THE EFFECT OF ARTIFICIALLY DEVELOPED ERYTHROMYCIN RESISTANCE ON THE STABILITY OF PHAGE TYPE AND BIOCHEMICAL CHARACTERISTICS OF *STAPHYLOCOCCUS AUREUS*

By

John Floris Eisses

AN ABSTRACT

Submitted to the College of Science and Arts
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Four defined cultures of Staphylococcus aureus were made resistant to erythromycin by means of successive transfers in increasing concentrations of the antibiotic. Three of the strains were made resistant to 2000 ug of erythromycin per ml. The fourth strain was made resistant to 100 ug of erythromycin per ml.

Phage typing and biochemical testing of the initial and final resistant cultures showed a reduction in hemolysin production and mannitol fermentation, but no significant change in the phage pattern.

When the final resistant strains were transferred daily through forty transfers in erythromycin free broth, the hemolysin production and mannitol fermentation properties returned to normal. Three strains initially made resistant to 2000 ug of erythromycin per ml did not lose this degree of resistance, whereas the fourth strain lost its degree of resistance.

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INTRODUCTION

Infections due to Staphylococcus aureus have recently been designated as the number two communicable disease problem in the United States. This bacterium, one of the first described during the "Golden Era" of bacteriology, has a varying ability to overcome the body defenses. As a result, the organism may live in a commensal state and the host becomes a staphylococcus carrier. The relationship may change to a parasitic one, when the organisms invade the tissues, with the pathogen producing infection and possible death. Certain strains of S. aureus are able to overcome the body defense mechanism and to resist the action of many drugs. It is this ability to adapt itself to unfavorable situations that has allowed S. aureus to become such a serious health problem.

A striking example of this organism's versatility is its ability to develop resistance to the commonly used antibiotics. Due to this characteristic, the selection of antibiotics is becoming quite limited. At this date, we are faced with a particular strain of the organism resistant to penicillin, streptomycin, and the tetracycline compounds, but sensitive to erythromycin and chloromycetin. This strain appears to be the primary agent in many epidemics of staphylococcic infection and

is designated the "Epidemic Strain" (Shaffer et al., 1957). The prevalence of outbreaks of staphylococcal infection due to this strain, particularly in maternity and surgical wards, has been the cause of much concern.

In recent years, a new laboratory method for the identification of S. aureus strains has been developed. This procedure employs staphylococcic bacteriophage as a means of identifying these organisms. The diagnosis is made by observing a pattern of reaction in which one or more phages will lyse a single strain of staphylococcus. This reaction is noted as a pattern rather than the specific type lysis observed in the phage typing of Salmonella typhosa (Craigie and Yen, 1938). The phage typing method has proven very useful in studying outbreaks in which carriers were found to have organisms with a pattern similar to the infecting strain.

As was noted above, we are able to observe strains of staphylococci exhibiting particular patterns of resistance with various antibiotics. This antibiotic pattern along with the phage pattern presents a more complete characterization of the particular strain. In routine diagnostic work done on S. aureus specimens from a Michigan hospital, we noted that many cultures were found showing the antibiotic and phage pattern of the "Epidemic Strain". In this case, erythromycin was administered as a

preventive therapy. Approximately a year later, organisms were isolated from this same hospital showing in addition a resistance to erythromycin and an altered phage pattern. In order to test the stability of the phage pattern, a study of the effect of artificial production of erythromycin resistance in staphylococci and its effect on the phage and biochemical characteristics of the organism was undertaken.

LITERATURE REVIEW

Since the discovery of bacteriophage by Twort (I9I5), rapid advances have been made in the characterization and subsequent use of this bacterial virus as a specific typing tool in the identification of several groups of organisms, particularly Salmonella typhosa. It was not until I935 that any positive information was made available as to the validity of phages of staphylococci as a diagnostic tool.

Burnet and Lush (I935) stated that the S. aureus phages could be divided into phage groups distinctly related to the staphylococcic serological types. The initial application by Williams and Timmins (I938) differentiated staphylococcal strains by means of their susceptibility to phage action in broth. This work also indicated that strains causing acute osteomyelitis were similar to those found in the nose and throat of the infected individual. They indicated that staphylococcic phage could be used as a practical diagnostic tool.

It became necessary to devise a method of isolating specific bacteriophages in order to obtain more consistent and accurate results. Fisk (I942a) found that strains of S. aureus, isolated from various sources, commonly carried latent or symbiotic bacteriophages. He observed that the incidence of lysogenic bacteria in his study

of 43 strains was 44.2 per cent. Staphylococci other than S. aureus could not be identified as lysogenic strains and consequently were not lysed by phages isolated from strains of S. aureus. This indicated that the action of the phages isolated was specific only for strains of S. aureus. Since these phages exhibited selective activity specific for strains of S. aureus, Fisk was able to isolate 24 different bacteriophages by matching a lysogenic staphylococcus with one sensitive to the latent phage. This method is called the "Cross Culture Technique". In further work with these phages, Fisk (1942b) was able to separate cultures of pathogenic staphylococci into groups by the differentiation of the phage pattern. He found that organisms isolated from related sources reacted with the same phages and could be differentiated from other cultures by this method. Fisk also noted that the reaction capacity of staphylococci to phage was not readily altered by changes in their environment. In order to re-emphasize the stability of phage patterns in staphylococci, Fisk and Mordvin (1944) isolated organisms from related sources at different intervals of time. They found that although the strains varied at times in their properties of hemolysis, pigmentation and toxogenicity, the phage pattern remained stable. They concluded that the bacteriophage pattern of an organism is quite stable under normal conditions,

even though other properties of the organism showed variation. Wilson and Atkinson (I945) modified the salmonella typing method of Craigie and Yen (I938) to fit the conditions for phage typing staphylococci. This technique, "The Plate Method", was more readily standardized because phages were applied to an evenly seeded surface by means of a pipette. By this method of typing, they obtained a more accurate reading of the lytic reactions.

Using these improved methods, many workers reported the use of staphylococcic phage typing in the investigation of various outbreaks in Australia (Rountree and Thompson, I952), England (Barber and Whitehead, I949), Canada (St. Martin et al., I95I), and later in the United States (Whysham snd Kirby, I957; Shaffer et al., I957). Although several articles from independent workers had appeared on phage typing, no logical methodology had been published until that reported by Williams and Rippon (I952). Detailed methods of propagating phages, testing for their critical dilution, typing, and interpreting the results were given. The data presented, showed that minor modifications in age of the cultures to be typed, the length of time to dry the seeded plates, the mode of spotting phages, the intervals of time between drying the plates and applying the phage and the mode of incubation did not seem to alter the final results.

Concurrently, Blair and Carr (I953) described the basic method of phage typing in the United States. Their material supported the method summarized by Williams and Rippon to a great extent.

The work of Blair in this country, Williams in England and Rountree in Australia resulted in a general increase in interest in the bacteriophage typing method. The application of this method to the epidemiology of staphylococcal infection allowed for more precise control measures to be developed.

