BACTERIOPHAGE TYPING OF *STAPHYLOCOCCUS AUREUS* ASSOCIATED WITH CASES OF BOVINE MASTITIS

by

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INTRODUCTION

During recent years, Staphylococcus aureus has been reported as a common cause of bovine mastitis. In epidemiological studies, strains which have no relationship to the disease under investigation are frequently encountered so that it is difficult to demonstrate the relationship of one strain to another. The biological characteristics of the staphylococci routinely identified are not helpful in deciding which of several cultures from related sources are identical and which are different. In the case of staphylococci of human origin, this problem has been solved by bacteriophage typing. The method is based on the determination of sensitivity or resistance of a particular bacterial isolate to each of several phages. The specific action of the phages is a relatively stable property, thus the phage typing will reveal minor differences between bacterial strains which are not detectable by the usual serologic procedures (Fisk, 1942). For routine typing of staphylococci of human origin, basic phages have been recommended by the Subcommittee on Bacteriophage Typing of Staphylococci, of the International Committee on Bacteriological Nomenclature, London, England (Blair, 1956).

In phage typing studies, the first essential is the preparation of suitable phages. Secondly, in order to be able to interpret the results obtained by various workers, it is necessary that a particular set of phages be used as basic phages. The uniformity of typing technique is also important so that comparable results may be obtained by all workers.

In the field of bacteriophage typing of staphylococci of animal origin, earlier workers (Williams-Smith, 1948; Edwards, 1954; Price et al., 1954; Barnum and Fuller, 1956; Edwards and Rippon, 1957), classified staphylococci of mastitis origin, utilizing mainly the human phages. Recently, Coles (1958)

and Seto et al. (1956), independently isolated two sets of phages for use in the typing of staphylococci associated with bovine mastitis. Of the six phages isolated by Coles, three were phages adapted from human type organisms and three were obtained from lysogenic cultures of Staphylococcus aureus of animal origin. These phages will hereafter be referred to as the Coles phages. Utilizing these six phages, Coles (1958) undertook the typing of staphylococci associated with bovine mastitis. He did not get good results in typing when the phages were used at their routine test dilutions, but observed that a greater percentage of cultures were lysed and wider patterns of lysis were produced when the phages were used in an undiluted form. A routine test dilution (or dose) (RTD) is the highest ten-fold dilution of the phage which will produce confluent lysis of the propagating host. Seto and Wilson (1958) typed micrococci of bovine origin using seven phages, six of which were isolated by Seto et al. (1956); these will be designated as the Seto phages. In their studies on bacteriophage typing of micrococci of bovine origin, Seto and Wilson used the phages at their routine test dilutions and found 93 per cent of the isolates sensitive to one or more of the seven phages.

The present study of bacteriophage typing of Staphylococcus aureus associated with bovine mastitis was undertaken with the view of comparing the action of the two sets of phages, the Coles phages and the Seto phages, and if possible to evolve a combination of the two which would give better results in typing. It was also a purpose of this work to compare the relation between the use of RTD and the concentrated phages in bacteriophage typing.

REVIEW OF LITERATURE

Bacteriophage Typing of Staphylococci of Human Origin

Burnet and Lush (1935) presented evidence to show that certain strains of

Staphylococcus aureus could be grouped according to their sensitivity to a series of phages. Fisk (1942) described the isolation of staphylococcal phages from the lysogenic cultures by the cross culture method; utilizing 27 different phages, he divided 95 culture strains of Staphylococcus aureus into specific groups. Fisk also demonstrated that cultures of probable identity, such as separate isolations from the same patient, reacted to the same phages and thus could be differentiated from other cultures. In another study, utilizing 35 different phages, Fisk and Mordvin (1944) confirmed these observations by showing that cultures isolated from a single source belonged to the same phage type even though they sometimes differed in other characteristics, such as chromogenesis, hemolytic properties or production of alpha hemotoxin. Wilson and Atkinson (1945), using Fisk's technique, prepared 18 different bacteriophage strains, seven of which were obtained by cross-culture method while the rest were prepared by growing one of the original phages on a different susceptible culture, thus producing a variant of the original phage. An important modification in typing, namely the use of a "test dilution", was introduced by these investigators. The test dilution was defined as the highest dilution of the phage which produces confluent lysis of the propagating strain. With 18 phages they typed 460 cultures and recognised 21 types or subtypes. These investigators described two outbreaks of pemphigus in maternity homes in which phage typing provided evidence that the infections originated with personnel who were staphylococcal carriers. The phages isolated by Wilson and Atkinson, along with their propagating strains are maintained by the National Collection of Type Cultures, Medical Research Council, London. Williams and Rippon (1952) presented a careful analysis of the results of the phage typing of strains of Staphylococcus aureus collected over a period of three years at the

Staphylococcal Reference Laboratory, Colindale, England. They pointed out that it is more important to make fine distinctions between a number of strains from one investigation than to recognise as identical those strains recovered from widely separated areas. It was also observed that about 40 per cent of the staphylococci could not be lysed by the phages used at their test dilutions; about half of these untypable strains were lysed by undiluted phage filtrates. In a later publication Williams and Rippon (1953) reported lysis of 50 to 80 per cent of the coagulase-positive staphylococci by one or more of the phages used at their routine test dilutions. The staphylococci obtained from pathological lesions as well as from healthy persons were classified into three broad groups. They reported that fulminating pneumonia was very commonly due to staphylococci of group I; food poisoning was almost always due to strains of group III. Blair and Carr (1953) determined the sensitivity of 539 coagulase-positive staphylococci and found 53.6 per cent of the strains susceptible to one or more of the 25 phages; the strains could be divided into six broad subdivisions. Rountree (1953), in Australia, typed human staphylococci obtained from various sources and observed that when staphylococci are recovered from urine, ears and wound surfaces their presence could be correlated with the carrier state of the patient. A predominance of group III strains was found in boils, abscesses and osteomyelitis which were caused by penicillin-sensitive strains. Wood (1953), with a view to reducing manipulations necessary in conventional methods of phage typing, suggested the use of pooled phages for initial screening. Levy et al. (1953) carried out phage typing of staphylococci that had been examined for their production of diffusible antigens, hemolysins, fibrinolysin and pigment formation. They concluded that there was no significant correlation between the phage group and the other properties. The animal

