Identification of Salmonella with the O-1 Bacteriophage

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Received for publication 18 June 1974

The O-1 bacteriophage test of Cherry et al. (1954) for the presumptive identification of salmonellae in the diagnostic laboratory was investigated. A phage lysate with a titer of $10^{12}$ plaque-forming units per ml was found to be optimal. This preparation lysed 98.2% of Salmonella strains tested, while maintaining its high specificity for salmonellae. Gram-negative organisms other than salmonellae were resistant to the O-1 phage; however, 5.9% of Escherichia coli strains tested were susceptible. The O-1 phage test is a simple, rapid, inexpensive, sensitive, and specific procedure for the identification of salmonellae in the diagnostic laboratory. A presumptive identification is obtained 1 day earlier than with conventional biochemical tests.

Methods currently used for the detection of Salmonella in clinical specimens include biochemical tests, slide agglutination, and fluorescent antibody techniques. Cherry et al. (5) first demonstrated the value of the bacteriophage O-1 of Felix and Callow (6) for the identification of salmonellae. A number of subsequent reports (7, 11–13, 15, 17) concluded that the simplicity and rapidity, as well as specificity, of the O-1 phage test contributed significantly to an improved screening procedure for these pathogens. In a number of European laboratories (7, 12, 15), the O-1 phage is used routinely for the presumptive identification of salmonellae. In view of the continued or even increasing importance of salmonellae infections, it was considered worthwhile to examine the usefulness of the O-1 phage test in our clinical laboratory. In addition, optimal conditions for performing the test were examined.

MATERIALS AND METHODS

The bacteriophage O-1 and its propagating strain Salmonella paratyphi B76 were obtained from W. B. Cherry, Center for Disease Control (CDC), Atlanta, Ga.

Test organisms. A total of 603 Salmonella strains was received from the Bureau of Laboratories, Florida State Board of Health; 13 strains were obtained from the CDC; and 36 strains were obtained from the Clinical Microbiology Laboratories, Shands Teaching Hospital and Clinics (STH), Gainesville, Fla. A total of 1,463 strains of freshly isolated gram-negative bacteria other than salmonellae was obtained from the clinical laboratories of Shands Teaching Hospital; 12 strains of E. coli O12 were received from George Herman of the CDC.

Propagation of the phage. The bacterial inoculum was standardized spectrophotometrically. Portions of a growing broth culture of the propagating strain were removed at timed intervals, and the adsorbancy was read on a Coleman Junior II spectrophotometer. Viable counts were performed in Trypticase soy agar (TSA; Difco) pour plates.

For propagation in broth, overnight cultures of S. paratyphi B76 in Trypticase soy broth (TSB; Difco) were diluted in TSB to approximately $0.5 \times 10^{9}$ to $1.0 \times 10^{8}$ bacteria/ml. Phage was added to the broth culture of the propagating strain to yield an input multiplicity of 1, and the mixture was incubated for 6 h at 37 °C in a shaking water bath (60 rpm) (8). The lysate obtained was then centrifuged at 10,000 rpm for 30 min to remove cells and debris. The supernatant fluid was decanted and filtered, and 1% chloroform was added. The lysate was titrated on the propagating strain by using 1.5% TSA and stored at 4 °C as the stock suspension (8).

For propagation in soft-agar overlay, the propagating strain was grown in TSB to a concentration of approximately $2 \times 10^{9}$ bacteria/ml. A phage suspension was diluted to $0.5 \times 10^{4}$ to $1.0 \times 10^{7}$ plaque-forming units (PFU)/ml, and 0.1 ml of this dilution was added to tubes containing 2 ml of molten 0.6% TSA; 1 drop of bacterial broth culture was added to each tube, yielding a multiplicity of $0.5 \times 10^{-3}$ to $1.0 \times 10^{-3}$ phage/bacterium. The mixture of phage and bacteria in soft agar was poured over the surface of a 1.5% TSA plate. The agar overlays were allowed to solidify, and the plates were then incubated at 37 °C. After overnight incubation, semiconfluent to confluent lysis was observed. The soft-agar layers were scraped into centrifuge tubes by using a Teflon policeman. Two milliliters of M-9 medium per plate (1) was added, and the tubes were placed on a Vortex mixer to disrupt pieces of agar. The lysates were centrifuged at 10,000 rpm for 30 min and filtered, and 1% chloroform was added. These lysates were then titrated on the propagating strain and kept at 4 °C.

When necessary, the lysates were concentrated by
dialysis against polyethylene glycol 20,000 (Fisher
Scientific Co.) (16). The concentrate was then
dialyzed against phosphate-buffered saline (pH 7.2 to
7.4) for several hours, filtered, and stored at 4 C with
chloroform.

