Comparison of Heat Shocking and Acridine Orange Treatment in Phage Typing of Nontypable Strains of *Staphylococcus aureus*

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Forty-five percent of 108 nontypable strains of *Staphylococcus aureus* from clinical and environmental sources were phage typable after heat shock and acridine orange treatment. Although phage patterns were produced by different types of treatment, the same phage groups were present, showing specificity. The newly typable strains appear to be similar in phage group distribution to the primary typable strains typed at the Peter Bent Brigham Hospital during the same time period. Ten of the originally nontypable *S. aureus* strains that showed lytic patterns after heat shock or acridine orange treatment were subcultured four times from the treated culture and then tested again with bacteriophages. Phage patterns were still present in 8 of the 10 strains without any further treatment. These findings are compatible with the findings of other workers, who have shown that the insensitivity of primary nontypable *S. aureus* strains to phage is linked to phage-resistant genes in extrachromosomal genetic elements that can be "cured" by both heat shock and acridine treatment.

Fifty percent of *Staphylococcus aureus* strains that have been isolated from the environment and from clinical material are nontypable using routine phage-typing methods at the Peter Bent Brigham Hospital. Nontypable *S. aureus* strains have prevented the establishment of important epidemiological associations. There are many known and theorized reasons for the sensitivity of *S. aureus* to bacteriophages.

Asheshov and Jevons (2) and Milch and Laszlo (10) advanced a working hypothesis for explaining changes in phage sensitivity after heat treatment. They assumed that heat exerts a prophage-inducing effect. The intracellular prophage entering the vegetative phase recombines with the type phage, resulting in the production of a phage with new lytic properties active against the formerly phage-insensitive strain.

The studies of Richmond (11) and other investigators (5, 9) have shown that extrachromosomal genetic elements, plasmids, contain phage genes. Clecner and Sonea (5) demonstrated that phage genes carried on an extrachromosomal element were responsible for the synthesis of staphylococcal delta-hemolysin. The association of this element with phage genes is suggested by the observation that transfer of the ability to synthesize delta-hemolysin is associated with the acquisition of

resistance to one of the staphylococcal phages—71, 81, or 97 (5).

Wu and Park (13) theorized that the phage insensitivity of a previously phage-sensitive *S. aureus* strain was due to the acquisition of a new surface polymer, *N*-acetyl-D-fucosamine and *N*-acetyl-D-mannosaminuronic acid, which presumably masked the phage receptor sites.

To increase the number of typable strains, the modified heat shock method of Ma and Mandle (P. Ma and R. J. Mandle, *Bacteriol. Proc.*, p. 111, 1961) and the acridine dye treatment as described by Arditti and Coppo (1) and Weiss and Nitzin (12) were investigated.

The mechanisms of heat shock have been explained in part previously (2, 10). Fairbrother et al. (7) also showed that the probability of loss of some but not all plasmids can be achieved by elevated temperatures.

Acridine dyes have given varied results in "curing" *S. aureus* cells of plasmids (8, 11). Acridine dyes have also been shown to be mutagens (1, 3, 6, 11, 13).

MATERIALS AND METHODS

***S. aureus* strains.** A total of 108 nontypable *S. aureus* strains were studied. Several *S. aureus* strains that produced phage patterns under normal test conditions were treated with acridine orange and heat shocked to determine the effect of treatments on known patterns. The strains used in the

study were collected from patients and surgical team members in the course of epidemiological studies of postsurgical *S. aureus* infections, clinical material, and environmental surveys. A staphylococcal strain was determined to be *S. aureus* if it gave a positive coagulase reaction either by the tube or slide method.

Bacteriophages. *S. aureus* bacteriophages of the basic international set at the time of testing and several that have been found useful in studying hospital *S. aureus* strains were used. These are: group I, 29/52/52A/79/80; group II, 3A/3C/55/71; group III, 6/42E/47/53/54/75/77/83A/84/85; group IV, 42D; miscellaneous, 81/187; additional set, 42B/83B/47C/82/86.

Bacteriophages were used throughout the study at a routine test dilution (RTD), and 94 of the same 108 strains were tested with preparations of phage 1,000 times stronger (RTD $\times$ 1,000).

In our experience over the last 15 years, inhibition of the 83A/84/85 complex phages and other phages at RTD $\times$ 1,000 has not been a problem in interpreting results. Although RTD and RTD $\times$ 100 concentrations of phages are now employed in our routine testing, RTD $\times$ 1,000 preparations were used in this study to obtain the optimum percentage of "newly" typable strains of *S. aureus* possible.

Phage-typing method. The method of Blair and Williams (4) was used to phage type *S. aureus* strains throughout the study.

Heat shocking. The effect of heat shocking on normally nontypable *S. aureus* strains was studied by growing a loopful of organisms from a Trypticase soy agar slant in 5 ml of Trypticase soy broth for 4 h at 37°C. Although heat shocking was tried at temperatures of 49 to 56°C in a water bath over a range of 2- to 10-min time periods, 56°C for 3 min gave the optimum results as evidenced by new phage patterns.

Acridine orange treatment. The same 108 *S. aureus* strains studied for the effect of heat shocking were treated with acridine orange dye. A stock solution of 1 mg of acridine orange (Allied Chemicals) per ml was prepared in distilled water. The stock solution was filtered through a 0.45- μ m membrane filter (Millipore Corp.) and stored at 4°C in the dark. For testing the effect of the dye on nontypable strains, 0.1 ml of the acridine orange stock solution (1 mg/ml) was incorporated in 9.9 ml of Trypticase soy broth, so that the final concentration was 10 μ g/ml. A loopful of the *S. aureus* strain to be tested was grown in the 10- μ g/ml acridine dye-Trypticase soy broth medium for 18 h at 37°C in preparation for phage typing.

