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Reduced Set of Phages for Typing Salmonellae

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A set composed of 27 phages is described for differentiating *Salmonella* spp. representative of groups A, B, C_1, C_2, D_1, D_2, E_1, E_2, E_3, E_4, G_1, K, and N. All of the 1,245 cultures used in this effort were typable and were differentiated on the basis of the 420 phage patterns observed. All results were reproducible. Characteristic phage patterns were produced by a variety of *Salmonella* serovars isolated from campus incidents and a number of hospital, family, restaurant, and processing plant outbreaks to indicate an existing epidemiological relationship.

Salmonella spp. were first identified in the last half of the 19th century and have since troubled health authorities everywhere. Salmonellosis is a widely disseminated disease affecting both man and animals and is far more widespread than is generally appreciated. An estimated two to four million people are infected annually in the United States alone, and according to recent reports, the incidence of *Salmonella* infection is in ascendancy around the world. Though the ubiquitous nature of these microorganisms appears to emphasize the futility of total eradication, these infections can be prevented if the presence of the offending organism is established and control measures are implemented accordingly.

More than 2,000 *Salmonella* serovars have now been recognized and cataloged, and new isolates continually supplement this already numerically impressive collection. The number of existing serovars in itself is not particularly disturbing, for it can actually be epidemiologically useful, especially if the serovar is uncommon and not widely encountered. Some cultures, however, are quite common and cannot be adequately characterized serologically for epidemiological application. Under these circumstances, measures must be taken to further delineate the types involved, and here, phage typing is an invaluable adjunct. The procedure is, however, complex and probably most appropriate for a reference laboratory.

In keeping with our own diagnostic interests and to contribute to a growing technology, we developed phage sets for a number of serovars such as *Salmonella anatum, S. binza, S. enteritidis, S. heidelberg, S. newport, S. senftenberg,* and *S. thompson* (3–9). In the course of our activities it was observed that a phage intended for a given serovar could also be used to characterize isolates that were not serologically identical or that did not even belong to the same serological group. Given this revelation, phages that were originally intended for specific serovars were incorporated into a single set consisting of 50 phages and used to characterize *Salmonella* isolates indiscriminately (10).

In recent months, in an effort to make our phage typing procedure more cost effective and less time-consuming, a combinatorial evaluation of our phages and phage patterns was conducted, and it was determined that a reduced set of 27 phages would be more practical for our purpose and could be used without compromising any significant attributes (Table 1).

MATERIALS AND METHODS

Media. Nutrient agar and broth were used exclusively for testing phage filtrates and phage typing. Before use, agar plates were dried in an incubator for 2 h with lids partially opened. Nutrient agar, nutrient broth, and nutrient broth with 0.5% NaCl and 0.7% agar were used for phage propagation. In all of our procedures the incubation temperature was 37°C.

Bacterial cultures. The host cultures used for phage recovery were isolated from cases of human gastroenteritis and diseased animals of avian and bovine origin, and recognizing that phages do exhibit indigenous preferences, efforts were made to use cultures (and sewage samples) of wide and diverse geographic origins to enhance the characterizing potential of our phages. Seven of the cultures used to isolate phages came from the National Animal Disease Laboratory, Ames, Iowa; one from the Pasteur Institute, Paris, France; seven from the Maine State Department of Health, Augusta, Maine; and twelve from our own University of Maine Diagnostic Service, where *Salmonella* isolates are occasionally received from various states (New England, New York, Pennsylvania, Ohio, Georgia, Arizona, etc.) and abroad (South America).

Phage isolation and propagation. Most of the sewage samples used were procured locally, but many were

TABLE 1. Phages and their propagating strains

Phage	Propagating strain
1	*S. heidelberg* (14H)
2	*S. heidelberg* (22H)
3	*S. heidelberg* (25H)
4	*S. thompson* (6)
5	*S. thompson* (14)
6	*S. thompson* (16)
7	*S. thompson* (57A)
8	*S. newport* (96)
9	*S. newport* (57)
10	*S. newport* (20)
11	*S. newport* (8)
12	*S. newport* (52)
13	*S. newport* (10)
14	*S. enteritidis* (4564)
15	*S. enteritidis* (3408)
16	*S. enteritidis* (2444)
17	*S. enteritidis* (3692)
18	*S. enteritidis* (3137)
19	*S. anatum* (20)
20	*S. anatum* (11)
21	*S. anatum* (8)
22	*S. anatum* (9)
23	*S. binza* (1B-13-99)
24	*S. senftenberg* (99S)
25	*S. senftenberg* (92S)
26	*S. senftenberg* (109S)
27	*S. senftenberg* (3230S)

obtained from Florida, New York, and Canada. Phages were isolated by enriching individual, untreated sewage samples (100 ml) with 6 ml of a 1.5-h incubated broth culture of one of the serovars under investigation. After 18 h of incubation, broths were passed through a 0.45-μm membrane filter and assayed for the presence of phages by spotting the filtrate on the culture used in the enrichment process.