strains, in their view, formed a different biological group. The animal strains were less often typable than the human strains which, according to Levy et al., was due to the fact that the phages were collected principally for the identification of human strains. They also found that the sheep strains too were insusceptible to the action of human phages. Using 20 different phages, Jackson et al. (1954), were able to demonstrate lysis of 35.2 per cent of coagulase-positive staphylococci of human origin, the phage sensitive cultures of which exhibited 129 different reaction patterns. They observed that transfer of specific strains of Micrococcus pyogenes from one person to another often can be documented by the use of bacteriophage typing with a high probability of validity.

Bacteriophage Typing of Staphylococci of Animal Origin

One of the early studies on the role of staphylococcal phages in bovine mastitis was done by Slanetz and Jawetz (1941). These workers isolated phages from milk and attempted classification of staphylococci obtained from cases of bovine mastitis. Williams-Smith (1948b) applied the bacteriophage typing method to study the epidemiology of staphylococcal infections in animals using mainly the human phages for typing. He found that a high proportion of coagulase-positive strains obtained from cattle were susceptible to one single human phage, phage 42D. At first it appeared that all the 42D-type strains were identical but, using the cross-culture method of Fisk (1942), he was able to subdivide the group. In another investigation Williams-Smith (1948a) typed 1,016 coagulase-positive strains obtained from milk samples of individual cows and found that 93.3 per cent of the strains were typable. Of the typable strains 83.8 per cent were susceptible to one or more of the phages of the 42D group

and 9.5 per cent to one or more of the 29 group. Of particular interest in his work are three strains of staphylococci, two of human origin and one obtained from a case of bovine mastitis. Antigenic analysis of these strains did not reveal any differences and the three strains were shown to be identical; however with phage typing it was shown that they were different strains.

Edwards (1954), with the aid of human phages, studied staphylococci isolated from samples of milk obtained from 37 cows. Thirty-five of the cows in his experiment belonged to one herd. The staphylococci isolated from these cows were classified as belonging to the phage type 42D and conformed usually to the undiluted pattern 79/42D/54/77. The staphylococci isolated from the remaining two cows were of different phage type. He particularly noted that continued treatment of an infected quarter did not produce any change in the phage type of the organism, Price et al. (1954) undertook the phage typing of staphylococci obtained from milk samples from 186 cows with a view to examine the value of phage typing, antibiotic sensitivity and hemolysis on blood agar plates as aids in distinguishing new infections from relapses. They utilized human phages for typing and found that staphylococci with pattern A, which included the 42D type, were very common. Staphylococci persisting after treatment showed no significant differences in phage type. They also found a fairly clear relation between phage type and penicillin sensitivity. In six cases, following successful treatment, organisms could be recovered which exhibited a different phage type which presumably indicated reinfection of the udder.

Barnum and Fuller (1956) undertook the bacteriophage typing of staphylococci associated with bovine mastitis, in an attempt to determine if this method could differentiate pathogenic from non-pathogenic strains, and to further characterize the organisms as an aid to epidemiological studies.

The phages were secured from a set of 42 lysogenic cultures of staphylococci of human origin which were maintained at the Ontario Public Health Laboratory. The relation of these cultures to phages used elsewhere was not known. They found that the use of phages did not serve to distinguish between strains isolated from healthy and diseased udders; but the method afforded evidence to show that the staphylococci capable of exciting infection in the mammary gland existed in the "carrier" cow. Barnum and Fuller observed that the greatest percentage of the strains producing white pigment belonged to Group B, strains producing cream pigment to Group A, and those producing golden pigment to Group A1. The strains producing alpha and beta hemolysis were distributed among all phage groups.

With a view to classifying staphylococci obtained from milk of cows, Edwards and Rippon (1957) employed phage typing as well as other tests; e.g., the coagulase test, hemolysin production, sugar fermentation reactions, etc. Utilising the human phages, they distinguished five different phage patterns. Most typical patterns formed only when undiluted phages were used. Of the 381 strains 77 per cent were lysed predominantly by phage 42D. A representative number of strains from each of the five phage types was examined by further tests. Edwards and Rippon observed that the strains belonging to the five different phage groups were pathogenic, since strains from all the types were found in cases of acute as well as chronic mastitis.

Seto and Wilson (1958) typed 429 cultures of micrococci of bovine origin with the help of seven phages, including phage 42D, a human phage, the rest being those isolated by Seto et al. (1956). None of the coagulase-negative cultures were susceptible to any of the seven phages. Of the 379 coagulase-positive cultures 93 per cent were susceptible to one or more of the seven phages.

The phage-sensitive cultures were divided into twelve types and patterns. Of 84 cultures from 11 herds, 78 were sensitive to only phage S2. The same phage which they found as the predominating phage lysed 49 per cent of all the sensitive cultures. The S2 type cultures were subdivided, by determining their lysogenicity, into three subtypes.

Coles (1958), utilizing the six phages that he isolated, typed 127 cultures of staphylococci obtained from samples of milk from six different herds. He observed that in all the herds, with the exception of one, more than one phage group existed. A greater diversity of phage patterns was demonstrated when the subtyping phages A10, P52, and P87 were utilized for typing. In his opinion, phage pattern of a particular strain of Staphylococcus aureus of animal origin depended primarily on the phages used.

