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Pour Plate Study of Bacteriophage.

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A simple method for direct and accurate enumeration of bacteriophage is still lacking. In many instances it is desirable to determine the bacteriophage unit directly. For this purpose 2 general methods are now in use, (1) the counting of plaques formed when bacteriophage and susceptible bacteria are spread on the surface of agar plates, and (2) the determination of the highest dilution in broth for a given bacteriophage to cause complete lysis of susceptible bacteria. The best agreement by the dilution method of titration has been calculated by Clark[1] to be 60%. The difficulty of spreading evenly on an agar surface and the adsorption of a variable amount of bacteriophage by the spreader are the disadvantages of the streak method. If bacteriophage and susceptible bacteria are mixed thoroughly in meat infusion agar and then poured into plates, we find that the technique is not only simplified but its accuracy is also increased. It seems worthwhile, therefore, to test the practicability of the pour plate method and the optimal conditions for the demonstration of bacteriophage plaques under various factors of growth.

A dysentery Shiga bacteriophage isolated from a single plaque was used. Pour plates were made by mixing 1 cc. of diluted bacteriophage, 0.5 cc. of an 18-hour agar slant growth of susceptible bacteria in concentration of 1:50 in saline and 15 cc. of 2% meat infusion agar (pH 7.6). Upon incubation at 37°C. for 24 hours, 2 kinds of plaques appear: (a) surface plaque, which is large, clear, and extending through the depth of the medium, and, (b) deep plaque, which is small, less clearly outlined, and situated below the surface.

In agreement with Bronfenbrenner and Korb[2] we found the size of the individual plaque to be bigger as the concentration of agar is decreased (Fig. 1). It was also found that the number of visible plaques differed within 10% when the agar concentration used varies from 0.5 to 2%. When, however, the concentration of agar

[1] Clark, H., *J. Gen. Physiol.*, 1928, **11**, 71.

[2] Bronfenbrenner, J. J., and Korb, C., *J. Exp. Med.*, 1925, **42**, 483.

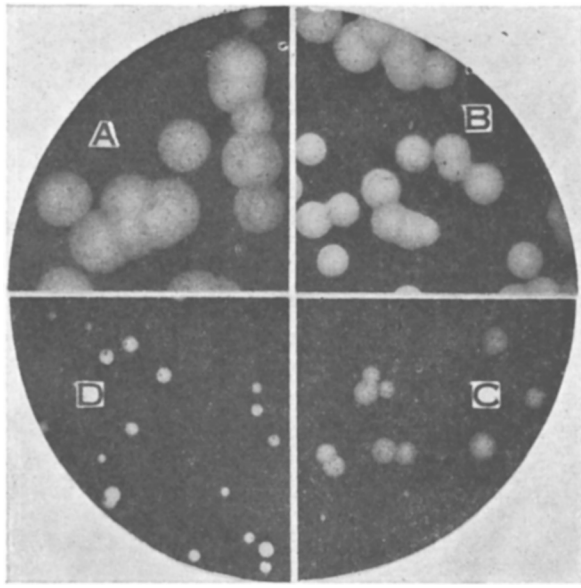

FIG. 1.
Showing size of plaques in different concentrations of agar using 1 cc. of a 1×10^{-7} dilution of bacteriophage (A) 0.5% agar, (B) 1% agar, (C) 2% agar, (D) 3% agar, natural size.

is increased over 2%, the number of plaques became considerably lower and finally no plaques could be detected when 5% agar is employed. The plaques are found to be distinct and large when the concentration of the susceptible bacterial suspension is between 1×10^9 and 5×10^9 organisms per cc. When the susceptible bacteria is decreased to 5.0×10^7 organisms per cc., the plaque appears markedly less clear cut and smaller in size and in number.

The thickness of media in the plate is also found to influence markedly the size of the plaque. In general, the thinner the plate the bigger is the plaque. When 5 to 10 cc. of medium were poured to a glass plate of 15 cm. diameter, practically all plaques are large, clear and extending to the surface, but when 15 to 30 cc. of medium were poured the average size of the plaque diminishes, and many plaques are situated below the surface so that counting becomes difficult.

When plates are incubated at 20°C. for 24 hours or grown anaerobically the plaques are smaller and less distinct (Fig. 2, A and D) than those incubated at 37°C. for 24 hours. It appears from these experiments that the slower the rate of bacterial growth, the smaller is the size of the plaque. In making the dilution of bac-

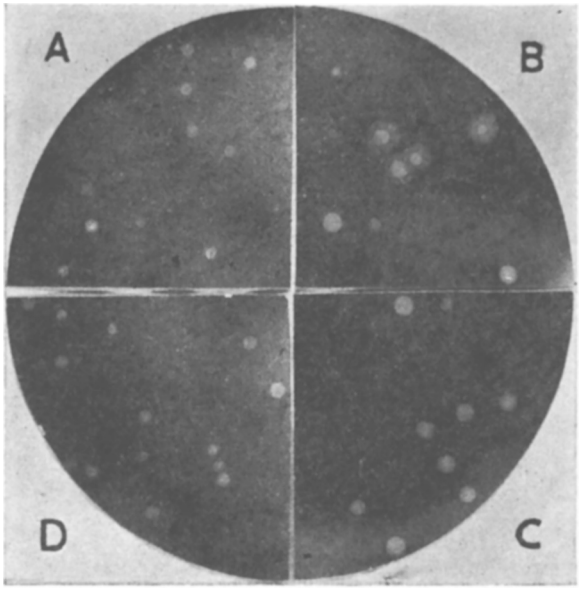

FIG. 2.
Showing plaques and ring formation (A) in 2% agar, 20°C., aerobic, 24 hours, (B) in 2% agar at 37°C. for 48 hours, concentric rings of lysis around plaques, (C) similar plate as B, but the media is thicker than in B, no ring around plaques, (D) in 2% agar at 37°C. anaerobic for 24 hours, natural size.

teriophage for plaque counting, it was found that meat infusion broth (pH 7.6) serves as a better diluent than saline which tends to give a lower count.

