation between toxigenic and nontoxigenic strains (7, 13, 14). Recently, we demonstrated an excellent correlation between culture-confirming PCR and the Elek immunodiffusion assay with 250 diphtheria isolates from diphtheria patients and carriers in Russia (10). In order to allow for the more rapid identification of toxigenic *C. diphtheriae*, we developed, evaluated, and standardized a PCR assay for the detection of the *tox* gene directly from clinical specimens.

MATERIALS AND METHODS

Bacterial strains and clinical samples. (i) Control strains. *C. diphtheriae* NCTC 10648 (*tox* positive) and NCTC 10356 (*tox* negative) were used as PCR-positive and -negative controls, respectively. The following organisms were used as negative controls: *Staphylococcus aureus* ATCC 12598; beta-hemolytic *Streptococcus* group A (CDC 2373-96), group B (CDC SS615), group C (CDC SS1344), group D (CDC SS498), and group G (CDC SS1175); *Neisseria meningitidis* serogroup A (CDC 318) and serogroup W135 (CDC 327); and the *Corynebacterium* strains *C. accolens* CDC 1455, *C. aquaticum* CDC 1443; *C. bovis* CDC 529, *C. jeikeium* CDC 1457, *C. kutscheri* CDC 1459, *C. minutissimum* CDC 536, *C. mycetoides* CDC 1460, 4 *C. pseudodiphtheriticum* CDC G2486, G2736, G2602, and F12487, *C. striatum* CDC 530, and *C. xerosis* NCTC 12078. All strains were maintained in sterile defibrinated sheep blood at −70°C until needed. Prior to use, the strains were streaked onto blood agar plates (tryptic soy agar II with 5% sheep blood; Becton Dickinson and Co., Paramus, N.J.) and were incubated overnight at 37°C. The same standard dilutions were used with all of these control strains.

(ii) Specimen collection and processing. Two different types of swabs were used: Falcon polyester-tipped swabs (PSs) with a wooden stick (362069; Becton Dickinson) and Puritan dacron polyester-tipped swabs with a plastic stick (PDSs; Hardwood Products, Guilford, Maine). The swabs were spiked with the control strains, as follows. A single colony of each control strain was suspended in 1 ml of sterile water, and this suspension was then diluted in a 10-fold series (from 10^{-1} to 10^{-4}). A standard plate count confirmed the final bacterial density (1). Different swabs were soaked with 50 and 25 μl of the suspension. Duplicate samples were prepared for each count. One set of swabs was kept at room temperature and the other was kept at 4°C until use.

The effects of storage temperature on the sensitivity of the direct PCR were evaluated by using PDSs and toxigenic *C. diphtheriae* NCTC 10648 (positive control). Four different bacterial dilutions were prepared (10^{-1} to 10^{-4}). Ten swabs were soaked in a solution of each dilution (50 μl per swab). All swabs were then placed in silica gel packages (Grace Davison, Baltimore, Md.). Five swabs soaked in each solution were kept at 4°C, and the remaining five swabs were kept at room temperature. Pairs of swabs soaked in identical bacterial solutions, but kept at different temperatures, were processed after 1, 4, and 7 days and after 2 and 3 weeks. The swabs were then placed into 1.5-ml Eppendorf tubes with 1 ml of sterile water and vortexed for 5 min. The organisms were collected by centrifuging the tube at 16,000 × *g* for 5 min.

A separate pilot study evaluated the effect of preincubation on the sensitivity of this direct PCR. The bacterial dilutions of the positive control strain were identical to those used in the storage evaluation assays; the only modification was that the swabs were briefly incubated in the Elek broth (6) for 4 h at 37°C prior to being stored in silica gel packages at 4°C for 1, 4, and 7 days and 2 weeks.

