USE OF BACTERIOPHAGE IN THE STUDY OF STAPHYLOCOCCIC BOVINE MASTITIS

by

Carlos Heinz Contag

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TABLE OF CONTENTS

INTRODUCTION

Staphylococcus aureus has been reported by many workers as a cause of both acute and chronic infections of the bovine udder and its significance is widely recognized.

The lack of differentiation of strains of Staph. aureus has been one of the principal obstacles in the study of the epizootiology of staphylococcal mastitis.

Phage typing has become an important tool in the differentiation of various strains of Staph. aureus. It has contributed to the epidemiological studies of infections of man caused by this agent. The bulk of the literature has been concerned with the typing of strains of human origin, whereas little attention has been paid in this respect to study of the bovine strains. The epizootiology of infectious staphylococcal bovine mastitis has yet to be completely elucidated, and a promising tool which would be used for such studies is bacteriophage typing, since it furnished a way of differentiation of strains not satisfactorily provided by other means.

The capacity of causing lysis of the susceptible strains in vitro has led to attempts to reproduce this same condition in vivo.

The known resistance of Staph. aureus to antibiotics and other therapeutic agents; the frequent failure to obtain a cure of bovine mastitis by their application; the objection

of sanitary officials and of the dairy industry to the presence of antibiotics in the market milk has led to investigation of the possibility of another and more successful method for treatment of the disease. The bacteriophage, a highly active agent in the test tube could provide this method if the obstacles that hinder its activity in vivo, could be overcome.

REVIEW OF LITERATURE

Bacteriophage Typing of Staphylococci

The differentiation and classification of various bacterial species into individual strains by means of their phage susceptibility has been applied to the grouping of staphylococci. Burnet and Lush in 1935 were able to demonstrate the grouping of certain staphylococci by analogous phage sensitivity. Williams and Timmins in 1938 suggested that phages might be used for typing. They could distinquish six phage types among staphylococci isolated from cases of osteomyelitis, by the use of four different phages obtained from Burnet and Lush. The phage sensitivity was detected by the lysis of the broth cultures.

The isolation of phages from lysogenic strains of staphylococci was described by Fisk (1942a). He found that 42.2 per cent of 43 strains tested were lysogenic. Groups of staphylococci were established in accordance with the susceptibility to phage combinations. Fisk propagated the phages on agar cultures of specific susceptible strains. Sensitivity of the coagulase positive staphylococci to the phages was indicated by the amount of lysis observed on the cultures of agar plates when the phage was "spotted" on them.

Staphylococci isolated by Fisk (1942b) from related sources that would likely yield the same strain, exhibited the same

phage susceptibility, while strains from unrelated sources showed a different phage pattern. The susceptibility of the staphylococci to phages is not readily altered by changes in environment.

Fisk's technique for the grouping of staphylococci was modified by Wilson and Atkinson in 1945 by the use of a "test dilution" for each phage. A "test dilution" was defined as the highest dilution of the phage which produced confluent lysis of its propagating strain. In addition Wilson and Atkinson applied a system to differentiate the staphylococci similar to that used in the Vi phage typing of typhoid bacilli by Craigie and Yen (1938).

Of 460 strains which were submitted by Wilson and Atkinson to the action of 19 phages, 278 (60.4%) were placed into various phage types and subtypes. One hundred four (22.6%) were susceptible to some phages that were not included in any of the types and 78 (17%) were not lysed by any phage. The authors stressed the importance of phage typing in epidemiological studies.

Smith in 1948 used the Wilson typing series of 21 phages plus 11 other phages that were isolated during his work. These phages were placed into five groups according to their action on staphylococci which had acquired phage resistance. Of the 1,016 strains of coagulase positive staphylococci isolated by Smith 948 (93.3%) could be phage typed. Eight hundred

and fifty-two (83.2%) of the 1,016 were susceptible to one or more phages of the phage group designated as 42D.

Rountree (1949a) indicated that most strains of staphylococci are lysogenic.

Rountree (1949b) reported that the differentiation of phages by serological methods is the most reliable. She classified the phages with antiphage sera of rabbit origin, and divided the 39 phages into at least six serological groups, which were designated as A, B, C, D, E, and F. Phages belonging to the A group lysed coagulase positive staphylococci of human origin. Group B lysed coagulase positive staphylococci of human and bovine origin. The author stressed the importance of the serological examination of the phages appearing during adaptation of phage from one strain of staphylococcus to another.

Williams and Rippon, (1952) stressed the greater value of phages for the elucidation of particular epidemics rather than for a general and stable classification of staphylococci.

The typing of 567 strains of staphylococci from various sources by Williams and Rippon (1952) revealed that 229 of these showed complete lysis by one or more phages. There were not fewer than 82 distinct phage patterns. Only 118 strains were lysed completely by a single phage. Forty percent of the strains were not lysed by the R.T.D.[1], but one half of these

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[1]R.T.D. is the highest test dilution that produces confluent lysis on the propagating strain.

were typable by undiluted filtrates. Three main phage groups were distinquised: 3A, 6/47 and 52. A given culture could be susceptible either to one phage or to a set of phages acting in various combinations.

Williams, Rippon and Dowsett (1953) tested 1,349 strains of Staph. aureus that were isolated from normal persons and from patients suffering from staphylococcal infections. They were able to classify the staphylococci into three phage groups and numbered them to correspond to the three serological groups of staphylococci. Staphylococci of type I commonly caused fulminating pneumonia, and type III food poisoning. Strains of this latter group were frequently penicillin resistant. Type II was less frequently isolated than types I and III and occurred in various types of infections.

Staphylococci isolated from related sources and from chronic infections at different time intervals often yielded strains with identical patterns of lysis, according to Blair and Carr (1953).

Price and Neave (1954) typed strains of staphylococci of bovine origin by the bacteriophage methods described by Williams and Rippon (1952). They indicated that it was not always possible to be certain when two given strains belonged to the same type. Twelve different phage patterns were recognized and referred to in their report with the code letters from A to M. The phages used were those

employed in the typing of human strains. The staphylococci were obtained from 186 milk samples from 15 dairy herds, and the most common phage type among others was 42D which is regarded as characteristic of bovine strains. In heavily infected herds, the infections were due to more than one phage type. The occurrence of two phage types of staphylococci in one udder was found in 2.5% of the udders when the teats were healthy. Infections by two phage types were present in 6.7% of the quarters with teat erosions and as high as 50% in 18 quarters that had a small sore at the teat orifice.

Thirty-five quarters sampled on two or three different occasions at monthly intervals indicated that all of the staphylococci isolated from the same quarter had the same phage pattern. A correlation was found between phage type and penicillin sensitivity. The authors indicated that the use of a set of phages for the typing of bovine strains would be more satisfactory than the one employed by them. The persistency in the udder of staphylococci of one phage type is very stable. There were only two spontaneous changes of phage type in two quarters among forty infected but untreated quarters. It was indicated that both quarters had unhealthy teats.

Since available bacteriophages were mainly active against human strains, Seto in 1955 was able to produce two adapted phages 5B and 104 from phage 42D. Phage types 253, 523, and 536 were adapted from spontaneous phage 19B. This series of

adapted and spontaneous phages was used by Seto to type 380 coagulase positive and 50 coagulase negative strains from 25 dairy herds. Ninety-three of the 380 cultures were sensitive to one or more phages and were divided into 12 different patterns of lysis. The coagulase negative staphylococci were not susceptible to the phages.

