Phage typing set for differentiating *Staphylococcus epidermidis*[1]

M. GERSHMAN[2]
Departments of Microbiology and Animal and Veterinary Sciences, University of Maine, Orono, ME 04469, U.S.A.
J. A. HUNTER
Department of Animal and Veterinary Sciences, University of Maine, Orono, ME 04469, U.S.A.
R. J. HARMON
Department of Animal Science, University of Kentucky, Lexington, KY 40546, U.S.A.
R. A. WILSON
Department of Veterinary Science, Pennsylvania State University, University Park, PA 16802, U.S.A.
AND
G. MARKOWSKY
Department of Computer Science, University of Maine, Orono, ME 04469, U.S.A.

Received April 14, 1988
Accepted August 19, 1988

GERSHMAN, M., HUNTER, J. A., HARMON, R. J., WILSON, R. A., and MARKOWSKY, G. 1988. Phage typing set for differentiating *Staphylococcus epidermidis*. Can. J. Microbiol. **34**: 1358–1361.

A phage typing set composed of 13 phages is described for characterizing *Staphylococcus epidermidis*. Isolates (372) from cases of bovine mastitis were used in this study. Of these, 350 or 94% were successfully delineated, and 63 phage types were observed. Twenty two cultures were not typeable.

GERSHMAN, M., HUNTER, J. A., HARMON, R. J., WILSON, R. A., et MARKOWSKY, G. 1988. Phage typing set for differentiating *Staphylococcus epidermidis*. Can. J. Microbiol. **34** : 1358–1361.

On décrit ici une batterie de 13 bactériophages utilisés pour typer des souches de *Staphylococcus epidermidis*. Cette étude a porté sur 372 souches isolées de cas de mastite bovine. Trois cent cinquante souches soit 94% ont été clairement caractérisées et on a observé 63 lysotypes. Vingt-deux cultures n'ont pu être typées.

[Traduit par la revue]

Although bovine mastitis is the most prominent problem plaguing the dairy industry today, progress toward its control has lagged for many reasons. Foremost among these is the realization that the disease can be initiated by a variety of microorganisms, some of which have yet to be placed in proper perspective.

We frequently isolate *Staphylococcus epidermidis* from cows with elevated leucocyte counts. Indeed, on many occasions these have been the only cultures we have been able to recover. These common inhabitants of the skin of mammals have drawn relatively little attention in the past, but recent observations suggest they are potential human pathogens (McDonald 1977), which are being implicated in bovine mastitis (Daniel et al. 1986; Hodges et al. 1984; Devriese and De Keyser 1980; Brown 1973; McDonald 1977, Brown 1983).

Crucial to the control and (or) eradication of a communicable disease is the identification of the causative agent. In the early days of microbiology, bacterial identifications were based primarily on form and function. Unfortunately, these traditional procedures, aside from being time consuming, were not always adequate in detecting the microbial variations that could be used for epidemiological application.

Bacteria are susceptible to viral infections. By exposing a bacterial isolate to appropriate dissimilar phages (bacterial viruses), a pattern will develop contingent upon the susceptibility or resistance of the culture under study to the phages employed. By this procedure numerous types or strains can be differentiated within a given species.

In 1938 Craigie and Yen (1938*a*, 1938*b*) introduced a phage typing system for *Salmonella typhi*. Its success led to the development and acceptance of this technique as a reliable and desirable delineating laboratory procedure.

To date, phage sets have been reported for a number of microorganisms, including *Staphylococcus aureus* of both human (Fisk 1942*a*, 1942*b*; Felix and Callow 1956; Blair and Williams 1961) and animal origin (Davidson 1961; Blouse and Meekins 1968; Carrol and Francis 1985; Shimizu 1977), *S. epidermidis* (Talbot and Parisi 1977; Verhoef et al. 1972), *Escherichia coli* (Gershman et al. 1984), *Salmonella typhimurium* (Callow 1959; Wilson et al. 1971), and a single set for characterizing salmonellae in general (Gershman and Markowsky 1983).

Pursuing our own interests, we have developed a phage typing system for *S. epidermidis* and species resistant to *S. aureus* phages (Brown et al. 1967), and we elected to use cultures identified by the latest diagnostic techniques available (Langlois et al. 1984).

Trypticase soy agar (BBL Microbiology Systems, Cockeysville, MD) and nutrient broth (Difco Laboratories, Detroit, MI) were used for isolating phages and phage typing. Prior to use, the trypticase soy agar plates were dried in an incubator for 2 h with lids partially opened. Trypticase soy agar, trypticase soy broth (BBL) with 0.6% agar (Difco), and nutrient broth were used for phage propagation. In all of our procedures the incubation temperature was 37°C.

