Microtitration of *Bacillus cereus* Hemolysin

JAMES C. COOLBAUGH, REUBEN D. WENDE, AND ROBERT P. WILLIAMS

Department of Microbiology, Baylor College of Medicine, and City of Houston Health Laboratories, Houston, Texas 77025

Received for publication 9 August 1972

A microtiter procedure for the quantitation of *Bacillus cereus* hemolysin is described.

One of the common assay methods for the various bacterial hemolysins described in recent literature (2-4, 5-7, 9, 11) is titration by serial dilution with the end point determined visually or spectrophotometrically. We report here the adaptation of the microtiter technique (8) to the quantitation of cereolysin, an extracellular hemolysin of *Bacillus cereus* (3). The procedure maintains the accuracy and eliminates many shortcomings of tube titrations read visually.

For routine production of cereolysin, *B. cereus* strain B-48 was grown in yeast extract broth (YEB) containing in percent (w/v): K$_2$HPO$_4$, 1.0; ferric ammonium citrate, 0.005; MgSO$_4$, 0.05; NaCl, 0.5; (NH$_4$)$_2$H$_2$O, 0.5; dextrose, 1.0; and yeast extract (BBL), 1.0. The pH was adjusted to 7.2 with 1 N HCl. Other media used in the studies were casein hydrolysate broth consisting of the above glucose-salts base plus, instead of yeast extract, 0.2% (w/v) or 1.0% (w/v) vitamin-free casein hydrolysate (Sigma), and 1.0% neopeptone beef infusion broth (Difco).

Bacteria were incubated at 37 C overnight in 5.0 ml of the appropriate broth in test tubes in a New Brunswick Scientific model G76 water bath shaker set at 120 rev/min. Then, 1.0 ml of this culture was transferred to 50 ml of the same broth in a 250-ml flask. These secondary cultures were incubated at 37 C on a New Brunswick Scientific model G53 rotary shaker set at 200 rev/min. After 6 to 7 hr of growth, samples were withdrawn and centrifuged at 1,000 x g for 10 min, and the supernatant was used for titrations.

Microtitrations were performed using 96-well styrene microtiter U-plates (Cooke Engineering Co., Alexandria, Va.). In each of the first and second wells of a 12-well row was placed 0.05 ml of the sample. Twofold dilutions in phosphate-buffered saline (PBS), pH 7.2, were begun with well 2 and continued through well 12 by using 0.05-ml microdiluters (Cooke Engineering Co.). Rabbit blood (in modified Alsevers solution) and sheep blood (defibrinated) used for the hemolytic assay were obtained in 100-ml quantities from Colorado Serum Co. (Denver, Colo.). The blood was stored at 4 C and not used beyond 3 weeks, unless, prior to that time, the supernatant from the second wash showed hemolysis (10). As needed for each assay, red blood cells (RBC) were washed three times in PBS and then suspended in PBS to the desired concentration.

For comparison to microtitration, 0.5-ml samples were titered by twofold serial dilution in PBS using test tubes (13 by 100 mm). After addition of 0.5 ml of a washed 0.5% (v/v) suspension of rabbit RBC to each tube, the series was incubated for 1 hr at 37 C in a stationary water bath and then centrifuged to pellet the RBC. The titer of cereolysin in the sample was read as the highest dilution showing complete hemolysis.

There exist inter- and intraspecies differences in sensitivity of RBC to hemolysin (1). We assayed samples of supernatant fluids containing cereolysin by microtitration using 0.5% and 1.0% sheep RBC (v/v), and 0.2, 0.5, 1.0, and 2.0% rabbit RBC (v/v). The best-defined end points were obtained with 0.5% (v/v) rabbit RBC. The most reproducible titers resulted when plates were incubated at 37 C for 60 min, as opposed to temperatures of 4 or 25 C and times of 15 or 30 min. To avoid difficulties caused by variations in hemolytic sensitivity of RBC from different lots of blood, all comparative assays were performed with RBC from one lot.