Bacteriophage in the Treatment of Infections

In 1917 d'Herelle discovered that when a faeces emulsion of a patient of bacillary dysentery, was filtered through a Chamberland filter, it had the capacity to dissolve the etiological agent. He found that this lysis was transmissible to broth cultures of susceptible organisms, caused its lysis and even increased in virulence, which was comparable to the increase in virulence of bacteria when passed through susceptible animals. Also in other infectious diseases bacteriophages were detected. The most active ones were recovered during the period of convalescence. This led d'Herellle to conclude that the phages that normally were present in the intestinal tract adapted themselves to the invading organism and resulted in the recovery of the patient. In the same way as a patient is able to infect with his secretions and excretions a normal individual; a convalescent is able to transmit this healing principle (bacteriophage) to the sick. On this he based his doctrine of the "infecting immunity". Prophy-

lactic and therepeutic investigations with certain pathogenic bacteria and virulent bacteriophages strengthened the idea of d'Herelle that these bacterial viruses played a significant role in the origin and cause of many infectious diseases.

With repeated subeutaneous injections of an active filtrate into rabbits he obtained serum that neutralized the action of the bacteriophages.

The conditions that would lead to recovery from an infectious disease caused by bacteria would be according to d'Herelle (1930):

1. The lysis of the micro-organism by the bacteriophage.

2. The action of bacteriophages as potent opsonins. This effect was exerted by the lysins secreted by the bacteriophage corpuscles during the process of bacterial dissolution.

3. During the process of dissolution the bacterial proteins are degraded to a particular state which possesses the power of inducing in the host a refractory state of more or less duration.

Chronic infections are less likely to respond to bacteriophage treatment than acute infections. d'Herelle indicated that bacteriophage was used in bacillary dysentery in Brazil and in the Sudan. In the first country two failures were recorded among the 10,000 treated cases. In India phage was used for the treatment of Asiatic cholera. Other phage treatments in infectious diseases such as typhoid and paratyphoid

fevers, colibacillosis, staphylococcosis, streptococcosis, and bubonic plague were described by d'Herelle. In order to have a favorable therapeutic effect d'Herelle expressed the great importance of the use of phages of high virulence. d'Herelle stated that "In certain respects an almost complete analogy obtains between bacteriophage therapy and serum therapy; once lesions have developed neither serum nor bacteriophge therapy is competent to restore, or through direct action heal the lesions."

The polyvirulent race "h" of Gratia has been found to be effective on all phage susceptible strains of staphylococci.

Staphylococcus infections of various types have been phage treated in one way or another with results that were quite generally satisfactory.

Schuurman in 1927, described the significance of bacteriophages in therapy. He considered several factors that would influence the bacteriophagy in a negative sense, such as: 1. Susceptibility of the bacteriophages to lose their virulence, 2. That a certain relationship between bacteria and bacteriophage had to exist to cause total lysis. 3. That mucus, antiseptics and other substances could protect the bacteria. 4. That bacteriophages could be phagocytized. 5. The resistance of the bacteria. 6. The necessity of a close contact between phage and bacteria. 7. The injection of too large quantities. 8. The use of insufficiently controlled or

avirulent bacteriophages and the application of inhibiting substances.

Larkum in 1928 reported that from 66 patients that were treated for furunculosis by subcutaneous injection of 2 ml. of polyvirulent phage, recovery or marked improvement occurred in all but one case, and in the majority of cases there has been no evidence of recurrent infection. He claimed that the treatment had been highly effective.

In carbunculosis, phage was less effective in its treatment, but there was no doubt as to its effectiveness. The phage injections were made subcutaneously, into the lesion and also by application of dressings moistened with bacteriophage.

Very frequently the instillation in situ reduced the pain completely in a few minutes. Unquestionably many of the failures recorded for bacteriophage treatment are referable to inhibitory factors such as the presence of antiphage serum.

Paronychia and infections of the hand responded well to phage therapy by subcutaneous inoculation of 2 ml. of filtrate. Forty two of the 50 cases were completely healed within less than 3 weeks.

Krueger and Scribner in 1941 indicated that the limitation of the clinical usage of phage was due to the inactivting effect of those products which occur in tissues as a constant part of the phenomenon of inflammation. Blood,

serum, white cells, bacterial polysaccharides, whole dead bacteria, tissue debris, and colloidal suspensions exert either a direct destructive action on phage or an indirect inhibitory effect on the interaction of phage and bacteria. The addition of serum to a mixture of bacteriophage and susceptible bacteria interferes to a marked degree, or prevents entirely the lytic action of the bacteriophage upon the bacteria.

The phage preparations according to Krueger and Scribner contained constituents of the media, the accumulated by-products of bacterial metabolisms, substances released by lysis of the microbial substrate, and a small amount of phage (at the most 1 or 2 mg/liter). The effects observed following inoculation were not due to phage alone but also to the other ingredients that were biologically active and were therapeutically significant. They were classified into two groups:

1. Bacterial derivatives serving as antigens and stimulating the production of specific antibacterial immunity of great therapeutic significance.

2. Medium proteins and peptones capable of initiating the common non-specific protein reaction, which is of some therapeutic significance.

Krueger and Scribner did not consider the lysis of bacteria <u>in vivo</u> of great significance, as well as the production of avirulent variants due to phage action, but they

claimed that the production of local immunity of the tissues due to the application of the lysate was of therapeutic value.

Specific immunization due to antigenic fractions of the lysed bacteria and the increased phagocytosis was regarded of great therapeutic significance by Nelson (1928) and MacNeal, McRae and Colmers (1938).

MacNeal, McRae, and Colmers (1938) observed that phagocytosis not only was increased in aqueous solution but in blood serum as well. A substantial opsonic effect was demonstrated when optimal quantities of specific phage were mixed with Staphylococcus aureus and human blood. This opsonic phenomenon is the most favorable effect of phage therapy and probably is responsible for the success obtained in early phage therapy.

Krueger and Scribner indicated that some unfavorable reactions were due to:

"a. Uncontrolled nonspecific protein shock.

b. Toxic fractions of lysate.

c. Spreading factors enhancing bacterial invasiveness."

Several negative reports on treatment of staphylococcus infections can be found in the literature. Zaytseff-Jern, Howes and Meleney in 1934, recovered phage from the exudate of only one lesion when large amounts of phage were inoculated at a distance from the site of infection. In a separate group of 8 patients satellite furuncules developed around the original

lesion that had been treated with phage. In spite of the admittedly close relationship between the primary and secondary sites of infection, no phage could be recovered from the latter. In 110 patients suffering from carbuncles and furuncules, the repeated local use of phage did not accomplish a complete destruction of the bacteria. Staphylococci persisted even after the slough and exudate had disappeared. However, the isolated colonies from the lesion were altered in their cultural characteristics by the phage application. These consisted in "moth-eaten", degenerative and roughened colonies, sometimes with changes in chromogenic and hemolytic properties. Phage resistant strains of staphylococcus were more readily phagocytized than the normal phage susceptible strains. The bacteriophage persisted in the lesion and still had a destructive action after 24 hours, but it did not increase in potency as it did in vitro, instead it grew progressively weaker.

Bronfenbrenner and Sulkin in 1939, and Slanetz and Jawetz (1941) found that the intravenous inoculation of phage was of no value in the treatment of cutaneous staphylococcus infections in rabbits. The results following the instillation of phage or the use of phage moistened dressings, were not uniformly favorable. Staphylococcus lesions of the skin and eye responded with an increased inflammatory reaction. The authors believed that the filtrates might contain Reynal's spreading

factor and could be dangerous to the host by promoting the influx of bacteria into the tissues. Lysates from non-invasive strains were recommended for therapeutic use.