The cultures used to isolate phage were obtained from the following universities: University of Kentucky, Lexington;

[1]Supported by the Maine Agricultural Experiment Station No. 1319, Hatch NE-112, Orono, ME 04469-0131.

[2]Author to whom all correspondence should be addressed.

Printed in Canada / Imprimé au Canada

TABLE 1. Lytic pattern of *Staphylococcus epidermidis* phages at RTD

Cultures	Phages												
	1[a]	2[b]	3[a]	4[b]	5[a]	6[b]	7[b]	8[c]	9[a]	10[c]	11[b]	12[a]	13[b]
1	CL	−	−	−	−	−	+++	−	+	−	−	−	−
2	−	CL	−	−	−	−	+±	+	−	−	−	−	−
3	−	−	CL	CL	−	+±	+	−	OL	−	−	SCL	−
4	−	−	OL	CL	−	+±	−	−	−	−	−	SCL	−
5	−	−	±	SCL	CL	CL	++	−	−	−	−	CL	±
6	+	+	−	−	−	CL	−	CL	−	SCL	−	−	−
7	+	±	±	±	±	±	CL	CL	CL	+	−	±	−
8	±	±	−	+	±	−	CL	SCL	CL	CL	−	−	−
9	−	−	−	−	CL	<SCL	−	−	−	CL	−	CL	−
10	−	−	−	−	−	−	−	−	−	−	CL	−	OL
11	+	+	−	−	−	−	CL	±	CL	+	±	CL	−
12	−	−	CL	−	−	CL	±	SCL	+++	−	OL	CL	CL

NOTE: CL, Confluent lysis; OL, opaque lysis (opacity due to secondary growth); SCL, semiconfluent lysis; <SCL, less than semiconfluent lysis; +++, 121 plaques; ++±, 81−120 plaques; ++, 61−80 plaques; +±, 41−60 plaques; +, 21−40 plaques; ±, 6−20 plaques; −, 0−5 plaques.
[a]RTD 10^{-3}.
[b]RTD 10^{-4}.
[c]RTD 10^{-5}.

the Pennsylvania State University, College Park; and our own University of Maine Diagnostic Service. Furthermore, the cultures used came from widely distributed geographical locations to minimize indigenous influences that might affect a native population of organisms relative to their susceptibility to phage (Carrol and Francis 1985).

Phages were isolated by enriching individual 100-mL, untreated sewage samples, obtained from local treatment plants during different seasons of the year. Each sample was inoculated with 6 mL of a single, barely turbid, 1.5-h nutrient broth culture of *S. epidermidis*. After 18 h of incubation, broths were passed through a 0.45-μm membrane filter and assayed for phage by spotting the filtrate onto the same culture that was used in the enrichment process. A lawn of this culture was prepared by evenly distributing 2 mL of a 1.5-h nutrient broth culture of *S. epidermidis*, containing a microbial population similar to the one stated above, over the surface of an trypticase soy agar plate. This plate then dried at room temperature for 15 min and was spotted with the filtrate under study using a Pasteur pipette. After the drop had been thoroughly absorbed (approximately 15−20 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 were then propagated by a method described by Swanstrom and Adams (1951). In essence, this procedure involved the lysis of a culture by a homologous phage suspended in a soft, thin agar matrix resting on a thicker base of trypticase soy agar. A 60-mL aliquot of melted trypticase soy agar was poured into a 150 × 15 mm petri dish and allowed to harden on a leveled support. Trypticase soy broth containing 0.6% agar was prepared in 15-mL quantities, cooled to 45°C, and inoculated with a mixture of the growth of an overnight trypticase soy agar slant suspended in 1 mL of nutrient broth and 2 mL of the phage to be propagated. Density of the broth culture was adjusted to equal the concentration used to isolate phage. This combination was gently agitated and poured over the surface of the base layer, allowed to harden, and incubated overnight. The next day, 10 mL of nutrient 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 × *g* for 20 min. The supernatant then was decanted, filtered through a 0.45-μm membrane filter, and assayed for phage content.

TABLE 2. Mnemonic for reporting phage types

Triplet	Number or letter representation
−−−	0
+++	1
++−	2
+−+	3
−++	4
+−−	5
−+−	6
−−+	7
+	A
−	B

The testing procedure involved 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 is the highest dilution of phage that produces a complete or confluent lysis of its propagating strain. Its use minimizes the occurrence of confusing cross-reactions. The RTD 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 determined by testing a phage, at its RTD, against all of the propagating strains that were used to maintain the phages that constituted our typing set. The lytic patterns of the typing phages are noted in Table 1. When RTD phage stocks were renewed, the lytic spectra of new and proceeding batches were compared to ensure that intrinsic properties were maintained; the phage pattern of each propagating strain was also checked.

