JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1993, p. 2720–2723
0095-1137/93/102720-04$02.00/0

Vol. 31, No. 10

Polymerase Chain Reaction Assay for Diagnosis of Potentially Toxinogenic *Corynebacterium diphtheriae* Strains: Correlation with ADP-Ribosylation Activity Assay

DANIEL HAUSER,[1]* MICHEL R. POPOFF,[1] MARTINE KIREDJIAN,[2] PATRICE BOQUET,[1] AND FRANÇOIS BIMET[2]

Unité des Toxines Microbiennes,[1] and Laboratoire des Identifications Bactériennes,[2] Institut Pasteur, 28 rue du Docteur-Roux, F-75724 Paris Cedex 15, France

Received 19 May 1993/Returned for modification 28 June 1993/Accepted 26 July 1993

We have developed a polymerase chain reaction assay for the clinical diagnosis of potentially toxinogenic strains of *Corynebacterium diphtheriae*, the causative agent of diphtheria. A 910-bp amplification product, overlapping a DNA portion encoding both fragments of the diphtheria toxin, has been found in 28 among the 36 strains tested. In addition, effective toxin production, as evidenced by the ability of bacterial culture supernatants to ADP ribosylate eukaryotic elongation factor 2, was determined. In every case, the presence of an amplification product correlated with an ADP-ribosylation activity, thus confirming the diagnosis. The polymerase chain reaction assay herein described is very rapid (2 h) compared with the Elek immunodiffusion test or the guinea pig lethality test. It can provide a convenient and reliable method for laboratories involved in the identification of toxinogenic corynebacteria.

Pathogenic *Corynebacterium diphtheriae* strains secrete a potent protein toxin called diphtheria toxin (DT). The ability to produce DT has been observed only in *C. diphtheriae* and closely related species (1, 6).

DT, a 58-kDa polypeptide, kills eukaryotic cells by introducing into their cytosol its 21-kDa N-terminal fragment (fragment A). Fragment A is an enzyme which blocks protein synthesis by ADP-ribosylation of the eukaryotic elongation factor 2 (2, 5). Although vaccination has almost completely eradicated diphtheria from developed countries, *C. diphtheriae* is still sometimes found in clinical isolates.

Classical tests commonly used to demonstrate the pathogenicity of a *C. diphtheriae* strain are based on the detection of DT, either by a lethal effect upon injection of a culture filtrate into guinea pigs (12) or by direct immunoprecipitation on agar plates (the Elek test) (3). Both tests give a belated answer to the diagnosis (usually more than 48 h) and are time-consuming to set up.

A rapid screening of toxinogenic *C. diphtheriae* based on the polymerase chain reaction (PCR) has recently been described (10). However, we failed to obtain an amplification product with the reported primers.

In this report, we describe a PCR assay involving two other primers, which allows for a rapid and easy identification of bacteria harboring the DT gene. We also clearly establish a relationship between the presence of the DT gene and an ADP-ribosylation of eukaryotic elongation factor 2 activity in culture medium. Our method should provide a fast and accurate test to differentiate toxinogenic from nontoxinogenic *C. diphtheriae*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 36 *Corynebacterium* strains subdivided into three species have been studied: *C. diphtheriae* (32 strains), *Corynebacterium diphtheriae* subsp. *ulcerans* (3 strains), and *Corynebacterium pseudotuberculosis* (1 strain). Some of these strains were obtained from the Collection de l'Institut Pasteur; the others were clinical isolates from the Laboratoire des Identifications Bactériennes (Institut Pasteur). Bacteria were grown on Columbia agar slants (Sanofi Diagnostics Pasteur) for 24 h at 35°C and used for PCR.

