BACTERIOPHAGE TYPING OF SALMONELLA PULLORUM

by

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B. S., Brooklyn College, 1950

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1952

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INTRODUCTION

Many investigators have noted that phages often demonstrate a selective action for certain strains of organisms within a given species, and evidence has accumulated which shows this specificity to be related to the antigenic pattern of the susceptible bacteria. Through the use of phages, it is sometimes possible to demonstrate minor differences between bacterial strains which are not detectable by the usual serological procedures. A suitable collection of phages should therefore provide a sensitive method of differentiating culture strains.

Craigie and Yen (10) devised a useful system of typing cultures of Salmonella typhosa by means of phage. They noted the specificity of V form typhoid phages for cultures possessing Vi antigen and that one such phage adapted itself readily to many culture strains. The latter characteristic made it possible to divide cultures of Salmonella typhosa into 24 distinct types according to their reaction to a critical test dilution of the adapted phage.

The method of bacteriophage typing offers certain advantages over the serological tests and the carbohydrate fermentation reactions. It is quicker, the bacteriophage adapting itself to a bacterial substratum in much less time than an animal can be immunized; it does not require a large number of different and complicated media, as carbohydrate test do; and no laboratory animals are required, the bacteria being the experimental organisms.

Once adapted to a certain species or race of bacteria, bacteriophage can be kept in the refrigerator the same as is done with sera, and used for typing at a later time.

Since *Salmonella pullorum* is susceptible to bacteriophage and has an antigenic likeness to *Salmonella typhosa* and since the present system of classification and identification of strains of *S. pullorum* is not wholly satisfactory, it was felt desirable to explore the possibility of using bacteriophage typing as a means of classifying or identifying strains of *S. pullorum*.

REVIEW OF THE LITERATURE

Many attempts have been made with varying degrees of success to use bacteriophage as an aid in the classification of closely related groups or species of bacteria, or for the differentiation of strains of a given species. The literature on the subject is extensive, hence, only the more pertinent work will be reviewed.

Craigie and Brandon (6) discovered the existence of bacteriophage which was specific for the V form of *Bacillus typhosus** (*Salmonella typhosa*) in cultures of the organism derived from the stools of cases and carriers. Exposure of the V form of *B. typhosus* (*S. typhosa*) to V phage resulted in its conversion to the phage-resistant W form, with loss of both V agglutinogen and the capacity to absorb the phage. Since the V form of *B. typhosus* (*S. typhosa*) is present in a very high percentage of carriers of this organism and of cases of typhoid fever, Craigie and Brandon (7) concluded that V bacteriophage might prove to be of definite diagnostic value. A complete parallelism between the sensitivity of phage Q 151 and the development of Vi antigen was observed by Craigie and Yen (9). Six hundred and forty-eight cultures of *B. typhosus* (*S. typhosa*) were examined and

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* The names of organisms are presented in the form used by the original author followed by the current name as used in the sixth edition of Bergey's Manual of Determinative Bacteriology (2), in parenthesis. Otherwise all nomenclature conforms with the sixth edition of Bergey's Manual of Determinative Bacteriology.

without exception were specific for phage Q 151. Craigie and Yen (10) found that strains of typhoid bacilli which contained the Vi antigen could be classified into separate types depending on their susceptibility to the lytic action of specific "races" of bacteriophage. The types and subtypes were designated by letters, A, B_1, B_2, C, etc. through T to a total of 24 types. They found some cross reaction between subtypes but very little between types. Enteric phage typing was found to depend not on the use of a variety of phages but rather on selected mutants of one serological species of specific Vi phage.

The possibility of tracing sources of typhoid cases by associating the "bacteriophage types" of the bacilli found in carriers was explored by Yen (47,48), Brandon (3), and Lazarus (30) in 1940. Bacteriophage typing is considered by Schlesinger (37), Felix (14), Frantz and Mason (20), and Vener and Stevens (43) as a valuable epidemiological tool in the investigation of outbreaks or sporadic cases of typhoid fever. In 1947, a standardization procedure for phage typing to assure uniformity in results was suggested by Craigie and Felix (8).

Attempts have been made to distinguish among other members of the genus Salmonella. Naidu (36) claimed that bacteriophage which was specific for Bacillus pullorum (Salmonella pullorum) regularly lysed cultures of this organism, but had no effect on cultures of Bacillus gallinarum (Salmonella gallinarum). Thus, these two species could be differentiated on the basis of their susceptibility to phage. In 1937, Munné (35) claimed

that S. pullorum and S. gallinarum were both equally susceptible to the action of bacteriophage. Felix (15), and Felix and Callow (16) have extended the principle to the typing of Salmonella paratyphi B (Salmonella schottmuelleri) and Salmonella aertrycke (Salmonella typhimurium). The effect of bacteriophage on "regular" and "variant" strains of Salmonella pullorum was studied by Gwatkins (24). There was no indication that the serological types could be differentiated by bacteriophagy. Lilleengen (31) typed Salmonella dublin and Salmonella enteritidis by means of phage isolated from sewage, feces, and phage-contaminated cultures. Eight selected "dublin" phages and six selected "enteritidis" phages were tested against 129 strains of S. dublin and 116 strains of S. enteritidis. S. dublin could be divided into six types and subtypes by means of five "dublin" phages, while S. enteritidis could be differentiated into eight types by four "enteritidis" phages. All strains could be typed.