Slanetz and Jawetz in 1941 inoculated six kittens intraperitoneally with staphylococci of bovine origin. Four of these were given 3 ml of phage and the two remaining kittens served as controls. One control died from staphylococcal peritonitis, the other control showed a purulent peritonitis 20 days after inoculation at autopsy. One of the four remaining showed mesenteric adhesions but the rest did not show any lesions at the 20th day.

Fisk (1942a) reported that staphylococcal phages were difficult to isolate due to their common inactivation, possibly by absorption by tissue cells and various micro-organisms, including susceptible and resistant staphylococci.

Sulkin, Douglass and Bronfenbrenner in 1942 had unfavorable results with phage treatment in experimental staphylococcal septicemia in rabbits. They attributed the failure to Reynal's spreading factor.

Rakieten and Rakieten in 1943 protected 10 day old chick embryos from a fatal infection with Shigella flexneri by a single dose of specific phage, and found an association between survival of the embryos and a demonstrable increase in their phage content. The developing chick embryo was an unusually good host for the demonstration of bacteriophages in vivo.

Morton and Perez-Otero in 1945 found that the concentration of dysentery phage in the blood stream of Swiss mice injected intraperitoneally remained high for about 24 hours and diminished slowly until it was barely detectable after 5-7 days. Lytic action in vitro was accompanied by protective action in vivo. Instances where there was no lytic action in vitro there was no protective action in vivo.

Morton and Engley (1945b) could protect mice in the ratio of one phage to eight dysentery bacilli where 10,000 mimimal lethal doses of organisms were employed. The treatment could be delayed for 3 hours after the infecting dose of organisms was given and was still effective in protecting the mice. If given eight hours after infection, little protection was afforded. Prophylactic action was obtained when the phage was given seven days before infection with 10,000 minimum lethal doses of dysentery bacilli.

Several authors have reported favorable results with phages as a prophylactic of human cholera in field trials in India, but on a large scale the results have not been encouraging, Wilson and Miles (1955).

For phage treatment the following steps taken from Wilson and Miles, have to be taken into consideration:

a. Bacteriological identification of the organism
b. Susceptibility to the phage used
c. Bacteriological proof of cure
d. Freedom of naturally occurring phages of the controls
e. Exclusion of the effect of other micro-organisms
f. Proof that the administered phage reacted with the

micro-organism.

Further reference material on phage experiments on Salmonella typhi, Salmonella typhi-murium, salmonellosis in fowl, hemolytic streptococci, anthrax, and Escherichia coli, can be found in Wilson and Miles 1955.

Wilson and Miles (1955) referring to the role of the bacteriophage in infection and resistance state that: "Their applicability to the therapy of natural infective disease is still to be determined, and the problem of the great discrepancy between the high in vitro and the low in vivo activity of a given phage is still unsolved."

MATERIALS AND METHODS

Bacteriophage Typing of Staphylococci

Sources of staphylococci and typing phages

The staphylococcus cultures were originally isolated from milk samples from individual cows. These samples were submitted from eleven Iowa farms to the Department of Veterinary Hygiene of Iowa State College for bacteriological examination. Additional staphylococci of bovine origin were isolated from the Iowa State College Dairy Herd and from the herd of the Department of Obstetrics and Radiology. Others of canine and human origin were obtained from various specimens also sent to this laboratory for bacteriological examination. All the staphylococci were tested for hemolysis on 5 per cent bovine blood agar and for the production of coagulase as indicated by Chapman, Berens and Stiles (1941). A clone of each isolated strain was maintained in duplicate as a stock culture on tryptose agar slants. The stock cultures of staphylococci were kept at room temperature. Before typing, each strain was tested again for purity, hemolysis and coagulase production.

A total of 190 strains of staphylococci were submitted to bacteriophage typing. Of these 173 were of bovine origin, 12 of canine and 5 of human origin.

A series of seven types of staphylococcus bacteriophages[1] designated as 42D, 5B, 104, 19B, 253, 523, and 536 were used for typing. Their propagating strains were 1336, 5B, 104, 19B, 253, 523, and 536 respectively. A new code system, as proposed by Seto (1956), has been applied to these phages and corresponding propagating strains and is to be used throughout this work.

A comparison of the old and new nomenclature is given below.

Designation of indicator strains of staphylococci		Designation of bacteriophages	
New	Old	New	Old
1363	1362	42D	42D
10	5B	S1	5B
11	104	S2	104
12	19B	S3	19B
13	253	S4	253
14	523	S5	523
15	536	S6	536

The original phages were kept frozen, and the original propagating strains maintained at 5 C. The phages were propagated as described on page 21 under the subtitle "Preparation of the routine test dilution and of high titer phage stocks". The original propagating strains were tested for purity, hemolysis, coagulase production and carefully transferred to a

[1]The bacteriophages and their propagating strains were obtained by the courtesy of Mr. J. T. Seto, The University of Wisconsin, Madison, Wisconsin.

double series of tryptose agar slants. One series was kept at 5 C in conjunction with the original propagating strains. The other part of the complete series was kept at room temperature and used for the routine transfers. Each three months the propagating strains that were kept at room temperature were replaced by new transfers from the whole series that was kept under refrigeration.

Media and diluents

Tryptose agar and tryptose broth were used throughout all the experiments. The tryptose agar was prepared according to the following formula:

Bacto-tryptose	10g.
Sodium Chloride	5g.
Proteose-Peptone #3	10g.
Bacto-Beef Extract	3g.
Yeast Extract	5g.
Bacto-Agar	18g.
Distilled water	1000 ml

For the preparation of the tryptose broth the same formula was used except that no agar was added. Tryptose agar and tryptose broth were chosen because they produced the highest phage yield by the pour plate method in comparison with nutrient agar, nutrient broth, and/or trypticase agar and trypticase

broth, Contag (1955).

Tryptose agar plus 5% citrated bovine blood constituted the blood agar used in this work.

Assay of phage by the double agar layer method

In order to estimate the amount of phage present in a certain solution or filtrate, properly diluted phage was placed in quantities of 0.1 ml or 1 ml into a test tube containing 2.5 ml of liquid 0.7% agar at ca. 50 C. To this suspension one drop of a 6 hour culture of the corresponding propagating strain was added and after mixing the agar-phage-staphylococcus suspension was poured over the 1.8% agar base layer of a petri dish. It was then incubated for 4-6 hours at 37 C and for 8 additional hours at room temperature. All of the dilutions used were plated in duplicate in order to obtain more accurate results in the counting of the plaques. The plaques were counted with the aid of a Quebec colony counter.

Preparation of the routine test dilution of high titer phage stocks

The R.T.D. was prepared by the method that follows:

Seven tubes containing 2 - 3 ml of tryptose broth were individually inoculated with the seven propagating strains of staphylococci that were kept at room temperature. The cultures were incubated at 37 C for a period of 4 to 6 hours after which they were checked for purity and 1 ml of each was delivered onto the tryptose agar surface of an individual petri dish that had been poured the day before[1]. The excess of inoculum was removed from each plate with a sterile pipette. The inoculated plates were then turned over and the bottom part of the petri dish was slightly elevated and tilted to permit aeration and drying of the inoculum.

