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**RAPID IDENTIFICATION OF BACILLUS ANTHRACIS  
AND THE STRAINS OF BRUCELLA BY  
MICROSCOPICAL OBSERVATION  
OF BACTERIOPHAGE LYSIS.**

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**Introduction**

*Bacillus anthracis* maybe distinguished from the closely related *B. cereus* by its susceptibility to a specific bacteriophage (McCloy, 1951). The usual technique for testing a suspected strain with phage consists in spotting the undiluted phage on a bacterial lawn on agar, and observing for lysis after incubation overnight (McCloy, 1951, Brown and Cherry, 1955). It seemed that identification could be accelerated by applying the phage to the bacteria at an earlier stage of their growth and observing lysis microscopically. The experiments reported by (P. Chadwick, 1959) Confirmed this prediction. When young growing filaments or microcolonies of *Bacillus anthracis* were treated with specific bacteriophage, fragmentation of the filaments followed by complete disintegration of the microcolony were visible microscopically within 2-3 hr. Other *Bacillus* species were unaffected by the phage, This technique is a simple one for accelerating the identification of *Bacillus anthracis*.

The presence of a bacteriophage capable of lysing strains of *Brucella* was reported by (Pickett and Nelson in 1950, and confirmed by Parnas and his associates in 1958. This phage, whose host propagating strain is R 19, lyses only smooth and intermediate strain of *Br. abortus*. Rough or mucoid strains of *Br. abortus* and all strains of *Br. suis* and *Br. melitensis* were resistant (Kessel and Braun 1961).

## Methods and Results

*Bacteriophage:* The bacteriophage used in the experiments was a virulent mutant ( $\alpha$ ) of a temperate phage ( $\beta$ ), derived from a *Bacillus cereus* strain W (McCloy, 1958). The phage was used undiluted a titre of  $1.5 \times 10^{10}$  particles/ml. The phage may be propagated on any strain of *B. anthracis* and *Brucella*.

### *Propagation Technique:*

A  $10^2$  dilution in broth of an overnight cultures of *Bacillus anthracis* and strains of *Brucella* were incubated for 3 hr. at  $37^\circ$ . The cultures tubes were held in a perspex drum rotating at about 2 rev./sec. inclined at  $45^\circ$  and fixed in a wooden box the temperature inside which was controlled thermostatically. The drum was rotated by a low-power motor. The source of heat was a 100 W. electric light bulb placed beneath a metal reflector. To the resulting logarithmic phase cultures were added ( $\alpha$ ) phage in the proportion of  $10^9$  particles/10 ml. cultures, and incubation continued until clearing was complete, usually about 2-3 hr. after addition of the phage. The bacterial debris was centrifuged, and the supernatant liquid treated with 1 ml. chloroform. The lysates were left in contact with chloroform for 30 min. on the bench, and the chloroform then removed by suction. A sterility test was performed on the lysates, which was afterwards ready for use.

### *Technique of the phage - Lysis microtest:*

Light inocula of *Bacillus anthracis* spores were allowed to germinate on plates of nutrient agar, with or without 5% (V/V) peptic extract of sheep blood (PSB) added. Same procedure were made with the strains of *Brucella* (*Br. abortus*, *Br. melitensis* and *Br. suis*). After 4-5 hr. incubation at  $37^\circ$ , single strand filaments of *B. anthracis* and colonies of the *Brucella* strains were visible under a binocular dissecting microscope, or a monocular microscope with transmitted light and 25 mm. or 16 mm. objectives.

The position of the filament and colonies on the agar surface was marked with a straight wire. A small loopful of undiluted ( $\alpha$ ) phage was applied to the area of agar plates carrying the filament and colonies, and allowed to soak into them. There was some advantage in watching the application of the phage through the microscope to note any mechanical disturbance of the filament and colonies. It was also desirable to examine the treated area microscopically.

pically after the liquid had dried in, to ensure that the filament and colonies had not been removed accidentally on the loop. The risk of this happening was small.

The plates were returned to the 37° incubator for about 1 hr. At this time, and on several occasions during the subsequent hour, the treated areas were examined microscopically for evidence of lysis in the filament and colonies. Between examinations the plates were returned to the incubator or kept in a hotbox (37°) equipped with a microscope. During the second hour after application of (a) phage, breaks appeared in the filament and colonies, due to the action of phage, and at about the end of this second hour, the filament and colonies had been completely lysed, and were represented by a thin groove in the agar corresponding to the position of the original filament and colonies. Disintegration of the microcolonies were, however, complete, within 3 hr. of application of the phage.