Phages were stored at 4°C and routinely tested for potency

TABLE 3. Phage types isolated

Culture number	Phage pattern	Mneumonic
1	1	500 0B
2	1/2/3/4/5/7/12/13	125 7A
3	1/2/3/10/12/13	100 3A
4	1/2/4/9	257 0B
5	1/2/4/10	250 5B
6	1/2/5/7/9/10/11/12/13	263 1A
7	1/2/5/7/9/12/13	263 7A
8	1/3/11	300 6B
9	1/4/8/10	556 5B
10	1/4/10/12	550 3B
11	1/7/8/9/11	501 6B
12	1/7/8/11/13	502 6A
13	1/7/10/11/12	505 1B
14	1/12/13	500 7A
15	1/13	500 0A
16	2	600 0B
17	2/4/9	657 0B
18	2/4/9/11	657 6B
19	2/4/10/12	650 3B
20	2/5/6	640 0B
21	2/5/9/10	667 5B
22	2/7/9	603 0B
23	2/8/11	606 6B
24	2/11	600 6B
25	2/11/13	600 6A
26	2/13	600 0A
27	3/4/9/12	757 7B
28	3/4/13	750 0A
29	3/6	770 0B
30	3/10/13	700 5A
31	3/12	700 7B
32	4/5/6/12	10 7B
33	4/7/8/9	51 0B
34	4/7/8/9/10/11	51 2B
35	4/9/11/13	57 6A
36	4/10	50 5B
37	4/10/12	50 3A
38	4/10/13	50 5A
39	5	60 0B
40	5/6/12	40 7B
41	5/7/8/10/12/13	62 3A
42	5/7/8/11/12	62 4B
43	5/10	60 5B
44	5/12	60 7B
45	5/12/13	60 7A
46	6	70 0B
47	6/8/12/13	76 7A
48	6/12	70 7B
49	7/8	2 0B
50	7/8/9	1 0B
51	7/8/9/10	1 5B
52	7/8/9/10/12	1 3B
53	7/8/9/13	1 0A
54	7/9	3 0B
55	7/9/12	3 7B
56	8/9/13	4 0A
57	10/11	2B
58	10/11/13	2A
59	11	6B
60	11/13	6A
61	12	7B
62	12/13	7A
63	13	A

at least once a week. The RTD was considered satisfactory 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 4–6 weeks and occasionally longer. In any event, the longevity of a RTD was not predictable, and frequent periodic checks were warranted.

The mastitis isolates typed in this investigation were all identified using the DMS Staph-Trac system (Langlois et al. 1984). Each was inoculated lightly into 3 mL of broth and incubated for 1.5 h or until turbidity constituted a population of 9×10^{-7} organisms/mL. A small quantity of the broth culture was flooded onto two 100 × 15 mm trypticase soy agar plates and handled in much the same way as described for the preparation of bacterial lawns. However, after each plate was dried, it was refrigerated for 30 min before being spotted with single drops of the phages that constituted our set. One plate was spotted with eight and the second with the remaining five dissimilar phages using a 26 gauge needle and incubated overnight. The following day, cultures were examined with a hand lens and viewed through the bottom of the plate.

Susceptibility to a phage was demonstrated by areas of clearing that ranged from isolated plaques to confluent lysis. In an effort to standardize our protocol, the readings were interpreted by the criteria we used in phage typing other organisms (Anderson 1964). Only strong reactions, i.e., 121 or more plaques per drop, were used in characterizing and reporting isolates, and the resulting phage types or patterns were reported on the basis of a mnemonic devised by Farmer (1970) that is particularly convenient for recording cultures with lengthy representations. With this system (Table 2), an isolate is first delineated by strong positive reactions (+++ or above) or weak reactions (−). Then, going from left to right a number was assigned to each type of triplet observed and a letter to the remaining digit. Using this procedure, phage type 1/2/5/7/9/10/11/12/13 is recorded as 263 1A. In cases where an isolate only responds to higher numbered phages, readings commence with the first encountered triplet or phage registering a reaction. Phage type 11/13, as an example, becomes 6A, and type 13, simply A. Sixty three phage patterns were observed in our present study (Table 3). Repeated trials revealed that our results were consistent and reproducible.

Aside from relating an isolate to an outbreak, phage typing can be used for surveillance and assessing strain distribution. Given the variety of *S. epidermidis* isolates, phage typing can contribute significantly to the control of mastitis by assisting the practitioner and health-related personnel in identifying potential pathogens and monitoring their response to therapy, or may be used for other appropriate applications.

ANDERSON, E. S. 1964. Phage typing of *Salmonella* other than *S. typhi*. *In* The world problem of salmonellosis. *Edited by* Van Oye. Junk, The Hague. pp. 89–110.

BLAIR, J. E., and WILLIAMS, R. E. O. 1961. Phage typing of staphylococci. Bull W.H.O., **24**: 771–784.