The use of a polyvalent bacteriophage for the identification of cultures of dysentery bacilli was recommended by Miller (34). Ninety-two percent of known strains were found to be susceptible to the phage and only one-tenth of one percent of cultures of non-dysentery bacilli were also found to be susceptible. The value of bacteriophage determinations in the diagnosis of bacillary dysentery was also recognized by Wheeler and Burgdorf (44). Bacteriophage strains specific for Shigella paradysenteriae formed a homogeneous group with respect to plaque morphology, specificity and cross resistance tests. The procedure was found to be of most

value as a supplemental method to cultural examination, especially in outbreaks where examinations could not be made immediately after onset of the disease. With the use of a suitable bacteriophage, Archer (1) found that the majority of strains of S. paradysenteriae types I to V could be identified as such by their susceptibility to lysis. Thomen and Frobisher (41) made an analysis of the serological and cultural relations among the various component species and varieties of the genus Shigella by the use of specifically adapted bacteriophage. The method of phage typing was found to be neither more accurate nor more sensitive than the serological tests or carbohydrate fermentation tests which are, at present, used for the genus. One hundred and eight strains of Shigella sonnei were divided into 14 distinct types by Hammarstrom (25) with the aid of bacteriophage. Epidemiologically related strains and strains from the same individual displayed constancy of phage types.

Fisk (17) investigated the lysability of strains of Staphylococcus aureus (Micrococcus pyogenes var. aureus) recovered from various human lesions. Nineteen out of 43 coagulase-positive strains were found to be phage carriers; none of these phages was lytic for any of the coagulase-negative strains. The phage susceptibility of 95 strains of S. aureus (M. pyogenes var. aureus) cultures was determined with 27 different bacteriophages by Fisk (18). The differential action of these phages made it possible to divide cultures of pathogenic staphylococci into groups. Strains isolated from

related sources were found to react to the same phages and could be differentiated from other cultures by this method. A series of 78 strains of _S. aureus_ (_M. pyogenes var. aureus_) cultures isolated from 30 patients was studied by Fisk and Mordvin (19) to compare certain cultural characteristics and alpha hemotoxin production with bacteriophage susceptibility. Strains of the same phage type sometimes differed in hemolytic properties and chromogenesis and not infrequently varied in toxogenicity. The use of a technique similar to that for the phage typing of typhoid bacilli enabled Wilson and Atkinson (46) to recognize 21 staphylococcal types or subtypes. The number of types and the comparative rarity of most of them, indicated the possible epidemiological application of the method. Williams (45) made use of bacteriophage typing in the study of the association of nose and skin carriage of _S. aureus_ (_M. pyogenes var. aureus_). The coccus was isolated from the hands and nose of 65 patients, both strains were typable in 36 cases and were of the same type in 31 cases. The majority of the coagulase-positive strains of _S. aureus_ (_M. pyogenes var. aureus_) which were isolated from samples of accredited milk and cases of bovine mastitis were found by MacDonald (32) to belong to phage type 42D. This type was not obtained from human sources in the same locality except in the hands of dairy workers. A slight modification of the bacteriophage method employed by Wilson and Atkinson (46) for typing staphylococci was employed by Smith (38). Previously untypable strains and strains considered as belonging to type 42D were classified by the use of additional phages. Acquired

phage resistance was shown by Smith (39) to be responsible for the classification of many strains of staphylococci as different phage types. Frisken and Johnson (22), and Millar (33) found bacteriophage typing of staphylococci of great value in their investigations of breast abscesses and food poisoning outbreaks.

Bacteriophages specific for certain strains of hemolytic streptococci were noted by Lancefield (29). The susceptibility of different strains of hemolytic streptococci to four races of bacteriophage which had been classified according to their serological reactions was studied by Evans (11,12). According to their sensitivity to the four races of phage, the strains of hemolytic streptococci fell into eight groups, the largest of which agreed in a general way with groups already recognized as species on the basis of other characteristics. The enhanced potency of streptococcus bacteriophage in the nascent stage was described by Evans (13). Strain 890, of Lancefield's group A, which is resistant to phage B/563 filtrate, was shown to be sensitive to the lytic principle in the presence of a growing culture of the sensitive strain 563. Hunter (27) justified the division of the lactic streptococcus into *Streptococcus cremoris* and *Streptococcus lactis* on the basis of phage sensitivity tests.

Keogh, Simmons and Anderson (28) have shown that susceptibility to phage can be used as an auxiliary method in classifying strains of *Corynebacterium diphtheriae*. They found that "gravis" type I strains carry a phage which lyses "gravis" type II and

intermediate strains. They concluded that phage may play a role in the spontaneous or induced transformation of the diphtheria bacillus. Toshach (42) noted that strains of *Corynebacterium* which were related as to type or source had no definite pattern of susceptibility to bacteriophage. Freeman (21) has reported that avirulent strains of *C. diphtheriae* infected with bacteriophage have yielded virulent *C. diphtheriae* strains.

The bacteriophage method for classifying closely related types of soil bacteria has its value according to Conn, Bottcher and Randall (4). Although, used alone, the method probably cannot separate one species from another, it is valuable as a supplementary test in cases of doubtful species status. The bacteriophage technique of Hofer and Campbell (26) was used to distinguish relationships within the genus *Rhizobium* by Conn, Bottcher and Randall (5). Their results were reasonably constant and clear-cut. Close relationships were shown among the pea, bean, and clover organisms.

The use of the phage reaction as a quick means of identification to confirm the presence of a bacterial plant pathogen has been suggested by Thomas (40).

The possible use of bacteriophage in classifying the aerobic spore-formers was investigated by Gordon (23).

The uses of bacteriophage for species and strain identification will be extended as suitable species of phage and appropriate techniques are discovered and independent and concomitant variations of phage and host cell, relevant to the problem, have been elucidated.

EXPERIMENTAL MATERIALS
BACTERIOPHAGE AND CULTURES

The use of the bacteriophage typing method for the identification or classification of strains of *Salmonella pullorum* was explored in this work.

Bacteriophage

The bacteriophage used was isolated from the caecal contents of a pullorum infected chicken.