A new field of drug therapy was opened with the discovery of penicillin by Fleming (I929) and its subsequent use as a bactericidal agent. In a relatively short period following the initial use of penicillin, it became apparent that many of the organisms exposed to this drug developed resistance. This was particularly evident with S. aureus. Rodgers (I956) has graphically presented the increasing occurrence of infections due to penicillin resistant strains of S. aureus (figure I).

The increasing prominence of penicillin resistant strains led to the study of the mechanisms of resistance. It was found that strains made resistant to a high concentration of penicillin showed various morphological and chemical changes (Klimek, Cavallito and Bailey, I948). These authors noted that as antibiotic resistance was

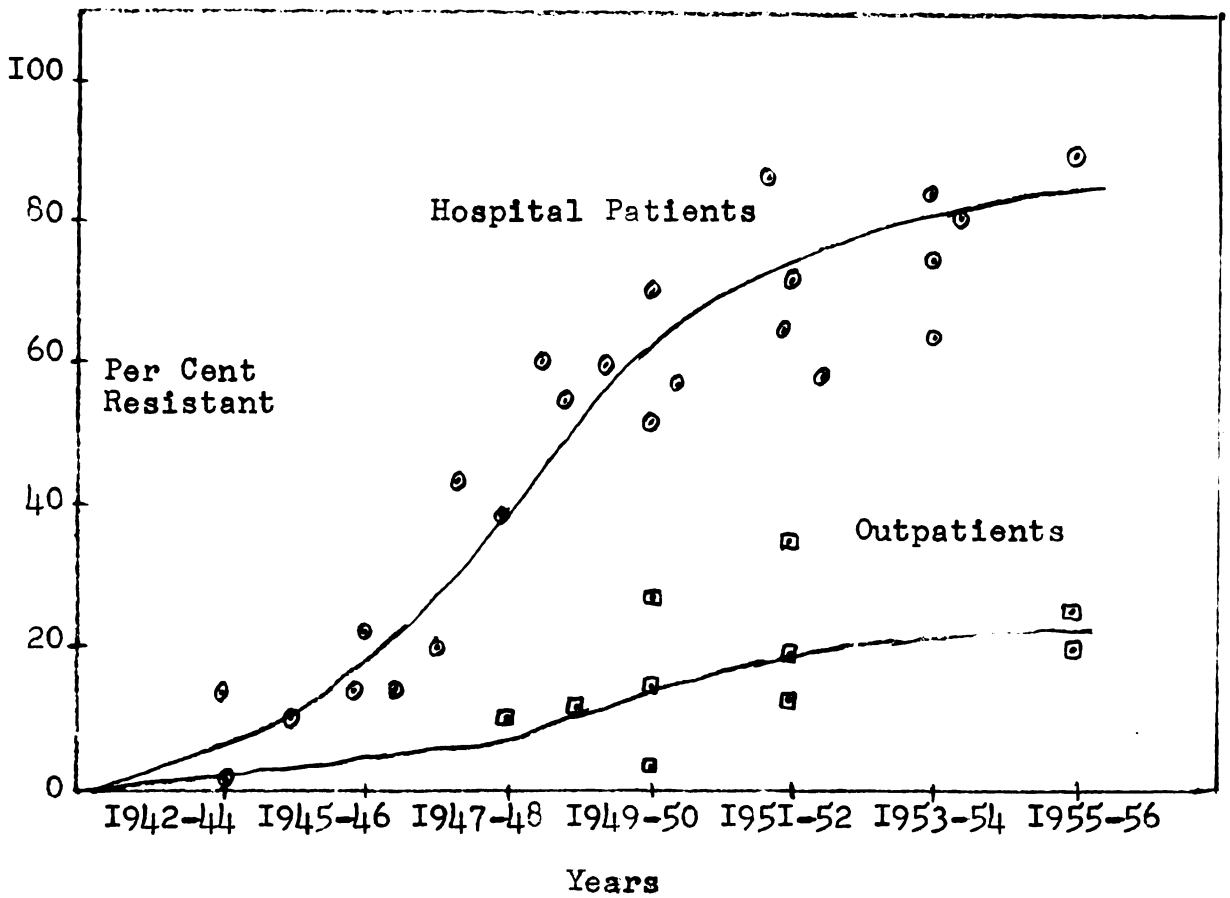

Figure I. The increasing penicillin resistance of staphylococci isolated from hospitalized patients and outpatients (Taken from Rodgers, 1956).

developed, hemolysin production was retarded, pigment production was lost and the cultures became pleomorphic and gram negative. They found that with reversal of resistance these characteristics returned to normal. Bellamy and Klimek (1948) summarized the properties exhibited by penicillin resistant staphylococci. The resistant cultures examined were inhibited in the growth phase by prevention of the assimilation of glutamic acid. A progressive loss of fermentative ability was noticed. The organisms also varied in morphology, gram stain reaction, production of the enzyme penicillinase, and utilization of nicotinic acid. When the organisms were grown in a deficient medium, these acquired characteristics were lost. The mechanism of antibiotic resistance was also studied by Garrod (1950) who observed that staphylococci exhibited either a natural or an acquired resistance. He found that naturally resistant organisms were penicillinase producers while those acquiring resistance in vitro by adaptation did not have the ability to produce this enzyme.

At present, the resistance of staphylococci to antibiotics is not confined to penicillin alone, but can be demonstrated with any new antibiotic introduced and regularly used. Organisms are commonly isolated in which multiple antibiotic resistant patterns are exhibited. These cultures are most commonly found in

environments where antibiotics are routinely used. One particular strain is now of prime importance, not only because of its degree of infectiveness, but also because of its marked resistance to many of the more commonly used drugs. This is the so-called "Epidemic Strain" previously referred to (Shaffer *et al.*, I957). However, the prevalence of staphylococcal infections has been little influenced by antibiotics (Waisbren, I958). Recent work done with erythromycin seemed to indicate this antibiotic as a drug of choice. Manten (I956) studied the action and interaction of various antibiotics. He found that erythromycin was bacteriostatic and showed a synergistic action with streptomycin. Garrod (I957) studied the cross resistant relationship of the erythromycin group of antibiotics. Strains of staphylococci grown *in vitro* , developed a complete cross resistance when treated with erythromycin. This was not found to be the case in organisms isolated from infections. Erythromycin resistant organisms could be either resistant or non-resistant to spiramycin and oleandomycin.

The mechanism of the development of resistance to antibiotics is not entirely understood. It has been observed that the emergence of resistance varies widely, even among strains of a given species. Bacterial resistance in active infections is thought to result from selection and not their mutation (Finland, I958).

Although attempts have been made to identify the mode of resistance, the fact remains that the resistance of S. aureus to antibiotics poses a serious public health problem.