(iii) Isolation and purification of DNA from the swabs. The following six standard protocols and three modifications of standard protocols were used and the results were compared: boiling of the swab for 20 min (10), the method of Dawson et al. (4), the method of Schoolnik (18), the method of de Lamballerie et al. (5), use of the QIAamp Tissue Kit and the QIAamp Blood Kit (both from QIAGEN GmbH, Hilden, Germany), a combination of lysozyme treatment and boiling, a combination of lysozyme treatment and the method of Dawson et al. (4), and a combination of the use of a microwave oven (2) and the QIAamp Blood Kit.

The modified extraction of the DNA from the organisms done by using the QIAamp Blood Kit was performed according to the manufacturer's protocol, except that the incubation conditions were as follows. The pellet was suspended in 170 μl of Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), and 10 μl of 100 mg of lysozyme solution per ml was added to the suspension. After 30 min of incubation at 37°C, 25 μl of 17.9 mg of proteinase K solution per ml and 200 μl of Buffer AL (QIAGEN GmbH) were added, and the contents were

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mixed by vortexing and were incubated at 70°C for 2 h and at 95°C for 30 min. Finally, 200 μl of DNA solution was obtained after purification with a QIAamp spin column.

The same bacterial dilutions of the positive control described earlier, soaked in PDSs and kept at room temperature for 1 day, were used with all nine different isolation procedures.

(iv) Clinical specimens. Twenty-six paired nasopharyngeal (NP) and throat swabs from 13 patients with clinical diphtheria from Tbilisi, Republic of Georgia, were collected with PDSs on-site and were transported to the Centers for Disease Control and Prevention (CDC) in silica gel packages at room temperature.

One hundred twenty-three throat and three NP swab specimens, nine pieces of the membranes from the throat, and one neck tissue specimen were collected from patients with respiratory infections from 1 January 1995 to 28 February 1997 throughout the United States. These clinical samples were transported to CDC in silica gel packages or in various transport media (Amies and Stuart media) at 4°C.

PCR. Two sets of primers targeting the A and B subunits of the diphtheria toxin gene were used to detect *C. diphtheriae*: primers Tox 1 (ATCCACTTTT AGTGCGAGAACCTTCGTCA) and Tox 2 (GAAAACTTTTCTTCGTACCA CGGGACTAA) (248 bp; A subunit) and primers Dipht 6F (ATACTTCCTGG TATCGGTAGC) and Dipht 6R (CGAATCTTCAACAGTGTTCCA) (297 bp; B subunit) (10–12). These primers were synthesized on a DNA synthesizer (ABI model 380A The Perkin-Elmer Corp., Norwalk, Conn.). This PCR was optimized by using the PCR Opti-Prime Kit (Stratagene, La Jolla, Calif.), and the conditions were subsequently modified for each primer set. The PCR amplification was performed in the Cetus DNA Thermal Cycler 480 or GeneAmp PCR System 9600 (Perkin-Elmer), as follows. A total of 10 μl of DNA solution, 5 μl of Opti-Prime 10× Buffer #6 (100 mM Tris-HCl [pH 8.8], 15 mM $MgCl_2$, and 750 mM KCl; Stratagene), 1 μl of 12.5 μM (each) appropriate primer, 4 μl of deoxynucleoside triphosphate mixture (2.5 mM each), and 1.25 U of *Taq* DNA polymerase (Perkin-Elmer) were added to a 0.5-ml centrifuge tube or a 0.2-ml thin-wall tube. Sterile Milli-Q water was added up to 50 μl. For the Cetus DNA Thermal Cycler 480, this PCR mixture was overlaid with a drop of mineral oil. The mixture was initially denatured at 95°C for 2 min, followed by 35 amplification cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min (for Thermal Cycler 480) or 95°C for 20 s, 55°C for 30 s, and 72°C for 1 min (for PCR System 9600), ending with a final 10-min extension at 72°C. To verify the amplification, 15 μl of the amplified product was electrophoresed on a 1.2% SeaKem GTG agarose gel (FMC Corp., Philadelphia, Pa.) for 1 h at 150 V. The gels were stained with ethidium bromide, and the amplicons were visualized on a UV transilluminator.