Therefore, it may be seen from a review of the literature, that different workers have utilized different phages and that there is not a single set of specific phages which can be used as basic phages for typing of cultures of staphylococci of bovine origin. Since so many studies have now been made with staphylococcus phage, it would seem that the time is ripe for the selection of a single set of phages that could be utilized routinely for the typing of staphylococci of animal origin.

MATERIALS AND METHODS

Culture Media

Tryptose-dextrose broth consisted of tryptose (Difco) 20 gms, sodium chloride 5 gms, dextrose 1 gram and distilled water 1,000 ml. The pH was adjusted to 7.4 by the addition of 2.5 N sodium hydroxide and the medium was finally sterilized in the autoclave for 30 minutes at 15 lbs. pressure. Tryptose-dextrose agar was prepared by adding 1.5 per cent agar to tryptose-

dextrose broth. The pH was adjusted to 7.6 prior to sterilization. The medium was used for preparing base layer in petri plates as well as for making slants. Soft agar for top layer was prepared by the addition of 0.8 per cent agar to tryptose-dextrose broth. The pH was adjusted to 7.6. Prior to sterilization the medium was distributed in test tubes in 10 ml. and 4 ml. aliquots.

Characterization of Cultures

Isolations were made on 5 per cent sheep-blood agar from samples of milk obtained from clinical or suspected cases of bovine mastitis. The degree of hemolysis was recorded for each culture. After studying the morphology, a single colony was transferred to an agar slant. Only those cultures which fermented mannitol and produced coagulase were used for bacteriophage typing. Coagulase tests were performed by the method of Fisk (1940), using rabbit plasma. The cultures were kept at room temperature and subcultured every month.

Cultures of Staphylococcus aureus were also obtained from other laboratories. The sources of all the cultures used are as follows:

Source	No. of Cultures
Clinical Pathology Laboratory, Kansas State College.	127
Department of Bacteriology, School of Veterinary Medicine, Davis, California.	39
Department of Bacteriology, Ohio State University, Columbus, Ohio.	15
Jen-Sal Laboratories, Kansas City, Missouri.	3
Diagnostic Laboratory, College of Veterinary Medicine, Fort Collins, Colorado	9

Source	No. of Cultures
Department of Medicine, New York State Veterinary College, Ithaca, New York.	17
Cultures from Compton, England. (Through the courtesy of Dr. I. M. Paton.)	15
Department of Bacteriology, Ontario Veterinary College, Guelph, Canada	43

The Phages

Two sets of phages were used during this work. One set, the Coles series, consisting of six phages was supplied by Dr. E. H. Coles, Department of Pathology, School of Veterinary Medicine, Manhattan, Kansas. The other set of six phages, the Seto phages, was obtained from Dr. J. B. Wilson, Department of Bacteriology, University of Wisconsin, Madison.

The Coles Phages. The six phages along with their propagating hosts are listed below.

Phage	Propagating Host
A8	H8
A10	H8
A13	H89
P52	H34
P87	H27
P102	H27

The three phages, phage A8, A10 and A13 are adapted phages which were obtained by propagating the human phages 42D, 7, and 81, respectively, on two different strains of Staphylococcus aureus of animal origin. Phages P52, P87 and P102 were isolated from three different lysogenic strains of Staphylococcus aureus of animal origin and propagated on two different strains of Staphylococcus aureus, also of animal origin (Coles, 1958).

The Seto Phages. Six phages, phage S1, S2, S3, S4, S5, and S6, were used for bacteriophage typing. The propagating hosts for these phages are also numbered as S1, S2, S3, S4, S5, and S6 and are all micrococci of bovine origin. Phage S1 and S2 were originally isolated by propagating phage 42D, a human phage, on two different strains of staphylococci of animal origin (Seto and Wilson, 1958); therefore, these are adapted phages. Phage S3 was initially isolated by Seto et al. (1956) from a lysogenic culture of Micrococcus pyogenes of bovine origin. Phages S4, S5, and S6 were derived from phage S3 by propagating the latter on three different strains of Micrococcus pyogenes of bovine origin.

Preparation of Phages

The phages were propagated by the agar layer method of Swanstrom and Adams (1951), with a slight modification. Agar plates were prepared a day previous to their use. To each of four sterile test tubes was added 0.4 ml. of an 18-hour broth culture of the propagating strain and 0.4 ml. of the stock phage. The mixture was allowed to stand for about ten minutes, and then added to a tube containing ten ml. soft agar, which had been melted and cooled to 47° C. The tube was rotated well between the hands and the contents quickly poured over the base agar in a Petri dish. In this way four plates were prepared for each phage. The plates were incubated for about six hours at 37° C and then left at room temperature overnight. To each of these plates, on the following day, 4 ml. of sterile broth was added and the soft agar and broth were mixed by means of a sterile glass rod. The plates were then placed in the deepfreeze for about six hours. Afterwards the plates were removed, thawed at room temperature and the fluid thus collected was transferred to a

sterile test tube. The phage was filtered through Selas candle filters. Sterility was tested by inoculating a tube of broth with 0.5 ml. of the phage and incubating the tube for 48 hours at 37° C. For determining the titer, serial tenfold dilutions of the phage were prepared in broth. From each dilution 0.1 ml. of the phage suspension was taken and mixed with one ml. of an 18-hour broth culture of the propagating strain. After about ten minutes, the phage-cell mixture from each tube was added to a tube containing four ml. of soft agar which was melted and cooled to 47° C. The contents were mixed and poured over plates containing agar base layers. The plaques were counted using plates of suitable dilutions and the titer recorded. Phages having a titer of 10^8 to 10^9 particles per ml. were prepared.