PCR. Two 18-mer oligodeoxynucleotide primers, DT1 and DT2, were synthesized on an Applied Biosystems DNA synthesizer, PCR-Mate. DT1 (upper strand), 5′-CGGGGAT GGTGCTTCGCG-3′, and DT2 (lower strand), 5′-CGCGAT TGGAAGCGGGGT-3′, correspond, respectively, to nucleotides 456 to 473 and 1348 to 1365 of the DT gene sequence, nucleotide 1 being the G of the first GTG codon (formylmethionyl). One loopful of cells was harvested from a 24-h slant agar culture and resuspended in 100 μl of distilled water. This bacterial suspension was boiled for 10 min to destroy some nucleases and PCR inhibitors as described previously (10) and centrifuged for 1 min at 7,600 × *g* to pellet the nonsoluble cellular fragments. PCR was performed with 5 μl of supernatant in a final volume of 100 μl containing 10 mM Tris-HCl (pH 8.2), 50 mM KCl, 4 mM $MgCl_2$, 100 μM (each) the four deoxynucleoside triphosphates (Pharmacia LKB, Bromma, Sweden), 0.01% bovine serum albumin (Sigma, St. Louis, Mo.), 14 mM 2-mercaptoethanol (Sigma), 0.5 μM (each) primer DT1 and primer DT2, and 2.5 U of *Taq* DNA polymerase (lot no. 10713; Amersham, Les Ulis, France). The mixture was overlaid with 2 drops of paraffin liquid (Merck, Darmstadt, Germany), and the vial was placed into the PCR apparatus (Programmable Restriction Enzyme Module version 2003; Flobio SA, Courbevoie, France) and submitted to an initial period of 2 min at 94°C (long denaturation step; ramp, 2 K/s) followed by 30 cycles of 20 s at 94°C (denaturation), 30 s at 62°C (annealing), and 30 s at 72°C (elongation). Once the PCR amplification was completed, 10 μl of each reaction mixture was loaded on a 1% agarose gel and electrophoresed at 5 V/cm. As a molecular size marker, the 1-kb DNA ladder (Bethesda Research Laboratories, Gaithersburg, Md.) was used.

* Corresponding author. Electronic mail address: dhauser@pasteur.fr.

ADP-ribosylation assay. For DT production, bacteria were grown as described by Pappenheimer et al. (11). Each strain tested was cultured in 2 ml of deferrated Casamino Acids-yeast extract liquid medium (11) for 2 days at 35°C under agitation. Then, 1.5 ml of the culture was centrifuged for 2 min at 7,600 × *g* and 1 ml of supernatant was dialyzed against distilled water for 18 h at 4°C in a microdialysis system (Bethesda Research Laboratories) with a 6,000-to-8,000 cutoff membrane (Bethesda Research Laboratories). One hundred microliters of each dialysate was used in the ADP-ribosylation reaction. An ADP-ribosylation mixture containing 90 μl of mouse liver extract (as a source of eukaryotic elongation factor 2), 10 μl of 100 mM dithiothreitol, and 0.1 μl of [adenylate-^{32}P]NAD (30 mCi/mmol, NEN Research Products, Du Pont de Nemours, Boston, Mass.) was prepared. To 100 μl of each dialyzed bacterial culture supernatant, 100 μl of the ADP-ribosylation mixture was added, and addition was followed by incubation at 37°C for 1 h. At the end of the incubation, samples were precipitated by addition of 200 μl of 20% trichloroacetic acid and then filtered on Whatman GF/C glass filters (Whatman, Maidstone, England) with a Millipore filtering apparatus (Millipore Corp., Bedford, Mass.). Filters were dried and counted in a scintillation counter. The ADP-ribosylating activity of each strain was expressed relative to that detected for the toxinogenic strain CIP A102 (Park-Williams no. 8, strain no. 1 [Table 1]) and was calculated by the formula $(cpm_{strain} - cpm_{no.\ 2}) \times (cpm_{no.\ 1} - cpm_{no.\ 2})^{-1} \times 100\%$, where $cpm_{no.\ 2}$ refers to the activity of the nontoxinogenic *C. diphtheriae* type strain, ATCC 27010/NCTC 11397/CIP 100721.

RESULTS

Specificity of PCR. Figure 1 shows the electropherogram obtained for 8 *Corynebacterium* strains out of the 36 tested plus 1 *C. diphtheriae* strain from a clinical isolate, not tested for its ADP-ribosylating activity. The primers selected for the detection of the DT gene led to the amplification of the expected 910-bp fragment. In bacterial strains in which the 910-bp amplimer was absent, no amplification product was found (Fig. 1). To confirm the specificity of the PCR test, the amplified product from strain A102 (10 μl) was hydrolyzed by *Mbo*I for 15 min directly in the PCR buffer and produced the two predicted restriction fragments of 710 and 200 bp (Fig. 2).

