BACTERIOPHAGE DETERMINATION OF STAPHYLOCOCCI

IN

COWS' MILK AFTER IMMUNIZATION TREATMENTS

by

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ABSTRACT

A preliminary investigation was conducted to determine the simplest method for propagating phages, which also would yield high titers. It was found that 40 ml. volumes of freshly prepared Trypticase soy broth to which was added 5 ml. of 12-18 hr. broth culture of the staphylococcal propagating strain and 5 ml. of homologous phage, the whole incubated at 28 degrees C. for 4-8 hours on a rotary shaker yielded best results. Incubation times differed for some of the phages, but visual controls showed the reaction to be complete within 4-8 hours.

Separation of unlysed cells and cellular debris from all phages propagated by this method was effected by refrigerated centrifugation of the phage suspensions at 17,300 x g for thirty minutes; aseptic technique was used throughout.

A six-week sampling period was conducted during which time milk samples were tested for coagulase-positive staphylococci. The samples were streaked on Tellurite-Glycine Agar, and also on mammalian blood-agar as a control, to determine any inhibitory effect of the Tellurite-Glycine Agar.

During this study 72 coagulase-positive and 71 coagulase negative isolates were obtained. Of the former, one was spontaneously lysed after coagulase testing; thus 71 were available for phage typing. Of these 68 were typable by the phages used; three were not. Using phages of bovine origin, the predominant reaction was type/pattern S3. With the human phages, the predominant reaction was type/pattern 42C.

Sixteen of the coagulase-negative isolates were tested with the phages, none reacted with any of the phages at either of the two dilutions used.

From the results obtained, four staphylococcal-carriers and four non-carriers were given intramuscularly, two 5 ml. injections of somatic antigen one week apart; one carrier animal was given similarly, equal volumes of a

mixture of antigen and toxoid, and four non-carrier and two carrier animals were retained as controls.

A further testing period to isolate coagulase-positive staphylococci showed that the romatic antigen was more successful in the removal of these bacteria than was the toxoid. Of the control animals, the four non-carriers remained unchanged and the two carriers continued to yield staphylococci.

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INTRODUCTION

INTRODUCTION

Bovine infections due to the staphylococci seem to be on the ascendancy in widely scattered areas of the world (5, 13, 18, 19). This upswing probably is due to the irrational use of antibiotics which have virtually eliminated the streptococci, at one time the predominant type, and have enabled the more adaptable staphylococci to proliferate. Whatever the reason, there is urgent need for a means by which either complete eradication or the suppression of these organisms may be accomplished. This need is all the more urgent when it is considered that during the life-span of a normal person there is hardly one that has not been stricken by some form of food-poisoning.

Staphylococcal food-poisoning is caused by the enterotoxin produced by toxigenic strains growing in a suitable food. Many cases have been traced to foods containing milk. According to Reid (15), however, the presence of staphylococci in the bovine udder is not a case for alarm because these organisms may be found in

apparently healthy udders. It may be worth adding that staphylococci from an apparently healthy udder on injection into a normal udder may be responsible for acute mastitis. Thus the severity of a staphylococcal mastitis infection may depend on high virulence of the pathogen or low resistance of the udder tissue.

As a result of the large number of existing staphylococcal strains, accurate identification on the basis of physiological characteristics has proven inadequate. Tests based on hemolysis, pigmentation, coagulase production, mannitol fermentation or high sodium chloride tolerance have served only to distinguish broad groups within the species, and to indicate actual or potential pathogens. A more fundamental procedure was required, and phage-typing has become the standard technique used in laboratories for the identification of staphylococcal strains.

Phage-typing is based on the response of a bacterial strain when tested with several phages. The response given determines the phage type of the strain.

The technique, however,has several limitations.

(1) The reaction is known to occur only with coagulase positive staphylococci.

(2) There is no type specific phage, that is, a phage which lyses a single staphylococcal strain only. Consequently, identification is based upon phage pattern.

(3) Phages are unstable, and as a result, titers once above a certain critical level (10^{-3}) must be routinely checked once a week. Phages below this level cannot be used in phage-typing.

(4) A phage is liable to undergo variation, and after each propagation, its lytic spectrum must be determined.