Cultures

Seventy-nine cultures of *S. pullorum* were selected for study. Gram's stain reaction, microscopic examinations, and biochemical and serological tests were employed as a means of ascertaining the purity and identification of each culture. Those which gave the specified reactions for *S. pullorum* according to the sixth edition of Bergey's Manual of Determinative Bacteriology (2) were used. Table 1, which follows, lists the history of these culture strains.

Table 1. History of culture strains.

Strain number as used in this work	Antigenic group	Original strain number	Source
1	undetermined	5776_1*	Strains 1 to 19 and 21 to 26 were obtained from Dr. Lester E. Erwin, Kansas State College. They were recent isolations from chickens and turkeys which had shown typical pullorum disease symptoms.
2	undetermined	5776_2	
3	undetermined	5776_3	
4	undetermined	5761_1	
5	undetermined	5761_2	
6	undetermined	5761_3	
7	undetermined	5779_1	
8	undetermined	5779_2	
9	undetermined	5779_3	
10	undetermined	5772_1	
11	undetermined	5772_2	
12	undetermined	5772_3	
13	undetermined	5788	
14	undetermined	5740	
15	undetermined	2	
16	undetermined	57	
17	undetermined	779	
18	undetermined	70	
19	undetermined	94	
21	undetermined	102	
22	undetermined	60	
23	undetermined	63	
24	undetermined	154	
25	undetermined	235	

Table 1. (cont.)

Strain number as used in this work	Antigenic group	Original strain number	Source
26	undetermined	250	
20	regular	BAI 20	Strains 20, 79 and 296 were obtained from the Bureau of Animal Industry
79	variant	BAI 79	
296	variant	BAI 296	
27	variant	3558	Strains 27 to 36 were obtained from the stock collection of the Department of Bacteriology, Kansas State College.
28	intermediate	B123T441	
29	regular	C1	
30	variant	C2	
31	variant	M61	
32	regular	17	
33	variant	M30	
34	variant	4016	
35	variant	4803	
36	regular	3245	
37	no record	Mass. 1	Strains 37 to 44 were obtained from Dr. G. H. Snoeyenbos, University of Massachusetts.
38	no record	Mass. 2	
39	no record	Mass. 3	
40	no record	Mass. 4	
41	no record	Mass. 5	
42	no record	Mass. 6	
43	no record	Mass. 7	
44	no record	Mass. 8	

Table 1. (cont.)

Strain number as used in this work	Antigenic group	Original strain number	Source
45	no record	51- 2- 29	Strains 45 to 50 were obtained from the Department of Bacteriology, Michigan State College.
46	no record	51- 2- 40	
47	no record	51-52- 5	
48	no record	51-52- 152	
49	no record	51-52- 175	
50	no record	65- 3	
51	no record	84	Strains 51 to 56 were obtained from Dr. Stanley E. Wedberg, University of Connecticut.
52	no record	85	
53	no record	86	
54	no record	98	
55	no record	149	
56	no record	150	
57	variant	P-514	Strains 57 to 77 were obtained from Dr. D. G. McKercher, University of California.
58	variant	P-542	
59	variant	P-551	
60	variant	P-552	
61	variant	P-571	
62	variant	P-572	
63	variant	P-575	
64	variant	P-554	
65	regular	P-516	
66	regular	P-553	
67	regular	P-555	

Table 1. (concl.)

Strain number as used in this work	Antigenic group	Original strain number	Source
68	regular	P-556	
69	regular	P-557	
70	regular	P-558	
71	no record	P-161	
72	no record	P-280	
73	no record	P-512	
74	no record	P-515	
75	no record	P-581	
76	no record	P-582	
77	no record	P-583	

* Strains which have identical numbers, but different subscripts were isolations from the same infected poultry flock.

EXPERIMENT I
ISOLATION OF BACTERIOPHAGE

It has been reported in the literature that bacteriophage isolated from chicken feces would show lytic activity against _S. pullorum_. The procedure used in this experiment is considered to be an adequate one for the isolation of _S. pullorum_ bacteriophage.

Media

Beef-extract broth, which consisted of Bacto beef-extract three grams, Bacto peptone five grams, and distilled water one thousand milliliters, adjusted to pH 7.0 and one percent beef-extract agar, which was of the same composition plus ten grams of agar, both served as the basic media in this and in the following experiments.

Procedure

The caecal contents of a pullorum infected chicken was emulsified in beef-extract broth in the proportion of approximately one gram of feces to ten milliliters of broth and was incubated at 37° C. for one hour. The supernatant fluid was filtered through a Seitz EK filter. By means of a sterile, bent glass rod, a one-half hour broth culture of _S. pullorum_ was

evenly distributed on the surface of a one percent beef-extract agar plate. After the inoculum had dried, several drops of the crude feces filtrate were scattered upon the agar plate. The crude feces filtrate was stored in the refrigerator and the plate was incubated at 37° C. until bacteriophage plaques were observed.

Results

Attempts were made to obtain bacteriophage from twenty-four individual samples of crude feces filtrates. Bacteriophage was isolated from filtrates twelve, fourteen and twenty-four.

EXPERIMENT II
HEAT KILLING vs. FILTRATION OF LYSED BACTERIAL CULTURES

The lytic action of bacteriophage against bacteria is rarely 100 percent complete. There is always assumed to be a few phage-resistant bacterial host cells present which will cause secondary growth. In the process of bacteriophage typing it is essential that the lysed bacterial cultures be free of any phage-resistant bacteria. Therefore, filtration or some other method of removing the phage-resistant bacteria becomes necessary.

Craigie and Yen (10), and Thomen and Frobisher (41), observed that in the process of filtering lysed bacterial cultures through Seitz, Berkfeld, Chamberland and similar-type filters much of the bacteriophage was lost due to the adsorption of the bacteriophage on the filter. It was found by Craigie and Yen (10) that heating the lysed culture, at the proper temperature for a period of time, would kill the few remaining phage-resistant bacteria and not materially affect the activity of the phage which had a thermal death point a few degrees higher than that of the bacteria.