The culture used to detect phages was prepared by inoculating 6 ml of broth that was subsequently incubated for 1.5 h or until growth was barely evident. At this stage, the microbial population was about 9×10^7 organisms per ml. A 2-ml amount of this broth then was applied evenly over the surface of an agar plate, allowed to dry for 15 min, and spotted with a drop (0.04 ml) of the filtrate from a Pasteur pipette. After the drop had been absorbed thoroughly (approximately 15 to 20 min), the plate was inverted, incubated overnight, and examined the following morning. If isolated plaques appeared, they were purified three times by serial, single-plaque passage. In cases where phage activity was too extensive to permit single-plaque isolations, the assaying procedure was repeated with a series of diluted filtrates. Phages then were propagated on the basis of a method described by Swanstrom and Adams (11). In essence, this procedure involves lysis of a culture by a homologous phage suspended in a soft, thin agar matrix resting on a thicker base of nutrient agar. A 60-ml amount of melted agar was poured into a 15-cm petri dish and allowed to harden on a leveled support. Nutrient broth with 0.5% NaCl and 0.7% agar was prepared in 15-ml quantities, cooled to 45°C, and inoculated with a mixture of the growth of an overnight agar slope suspended in 1 ml of broth and 2 ml of the phage to be propagated. Density of the broth culture was adjusted to equal the concentration used for phage isolations. This combination was agitated gently and poured over the surface of the base layer, allowed to harden, and incubated overnight. The next day, 10 ml of broth was added to the plate, and the soft-agar layer was removed with a sterile tongue depressor, transferred to tubes, shaken vigorously to break up the agar-phage complex, and centrifuged at $60 \times g$ for 20 min. The supernatant then was decanted, filtered through a 0.45-μm membrane filter, and assayed for phage content.

Testing of phage filtrates. The testing procedure involved a preliminary titration to determine the routine test dilution (RTD) and a lytic pattern to ascertain the novelty, stability, and usefulness of a phage isolate. The RTD, as defined by Anderson (1), is the highest dilution of phage that produces complete or confluent lysis on its propagating strain or a reaction approaching that order. Its use minimizes the occurrence of confusing cross-reactions. The RTDs of our typing phages varied from 10^{-3} to 10^{-5}. They were established by titrating phage in 10-fold serial dilutions. Only phages with an RTD of not less than 10^{-3} were used.

The lytic pattern was ascertained by testing a phage against all of the propagating strains that were used to maintain the phages that constitute our typing set. When RTD phage stocks were renewed, the lytic spectra of new and proceeding batches were compared to insure that intrinsic properties were maintained. The phage pattern of each new subculture was also checked for similar reasons.

Storage. The RTDs were stored at 4°C and tested for potency at least once a week. A test dilution was satisfactory for phage typing as long as it produced confluent lysis on its propagating strain. In general, the test dilutions of the majority of phages retained their effectiveness for 4 to 6 weeks and, occasionally, longer. In any event, the longevity of a test dilution was not predictable, and frequent periodic checks were required.

Typing technique. Cultures to be typed were lightly inoculated into 3 ml of nutrient broth and incubated for 1.5 h or until turbidity was barely perceptible. A small quantity of the broth culture was then flooded onto a nutrient agar plate and allowed to dry at room temperature. The plates were then refrigerated for 30 min, spotted with drops of phage using a 26-gauge needle, and incubated overnight. The following day the cultures were examined with the aid of a hand lens and viewed through the bottom of the plate. Susceptibility

TABLE 2. Mnemonic for reporting phage types

Triplet	No. representation
− − −	0
+ + +	1
+ + −	2
+ − +	3
− + +	4
+ − −	5
− + −	6
− − +	7

TABLE 3. Summary of phage patterns observed for serovars typed

Serovar	Group	No. of isolates typed[a]	No. of phage types observed[b]
Salmonella abortus canis	B	2	1
S. agona	B	25	7
S. alamo	C_1	1	1
S. amsterdam	E_1	47	14
S. anatum	E_1	39	21
S. aragua	N	10	1
S. bareilly	C_1	10	4
S. berta	D	2	1
S. binza	E_2	35	17
S. blockley	C_2	11	6
S. bloemfontein	C_1	2	2
S. braenderup	C_1	4	3
S. bredeney	B	5	1
S. california	B	6	3
S. cerro	K	1	1
S. chester	B	7	3
S. chittagong	E_4	6	4
S. cholerae-suis	C_1	3	1
S. cholerae-suis subsp. *kunzendorf*	C_1	2	1
S. concord	C_1	6	3
S. dar-es-salaam	D_1	2	1
S. decatur	C_1	5	2
S. derby	B	10	5
S. dessau	E_4	2	2
S. drypool	E_2	39	14
S. dublin	D_1	5	1
S. eastbourne	D_1	4	2
S. eimsbuettel	C_1	11	7
S. emek	C_2	1	1
S. enteritidis	D_1	129	26
S. essen	B	2	2
S. ferruch	C_2	6	1
S. gateshead	D_2	5	1
S. give	E_1	17	11
S. glostrup	C_2	2	2
S. hadar	C_2	3	2
S. halmstead	E_2	6	2
S. heidelberg	B	50	28
S. hillsborough	C_1	1	1
S. illinois	E_3	19	7
S. infantis	C_1	10	4
S. java	B	5	3
S. javiana	D_1	1	1
S. jerusalem	C_1	6	2
S. kentucky	C_2	5	1
S. kottbus	C_2	13	4
S. lexington	E_1	5	2
S. lille	C_1	1	1
S. livingstone	C_1	1	1
S. london	E_1	4	1
S. manhattan	C_2	1	1
S. manila	E_2	4	1
S. meleagridis	E_1	5	1
S. miami	D_1	4	1
S. mikawasima	C_1	1	1
S. minneapolis	E_3	15	6
S. montevideo	C_1	11	5
S. moscow	D_1	2	2
S. muenchen	C_2	2	2
S. muenster	E_1	17	8
S. newington	E_2	29	12
S. newport	C_2	26	14