#### *Optical Apparatus:*

The lytic changes were observed by using a monocular microscope with 16 and 4 mm. objectives and X 10 ocular, the source of illumination being transmitted light from a high - power filament lamp. The vertical illumination method described by Pearce and Powell (1951) was also tried. Observations were made with (I) a 16 mm. objective, using a light background (II) a 4 mm. objective, using a dark background.

During the action of the phage, the grooves left in the agar by the lysed portions of the filaments and colonies contrasted strongly with the segments and colonies not yet lysed. But vertical illumination with this objective was not suitable for observation of fine detail, because of the frosted appearance of the agar after application of the phage. By transmitted light the background was relatively clear.

The dark background for use with the 4 mm. objective was provided by incorporating nigrosin 0.5 % (W/V) in the medium. When bacterial growths and phage lysis were allowed to take place on this medium, examination with the 4 mm. objective revealed very striking patterns of lysis. The irregular distribution of damage by the phage was very obvious, and when observation was continuous, individual cells could be seen to explode as lysis took place.

*Reproducibility of phage - lysis with different strains of Bacillus anthracis and Brucella.*

Microcolonies of 8 virulent *B. anthracis* strains and 3 strains of *Brucella* were allowed to develop on nutrient agar, and one colony of each strain tested with (a) phage. Signs of disintegration of the colonies were evident with each strain within 3 hr. of application of the phage.

*Tests with other species of the genus Bacillus and Bacteria:*

The (a) phage caused no damage to filaments or microcolonies of one strain each of *Bacillus cereus*, *B. mycoides*, *B. subtilis*, and *Bacterium coli*, 3 strains of *Mycobacterium tuberculosis*, when these were tested in parallel with strains of *B. anthracis*, and *Brucella*.

*Other Control Measures:*

When (a) phage was applied to a filament or microcolony, a similar filament or microcolony on the same plate was treated with a loop of sterile broth in the same way. Such control filaments and colonies always grew normally into microcolonies which never showed any fragmentation of this constituent filaments and colonies such as might have been produced by mechanical means.

*Total time for identification of Bacillus anthracis and 3 strains of Brucella by the phage - lysis microtest:* The time elapsing between inoculation of agar with a light spore and bacteria suspension and the observation of convincing lysis of the resulting microcolonies was 4-8 hr. The shorter times were observed on medium enriched with PSB, where growth and lysis were slightly faster than on plain nutrient agar.

## Discussion

The specificity of the (a) phage was established by McClay (1951) who examined 171 strains of *Bacillus anthracis* and 244 other strains of other species of the genus *Bacillus*, by spotting a drop of the undiluted phage on a quarter plate spread previously with the bacterial strain to be tested. All the strains of *B. anthracis* were attacked by the phage, and only 2 of the 244 strains of other species were susceptible. These Two were both *B. cereus* strains, another 54 strains of *B. cereus* were not attacked by the phage. Brown and cherry (1955) found that (a) phage lysed all of 41 naturally occurring rough strains of *B. anthracis*, 2 of 89 strains of *B. cereus*, and none of 134 strains of other *Bacillus* species.

The presence of a bacteriophage capable of lysing strains of *Brucella* was reported by Pickett and Nelson in 1950, and confirmed by Parnas and his associates in 1958. This phage, whose host-propagating strain is R 19, lyses only smooth and intermediate strains of *Br. abortus*. Rough or mucoid strains of *Br. abortus* and all strains of *Br. suis* and *Br. melitensis* were resistant (Kessel and Braun 1961). However, in our experiments, lysis was observed in all 3 strains (*Br. abortus*, *Br. Melitensis* and *Br. suis*) of *Brucella*, and we obtained a result that 3 strains of *Brucella* can be identified by this technique.

The evidence presented in this paper indicates that it should be possible to identify *Bacillus anthracis* and 3 strains of *Brucella* by means of specific bacteriophage within 8 hr. of sowing agar plates with suspected materials.

### Summary

When young growing filaments or microcolonies of *Bacillus anthracis* and 3 strains of *Brucella* (*Br. abortus*, *Br. melitensis* and *Br. suis*) were treated with specific bacteriophage, fragmentation of the filaments and lysis followed by complete disintegration of the microcolonies were visible microscopically within 8 hr. of sowing agar plates with suspected materials. Other some *Bacillus* and *Bacteria* species were unaffected by the phage. This technique is a simple one for accelerating the identification of *Bacillus anthracis* and strains of *Brucella*.

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