RECENT STUDIES ON METHODS OF ISOLATING A BACTERIOPHAGE FOR BACILLUS DIPHTHERIÆ.

By JULIUS A. KLOSTERMAN and KATHRYN W. SMALL, M.D.

(*From the Laboratories of the Department of Bacteriology, College of Dentistry, New York University, New York.*)

(Received for publication, November 1, 1927.)

D'Hérelle, Fejgin and Blair (1–3) have published accounts of their success in isolating a lytic principle for *B. diphtheriæ*. In the light of increasing interest in studies of bacteriophagy in general, it appears advisable to contribute to the available literature additional experimental data pertaining to the isolation and activity of a true bacteriophage for *B. diphtheriæ*.

The writers have exercised particular care to utilize only toxic strains of *B. diphtheriæ* throughout their entire investigation. The purpose was to secure a phage active against virulent strains, to be used in proposed subsequent experiments.

Unfortunately, d'Hérelle has given insufficient information concerning the details of his investigation on antidiphtheria bacteriophages. Hence duplication of his researches for corroboration could not be attempted. However, the two phages which he reported to have isolated from the feces of diphtheria antitoxin horses were active only upon atoxic strains of *B. diphtheriæ*. The mode of detecting the bacteriophagic activity was not disclosed in the instance of this particular phage.

Fejgin briefly describes having isolated a diphtheria phage from a Martin bouillon culture of *B. diphtheriæ* several weeks old. In an effort to demonstrate the activity of this phage on agar, she secured but a single plaque, in the center of which one bacterial colony subsequently developed. Unfortunately she was unable to proceed with her investigation as the tube was broken in handling.

Blair, on the other hand, reported isolations of antidiphtheria phages with comparative ease from three distinct sources. He based these findings entirely upon the degree of clearness of broth cultures containing the filtrate to be tested for phage, as compared to the turbidity of the broth culture controls. In no instance

has he included accounts of antidiphtheria phage activity on agar. The latter characteristic, according to the most recently developed technical procedure, appears to be absolutely necessary in establishing the fact of true bacteriophagy. Lytic action based wholly upon apparent lysis in broth cultures may be due, particularly if not capable of serial transfer, to inhibitory or enzymatic substances which by chance are present in the suspected filtrates rather than to a real bacteriophage. Another extremely important factor which also decides the genuineness of a bacteriophage is the well known characteristic of cultivation by successive transferring with the microorganism for which it is active. Blair gave no indication that his phages were propagatable.

The following protocols outline the procedure used in the attempt to isolate an antidiphtheria phage. Four cardinal principles were rigidly adhered to—first, the use of only definitely toxic strains, second, requiring all suspected phages to show activity on agar slants as well as in broth, third, carrying all tests on filtrates through two or more transfers before discarding because of lack of visible phage action, fourth, requiring that any suspected phage be capable of propogation by subculturing in the presence of a toxic strain of *B. diphtheriæ*. Fecal material collected from either human or animal sources was treated as follows. Approximately a cc. of feces was placed in 20 cc. of broth (pH 8.2) and allowed to incubate overnight at 37°C. It was then filtered through infusorial earth packed in a Buchner funnel. The filtrate was finally passed through a Berkefeld filter (*w*).

The first attempt at isolation was made by using the feces of an active case of diphtheria.[1] The infecting organism was isolated from the throat of this patient and was made use of in the following procedure. It is referred to as the homologous strain. Eleven stools were examined. The first specimen was taken on the 5th day of illness and the others at intervals for the next 2 weeks covering course and convalescence. Of the eleven stool filtrates eight were tested upon one homologous and two heterologous strains. Two were tested upon one homologous and five heterologous strains. One was tested upon one homologous and six heterologous strains. In no instance was there any indication of the presence of a phage.

Next a 33 day old culture in 30 per cent serum broth of a recently isolated virulent strain of *B. diphtheriæ* (Strain *A*) was filtered and used

[1] The authors wish to thank Dr. W. H. Park for his kindness in making arrangements to secure the materials used in these experiments.

as a potential source of a phage. It was tested against the seven strains of *B. diphtheriæ* which included its homologous strain and gave entirely negative results.

The third source tested for the presence of an antidiphtheria phage was the intestinal contents and peritoneal washings of guinea pigs inoculated with diphtheria cultures (Tables I and II).

TABLE I.

Test Animals.

Showing animal inoculations, time of death, result at autopsy with respect to signs of diphtheria toxemia and result of test for phage activity on intestinal contents and peritoneal washings.