RESULTS

Effects of processing methods, collection, transport, and storage conditions on the sensitivity of direct PCR. Initially, the sensitivity of direct PCR was tested by using nine protocols to extract and purify DNA directly from the throat and NP swabs spiked with a different dilution of the control strain. Primer set Tox 1 and Tox 2, which detects the A subunit of the diphtheria toxin gene, was used in this part of our study. Three methods did not yield any DNA that could be detected by this PCR: the method of Schoolnik (18), a combination of lysozyme treatment and boiling (10), and a combination of lysozyme treatment and the method of Dawson et al. (4). Boiling of the samples (10), the method of de Lamballerie et al. (5), and the method of Dawson et al. (4) allowed for the detection of 150,000, 62,300, and 15,000 CFU/sample, respectively. With the use of the QIAamp Tissue Kit, 7,500 CFU/sample could be detected. A comparison of the sensitivities of direct PCR following DNA isolation and purification by use of the QIAamp Blood Kit and the method of Dawson et al. (4) is presented in Fig. 1. The best results were obtained with the QIAamp Blood Kit and a combination of microwaving the sample and subsequently using the QIAamp Blood Kit column without the enzyme treatment; 1,200 and 1,400 CFU/sample, respectively, were detected by these methods. Subsequent modification of the manufacturer's protocol (see Materials and Methods) resulted in a 10-fold increase in the sensitivity of this direct PCR.

The storage of swabs at 4°C resulted in a more sensitive PCR than storage at room temperature. After 3 weeks of storage of the swabs in the silica gel packages at 4°C, 750 CFU/sample could have easily been detected, while barely visible amplicons were present after incubation at room temperature. A 10-fold increase in the sensitivity of this PCR was observed when the PDSs were used compared with that when the PSs were used (Fig. 2). Preincubation of the swabs in the Elek medium for 4 h at 37°C decreased the sensitivity of the PCR by a factor of 10.

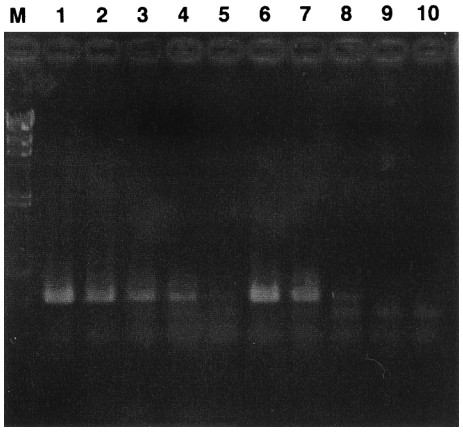

FIG. 1. Comparison of sensitivities of direct PCR following DNA isolation and purification by use of the QIAamp Blood Kit (lanes 1 to 5) and the method of Dawson et al. (lanes 6 to 10). Lane M, molecular size marker (bacteriophage λ *Hin*dIII digest); lanes 1 and 6, 1,500,000 CFU (×1 dilution); lanes 2 and 7, 150,000 CFU ($\times 10^{-1}$ dilution); lanes 3 and 8, 15,000 CFU ($\times 10^{-2}$ dilution); lanes 4 and 9, 1,500 CFU ($\times 10^{-3}$ dilution); and lanes 5 and 10, 150 CFU ($\times 10^{-4}$ dilution).

Optimization of the direct PCR. In addition to using *Taq* DNA polymerase (Perkin-Elmer), Expand High Fidelity PCR System (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and *TaqPlus* Long PCR System (Stratagene) were used in two separate experiments; in both instances the use of the *Taq* DNA polymerase resulted in the strongest signal. The Stratagene Opti-Prime PCR Optimization Kit indicated that the same PCR conditions for both primer sets were necessary. With these optimized PCR conditions, the levels of sensitivity of this direct PCR were 50 and 500 CFU/sample for detection of the A and B subunits of the diphtheria toxin gene, respectively. In addition, three different batches of this enzyme were tested, with no apparent differences in intensity of the amplicon's appearance. The subsequent PCR used to test the clinical specimens was then carried out accordingly.

