Reversed Passive Latex Agglutination Assay for Detection of Toxigenic Corynebacterium diphtheriae

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A reversed passive latex agglutination (RPLA) assay for determining the toxigenicity of Corynebacterium diphtheriae is presented. Rabbit antitoxin antiserum was raised by using commercially available diphtheria toxin. This antiserum reacted with the diphtheria toxin when the culture supernatant was assayed by Western blotting, and it did not cross-react with other extracellular antigens. Affinity-purified antibodies for latex sensitization were obtained by using a Hi Trap N-hydroxysuccinimide-activated column. Demonstration of toxin in five of seven clinical isolates was in accordance with the PCR assay and the Vero cell cytotoxicity test. Culture of the bacteria for 6 h was sufficient for toxin production, and an additional 6 h was needed to observe latex agglutination. Therefore, diphtheria toxin can be detected in 12 h by this method. The lowest concentration of diphtheria toxin detectable by the RPLA assay was about 5 ng/ml. The RPLA assay can provide a convenient and reliable method for laboratories involved in the identification of toxigenic corynebacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Seven C. diphtheriae strains isolated in 1996 from patients in the Lao PDR were used. Strain Park-Williams no. 8 (PW-8) was used as a positive control. Bacteria were grown on a blood agar plate. For the toxin production assays, strains were grown in 10 ml of Elek broth medium (2% Proteose Peptone no. 3 [Difco], 0.3% maltose, 0.5% NaCl, 0.07% lactic acid, 10% calf serum, pH 7.8) in a 100-ml Erlenmeyer flask and incubated at 34°C with shaking for 17 h. Aliquots were taken at 4, 6, and 8 h for studying the kinetics of toxin production.

Antiserum preparation. A rabbit was immunized with diphtheria toxoid (Td: Wako Pure Chemical Co., Osaka, Japan) every 2 weeks. One milliliter of Td (100 μg/ml) emulsified with an equal volume of Freund’s incomplete adjuvant was injected subcutaneously into multiple sites. For the booster injection, Td (25 μg/ml) and Freund’s incomplete adjuvant were used.

Preparation of a toxoid coupled affinity gel column. The Td solution was dialyzed against coupling buffer (0.2 M NaHCO3, 0.5 M NaCl, pH 8.3), and 1 ml containing 10 mg of Td was applied to a 1-ml N-hydroxysuccinimide-activated Hi Trap Affinity Column (Pharmacia Biotech). The column was then washed with buffer A (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3) and then with buffer B (0.1 M sodium acetate, 0.5 M NaCl, pH 4.0). The column was equilibrated with storage buffer (0.05 M Na2HPO4, 0.1% NaN3, pH 7.0) and kept in a cold room until use.

Preparation of affinity-purified antibody. Antitoxin antiserum was heated at 56°C for 30 min. The 20 to 33% ammonium sulfate-insoluble material was suspended in 10 mM phosphate-buffered saline (PBS), pH 7.4, and dialyzed against the same buffer. One milliliter of this crude immunoglobulin fraction was applied to the Td-coupled Hi Trap Affinity Column equilibrated with PBS. The column was washed with PBS until the optical density at 280 nm returned to the baseline. Antitoxin-specific antibodies were eluted with elution buffer (0.2 M glycine, 0.5 M NaCl, pH 2.7). The eluted sample was neutralized with 0.1 M NaOH.

Preparation of sensitized latex. Procedures for latex sensitization have been described previously (11). SLDS95 latex (Takeda Pharmaceutical Co., Osaka, Japan) prepared in a 0.1% suspension was mixed with an equal volume of 5% glutaraldehyde and incubated overnight at 4°C with shaking. The latex was washed and suspended in PBS to a final concentration of 0.5%. Different concentrations (5, 10, 20, and 30 μg/ml) of the affinity-purified antibodies in 3% sucrose PBS were mixed with 0.1% latex. The mixture was incubated at 4°C for 1 h with shaking. The sensitized latex was then washed with PBS containing 0.8% bovine serum albumin (BSA) and 0.1% sodium azide. Finally, the latex was suspended in adjusting buffer (PBS containing 0.8% BSA, 0.1% sodium azide, and 0.005% polyvinyl alcohol) at a concentration of 0.5% (stock suspension). Before use, the stock latex suspension was diluted 20 times with diluent buffer (PBS containing 0.8% BSA, 0.01% polyvinylpyrrolidone, 0.1% sodium azide).