RESULTS

RTD testing. Study results showed (Table 1) that of the 108 originally nontypable *S. aureus* phage typed at RTD, 8 (7.4%) were only typable after acridine orange treatment. Twelve (11.1%) of the 108 strains showed lytic patterns only after heat shocking at 56°C for 3 min. Eleven (10.2%) of the 108 *S. aureus* strains

were typable by both acridine dye and heat shock treatments independently. By testing all 108 nontypable strains, using both treatments independently, phage patterns could be demonstrated in a total of 31 (28.7%) of the previously nontypable strains.

RTD $\times$ 1,000 testing. A total of 94 of the 108 *S. aureus* strains were tested with RTD $\times$ 1,000 concentrations of bacteriophages after treatment with acridine orange and heat shocking independently. As seen in Table 2, only 7 (7.4%) of the 94 nontypable *S. aureus* tested were typable after acridine orange treatment only, but 17 strains (18.1%) were typable by heat shocking only. A total of 18 (19.2%) of the 94 *S. aureus* strains were typable by both acridine dye and heat shock treatments independently. After using both types of treatments and testing with stronger phages, RTD $\times$ 1,000, 42 (44.7%) of the originally nontypable *S. aureus* strains showed lytic patterns.

Specificity. Although phage patterns were produced by different types of treatment in the same primary nontypable *S. aureus* strains, the same phage groups were present, showing specificity. The newly typable strains appear to be similar in phage group distribution to the primary typable strains typed at the Peter Bent Brigham Hospital during the same time period. Several primary typable *S. aureus* strains were treated with acridine orange and heat shocked.

TABLE 1. Effect of treatments on 108 nontypable *S. aureus* strains at RTD concentration of bacteriophages

Treatment	No. tested	No. typable
Phage typable only after heat shocking for 3 min at 56°C	108	12 (11.1) ^a
Phage typable only after acridine orange, 10 μ g/ml	108	8 (7.4)
Phage typable for both, independent, heat shock and acridine orange	108	11 (10.2)

^a Number in parentheses indicates percentage.

TABLE 2. Effect of treatments on 94 nontypable *S. aureus* strains at RTD $\times$ 1,000 concentration of bacteriophages

Treatment	No. tested	No. typable
Phage typable only after heat shocking for 3 min at 56°C	94	17 (18.1) ^a
Phage typable only after acridine orange, 10 μ g/ml	94	7 (7.4)
Phage typable by both, independent, heat shock and acridine orange	94	18 (19.2)

^a Number in parentheses indicates percentage.

In some cases treatment increased the number of plaques as well as the number of phages that caused lysis without substantially changing the original typing spectrum. These findings are compatible with the findings of Weiss and Nitzin (12), who reported acridine orange treatment of 20 primary typable *S. aureus* strains. Also Milch and Laszlo (10) examined standard staphylococcal strains used for phage propagation and found that with the exception of strains 3A, 3C, and 42D, heat treatment induced no change into another phage group.

"Curing" effect of treatment. Seven of the originally nontypable *S. aureus* strains that were typable after heat shocking only were subcultured from the treated culture onto Trypticase soy agar slants. After four transfers on Trypticase soy agar, the seven strains were retested without further treatment for phage patterns. The same procedure was repeated for three originally nontypable strains that were typable after acridine orange treatment only.

After retesting with bacteriophages, all of the *S. aureus* strains typable after acridine orange treatment only still showed lytic patterns and five of the seven *S. aureus* strains that were typable after heat shock only were still typable. These findings are compatible with the findings of other workers (1, 3, 8, 11, 12), who have shown that the insensitivity of primary nontypable *S. aureus* strains to phage is linked to phage-resistant genes in extrachromosomal genetic elements that can be "cured" by both heat shock treatment and acridine orange treatment.

DISCUSSION

Heat shocking (1, 2, 10, 11; Ma and Mandle, Bacteriol. Proc., p. 111, 1961) and acridine orange treatment (1, 5, 8, 11, 12) have been studied by many investigators separately, but never together. In this study both methods of treating nontypable *S. aureus* were studied to determine comparative merits of each treatment and the possible advantage of treating nontypable strains by both methods, independently, to obtain the optimum percentage of newly phage-sensitive strains.

Study results showed that heat shocking appeared to produce more hidden phage patterns than did acridine orange treatment. When both independent types of treatment were used on nontypable *S. aureus* strains and RTD $\times$ 1,000 phages were employed, it was possible to type 45% of previously nontypable strains. Along with the 50% of the *S. aureus* strains that were primarily typable, 72.4% of our strains now

showed phage patterns and could be legitimately used in establishing epidemiological relationships. The phage patterns of newly typable strains appeared to be extremely specific and similar in phage group distribution to primary typable strains of the hospital. Eight out of ten of the newly typable strains tested showed phage patterns after four transfers without further treatment. This finding is compatible with the findings of other investigators (1, 3, 8, 11, 12), who showed that the insensitivity of *S. aureus* to bacteriophage is linked to resistant phage genes in plasmids. This insensitivity of *S. aureus* to bacteriophage can be "cured" by eliminating plasmids by both heat shock and acridine orange treatments.

In a laboratory primarily concerned with the epidemiology of hospital-acquired cross-infections, the typing of nontypable *S. aureus* is of great value in pinpointing the source of these infections. Once the source of the infection is established, appropriate measures can be taken to control future outbreaks. The findings presented here indicate that the routine employment of both heat shocking and acridine orange treatment in phage typing of primary nontypable *S. aureus* strains will demonstrate the presence of phage patterns in at least 45% of previously nontypable strains. These newly typable strains can be used in the establishment of important epidemiological associations.

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