Serial tenfold dilutions from 10^{-2} to 10^{-5} of the seven original phage filtrates were made in tryptose broth. A loopful from a 2 mm inoculating needle of each of the dilutions of the corresponding phages was spotted on the marked area[2] of the previously seeded plates. After the spotted areas had dried, the plates were incubated at 37 C for 4 to 6 hours and then left at room temperature overnight. The reading was performed the next morning, and consisted in the determination of the highest dilution which produced confluent lysis of the

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[1]The object of preparing the agar plates the day before was to allow the agar surface to dry by incubating at 37 C and at the same time to permit the growth of possible contaminants on the plates.

[2]The bottoms of the petri dishes were marked with a wax pencil into as many squares as needed for the spotting of phage dilutions.

spotted area. This was considered the R.T.D. The R.T.D. varied for the various phages and was found to be between the dilutions of 10^{-2} and 10^{-5}.

All the dilutions were stored at 5 C until used.

The R.T.D. of each bacteriophage was used for the propagation and also for the typing of staphylococci.

In order to propagate the various phages to obtain high phage stocks four "dried" tryptose agar plates for each bacteriophage were employed. Each series of four plates were seeded with 1 ml of a 4 to 6 hour culture of the propagating strains of staphylococci. The excess inoculum was pipetted off. The plates were turned over, bottoms tilted, and allowed to dry for an hour. Then 1 ml of the R.T.D. of each phage was applied to the corresponding propagating strains in the petri dishes. The plates were flooded in such a way that a small strip of the agar was left without phage inoculum in order to serve as a control. The incubation temperature and time were the same as those described previously. After incubation the control areas of agar which did not show any bacterial lysis were cut out with a sterile spatula and removed, leaving the rest of the lysed culture in the petri dishes. The plates were placed in the refrigeration at -20 C for 24 hours. After this period the bacteriophages were harvested by letting the plates thaw and the exuding fluid which contained the bacteriophage was removed by means of a pipette. The phage-containing

fluid from the four petri dishes was pooled and centrifuged at approximately 1,500 rpm for 20 minutes and decanted. Ten-fold dilutions of this supernatant were diluted in tryptose broth and titered on the appropriate propagating strain to determine the potency. Supernatants that produced confluent lysis at the dilutions of 10^{-3} to 10^{-5} were filtered through Selas 02 filters. If the titer was below 10^{-3}, this dilution was used for a new phage propagation in order to increase its virulence and titer. When the titer was satisfactory, the phage suspension was submitted to filtration. All of the filtrates were tested to determine the R.T.D. which was usually between 10^{-2} and 10^{-4} for the various phages. The filtrates were checked for sterility and cross-spotted on the other propagating strains to determine the lytic spectrum, and the abscence of a staphylococcus inhibiting substance and then stored at 5 C.

The R.T.D. of the filtrates was checked twice a week on the corresponding propagating strains and it was found that the titer of the majority of the filtrates remained quite stable. In case the titer decreased the next lower dilution was used as the R.T.D. If the titer continued to decrease, a new phage propagation was performed.

Typing technique

The various strains of staphylococci were kept on tryptose

agar slants until they were to be typed. Before the typing was performed, they were again tested for hemolysis on blood agar and for coagulase production. The pure cultures were grown in tryptose broth for 18 hours at 37 C, and then transferred to another tube of tryptose broth and incubated for 4 to 6 hours at 37 C. One milliliter of the broth culture was uniformly distributed over the surface of a "dried" tryptose agar plate and the excess of culture was pipetted off. Usually 20 strains of staphylococci were typed at a time.

When the inoculum on the plates had dried, a drop of the R.T.D. of each of the seven phages was spotted on a marked square by means of a tuberculin syringe with a 25 gauge needle. The presence or absence and the types of lysis were recorded for each strain after an incubation at 37 C for 4 to 6 hours and for 8 additional hours at room temperature. The plates were left at room temperature and a final observation was made 24 hours after the plates had been inoculated. This second recording was performed in order to check the first readings and also a probable additional lysis.

Bacteriophage in the Treatment of Staphylococcal Bovine Mastitis

Selection of cows and detection of inflammatory changes in the bovine udder

Two cows affected with chronic staphylococcal mastitis

were selected for phage treatment, since both were affected with staphylococci which were susceptible to the phage type S6. Cow No. 10 had two infected quarters (LF and RF), whereas cow No. 11 had one infected quarter (RF). All of the infected quarters showed slight fibrosis, which was detected by the method for the physical examination of the udder described by Udall (1943, pp. 629-631).

Milk samples were taken as aseptically as possible from all four quarters of the two cows and bacteriologically tested. The brom cresol purple, the Whiteside and the catalase tests were performed as indicated by Merchant and Packer (1952, pp. 35-43). The body temperature of both cows was taken each time when the samples were obtained. Milk samples and body temperatures were taken eight days before phage treatment, 1 hour before and then 12 hours, 1, 2, 4, 8, 13, 35 and 48 days after treatment. The milk samples were taken shortly before the cows were milked, and the palpation was performed afterwards.

Prior to taking the milk samples the udder was prepared as follows: 1. the adhering dirt was removed by brushing; 2. the udder was wiped with a towel, that had previously been submerged in 200 ppm of calcium hypochlorite; 3. the teat orifices were disinfected with tincture of iodine. The first streams of milk were collected in a strip cup to detect any physical changes in the milk. Then ca. 16 ml of milk were withdrawn aseptically from each quarter for the catalase and

Whiteside tests. Additional 15 ml were collected for the Hotis test in screw-cap vials that contained 0.5 ml of brom cresol purple. As soon as these samples were collected the number of hemolytic staphylococci per ml of milk was determined for each quarter of the two cows by the viable plate count method. After 24 hours of incubation at 37 C of the brom-cresol-purple-milk samples, a loopful of each was streaked on a blood agar plate. Both plates were incubated at 37 C for 48 hours and two representative hemolytic colonies of Staph. aureus were picked from each plate. These colonies were transferred to separate tryptose agar slants and tubes of tryptose broth. The broth tubes were incubated for 18 hours at 37 C. A smear from the broth-culture was stained by Gram's method in order to check the cell morphology and staining characteristics as well as the cell arrangement. Finally the culture was checked for the production of coagulase.

When a sufficient number of strains of staphylococci accumulated they were phage typed.

Preparation of bacteriophage for intramammary infusion

Since the plate method for the propagation of phage did not render large volumes of a phage suspension, a propagation was performed in liquid medium by inoculating tubes containing 10 ml of tryptose broth with one drop of a six hour culture of Staph. aureus strain 15. After one hour of incubation

at 37 C, 0.5 ml of a R.T.D. of phage S6 was added. The tubes were incubated for 8 hours and then left at room temperature for additional 4 hours. Complete lysis occurred between 10 and 12 hours following phage inoculation. The tubes were centrifuged for 25 minutes at 2,000 rpm and the lysate was filtered through Selas 02 filters. The filtrates were pooled and phage assayed in order to determine the number of phage particles per ml of phage suspension.

The filtrate was tested for the presence of hemotoxin and coagulase. The hemotoxin was detected by setting up a series of seven tubes each containing 0.5 ml of saline. To the first tube 0.5 ml of the filtrate was added and then serially diluted by passing 0.5 ml of a well mixed sample from tube to tube through tube No. 6 and then 0.5 ml from tube No. 6 was discarded. Tube No. 7 served as control. To each of the tubes a 0.25 ml of a 1 per cent suspension of washed sheep erythrocytes was added. All tubes were incubated for two hours at 37 C. Following a centrifugation for two minutes at approximately 1,500 rpm, the test was observed for the presence of hemolysis.