The purpose of this experiment was, first, to determine the temperature and length of time needed to kill the phage-resistant bacteria and leave the bacteriophage unaffected and, secondly, to determine whether or not the use of Seitz EK filters entailed any loss of phage.

Procedure

<u>Effect of Heat</u>. Three tubes were prepared as follows. Tube one contained 1 ml of a five-hour old culture of *S. pullorum*. Tube two contained 1 ml of bacteriophage and tube three contained 1 ml of each. A second series of three tubes (tubes four, five and six) was prepared in the same manner.

Tubes one, two, and three were heated at 60° C. for one-half hour, and tubes four, five and six were incubated at 37° C. for the same length of time. With the aid of a sterile, bent glass rod, 0.5 ml of the contents of tubes one, three, four, and six were smeared on the surface of agar plates numbered one, three, four and six. Several drops of the contents of tubes two and five were scattered on agar plates numbered two and five which had previously been seeded with a five-hour old broth culture of *S. pullorum*. The plates were observed after incubation at 37° C. for 24 hours.

<u>Seitz Filtration</u>. A broth tube was inoculated with 1 ml of a 24 hour old culture of *S. pullorum* (strain 22). After shaking the broth tube, each of five loopfuls was transferred to individual, circled areas on an agar plate. When the agar had absorbed the fluid which had been applied (optimum five to ten minutes), drops from the "critical test dilution" tube of phage 12 adapted to strain 22 were superimposed on each of

the drops of bacteria. When the fluid had dried sufficiently to permit the plates to be safely moved, they were incubated for two hours at 37° C. and then transferred to the refrigerator overnight. They were returned to the incubator and after five hours the extent of the lytic activity of phage 12 against strain 22 was noted.

The "critical test dilution"* tube of phage 12 was filtered through a Seitz EK filter and the above procedure was repeated using the Seitz-filtered phage. The extent of the lytic activity was again noted.

Results

<u>Effect of Heat</u>. After incubation, growth was observed on plate four but not on plates one and three. Phage plaques were present on plates two, five and six. A thermal death time of 60° C. for one-half hour appeared to kill all the bacteria and to leave the activity of the phage unaffected.

<u>Seitz Filtration</u>. The unfiltered "critical test dilution"

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* The "critical test dilution" is that dilution of phage which under the conditions of the test just produces a sufficient number of plaques to give an area of confluent lysis on its homologous strain. The procedure for obtaining the "critical test dilution" is discussed in Experiment 4.

of phage 12 produced confluent lysis on all five drops of *S. pullorum* (strain 22). The filtered phage produced confluent lysis on only one of the five drops of strain 22. The other four drops each showed only individual phage plaques.

The use of Seitz EK filters does entail some loss of phage.

EXPERIMENT III
ADAPTATION OF PHAGES TO STRAINS OF *SALMONELLA PULLORUM*

Bacteriophages are specific in their action with respect to the kind of bacteria which are susceptible to lysis. In some instances, more than one species of bacteria may be lysed by a single phage and in other cases the activity is highly specific and only a single strain may be lysed. The specificity of certain bacteriophages can be conditioned by the particular strain of bacteria on which they are propagated. By changing the substrate strain a number of differently reacting preparations of bacteriophage may be obtained which show a high relative affinity for their homologous strains. Such preparations, diluted to contain only an effective number of particles, are employed to identify strains similar in type to that on which they were grown.

In this experiment, an endeavor was made to increase the specificity of phages 12, 14, and 24 for various strains of *S. pullorum* by the process of adaptation (selective cultivation) of the phages.

Procedure

A phage plaque for phage 12, 14, and 24 (which were obtained in Experiment I) with the underlying agar and a little of the surrounding intact culture was transferred to

ten ml of broth and heated for 30 minutes at 60° C. Two-tenths ml of each of these heated phage cultures were added to five-hour broth cultures of strains 1 to 24, 79 and 296 of S. pullorum and incubated for 12 hours. At the end of this time the lytic effect of phages 12, 14 and 24 on strains 1 to 24, 79 and 296 was noted. The broth tubes were heated for 30 minutes at 60° C. and two-tenths ml of the lysed bacterial cultures were again added to five-hour broth cultures of strains 1 to 24, 79 and 296. This process of adaptation was repeated three more times and each time the lytic effect was noted.

After the fourth adaptation passage, drops from the heated lysed bacterial cultures were added to agar plates previously seeded with the homologous strains of S. pullorum. The plates were incubated at 37° C. for eight hours, and it was noted on which strains phages 12, 14 and 24 had produced phage plaques.

A phage plaque, produced by adaptation from phage 12, with the underlying agar and surrounding culture was transferred to 10 ml of broth and heated at 60° C. for 30 minutes. Serial ten-fold dilutions of the heated phages from 1 in 10^1 to 1 in 10^{10} were made in 9 ml of broth previously seeded with 1 ml of the homologous strain, diluted so that an estimated 1,000 organisms were added to each tube. The series of tubes was incubated for 12 hours. The last clear tube in the series, representing the highest dilution of phage 12 giving complete

lysis on its homologous strain was heated at 60° C. for 30 minutes and was stored in the refrigerator.

Results

In each of the four steps in the process of adaptation it was noted that complete clearing of the culture tubes occurred in all strains except strains 1, 3, 10, 14, and 79. Culture tubes of strains 1, 3, and 14 showed no lysis whatsoever. These tubes were as turbid as the control tube. Culture tubes of strains 10 and 79 were slightly less turbid than the control tube and thus showed some susceptibility to the phages.

The phages produced plaques on all strains except strains 1, 3, and 14.

The lytic action of phages 12, 14, and 24 in the above lytic tests was the same for all strains of S. pullorum used, indicating that these phages may have been of the same type.

