Activity and Characteristics of a New Staphylococcus Phage 94[1] (37069)

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(Introduced by G. J. Buddingh)
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Recently, the authors reported apparent spontaneous induction of *Staphylococcus aureus* strains from clinical sources (1). As a result of these studies two new phages were identified. One of these phages, 94 (WH-1), showed particular promise for typing a number of staphylococcus strains refractile to the currently accepted basic set of typing phages.

Recent staphylococcal outbreaks in hospital nurseries (2) following discontinuation of routine hexachlorophene baths have renewed interest in staphylococcus phage typing. Therefore, it is appropriate that a new and useful typing phage 94 be documented at this time since there is a continuing requirement for additional typing phages of value in differentiating staphylococcus strains for epidemiologic purposes. This is a report of the initial isolation of phage 94 and those characteristics (morphology, serology, buoyant density, and lytic spectrum) necessary for classification of this phage in relation to other typing phages. Preliminary findings, relative to the efficacy of phage 94 in typing otherwise untypable staphylococcus strains, are also presented.

Materials and Methods. Phage and host strain. Lysogenic host strain 94 was received from Wilford Hall USAF Medical Center, Lackland AFB, Texas. This strain was isolated from a routine bacteriologic culture of the cord of a newborn. Neither the newborn nor the mother experienced clinical staphylococcal infection during hospitalization.

Phage 94 was isolated from lysed areas on the bacterial lawn which apparently resulted from spontaneous induction of the lysogenic host strain. Phage purification was accomplished by four single-plaque passages using the agar overlay method described by Adams (3). Trypticase soy agar and broth were used for passage, propagation and other experimental procedures. Mueller–Hinton agar was used for antibiotic susceptibility testing with penicillin (10 U), ampicillin (10 μg), methicillin (5 μg), cephalothin (30 μg), erythromycin (15 μg), chloramphenicol (30 μg), tetracycline (30 μg), kanamycin (30 μg), lincomycin (2 μg), and colymycin (10 μg) using the method of Bauer *et al.* (4).

Phage typing of strains. S. aureus isolates for phage typing were received from Air Force hospitals throughout the United States and from a limited number of local civilian hospitals. Phages used for typing staphylococcal strains were those of the currently accepted international basic set, 29, 52, 52A, 79, 80, 3A, 3C, 55, 71, 6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85, 42D, 81, and 187. Also included were phages 86, 87, 88, 7 (US), 3B, 42B, 47C, 52B and 83B. Phage typing was performed using standard methods at routine test dilution (RTD) or, when appropriate, at

[1] Phage 94 was designated previously as WH-1. Subsequently, it was assigned the official number 94 by the Subcommittee on Phage-Typing of Staphylococci of the International Committee on Nomenclature of Bacteria.

The research reported in this paper was conducted by personnel of the Epidemiology Division, USAF School of Aerospace Medicine, Aerospace Medical Division, AFSC, United States Air Force, Brooks AFB, Texas. Further reproduction is authorized to satisfy the needs of the U. S. Government.

The animals involved in this study were maintained in accordance with the "Guide for Laboratory Animal Facilities and Care" as published by the National Academy of Sciences, National Research Council.

concentrations of 100 × RTD (5).

Lytic spectrum determination. The lytic spectrum of phage 94 against 26 standard staphylococcus test strains (3A, 3B, 3C, 6, 7, 29, 42C, 42D, 42E, 47, 52, 52A/79, 53, 54, 55, 71, 75, 77, 80, 81, 83A, 84, 85, 187, 8719, and 2009) was determined in order to detect phage variation due to mutation or modification during subsequent propagation. The methods used were those recommended by the Center for Disease Control, Atlanta, Georgia (5).

Preparation of antisera. Anti-94 hyperimmune serum was prepared in adult Dutch rabbits. Phage suspensions containing 2.6 × 10^{11} plaque-forming units (PFU)/ml were emulsified with 20% (v/v) Freund's complete adjuvant (Difco) for immunization. The regimen consisted of 2 ml subcutaneous injections administered biweekly for five weeks. After a one-week rest, trial bleedings were made to determine antiphage neutralization levels.

Group A and B antiphage sera were previously supplied by Dr. E. D. Rosenblum (Southwestern Medical School, Dallas, Texas). Sera for groups F and L were prepared in this laboratory using essentially the same procedures described above.