BLOUSE, L., and MEEKINS, W. E. 1968. Isolation and use of experimental phages for typing *Staphylococcus aureus* from sentry dogs. Am. J. Vet. Res. **29**: 1817–1822.

BROWN, R. W. 1973. Intramammary infections produced by various strains of *Staphylococcus epidermidis* and *Micrococcus*. Cornell Vet. **63**: 630–645.

——— 1983. Biotypes of *Staphylococcus epidermidis* and *Micrococcus* organisms, isolated from intramammary infections, reclassified into species of the genus *Staphylococcus* (*epidermidis*, *hyicus*, *xylo-*

sus and *sciuri*). Cornell Vet. **73**: 109–116.

BROWN, R. W., SANDVIK, O., SCHERER, R. K., and ROSE, D. L. 1967. Differentiation of strains of *Staphylococcus epidermidis* isolated from bovine udders. J. Gen. Microbiol. **47**: 273–287.

CALLOW, B. R. 1959. A new phage-typing scheme for *Salmonella typhimurium*. J. Hyg. **57**: 346–359.

CARROL, P. J., and FRANCIS, P. G. 1985. The basic phage set for typing bovine staphylococci. J. Hyg. **95**: 665–669.

CRAIGIE, J., and YEN, C. H. 1938*a*. The demonstration of types of *B. typhosus* by means of preparations of type II Vi phage. I. Principles and technique. Can. J. Public Health, **29**: 448–484.

——— 1938*b*. The demonstration of types of *B. typhosus* by means of preparations of type II Vi phage. II. The stability and epidemiological significance of V form types of *B. typhosus*. Can. J. Public Health, **29**: 484–496.

DANIEL, R. C. W., BARNUM, D. A., and LESLIE, K. E. 1986. Observations on intramammary infections in first calf heifers in early lactation. Can. Vet. J. **27**: 112–115.

DAVIDSON, I. 1961. A set of bacteriophages for typing bovine staphylococci. Res. Vet. Sci. **2**: 396–407.

DEVRIESE, L. A., and DE KEYSER, H. 1980. Prevalence of different species of coagulase-negative staphylococci on teats and in milk samples from dairy cows. J. Dairy Res. **47**: 155–158.

FARMER, J. J., III. 1970. Mnemonic for reporting bacteriocin and bacteriophage types. Lancet, **1**: 96.

FELIX, A., and CALLOW, B. 1956. Phage typing of *Salmonella typhimurium*: its place in epidemiological and epizootiological investigations. J. Gen. Microbiol. **14**: 208–222.

FISK, R. T. 1942*a*. Studies on staphylococci. I. Occurrence of bacteriophage carriers among strains of *Staphylococcus aureus*. J. Infect. Dis. **71**: 153–160.

——— 1942*b*. Studies on staphylococci. II. Identification of *Staphylococcus aureus* strains by means of bacteriophage. J. Infect. Dis. **71**: 161–165.

GERSHMAN, M., and MARKOWSKY, G. 1983. Reduced set of phages for typing salmonellae. J. Clin. Microbiol. **17**: 240–244.

GERSHMAN, M., MARKOWSKY, G., and HUNTER, J. 1984. Reduced set of phages for typing *Escherichia coli*. J. Dairy Sci. **67**: 1306–1315.

HODGES, R. T., JONES, Y. S., and HOLLAND, J. T. S. 1984. Characterization of staphylococci associated with clinical and subclinical bovine mastitis. N.Z. Vet. J. **32**: 141–145.

LANGLOIS, B. E., HARMON, R. J., and AKERS, K. 1984. Identification of *Staphylococcus* species of bovine origin with the DMS Staph-Trac system. J. Clin. Microbiol. **20**: 227–230.

MCDONALD, J. S. 1977. Streptococcal and staphylococcal mastitis. J. Am. Vet. Med. Assoc. **170**: 1157–1159.

SHIMIZU, A. 1977. Establishment of a new bacteriophage set for typing avian staphylococci. Am. J. Vet. Res. **38**: 1601–1605.

SWANSTROM, M., and ADAMS, M. H. 1951. Agar layer method for production of higher titer phage stocks. Proc. Soc. Exp. Biol. Med. **7**: 372–375.

TALBOT, H. W., JR., and PARISI, J. T. 1977. Phage typing of *Staphylococcus epidermidis*. J. Clin. Microbiol. **3**: 519–523.

VERHOEF, J., VAN BOVEN, C. P. A., and WINKLER, K. C. 1972. Phage-typing of coagulase-negative staphylococci. J. Med. Microbiol. **5**: 5–19.

WILSON, V. R., HERMANN, G. J., and BALOWS, A. 1971. Preliminary report of a new system for typing *Salmonella typhimurium* in the United States. Appl. Microbiol. **21**: 774–776.