The problem of antibiotic resistance was observed at approximately the same time that the staphylococcus phage typing method was being developed. It was a logical conclusion that the various relationships of antibiotic resistance and the staphylococcus phage pattern would be studied. Himmelweit (1945) studied the action of penicillin on bacteriophage and their combined action on strains of staphylococci. He found that penicillin did not affect the multiplication of phage K, nor did it interfere with its lethal and lytic action. The combination of phage and penicillin produced more rapid lysis on the bacteria than either alone. Together, they also affected rapid and complete sterilization, indicating that penicillin-resistant organisms were killed more rapidly by the combined action of phage and the antibiotic. In order to explain this rapid lysis resulting from the combined action of phage and antibiotic on the sensitive organisms, Rountree (1947) studied the effect of penicillin on a lysogenic strain of S. aureus. She noted that the lytic phage action did not become evident after the lysogenic cell had been treated with

penicillin. She believed that possibly the lysis of the bacterial cell by penicillin destroys the surface molecular configuration of the cell which is involved in phage absorption. Thus the lysis of staphylococcus strains by penicillin did not release the latent phage, nor were treated cells able to absorb latent phage. Phage in itself did not seem to be affected by the presence of penicillin. The possibility remained, however, that the phage pattern might be altered in an organism with a developed resistance to an antibiotic. Fouace (1953) developed three strains of pathogenic S. aureus, resistant in vitro to penicillin. He found that the variation in phage pattern from the initial to the final resistant strain was so slight as to be considered insignificant. The changes observed in biochemical reactions were considered to be a result of the slow growth of the resistant strain rather than a change in the physiological system of the organism itself. The slight variations in the phage pattern were also explained on the basis of poor growth of the bacteria rather than the result of the developed resistance. Although the in vitro resistance studies of Fouace (1953) seemed to indicate the phage pattern as a stable characteristic, evidence was presented showing a possible correlation between the antibiotic and phage pattern of a given strain. Jackson et al. (1954) attempted to compare

the phage patterns with the antibiotic resistance patterns of bacteria. Even though they were able to draw a rough correlation between the two characteristics, they suggested that this classification could be produced by selection of naturally resistant strains by antibiotic therapy and not by an adaptive mechanism of resistance. Barber and Burston (I955) noticed, in their study of antibiotic and phage pattern correlation, that all the strains showing multiple resistance to antibiotics reacted with the bacteriophages of group III.

Wise et al.(I956) confirmed the findings of Jackson and Barber on phage and antibiotic correlation. They indicated that the appearance of antibiotic-resistant staphylococci is a property of certain strains particularly those of group three and the untypable strains. They observed, however, that though the above resistance indicated natural selection, there was also the possibility of resistance due to either mutation or adaptation.

Although there has been work done which seems to indicate that resistance could be mutational or adaptive, the present trend places the emphasis on natural selection. In this instance, phage pattern could very well be altered by resistance acquired through natural selection (Pakula, I956).

MATERIALS

Erythromycin

A stock solution of erythromycin was prepared from erythromycin base supplied by Dr. B.H. Olson of the Michigan Department of Health Laboratories. The stock solution consisted of 2000 ug of erythromycin per ml of trypticase soy broth with 2 per cent ethanol added to keep this concentration of antibiotic in solution at 4 C.

Bacteriophage

The bacteriophage preparations used were dilutions of those received from Dr. J.E. Blair of the Hospital of Joint Diseases in New York City by Dr. W.W. Ferguson. The 24 phages are used routinely at the Michigan Department of Health Laboratories in Lansing. They include the "basic" phages that are recommended by the International Committee, plus 4 additional phages that are in use at the Hospital of Joint Diseases. These phages were chosen as being the most significant for accurate identification in routine typing. The phages used in this study are grouped as follows:

Group I. 29, 52, 52A, 79, 80

Group 2. 3A, 3B, 3C, 55

Group 3. 6, 7, 42E, 47, 53, 54, 70,
73, 75, 77, 42B, 47C, Val4

Group 4. 42D; Miscellaneous 81

Trypticase Soy Media

Trypticase soy broth and agar were used in the typing procedure, as recommended by Blair (I953). This broth was also used as a diluent in the tube dilutions and preparation of the erythromycin stock solution. The agar was used to prepare slants for stock culture and for phage plates. These media were prepared according to the following formulae:

Trypticase Soy Broth

Trypticase	I5 gm
Phytone	5 gm
Sodium chloride	5 gm
Distilled water	I000 ml

To this broth, I5gm/l of agar were added to prepare the trypticase soy agar.

Coagulase-hemolysis Medium

Material used for the testing of hemolysis and coagulation in a single tube test is that employed in the routine examination for staphylococci at the Michigan Department of Health Laboratories (Graham, I957). It is prepared as follows:

Step I. Veal infusion is prepared from:

Lean ground veal	I lb
Distilled water	I000 ml

Step 2. A veal infusion broth is made by combining:

Veal infusion (step I)	I000 ml
"Bacto" peptone	I0 gm
Sodium chloride	5 gm

Step 3. The coagulase-hemolysis medium is then prepared by using:

Veal infusion broth (step2)	50 ml
Citrated rabbit blood	3 ml
Citrated rabbit plasma	7.5 ml

Mannitol Broth

A proteose peptone broth with mannitol added to a concentration of I per cent and containing brom cresol purple as an indicator was used.

Sheep Blood Veal Infusion Agar

This medium was used as a growth medium for the determination of the antibiotic sensitivity pattern of the organism. The blood plates were made by preparing a 2 per cent veal infusion agar as follows:

Veal infusion	I000 ml
"Bacto" peptone	I0 gm
Sodium chloride	5 gm
Agar	20 gm

To a 300 ml volume of this 2 per cent veal infusion agar were added I5 ml of sterile defibrinated sheep blood.

Antibiotic Sensitivity Disks*

Sensitivity disks were used to identify the antibiotic pattern and contained the following antibiotics with concentrations per tab as indicated:

Erythromycin	I0 ug
Terramycin	I0 ug
Tetracycline	I0 ug
Chloromycetin	I0 ug
Penicillin	I.5 units
Streptomycin	I0 ug

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* "Multidisk" (7-64A)

Case Laboratories
5I5 N. Halsted St.
Chicago 22, Illinois

METHODS

Acquired resistance to erythromycin was developed using daily serial transfers in increasing concentrations of the antibiotic. Procedures used were similar to those described by Rammelkamp and Maxon (I942) in testing the sensitivity of organisms to penicillin. They prepared a series of doubling dilutions by adding 0.2 ml of stock penicillin with 0.2 ml of broth. A 0.2 ml volume of this mixture was added to 0.2 ml of broth and this procedure repeated to obtain the desired dilution series. To test for sensitivity, they used 0.5 ml of inoculum.

This method was modified to fit the resistance build-up procedure by mixing 2 ml of trypticase soy broth and 2 ml of erythromycin to begin the dilution series. Of this mixture, 2 ml were added to 2 ml of broth and this step was repeated to make the desired doubling dilution series. An inoculum of 0.05 ml was employed to reduce the amount of inoculum per volume of broth mixture.

