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Application of PCR for Detection of Toxigenic *Corynebacterium diphtheriae* Strains Isolated during the Russian Diphtheria Epidemic, 1990 through 1994

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A total of 250 *Corynebacterium diphtheriae* isolates from clinical cases and carriers in Russia were assayed by PCR directed at the A subunit of the diphtheria toxin gene to distinguish toxigenic from nontoxigenic strains; 170 strains were positive as indicated by the presence of the 248-bp amplicon. The results of this PCR assay were in complete concordance with those of the standard immunoprecipitation assay (Elek), and the PCR assay is a useful tool for rapid identification in clinical laboratories.

Following 3 decades of excellent control of the disease, epidemic diphtheria has again emerged in Russia; between 1990 and the end of 1994, over 80,000 cases were reported. By 1993 and 1994, epidemic diphtheria spread to all of the new independent states and made up the largest diphtheria outbreak in the developed world in recent years (1). At least 20 imported cases of diphtheria in adults have been reported in neighboring central and western European countries, and once again diphtheria is a cause for global concern (2, 10). The major virulence factor produced by *Corynebacterium diphtheriae* is diphtheria toxin, which consists of two major functional domains: the enzymatically active amino-terminal A domain and the binding carboxyl-terminal B domain. The current diphtheria epidemic emphasizes the need for development of rapid, simple, and reliable methods that distinguish toxigenic from nontoxigenic isolates. Such methods could assist clinicians in achieving the correct diagnosis, leading to prompt and specific treatment with diphtheria antitoxin. Two PCR assays capable of detecting sequences of the diphtheria toxin gene (*tox*) that code for the A and/or B subunits of diphtheria toxin were recently developed (6–9). In this study, we evaluated the applicability of the PCR assay, as developed by Pallen et al. (9), directed at the A subunit of the diphtheria toxin gene to distinguish toxigenic from nontoxigenic strains. In addition, results of this PCR were compared with those of the standard Elek assay for a large number of *C. diphtheriae* isolates from clinical cases and carriers.

Isolates. A total of 250 *C. diphtheriae* isolates were included in this study. Two hundred and twenty were from the collection of the Russian Federal Reference Laboratory for Diphtheria; of these, 194 were collected from six distinct geographic regions within Russia since 1991 (Fig. 1); 26 isolates were collected from 1985 to 1990 (the pre-epidemic period). These 220 isolates were recovered from 94 diphtheria patients, 81 carriers and 45 patients with nondiphtherial pharyngitis. A further 30 isolates were recovered in 1994 from throat swabs taken from 21 diphtheria patients and nine previously verified *C. diphtheriae* carriers at Clinical Infectious Hospital 1 (Moscow, Russia). All *C. diphtheriae* isolates were identified and biotyped by using the standard microbiological procedure for isolation and identification as described previously (3); 153 were of the *gravis* biotype, and 97 were of the *mitis* biotype.

Elek assay. All isolates were initially assayed by the Elek test, performed as described earlier (3) with purified antitoxin (Biomed, Moscow, Russia). *C. diphtheriae* G 4230 (CC UG 17398; Culture Collection of the University of Göteborg, Göteborg, Sweden), which is toxin positive, and G 4224 (CC UG 17024), which is toxin negative, were used as standard controls.

PCR. Subsequently, all isolates were assayed by PCR in a blind fashion. All throat swabs from patients at Clinical Infectious Hospital 1 were plated directly onto Hoyle's tellurite medium; after 18 to 20 h of incubation at 37°C, three to five suspect colonies were transferred into a 1.5-ml Eppendorf vial containing 0.5 ml of sterile distilled water. For all other *C. diphtheriae* isolates, a single colony from the pure culture grown on blood agar was transferred into a 1.5-ml Eppendorf vial containing 0.5 ml of sterile distilled water. Samples were boiled for 20 min and then centrifuged at 8,000 × *g* for 1 min. Three microliters of the supernatant was used in a 50-μl PCR mixture as described by Pallen et al. (9). The primers used for amplification corresponded to nucleotides 43 to 71 and 362 to 391 of the diphtheria toxin gene sequence, as determined by Greenfield et al. (4), and spanned a region of 248 nucleotides. DNA was initially denatured at 95°C for 5 min and then subjected to 35 amplification cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final 10-min extension at 72°C. An Ampli 2 thermocycler was used (Biocom, Moscow, Russia). Twenty microliters of the reaction mixture was elec-