Antiphage serum assay. Typing phages 6, 29, 42D, 187, and 94 were used in assaying the phage-neutralizing capacity of grouping sera, A, B, F, L, and homologous 94 serum. Serologic grouping was established by comparison of the serum constant (K) values for neutralizing phage. Determination of serum K values was performed at 37° by the methods described by Adams (3). Each respective phage suspension was adjusted to approximately 10^6 PFU/ml, mixed with an equal volume of appropriately diluted antiserum, and assayed for surviving phage at 5-min intervals during a 30-min reaction period. Serum constant values for homologous serum-phage reactions were established using serum dilutions representing $\geq$ 95% neutralization after 30 min exposure.

Buoyant density determination. Four milliliters of CsCl solution of buoyant density 1.49 g/cm^3 (25°) were overlayed with 0.5 ml of a partially purified phage suspension containing 10^{10} PFU/ml. Banding of the phage was accomplished using a Spinco Model L centrifuge (SW 39 swinging-bucket rotor) and centrifuging for 17 hr at 22,000 rpm. Cesium chloride densities were calculated from refractive indices determined on an Abbe-3L refractometer (Bausch and Lomb) (7). Fractions of 10 drops each were collected and assayed for infective phage by the agar overlay method. The fraction yielding maximum infectivity was correlated with density.

Electron microscopy. Fractions showing the highest phage titers were pooled, dialyzed against 2% $NH_4C_2H_3O_2$, and negatively stained with a 2% solution of phosphotungstic acid (pH 7.2). Electron micrographs were taken with an RCA EMU-3G electron microscope.

Results and Discussion. The bacterial lawn of strain 94 demonstrated a noticeable mottled appearance after the second subculture for purity (Fig. 1). Select areas of apparent lysis yielded typical phage plaques when eluted in TSB and passaged on the parent host strain. This phenomenon indicated the occurrence of spontaneously induced lysis similar to that noted by Fisk (8) and reported by Wallmark and Finland (9). Subsequent subcultures of the host strain did not reveal spontaneous lysis. On further examination, host strain 94 conformed to the characteristic identity reactions (deoxyribonuclease, mannitol, and coagulase) of *S. aureus* and was untypable with the standard series and additional typing phages.

When passaged on TSA medium with a semisolid (0.6%) agar overlay, phage 94 produced small, clear plaques of 1 to 1.5 mm diameter. During subsequent propagation in TSB at 37°, it was not uncommon to note complete lysis during logarithmic growth of the host about 2 1/2 hr after phage inoculation at a multiplicity of infection equal to 10. These propagates yielded titers of 10^{10} PFU/ml, thus demonstrating the relative ease with which phage 94 can be propagated.

The lytic spectrum for phage 94 was determined before additional experimental studies were undertaken. Using 26 standard staphylococcus test strains and host strain 94, lytic reactions were graded 5 for propagating

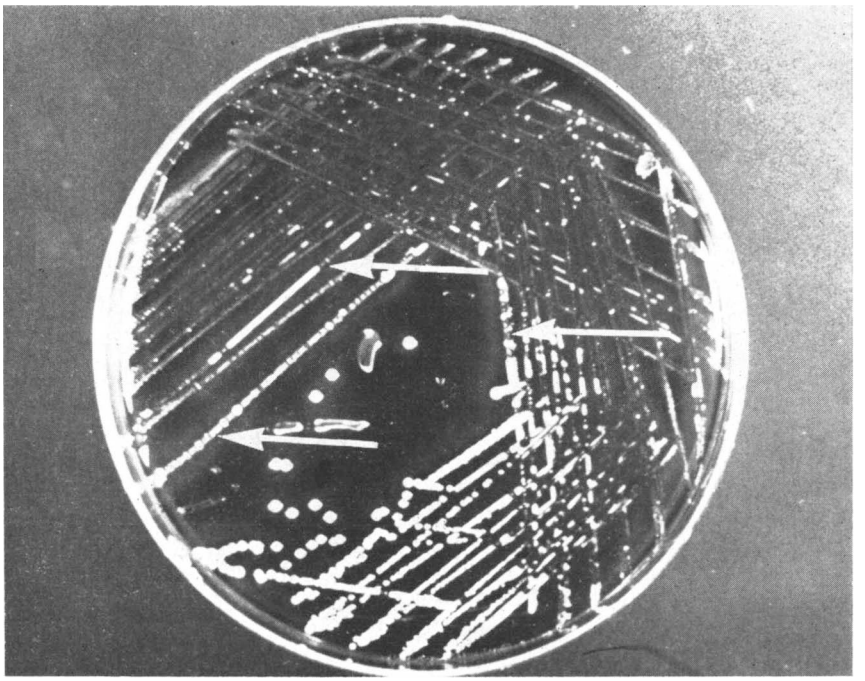

FIG. 1. Subculture of *S. aureus* host strain 94 showing apparent spontaneously induced lytic areas.

strain 94, 3 for PS 3A, 2 for PS 3B, 2 for PS 3C, 4 for 42C, 2 for PS 7, 2 for PS 47, 3 for PS 54, 1 for PS 55. No reactions were elicited with the remaining test strains. Reactions with PS 3A, 3C, 42C, 7, 47, and 54 were compatible with those determined by Dr. A. E. Asheshov, WHO International Center for Staphylococcus Phage Typing, London. In addition, a reaction of 2 for PS 6 was recorded by Dr. Asheshov. Subsequent propagates of phage 94 were tested for general conformity with this spectrum.