After having established the sensitivity of an organism, a series of dilutions was prepared which bracketed the sensitivity level of the organism. From the Michigan Department of Health Laboratory stock cultures, four cultures of S. aureus were selected which were originally sensitive to 0.I5 ug/ml of erythromycin. One drop (0.05 ml) of a 24 hour broth culture was added to each serial dilution by means of

a 2 ml pipette. After incubation at 37 C for I8-24 hours, the lowest dilution showing definite growth was used to inoculate the next series of dilutions. This procedure was repeated daily without allowing the bacteria to grow in the absence of erythromycin at any time.

The method used for phage typing is that described by Blair and Carr (I953), and modifications in the method are those recommended by Blair in a work sheet forwarded to Dr. W.W. Ferguson (I957) at the Michigan Department of Health Laboratories. The cultures used were incubated at 37 C for 4-6 hours in trypticase soy broth. Approximately I ml of the broth culture was used to seed the surface of square plastic plates containing trypticase soy agar, the surface of which had been allowed to dry for approximately 2 hours at 37 C. Excess broth culture was removed from the plate by means of a pipette. After the seeded surface had dried, the phage preparations were applied by means of a 2 ml tuberculin syringe fitted with a blunted 27 guage needle. The plate had been previously marked with 25 squares. The phage spots were then allowed to dry and the plates were incubated at 37 C for I8-24 hours. The phage spots on the plate were then read for lysis or clearing of growth.

RESULTS

Three cultures of S. aureus (308, 689 and 736) were selected from among cultures isolated in the routine examination for staphylococci at the Michigan Department of Health Laboratories in Lansing, Michigan. The fourth culture (I6) was that submitted for phage typing by a Michigan hospital. This particular culture was also typed by Blair in New York and Wentworth in Columbus, Ohio. Identical phage patterns were obtained in each laboratory.

Cultures were selected by testing for the following characteristics:

1. Alpha hemolysis
2. Mannitol fermentation
3. Coagulase production
4. Pigment production
5. Antibiotic pattern
6. Phage pattern

The initial cultures were given three successive 24 hour transfers in trypticase soy broth. A 6 hour broth culture was then grown from the third 24 hour broth culture. A loopful of this 6 hour broth culture was streaked on a blood agar plate. A single isolated colony of each strain was picked to broth. This broth culture was characterized for the above criteria (table I). Duplicate stock cultures and I2 lyophilized cultures were made from the initial broth cultures of each of the strains.

TABLE I.

The Initial Reactions of the
Four Selected Cultures

Culture	Erythromycin Resistance ug per ml	Biochemical Reactions*			Antibiotic Pattern**						Phage Pattern
		H	C	M	E	T	Te	C	P	S	
I6	0.I5	+	+	+	S	R	R	S	R	S	42B/52/80/8I
308	0.I5	+	+	+	S	R	R	S	R	R	42B/47C/52/80/8I
689	0.I5	+	+	+	S	S	S	S	S	S	29/42B/47/47C/52/52A/79/80
736	0.I5	+	+	+	S	S	S	S	R	S	Val4/42B/42D/42E/47C/52/8I

* H= Hemolysin
C= Coagulase
M= Mannitol

** E= Erythromycin
T= Terramycin
Te=Tetracycline
C= Chloromycetin
P= Penicillin
S= Streptomycin

\+ = Positive

S = Sensitive
R = Resistant

The initial broth cultures of the 4 strains selected were made resistant to erythromycin by means of the serial tube dilution transfer method. Approximately 0.05 ml of the initial broth culture was transferred to each of the tubes in the dilution series. The tubes were then incubated overnight at 37 C. The following morning, the tube with the highest concentration of erythromycin showing good growth was selected for transfer to the next series of dilutions. Daily transfers were made in this manner. Each daily broth culture was also used for antibiotic sensitivity determinations, biochemical testing and phage typing (tables 2, 3, 4 and 5). Daily stock cultures were also made on agar slants containing erythromycin in a concentration of at least 10 per cent of that to which the organism was resistant. This was found adequate to maintain the resistance level of the particular strain.

In order to observe these cultures under standard conditions, the initial and final stock cultures were transferred to broth and a 6 hour broth culture was made and tested for biochemical reactions, antibiotic and phage patterns. Two sets of cultures were typed in duplicate and incubated overnight at 30 C for the one set and 37 C for the other (table 6).

TABLE 2.

Development of Resistance to Erythromycin of *S. aureus*, Culture I6

Transfer Day	Erythromycin Resistance ug per ml	Biochemical Reactions* H	C	M	Phage Pattern 30 C Incubation**
0	0.I5	+	+	+	42B/52/80/8I
I	0.I5	+	+	+	42B/52/80/8I
2	0.I5	+	+	+	42B/52/80/8I
3	0.60	+	+	+	42B/52/80/8I
4	I.20	+	+	+	42B/52/80/8I
5	5.00	±	+	-	42B/52/80/8I
6	5.00	±	+	±	42B/52/80/8I
7	5.00	±	+	-	42B/52/80/8I
8	I0.00	±	+	-	42B/52/80/8I
9	20.00	±	+	±	42B/52/80/8I
I0	25.00	±	+	-	42B/52/80/8I
II	25.00	±	+	-	42B/52/80/8I
I2	25.00	±	+	-	42B/52/80/8I
I3	50.00	±	+	-	42B/52/80/8I
I4	50.00	±	+	+	42B/52/80/8I
I5	50.00	±	+	-	42B/52/80/8I
I6	50.00	±	+	-	42B/52/80/8I
I7	50.00	-	+	+	42B/52/80/8I
I8	50.00	-	+	±	42B/52/80/8I
I9	50.00	-	+	-	42B/52/80/8I
20	50.00	-	+	±	42B/47C/52/80/8I
2I	I00.00	-	+	±	42B/47C/52/80/8I
22	I0C.00	±	+	±	42B/47C/52/80/8I
23	I00.00	-	+	-	42B/47C/52/80/8I
24	I00.00	-	+	-	42B/47C/52/80/8I
25	I00.00	-	+	-	42B/47C/52/80/8I
26	I00.00	-	+	-	42B/47C/52/80/8I
27	I00.00	-	+	-	42B/47C/52/80/8I

* H= Hemolysis
C= Coagulase
M= Mannitol

\+ = Positive
± = Doubtful
\- = Negative

** Due to slow growth, phage plate cultures from transfer 20 through 27 were incubated at 37 C.

TABLE 3.