Four strains, instead of producing a 910-bp band, gave a very weak signal around 780 bp (data not shown). In order to see whether or not this weak amplified DNA fragment was related to the DT gene, this unexpected 780-bp band was cut out from the gel, eluted, reamplified with DT1 and DT2 (to enhance its quantity for further cloning), and cloned as a blunt-ended fragment into pUC19 previously hydrolyzed by *Sma*I. The subsequent nucleotide sequence of both extremities of this 780-mer, performed with the forward and reverse primers of pUC19, corresponded to DT1, showing that only one primer (DT1) served for the amplification of that product (data not shown). At both ends, the nucleotide sequence immediately downstream of DT1 was completely different from that of the DT gene and unrelated to any known sequence (data not shown).

Correlation between the presence of the PCR product and the ADP-ribosyltransferase activity. The identification number, source and origin, the results of the PCR test, and the ADP-ribosylating activity of each of the 36 strains studied are presented in Table 1. The strains which gave a positive PCR result but very low ADP-ribosylating activity in a 2-day culture supernatant were then allowed to grow for four additional days. Under these conditions, they all showed significant ADP-ribosylating activities. In contrast, a 6-day culture of strain no. 35 was still negative (less than 5% activity).

When first tested, three strains, known to be toxinogenic by the guinea pig lethality test or by the Elek test, did not reveal significant ADP-ribosylation activity. As a matter of fact, strains no. 3, 12, and 13, first deposited at the Collection de l'Institut Pasteur in 1952 as toxinogenic strains, gave no band either by the PCR method or by ADP-ribosylated elongation factor 2 (Fig. 1, lanes 7 and 8). The lyophilized stock cultures used in our study were prepared in 1974. When performed again on culture supernatants of these strains derived from original sealed vials of 1952, both the PCR and the ADP-ribosylation assays gave positive results

TABLE 1. Summary of the tests performed on the 36 *Corynebacterium* strains

Strain no.	Identification no.	Source[a]	Origin	Result of toxinogenicity test:	
				PCR[b]	ADP-ribosylating activity (%)
1[c]	A102	CIP	Unknown	+	100
2[d]	100721	CIP	Unknown	−	0.0
3	52.112	CIP	Unknown	+	46.8
4	52.113	CIP	Unknown	+	58.7
5	60.82	CIP	Throat	+	24.5
6	66.3	CIP	Throat	+	40.2
7	A97	CIP	Unknown	+	123.5
8	A98	CIP	Unknown	+	81.0
9	A99	CIP	Unknown	+	93.0
10	A100	CIP	Unknown	+	270.0
11	A101	CIP	Unknown	+	67.1
12	52.110	CIP	Unknown	+	68.0
13	52.111	CIP	Unknown	+	69.2
14	80.04	CIP	Throat	+	61.9
15	80.05	CIP	Unknown	+	64.8
16	100163	CIP	Throat	+	77.4
17	101317	CIP	Throat	+	129.9
18	101434	CIP	Pharynx	+	255.6
19	63.77	CIP	Unknown	+	117.7
20	477.78	CIP	Unknown	+	77.1
21	713.79	CIP	Throat	+	71.6
22[e]	54.53	CIP	Tonsillitis	+	48.0
23[e]	54.67	CIP	Tonsillitis	+	50.4
24	69.28	CIP	Unknown	+	71.1
25[e]	54.59	CIP	Lung	+	83.6
26	53.80	CIP	Throat	+	73.8
27	440.75	CIP	Leg ulcer	+	50.3
28	419.76	CIP	Throat	+	84.1
29[f]	52.103	CIP	Equine ulcerative lymphangitis	−	0.2
30	684 91	LIB	Knee	−	4.8
31	805 91	LIB	Vagina	−	2.6
32	862 91	LIB	Blood	−	4.5
33	875 91	LIB	Blood	−	2.6
34	247 92	LIB	Blood	−	0.2
35	258 92	LIB	Expectoration	−	3.9
36	308 92	LIB	Bone	−	1.5

[a] CIP, Collection de l'Institut Pasteur; LIB, Laboratoire des Identifications Bactériennes.
[b] +, positive; −, negative.
[c] Toxinogenic reference strain CIP A102 (Park-Williams no. 8).
[d] *C. diphtheriae* type strain, ATCC 27010/NCTC 11397/CIP 100721.
[e] *C. diphtheriae* subsp. *ulcerans*.
[f] *C. pseudotuberculosis*.