Performance of phage test. Strains of Salmonella
were tested for susceptibility to the O-1 phage by a
spot test. TSA plates containing 1.0% agar (12) were
marked with a grid of eight sections, and a drop of
TSB was added to each section. An isolated colony
of each test strain, growing either on MacConkey or SS
agar, was picked with a loop and emulsified in a drop
of broth on the TSA plate. The bacterial suspension
was spread to cover an area of about 2-cm diameter
and allowed to dry. A small drop of phage lysate was
placed on the center of the inoculated area by using a
Pasteur pipette with a fine tip. The phage lysate
usually covered about one-half of the diameter of the
area inoculated with bacteria. After incubation at
37 C for 4 to 5 h, the plates were examined for lysis.
Lysis reactions were recorded as follows: 4+, confluen-
tent lysis; 3+, nearly confluent lysis; 2+, semiconfluen-
tent lysis; 1+, irregular inhibition of growth; 0+, a few
plaques or some thinning of growth; and 0, no lysis.
Nearly confluent lysis was defined as complete lysis
except for a slight haze of secondary growth, probably
due to resistant organisms. Semiconfluent lysis con-
sisted of a uniform thinning of bacterial growth. This
thinning of growth was not as extensive as observed
with a 3+ reaction. Irregular inhibition of growth was
rarely observed. In the few cases where it occurred,
one-half to two-thirds of the spot test area revealed
normal bacterial growth, but semiconfluent to confluen-
tent lysis was observed in the remaining area (12). A
2+ to 4+ lytic response was considered a positive
result, and equivocal or doubtful results were re-
peated. The area of undisturbed bacterial lawn at the
periphery of the area of growth was used for confirma-
tory biochemical tests.

RESULTS

Titers of lysates. Propagation of the bacte-
riophage O-1 in broth yielded lysates with titers
ranging from $0.1 \times 10^{11}$ to $1.5 \times 10^{11}$ PFU/ml.
Attempts to obtain lysates of higher titer by
variation of input multiplicity, addition of yeast
extract and CaCl$_2$ (1) to the medium, and
incubation of the flasks containing the propaganda
tion mixture with shaking and/or aeration were
unsuccessful.

In contrast, when the soft-agar overlay
method (1) was used, lysates with a titer of
$10^{12}$ PFU/ml could be obtained consistently. Further
concentration was readily achieved by dialysis
against polyethylene glycol. Several lysates con-
taining $10^{12}$ PFU/ml were kept at 4 C for 6
months without loss of titer.

Factors influencing the spot test results.
Spot tests performed on TSA plates contain-
ing 1.0 and 1.5% agar were compared. It was found
that at the lower agar concentration,
positive reactions were detected more readily
because lysis was more complete due to forma-
tion of larger plaques, and the medium was
more translucent.

The optimal time for incubation of spot tests
was investigated. Fifty Salmonella strains with
a range of 0 to 4+ reactions to the phage were
tested again with a phage lysate of $10^{11}$ PFU/ml.
The results were read after 5 h of incubation and
at 2-h intervals thereafter through a 24-h pe-
period. None of the strains tested showed a
stronger lytic response after incubation for more
than 5 h. A decrease in the number of positive
results from 64 to 48% was actually ob-
erved upon prolonged incubation due to over-
growth by phage-resistant salmonellae. Most of
the strains demonstrated a gradual decrease in
lysis reaction; however, the reaction of one
group D organism decreased from 4+ after 5 h of
incubation to 1+ after 7 h of incubation.

Sensitivity of Salmonella to the O-1 phage.
Three lysates of increasing titer were tested
against 653 Salmonella strains belonging to
several O groups (Table 1). Although most
groups were highly susceptible to lysis by prepa-
rations of $6 \times 10^{10}$ and $1.0 \times 10^{11}$ PFU/ml, the
majority of the group E strains were lysed only
by the lystate with the highest titer. The overall
sensitivity of Salmonella strains increased to
98.2% positive when a lystate of $10^{12}$ PFU/ml was
used. The difference between the results ob-
tained with lysates of $10^{11}$ and $10^{12}$, respec-
tively, was significant ($P < 0.001$), using the
chi-square test (10). The 27 Salmonella strains
found to be resistant to a phage lysate with $10^{11}$
PFU/ml were reexamined by using lysates of
higher titer. A lystate with $10^{12}$ PFU/ml resulted
in a significant increase in lysis of group E
strains (Table 2). The use of lysates with a titer of
greater than $10^{12}$ PFU/ml, obtained by poly-
ethylene glycol concentration, resulted in the
lysis of only one additional strain.