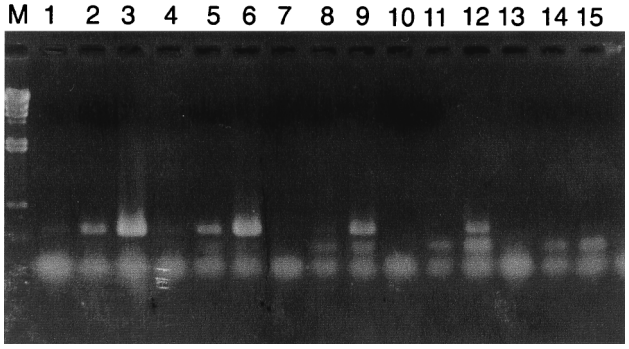

FIG. 2. Effects of swab type on the sensitivity of PCR for detection of diphtheria toxin gene. DNA was isolated and purified by the QIAamp Blood Kit from two kinds of swabs, PSs (lane 2, 5, 8, 11, and 14) and PDSs (lanes 3, 6, 9, 12, and 15), or directly from cell suspension (lanes 1, 4, 7, 10, and 13). Lane M, molecular size marker (bacteriophage λ *Hin*dIII digest); lanes 1 to 3, 150,000 CFU ($\times 10^{-1}$ dilution); lanes 4 to 6, 15,000 CFU ($\times 10^{-2}$ dilution); lanes 7 to 9, 1,500 CFU ($\times 10^{-3}$ dilution); lanes 10 to 12, 150 CFU ($\times 10^{-4}$ dilution); and lanes 13 to 15, 15 CFU ($\times 10^{-5}$ dilution).