(5) A phage to be stored should be sterile; this is due to the susceptibility of propagating and harvesting methods to contamination. Sterilization usually is accomplished by filtration, but other methods have been suggested (23).

In epizootic studies of the staphylococci, phages of human origin have been used, but without complete success. Williams and Rippon (21) suggested the adaptation of human phages for this purpose. According to Coles (5), Seto was the first to report the use of "adapted" staphylococcal phages in the identification of staphylo-

cocci of animal origin.

Several workers (5, 13, 18, 19) have reported phage type 42D to be the most common type isolated from cow's milk. However, McLean (14), found that type 44A constituted 44.3% of the isolates identified and 42D, 30%. Seto (16), also in Wisconsin, found 78 of 84 cultures to be sensitive to phage S2. This phage, which was the predominant type, lysed 49% of all the sensitive cultures. He reported that all cultures found to be sensitive to his adapted phage S2 were sensitive to phage type 44A, and approximately 80% of the 44A type cultures were sensitive to phage S2. According to Fung (8), phage type 8I seems to be predominant in the environs of Winnipeg. However the same worker later reported type SI/S3 to be predominant in the university herd.

The fact that staphylococci may be present in the healthy udder has been established, and, because of the potential infectivity of these organisms, it is imperative that a method of suppressing or of removing them from infected as well as from healthy udders be found.

As a result of the production and testing of a

somatic antigen found useful in controlling staphylococcal infections in experimental animals and in humans (11, 12), an attempt was made by Crawley (7), of the Connaught Medical Laboratories in Toronto to evaluate the effect of this antigen in controlling staphylococcal mastitis in cattle. From field trials, indications were that bovine staphylococcal mastitis might be prevented by this procedure.

In this study, an attempt was made to evaluate the effectiveness of this antigen in suppressing staphylococci in animals in the university herd. Simultaneously, some of the animals were treated with α and β toxoid, and others with a mixture of antigen and toxoid. It was hoped in this way to ascertain whether the method of Crawley would prove more useful for controlling staphylococci of bovine origin than these other methods.

MATERIALS AND METHODS

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MILK SAMPLES

The milk samples used in this investigation were obtained from 25 cows selected at random from the university herd. In a six week preliminary study, coagulase-positive isolates were obtained from some, but not all animals. The staphylococcal carriers were given intramuscularly, two 5 ml. injections at weekly intervals, of either a somatic antigen, or of α and β toxoid, or of equal parts of a mixture of the two. Four of the animals from which no coagulase-positive staphylococci were obtained during the preliminary study were treated also with somatic antigen. It was felt that these latter animals would be exposed to the staphylococci carried either by the other animals of the herd, or by human handlers. In the event that staphylococci were cleared from carriers these non-carrier animals could show whether this was an induced immune reaction, or a natural defence reaction.

Inoculations were arranged so as not to disrupt a

weekly sampling schedule. The cow's udder was washed with a warm chlorine solution and then swabbed with 70% ethanol; and a sample obtained aseptically from each of the four quarters. Samples were plated immediately, or in some cases held at 4 degrees C. for 2-3 hours before plating.

ISOLATION OF STAPHYLOCOCCI

The staphylococci were isolated by streaking 0.005 ml. of the well-shaken sample on one segment of a previously prepared plate of Potassium-Tellurite-Glycine agar, the other three segments being used for the samples from the other three quarters of the udder. This medium was suggested by Zebovitz, Evans and Niven (22) as a selective plating medium for the quantitative detection of coagulase-positive staphylococci. After inoculation the plates were incubated in an inverted position at 37 degrees C. for 24 hours, coagulase-positive staphylococci producing black colonies within this time. Colonies usually were discrete. According to the number, several isolates were selected on the basis of colony size, morphology and color intensity,

inoculated into 5 ml. trypticase soy broth and incubated at 37 degrees C. overnight. The following day Gram stains were made and those isolates appearing morphologically to be staphylococci were streaked on trypticase soy agar slants and incubated at 37 degrees C. overnight. These cultures were then stored at 4 degrees C. It was found that this medium was not specific for coagulase-positive staphylococci, since with some samples Gram variable pleomorphic organisms, as well as Gram positive cocci in chains, also appeared black within 24 hours.