Since these results show that size and number of bacteriophage plaques in pour plates may vary greatly depending upon percentage of agar, concentration of susceptible bacteria, nature of diluent, and thickness of plates, the pour plate method can not be used for accurate quantitative determination of bacteriophage unless optimal conditions are observed. Such conditions are obtained when the following procedure is adopted: 1 cc. of bacteriophage diluted in meat infusion broth (pH 7.6) and 0.5 cc. of an 18 hour agar slant growth of susceptible bacteria in concentration 1:50 in saline are placed in a glass plate of 15 cm. diameter and 10 cc. of 1.5% meat infusion agar is added; the contents in the plate are thoroughly mixed and incubated at 37°C. aerobically for 24 hours before the plaques are counted.

When the plaque counts were determined in a serial dilution from undiluted bacteriophage, it was found that the number of plaques produced are not directly proportional to the respective concentra-

tion of bacteriophage used. The results are in agreement with Dreyer,[3] who also found that a relatively higher figure is obtained when higher dilution is used. Our results are given in Table I, which illustrates the applicability of Dreyer's standard curve showing the relation of bacteriophage units and plaque count. The K calculated shows a close agreement.

TABLE I.

Bacteriophage dilution, factor, B	1/B x 10^5	Plaques counted	Units from Dreyer's curve	K	B x plaques
1 x 10^6	.1	750	1337	7.310 x 10^{-5}	750 x 10^6
2	.05	450	652	7.669 "	900 "
4	.025	265	315	7.937 "	1060 "
8	.0125	155	154.5	8.090 "	1240 "
16	.00625	86	74.14	8.430 "	1376 "
32	.003125	51	39.30	7.952 "	1632 "
64	.001563	27	19.13	8.171 "	1728 "
128	.0007813	14	9.56	8.173 "	1792 "
256	.0003906	7	4.79	8.155 "	1792 "

Bacteriophage plaques in the poured plates presented another interesting phenomenon (Fig. 2, B and C). In some of the plaques it was found to be surrounded by 2 to 4 concentric rings. The first ring is a narrow zone of partial clearing immediately next to the perfectly clear central plaque. The second ring is a zone of comparatively dense bacterial growth. The third ring is another zone of partial clearing more opaque than the first ring and a little wider. The fourth ring is a zone of dense bacterial growth thicker than the surrounding parts of homogeneous bacterial growth in the plate. Often not all of these rings are conspicuous, but two rings, namely, a central plaque surrounded by a ring of dense bacterial growth which is in turn encircled by a very narrow zone of partial clearing may be seen. In this case the first ring can not be distinguished from the central plaque and the fourth ring is absent. The width of these rings varies according to the percentage of agar employed. It is wider when the percentage of agar is low. The occurrence of these rings depends upon several factors. It is seen when inoculum of susceptible bacteria is heavy (5x10^9 organisms per cc.) and the media is poured thin (5 to 8 cc. of agar in a plate of 15 cm. diameter). It is better seen when incubated aerobically at 37°C. for 2 to 4 days. They are absent when incubated at 20°C. or anaerobically.

The mechanism in the production of these rings is not clear.

[3] Dreyer, C., and Campbell-Tenton, M. L., *J. Path.*, 1933, **36**, 399.

Bronfenbrenner[4] stated that the diffusion of bacteriophage occurred even at 4°C. This diffusion factor in itself seems to us inadequate to account for the occurrence of rings of lysis. The following theory is presented as a possible explanation. The central plaque ceases to increase in size after 24 hours because by then, some inhibitory product resulting from bacterial lysis might have been accumulated. If one may be permitted to assume that this inhibitory product cannot diffuse in the agar as rapidly as the bacteriophage, then one might expect a concentric ring of partially lysed bacteria immediately surrounding the central clear plaque, to be surrounded by another zone of clearing. Thus alternate zones of clear and cloudy rings corresponding to presence and absence of bacteriolysis can be seen. The increased density of bacterial growth occurring at the outermost ring might be due to the stimulating action of the bacteriophage.

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Resistance In Vitro of Leishmania Donovani to Contamination with Bacteria.

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Leishmania donovani, the causative agent of Kala-azar, is generally believed to be a delicate organism which cannot survive in the presence of common bacteria. The studies of Spagnolio[1] and those of Giugni and Benoni[2] tended to indicate that contamination with various common bacteria is detrimental to the culture of this parasite. The recent demonstration by Forkner and Zia[3, 4] of viable and infective *Leishmania donovani* in the midst of numerous bacteria in the oral and nasal secretions of 13 out of 14 patients suffering from Kala-azar has, however, led us to alter our concept con-

[4] Bronfenbrenner, J., "The Newer Knowledge of Bacteriology and Immunology," The University of Chicago Press, Chicago, 1928, p. 526.

[1] Spagnolio, G., *Malaria e Malat. d. Paesi Caldi,* 1912, **3**, 151. *Abs. Trop. Dis. Bull.,* 1912, **1**, 8.

[2] Giugni, F., and Benoni, F., *Malaria e Malat. d. Paesi Caldi,* 1915, **6**, 89. *Abs. Trop. Dis. Bull.,* 1915, **6**, 220.

[3] Forkner, C. E., and Zia, L. S., *J. Exp. Med.,* 1934, **59**, 491.

[4] Forkner, C. E., and Zia, L. S., *J. Exp. Med.,* 1935, in press.