The results of the highest dilution of phage 12 which gave complete lysis in the broth tubes are recorded in Table 2.

Table 2. Results of highest dilution test of adapted phage 12 on 26 strains of *S. pullorum*.

Homologous phage dilutions used	Culture strains																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	79	296
1×10^1	G	-	G	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
1×10^2	G	-	G	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
1×10^3	G	-	G	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
1×10^4	G	-	G	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
1×10^5	G	-	G	-	-	-	-	-	G	-	-	-	-	G	-	-	G	-	-	G	-	-	-	-	-	-
1×10^6	G	-	G	-	-	-	-	-	G	G	-	-	-	G	-	-	-	-	-	G	-	-	-	-	-	-
1×10^7	G	-	G	-	-	G	G	G	G	G	-	-	G	G	-	G	-	G	-	G	G	-	-	-	G	-
1×10^8	G	G	G	G	G	G	G	G	G	G	-	G	G	G	G	G	G	G	G	G	G	G	G	-	G	G
1×10^9	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
1×10^{10}	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G

G = growth indicating absence of complete lysis.

- = clear tubes indicating complete lysis.

EXPERIMENT IV
DETERMINATION OF THE "CRITICAL TEST DILUTION" OF ADAPTED PHAGES

By maintaining a given phage on certain selected strains, the phage becomes so highly adapted or specific for that strain that it will not act on certain other strains of the same species when diluted to its "critical test dilution". The "critical test dilution" is that dilution of phage which under the conditions of the test just produces a sufficient number of plaques to give an area of confluent lysis on its homologous strain. If kept refrigerated when not actually in use the phage, diluted to the "critical test dilution", retains its activity for at least several weeks.

Procedure

Serial ten-fold dilutions of the highest dilution tubes of phage 12 which showed complete lysis from 1 in 10^1 to 1 in 10^{10} were made in 9 ml of broth. For each strain used, 1 ml from a turbid, 24-hour broth culture was inoculated into another tube containing 9 ml of broth. Loopfuls from this latter tube were transferred to ten individual, circled areas on agar plates. When the agar had absorbed the culture, loopfuls from each of the ten serially diluted phage tubes were superimposed on each of the drops of culture. When the plates could be safely

moved, they were incubated for two hours at 37° C. and transferred to the refrigerator overnight. They were returned to the incubator and were ready for reading after five hours. The serial dilutions of phages were refrigerated. It was noted which dilutions of phage produced an area of confluent lysis on its homologous strain.

Results

The results of determining the "critical test dilution" of the phages are tabulated in Table 3.

Table 3. Determination of "critical test dilution" for each adapted phage.

Dilutions of phages used	Adapted phage strains																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	79	296
1×10^{1}	-	C	-	C	C	+	C	+	C	+	+	C	C	-	C	C	C	C	+	+	C	C	+	C	+	+
1×10^{2}	-	C	-	C	C	+	C	+	C	+	+	C	C	-	C	C	C	C	+	+	C	C	+	C	+	+
1×10^{3}	-	C	-	C	C	+	C	+	C	+	+	C	C	-	C	-	C	C	+	+	C	C	+	C	+	+
1×10^{4}	-	C	-	C	C	+	C	+	C	+	+	C	C	-	C	C	C	C	+	+	C	C	+	C	+	+
1×10^{5}	-	C	-	C	C	+	C	+	C	+	+	C	C	-	±	±	C	±	+	±	C	C	+	C	+	+
1×10^{6}	-	±	-	C	C	±	±	±	C	±	+	±	C	-	-	-	C	±	+	-	±	±	+	C	±	+
1×10^{7}	-	±	-	±	-	-	±	±	±	±	±	±	±	-	-	-	±	±	±	-	-	-	±	±	±	±
1×10^{8}	-	-	-	-	-	-	-	-	-	-	-	-	±	-	-	-	-	-	-	-	-	-	-	±	-	-
1×10^{9}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1×10^{10}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

C = confluent lysis.
+ = numerous discrete plaques, subnormal in size.
± = few discrete plaques, normal in size.
- = no lysis.

EXPERIMENT V
BACTERIOPHAGE TYPING OF S. PULLORUM

Having determined the "critical test dilution" for bacteriophage 12 on the homologous strains it has been adapted to, it is now possible to attempt bacteriophage typing of *S. pullorum*.

Procedure

The "critical test dilution" determined for the phages adapted to strains 2, 4, 5, 7, 9, 12, 13, 15, 16, 17, 18, 21, 22, and 24 were the only ones used. One ml of a 24-hour broth culture of the unknown strain, to be tested, was transferred to 9 ml of broth and one loopful of this culture was used in the typing tests.

While the technique for carrying out these tests was simple, a strict attention to certain details seemed to be essential. In brief, the technique was designed to obtain areas of uniform inoculation of the strain on an agar plate on which standard volumes of the phage preparations were superimposed as soon as possible after inoculation and without significantly disturbing the uniformity of bacterial distribution. The agar plates which were used for the tests were free from defects which, causing irregularities in the thickness of the medium, would have resulted in uneven growth of different areas of the culture being

tested. About 15 ml of beef-extract agar were poured in plates and when the agar had solidified the plates were dried at 37° C. for three to five hours and were then stored in the refrigerator. Insufficient drying caused delay in setting up the tests and excessive drying resulted in an uneven distribution of culture and phage.

Two standardized Nichrome wire loops, 2.75 mm in internal diameter, were used alternately in applying the culture and phage to the plate. While one loop was in use, the other was cooling after sterilization. In transferring a loopful of broth culture to the plate, the loop was used merely to direct the natural spreading of the broth and care was taken not to rub it over the agar. The shank of the loop was angled so that the loop was lying parallel with the agar surface during inoculation. A full loopful of broth culture was obtained by sharply moving the culture tube so that the loop quickly broke the surface of the fluid. Then the loop was lowered until the drop held in it just touched the surface of the agar. At this point, the loop was given a slight horizontal circular movement by moving the fingers, guiding the spreading of the drop over an area approximately 15 mm in diameter. A number of circled area, corresponding to the number of phage preparations used in the test, were inoculated with each strain.