Determination of the Routine Test Dilution

The routine test dilution (RTD) was determined by the method of Wilson and Atkinson (1945). Tenfold dilutions of the phage were prepared in broth. Usually five dilutions were sufficient. An 18-hour culture of the propagating strain was streaked uniformly on an agar plate by means of a sterile cotton swab. After allowing about twenty minutes for drying, each dilution of the phage was spotted on the plate. The plate was incubated at 37° C for about six hours and then kept at room temperature overnight. The highest dilution which produced confluent lysis was regarded as the routine test dilution. The RTD was usually found between 10^{-3} to 10^{-4}. No phage was used for typing unless it had an RTD of at least 10^{-3}. The phages were always stored in a deepfreeze.

Phage Typing

Plates containing agar base layer were poured on the previous day and left at room temperature overnight. The plates were marked with wax pencil on the bottom sides to facilitate reading. The surface of the agar medium was swabbed with a 12- to 18-hour broth culture of the organism by means of a sterile cotton swab and the plates were allowed to dry for about 45 minutes. The phages were spotted on the culture with one ml. tuberculin syringes equipped with 27 guage hypodermic needles. After the spots were dry the plates were incubated for about four to five hours at 37° C and then left at room temperature overnight. One set of six phages was used at a time. Each phage was used in a concentrated form as well as at its RTD so that each plate contained twelve spots. Along with each batch of cultures the dilute phages were spotted on their propagating strains to serve as control on RTD. If the testing dilution of any phage did not produce confluent lysis of the propagating strain a fresh dilution, using stock phage, was prepared.

Results of phage typing were recorded as follows:

CL Confluent lysis.

3+ Almost complete lysis with only small islands of growth.

2+ Equal amounts of lysed area and growth.

1+ Only a few plaques in the center.

Lysis scored as 2+, 3+, or CL was considered a positive response. For reporting the results of typing, the terminology suggested by Jackson et al. (1954), and Seto and Wilson (1958), was used.

RESULTS

Comparison of Results Upon Use of RTD and Use of Undiluted Phage Suspensions

A total of 268 cultures of Staphylococcus aureus were subjected to bacteriophage typing. Of these, 210 were typed utilizing the twelve phages at their RTD as well as in concentrated form, and the remaining cultures were typed with six selected phages from the two sets. Of the 210 cultures, 158 or 75.2 per cent were sensitive to one or more of the Coles phages and 195 or 92.8 per cent to one or more of the Seto phages. These results were obtained only when the phages were used in a concentrated form. The number of cultures typable with the use of RTD was very low; with the Coles phages only 96 or 45.7 per cent of the cultures and with the Seto phages, 86 or 40.9 per cent of the cultures were typable. Phage patterns were produced only when the cultures were typed with the use of concentrated phages. A comparison of the results of typing of cultures from each herd, with the use of concentrated phages as well as with phages at their RTD, is shown in Tables 1 and 2. The results of phage typing with the concentrated phages of the two series are compared in Table 3.

Phage Types and Patterns

The total phage-sensitive cultures were divided into 19 phage types with the Coles phages and into 26 phage types with the Seto set. (Tables 4 and 5.) With the Seto phages, cultures belonging to phage pattern S2/S5 and phage type S2, were found to be common. With the Coles phages, the phage pattern, A8/A10/A13/P87/P102 was predominant. In each herd, cultures belonging to more than one phage type or pattern were seen. In the case of typing with the

Table 1. Cultures typable with the Coles phages when used as concentrated as well as at their RTD.

Herd	No. of cultures used	Cultures typable with concentrated phages		Cultures typable with phages used at their RTD	
		No. of cultures typable	Per cent typable	No. of cultures typable	Per cent typable
A	12	12	100	9	75
B	17	9	52.9	3	17.6
C	38	9	23	6	15.7
D	9	9	100	9	100
E	63	60	95.2	37	58.7
F	17	17	100	17	100
G	25	19	76	2	8
H	14	8	57.1	6	42.8
J	15	15	100	7	46.6
Total	210	158	75.2	96	45.7

Table 2. Cultures typable with the Seto phages when used as concentrated as well as at their RTD.

Herd	No. of cultures used	Cultures typable with concentrated phages		Cultures typable with phages used at their RTD	
		No. of cultures typed	Per cent typable	No. of cultures typable	Per cent typable
A	12	12	100	4	33.3
B	17	17	100	9	52.9
C	38	38	100	7	18.4
D	9	8	88.8	1	11.1
E	63	60	95.2	38	60.3
F	17	17	100	0	0
G	25	22	88	20	80
H	14	8	57.1	4	28.5
J	15	13	86.6	3	20
Total	210	195	92.8	86	40.9

Table 3. Comparison between cultures typable with the concentrated Coles phages and the concentrated Seto phages.

Herd	No. of cultures	Coles phages		Seto phages		Six selected phages	
		No. of cultures typable	Per cent typable	No. of cultures typable	Per cent typable	No. of cultures typable	Per cent typable
A	12	12	100	12	100	12	100
B	17	9	52.9	17	100	17	100
C	38	9	23	38	100	33	86.8
D	9	9	100	8	88.8	9	100
E	63	60	95.2	60	95.2	62	98.4
F	17	17	100	17	100	17	100
G	25	19	76	22	88	22	88
H	14	8	57.1	8	57.1	8	57.1
J	15	15	100	13	86.6	15	100
Total	210	158	75.2	195	92.8	195	92.8

Coles phages, the majority of the phage-sensitive cultures was lysed by three or more of the phages. The number of cultures susceptible to the action of only one or two phages was very low. With the Seto phages, on the contrary, the majority of cultures was lysed by one or two phages only. Cultures resistant to the action of the Coles phages were found to be sensitive to the Seto phages. A few of the cultures which were untypable with the Seto phages were found to be sensitive to the Coles phages. A very high percentage of the cultures was susceptible to the Seto phages but these phages did not usually reveal subtypes within the group.