Infecting strain used	Broth suspension, 1 cc.										Broth culture, 1 cc.				
	Intraperitoneal					Subcutaneous					Subcutaneous				
	Guinea pig No.	Death	Autopsy findings	Phage activity: Intestinal contents	Phage activity: Peritoneal washings	Guinea pig No.	Death	Autopsy findings	Phage activity: Intestinal contents	Phage activity: Peritoneal washings	Guinea pig No.	Death	Autopsy findings	Phage activity: Intestinal contents	Phage activity: Peritoneal washings
		hrs.					*hrs.*					*hrs.*			
B. diphtheriæ Strain *M* 1314	1	48	+	−	−	4	48	+	−	−	6	48	+	−	−
B. diphtheriæ Strain *A*	2	24	+	−	−	5	48	+	−	−	7	Killed at 48	−	−	−
B. diphtheriæ Strain *F*	3	48	+ (also peritoneal abscess)	−	−						8	48	+	−	−

Eleven animals were used of which eight served as test pigs and three as controls. Three toxic strains of *B. diphtheriæ* were employed, namely Strain *M* 1314, Strain *A* and Strain *F*. The first two had been isolated 7 months ago while the third was isolated 3 weeks previously. Three different types of inoculations were made in the case of two strains, while only two of these were employed with the third strain. Hence altogether eight guinea pigs were used as test animals. The types of inoculations resorted to were first, 1 cc. of a suspension made by emulsifying the bacterial mass of an 18 hour serum agar slant in 5 cc. of broth, adminis-

tered intraperitoneally; second, the same material administered subcutaneously and third, 1 cc. of a 48 hour broth culture given subcutaneously.

One control pig was used for each of the three strains of *B. diphtheriæ*. In each case the pig received a subcutaneous inoculation of 1 cc. of a 48 hour broth culture of its assigned strain and in addition two hundred units of diphtheria antitoxin given intraperitoneally. These controls served as an indirect check on the toxin-producing property of the listed strains. In other words they were included for the purpose of demonstrating that all of the strains used produced toxin which was neutralized by a specific antitoxin.

The results were as follows. One test animal (No. 2) receiving an intraperitoneal inoculation of a suspension of Strain *A* died in 24 hours. The autopsy revealed signs of diphtheria toxemia which were the presence of a serous exudate in the peritoneal cavity, congestion of the peritoneum and enlargement and congestion of the adrenals. Six other test pigs (Nos. 1, 3, 4, 5, 6, 8) died in 48 hours with

TABLE II.

Control Animals.

Showing animal inoculations used to control specificity of toxin produced by listed strains.

Infecting strain used	Diphtheria antitoxin administered subcutaneously	Guinea pig No.	Broth culture (subcutaneous)	
			Death	Autopsy findings
B. diphtheriæ Strain *M* 1314	200 units	9	11 days	Cause of death undetermined (no signs of diphtheria toxemia)
B. diphtheriæ Strain *A*	200 units	10	4 days	Streptococcus infection (no signs of diphtheria toxemia)
B. diphtheriæ Strain *F*	200 units	11	Alive	

positive signs at autopsy. The remaining test pig (No. 7) having received a subcutaneous broth inoculation of Strain *A* was killed and autopsied with negative results, however.

One control (No. 11) was alive on the 20th day. A second control (No. 10) died on the 4th day. Before inoculation the latter pig showed a fluctuating swelling of the perineal region. At autopsy there were no signs of diphtheria toxemia. However, there were soft swollen inguinal and retroperitoneal lymph nodes (bilateral) and an enlarged, congested spleen. Smears from the lymph nodes showed many Gram-positive cocci in long chains. Hence the animal probably succumbed to an acute streptococcus infection, the focus of which was the lesion observed before inoculation. The third control (No. 9) died on the 11th day and autopsy failed to reveal the cause of death but certainly there was no indication that it was due to diphtheria toxemia.

At autopsy one of the intraperitoneal suspension pigs (No. 3) showed a small peritoneal abscess at the site of inoculation. A smear and culture from the lesion showed *B. diphtheriæ*. The abscess and surrounding wall were emulsified in broth, incubated and filtered.

This filtrate and those made from intestinal contents and from peritoneal washings of the eight pigs which received culture without antitoxin were examined

TABLE III.

Results of phage activity of filtrates of feces from antitoxin horses on strains of *B. diphtheriæ*.

Results indicated are those recorded after two transfers.

