Plaque Assay for Measuring Serum Bactericidal Activity Against Gonococci

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A new semiquantitative plaque assay was developed to measure killing of gonococci by serum. Results compare well with an established quantitative method, but the plaque assay is faster, simpler, and less expensive.

Insight into the role of serum killing in resistance to gonococcal infection has been highlighted by two recent observations. First, strains from disseminated gonococcal infection are serum resistant (7), and second, people with deficiencies in the terminal components of complement are more susceptible to *Neisseria* infections than are members of the normal population (6). Available methods for measuring serum killing of gonococci are, however, cumbersome, time consuming, and expensive. Currently there are two approaches to measuring these interactions. In one type of assay, minimal amounts of serum are required because a fixed number of organisms are exposed to serial dilutions of serum. Much more serum is required for the other type, which uses undiluted serum and variable dilutions of organisms. The advantage of the second approach is that in vivo conditions are simulated by using endogenous complement and preserving concentrations of other bactericidal factors and cofactors (such as β -lysin, lysozyme, bacteriocins, Mg^{2+} , and Ca^{2+}). To capitalize on the second approach and yet use less serum and simplified procedures, we developed a semiquantitative plaque assay to measure serum killing of gonococci by modifying the qualitative method of Fierer et al. for enteric bacilli (1). We tested this method by comparing it with an established quantitative assay for measuring killing of gonococci with undiluted serum.

A standard laboratory strain of *N. gonorrhoeae*, F62 (2), was compared with two serum-sensitive strains from local genital infections (N24 and 3011) and three serum-resistant strains from disseminated gonococcal infection cases (DD, MW, WK). Organisms were grown for approximately 20 h on GC agar base supplemented with IsoVitaleX (Baltimore Biological

Laboratory, Cockeysville, Md.) in 5% CO₂ at 37°C.

Serum was processed to preserve complement. Rabbit and human blood were collected aseptically and held at room temperature for 30 min before centrifugation at 4°C to remove serum. Because of the extreme lability of mouse complement, however, blood from Caw:CF1 mice (Carworth, Div. Charles River Breeding Laboratories, Inc., Kingston, N.Y.) was placed immediately in tubes in an ice bath and held for 30 min before removing serum or plasma. Because mouse sera gave results similar to heparinized mouse plasma, serum results are given. All serum and plasma samples were frozen in small samples at -70°C and thawed only once. Heat-decomplemented controls (56°C for 30 min) were present in all assays.

Plates for the plaque assay contained 0.5 mg of diethylaminoethyl-dextran (sterilized by M membrane filtration [Millipore Corp., Bedford, Mass.]) per ml of GC agar base plus IsoVitaleX. They were dried enough to absorb fluids by storing them at room temperature for at least 4 days before use.

The plaque assay was made semiquantitative by seeding plates with known concentrations of bacteria. Gonococci were scraped off plates and suspended in Dulbecco medium (Grand Island Biological Co., Grand Island, N.Y.) at a concentration of 10⁸ organisms per ml (estimated spectrophotometrically and confirmed by plate counts). Serial 10-fold dilutions were made, and 0.1-ml portions were dropped on diethylaminoethyl-dextran plates with an Oxford pipette (G. D. Searle & Co., Foster City, Calif.). Immediately the drop was spread evenly to dryness over the entire plate with a sterile bent-glass rod. Then 0.05-ml portions of serum were dropped onto the lawn and allowed to soak into the agar for 15 min. Each serum was dropped on a series of lawns containing serial dilutions of each iso-

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late of gonococci (10^7 , 10^6 , 10^5 , or 10^4 colonies per plate; less than 10^4 colonies per plate was unreadable due to the very sparse lawn).

After overnight incubation in 5% CO_2 at 37°C , plates were examined for plaques. Plaques were graded according to the density of colonies in the plaque compared with those in the lawn (partial plaques [$<50\%$ of the density of the lawn], or total plaques (no colonies), as shown in Fig. 1). Quantitative killing was calculated from the lawn with the highest concentration of organisms where total plaques were seen. Because the area of the plaque was approximately $1/40$ of the area of the plate, the number of organisms killed within the plaque equaled $1/40$ of the number of organisms on the plate; and because the amount of serum in one plaque was 0.05 ml, the total number of gonococci killed by 1 ml of serum was $1/40 \times 20$ or 0.5 times the number on the plate.