The coagulase test was conducted by the method previously indicated.

Determination of the numbers of staphylococci before and after bacteriophage treatment

As soon as possible after the samples had been taken one milliliter from each of the brom cresol purple samples was diluted 1 to 100 and 1 to 1,000 and plated in duplicate on blood agar. The number of staphylococci per ml of milk was estimated after incubation at 37 C for 24 and 48 hours. The number of viable staphylococci was reported as the average number of colonies on two plates of the same dilution.

Intramammary infusion of phage filtrates

Immediately after milking the teat orifices were disinfected with tincture of iodine and the phage filtrate was inoculated into the teat cystern. All four quarters of cows No. 10 and 11 were inoculated with 5 ml of phage S6 filtrate which contained 3.73×10^9 phage particles per ml. The injection was performed with the aid of a syringe and a separate teat canula.

Recovery of the inoculated bacteriophage

Twenty-four hours following phage administration a milk sample of approximately 15 ml was taken from each quarter of cow No. 11 and coagulated with rennet. The whey was removed, centrifuged and filtered through a selas 02 filter. The whey

filtrate was tested by the phage assay method on the propagating strain 15 for the detection of phage S6.

Survival Bacteriophage in Non-heated and Heated Milk

The following experiments were carried out in order to establish possible differences in the survival of phage in milk from individual cows when placed in non-heated or heated milk:

1. Non-heated milk with diluted phage S3 filtrate
2. Non-heated and heated milk with undiluted phage S3 filtrate
3. Non-heated and heated milk with undiluted phage S6 filtrate. The milk was obtained from two phage treated and two non-phage treated cows.

The milk samples for each experiment were collected with the same technique that was used in the obtaining of milk for the chemical and bacteriological tests. Soon after the milk samples were drawn, they were refrigerated at 5 C until used. The milk samples were obtained from the cows and quarters that are indicated in table 1.

The first experiment was carried out to determine the phage survival in non-heated milk. Eight milk samples of 9 ml each were placed in rubber stoppered test tubes. One tube containing 9 ml of broth was used as a control. To each of

Table 1. Source of the milk samples used for the phage survival experiments[a]

Number of cow	Quarters from which the samples were drawn				Quarters infected with Staph. aureus			
	RH	LH	LF	RF	RH	LH	LF	RF
1	S		S	S	-	-	+	+
4	S	S			+	+	+	+
9		S			-	-	-	-
10[b]	S	S	S	S	-	-	+	+
11[b]				S	-	-	-	+
2963		S			-	-	-	-

[a]RH - right hind quarter.
LH - left hind quarter.
LF - left front quarter.
RF - right front quarter.
S Indicates quarters from which the milk samples were drawn.
+ Indicates infected quarters.
- Indicates non-infected.

[b]Cows No. 10 and 11 were previously used for the phage therapy experiment.

the nine tubes 1 ml of a dilution of 10^{-4} of phage S3 was added. The samples were incubated at 37 C and submitted to phage assay on staphylococcus strain 13 at 0, 2, 6, and 24 hour intervals.

The number of phage particles per ml of milk, obtained at the various time intervals, was multiplied by 10,000 in

order to compare these results with those obtained with the undiluted phage.

The second experiment was designed to compare the survival of undiluted phage S3 in non-heated and heated milk[1]. A series of eight tubes containing 9.9 ml of milk and one additional tube with an equal amount of broth were inoculated with 0.1 ml of undiluted phage S3 filtrate. Of the eight tubes four contained non-heated milk and the other four heated milk. All the tubes were incubated at 37 C and the phage was assayed as described for the first experiment.

The third experiment was performed in two parts. Both were designed to compare the differences in survival of phage S6 in milk from a cow treated with phage S6 with the milk from non-treated cows. The first part was performed by using non-heated milk from two quarters of a treated cow and two quarters from two non-treated cows. These four milk samples and one broth control which contained 9.9 ml of milk and broth respectively were inoculated with 0.1 ml of undiluted phage S6, incubated at 37 C and phage assayed at 0, 5, 8 and 26 hour intervals.

The second part of this experiment was a duplicate of the first part, except that heated milk was used.

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[1]Raw milk was heated to 96 C in a water bath for 7 minutes, cooled and kept at 5 C. All samples were allowed to reach room temperature before phage inoculation.

The third experiment also provided data on the survival of phage S6 in non-heated and heated milk.

RESULTS

Typing of Staphylococci and Phage Patterns

Preceeding the bacteriophage typing of staphylococci the lytic spectrum for the seven bacteriophages was determined. This spectrum is given in table 2.

Table 2. Lytic spectrum of the seven staphylococcal bacteriophages when applied to the propagating strains in form of undiluted and diluted filtrates[a]

Propagating strains	Bacteriophages													
	42D		S1		S2		S3		S4		S5		S6	
	U	D	U	D	U	D	U	D	U	D	U	D	U	D
1363	++	++	-	-	+	-	-	-	-	-	-	-	-	-
10	++	-	++	++	-	-	++	++	-	-	++	++	-	-
11	-	-	-	-	++	++	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	++	++	-	-	-	-	-	-
13	-	-	-	-	-	-	+	-	++	++	-	-	++	++
14	-	-	-	-	-	-	+	-	-	-	++	++	-	-
15	±	-	-	-	+	-	±	-	±	-	±	-	++	++

[a] U indicates undiluted filtrates
D indicates diluted filtrates (R.T.D.)
++ indicates confluent lysis
- indicates no lysis
+ indicates semiconfluent lysis
± indicates less than 20 plaques (weak lysis)

Table 2 indicated that a more defined phage specificity can be obtained when the R.T.D. of each of the bacteriophages is applied. An overlapping in specificity can be observed with the phages S3 on propagating strain 10, S5 on 10 and S6 on 13.

In table 3 the number of strains lysed by each bacteriophage is given. Some strains of Staph. aureus were susceptible to the action of more than one phage, while others were lysed by only a single phage. The percentages given in the table will therefore total more than a 100.

Table 3. Susceptibility of 173 strains of Staph. aureus of bovine origin to phage typing

Bacteriophages	Number of strains lysed	Percentage of the total of 173
42D	53	30.6
S1	18	10.4
S2	32	18.5
S3	19	12.0
S4	26	15.0
S5	3	1.7
S6	37	21.4

In table 3 it appears that phage 42D lysed the greatest number of strains. However, it must be noted that forty of the fifty strains were isolated from herd "E" as shown in table 5. Therefore phage S2, which lysed thirty-two of the strains from other herds, can be considered as the predominant phage type among the 173 bovine strains.

Table 4. Frequency of weak reactions[a] to the seven bacteriophages expressed in percentage of the total of 173 tested bovine strains of Staph. aureus

Bacteriophages	42D	S1	S2	S3	S4	S5	S6
Percentage of weak reactions	5.8	3.7	2.7	4.8	2.6	3.7	5.8

[a]Those that did not produce confluent lysis.

In table 4 it can be noted that the bacteriophages S2 and S4 produced a smaller percentage of weak reaction than the rest of the bacteriophages of the typing set.

From table 6, it can be observed that of 164 hemolytic and coagulase positive strains of bovine origin 151, 92.07%, were susceptible to one or more phages of the typing set and 13 to none of the phages. Nine additional strains of non-hemolytic or coagulase negative staphylococci were not susceptible to any phage.