Strains of *B. diphtheriæ*	Filtrates from feces of antitoxin horses					
	149	152	161	87	143	144
B	—	—	—	—	—	—
C				—	—	—
D				—	—	—
E				—	—	—
Park 8	—	—	—	—	—	—
M 1314	—	—	—	—	—	—

Blank space indicates these combinations were not tested.

TABLE IV.

Results of phage activity of filtrates of feces of five additional antitoxin horses on seven strains of *B. diphtheriæ*.

Results indicated are those recorded after two transfers.

Strains of *B. diphtheriæ*	Filtrates from feces of antitoxin horses				
	165	142	156	136	154
A	—	—	—	—	—
B	—	—	—	—	—
C	—	—	—	—	—
D	—	—	—	—	—
E	—	—	—	—	—
Park 8	—	—	—	—	—
M 1314	—	—	+	—	—

for the presence of phage. Each filtrate was tested on its homologous strain and gave negative results. Then all filtrates were tested on two heterologous strains of *B. diphtheriæ* and six of the filtrates were additionally tried on six other heterologous strains of *B. diphiheriæ*. All results were negative (Table I).

The final attempt to isolate an antidiphtheria phage was conducted by using the filtrates of feces emulsions of antitoxin horses. The feces of six horses[2] retained at Otisville (for the New York City Department of Health) for the production of diphtheria antitoxin were emulsified, incubated and filtered according to the usual procedure. Of these six filtrates three were tested on three strains of diphtheria and three were tried on six strains. As the toxin of Strain Park 8 is used for the stimulation of antitoxin in these horses, it is regarded as the homologous strain. All efforts to isolate a phage from these filtrates failed (Table III).

TABLE V.

Observations on specificity of two generations of antidiphtheria Phage 156 *M* 1314 on Strains of *B. diphtheriæ*.

Results indicated are those recorded after two transfers.

Strains of *B. diphtheriæ*	Antidiphtheria Phage 156 *M* 1314 Generation 11	Antidiphtheria Phage 156 *M* 1314 Generation 23
A	−	−
B	−	−
C	−	−
D	−	−
E	−	−
F		+
G		+
Park 8	−	−
M 1314	+	+

Blank space indicates these combinations were not tested.

Filtrates from five other horses immunized with diphtheria toxin showed activity against one of these strains. This is the only instance in which the authors secured an antidiphtheria phage (Table IV).

The filtrate of Horse 156 showed lysis of *M* 1314 in broth and on agar in the second generation. The test broth was only slightly less turbid than the control and on agar phage activity was evident only in the form of isolated plaques. The fourth generation showed confluent plaques on the agar. The successive generations in broth gradually showed more and more lysis but even in the twenty-second

[2] The toxin used to immunize these horses consisted of the supernatant fluid of broth cultures which were allowed to sediment.

generation lysis is not complete. 500 units of concentrated diphtheria antitoxin were mixed with culture and phage in broth to counteract any undesirable action of toxin upon phage activity. There was

TABLE VI.

Observations on specificity of antidiphtheria Phage 156 *M* 1314 tested on eighteen strains of microorganisms other than toxic *B. diphtheriæ*.

Results indicated are those recorded after two transfers.

Microorganisms	Antidiphtheria Phage 156 *M* 1314 (Generation 16)
Diphtheria-like Strain 1154 (non-toxic)	—
Diphtheria-like Strain 1175 (non-toxic)	—
Diphtheria-like Strain 1178 (non-toxic)	—
Diphtheria-like Strain *U B* (non-toxic)	—
B. xerosis	—
B. hoffmanii	—
Staphylococcus aureus	—
Staphylococcus albus	—
Staphylococcus citreus	—
B. coli communior	—
B. dysenteriæ Shiga	—
B. dysenteriæ Flexner	—
B. dysenteriæ Mt. Desert	—
B. typhosus (Pfeiffer)	—
B. paratyphosus A	—
B. paratyphosus B	—
Cholera-like	—
B. subtilis	—

TABLE VII.

Observations on thermal inactivation point of antidiphtheria Phage 156 *M* 1314.

Temperature	30 min. exposure	45 min. exposure
°C.		
50	Active	Active
55	—	—
60	—	—

no apparent difference in end-result between the test containing antitoxin and the control without antitoxin as far as lysis due to phage activity was concerned.

Two later specimens of feces from Horse 156 were tested for the presence of phage and were found negative.

The blood serum of Horse 156 whose first specimen of feces gave positive results on *M* 1314 was tested on this culture but failed to give evidence of a phage.