The results with the plaque assay were compared with the quantitative bactericidal assay of McCutchan et al. (3, 4), in which undiluted serum is incubated with equal volumes of serial dilutions of gonococci. Quantitative results were obtained by plate counts (5). Comparable results

were obtained with the two methods (Table 1). Gonococci which were not killed by human serum in the assay of McCutchan et al. were not killed in the plaque assay. Similar numbers of serum-sensitive gonococci (N24 and 3011) were killed by normal human serum in both assays. The amount of killing of strains of intermediate serum sensitivity (e.g., F62) was not determined in the semiquantitative plaque assay because the lower limit of the test was 4 logs. However, the plaque assay could still be used for screening such organisms because partial plaques were seen (Fig. 1) when 2 to 4 logs of killing were detected in the assay of McCutchan et al.

We also studied the effect of rabbit and mouse serum in the plaque assay. All strains were killed by rabbit serum as reported earlier (4). Because the assay of McCutchan et al. was found to be unsuitable for mouse serum due to the large volumes of serum required, only the plaque assay was used for mouse serum (Table 1). Of the six strains of gonococci tested, all but N24 were killed to some extent by normal CF1 mouse serum. Another difference was in heat stability of the bactericidal factors. Heated (56°C for 30 min) human and rabbit serum did not kill gon-

FIG. 1. Plaque assay plate with 10^6 F62 gonococci in the lawn, stained by spraying with oxidase reagents. A total plaque is seen at the left with rabbit serum, a partial plaque is at the right with human serum and no plaques are seen at the top and bottom with heated rabbit and human serum, respectively.

TABLE 1. Comparison of the bactericidal activity of normal human, rabbit, and mouse sera measured by the assay of McCutchan *et al.* and by the plaque assay

Strain of gonococcus	Average log kill by ^a :				
	Human serum		Rabbit serum		Mouse serum
	M.A. ^b	P.A. ^c	M.A.	P.A.	P.A.
F62	3.0	<4 ^d	>6	>7	4.9
N24	4.7	5.1	>6	>7	<4
3011	3.8	5.1	>6	>7	4.8
DD	0.4	<4	>6	>7	7
MW	0.4	<4	>6	>7	4.7
WF	0.5	<4	>6	>7	4.2

^a Killing is expressed as the log₁₀ of gonococci killed by 1 ml of fresh serum.

^b M.A., Assay of McCutchan *et al.*

^c P.A., Plaque assay.

^d Partial plaques were seen with F62, but with the disseminated gonococcal infection strains (DD, MW, and WF) not even partial plaques were seen.

ococci in either assay, but heated mouse serum was bactericidal, although less so than fresh unheated mouse serum or plasma. Thus, it appears that a heat-stable bactericidal system exists in mouse serum but not in human or rabbit serum.

In summary, a simple, inexpensive, semiquantitative plaque assay was developed for measuring serum bactericidal activity against gonococci. It requires only 0.2 ml of serum for each strain of gonococcus tested and is faster to per-

form than conventional bactericidal assays. It can detect both heat-stable and heat-labile bactericidal systems. This method should be useful in screening gonococcal strains for serum resistance and in screening sera for bactericidal activity.

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LITERATURE CITED

1. Fierer, J., F. Finley, and A. I. Braude. 1972. A plaque assay on agar for detection of gram-negative bacilli sensitive to complement. *J. Immunol.* **109**:1156-1158.
2. Kellogg, D. S., Jr., W. L. Peacock Jr., W. E. Deacon, L. Brown, and C. I. Pirkle. 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J. Bacteriol.* **85**:1274-1279.
3. McCutchan, J. A., S. Levine, and A. I. Braude. 1976. Influence of colony type on susceptibility of gonococci to killing by human serum. *J. Immunol.* **116**:1652-1655.
4. McCutchan, J. A., D. Katzenstein, D. Norquist, G. Chikami, A. Wunderlich, and A. I. Braude. 1978. Role of blocking antibody in disseminated gonococcal infection, p. 181-186. In G. F. Brooks, E. C. Gotschlich, K. K. Holmes, W. D. Sawyer, and F. E. Young (ed.), *Immunobiology of Neisseria gonorrhoeae*. American Society for Microbiology, Washington, D.C.
5. Miles, A. A., and S. S. Misra. 1938. The bactericidal power of the blood. *J. Hyg.* **38**:732-748.
6. Peterson, B. H., J. A. Graham, and G. F. Brooks. 1976. Human deficiency of the eighth component of complement. *J. Clin. Invest.* **57**:283-290.
7. Schoolnik, G. K., T. M. Buchanan, and K. K. Holmes. 1976. Gonococci causing disseminated gonococcal infection are resistant to the bactericidal action of normal human sera. *J. Clin. Invest.* **58**:1163-1173.