Cultures obtained from different quarters of the same cow were not always identical. For instance, cultures 70 and 71 were isolated from different quarters of the same cow, but were shown to belong to two different phage patterns. Although cultures 45, 46, 47 and 48 were obtained from four different quarters of another cow, only culture 48 was susceptible to the Coles phages. All the four cultures, however, were sensitive to the Seto phages and typing with these phages revealed the presence of three different strains.

Table 4. Phage types and patterns with the concentrated Coles phages.

Herd	No. of cultures used	No. typable	Untypable	Phage types and patterns																						Total patterns
				A8 A10 A13 P87 P102	A8 A10 A13 P87 P52 P102	A8 A13 P87 P102 P52	A8 A13 P87 P102	A8 A13 P87 P102	A8 A10 P102 P87	A8 P87 P102	A8 P52 P102 P87	A8 A13 P102 P87	A10 A13 P102 P87	A8 A10 P87	A8 P52 P102	A8 P102	A8 A10 A13 P102	A8 A13 P102 P52	A8 A10 A13 P52	A8 A10 P52	A8 A10 A13 P102						
A	12	12	-	5	4	1	2																			4
B	17	9	8	1	-	1	2	-	1	2	1	1														7
C	38	9	29	-	-	1	-	4	-	1	2	-	1													5
D	9	9	-	4	2	1	-	-	2	-	-	-	-	-												
E	63	60	3	16	16	3	3	-	1	3	1	2	-	1	1	2	1	1	3	5		1	16			
F	17	17	-	10	6	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	3			
G	25	19	6	2	-	1	4	-	-	10	-	-	-	-	-	1	-	1					6			
H	14	8	6	4	-	3	1																3			
J	15	15	-	3	1	1													6	3	1		6			
Total	210	158	52	45	29	12	12	4	5	16	4	3	1	1	1	3	1	2	9	8	1	1				

Table 5. Phage types and patterns with the concentrated Seto phages.

Herd	No. of cultures used	No. typable	Untypable	Phage types and patterns																										Total patterns
				S2:S5	S2:S5:S6	S2	S1:S2:S3:S5	S2:S4:S5:S6	S2:S3:S4:S5:S6	S3:S5	S2:S3	S5	S5:S6	S1:S3:S5	S4:S6	S1:S4:S5:S6	S1:S2:S3:S4:S5	S2:S3:S5	S1:S3:S4:S5:S6	S1:S3	S1:S2:S3:S5:S6	S3	S2:S4:S5	S2:S3:S5:S6	S4:S5:S6	S4:S5	S2:S4	S6	S1:S2:S3	
A	12	12	-	7	-		1	1										2					1							5
B	17	17	-	2	8		-	4	2															1						5
C	38	38	-	15	4		3				2	1	1	3											1	1	1	5	1	12
D	9	8	1	1	-	3	-	-			1											3								4
E	63	60	3	18	6	11	3	1	2	1	-		2	1			3	1	2	7	1							1		15
F	17	17	-	-	-	16	-	-			1																			2
G	25	22	3	-	-	-	13	-		5		1	3																	4
H	14	8	6	-	-	-	-	-				6		1	1															3
J	15	13	2	2	1	6	-	-			1																		3	5
Total	210	195	15	45	19	36	20	6	4	6	5	8	3	7	1	1	3	3	2	7	1	3	1	1	1	1	1	6	4	

Cultures 47 and 48 were shown to be identical while cultures 45 and 46 formed two distinct strains.

Phage Typing of Known Identical Cultures

When the phage patterns produced by the concentrated phages of the two series were compared, it was observed that the grouping of cultures was not identical. In order to find out the factors responsible for this difference the following experiment was conducted. Four cultures of known phage pattern were selected. They were streaked on tryptose-dextrose agar plates and single colonies were isolated. A single colony from each culture was restreaked to obtain separate colonies. Five such isolated colonies from each culture were obtained and transferred to agar slants. These represented five known identical cultures for typing. A total of twenty single-colony isolates were subjected to typing with the concentrated Coles phages and the Seto phages. The findings were compared with those of the original cultures. The results, summarized in Table 6, show that the single-colony isolates revealed exactly the same phage patterns as were revealed by the original cultures. A slight difference however, was noted in the phage pattern of the original culture, culture No. 186 and its single-colony isolates. This difference was revealed due to the variable reactions produced by phage S4. The original culture gave a 2+ reaction to the concentrated phage S4, while the single-colony isolates from the same culture gave either a 1+ reaction or a negative reaction. No significant differences in reaction were observed in the case of confluent lysis.

The action of the two sets of phages was further tested on separate isolations made from one quarter of the same cow. Samples of milk from left-hind quarter of cow No. 20B, College Dairy Farm, were obtained. A total of five

Table 6. Typing of known identical cultures with the two series and the six selected phages. (The phages were used in concentrated form.)