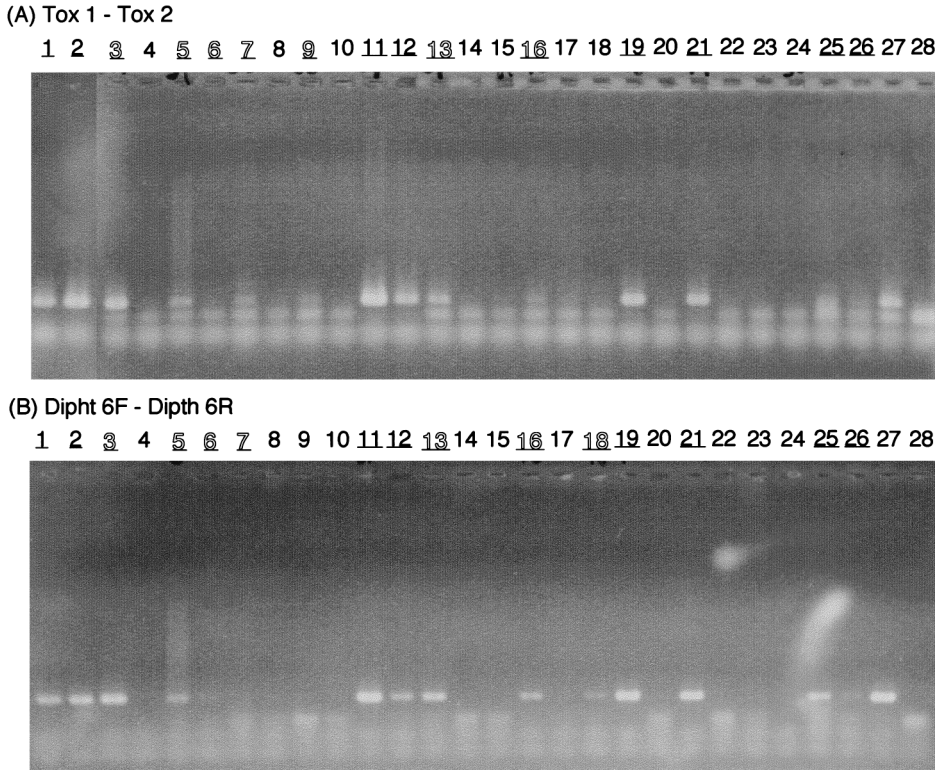

FIG. 3. Detection of diphtheria toxin gene directly from clinical samples (paired throat and NP swabs) from patients with clinical diphtheria in Tbilisi, Republic of Georgia. Two PCR primer sets were used for the detection of the A (A) and the B (B) subunits of the diphtheria toxin gene. Lanes 1 to 26, paired swab specimens from 13 patients; DNA was prepared from throat swabs (odd-numbered lanes) and NP swabs (even-numbered lanes); lane 27, *C. diphtheriae* NCTC 10648 (positive control); lane 28, *C. diphtheriae* NCTC 10356 (negative control). Underlined numbers indicate PCR-positive clinical samples from which *C. diphtheriae* was also isolated, and outlined numbers indicate PCR-positive, but culture-negative samples.

Specificity of the direct PCR. Numerous other species (pathogenic and part of the respiratory flora) were tested. The results of the PCR with both sets of primers were consistently negative; no nonspecific amplicons were detected.

Performance of PCR with clinical samples. Two different kinds of clinical samples were tested: 26 paired throat and NP swab specimens were collected in the Republic of Georgia from 13 patients with a clinical diagnosis of diphtheria. From 10 of those swabs (five patients) toxigenic *C. diphtheriae* was isolated. For 8 of the swabs direct PCR was positive with both primer sets; 2 NP swabs remained negative. However, for five additional patients (four throat swabs and two NP swabs) PCR with both primer sets was also positive. For two more patients (one throat swab and one NP swab) PCR was positive with only one primer set. Only for samples from the single remaining patient was PCR negative with both sets of primers (Fig. 3; Table 1).

An additional 136 samples (126 throat and NP swabs, 9 pieces of the throat membrane, and 1 neck tissue specimen) from the patients in the United States who sought medical treatment for their upper respiratory symptoms in the period from 1 January 1995 to 28 February 1997 were tested by using these two sets of primers. For one patient only, amplicons from both subunits of the diphtheria toxin gene were observed, but cultures for *C. diphtheriae* were negative. However, only one of the two subunits of the diphtheria toxin gene was detected in clinical samples from 5 of 13 contacts. The remaining 130 samples were all negative for both sets of primers. *C. diphtheriae* was not isolated from any of these clinical samples.

DISCUSSION

Since 1990, epidemic diphtheria has reemerged in the newly independent states of the former Soviet Union, and by 1995 the epidemic had spread to all 15 of these countries. The main causes for this epidemic are thought to be the waning of immunity, a lack of the booster vaccination in the adult population, and falling immunization coverage among children; as the epidemic developed in Russia, specific clones emerged (15). Even though diphtheria is now rare in the developed countries of Europe and North America, serologic studies in developed countries have demonstrated significant susceptibility to diphtheria, particularly in the adult population (8, 9, 17). This presents the potential for diphtheria to reemerge as a public health problem even in countries where it has been well controlled for decades. The current diphtheria epidemic and reports of at least 20 imported cases of diphtheria in Europe suggest that the populations of other countries remain at risk. Because prognosis improves with early treatment with diphtheria antitoxin and the disease may not be recognized clinically in the index patient of an outbreak in countries where the disease is not epidemic, rapid methods for identification of toxigenic *C. diphtheriae* strains are needed. In most cases, suspect organisms are assayed for toxin production with the use of the Elek immunodiffusion assay (6). Recently, an excellent correlation has been demonstrated between the Elek assay and the PCR that detects the A subunit of diphtheria toxin gene (10, 14). When compared with culture, this PCR has proven to be a reliable and reproducible tool for the detection of toxi-