Susceptibility of various gram-negative or-
ganisms to the O-1 phage. To determine the
specificity of the bacteriophage O-1 assay, vari-
gous gram-negative organisms other than sal-
monellae were tested for their susceptibility to
the phage. A total of 1,463 clinical isolates were
tested with lysates of $6 \times 10^{10}$ and $1 \times 10^{12}$
PFU/ml. The following strains were tested and
found to be resistant to lysis: one strain of
Acinetobacter lwoffi; 12 of Acinetobacter ani-
tratus; three of Aeromonas spp.; three of Al-
calescens dispar; 10 of Citrobacter freundii; 85
of Enterobacter cloacae; six of E. liquefaciens;
60 of E. aerogenes; two of E. hafniae; 238 of
Klebsiella pneumoniae; two of K. ozanense; 308 of
Proteus spp.; three of Providencia spp.; 246 of
Pseudomonas spp.; 18 of Serratia marcescens; and five of unclassified gram-negative organisms.

Of 222 strains of Escherichia coli tested with a phage preparation of $6 \times 10^{10}$ PFU/ml, 10 (4.5%) were positive. Fourteen of 239 (5.9%) different strains of E. coli tested with $10^{12}$ PFU/ml were susceptible. These susceptible strains were biochemically typical, with the exception of one indole-negative strain, and all except three strains fermented lactose promptly. Of importance was the observation that only two strains were lactose negative on MacConkey agar. The 24 E. coli strains were highly sensitive (3+ to 4+) to undiluted phage lystate, but their sensitivity to 10-fold dilutions of the phage preparations varied significantly; some strains were as susceptible to the O-1 phage as the propagating strain, paratyphi B76. In contrast, a few E. coli strains gave confluent lysis with the undiluted lysates only. Among the 24 O-1 phage susceptible E. coli strains, 18 had undetermined O antigens or were rough strains; one strain each was of serotype O148 and O10, and four strains had the O12 antigen. Of 12 additional E. coli O12 strains received from the CDC, four (33.3%) were lysed by the phage. The phage susceptibility of these strains was significantly greater than that of the general population of 461 E. coli strains ($P < 0.05$) by the binomial test (10).

**DISCUSSION**

Our results confirmed the previously reported (7, 11–13, 15, 17) sensitivity, specificity, and rapidity of the bacteriophage O-1 test for the screening of salmonellae. A high-titer preparation of phage lysed 98.2% of Salmonella tested, whereas only Arizona and 5.9% of E. coli strains examined gave false positive results. The test was simple to perform and gave results after only 4 to 5 h of incubation.

Although most investigators use some form of spot test assay, a number of variations in the methodology have been reported, including the titer of the phage preparation, the medium used, the length of incubation, and the interpretation of the results (3, 5, 7, 12, 15). Although

| Table 1. Lysis of Salmonella strains by O-1 phage preparations |
|---------------------------------------------------------------|
| Group | No. positive strains/total no. at lysate concn of: |
|       | $6 \times 10^{10}$ PFU/ml | $10^{11}$ PFU/ml | $10^{12}$ PFU/ml |
|-----------------|-----------------|-----------------|-----------------|
| A               | 2/2 (100)*      | 2/2 (100)       | 2/2 (100.0)     |
| B               | 92/104 (88.5)   | 173/176 (98.3)  | 261/263 (99.2)  |
| C               | 27/27 (100)     | 48/48 (100)     | 71/71 (100.0)   |
| C'              | 33/41 (80.5)    | 75/76 (98.7)    | 122/122 (100.0) |
| D Salmonella typhi | 30/36 (83.3)   | 94/100 (94)     | 136/141 (96.4)  |
| E               | 4/4 (100)       | 38/38 (100)     | 43/43 (100.0)   |
| F-H             | 2/9 (22.2)      | 8/23 (34.8)     | 26/30 (86.7)    |
| Miscellaneous Salmonella strains | 4/4 (100) | 10/10 (100)    | 13/13 (100)     |
| Arizona strains | 1/1 (100)       | 4/6 (66.7)      | 8/9 (88.9)      |
| Total Salmonella strains | 191/224 (85.3) | 414/441 (93.9) | 640/652 (98.2)  |

* Numbers in parentheses indicate percentage of positive strains.