Culture No.	The Coles phages							The Seto phages						The six selected phages					
	A8	A10	A13	P52	P87	P102		S1	S2	S3	S4	S5	S6	A8	A10	P87	P102	S2	S5
Original culture No. 192	CL	CL	CL	-	CL	CL	-	CL	-	-	-	-		CL	CL	CL	CL	CL	-
Single colony isolates 192/1	CL	CL	CL	-	CL	CL	-	CL	-	-	-	-		CL	CL	CL	CL	CL	-
192/2	CL	CL	CL	-	CL	CL	-	CL	-	-	-	-		CL	CL	CL	CL	CL	-
192/3	CL	CL	CL	-	CL	CL	-	CL	-	-	-	-		CL	CL	CL	CL	CL	-
192/4	CL	CL	CL	-	CL	CL	-	CL	-	-	-	-		CL	CL	CL	CL	CL	-
192/5	CL	CL	CL	-	CL	CL	-	CL	-	-	-	-		CL	CL	CL	CL	CL	-
Original culture No. 175	CL	CL	CL	CL	-	CL	-	CL	-	-	CL	-		CL	CL	-	CL	CL	CL
Single colony isolates 175/1	CL	CL	CL	CL	-	CL	-	CL	-	-	CL	-		CL	CL	-	CL	CL	CL
175/2	CL	CL	CL	CL	-	CL	-	CL	-	-	CL	-		CL	CL	-	CL	CL	CL
175/3	CL	CL	CL	CL	-	CL	-	CL	-	-	CL	-		CL	CL	-	CL	CL	CL
175/4	CL	CL	CL	CL	-	CL	-	CL	-	-	CL	-		CL	CL	-	CL	CL	CL
175/5	CL	CL	CL	CL	-	CL	-	CL	-	-	CL	-		CL	CL	-	CL	CL	CL
Original culture No. 186	CL	CL	CL	CL	CL	CL	CL	CL	CL	2+	CL	-		CL	CL	CL	CL	CL	CL
Single colony isolates 186/1	CL	CL	CL	CL	CL	CL	CL	CL	CL	-	CL	-		CL	CL	CL	CL	CL	CL
186/2	CL	CL	CL	CL	CL	CL	CL	CL	CL	1+	CL	-		CL	CL	CL	CL	CL	CL
186/3	CL	CL	CL	CL	CL	CL	CL	CL	CL	-	CL	-		CL	CL	CL	CL	CL	CL
186/4	CL	CL	CL	CL	CL	CL	CL	CL	CL	1+	CL	-		CL	CL	CL	CL	CL	CL
186/5	CL	CL	CL	CL	CL	CL	CL	CL	CL	-	CL	-		CL	CL	CL	CL	CL	CL
Original culture No. 190	CL	CL	CL	CL	CL	CL	-	CL	-	-	CL	-		CL	CL	CL	CL	CL	CL
Single colony isolates 190/1	CL	CL	CL	CL	CL	CL	-	CL	-	-	CL	-		CL	CL	CL	CL	CL	CL
190/2	CL	CL	CL	3+	CL	CL	-	CL	-	-	CL	-		CL	CL	CL	CL	CL	CL
190/3	CL	CL	CL	CL	CL	CL	-	CL	-	-	CL	-		CL	CL	CL	CL	CL	CL
190/4	CL	CL	CL	3+	CL	CL	-	CL	-	-	CL	-		CL	CL	CL	CL	CL	CL
190/5	CL	CL	CL	2+	CL	CL	-	CL	-	-	CL	-		CL	CL	CL	CL	CL	CL

samples were obtained at a day's interval. About a month and a half prior to obtaining these samples, the milk of this cow was examined and a phage-sensitive staphylococcal culture was obtained from the left-hind quarter. The separate isolations, on testing, revealed phage patterns identical to that of the earlier culture. The results are shown in Table 7.

Table 7. Phage typing of separate isolations from the same cow.

Culture No.	Date	Phage type or pattern with concentrated phages											
		Coles phages						Seto phages					
		A8	A10	A13	P52	P87	P102	S1	S2	S3	S4	S5	S6
109	9/3/58	CL	CL	CL	-	CL	CL	-	CL	-	-	-	-
109/1	10/13/58	CL	CL	CL	-	CL	CL	-	CL	-	-	-	-
109/2	10/14/58	CL	CL	CL	-	CL	CL	-	CL	-	-	-	-
109/3	10/15/58	CL	CL	CL	-	CL	CL	-	CL	-	-	-	-
109/4	10/16/58	CL	CL	CL	-	CL	CL	-	CL	-	-	-	-
109/5	10/17/58	CL	CL	CL	-	CL	CL	-	CL	-	-	-	-

CL = confluent lysis

Typing of Cultures With Few Selected Phages

Of the 12 phages used for typing in the present studies, six phages were selected for a new typing scheme. These include, phage A8, A10, P87, P102 from the Coles series and phage S2 and S5 from the Seto set. A total of 48 cultures, 15 from Compton, England, and 43 from Canada, were typed with the help of these six phages. The phages were used only in concentrated form. In the interpretation of the results, confluent lysis and 3+ lysis alone were considered as positive responses. The results of typing with these phages are shown in Table 8.

Table 8. Typing of cultures obtained from Canada and England with the six selected phages.

Source of cultures	No. of cultures used	No. typable	Phage types or patterns													
			A8 A10 P102 S2	A8 P87 P102 S5 S2	A8 A10 P102 S5	A8 P87 S2	A8 A10 P102 S2	A8 P87 P102 S5	A10 A8 P102 P102 S5	A8 P102 S5	P102 A8 S2	P87 P102 S2	A8 S5	A8 P102 S5	A8 A10 P102	A8 P102 S2 S5
Canada	43	36	11	4	1	3	1	3	1	2	1	3	1	2	1	1	1
England	15	7	1	-	-	2	-	-	-	4	-	-	-	-	-	-	-