When the agar had absorbed the fluid which had been applied (optimum five to ten minutes) the phage was applied from the standard loop to the center of the inoculated area. The drop of phage was allowed to spread naturally on the plate and actual contact of the loop and inoculated area was avoided.

When the fluid which had been applied had dried sufficiently to permit the plates to be safely moved, they were incubated for two hours at 37° C. and then transferred to the refrigerator overnight. They were returned to the incubator and were ready for reading in four to six hours. The method of interrupted incubation had two advantages. It permitted the phage reactions to be observed at their optimum and before they were obscured by late growth and also the intermediate period in the refrigerator permitted diffusion of the phage into the surrounding normal culture, thus enhancing the clarity of the reactions.

Results

The results of the reactions, which were observed by oblique artificial illumination against a dark background, are recorded in Table 4.

Table 4. The reactions of strains of Salmonella pullorum to "critical test dilutions" of phage twelve preparations.

Phage twelve preparations	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Ø 12/2 *	-	C	-	C	C	+	C	+	C	+	+	C	C	-	C	C
Ø 12/4	-	C	-	C	C	±	±,	±	C	±	-	C	±	-	C	C
Ø 12/5	-	C	-	C	C	±	C	±	C	+	+	C	C	-	C	C
Ø 12/7	-	C	-	C	C	+	C	+	C	+	+	-	C	-	-	C
Ø 12/12	-	C	-	C	C	+	C	+	C	+	+	C	C	-	C	C
Ø 12/13	-	±	-	±	C	±	±	±	±	±	-	±	C	-	±	±
Ø 12/15	-	C	-	C	C	±	C	+	C	±	±	C	C	-	C	C
Ø 12/16	-	C	-	C	C	+	C	+	C	+	+	C	C	-	C	C
Ø 12/17	-	C	-	C	C	+	C	+	C	+	+	C	C	-	C	C
Ø 12/18	-	C	-	C	C	+	C	+	C	+	+	C	C	-	C	C
Ø 12/21	-	C	-	+	C	+	C	-	C	+	+	C	C	-	C	C
Ø 12/22	-	C	-	C	C	+	C	+	C	+	+	C	C	-	C	C
Ø 12/24	-	C	-	C	C	+	C	+	C	+	+	C	C	-	C	C

Phage twelve preparations	17	18	19	20	21	22	23	24	79	296	25	26	27	28	29	30	31	32	33	34	35	36	37
Ø 12/2 *	+	C	C	±	C	C	C	C	+	+	C	C	+	C	-	C	C	-	C	C	C	C	C
Ø 12/4	+	C	+	+	±	±	±	±	+	+	C	C	+	C	-	C	C	-	C	C	C	C	C
Ø 12/5	+	C	+	+	C	C	+	C	+	+	C	C	+	C	-	C	C	-	C	C	C	C	C
Ø 12/7	+	C	+	+	C	C	+	C	+	+	C	C	+	C	-	C	C	-	C	C	C	C	C
Ø 12/12	C	C	+	+	C	C	+	C	+	+	C	C	+	C	-	±	±	-	C	C	C	C	C
Ø 12/13	±	±	±	+	C	C	+	±	+	+	C	C	+	C	-	C	C	-	C	C	C	C	C
Ø 12/15	C	C	+	+	C	C	+	C	+	+	C	C	+	C	-	C	C	-	C	C	C	C	C
Ø 12/16	+	C	+	+	C	C	+	C	+	+	C	C	+	C	-	C	C	-	C	C	C	C	C
Ø 12/17	C	C	+	+	C	C	+	C	+	+	C	C	+	C	-	C	C	-	C	C	C	C	C
Ø 12/18	+	C	+	+	C	C	+	C	+	+	C	C	+	C	-	C	C	-	C	C	C	C	C
Ø 12/21	C	C	+	+	C	C	+	±	+	+	C	C	+	C	-	C	C	-	C	C	C	C	C
Ø 12/22	+	C	+	+	C	C	+	C	+	+	C	C	+	C	-	±	±	-	C	±	±	C	C
Ø 12/24	+	C	+	+	C	C	+	C	+	+	C	C	+	C	-	C	C	-	C	C	C	C	C

* Abbreviation for, bacteriophage 12 adapted to substratum strain 2.

C = confluent lysis.
+ = numerous discrete plaques, subnormal in size.
± = few discrete plaques, normal in size.
- = no lysis.