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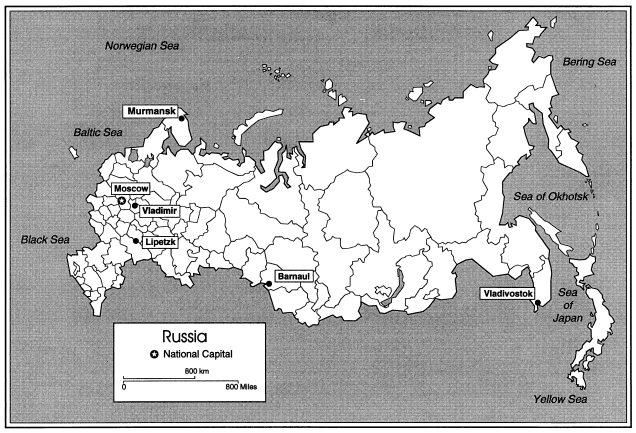

FIG. 1. Cities and regions within Russia where *C. diphtheriae* isolates were obtained.

trophoresed in a 0.8% agarose gel and run at 180 V for 3 h. The gels were stained with ethidium bromide, and the amplicons were visualized on a UV light transilluminator. In each PCR run, strains CCUG 17398 and CCUG 18645 were used as positive and negative controls, respectively. Forty-nine of the 250 isolates were randomly selected and PCR tested at the Centers for Diseases Control and Prevention under the same cycling conditions, with a Perkin-Elmer 480 thermocycler (The Perkin-Elmer Corp., Norwalk, Conn.).

Results and discussion. Of 220 *C. diphtheriae* isolates from the collection of the Russian Federal Reference Laboratory for Diphtheria, 140 isolates were positive when assayed by PCR, as indicated by the presence of the 248-bp amplicon (Fig. 2). The remaining 80 isolates were negative. All 30 *C. diphtheriae* isolates from throat swabs were positive when assayed by this PCR. A 100% correlation between the PCR assay and the Elek test was observed among all of the strains studied.

This report presents data on the largest and most geographically diverse collection of *C. diphtheriae* isolates comparatively assayed for toxigenicity by both PCR and the standard Elek test. Our data are in agreement with those of Pallen et al., who analyzed 87 clinical isolates of corynebacteria sent to the Diphtheria Reference Unit, Central Public Health Laboratory, London, England, in a blind survey (9). There was complete concordance between the results of the PCR and conventional tests for all but one Elek-positive isolate, which was PCR positive when collected from tellurite agar but negative when taken from blood agar. The same investigators also reported that only 1 of 33 nontoxigenic *C. diphtheriae* strains (as assayed by the Elek test) from the National Collection of Type Cultures was positive in the PCR assay (9). In another study, Hauser et al. used a PCR that amplified a 910-bp segment that contained partial sequences of the *tox* A and B subunit genes (6). Among the 32 *C. diphtheriae* strains studied, 24 were found to be PCR positive; culture supernatants of all 24 strains exhibited significant ADP-ribosylating activities. The remaining eight strains were negative in both PCR and ADP-ribosylation assays. Among 80 nontoxigenic strains (as initially determined by the Elek test), no false-positive PCR results were observed in our study. False-positive PCR results are generally a consequence of amplification of DNA sequences that are part of a mutated or partial, and therefore nonfunctional, *tox* gene. It has been previously shown that naturally occurring nontoxigenic corynebacteriophage may contain all or a portion of the *tox* gene cryptically and that nontoxigenic strains of *C. diphtheriae* may contain within their chromosome sequences homogeneous to the *tox* gene. However, such *tox*-bearing strains are incapable of achieving biological activity of the toxin, as demonstrated by animal inoculation. Additionally, variations within the B subunit of the toxin gene (unpublished data, Centers for Disease Control and Prevention) that leave the DNA sequence of the A subunit intact may be responsible for synthesis of the nonfunctional toxin. Data from our and other studies indicate that *tox*-bearing, nontoxigenic *C. diphtheriae* strains are rarely isolated from human clinical specimens (2a, 9). The only exception are the observations of Groman et al., who reported that 14 of 43 nontoxigenic U.S. isolates carried at least part of the *tox* gene when assayed with probes specific for the *tox* gene A and/or B subunits (5). However, Groman et al. analyzed a set of isolates that belonged to a common epidemiological cohort, i.e., from one particular location where a single nontoxigenic clone was prevalent, consistent with the hypothesis that such strains naturally occur less frequently than the raw data in the Groman study made it appear. A study focusing on the characterization of a panel of nontoxigenic strains that carry portions of the *tox* gene is currently under way.