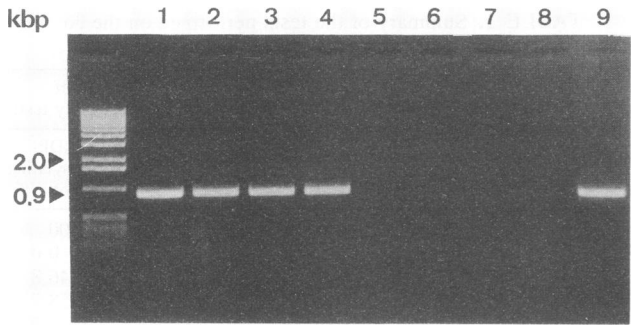

FIG. 1. Electropherogram of the PCR products from toxinogenic and nontoxinogenic *Corynebacterium* strains. Lane 1, strain no. 25; lane 2, strain no. 26; lane 3, strain no. 27; lane 4, strain no. 28; lane 5, strain no. 29; lane 6, strain no. 62 (isolated from the blood of a patient, not reported in this study); lane 7, strain no. 12 (lyophilized stock culture of 1974); lane 8, strain no. 13 (lyophilized stock culture of 1974); lane 9, strain no. 1 (Park-Williams no. 8); far left lane, molecular size standards.

(Table 1). Consequently, among the 36 strains studied, all (n = 11) that had previously been found lethal for the guinea pig were also positive by the PCR and ADP-ribosylation assays.

DISCUSSION

The results presented in Table 1 show that 27 strains were found positive by the PCR assay. Culture supernatants of these strains exhibited significant ADP-ribosylating activities. Some of these strains (n = 11) were also positive by the guinea pig lethality test. There is thus a relatively good agreement among the results of the PCR assay, the ADP-ribosylation test, and the guinea pig lethality test.

The PCR test for the detection of toxinogenic *Corynebacterium* strains proposed in the present study was performed on strains already identified by biochemical and morphological means as *C. diphtheriae*, but also as *C. pseudotuberculosis* or *C. diphtheriae* subsp. *ulcerans*, sometimes toxinogenic as well (8). As a consequence, our test is intended for pure bacterial cultures. Moreover, it is known that a given *C. diphtheriae* strain may be a mixture of both toxinogenic and nontoxinogenic bacteria (13). This potential bias is overcome either by using mass cultures or by harvesting several colonies with a loopful as a source of DNA for the PCR assay. This might be of importance in routine applications.

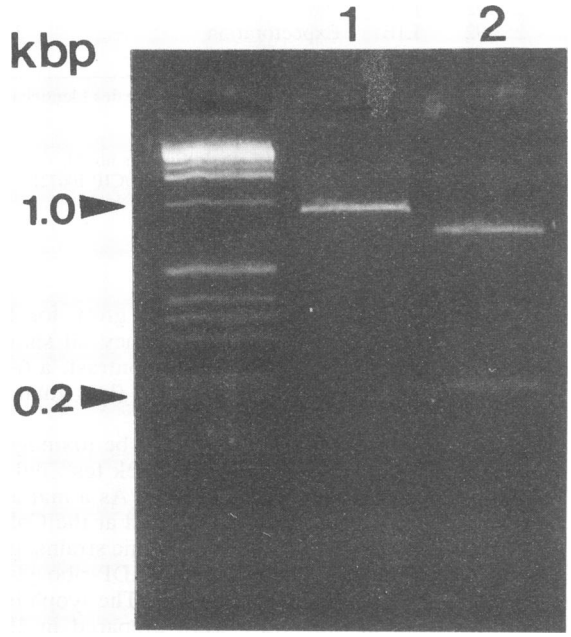

FIG. 2. Electropherogram of the PCR product of strain no. 1 (Park-Williams no. 8). Lane 1, uncut; lane 2, hydrolyzed by *Mbo*I; far left lane, molecular size standards.