Only one of the canine strains was susceptible to a group of phages (S1, S3, S6). However, three of the canine strains reacted with the undiluted filtrates of phages S5 and S6.

Of the five human strains, two reacted weakly with the R.T.D. of the phages S4 and S5 respectively. Two others showed partial lysis with the undiluted phage-filtrates S2, S5 and 42D, S1, S2, S3. The fifth strain was completely non-susceptible.

Table 5. Phage patterns among 144 strains of Staph. aureus isolated from various herds in Iowa.

Source	No. of strains	Phage patterns								
		42D[a]	42D, S2	42D,S1, S3	42D,S1,S3, S4,S6	S4,S6	S2[a]	S1,S3	S2,S6	S1,S2
Herd A	8	-	-	-	-	7	-	-	1	-
B	2	-	-	-	2	-	-	-	-	-
C	4	-	-	-	-	-	3	1	-	-
D	8	-	-	-	-	5	2	1	-	-
E	50	40	1	3	-	-	1	5	-	-
F	6	-	-	-	-	-	5	1	-	-
G	3	-	-	2	-	-	-	-	-	1
H	7	-	2	1	-	3	1	-	-	-
I	1	-	-	-	-	1	-	-	-	-
J	3	-	-	-	-	-	3	-	-	-
K	8	-	1	-	-	-	7	-	-	-
C.D.H.[b]	34	-	1	-	1	25	5	2	-	-
O.B.[c]	10	-	-	-	-	5	1	3	-	-

[a]Phage type

[b]C.D.H. - Iowa State College Dairy Herd.

[c]O.B. - Herd of the Department of Obstetrics and Radiology of Iowa State College. One strain was susceptible to 42D,S1.

Table 6. Results of bacteriophage typing of 190 strains of staphylococci

	Origin of strains	Strains susceptible to phage typing		Strains not susceptible to phage typing	
		No.	Percent	No.	Percent
Staph. aureus	Bovine	151	92.07	13	7.92
Staph. aureus	Canine	1	8.3	11	91.7
Staph. aureus	Human	0	0	5	100
Non-hemolytic coagulase negative staphylococci	Bovine	0	0	9	100

In tables 7 and 8 the results of phagotherapy in two cows of the Department of Obstetrics and Radiology are given. The data given correspond only to some of the tests that were performed, since these offered a more significant picture of the changes in the milk that occurred after phagotherapy.

A marked inflammatory reaction was revealed by the various tests as well as by palpation 12 hours after the intramammary infusion of bacteriophage S6. Approximately the same inflammatory response was observed in both phage treated cows. The results of the strip cup and bromcresol purple tests were more consistent during the inflammatory reactions in cow No. 11.

Table 7. Physical and chemical changes of the milk before and after intramammary phagotherapy in two cows with chronic staphylococcal mastitis[a]

Cow No. 10 with two fore-quarters infected with Staph. aureus

Days in respect to treatment	Strip cup				Brom-C-purp				Catalase				Whiteside			
	RH	LH	LF	RF	RH	LH	LF	RF	RH	LH	LF	RF	RH	LH	LF	RF
8 days before	-	-	-	+	-	-	-	+	3	3	4	13	-	-	-	-
1 hour before	-	-	+	+	-	-	-	+	1	1	17	11	-	-	+	+
12 hours after	+	+	+	+	+	+	±	±	20	14	17	20	+	+	+	+
1 day after	+	-	-	+	±	±	±	+	20	15	19	20	+	+	+	+
2 days after	+	+	+	+	-	-	+	+	10	7	12	12	+	+	+	+
4 days after	-	-	+	+	-	-	±	±	1	2	2	2	-	-	-	-
8 days after	-	-	-	-	-	-	+	-	1	1	2	3	-	-	-	±
13 days after	-	-	±	-	±	-	+	+	1	1	17	20	-	-	+	+
35 days after	-	+	-	-	-	-	-	-	1	1	3	4	-	-	-	+
48 days after	-	-	+	-	-	-	+	+	1	1	8	14	-	-	+	+

[a] RH - right hind quarter.
LH - left hind quarter.
LF - left front quarter.
RF - right front quarter.
\- Indicates negative results for the various tests.
\+ Indicates positive results for the various tests.
± Indicates doubtful results for the various tests.

Table 8. Physical and chemical changes of the milk before and after intramammary phagotherapy[a] in two cows with chronic staphylococcal mastitis

Days in respect to treatment	Strip cup				Brom-C-purp				Catalase				Whiteside			
	RH	LH	LF	RF	RH	LH	LF	RF	RH	LH	LF	RF	RH	LH	LF	RF
Cow No. 11 with RF quarter infected with *Staph. aureus*																
8 days before	-	-	-	+	-	-	-	+	2	1	3	6	-	-	-	±
1 hour before	-	-	-	-	-	-	-	+	3	4	4	10	-	-	-	+
12 hours before	+	+	+	+	±	±	+	±	17	13	20	20	+	+	+	+
1 day after	±	±	+	+	±	±	±	+	5	7	17	20	+	+	+	+
2 days after	±	±	+	+	±	-	-	+	6	4	15	19	+	±	+	+
4 days after	-	-	±	+	-	-	±	+	2	2	2	12	-	-	-	+
8 days after	-	-	-	-	-	-	-	+	1	2	2	7	-	-	-	+
13 days after	-	-	-	-	-	-	-	+	5	6	6	18	-	-	-	+
35 days after	-	-	-	-	-	-	-	+	1	2	2	6	-	-	-	+
48 days after	-	-	-	+	-	-	-	+	2	2	2	12	-	-	-	±

[a]The table symbols correspond to those explained in Table 7 footnote.

The inflammation subsided in both cows after the second day post-treatment. However the milk from the infected quarters continued to yield positive reactions to the indicated tests as shown in tables 7 and 8. Staph. aureus was isolated from the milk of the infected quarters throughout the period of investigation and the numbers of organisms shedded remained more or less constant, table 9.

In table 9 the phage type in both cows remained the same in both cows respectively during 35 days. At the 48th day the staphylococci isolated from the LF quarter of cow No. 10 were not susceptible to phage typing, whereas Staph. aureus from the RH quarter was susceptible to the phage S4 instead of to S6 as observed in the preceeding typings. The staphylococci from the RH quarter of cow No. 11 also changed in their phage susceptibility in being susceptible only to S4.

The number of staphylococci per ml of milk from the infected quarters of either cow did not change markedly after phage treatment.

In addition to the results, the administration of phage filtrate resulted in a decrease in milk production that was concomitant with the inflammatory stage. However, this was not accompanied with pyrexia in either cow.