Isolates analyzed in our study reflect significant temporal and geographical diversity within Russia. Complete correlation of the PCR assay results with the Elek test results suggests that *tox*-bearing but nontoxigenic *C. diphtheriae* isolates are not frequently encountered in Russia and that this PCR can be used as a rapid and reliable alternative to the Elek test. Important advantages of PCR over immune precipitation are rapidity, ease of performance, the large number of strains which can be simultaneously tested, and the fact that interpretation of the PCR assay is simple. Also, as shown here, PCR can be performed directly from the primary isolation media, without further subculturing and checking for the purity of the culture. This permits identification of toxigenic strains within 24 h. The use of PCR is becoming increasingly important in the

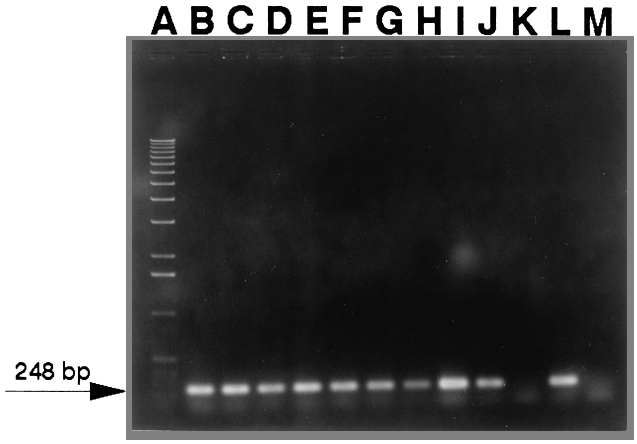

FIG. 2. PCR amplification of a 248-bp DNA product from the diphtheria toxin A subunit gene for detection of toxigenic *C. diphtheriae*. Lanes, including strain designation, clinical diagnosis, city of isolation, and year of isolation: A, molecular weight ladder; B, *C. diphtheriae* 302-94, carrier, Lipetsk, 1994; C, *C. diphtheriae* 613-93, diphtheria, Murom (Vladimir), 1993; D, *C. diphtheriae* 101-91, nondiphtheria pharyngitis, Murom (Vladimir), 1991; E, *C. diphtheriae* 91-88, diphtheria, Kovrov (Vladimir), 1988; F, *C. diphtheriae* 383-87, carrier, Selivanovo (Vladimir), 1987; G, *C. diphtheriae* 731-85, diphtheria, Vladimir, 1985; H, *C. diphtheriae* 911-86, carrier, Kovrov (Vladimir), 1986; I, *C. diphtheriae* 928-86, carrier, Vladimir, 1986; J, *C. diphtheriae* CCUG 17398 (positive control); K, *C. diphtheriae* CCUG 17024 (negative control); L, *C. diphtheriae* Parker-Williams 8, DNA phenol extract (positive control); M, water.

diagnosis of infectious diseases, and the PCR used in this study could be a simple and fast alternative to immunologic and other assays that are currently employed in microbiology laboratories.

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