Detection of DT by RPLA. The culture supernatants were serially twofold diluted with diluent buffer in a U-bottom microtitration plate. An equal volume (25 μl) of 0.025% sensitized latex was added to each well. Microdilution plates were shaken thoroughly and then allowed to stand at room temperature. Agglutination was judged visually after 6 h. The titer was defined as the reciprocal of the highest dilution in which agglutination was observed. Td at 200 ng/ml was used as the standard to determine the minimum detectable amount.

Pathogenic Corynebacterium diphtheriae strains secrete a potent toxin called diphtheria toxin (DT), which consists of two major functional domains: the enzymatically active amino-terminal A domain and the binding carboxyl-terminal B domain (7). Clinical diagnosis, particularly in countries where the disease is uncommon, is not made easily, and diphtheria may be confused with other infections such as tonsillitis and streptococcal sore throat. This highlights the important role of the diagnostic laboratory in providing rapid microbiological confirmation for prompt and specific treatment with diphtheria antitoxin.

Several methods have been described for the detection of DT, including the Elek immunoprecipitation test (5, 6), tissue culture using HeLa cells (16), biological activities in animals (2), sandwich enzyme-linked immunosorbent assay (19, 21), and PCR (1, 8, 17, 18, 20). All of these methods have the disadvantage of being technically demanding or expensive to carry out and therefore may not be applicable in developing countries, where resources are minimal.

A diphtheria outbreak occurred in 1996 in the Lao People’s Democratic Republic (PDR), and C. diphtheriae was isolated from the patients. However, DT production in the isolates was difficult to examine because of the laboratory capacity. The recommended in vitro Elek test is difficult to optimize because different batches of peptone and sera may vary considerably in suitability for the test (3, 4). Even if the technique is standardized, the test is technically demanding and needs to be rigidly quality controlled. Furthermore, a microbiological quality assessment trial for examining toxin production by in vitro methods showed that only 69% of the laboratories achieved the correct result (23). The large amount of effort needed to ensure satisfactory results with the Elek test suggests the necessity of a simple and reliable in vitro method for DT detection. We therefore developed a reversed passive latex agglutination (RPLA) assay which can demonstrate the production of toxin from C. diphtheriae.

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Electrophoresis and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were carried out by the methods of Laemmli (15) and Towbin et al. (24), respectively. The 40 to 70% ammonium sulfate-insoluble material of the culture supernatant (10 ml) was suspended in 0.5 ml of 0.02 M Tris-HCl buffer (pH 7.6) and dialyzed against the same buffer. This concentrated supernatant was used for DT detection by Western blotting. A prestained molecular size marker (New England Biolabs, Beverly, Mass.) was used as the standard.

PCR conditions. The primers described by Pallen et al. (20), directed at subunit A of the DT gene and spanning a region of 248 nucleotides, were used. An inoculum of the pure culture grown on blood agar was resuspended in 0.5 ml of distilled water. Samples were boiled for 20 min and then centrifuged. Five microliters of the supernatant was used in a 50-μl PCR mixture as described by Pallen et al. (20). Amplification conditions were as described by Mikhailovich et al. (17). The PCR product was electrophoresed in a 1.3% agarose gel with a 100-bp DNA ladder as a standard molecular mass marker (Gibco Laboratories, Grand Island, N.Y.).

Vero cell assay. Vero cells were grown in minimal essential medium (Gibco Laboratories) supplemented with 10% fetal calf serum. The cells were grown as monolayers in 96-well microtiter tissue culture plates. A twenty-microliter filtered test sample was added to each well and incubated under 5% CO₂ in air at 37°C. The cells were observed by microscopy for cytopathic effects for 2 days. For the neutralization test, a preincubated mixture of culture supernatant and antitoxin antiserum was used as a sample (13).

## RESULTS

Sensitization of latex. Various concentrations of affinity-purified antitoxin antibodies were mixed with a constant concentration of latex to find the optimal concentration to sensitize the latex. The detection limit decreased with increasing antibody concentrations, but high antibody concentrations tended to cause autoagglutination. Therefore, latex was sensitized with antibodies at a concentration of 5 μg/ml. The lowest Td concentration detectable was about 5 ng/ml.