38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77
C	C	C	C	C	-	C	C	C	C	C	C	C	+	C	C	C	C	+	C	C	C	C	C	-	+	C	C	C	+	C	C	C	C	+	C	C	C	+	+
C	C	C	C	C	-	C	C	C	C	C	C	C	+	C	C	C	C	+	C	C	C	C	C	-	+	C	C	C	+	C	C	C	C	+	C	C	C	+	+
C	C	C	C	C	-	C	C	C	C	C	C	C	+	C	C	C	C	+	C	C	C	C	C	-	+	C	C	C	+	C	C	C	C	+	C	C	C	+	+
C	C	C	C	C	-	C	C	C	C	C	C	C	+	C	C	C	C	+	C	C	C	C	C	-	+	C	C	C	+	C	C	C	C	+	C	C	C	+	+
C	C	C	C	C	-	C	C	C	C	C	C	C	+	C	C	C	C	+	C	C	C	C	C	-	+	C	C	C	+	C	C	C	C	+	C	C	C	+	+
C	C	C	C	C	-	C	C	C	C	C	C	C	+	C	±	±	C	+	C	+	C	C	C	-	+	C	C	C	+	C	C	C	C	+	C	C	C	+	+
C	C	C	C	C	-	C	C	C	C	C	C	C	+	C	C	C	C	+	C	C	C	C	C	-	+	C	C	C	+	C	C	C	C	+	C	C	C	+	+
C	C	C	C	C	-	C	C	C	C	C	C	C	+	C	C	C	C	+	C	C	C	C	C	-	+	C	C	C	+	C	C	C	C	+	C	C	C	+	+
C	C	C	C	C	-	C	C	C	C	C	C	C	+	C	C	C	+	+	C	C	C	C	C	-	+	C	C	+	+	C	C	C	C	+	C	C	C	+	+
C	C	C	C	C	-	C	C	C	C	C	C	C	+	C	C	C	+	+	C	C	C	C	C	-	+	C	C	C	+	C	C	C	C	+	C	C	C	+	+
C	C	C	C	C	-	C	C	C	C	C	C	C	+	C	C	C	C	+	C	C	C	C	C	-	+	C	C	C	+	C	C	C	C	+	C	C	C	+	+
C	C	C	C	C	-	C	C	C	C	C	C	C	+	C	C	C	C	+	C	C	C	C	C	-	+	C	C	C	+	C	C	C	C	+	C	C	C	+	+
C	C	C	C	C	-	C	C	C	C	C	C	C	+	C	C	C	C	+	C	C	C	C	C	-	+	C	C	C	+	C	C	C	C	+	C	C	C	+	+

DISCUSSION

The main objective of this work was to identify or classify strains of Salmonella pullorum by the use of the bacteriophage typing method. "Critical test dilutions" of bacteriophage 12 which had undergone the process of "adaptation" to thirteen strains (i.e. strains 2, 4, 5, 7, 12, 13, 15, 16, 17, 18, 21, 22 and 24) of S. pullorum were used in the actual typing test.

As was shown in Table 4, these supposedly different "adapted" phage preparations did not exhibit any specificity to their homologous strains in preference to the heterologous strains upon which they were tested. For example, phage preparation $\frac{\phi 12}{2}$ produced confluent lysis on strain 2, which was to be expected, but also produced confluent lysis on heterologous strains 4, 5, 7, 9, 12, 13, 15, 16, 18, 19, 21, 22, 23, and 24. All the other phage preparations exhibited similar results. If the phage preparations had been highly specific only for their homologous strains and not for the heterologous strains, then $\frac{\phi 12}{2}$ phage preparation would have produced confluent lysis only on strain 2. Thus, distinguishing strains similar to strain 2 would be highly practicable. Since those strains of S. pullorum which were very susceptible to lysis seemed to exhibit confluent lysis to most, and in some cases to all, of the phage preparations

used, it must be concluded that it is almost impossible to distinguish type differences among those strains which are easily lysed.

There may be some explanation for the production of confluent lysis by all the phage preparations on all the lysable strains of _S. pullorum_.

First, the bacteriophage used, bacteriophage 12, may not have been capable of developing a high lytic activity for the strains of _S. pullorum_ on which it was propagated. If this be correct, then by changing the substrate strain of _S. pullorum_ a number of differently reacting preparations of the original phage would not be obtained. The different phage preparations used may have actually been one distinct type of phage (i.e. the originally isolated bacteriophage 12). As was mentioned in Experiment III, difficulty was encountered in adapting bacteriophage 12 to strains 1, 3, 10, 14, and 79. At that time, further attempts were made to isolate bacteriophage from chicken feces. However, phages 14 and 24, which were eventually isolated, proved to be similar to phage 12 in their inability to adapt themselves to strains 1, 3, 10, 14, and 79. Lack of time and the difficulty encountered in isolating bacteriophage prevented the isolations of other native phages. The inability of bacteriophage 12 to become adapted to strains was also encountered

in the determination of the "critical test dilutions". "Critical test dilutions" were easily obtained for strains 2, 4, 5, 7, 12, 13, 15, 16, 17, 18, 21, 22, and 24. The "critical test dilutions" could not be obtained from the other strains as is shown in Table 2.

The use of a more adaptable phage, if it exists, which would exhibit specificity only for its homologous strains might enable one to distinguish strain differences among the lysable strains of _S. pullorum_.

A second explanation which might be offered is that the process of tenfold dilution of the phage used to obtain the critical test dilution may not have been the highest dilution of the phage needed to produce confluent lysis on its homologous strain. The "critical test dilutions" used may have been overly concentrated with phage to the degree that it was active on all lysable strains.

A dilution intermediate between the "critical test dilution" and the next highest tenfold dilution may have made the phage preparations more selective.

If one assumes that all the strains of _S. pullorum_, on which most of the phage preparations had produced confluent lysis, are one and the same strain, then no explanation would be necessary.

As is shown in Table 4, most of the phage preparations used classified 79 strains of S. pullorum into three, more or less, distinct types. Type I which shows confluent lysis by most of the preparations; and Type II which shows partial lysis by most of the preparations; and Type III which is not lysed by any of the phage preparations.

Upon comparison of Table 4 with Table 1, two facts are to be noted. First, the three serological types of S. pullorum have no relationship with the three types as distinguished by bacteriophage typing in this work. Of the 79 strains used, one strain (strain 28) was an "intermediate" type; ten strains (strains 20, 29, 32, 36, 65, 66, 67, 68, 69, and 70) were "regular" types; and sixteen strains (strains 79, 296, 27, 30, 31, 33, 34, 35, 57, 58, 59, 60, 61, 62, 63, and 64) were "variant" types. The one "intermediate" strain used belonged to Type I. Of the ten "regular" strains used, six strains belonged to Type I, two strains belonged to Type II and the remaining two strains belonged to Type III. Of the sixteen "variant" strains used, eleven belonged to Type I, four belonged to Type II, and one belonged to Type III.