Development of Resistance to Erythromycin of S. aureus, Culture 308

Transfer Day	Erythromycin Resistance ug per ml	Biochemical Reactions* H	C	M	Phage Pattern 30 C Incubation
0	0.I5	+	+	+	42B/47C/52/80/8I
I	0.I5	+	+	+	42B/47C/52/80/8I
2	0.I5	+	+	±	42B/47C/52/80/8I
3	0.I5	+	+	±	42B/47C/52/80/8I
4	0.30	+	+	+	42B/47C/52/80/8I
5	0.60	±	+	+	42B/47C/52/80/8I
6	I.20	-	+	±	42B/47C/52/80/8I
7	I.20	+	+	±	42B/47C/52/80/8I
8	20.00	±	+	±	42B/47C/52/80/8I
9	40.00	±	+	±	42B/ - /52/80/8I
I0	63.00	+	+	±	42B/47C/52/80/8I
II	250.00	+	+	-	42B/ - /52/80/8I
I2	I25.00	+	+	-	42B/ - /52/80/8I
I3	I25.00	+	+	-	42B/ - /52/80/8I
I4	250.00	+	+	-	-not tested-
I5	300.00	+	+	-	42B/ - /52/80/8I
I6	400.00	+	+	-	42B/ - /52/80/8I
I7	500.00	+	+	-	42B/ - /52/80/8I
I8	I000.00	+	+	-	42B/ - /52/80/8I
I9	I500.00	+	+	-	42B/ - /52/80/8I
20	I500.00	+	+	-	42B/ - /52/80/8I
2I	I500.00	+	+	+	42B/ - /52/80/8I
22	I000.00	+	+	±	42B/ - /52/80/8I
23	I500.00	+	+	-	42B/ - /52/80/8I
24	2000.00	+	+	±	42B/ - /52/80/8I

* H = Hemolysis
C = Coagulase
M = Mannitol

\+ = Positive
± = Doubtful
\- = Negative

TABLE 4.

Development of Resistance to Erythromycin
of S. aureus, Culture 689

Transfer Day	Erythromycin Resistance ug per ml	Biochemical Reactions* H	C	M	Phage Pattern 30 C Incubation**
0	0.I5	+	+	+	29/42B/47/47C/52/52A/79/80
I	0.I5	+	+	+	29/42B/47/47C/52/52A/79/80
2	0.I5	+	+	+	29/42B/47/47C/52/52A/79/80
3	0.60	+	+	±	29/42B/47/47C/52/52A/79/80
4	I.20	±	+	±	29/42B/47/47C/52/52A/79/80
5	5.00	±	+	-	29/42B/- /47C/52/52A/79/80
6	2.50	±	+	+	29/42B/- - /52/52A/79/80
7	5.00	±	+	-	29/42B/- - /52/52A/79/80
8	5.00	±	+	-	29/42B/- - /52/52A/79/80
9	I0.00	±	+	±	29/42B/47/ - /52/52A/79/80
I0	I2.50	±	+	±	29/42B/47/ - /52/52A/79/80
II	25.00	±	+	-	29/42B/- - /52/52A/79/80
I2	25.00	±	+	-	29/42B/- - /52/52A/79/80
I3	25.00	±	+	-	29/42B/- - /52/52A/79/80
I4	25.00	±	+	-	29/42B/- - /52/52A/79/80
I5	25.00	±	+	-	29/42B/- - /52/52A/79/80
I6	25.00	±	+	-	29/42B/- - /52/52A/79/80
I7	50.00	-	+	-	29/42B/- - /52/52A/79/80
I8	50.00	-	+	-	29/ - - /47C/52/52A/79/80
I9	50.00	-	+	-	29/ - - - /52/52A/79/80
20	50.00	-	+	-	Va4/29/47C/52/52A/79/80
2I	200.00	-	+	-	Va4/6/29/47/47C/52/52A 79/80
22	250.00	-	+	-	Va4/6/29/47/47C/52/52A 79/80
23	I000.00	±	+	-	Va4/6/29/42B/47/47C/52 52A/79/80
24	2000.00	-	+	-	Va4/6/29/42B/47/47C/52 52A/53/79/80
25	I000.00	-	+	-	Va4/6/29/42B/47/47C/52 52A/79/80
26	2000.00	-	+	-	29/42B/47C/52/52A/79/80
27	2000.00	-	+	-	Va4/29/42B/47/47C/52/52A 53/79/80

* H = Hemolysis, C = Coagulase, M = Mannitol
+ = Positive, ± = Doubtful, - = Negative

** Due to slow growth, phage plate cultures from transfer 20 through 27 were incubated at 37 C.

TABLE 5.

Development of Resistance to Erythromycin of S. aureus, Culture 736

Transfer Day	Erythromycin Resistance ug per ml	Biochemical Reactions* H C M	Phage Pattern 30 C Incubation
0	0.I5	+ + +	Va4/42B/42D/42E/47C/52/8I
I	0.I5	+ + +	Va4/42B/42D/42E/47C/52/79/8I
2	0.I5	+ + +	Va4/42B/42D/42E/47C/52/79/8I
3	0.60	+ + +	Va4/42B/42D/42E/47C/52/79/8I
4	I.20	+ + +	Va4/42B/42D/42E/47C/52/8I
5	2.50	+ + +	Va4/42B/42D/42E/47C/52/8I
6	5.00	+ + +	Va4/42B/42D/42E/47C/52/79/8I
7	5.00	+ + +	Va4/42B/42D/42E/47C/52/8I
8	5.00	+ + +	Va4/42B/42D/42E/47C/52/8I
9	I0.00	+ + +	Va4/42B/42D/42E/47C/52/8I
I0	I2.50	+ + +	Va4/42B/42D/42E/47C/52/53 77/79/8I
II	50.00	+ + +	Va4/42B/42D/42E/47C/52/53 77/79/8I
I2	200.00	+ + +	Va4/42B/42D/42E/47C/52/8I
I3	I000.00	+ + +	Va4/42B/42D/42E/47C/52/52A 53/8I
I4	2000.00	+ + +	Va4/42B/42D/42E/47C/52/52A 53/8I

* H = Hemolysis + = Positive
C = Coagulase ± = Doubtful
M = Mannitol − = Negative

TABLE 6.