Previous studies (1) suggested the usefulness of phage 94 as an adjunct to the current standard series of typing phages. During a 9-month evaluation following the isolation of phage 94, 900 staphylococcus strains were received for phage typing from widely separated geographic areas of the United States and represented isolates from a variety of clinical sources. Of these, 542 (60.2%) were typed with one or more of the standard phages; while the remaining 358 (39.8%) were untypable at RTD or 100 × RTD. A total of 119 (13.2%) of the 900 strains tested were typed by phage 94 at RTD and were untypable with the basic series of phages. The number of strains susceptible to phage 94 and their geographic distribution suggests the endemic presence of type 94 strains in the current hospital environment.

The overall typability of strains by phage 94 (13.2%) compares favorably to other described experimental phages, *e.g.*, 84 (77Ad), 85 (B5), and 93 (6657) which lysed 17%, 15%, and 6% of 350 strains tested (10). Recent communication with Dr. A. E. Asheshov indicates that phage 94 at RTD lysed approximately 3.2% of 3000 strains tested. The majority of the strains lysed by phage 94 were untypable with the basic set of phages as was the experience in our own laboratory.

Examination of a randomly chosen subsample, 70 strains of the phage type 94, indicates that these strains are uniformly resistant to penicillin, ampicillin, and colymycin. Additionally, about 5% of the strains also showed resistance to methicillin. Multiple antimicrobic resistance patterns, often including methicillin resistance, have been reported with increasing frequency since the early

TABLE I. Homologous and Heterologous Serum Constant (K)[a] Values of Relative Neutralizing Capacity for Phage.

Phage	Antiserums				
	94	A	B	F	L
94	125	60	<1	<1	<1
6	230	225	<1	<1	<1
29	<1	<1	60	<1	<1
42D	<1	<1	<1	145	<1
187	<1	<1	<1	<1	200

[a] K value expresses the fractional rate of neutralization in min $^{-1}$.

1960's (11, 12). Barrett *et al.* found that the majority of methicillin resistant strains studied were untypable (11). The fact that phage 94 lyses strains exhibiting this multiple methicillin resistance pattern further enhances the epidemiologic value of this new phage.

Serum dilutions of 10^{-1} to 10^{-3} were used to determine serum constant (K) neutralization values for phage 94 and other selected phages representing major serologic groups to which staphylococcal typing phages have been assigned. Homologous or group specific K values ranged from 60 to 225 min^{-1} (Table I). Phage type 6, serologic group A, gave a K ratio (K value when tested with homologous group A antiphage serum/K value when tested with 94 antiphage serum) of 225/230, while phage 94 gave a comparative value of 125/60. The absence of detectable neutralization when Phage 94 was tested against groups B, F, and L antiphage serums and a similar absence of neutralization by anti-94 hyperimmune serum of phages representing these groups establish a group A serologic classification of phage 94.

Staphylococcal phages of a specific serologic group have shown a correlation between serology, buoyant density, and phage morphology (13). Three separate experiments established the buoyant density of phage 94 (1.470, 1.471, and 1.472 g/cm^3). The mean density of 1.471 for phage 94 is slightly higher than the density range of 1.451 to 1.469 reported for group A phages (13). It is suspected that this difference reflects experimental variation. Only a single group L phage,