DISCUSSION

Relation Between RTD and Concentrated Phages

Of a total of 210 cultures, 158 or 75.2 per cent were lysed by the Coles phages and 195 or 92.8 per cent by the Seto phages. These results were obtained with the use of concentrated phages only. When the phages were used at their RTD, distinct patterns of lysis were not produced and the number of cultures lysed was very small, e.g., 45.7 per cent with the Coles phages and 40.9 per cent with the Seto phages. This shows a significant difference in the results of typing obtained by the use of RTD and the use of concentrated phages. Typical phage patterns were produced only by the use of concentrated phages. The phages used at their RTD did not reveal such patterns. Similar findings were reported by Williams-Smith (1948), Edwards and Rippon (1957), and Coles (1958). They reported that more cultures were lysed and patterns of lysis produced when the phages were used in an undiluted form. Seto and Wilson (1958), however, were able to type 93 per cent of the cultures with the use of RTD. Even with the Seto phages used at their RTD, in the present study,

only 40.9 per cent of the cultures were lysed. In phage-typing of cultures of staphylococci associated with bovine mastitis, therefore, undiluted phage suspensions alone should be used. Suspensions with a titer of at least 10^8 phage particles per ml. should be prepared.

Phage Types and Patterns

In all the herds studied, more than one phage pattern was observed with the number of patterns in different herds varying from 2 to 7 depending on the size of the herd. For example, herd C from which 38 cultures were obtained, revealed the presence of a large number of phage types and patterns. Herd E consisted of cultures obtained from various sources, but for convenience they were grouped together. Cultures isolated from different quarters of the same cow were not identical; usually each quarter excreted a different type of organism. This suggests that the infection is transmitted from one animal to another either by milking machines or by the milkers hands. These findings are in agreement with those of Williams-Smith (1948a), Price et al. (1954), Coles (1958), and Seto and Wilson (1958). From the information obtained by typing of cultures from a herd, it is possible to find out the spread of infection within that herd. Carrier animals can also be detected.

Phage Typing of Known Identical Cultures

When the results of typing with the two sets of phages were compared, it was observed that the same culture, when it was subjected to typing with the two series of phages, appeared to belong to two different groups. Similar discrepancy was noted by Coles (1958). He indicated that the results of typing depended upon the phages utilised and that different classifications

and grouping may result when different sets of phages were used. With a view to clarifying this particular point, known identical cultures were used for typing. These were of two kinds. In one case separate isolations were made from the same cow; in another instance single colonies were obtained from a few selected cultures. The results indicated that the cultures were identical (Table 6). The action of phage 84 on culture 186, however, gave variable results. On the original culture this phage produced a 2+ reaction while on the single-colony isolates either there was a 1+ reaction or no reaction at all. From these findings it is apparent that in phage typing a 2+ reaction is of little significance. If a 2+ reaction is considered as a positive response even identical cultures may appear different. The only reactions to be considered are, therefore, confluent lysis and 3+ lysis. These two responses will mostly be obtained when the concentrated phages are used. This again emphasizes the necessity of using undiluted phages in typing studies. Separate isolations from the same cow were typed with the phages and were shown to be identical (Table 7). This illustrates the point that phage typing will reveal the true relation between several strains.

New Combination of Phages Suggested for Typing of Staphylococcal Cultures of Bovine Origin

The ability of a typing phage to produce lysis of a bacterial cell will depend, besides other factors, upon the type of phage which the bacterium carries. Bacteria are generally immune to the phage which they already carry (Luria 1956). Lysogeny appears to be a rule rather than exception. Rountree (1949) demonstrated lysogeny in 27 out of 30 cultures of coagulase-positive staphylococci. Coles (1958) examined 95 cultures of Staphylococcus aureus of animal origin and reported lysogeny in one hundred per cent of the cultures.

In preparing phages for use in bacteriophage typing studies, it appears necessary to screen the cultures for the presence of lysogenic phages and to select those phages which will be active on a large number of cultures. Adapted phages can also be used for typing.

The results of typing obtained in the present study indicated that each series of phages, when used alone, had certain limitations. The Coles phages, for example, produced typical patterns of lysis but lysed only about 75 per cent of all the cultures used. The Seto phages, on the other hand, lysed 92.8 per cent of all the cultures but did not produce typical patterns of lysis; there were many cultures which were lysed by phage S2 alone. Besides, both series contained certain phages, the use of which did not serve to give additional information in typing. It was therefore decided to set up a new combination, utilising few phages from each of the series. Of a total of 12 phages, six were selected for inclusion in the new typing scheme. These are phage A8, A10, P87 and P102 from the Coles series, and phage S2 and S5 from the Seto set. These phages combined the advantages of the two series in that they lysed large number of cultures and at the same time produced typical patterns of lysis. In making this selection, two properties of each phage were mainly considered, namely the ability to lyse a large number of cultures and the ability to differentiate one culture from the other. Of the phages not included in the new combination, phage P52, S1, S3, S4, and S6 lysed only few cultures and secondly, they did not contribute much towards producing typical patterns of lysis. Another phage, phage A13, was an active phage, but it duplicated the action of other phages in the Coles series. The results of typing that can be obtained by using the few selected phages are summarised in Table 9. As may be seen from this table, these six phages were capable of

Table 9. Phage types and patterns that would be produced if only the six selected phages were used.