RPLA. Preliminary studies of toxin detection were done with overnight culture supernatants. Five of the seven strains isolated in the Lao PDR were positive by the RPLA assay (Table 1), with titers ranging from 4,096 to 16,384. Because isolation of toxigenic *C. diphtheriae* must be reported at an early stage, we investigated whether a shorter culture incubation time can produce detectable levels of DT. Although 4 h of incubation was not enough for DT production in slow toxin producer strains, 6 h of incubation was enough to obtain a clearly positive result (Table 1). The approximate cell density of the broth cultures at 6 h was 3 × 10⁸ CFU/ml (optical density at 600 nm, 1.2).

Western blotting. Western blotting with antitoxin antiserum was performed to determine if cross-reactive proteins were present in the culture supernatant of strain PW-8. A 20-fold concentrated culture supernatant was mixed with sample buffer without 2-mercaptoethanol and subjected to sodium dodecyl sulfate–9% polyacrylamide gel electrophoresis. A single polypeptide of about 60 kDa reacted with the antiserum. No other reactive protein was observed (Fig. 1).

## DISCUSSION

Difficulties in detecting DT production by *C. diphtheriae* isolates from the Lao PDR encouraged us to develop an RPLA assay. A Td-coupled affinity column was used to purify the antibodies for latex sensitization. We expected a low coupling efficiency, because the Hi Trap N-hydroxysuccinimide-activated column is designed for covalent coupling of ligand-con
taining primary amino groups, and toxoid formation is supposed to block these groups. However, our result suggests that there are still enough primary amino groups that are able to react.

Five of the seven strains studied were positive by the RPLA assay (Table 1). There was agreement among the RPLA, PCR (Table 1 and Fig. 2), and Vero cell cytotoxicity assay results. The changes in cell morphology observed in the DT-affected cells were similar to those reported by Konowalchuk et al. for vero cytotoxin-affected cells (14). Both toxins are protein synthesis inhibitors and induce cell lysis in a manner characteristic of programmed cell death or apoptosis (10, 22).

The antitoxin antiserum reacted only with DT when concentrated culture supernatant of PW-8 was tested by Western blotting (Fig. 1). Misinterpretation of nonspecific precipitin lines in the Elek test remains common (6). On the contrary, secondary antigen-antibody reactions (false positive) will be avoided by using latex sensitized with affinity-purified antibodies. In the Outcherlony test, the amount of antigen needed to form a precipitin line was about 1,000 times the amount needed for latex agglutination (data not shown). Therefore, the higher sensitivity of the RPLA assay will avoid the false-negative results of the Elek test. The final report was available within 12 h after isolation of the organisms with the RPLA assay, while 48 h of incubation is recommended for the conventional Elek test (5) and 24 h is recommended for the modified Elek test described by Engler et al. (6).

Other agglutination tests have been described for DT detection. That reported by Holmes and Perlow (9) uses sensitized sheep erythrocytes, while that of Jalgaonkar and Saoji (12) uses the sensitized Cowan I strain of Staphylococcus aureus. Because of its stability, latex sensitization would be better than these two methods. Although the long-term stability of anti-DT sensitized latex has not been demonstrated, anti-cholera toxin-sensitized latex prepared in our laboratory (11) was stable for several years. On the other hand, the method described by Jalgaonkar and Saoji (12) that detects DT by a slide agglutination test is more rapid than the RPLA assay because these latex particles are not useful for slide agglutination.

The “gold standard” for detection of toxigenic C. diphtheriae is the guinea pig subcutaneous virulence test. However, it is not recommended for inexperienced laboratory personnel (5) and is not suitable as a first-line assay of toxigenicity. Although enzyme-linked immunosorbent assay and PCR are also reliable methods for toxin detection, the former requires many steps (first antibody, second antibody, and substrate incubations) and the PCR requires reagents and equipment that are not available in all laboratories. Because RPLA can be performed by simply mixing the culture supernatant and sensitized latex, we think that this will be the most suitable method in laboratories where DT detection is not performed frequently or where the resources are minimal.

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