The specificity of Phage 156 *M* 1314 was studied. The eleventh generation was tested on six diphtheria strains other than Strain *M*

TABLE VIII.

Results of using non-diphtheria phages in conjunction with *B. diphtheriæ* Strain *M* 1314 for determining whether or not these known bacteriophages are active on this strain.

Results indicated are those recorded after two transfers.

Name of phage	Source of phage	Organism on which phage is active	Generation of phage	Activity on *B. diphtheriæ* Strain *M* 1314
Coli	Not recorded*	*B. dysenteriæ* Mt. Desert	10	—
Coli	East River water	*B. coli communior*	5	—
Coli	Bellevue sewer water	*B. coli communior*	5	—
Coli	Willard Parker sewer water	*B. coli communior*	7	—
Coli	Normal cat feces	*B. coli communior*	8	—
Dysentery	Feces from case of typhoid	*B. dysenteriæ* Shiga	5	—
Dysentery	Sample from pail of milk just collected by farm hand	*B. dysenteriæ* Shiga	9	—
Dysentery	Human saliva pooled from six sources	*B. dysenteriæ* Shiga	12	—
Typhoid	Feces from case of dysentery	*B. typhosus* (Pfeiffer)	6	—

* This phage was supplied through the courtesy of Dr. J. J. Bronfenbrenner to whom the authors are also indebted for valuable suggestions and for his kindness in reviewing this paper.

1314, with negative results. The twenty-third generation was tested on the same six strains and two additional ones. It was active against the latter two. These two strains, namely Strain *F* and Strain *G* as well as Strain *M* 1314, were all recently isolated; that is within 6 months (Table V).

The sixteenth generation of antidiphtheria Phage 156 *M* 1314 was tested on eighteen different strains of microorganisms other than toxic *B. diphtheriæ* with negative results (Table VI).

The thermal inactivation point of Generation 23 of antidiphtheria Phage 156 *M* 1314 was found to lie between 50°C. and 55°C. (Table VII).

Generation 22 was titrated by a modified broth dilution method (4). In carrying out the final transfer, however, the authors substituted filtration for heating as this phage is inactivated at a comparatively low temperature. The phage showed activity when it was present in a 1×10^{-9} dilution.

Nine non-diphtheria bacteriophages of high titer previously isolated from various sources were tested for the purpose of determining whether or not they were active on *B. diphtheriæ* Strain *M* 1314 (Table VIII).

SUMMARY.

Of the attempts to isolate an antidiphtheria phage (1) from stools collected daily during the course of a case of the disease, (2) from a 33 day old broth culture of *B. diphtheriæ*, (3) from intestinal contents and peritoneal washings of guinea pigs inoculated with three different toxic strains of *B. diphtheriæ*, none yielded an antidiphtheria phage. However of eleven specimens of feces collected from eleven antitoxin horses one was found to contain a bacteriophage active against *B. diphtheriæ*. This phage was not observed in the first generation and did not show up until transferred the second time. Had the results of the first transfer been regarded as final in all certainty the existence of a phage would not have been recognized. Two additional specimens of feces from the positive horse (No. 156) were later tested to determine whether this bacteriophage was continually present in the intestinal tract of this animal. Both of these feces filtrates failed to yield a phage. Later a sample of freshly collected blood serum from the same horse was tested and found not to contain a phage.

The antidiphtheria phage was tested against eighteen non-diphtheria strains of microorganisms to determine whether it would show lytic activity for related or unrelated bacteria. There was no evidence of lysis in any of these types. The specificity of this phage was also tested on nine strains of *B. diphtheriæ* and found to be active on three heterologous strains (Strain *M* 1314, Strain *F* and Strain *G*). These incidentally were all recently isolated. It failed to lyse the remaining six strains of which five were recently isolated.

B. diphtheriæ Strain *M* 1314 was used in combination with nine heterologous bacteriophages isolated from various sources, to determine if any of these phages would by chance lyse this culture. The results were all negative.

To date this phage fails to show complete lysis although the twenty-second generation has a titer of 1×10^{-9}.

Additional proposed experiments involving this phage are being planned and will be undertaken in the near future.

BIBLIOGRAPHY.

1. d'Hérelle, The bacteriophage, Baltimore, 1922, 110.
2. Fejgin, *Compt. rend. Soc. biol.*, 1925, xciii, 365.
3. Blair, *J. Infect. Dis.*, 1924, xxxv, 401.
4. Bronfenbrenner, J. J., and Korb, C., *J. Exp. Med.*, 1925, xlii, 483.