In table 10 a marked loss in bacteriophage titer can be observed after the zero hour phage assay. The phage in all the samples was only partially inactivated after 24 hours of

Table 9. Number and phage type of Staph. aureus contained in the milk of two cows before and after phage treatment

Days in respect to treatment	Phage type				Number of staphylococci per ml of milk			
	RH	LH	LF	RH	RH	LH	LF	RH
Cow No. 10								
8 days before	-[a]	-	S6	S6	-	-	[b]	[b]
1 hour before	-	-	S6	S6	-	-	1,000	5,000
12 hours after	-	-	S6	S6	-	-	[c]	2,000
1 day after	-	-	S6	S6	-	-	200	4,000
2 days after	-	-	S6	S6	-	-	100	3,800
4 days after	-	-	S6	S6	-	-	[c]	2,300
8 days after	-	-	S6	S6	-	-	4,800	7,650
13 days after	-	-	S6	S6	-	-	7,450	3,300
35 days after	-	-	S6	S6	-	-	[b]	[b]
48 days after	-	-	-	S4	-	-	[b]	[b]
Cow No. 11								
8 days before	-	-	-	S4, S6	-	-	-	[b]
1 hour before	-	-	-	S4, S6	-	-	-	8,500
12 hours after	-	-	-	S4, S6	-	-	-	1,000
1 day after	-	-	-	S4, S6	-	-	-	1,500
2 days after	-	-	-	S4, S6	-	-	-	[c]
4 days after	-	-	-	S4, S6	-	-	-	5,000
8 days after	-	-	-	S4, S6	-	-	-	2,500
13 days after	-	-	-	S4, S6	-	-	-	[c]
35 days after	-	-	-	S4, S6	-	-	-	[b]
48 days after	-	-	-	S4	-	-	-	[b]

[a] Indicates no staphylococci could be isolated.

[b] Plate count not performed.

[c] No colonies were detected by direct plating. Staph. aureus was isolated from the incubated milk and phage typed.

Table 10. Survival of bacteriophage S3 at 37 C applied as a diluted filtrate to non-heated milk of various cows

Cow number and quarter	Phage survival in millions per ml of milk and at different time intervals after inoculation			
	Hours of exposure			
	0	2	6	24
10-RF	900	71	65	5.3
10-RH	1,430	260	17	14
10-LH	1,200	210	34	11
11-RF	1,070	99	66	102
4-RH	1.4	27	7	3
4-LH	50	7	14	4
1-RH	780	140	12	13
1-LF	780	51	15	5
Broth control	1,700	1,990	1,590	1,260

of incubation at 37 C. The titer of the control diminished to a much lesser extent than the rest of the eight milk samples. No significant difference in the inactivation of the bacteriophage in the various milk samples can be observed after 24 hours of incubation except in the milk sample 11 RF in which it was less. An immediate inactivation occurred in the milk of the two quarters of cow No. 4, whereas the inactivation in the milk of two quarters (RH and LH) of cow No. 10 was not immediate. The inactivation of the phage in the milk of cow No. 11 was less than in the rest of the milk samples.

From table 11 it can be noted that there is a marked

Table 11. Survival of bacteriophage S3 at 37 C applied as undiluted filtrate to non-heated and heated milk from various cows

Cow number and quarter	Phage survival in millions per ml of milk and at different time intervals			
	0	2	6	24
A. Non-heated milk				
10-RF	1,160	99	64	4
10-LF	1,500	101	42	18
1-RF	192	390	40	21
1-LF	1,660	380	88	65
Control[a]	4,900	4,300	3,160	1,750
B. Heated milk				
10-RF	2,820	2,400	2,950	3,490
10-LF	3,440	3,270	2,960	2,910
1-RF	3,770	3,200	4,820	3,400
1-LF	3,900	3,350	3,370	1,170
Control	4,900	4,300	3,160	1,750

[a]Broth control served for both experiments since they were performed simultaneously.

difference between the survival of bacteriophage S3 in non-heated and heated milk; suggesting that the phage is more stable in the latter.

The 24 hour sample of the milk of the LF quarter of cow No. 1 was contaminated and the plaques difficult to count. The 24 hour milk sample 10-RF was coagulated.

Table 12. Comparison of survival of bacteriophage S6 in millions per ml of milk at 37 C applied as undiluted filtrate to non-heated and heated milk from various cows

Hours	Number of cow and milk from quarter tested[a] 10-LH	10-LF	9-LH	2963-LH	Broth control
A. Non-heated milk					
0	455	390	515	310	390
5	145	10	500	435	565
8	85	5	230	345	610
26	13.8	6	270	265	460
B. Heated milk					
0	365	400	[b]	300[c]	370
5	430	520	560	520	760
8	650	875	620	750	700
26	415	670	595	510	470

[a]The numbers are given in millions of phage particles per ml.

[b]Plates were contaminated and no plaques could be counted.

[c]Plates were contaminated, thus reducing the number of plaques.

The bacteriophage S6 survival is also greater in the heated milk than in the non-heated milk as it has been described elsewhere for bacteriophage S3 survival. The phage inactivation in the non-heated milk of both quarters of cow No. 10 is more manifest than it is in the two other milk samples. This difference in inactivation among quarters

cannot be found in the heat treated milk. A tendency of increased phage activation can be observed in the milk samples, which were previously heat treated, as well as in both broth controls.

DISCUSSION

The results reported in this study indicate that a majority of strains of mastitis staphylococci are susceptible to the lysis by bacteriophage. It was found that of the 164 coagulase positive and hemolytic strains, 151, 92.07 per cent, were susceptible to one or more phages. This finding is in accordance with the results of bacteriophage typing reported by other workers. Seto (1955) was able to type 93.2 per cent and Smith (1948) 93.3 per cent of staphylococci of bovine origin. These percentages are higher than those obtained in phage typing of Staph. aureus of human origin. Wilson and Atkinson (1945) were able to type 60.4 per cent, Williams and Rippon (1952) about 60 per cent, Blair and Carr (1953) 73.5 per cent, and Desranleau et al. (1955) 72.6 per cent of the staphylococci of human origin. To judge from these various results it appears that the bovine strains are more readily typable than the human strains, indicating a more successful application of phage typing to bovine strains.

Nine coagulase negative and non-hemolytic strains of staphylococci of bovine origin were insusceptible to the action of any of the bacteriophages employed indicating that only the strains that produce coagulase and hemolysis are sensitive to these phages. This property could be useful as an additional tool to determine the pathogenicity of staphy-

lococci of bovine origin.

Strains of Staph. aureus of human and canine origin were generally insusceptible to the action of the seven bacteriophages used. This would indicate that the bovine strains differ from human and canine strains.

Prior to typing, the lytic spectrum of the seven bacteriophages was determined by cross-reactions on the seven propagating strains. This was performed in order to detect any possible changes in the behavior of the individual phages after they had been obtained. This spectrum is given in table 2 and is similar to the one reported by Seto. It reveals that the phages were quite specific in action in spite of some overlapping of lysis. The application of the undiluted filtrates of the phages resulted in some weak reactions on staphylococcus strain 15. However this action was not observed when the R.T.D. of the various phages was applied to this strain.

Complete lysis was the criterion that was followed to determine the susceptibility of the various strains of staphylococci to the action of the seven typing phages. The result of typing permitted the grouping of staphylococci of bovine origin into ten main phage patterns. Seto reported twelve phage patterns among staphylococci isolated from the area surrounding Madison, Wisconsin. However, these differed somewhat from the phage patterns of the strains isolated in Iowa. Phage S5 was present in Seto's patterns, whereas it was not

found in the phage patterns of the strains isolated from this geographical area. The action of S5 on the strains isolated in this study was not marked, except in the case of one strain of which a simultaneous lysis occurred with the phage S4. Some phage patterns such as the 42D; 42D,S2; and S4,S6 were common groups for both geographical areas. Staphylococcus aureus phage type S2 was the predominant strain amongst the 13 Iowa dairy herds studied. This is also true for the staphylococci isolated from the herds around Madison, Wisconsin. However, phage type 42D comprised 80 percent of the 50 strains of staphylococci isolated from herd E.

