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Spot Test Method for Rapid Serological Grouping of Streptomycete Bacteriophages

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Inactivation by homologous and heterologous antiphage sera is a useful diagnostic criterion for the characterization of bacteriophages. Procedures in common use (for example, see M. H. Adams, *Bacteriophages*, p. 465, Interscience Publishers, Inc., New York, 1959) are cumbersome in surveys where many heterologous cross-reactions must be determined. The rapid method described below does not substitute for inactivation kinetic studies in establishing degrees of relationship within a serological group. It does, however, facilitate preliminary placement of large numbers of phage isolates into cross-neutralizing groups. The investigator is saved time-consuming procedures where no cross-reaction exists.

Antiphage sera were prepared in rabbits according to procedures outlined by Adams (*Bacteriophages*, p. 461). Antigen consisted of whole bacteriophage separated from host material by low-speed centrifugation and by passage through a Selas 03 porcelain filter (Selas Corp. of America, Dresher, Pa.) or a membrane filter (pore size, 0.45 μ). The antigen was washed twice by centrifugation at 20,000 $\times$ *g* for 2 hr, followed by resuspension of the pellet. Final titer must be greater than 10^9 plaque-forming units (PFU) per ml. A washing and resuspending medium composed of 0.8% NaCl, 0.3% peptone (Difco), and 250 mg of $CaCl_2$ in distilled water maintained phage stability and did not contribute confusing antigenic stimulants. An initial intravenous injection of 2 ml of phage antigen was followed with: 1 ml by the same route in 2 days, rest for 7 days before the third injection consisting of 2 ml, and three successive injections of 2 to 3 ml at 3- to 5-day intervals. Blood was obtained by cardiac puncture 7 to 10 days after the final injection. It was allowed to clot at room temperature in sterile 50-ml polypropylene centrifuge tubes. After overnight refrigeration, the serum was decanted aseptically, clarified by centrifugation, and stored at -10 C in sterile screw-cap vials. Homologous-inactivation constant was determined as a measure of neutralizing potency (G. S. Stent, *Molecular Biology of Bacterial Viruses*, p. 58, W. H. Freeman and Co., San Francisco, 1963; M. H. Adams, *Bacteriophages*, p. 463). For the spot test, each antiserum was diluted appropriately (usually 1:10) in broth of the same composition as used for phage propagation; physiological saline is not recommended as a diluent.

Bacteriophage for the spot test was adjusted to a titer of 10^7 PFU/ml. Streptomycete host cells for this test were newly germinated spores prepared as follows. Aerial spores were harvested from a 7- to 14-day culture on tomato paste-oatmeal-agar (I. N. Asheshov, E. Strelitz, and E. A. Hall, Antibiot. Chemotherapy **2**:366, 1952) or Amidex agar (R. J. Hickey and H. D. Tresner, J. Bacteriol. **64**:891, 1952), and were suspended in a broth containing 0.2% peptone, 0.2% yeast extract, and 0.1% N-Z Amine (Sheffield Chemical, Norwich, N.Y.) in distilled water, final *p*H, 7.4. The spore concentration should be between 10^8 and 10^9 spores per milliliter. The suspension was incubated at 27 C on a rotary shaker with 160 oscillations per min. Samples withdrawn at hourly intervals were examined microscopically for germination. Streptomycete seed layers from spores with short, unbranched germ tubes generally gave the best results. In our experience, the germination time for a given species is constant under standardized conditions of incubation and need not be redetermined microscopically for each experiment.

Spot tests for rapid detection of heterologous antisera capable of inactivating a given phage were made in petri dishes containing a base layer of plaque-count medium (peptone-yeast extract broth as given above, plus 1.5% agar and $MgCl_2$, 0.001 M). Base layers were seeded by the overlay technique with 1 ml of 0.7% agar containing approximately 10^8 host cells per milliliter, and the seeded surface was allowed to solidify. An additional drying period (4 hr at 28 C for streptomycetes) improved the surface for application of droplets as described below.

Fine Pasteur pipettes or tuberculin syringes were used to deposit as many as five different single droplets of antiphage sera, one of which was the homologous antiserum control, at equal intervals around the seeded agar surface on dupli-

cate plates. Prelabeled marks on the bottom of the dish served as spotting guides to identify each antiserum spot. Immediately, 1 drop of a phage to be tested for inactivation was placed in the center of the agar surface, to serve as a phage control, and then a droplet of the same phage was superimposed on each of the antiserum spots. The petri dishes should not be moved until the combined drops have been absorbed by the agar.

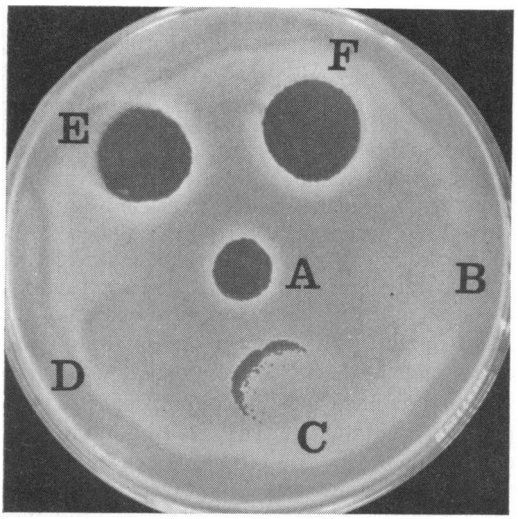

FIG. 1. *Comparison of the effects of homologous and heterologous antisera on a streptomycete phage, by use of the spot test method. (A) Spot of virulent phage (alone) on sensitive host; (B–F) mixed spots of same phage and selected antisera. (A) Confluent lysis by phage without antiserum; (B) complete inactivation of phage by homologous antiserum; (C) partial inactivation of phage by a heterologous antiserum; (D) complete inactivation of phage by heterologous antiserum for a related phage; (E and F) confluent lysis comparable to control (A), indicating no inactivation of phage by heterologous antisera for two unrelated phages.*

Time and temperature for incubation varied with the phage-host systems under study. The appropriate time was judged by appearance of confluent lysis of the host in the center spot where phage, without antiserum, was applied. Spots on streptomycete lawns were read after 36 to 48 hr of incubation (Fig. 1).

The antiserum-free central spot showed confluent lysis by the phage; the spot receiving both phage and its homologous antiserum showed no lysis. Locations where phage droplets were superimposed upon a heterologous antiserum showed lysis comparable to the center control spot when no cross-reaction existed, but showed complete protection of the host lawn (no lysed spot) when heterologous antiserum inactivated all the phage. Intermediate reactions, representing partial inactivation, were of three types: turbid spots, isolated plaques within the spot area (Fig. 1C), and plaques or lysis at the perimeter of the spot area (Fig. 1C).

Clear lysis (comparable to the antiserum-free control spot in the center of the lawn) indicated no inactivation and, hence, no significant serological relationship between the phage initially used as antigen for producing an antiserum and the phage tested at the antiserum spot (Fig. 1E and 1F). Complete inactivation (Fig. 1D) or partial inactivation (Fig. 1C) suggested serological relationship. The putative relationship was confirmed by conducting the reciprocal antiserum-phage spot test (cross-reaction). This simple test proved adequate for placing phages of streptomycetes into serologically related groups. The closeness of the relationship can now be established in the classical way by determining and comparing neutralization constants (M. H. Adams, *Bacteriophages*, p. 465).

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