In all cases, immediately after incubation, plates were refrigerated for at least 4 hr before reading (10) to stop the hemolysis and to allow settling of intact RBC. As with the tube titration, the titer was read as the highest dilution showing complete hemolysis.
Table 1. Comparative assays of cereolysin produced in four different media

| Culture medium* | Cereolysin titer* |
|-----------------|------------------|
|                 | Tube dilution | Microtiter# |
| 1.0% YEB        | 512           | (2) 512     |
| 1.0% NBIB       | 16            | (1) 1,024   |
| 1.0% CHB        | 16            | (3) 16      |
| 0.2% CHB        | 4             | (3) 4       |

*Samples taken after 6.5 hr of growth.
#Abbreviations: YEB, yeast extract broth; NBIB, neopeptone beef infusion broth; CHB, casein hydrolysate broth.

Reciprocal of highest dilution showing complete hemolysis.

Done in triplicate; number in parentheses shows frequency of titer indicated.

0.1 ml of supernatant from centrifuged sample

0.05 ml

1st well

2-fold dilutions in PBS

Add 0.05 ml of 0.5% rabbit RBC per well

Incubate one hr at 37°C

Refrigerate >4 hr

Read titer as highest dilution showing complete hemolysis

Fig. 1. Microtiter technique for quantitation of Bacillus cereus hemolysin. PBS, phosphate-buffered saline, pH 7.2; RBC, red blood cells.

In evaluations of the sensitivity of microtiter versus tube dilution methods, parallel titrations of the same samples were performed using each assay method. When samples taken from a 1.0% YEB culture at 0, 2, 3, 5, and 7 hr of growth were assayed by tube dilution and by microtiteration, the cereolysin titers were identical, being 0, 1:1, 1:8, 1:64 and 1:256, respectively. In other comparative assays of this type, we found the same good agreement between methods. Table 1 indicates the results of comparative titrations of portions of samples from 6.5-hr cultures of B. cereus in four different media.

The results demonstrate that the microtiter technique is readily adaptable to titration of cereolysin. Since no difference in titer was detected between crude and filter-sterilized supernatant fluids, there is no need for aseptic technique.

The procedure for cereolysin assay is summarized in Fig. 1. It is as sensitive as, yet requires only one-tenth the time and reagents of, the tube dilution method. Those assays requiring the precision of spectrophotometric analyses of hemolyzed RBC suspensions cannot be supplanted by the method described here. However, where a tube dilution assay would provide the necessary precision, as in our studies with cereolysin, microtiteration can be a valuable tool in the study of bacterial hemolysins.

LITERATURE CITED

1. Bernheimer, A. W. 1970. Cytolytic toxins of bacteria, p. 183–212. In S. J. Ajl, S. Kadis, and T. C. Montie (ed.), Microbial toxins, vol. 1. Academic Press Inc., New York.

2. Bernheimer, A. W., and L. S. Avigad. 1970. Nature and properties of a cytolytic agent produced by Bacillus subtilis. J. Gen. Microbiol. 61:361–369.

3. Bernheimer, A. W., and P. Gunshoff. 1967. Cereolysin: production, purification and partial characterization. J. Gen. Microbiol. 46:143–150.

4. Bernheimer, A. W., and L. L. Schwartz. 1963. Isolation and composition of staphyloccal alpha toxin. J. Gen. Microbiol. 30:455–468.

5. Fossum, K. 1963. Separation of hemolysin and egg yolk turbidity factor in cell-free extracts of Bacillus cereus. Acta Pathol. Microbiol. Scand. 59:400–406.

6. Fossum, K. 1963. The heat sensitivity of Bacillus cereus hemolysin. Acta Pathol. Microbiol. Scand. 60:523–527.

7. Njoku-Obi, A. N., E. M. Jenkins, J. C. Njoku-Obi, J. Adams, and V. Covington. 1963. Production and nature of Listeria monocytogenes hemolysins. J. Bacteriol. 86:1-8.

8. Sever, J. L. 1962. Application of a microtechnique to viral serological investigations. J. Immunol. 88:320–329.

9. Snyder, I. S., and N. A. Koch. 1966. Production and characteristics of hemolysins of Escherichia coli. J. Bacteriol. 91:763–767.

10. U.S. Department of Health, Education, and Welfare. 1968. Standardized diagnostic complement fixation method and adaptation to micro test. Pub. Health Monogr. No. 74, Atlanta, Ga. 34 p.

11. Zwadyk, P. J., and I. S. Synder. 1971. Purification and kinetic studies of the hemolysin from Escherichia coli. Can. J. Microbiol. 17:741–745.