Comparison of the Initial and Final Stock Cultures

Culture		Erythromycin Resistance ug per ml	Biochemical Reactions*			Antibiotic Pattern**						Phage Patterns	
			H	C	M	E	T	Te	C	P	S	30 C Incubation	37 C Incubation
I6	A	0.I5	+	+	+	S	R	R	S	R	S	42B/52/80/8I	42B/52/80/8I
	B	I00.00	-	+	-	R	R	R	S	R	S	42B/52/80/8I	42B/52/80/8I
308	A	0.I5	+	+	+	S	R	R	S	R	R	42B/47C/52/80/8I	42B/47C/52/80/8I
	B	2000.00	-	+	-	R	R	R	S	R	R	42B/ - /52/80/8I	42B/ - /52/80/8I
689	A	0.I5	+	+	+	S	S	S	S	S	S	29/42B/47/47C/52 52A/79/80	Va4/6/29/42B/47/47C 52/52A/79/80
	B	2000.00	-	+	-	R	S	S	S	S	S	29/42B/47/47C/52 52A/79/80	Va4/6/29/42B/47/47C 52/52A/75/79/80
736	A	0.I5	+	+	+	S	S	S	S	R	S	Va4/42B/42D/42E/47C 52/8I	Va4/42B/42D/42E/47C 52/ 8I
	B	2000.00	±	+	±	R	S	S	S	R	S	Va4/42B/42D/42E/47C 52/53/8I	Va4/42B/42D/42E/47C 52/8I

A = Initial Culture
B = Final Culture

* H = Hemolysis
C = Coagulase
M = Mannitol

\+ = Positive
± = Doubtful
\- = Negative

** E = Erythromycin
T = Terramycin
Te= Tetracycline
C = Chloromycetin
P = Penicillin
S = Streptomycin

S = Sensitive
R = Resistant

In order to eliminate variable factors (metabolites) from the broth culture as much as possible, the initial and final broth cultures were washed twice with unbuffered physiological saline and resuspended in broth. These cultures were typed in the same manner as was described above (table 7). In order to show the appearance of the various reactions studied, photographs were taken to show the appearance of the antibiotic patterns, biochemical reactions and phage patterns. The initial and final results of culture 308 were depicted (figures 2, 3 and 4). Six cultures of culture I6 and of culture 308, taken from stock cultures with varying degrees of resistance to erythromycin, were streaked on an agar plate containing erythromycin (figure 5).

An attempt was made to explain the mode of resistance. The disk assay method, used by Dr. Olson (I958) at the Michigan Department of Health Laboratories, was employed for this examination. The initial stock cultures of each strain were transferred to trypticase soy broth for two successive days. Six hour cultures were streaked on blood plates and incubated overnight at 37 C. Ten isolated colonies, picked from each strain, were again incubated overnight in individual tubes of broth. Two ml from each broth culture were added to 20 ml of tryticase soy agar and I0 ml transferred to each of 2 petri dishes. To each of 4 assay disks, I2 mm in

TABLE 7.

Comparison of the Initial and Final
Saline Washed Stock Cultures

Culture		Erythromycin Resistance ug per ml	Biochemical Reactions*			Antibiotic Pattern**						Phage Patterns	
			H	C	M	E	T	Te	C	P	S	30 C Incubation	37 C Incubation
I6	A	0.I5	+	+	+	S	R	R	S	R	S	42B/52/80/8I	42B/47C/52/80/8I
	B	I00.00	-	+	-	R	R	R	S	R	S	42B/52/80/8I	42B/ - /52/80/8I
308	A	0.I5	+	+	+	S	R	R	S	R	R	42B/47C/52/80/8I	42B/47C/52/80/8I
	B	2000.00	-	+	-	R	R	R	S	R	R	42B/ - /52/80/8I	42B/ - /52/80/8I
689	A	0.I5	+	+	+	S	S	S	S	S	S	6/29/42B/47/47C/52 52A/79/80	Val4/6/29/42B/47/47C 52/52A/75/79/80
	B	2000.00	-	+	-	R	S	S	S	S	S	-/29/42B/47/47C/52 52A/79/80	Val4/6/29/42B/47/47C 52/52A/53/75/79/80
736	A	0.I5	+	+	+	S	S	S	S	R	S	Val4/42B/42D/42E/47C 52/8I	Val4/42B/42D/42E/47C 52/8I
	B	2000.00	±	+	±	R	S	S	S	R	S	Val4/42B/42D/42E/47C 52/8I	Val4/42B/42D/42E/47C 52/8I

A = Initial Culture
B = Final Culture

* H = Hemolysis
C = Coagulase
M = Mannitol

\+ = Positive
± = Doubtful
\- = Negative

** E = Erythromycin C = Chloromycetin
T = Terramycin P = Penicillin
Te= Tetracycline S = Streptomycin

S = Sensistive
R = Resistant

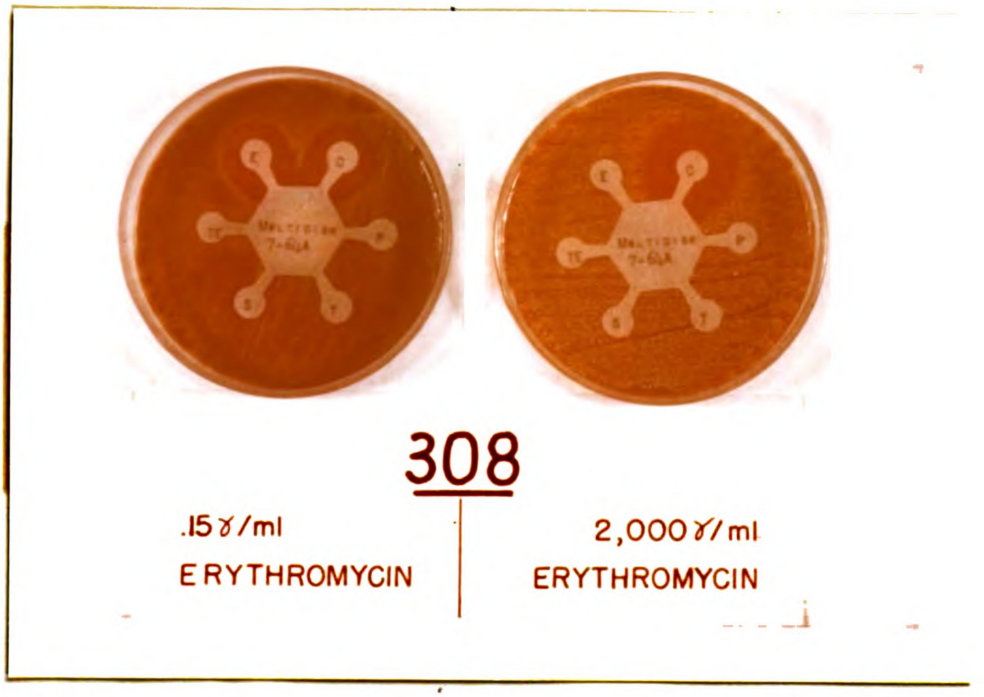

Figure 2. The appearance of the antibiotic patterns of the initial and final stock cultures of culture 308.