Staphylococcus aureus with phage pattern S4,S6 was the most frequent among all the isolated strains, with S1,S3 as the second in occurrence. In both phage patterns were included a few strains which were susceptible to S6 or S1 alone, since it was practical to include them in the two corresponding phage patterns, rahter than to place them into two separate phage types. The phage pattern S4,S6 was most common among the strains isolated from the Iowa State College dairy herd.

Weak reactions could be useful for detecting closer relationships between strains of staphylococci of the same phage pattern. This was particularly true among the strains isolated from the college dairy herd. Several strains were susceptible to the phage pattern S4,S6 and weakly to phage

S3. Other strains isolated from the same herd were susceptible to S4,S6 alone. The question arises whether the strains of both groups were identical or different. It appears that both groups are related. However, the first group of phage pattern S4,S6 with the weak reaction to type S3 can be considered as only slightly varying from the second. On the other hand, the reason for the weak reactions that occur among the staphylococci to the various phages is not entirely understood.

Finally it can be stated that the present set of adapted bacteriophages is quite satisfactorily for the typing of staphylococci of bovine origin and should be useful in epizootiological studies of staphylococcal bovine mastitis. Furthermore the typing staphylococci isolated from infected udders would help to determine the success of any treatment for this condition.

Phage Therapy

Phage therapy applied to two cows with chronic staphylococcal mastitis was not successful in effecting a cure as indicated by the results of various tests for the diagnosis of mastitis and by the absence of a marked reduction of the number of staphylococci shed in the milk.

The phage S6 was inoculated in form of a crude filtrate into the teat cystern of all quarters of two cows. The dose

consisted of 5 ml of filtrate containing 3.73×10^9 phage particles per ml. The result of the inoculation was an immediate transitory inflammation of all quarters, which lasted for two days. The inflammation was accompanied by a decrease in milk production that was, however, not concomitant with a general febrile reaction. The cause of the inflammation can be attributed to the irritating properties of the filtrate, which contained all the filtrable staphylococcal metabolites and lysates of the phage action. The presence of coagulase and some hemolysins were detected in the filtrate.

In order to avoid these undesirable reactions caused by the filtrate it would be desirable to use a purified phage suspension deprived from the irritating substances. The absence of staphylococcal by-products in the inoculum would permit the study of the phage action alone.

In spite of the continued recovery of pathogenic staphylococci from the phage treated quarters, there was some decrease in numbers of staphylococci after 12 hours following phage administration. However, the bacterial counts increased again after four days in one cow and after eight days in the other one. The drop in titer of staphylococci per ml of milk can either be attributed to the inflammatory reaction or to the lysis caused by the injected bacteriophages.

The viable counts of staphylococci failed on four occa-

sions to reveal the number of organisms present in the milk. This was attributed to the presence of a small number of cells not detectable by the counting method used. However, the organisms were not entirely absent, since they were recovered from the incubated milk samples.

A disadvantage of the applied viable plate count technique with blood agar is that in case of large number of colonies, the zones of hemolysis become confluent making the reading of the Staph. aureus colonies on the plates difficult.

On some occasions the recommendations of the American Public Health Association Inc. (1953, pp. 22-23) in reference to the number of colonies to be counted on each plate, could not be met.

The phage type of the staphylococci isolated from the infected quarters remained constant during 35 days after phage treatment was performed. A repeated typing performed 48 days after treatment indicated that a possible change in phage type had occurred in the Staph. aureus population of the infected quarters. This finding, however, could not be verified with an additional typing since the cows used in this experiment were sent to slaughter. It is also debatable whether the two isolated colonies from each infected quarter were or were not sufficient to determine the phage type of the entire population. It seems though, by experience of the author and by the report of Price and Neave (1954), that two

colonies isolated from an inoculated plate would suffice to detect the phage susceptibility.

The failure to eliminate the staphylococci from the udder could be attributed to many possibilities and three among others could be considered of importance.

1. The rapid inactivation of the phage in the milk of the udder. Evidence for this possibility is that only one phage particle per ml of filtered whey could be recovered from the milk from one of the treated cows 24 hours after phage application. Furthermore, the plaques that were observed differed in appearance from those usually produced by phage S6 on its propagating strain. The difficulty in the recovery of phage from milk from quarters infected with staphylococci is also reported by Slanetz and Jawetz (1941). The fate of the bacteriophage in the bovine udder is almost an impossibility to determine, however the fact of its inactivation suggests that there are various mechanisms involved.

2. The inaccessibility of the staphylococci to the bacteriophage. The probable deep location of the staphylococci in the glandular parenchyma did not give the phage the opportunity to come into contact with the susceptible pathogens, hence the phage that was inoculated into the teat cystern probably did not diffuse into all parts of the udder.

3. Possible low virulence of the phage administered. Only one propagation on solid media was performed, prior to

the propagation in liquid medium, which served for the preparation of the filtrate used for phage inoculation.

Phage Survival in Heated and Non-heated Milk

A gradual and progressing inactivation of phage S3 and S6 was observed in non-heated milk during a period of 24 hours of incubation at 37 C. The inactivation was markedly less in the heated milk and in tryptose broth, and in case of phage S6 even a slight activation occurred during the incubation period in the broth and in the heated milk. From this it is apparent that the heating process destroyed phage inactivating agents present in raw milk.

The rapid and greater inactivation of phage S6 in the milk from a cow treated with the homologous phage in comparison with the phage survival in milk from non-treated cows, suggests that the presence of possible antibodies to this particular phage may play some role in the inactivation of the phage. These antibodies were probably destroyed by the heating process since no difference in the phage survival could be detected in the heated milk from treated and non-treated cows.

Although these results indicate that a progressive inactivation of phage S3 and S6 takes place in the raw milk, this inactivation of the phage is not complete. If the phage undergoes a similar inactivation in the udder as in the raw

milk *in vitro*, it would be possible for the phage to act on the susceptible staphylococci within the 24 hours after phage administration, even though the number of active phage particles are reduced.

SUMMARY AND CONCLUSIONS

In this study 190 strains of staphylococci were tested for their susceptibility to lysis by seven bacteriophages which have been adapted to the bovine mastitis staphylococci. The 181 strains which were found to produce coagulase and hemolysin were classified as Staph. aureus. One hundred sixty-four of these strains were isolated from cases of bovine mastitis and 151 or 92.07 per cent were lysed by one or more of these phages. It was found that one phage type or phage pattern would predominate among the staphylococci isolated from a given herd. Phage type S2 and phage pattern S4,S6 staphylococci occurred more frequently than any other.

Although the phage types of staphylococci isolated from 13 dairy herds in Iowa were very similar to those reported from Wisconsin, there were some differences noted.

Twelve strains of Staph. aureus of canine origin and five from human sources were in general insusceptible to the action of the seven bacteriophages used. This suggests that the staphylococci which infect these hosts are different.

Nine strains of staphylococci which were neither hemolytic or coagulase positive were found to be completely insusceptible to any of the phages used.

In the limited trials and under the conditions of these experiments, therapy of staphylococcal bovine mastitis was

not successful. However, there was some indication that the numbers of staphylococci were reduced for a few days following the introduction of a specific phage into the udder.

The possible reason for failure of phage therapy are discussed. One of these possibilities, phage inactivation in the milk was investigated. It was found that raw milk apparently contains substances which progressively inactivate the bacteriophages in a few hours. It was also determined that the raw milk from a cow previously phage treated was even more detrimental than raw milk from a non-treated cow.

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