TABLE 1. Comparison of results obtained by culture and direct PCR from the clinical specimens from diphtheria patients from Tbilisi, Republic of Georgia[a]

Patient no.	Throat swab result			NP swab result		
	Culture	*toxA*	*toxB*	Culture	*toxA*	*toxB*
1	+	+	+	+	+	+
2	−	+	+	−	−	−
3	−	+	+	−	+	+
4	−	+	+	−	−	−
5	−	+	−	−	−	−
6	+	+	+	+	+	+
7	−	+	+	−	−	−
8	−	−	−	−	+	+
9	−	−	−	−	−	+
10	+	+	+	+	−	−
11	+	+	+	+	−	−
12	−	−	−	−	−	−
13	+	+	+	−	+	+

[a] For culture, *C. diphtheriae* biotype gravis was isolated in all cases by a standard microbiologic method described earlier (6). *toxA* indicates direct PCR targeted to amplification of a 248-bp product from the A subunit of the diphtheria toxin gene. *toxB* indicates direct PCR targeted to amplification of a 297-bp product from the B subunit of the diphtheria toxin gene.

genic *C. diphtheriae* strains. However, it still takes several days to culture and isolate the organism before the PCR can be performed. Given the current global developments, there is a clear need for a more rapid assay that can detect toxigenic *C. diphtheriae* strains within hours of collection of the clinical specimen in order to allow for timely and prompt treatment of patients and contacts and implementation of adequate preventive measures. Therefore, in this study we optimized and standardized the PCR assay that detects both the A and the B subunits of the diphtheria toxin gene, so that it can be applied in a test directly with the clinical specimen. These PCR results are available within hours from the moment of collection of the clinical specimen.

Given that numerous factors can influence the outcome of a direct PCR, we attempted not only to optimize the PCR conditions but also to evaluate the most crucial factors, such as transport and storage of the clinical specimen and procedures to isolate and purify the DNA.