TABLE 3—*Continued*

Serovar	Group	No. of isolates typed[a]	No. of phage types observed[b]
S. norwich	C_1	3	3
S. ohio	C_1	25	1
S. oranienburg	C_1	170	8
S. panama	D_1	4	2
S. paratyphi-A	A	6	3
S. paratyphi-A subsp. *durazzo*	A	7	2
S. paratyphi-B	B	3	2
S. paratyphi-B subsp. *odense*	B	2	1
S. paratyphi-C	C_1	5	2
S. poona	G	3	2
S. potsdam	C_1	2	2
S. pullorum	D_1	6	2
S. reading	B	3	3
S. rutgers	E_1	1	1
S. saint paul	B	11	5
S. sandiego	B	1	1
S. schwarzengrund	B	7	3
S. senftenberg	E_4	20	11
S. taksony	E_4	9	3
S. tennessee	C_1	8	3
S. thomasville	E_3	25	14
S. thompson	C_1	32	15
S. typhi	D_1	10	5
S. typhimurium	B	128	33
S. typhimurium subsp. *copenhagen*	B	7	3
S. uganda	E_1	3	1
S. westhampton	E_1	6	2

[a] A total of 1,245 isolates were typed.
[b] A total of 420 phage types were observed.

to a phage was demonstrated by areas of clearing that ranged from isolated plaques to confluent lysis.

RESULTS AND DISCUSSION

Only strong reactions, i.e., 121 or more plaques, were used in characterizing and reporting isolates, and when attempts were made to correlate the relationship of suspicious cultures, the readings involved were compared in exacting detail.

In the past, a phage type was recognized and reported on the basis of the phages it was susceptible to. In recent months we have adopted a procedure which enables us to report any conceivable phage type in nine digits. This method is based on a mnemonic devised by Farmer (2) (Table 2) and is particularly convenient when recording cultures with lengthy representations. With this system an isolate is first delineated on the basis of strong + or − reactions to the 27 phages used. Then, reading from left to right, a characteristic number is assigned to each type of triplet distinguished. Phage type 3/4/6/9/13/14/15/16/18/19/20/21/22/23/25/26/27 would thus become 737 013 121.

To date we have phage typed all of the 1,245 cultures procured from various state, national, and international sources. A summary of these results appears in Table 3. The 420 phage types observed were reproducible, and we have established epidemiological relationships in a number of disease outbreaks (Table 4). We have also

TABLE 4. Epidemiologically linked isolates

Serovar	Culture no.	Outbreak	Mnemonic
S. agona	4–20	Hospital	077 443 122
S. chester	2–4	Family	000 761 704
S. saint paul	5–8	Family	000 005 326
S. typhimurium	9–12	Family	500 543 522
	17–53	Restaurant	500 064 664
	59–62	Family	500 006 007
S. bareilly	1–4	Family	544 000 400
S. ohio	1–25	Processing plant	060 000 000
S. oranienburg	2–133	Hospital	577 706 400
	134–160	Hospital	500 706 707
S. thompson	14–26	Campus	073 700 400
S. ferruch	1–6	Processing plant	000 055 560
S. kottbus	4–12	Hospital	006 255 326
S. eastbourne	2–4	Family	577 463 100
S. enteritidis	27–89	Campus	500 442 322
S. aragua	1–10	Processing plant	500 050 007

been able to characterize a variety of phage types of *S. anatum, S. binza, S. newport*, and *S. senftenberg* among isolates that were previously untypable with homologous, serovar-specific phages.

Approximately 95% of all of the *Salmonella* isolates belong to serological groups A, B, C_1, C_2, D_1, D_2, E_1, E_2, E_3, and E_4. Already we have phage typed 86 different serovars representative of the above groups plus a group G_1 and two isolates typifying the further groups K and N.

Numerous *Salmonella* serovars exist, and any one serovar can suddenly assume a position of prominence. Under these circumstances, it is unlikely that serovar-specific phages will be available for laboratory use. Consequently, the convenience of a single, wide-spectrum, general-purpose phage typing set has obvious advantages.

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