Adaptation of a Microdilution Procedure to the *Limulus* Lysate Assay for Endotoxin

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Endpoint determinations of endotoxin by the *Limulus* amoebocyte lysate assay have been facilitated by use of a microdilution procedure.

In 1964, Levin and Bang (4) first reported that lysate made from the amoebocytes of *Limulus polyphemus* (horseshoe crab) coagulated in the presence of minute amounts of bacterial lipopolysaccharide (endotoxin). Since then the *Limulus* amoebocyte lysate test has been researched and developed by many investigators and currently can detect picogram quantities of bacterial lipopolysaccharide. *Limulus* assay kits are now commercially available from several companies, and the *Limulus* amoebocyte lysate test is used primarily for the detection of bacterial lipopolysaccharide in spinal fluid from patients suspected of having meningitis (3) and for the detection of pyrogenic substances in injectable pharmaceuticals (5). Despite recently reported modifications of the *Limulus* amoebocyte lysate test for quantitative assays which involve the use of microslides (8), spectrophotometers (2), protein assays (7), or radioisotopes (6), most assays are routinely performed in test tubes, since observation for gelation is simple and only involves inverting the tubes. However, tube assays for the quantitation of lipopolysaccharide require a series of dilution tubes and a considerable amount of lysate and technician time to perform. We report that the microdilution equipment available in most laboratories can be used to perform a quantitative *Limulus* amoebocyte lysate test.

Sterile microdilution U-bottom plastic plates, disposable plastic 50-μl pipettes, and 50-μl microdiluters (Dynatech Laboratories, Alexandria, Va.) were used. The microdiluters were rendered pyrogen-free by flaming the tips in a Fisher burner. The diluent was pyrogen-free sterile water (Travenol Laboratories, Deerfield, Ill.), and the test lipopolysaccharide was soluble *Escherichia coli* endotoxin (lot EC-2, Bureau of Biologics, U.S. Food and Drug Administration). The microdilution procedure was performed as previously described (1), and the lipopolysaccharide standard was serially diluted in twofold amounts (0.5 to 0.002 ng/ml) before the addition of the lysate. Three lots of lysate (kindly supplied by S. W. Watson, Cape Cod Associates, Woods Hole, Mass., A. L. Lane, Difco Laboratories, Detroit, Mich., and P. I. Griffin, Mallinckrodt, Inc., St. Louis, Mo.) were rehydrated with water, and 50 μl of lysate was added to wells containing diluted lipopolysaccharide. Additional wells containing water were included and served as negative controls. The plates were then covered with plastic lids, mixed, and incubated at 37°C for 1 h. Parallel tests were performed by the standard method in pyrogen-free glass test tubes (10 by 75 mm), using 0.1 ml of lysate per tube as described by the manufacturer. Eight comparative titration series were performed for each lysate lot.

After incubation, the presence or absence of gel formation in the standard tube test was determined by inversion of the tubes. Adherence of a firm gel to the bottom of the tube was read as a positive test, and lack of gelation was read as a negative test. For the microdilution plates, however, the presence or absence of gelation was determined by first adding 50 μl of a 0.005% aqueous crystal violet stain solution to each well. The bottoms of the plates were then viewed at an oblique angle (30 to 45°). Gelation was noted in wells in which the stain did not mix and color the contents. Lack of gelation was noted in wells in which the dye mixed and colored the contents completely. Figure 1 shows a typical titration series in which gelation, lack of gelation, and a definitive endpoint or titer are visible. The test results were read immediately after the addition of the indicator stain, since the stain penetrated the gel within 3 h and reading of gelation was then impossible. However, after addition of the stain, the plates could be frozen at −20°C and stored for several weeks with the endpoint still visible.

Microdilution *Limulus* amoebocyte lysate test results for each titration series and lysate lot
FIG. 1. Photograph of the bottom of a microdilution Limulus amoebocyte lysate test plate viewed at an oblique angle showing gelation (white wells) and no gelation (dark wells). Serial twofold dilutions of E. coli endotoxin range from 0.5 to 0.002 ng/ml in wells 1 through 9. Well 10 is a water (negative) control, and all other wells are empty. Endpoint in this titration series is 0.063 ng/ml (well 4). Bar represents 1 cm.

Tested were the same as or 1 dilution higher than those obtained by the comparative tube method. There were no significant differences between the mean endpoints for each test method (P > 0.05 by Student’s t test). Similar results were also obtained by using biological specimens other than reference lipopolysaccharide. These samples included intact E. coli and clinical urethral and cervical exudates known to contain Neisseria gonorrhoeae.

The microdilution Limulus amoebocyte lysate test procedure described above was rapid, economical, and easy to perform. The volume of lysate needed was reduced 50% over the tubes using 50-μl volumes and would be 75% if 25-μl volumes were used.

LITERATURE CITED