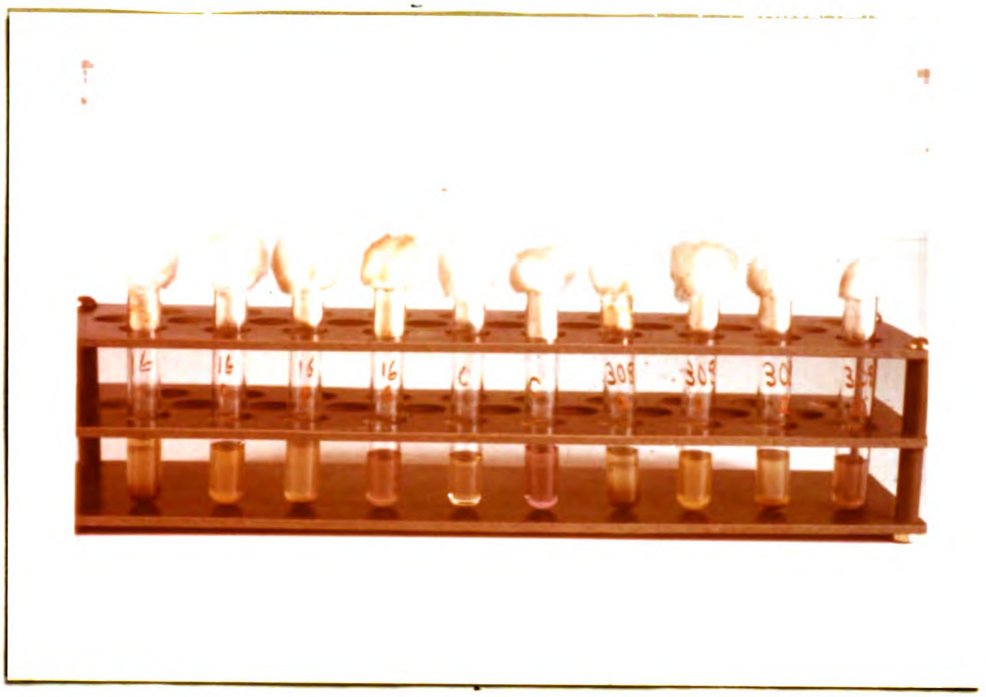

Figure 3. The appearance of the biochemical reactions of the initial and final cultures of culture I6 and 308. The letter C represents the initial cultures. The letter E represents the final resistant cultures. The control tubes are marked C. The first tube of each set represents the coagulase-hemolysis tube. The second tube of each set is the mannitol broth.

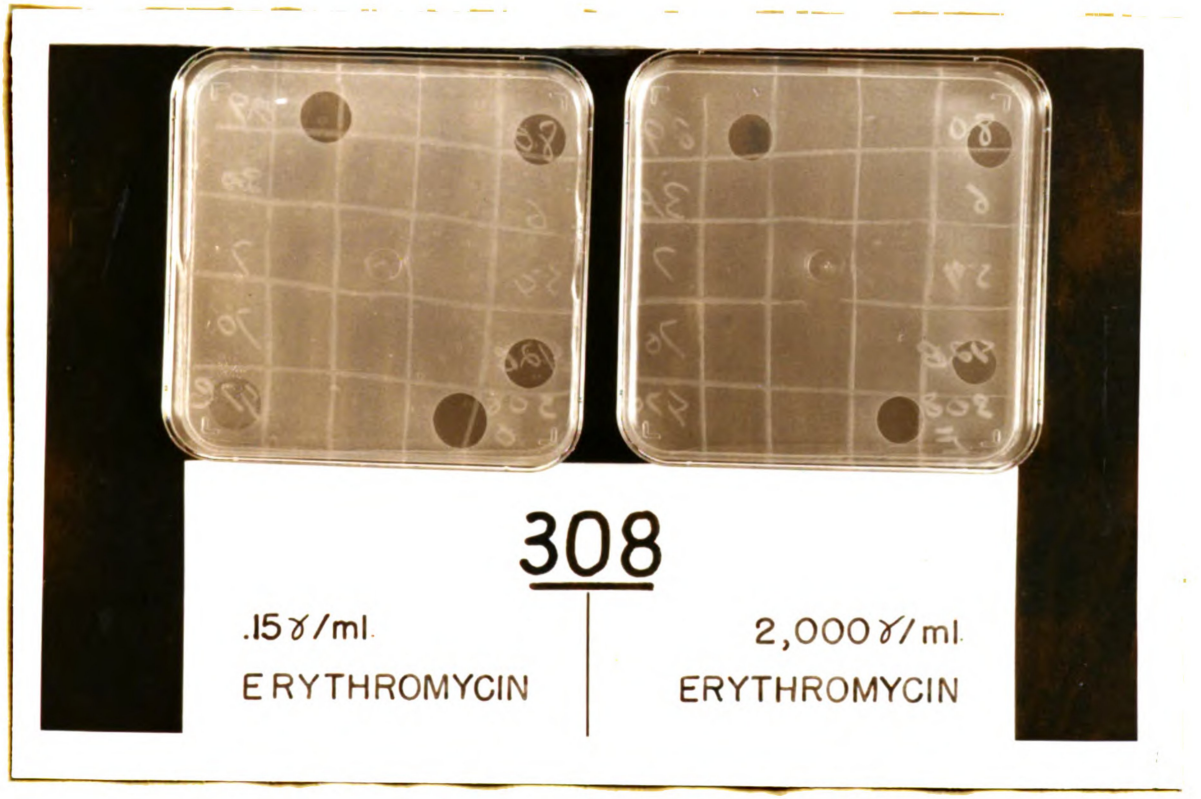

Figure 4. The phage patterns of the initial and final resistance stock cultures of culture 308.

Figure 5. The appearance of stock cultures of varying degrees of resistance when streaked on a veal infusion agar plate containing the above indicated amount of erythromycin per ml. The plates are read counter-clockwise begining with the top left section. The degree of resistance of each colony is listed with its respective section.

diameter, was added 0.09 ml of a solution containing I60 ug of erythromycin per ml. Four of these treated disks were placed on each of the seeded plates. These plates were incubated overnight at 37 C and the diameter of the zones of inhibition were read. The results showed a fairly regular zone of resistance for the colonies of each culture.

At the end of the build-up period, resistant cultures of each strain were transferred daily in erythromycin free broth. Forty successive transfers were made with daily testing for biochemical reactions, loss of resistance and antibiotic pattern. This was done in order to determine if resistance was lost when the resistant culture was grown in the absence of erythromycin (tables 8 and 9). Strain I6 did not entirely regain its biochemical characteristics and lost its high degree of resistance to erythromycin. Strains 308, 689 and 736 regained their biochemical characteristics, but did not lose their high degree of resistance to erythromycin.

TABLE 8.