Our ADP-ribosylation assay only detects the activity of fragment A in vitro, whereas the animal toxicity supposes a complete and unaltered DT (fragment A and fragment B). This is one of the reasons why we have chosen to amplify a DNA fragment overlapping part of the sequence for both fragments A and B of the DT. Although we have found that PCR and ADP-ribosylating activity are correlated, it must not be concluded that PCR-positive strains are toxinogenic, since no test has been performed with regard to an in vivo toxicity, but they remain potentially toxinogenic (7). In any case, it is highly recommended in each PCR test to include positive and negative controls. A known toxinogenic *Corynebacterium* isolate (positive control, e.g., Park-Williams no. 8) would serve both as a size marker and as a yield marker. In our work, the yields of amplified DNA were equivalent in all the PCR-positive strains tested, confirming the reliability of the PCR assay. Moreover, there is no known genetic variation of the DT gene (9).

The PCR is essentially a specific method. In the absence of repeated DNA sequences or particular sequence features, there is no reason for the appearance of nonspecific bands of any size. The nonspecific very weak amplification product of 780 bp that was obtained for four *C. diphtheriae* strains and that has been cloned and sequenced does not depart from this rule since it clearly results from a mismatch of DT1 on the total bacterial DNA (it is assumed that no contamination by exogenous DNA occurred). It is not known where and with which matching percentage a DNA sequence close to DT1 is present in the total DNA of the strains considered.

The PCR test described in this report is now routinely used at the Laboratoire des Identifications Bactériennes. It must be kept in mind that a nontoxinogenic strain potentially remains sensitive to toxinogenic phage infection (4). An epidemiological survey to identify *C. diphtheriae* in isolates and a systematic search of toxinogenic strains among these isolates remain to be carried out.

ACKNOWLEDGMENTS

We are indebted to M.-L. Quilici and C. Bizet for providing us with Collection de l'Institut Pasteur strains.

This work was supported by the Institut Pasteur. D.H. was supported by a doctoral fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche.

REFERENCES

1. **Buck, G. A., R. E. Cross, T. P. Wong, J. Loera, and N. Groman.** 1985. DNA relationships among some *tox*-bearing corynebacteriophages. Infect. Immun. **49**:679–684.
2. **Drazin, R., J. Kandel, and R. J. Collier.** 1971. Structure and activity of diphtheria toxin. Attack by trypsin at a specific site within the intact toxin molecule. J. Biol. Chem. **246**:1504–1510.
3. **Elek, S. D.** 1948. The recognition of toxicogenic bacterial strains *in vitro*. Br. Med. J. **1**:493–496.
4. **Freeman, V. J.** 1951. Studies on the virulence of bacteriophage-infected strains of *Corynebacterium diphtheriae*. J. Bacteriol. **61**:675–688.
5. **Gill, D. M., and A. M. Pappenheimer, Jr.** 1971. Structure-

activity relationships in diphtheria toxin. J. Biol. Chem. **246:** 1492–1495.
6. **Gill, D. M., T. Uchida, and R. A. Singer.** 1972. Expression of diphtheria toxin genes carried by integrated and nonintegrated phage beta. Virology **50:**664–668.
7. **Groman, N., N. Cianciotto, M. Bjorn, and M. Rabin.** 1983. Detection and expression of DNA homologous to the *tox* gene in nontoxinogenic isolates of *Corynebacterium diphtheriae*. Infect. Immun. **42:**48–56.
8. **Groman, N., J. Schiller, and J. Russel.** 1984. *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* responses to DNA probes derived from corynephage β and *Corynebacterium diphtheriae*. Infect. Immun. **45:**511–517.
9. **Murphy, J. R.** 1985. The diphtheria toxin structural gene. Curr. Top. Microbiol. Immunol. **118:**235–251.
10. **Pallen, M. J.** 1991. Rapid screening for toxigenic *Corynebacterium diphtheriae* by the polymerase chain reaction. J. Clin. Pathol. **44:**1025–1026.
11. **Pappenheimer, A. M., Jr., T. Uchida, and A. A. Harper.** 1972. An immunological study of the diphtheria toxin molecule. Immunochemistry **9:**891–906.
12. **Roux, E., and A. Yersin.** 1888. Contribution à l'étude de la diphthérie. Ann. Inst. Pasteur **2:**629–661.
13. **Simmons, L. E., J. D. Abbott, M. E. Macaulay, A. E. Jones, A. G. Ironside, B. K. Mandal, T. N. Stanbridge, and P. Maximescu.** 1980. Diphtheria carriers in Manchester: simultaneous infection with toxigenic and non-toxigenic *mitis* strains. Lancet **i:**304–305.