| Table 2. Effect of high-titer O-1 phage preparations on 27 Salmonella strains resistant to phage lystate with $10^{11}$ PFU/ml |
|----------------------------------------------------------------------|
| Group | Total no. of strains tested | 10$^{11}$ PFU/ml | $6 \times 10^{11}$ PFU/ml | $10^{12}$ PFU/ml | $3 \times 10^{11}$ PFU/ml | $6 \times 10^{12}$ PFU/ml |
|-------|-----------------------------|-----------------|-----------------|----------------|----------------|----------------|
| B     | 3                           | 0               | 2               | 2              | 3              | 3              |
| C'    | 1                           | 0               | 1               | 1              | 1              | 1              |
| D     | 6                           | 0               | 1               | 1              | 1              | 1              |
| E     | 15                          | 0               | 3               | 13             | 13             | 13             |
| Pool I| 1                           | 0               | 0               | 1              | 1              | 1              |
| Rough | 1                           | 0               | 0               | 0              | 0              | 0              |
| Total no. of strains: | 27                         | 0 (0.0%)*      | 7 (25.9%)      | 18 (66.7%)     | 19 (70.4%)     | 19 (70.4%)     |

* Numbers in parentheses indicate percentage of the 27 strains yielding positive reactions.
most reports demonstrated the O-1 phage test to be very sensitive and specific, there were variations in the number of resistant salmonellas encountered and in the number of positive results obtained with strains other than salmonellas. It is difficult to analyze the reported data with the intention of selecting an optimal technique for the O-1 phage test, since the results are obviously influenced by the selection of the Salmonella strains tested. If the test material contained a large proportion of epidemiologically related strains, the results would be biased depending on whether the epidemic strain happened to be sensitive or resistant to the O-1 phage. For this reason, a group of Salmonella strains was selected for this study that had been collected over a period of about 2 years in a large state Salmonella reference laboratory, to insure that strains from a single outbreak did not predominate.

On the basis of this study we feel that the following methodology for the O-1 phage is optimal.

(i) **Titer of phage preparation.** The best results were obtained with a lysate of a titer of 10^{12} PFU/ml, which gave a significantly higher number of positive results with Salmonella strains than the usually used lysate of about 1 \times 10^{11} to 2 \times 10^{11} PFU/ml. The superiority of the high-titer lysate was marked with Salmonella strains of group E. It seems likely that most group E strains were attacked by lysin from without, which requires very high phage titers (12). Further increases in the lysate titer over 10^{12} PFU/ml did not significantly increase the sensitivity of the assay. Of importance was the finding that a titer of 10^{12} PFU/ml did not produce a higher number of false positive results. Since the great majority of the susceptible *E. coli* strains were lactose positive on MacConkey agar, they did not constitute a significant problem in the routine application of the O-1 phage test. The majority of the phage-susceptible *E. coli* strains were O nontypeable, but four of six typeable strains had the O antigen 12. This increase O-1 phage susceptibility suggests that there may be antigenic similarities between the core region of the O antigen of *E. coli* O12 and of salmonellae (14).

It has been noted by several investigators (3, 5, 7, 12, 17) that diphasic Arizona strains are highly susceptible to lysis by bacteriophage O-1. Because of their close taxonomic relationship to Salmonella and the similar pathogenicity of these two organisms, a false positive phage test caused by Arizona strains appears to be of little consequence.

Another advantage of the use of the high-titer lysate was the observation that all susceptible Salmonella strains gave strong reactions of lysis. There were no reactions showing irregular lysis, thinning of growth, or the presence of individual plaques. This facilitated interpretation of the test results and avoided ambiguity.

Finally, a stable lysate with a titer of 10^{12} PFU/ml was obtained consistently by a standard method of phage preparation, the soft-agar overlay technique.

(ii) **Medium for O-1 phage test.** An agar concentration of 1.0% was somewhat superior to 1.5%, since the plaques tended to be bigger, the lysis reactions stronger, and the medium more translucent. Thus, the test was easier to read and interpret.

(iii) **Length of incubation.** Some authors have used an incubation of 18 to 24 h for the O-1 phage test. This negates one of the main assets of the test, i.e., furnishing results faster than the standard biochemical methods. Our results demonstrated that incubation beyond 4 to 5 h actually led to a reduction in the number of positive results, likely due to overgrowth of phage-resistant organisms. The short incubation period also avoids overgrowth of the test plates by swarming proteus (7, 8).

The advantages of the spot test for Salmonella identification can be summarized as follows. (i) The test has a high specificity and sensitivity for identification of salmonellae. (ii) The spot test is simple and practical to perform and interpret, and it is economical with respect to supplies and time required. Hence, a large number of cultures can be processed, and more colonies can be tested with the phage. (iii) The results of the phage test are available within 4 to 5 h, compared with 24 h for conventional biochemical tests.

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