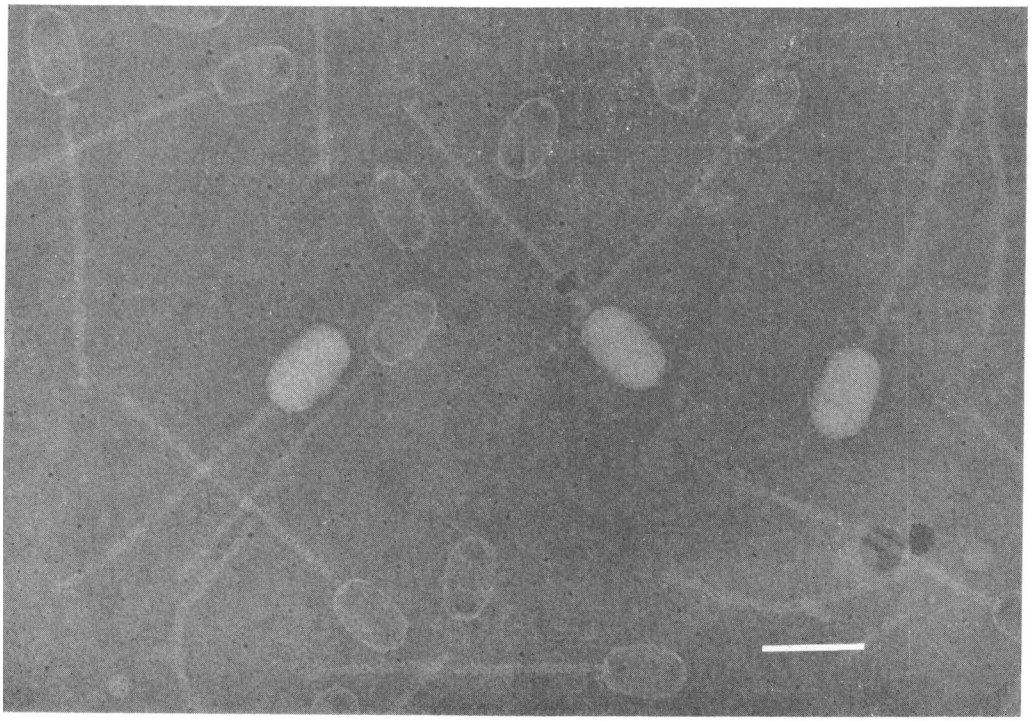

FIG. 2. Staphylococcus phage 94, negatively stained with phosphotungstic acid (×124,000). Bar represents 100 nm.

187, has been examined for density and found to be 1.473 (13). The mean density of phage 94 places it in a position between the phages of serologic group A and group L.

Phages of group A form a homogeneous group with respect to morphology and density features. Structurally, group A phages have oval, elongated heads with long tails terminating in a knob-like base plate. Phage 94 was shown to possess morphological features compatible with Group A phages (Fig. 2). The mean length by width measurement for the head silhouette of phage 94 is 95 by 55 nm. The tail structure measures 310 nm long and terminates in what appeared to be a tail knob base plate. No attempt was made to resolve the tail structure.

The general structure and dimensions of phage 94 closely approximate those reported for group A staphylococcal phages (14). The fact that the group L phage, 187, presents a radically different general structure, *i.e.*, regular hexagonal head with a tail structure approximately half the length of the group A phages, suggests that phage 94 should be classified with the group A phages.

Summary. Staphylococcus phage 94, formerly designated WH-1, was found to type 119 (13.2%) of 900 clinical isolates which were untypable with the basic series of typing phages. Strains typed by this phage were uniformly resistant to penicillin, ampicillin, and colymycin. A lytic spectrum for phage 94 is presented and the phage is shown to be antigenically related to the group A staphylococcus phages. Morphologically, phage 94 has an oval, elongated head (95 × 55 nm), a tail length of 310 nm, and a density of 1.471 g/cm^3.

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1. Blouse, L. E., Mauney, C. U., Marraro, R. V., and Dupuy, H. J., Appl. Microbiol. **23**, 1023 (1972).

2. Center for Disease Control, Morbid. Mortal. **21**, 37 (1972).

3. Adams, M. H., "Bacteriophages," pp. 450, 465. Interscience, New York (1959).

4. Bauer, A. W., Kirby, W. M. M., Sherris, J. C., and Turck, M., Amer. J. Clin. Pathol. **45**, 493 (1966).

5. Center for Disease Control, "Recommended Methods for Evaluation of Microbiological Reagents," B29-1-29-6 (1966).

6. Meselson, M., Stahl, F. W., and Vinograd, J., Proc. Nat. Acad. Sci. U.S.A. **43**, 581 (1957).

7. Ifft, J. B., Voet, D. H., and Vinograd, J., J. Phys. Chem. **65**, 1148 (1961).

8. Fisk, R. T., J. Infect. Dis. **71**, 153 (1942).

9. Wallmark, G., and Finland, M., Proc. Soc. Exp. Biol. Med. **106**, 73 (1961).

10. Bulow, P., Acta Pathol. Microbiol. Scand. **72**, 147 (1968).

11. Barrett, F. F., McGehee, R. F., and Finland, M., New Engl. J. Med. **279**, 441 (1968).

12. Parker, M. T., and Hewitt, J. H., Lancet **1**, 800 (1970).

13. Rosenblum, E. D., and Tyrone, S., J. Bacteriol. **88**, 1732 (1964).

14. Bradley, D. F., J. Ultrastruct. Res. **8**, 552 (1963).

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