The second fact to be noted is that some strains of S. pullorum which were isolated from the same infected poultry flock were found to belong to different bacteriophage types.

Four individual groups of three isolations from the same infected flock were used. In the first group (strains 1, 2, and 3), one belonged to Type I and the remaining two were Type III. In the second (strains 4, 5, and 6) and third groups (strains 7, 8, and 9), two were of Type I and one of Type II. In the last group (strains 10, 11, and 12), one was of Type I and two were of Type II.

Tabulation of the results listed in Table 4 gives some idea as to the incidence of the three bacteriophage types of S. pullorum. Of the 79 strains examined, 54 strains were of Type I; 18 strains were of Type II and 7 strains were of Type III.

SUMMARY

1. Bacteriophage was isolated which lysed most, but not all, of the 79 strains of *Salmonella pullorum* tested.

2. The bacteriophage typing method is outlined.

3. The 79 strains of *S. pullorum* tested with the "critical test dilutions" of "adapted" phage preparations were classified into three distinct types: Type I which showed confluent lysis by most of the phage preparations; Type II which showed only partial lysis by most of the preparations; and Type III which was resistant to the lytic action of all the phage preparations.

4. Explanations were presented as to why subdivisions of Type I strains were not elicited.

5. The three *serological* types of *S. pullorum* showed no relationship to the three *bacteriophage* types.

6. Strains of *S. pullorum* isolated from the same infected poultry flocks were found to belong to different bacteriophage types.

7. Because of the mixture of bacteriophage types found in the *same* infected poultry flock, it is doubtful if bacteriophage typing of isolated strains could be used for tracing epizootics of pullorum disease. If further expansion of this work reveals additional types, this method may then prove of value in epidemiological studies.

ACKNOWLEDGMENT

The writer gratefully expresses his sincerest gratitude to Dr. Thomas H. Lord for his highly constructive criticisms and kind assistance during the progress of this research and writing of the thesis.

Appreciation is also extended to Dr. Lester E. Erwin, Kansas State College; Dr. G. H. Snoeyenbos, University of Massachusetts; The Department of Bacteriology, Michigan State College; Dr. Stanley E. Wedberg, University of Conn.; and Dr. D. G. McKercher, University of California, who aided materially by furnishing the cultures used in this work.

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BACTERIOPHAGE TYPING OF SALMONELLA PULLORUM

by

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B. S., Brooklyn College, 1950

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1952

Many investigators have noted that phages often demonstrate a selective action for certain strains of organisms within a given species, and evidence has accumulated which shows this specificity to be related to the antigenic pattern of the susceptible bacteria. Through the use of phages, it is sometimes possible to demonstrate minor differences between bacterial strains which are not detectable by the usual serological procedures. A suitable collection of phages should therefore provide a sensitive method of differentiating culture strains.

The method of bacteriophage typing offers certain advantages over the serological tests and the carbohydrate fermentation reactions. It is quicker, the bacteriophage adapting itself to a bacterial substratum in much less time than an animal can be immunized; it does not require a large number of different and complicated media, as carbohydrate tests do; and no laboratory animals are required, the bacteria being the experimental organisms. Once adapted to a certain species or race of bacteria, bacteriophage can be kept in the refrigerator the same as is done with sera, and used for typing at a later time.

Since Salmonella pullorum is susceptible to bacteriophage and since the present system of classification and identification of strains of S. pullorum is not wholly satisfactory it was felt desirable to explore the possibility of using bacteriophage typing as a means of classifying or identifying strains of S. pullorum.

The caecal contents of a pullorum infected chicken was

emulsified in broth in the proportion of approximately one in ten and the mixture was incubated at 37° C. for one hour. At the end of this time it was filtered through paper and then through a Seitz EK filter. The crude feces filtrate obtained was scattered upon the surface of an agar plate which had previously been seeded with a young, actively growing culture of S. pullorum. After incubation, for about 18 hours, bacteriophage plaques were observed.

A thermal death time of 60° C. for 30 minutes appeared to kill all phage-resistant bacteria and not to affect materially the activity of the phage. Since the use of Seitz EK filters was found to entail some loss of phage, heating at 60° C. for 30 minutes was used instead of Seitz filtration to remove phage-resistant bacteria from the lysed bacterial cultures throughout the typing program.

The bacteriophages isolated were "adapted" (selectively cultivated) on 26 strains of S. pullorum. It was hoped that by changing the substrate strain, a number of differently reacting preparations of phage would be obtained which would show a high relative affinity for their homologous strains.

"Critical test dilutions" of phage 12 preparations which had undergone the process of "adaptation" to 13 strains of S. pullorum were used in typing 79 strains of S. pullorum. These supposedly different "adapted" phage preparations did not exhibit any specificity to their homologous strains in preference to the heterologous strains upon which they were

tested, making it almost impossible to distinguish type differences among those strains which are easily lysed.

The 79 strains of S. pullorum tested were classified into three, more or less, distinct types. Type I which showed confluent lysis by most of the phage preparations; Type II which showed only partial lysis by most of the preparations; and Type III which was resistant to the lytic action of all phage preparations. Explanations were presented as to why subdivisions of Type I strains were not elicited.

Of the 79 strains of S. pullorum examined, 54 strains were of Type I; 18 strains were of Type II; and 7 were of Type III.

It was found that the three serological types of S. pullorum showed no relationship to the three bacteriophage types as distinguished in this work.

Strains of S. pullorum isolated from the same infected poultry flocks were found to belong to different bacteriophage types. Because of the mixture of bacteriophage types found in the same infected poultry flock, it is doubtful if bacteriophage typing of isolated strains could be used for tracing epizootics of pullorum disease. If further expansion of this work reveals additional types, this method may then prove of value in epidemiological studies.