Reactions of Two Cultures of S. aureus With Daily Transfer in Erythromycin-free Broth

Transfer	Culture I6				Culture 308			
	Erythromycin Resistance ug per ml	Biochemical Reactions* H C M	Antibiotic Pattern** E T Te C P S		Erythromycin Resistance ug per ml	Biochemical Reactions* H C M	Antibiotic Pattern** E T Te C P S	
0	I00.00	- + -	R R R S R S		2000.00	- + -	R R R S R R	
5	I00.00	- + +	R R R S R S		2000.00	- + -	R R R S R R	
I0	I00.00	+ + ±	R R R S R S		2000.00	- + ±	R R R S R R	
I5	I00.00	- + ±	SR R R S R S		2000.00	- + +	R R R S R R	
20	50.00	- + +	SR R R S R S		2000.00	- + +	R R R S R R	
25	50.00	- + +	SR R R S R S		2000.00	+ + +	R R R S R R	
30	-	+ + +	S R R S R S		2000.00	+ + +	R R R S R R	
35	-	± + +	S R R S R S		2000.00	+ + +	R R R S R R	
40	-	± + +	S R R S R S		2000.00	+ + +	R R R S R R	

* H = Hemolysis
C = Coagulase
M = Mannitol

\+ = Positive
± = Doubtful
\- = Negative

** E = Erythromycin C = Chloromycetin
T = Terramycin P = Penicillin
Te= Tetracycline S = Streptomycin

S = Sensitive
SR = Partial Resistance
R = Resistant

TABLE 9.

Reactions of Two Cultures of S. aureus With Daily Transfer in Erythromycin-free Broth

Transfer	Culture 689										Culture 736									
	Erythromycin Resistance ug per ml	Biochemical Reactions*			Antibiotic Pattern**						Erythromycin Resistance ug per ml	Biochemical Reactions*			Antibiotic Pattern**					
		H	C	M	E	T	Te	C	P	S		H	C	M	E	T	Te	C	P	S
0	2000.00	-	+	-	R	S	S	S	S	S	2000.00	±	+	±	R	S	S	S	R	S
5	2000.00	-	+	±	R	S	S	S	S	S	2000.00	+	+	+	R	S	S	S	R	S
10	2000.00	-	+	+	R	S	S	S	S	S	2000.00	+	+	+	R	S	S	S	R	S
15	2000.00	±	+	+	R	S	S	S	S	S	2000.00	+	+	+	R	S	S	S	R	S
20	2000.00	-	+	+	R	S	S	S	S	S	2000.00	+	+	+	R	S	S	S	R	S
25	2000.00	±	+	+	R	S	S	S	S	S	2000.00	+	+	+	R	S	S	S	R	S
30	2000.00	+	+	+	R	S	S	S	S	S	2000.00	+	+	+	R	S	S	S	R	S
35	2000.00	+	+	+	R	S	S	S	S	S	2000.00	+	+	+	R	S	S	S	R	S
40	2000.00	+	+	+	R	S	S	S	S	S	2000.00	+	+	+	R	S	S	S	R	S

* H = Hemolysis
C = Coagulase
M = Mannitol

** E = Erythromycin
T = Terramycin
Te= Tetracycline
C = Chloromycetin
P = Penicillin
S = Streptomycin

\+ = Positive
± = Doubtful
\- = Negative

S = Sensitive
SR = Partial Resistance
R = Resistant

DISCUSSION

Of the four cultures of S. aureus picked, three developed resistance to 2000 ug of erythromycin per ml or a I3,000 fold increase in resistance. The resistance of the fourth culture (no. I6) was stabilized at I00 ug of erythromycin per ml or a 666 fold increase in 2I days. In general, the strains became resistant to erythromycin at a fairly rapid rate. However, there was a variability in the time required to develop a resistance of 2000 ug per ml. In the case of culture no. I6, this point was never reached. Culture no. 736 reached this 2000 ug per ml level of resistance in I4 days with excellent growth at all times and little change in the biochemical reactions. This difference in speed of increased resistance is attributed to the variable adaptability of the given strain to the antibiotic. Similar antibiotic patterns at each daily testing indicated that the organism was the same and stable in this respect.

Although culture no. 736 showed little evidence of reduction of growth or change in biochemical reactions, this was not true with the other strains. In all cases, the mannitol fermentation seemed the least stable, hemolysis production was next, while the coagulase reaction was never altered. At this point, using culture no. 736 as an example, it would appear that these biochemical alterations were due to a reduction in the

growth rate of the organism leading to a slow mannitol fermentation and a reduction in the production of hemolysin which was occluded by the coagulation of the plasma. The reappearance of these characteristics, as the growth rate increased, was demonstrated when these cultures were grown in media without erythromycin.

There was an apparent change in the characteristic phage pattern of the cultures as the resistance of the organisms to erythromycin increased. This can best be explained on the basis of variation due to the growth rate of the organism as well as possible daily variations in the phage typing technique. Any apparent gain or loss was due to a minor change of an already weak or questionable characteristic. The types showing strong reactions were not altered. The typing of the organism under standard conditions showed only slight variations in the phage pattern. In general, the lysis of the cells was more prominent in the resistant organisms. These changes, too, may be explained by the fluctuation of a poorly expressed characteristic. No significant alteration of the phage pattern occurred.

Similarity in the measurements of the zones of inhibition of I0 isolated colonies picked from each initial cultures showed that in general the population was fairly uniform with regard to resistance. However, there were one or two colonies in each case showing

significant difference in either resistance or sensitivity. This suggests that within a given population some organisms will show a greater tendency to develop resistance than others. The general population apparently decides the rate of the development of resistance.

When the resistant strains were transferred daily in erythromycin free broth, biochemical reactions, typical of the sensitive strains, were observed on the twenty to twenty-fifth day of transfer. This indicated the influence of the growth rate of the organism on the manifestation of its biochemical characteristics. Strains 308, 689 and 736 maintained a resistance level 2000 ug per ml through forty transfers. Strain I6 showed complete loss of resistance after twenty transfers and remained sensitive through the fortieth transfer. The permanence of the resistance level in strains 308, 689 and 736 indicates either the selection of a pure resistant strain or a semi-permanent adaptation to erythromycin. Strain I6 seems to show a resistance to erythromycin which is readily lost in the absence of this antibiotic.

SUMMARY

A study was made of the stability of the staphylococcal phage pattern with development of resistance of the organism to the antibiotic, erythromycin. The biochemical characteristics were also studied with a loss in the mannitol and hemolytic reactions being observed as the organisms acquired resistance. Little change was seen in the phage pattern. The minor changes observed were attributed to the slow rate of growth of the resistant organisms.

The resistant organisms were allowed to grow in the absence of erythromycin. The biochemical reactions were restored after twenty to twenty-five transfers. Three of the four strains tested maintained their resistance level through forty transfers. The fourth strain, which lost its resistance, did not attain as high a level of resistance to erythromycin as the other three strains.

A study of the resistance of individual colonies in the initial population showed a uniform degree of resistance with only a few colonies showing greater sensitivity or resistance.

CONCLUSIONS

1. The phage pattern of an organism is generally stable and is not altered significantly by the development of resistance to erythromycin.
2. The reduction of alpha hemolysin production and mannitol fermentation, associated with acquired resistance, is due to the slow growth rate of the organism.
3. The production of erythromycin resistance in strains is relatively rapid, but variable as to the level of resistance developed.
4. Selected colonies of the initial populations of the cultures studied, showed similar degrees of resistance with only a few exceptions.
5. Highly resistant organisms do not tend to revert to the original sensitivity level when grown in the absence of erythromycin.

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