Herd	No. of cultures used	No. typable	Untypable	Phage types and patterns														Cont. below
				A8 A10 P87 P102 S2 S5	A8 P87 P102 S2 S5	A8 A10 S2 S2 S2 S5	A8 P102 S2 S5	A8 A8 P87 P102 S2 S5	A8 P87 S2 S2	A8 P87 P102 S2 S5	A8 A10 P87 P102 S2 S5	P102 A10 P87 P87 S2 S5	A8 A10 P102 S2 S5	A8 P87 P102 S2 S5				
A	12	12	-	9	3	-	-	-	-	-	-	-	-	-	-	-	-	
B	17	17	-	2	4	8	-	2	1	-	-	-	-	-	-	-	-	
C	38	33	5	-	2	16	1	1	-	3	7	3	-	-	-	-	-	
D	9	9	-	-	1	-	-	-	-	-	-	-	8	-	-	-	-	
E	63	62	1	20	5	2	-	2	1	1	-	2	11	1	1	1	1	
F	17	17		-	-	-	-	-	-	-	-	-	17	-	-	-	-	
G	25	22	3	1	7	3	-	10	-	-	-	-	-	-	-	-	-	
H	14	8	6	2	4	-	-	-	-	-	-	-	-	-	-	-	-	
J	15	15	-					1		1			3					
Total	210	195	15	34	26	29	1	16	2	5	7	5	39	1	1	1	1	

Herd	No. of cultures used	No. typable	Untypable	Phage types and patterns														Total types
				A8 A10 P87 P102 S2	A8 A10 P87 S5	A8 A10 P102 P102 S5	A10 P102 S2	A8 A8 P102 S2 S5	A8 P102 S2 S5	A8 A10 A13 S5	A8 A10 P102 S5	A8 A10 P102 P102 S2	A8 A10 P102 P102 S2					
A				-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
B				-	-	-	-	-	-	-	-	-	-	-	-	-	-	5
C				-	-	-	-	-	-	-	-	-	-	-	-	-	-	7
D				-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
E				1	2	1	1	1	3	1	1	1	2	-	-	-	-	22
F				-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
G				1	-	-	-	-	-	-	-	-	-	-	-	-	-	5
H				1	1	-	-	-	-	-	-	-	-	-	-	-	-	4
J						3			1				1	1	4			8
Total				3	3	4	1	1	4	1	1	1	3	1	4			

typing 195 of 210 cultures. In addition, distinct patterns of lysis were observed in cultures from different herds.

Since the six selected phages give better results than any single set used previously, it is proposed that phages A8, A10, P87, P102, S2, and S5, selected from the two series, be used as basic phages for routine typing of cultures of Staphylococcus aureus associated with cases of bovine mastitis. Besides these, additional phages can also be used if a worker feels that by doing so additional information will be obtained. In order to be able to type a large number of cultures and to produce patterns of lysis, the phages should be used in a concentrated form. It is also suggested that in interpreting the results of typing, confluent lysis and 3+ lysis alone should be considered as positive responses. This procedure will assist in assigning the cultures to their proper phage groups.

Typing of Cultures Obtained from Canada and England With the Six Selected Phages

As may be seen in Table 8, a total of 58 additional cultures, 43 from Canada and 15 from England, were typed utilizing the six selected phages. The cultures from Canada revealed the presence of 15 different phage types or patterns, eleven of which belonged to phage pattern A8/P102/S2. This was an interesting feature since, out of 210 cultures from the United States, only one showed this pattern. Of the 15 cultures received from England, only seven were typable and were divisible into three different phage patterns. A new phage pattern A8/P102/S5 was also present in the cultures from Canada and England but absent from the rest of the cultures. This further illustrates the value of phage typing, since isolates from different geographic locations reveal different phage types.

SUMMARY

A total of 268 cultures of Staphylococcus aureus obtained from cases of bovine mastitis were typed by three sets of phages; the Coles series, the Seto series, and a third series of six phages selected from the two other groups. On the basis of the results, it is proposed that the third selected series is superior to either of the other two alone and therefore should be adopted for the routine typing of staphylococci of bovine origin. Further, it is proposed that these six phages should be used in concentrated form rather than at their routine test dilution.

In the course of testing these 268 cultures, certain other information was obtained. These include the observations that cultures belonging to more than one phage pattern could be isolated from a single herd and even from different quarters of the same cow. Also, cultures from Canada and England revealed phage types quite distinct from those isolated in the United States.

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BACTERIOPHAGE TYPING OF STAPHYLOCOCCUS AUREUS ASSOCIATED WITH CASES OF BOVINE MASTITIS

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In epidemiological investigations, valuable information can be obtained by the use of a bacteriophage typing scheme. Phage-typing will reveal differences among strains which are not detectable by the usual serologic procedures. In human medicine, basic phages are available for typing of strains of staphylococci. There are, however, no such basic phages at present for typing of staphylococci of bovine origin. The human phages are not helpful in typing of these strains.

In the present study two different sets of phages, the Coles phages and the Seto phages, were used for typing of strains of Staphylococcus aureus associated with bovine mastitis. The action of the two sets of phages was compared. Also, phages were selected from both groups to devise a typing scheme that might have merit over either one alone. In the process of devising this phage typing scheme, a comparison was made between the use of routine test dilutions (RTD) and concentrated phage.

A total of 268 cultures of Staphylococcus aureus, obtained from cases of bovine mastitis, were typed by use of the two sets of phage. With the Coles phages, 75.2 per cent and with the Seto phages 92.8 per cent of the cultures were typable when concentrated phage suspensions were used. With the use of routine test dilutions, only 45.7 per cent and 40.9 per cent of the cultures were typable with the Coles phages and the Seto phages respectively.

Each herd revealed the presence of cultures belonging to more than one phage pattern. Different quarters of the same cow usually excreted organisms of different phage patterns.

Cultures known to be identical with regard to other characteristics were typed by using the two sets of phages. The phages revealed that the cultures were identical with respect to phage type. Separate isolations from the same cow were shown to be identical by the phage typing method.

Cultures obtained from Canada and England revealed the presence of few cultures which belonged to a phage pattern, which was quite distinct from those found in the rest of the cultures.

From the Coles and the Seto sets of phages, six phages were selected. It is proposed that these six are superior to either set alone and therefore should be adopted in routine typing of Staphylococcus aureus of animal origin.