One of the most crucial factors in optimizing the direct PCR is overcoming the effect of numerous inhibitory factors that may be present in the clinical sample and the swab itself. Therefore, we began this work by evaluating methods that allow for the isolation and purification of DNA directly from the clinical samples. Since most such samples from patients with diphtheria would be in the form of a swab, either an NP or a throat swab, we concentrated on the isolation of DNA from the swabs. Initially, swabs with known numbers of CFU/sample were used and processed by nine different methods, which were tested earlier for the preparation of samples for PCRs that target other organisms and their specific genes. Significant differences were observed among these methods. The method of Schoolnik (18), which relies on the use of proteinase K and detergents (but which does not include the active removal of proteinase K), was not successful in recovering *C. diphtheriae* from our samples, suggesting that excess proteinase K may have acted as the PCR inhibitor and that a step that removes the proteinase K would be necessary. Similar observations held for the use of lysozyme treatment combined with boiling and the lysozyme treatment and the method of Dawson et al. (4), by which negative results could be attributed to the inhibitory effect of the excess lysozyme, which also needs to be removed before setting up the PCR. When boiling for 20 min was used, more than 150,000 CFU/PCR mixture was needed to visualize the amplicon. This result confirms observations in earlier reports that the main obstacle in this method is the resistance of the *C. diphtheriae* cell to the thermal shock (−196°C and 100°C) (2, 5) and that DNA could not be efficiently extracted from gram-positive bacteria (including *Corynebacterium*) and acid-fast bacteria by a simple thermal shock. The method of Dawson et al. (4), which relies on the use of detergents and glycogen, improved the sensitivity obtained by the boiling method 10-fold, but 15,000 CFU/sample was still required for detection. The method of de Lamballerie et al. (5), which uses chelating ion-exchange resin to protect DNA from heating and to help in disrupting external cellular structures at high temperature, was also unsuccessful in our application. This indicates that the low sensitivity of the direct PCR for *C. diphtheriae* may have been caused not by degradation of DNA during preparation but by other obstacles associated with the disruption of the cell wall. Finally, the QIAamp Tissue Kit was used for the isolation and preparation of DNA. This kit improved the sensitivity of our PCR, but it was still less effective than the QIAamp Blood Kit, which uses lysozyme. It appeared that the addition of the purification procedures in the QIAamp kits is just as crucial as efficient cell wall destruction for the sensitivity of the direct PCR. Bollet et al. (2) reported a simple method for the preparation of chromosomal DNA from gram-positive bacteria by using the microwave oven. To remove some inhibitory materials present in the clinical specimen, we combined this method with QIAamp column purification without any enzyme treatment, resulting in a level of sensitivity very close to that obtained with the QIAamp Blood Kit. Although this method provides rapid and high sensitivity for the DNA preparation, it may not be suitable for processing clinical samples. The cell wall destruction step achieved by microwaving can be inhibited by minimal remains of the supernatant. Since the clinical samples frequently contain materials such as pieces of cotton, removal of the supernatant is hampered, and therefore, the amount of liquid in the clinical sample varies. In addition, it is also adversely affected by the physical position of the sample in the microwave oven. Finally, the sensitivity of this method may be dependent on the microwave oven's size and power. In this study, modifications in the QIAamp Blood Kit manufacturer's protocol allowed for the best results. The total time required for the preparation of DNA from a clinical specimen was about 4 h. Because some inhibitory materials may remain even after the final column purification step, increasing the amount of DNA solution in the PCR mixture may cause decreased intensity or disappearance of the amplicons when more than 10 μl of DNA solution is used in the individual PCR. Repeating the purification step in the purification column was not helpful. However, some contaminants were successfully removed by the gel filtration column (Centri Sep; Princeton Separations, Inc. Adelphia, N.J.) (data not shown), intensifying the amplicons' appearance on the agarose gel, especially in cases when there was enough initial DNA. When the number of cells was near the limit of detection, the gel filtration step had no effect.

Once the DNA isolation and purification steps were standardized, we evaluated storage and transport conditions when different types of swabs were used. The best results were obtained with PDSs when they were stored in silica gel packages at 4°C. Apparently, preincubation of swabs in the broth (in our case, Elek medium without agar) was not encouraging but, rather, decreased the sensitivity of our PCR.

PCR conditions had to be optimized for both sets of primers. Better sensitivity levels were obtained with primers that detect

the A subunit of the diphtheria toxin gene. The Expand High Fidelity PCR System (Boehringer Mannheim) and *TaqPlus* Long PCR System (Stratagene) had no advantages in our direct PCR, although they are reported to be able to increase the yield of PCRs by reducing the mismatch pausing associated with *Taq* DNA polymerase.

These standardized conditions were subsequently used to test the clinical samples in our study. This PCR was more sensitive than the culture method when it was used to test specimens from 13 patients from Tbilisi, Republic of Georgia, with clinically diagnosed diphtheria. A total of 26 paired swab specimens were collected, and only 10 swabs from 5 patients yielded *C. diphtheriae*; such a low yield can presumably be attributed to the delay in the transportation of the collected swabs to CDC. However, this PCR detected both the A and the B subunits of the diphtheria toxin gene in 77% of the swabs, whereas the culture method detected the gene in 38% of the swabs. Among the 136 samples from U.S. patients that were assayed, PCR results with both sets of primers were positive for a single patient, but none of the patient's contacts were positive.

The PCR assay developed, optimized, and standardized for use with clinical specimens was shown in this study to be a reliable and reproducible tool for the rapid diagnosis of toxigenic *C. diphtheriae*. This method is now in routine use in the CDC Diphtheria Laboratory.

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