PRELIMINARY TESTS ON THE STAPHYLOCOCI

Coagulase Test

Because the available set of phages have been found capable of typing only coagulase-positive strains of staphylococci, this test was carried out on all staphylococcal isolates. The procedure involved reconstituting 1 ml. commercial lyophilized plasma* in 3 ml. sterile distilled water and preparing a 1:10 sterile saline dilution. A small inoculum of the isolate was cultured in 0.5 ml. of the plasma

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*Obtainable from Baltimore Biological Laboratory.

at 37 degrees for 1 to 4 hours. Readings were made hourly and with some isolates the results were positive in fewer than four hours. Only those isolates giving a positive reading were used for typing.

Mannitol Test

Each isolate was cultured in 5 ml. brom cresol purple mannitol broth under anaerobic conditions at 37 degrees C. for 96 hours; and examined for color change to lemon or yellow.

Hemolytic Test

Each staphylococcal isolate was streaked on freshly made (B.B.L.) Blood Agar Base containing 5% sterile citrated sheep blood in a plate, and incubated at 37 degrees C. for 24-48 hours. Each was rated according to its hemolytic characteristics. Only isolates showing hemolysis were typed.

PHAGES

The phages used for typing included the 21 basic phages recommended by the International Committee on Phage Typing of Staphylococci and kindly supplied by Dr. Bynoe

of the Lab of Hygiene in Ottawa, 5 "Adapted" Seto phages supplied by Dr. Wilson of the University of Wisconsin, and 8 others some of which had been found useful in earlier work at this university (9). The groups of phages follow.

Group	Phages
1	29, 52, 52A, 79, 80
2	3A, 3B, 3C, 55, 71
3	6, 7, 42E, 47, 53, 54, 75, 77
4	42D
Misc.	81, 187
Add'l. Phages	42B, 42C, 44A, 47C, 73, 82, 83, W
Adapted Phages	S2, S3, S4, S5, S6

Of the "adapted" phages S1 was omitted because in each trial during propagation the control underwent spontaneous lysis. It should also be pointed out that phage W, of the "additional phage" group, was isolated at the Provincial Laboratory in Winnipeg, and is better known as phage 57. The propagating strains for the phages were maintained on Trypticase Soy Agar slants in metal capped tubes at 4 degrees C. and were transferred monthly. A

reserve stock of the propagating strains maintained on the same medium and at the same temperature was transferred at intervals of about 3 months.

PHAGE PROPAGATION

In a preliminary study three methods of propagating phages were tested: the plate, soft-agar layer and broth methods.

The plate method was adapted from that of Blair and Carr (2). About 60 ml. Trypticase Soy Agar, pH 7.3, was poured in a 15 cm. Petri dish and dried at 37 degrees C. for 18 hours. Five milliliter of an 18-hour broth culture of the propagating strain was poured into each of two plates and the excess broth removed with a sterile Pasteur pipette. The agar surface was allowed to dry for a few minutes by slightly tilting the lid, and 0.6 ml. of the appropriate phage suspension at its routine test dilution distributed over the staphylococcal lawn, except in that portion designated as the control. The agar surface was once more allowed to dry for a few minutes. The plates were then incubated at 37 degrees C. for 4-6 hours, and

held at 4 degrees C. overnight. The following morning, the control region was removed, and the plates held in the freezer overnight. They were thawed in a tilted position at room temperature; and the expressed liquid transferred aseptically and centrifuged under refrigeration at 17,300 x g for 30 minutes. Filtration was not necessary if care were taken to place the thawed fluid in the middle of the tube and after centrifugation not to disturb the lower 5-10 ml. This liquid was titered, checked for lytic activity on the appropriate strains and tested for sterility by inoculating 5 ml. thioglycollate broth with 0.2 ml. of phage suspension and incubating at 37 degrees C. for 24-36 hours. If the titer was below the Critical Test dilution of 10^{-3}, another passage was made. Usually at least four passages were necessary for phages from freshly opened lyophilized stocks.

The procedure adapted in the soft Agar Layer method, with minor modifications, was that of Blair and Carr (3). About 40 ml. of agar, were poured into a Petri dish. This constituted the basal medium which was dried at 30 degrees C. for 24 hours. Two-tenths ml. of a 37 degrees C. -18

hour Trypticase Soy Broth culture was added to 10 ml. semi-solid (0.7%) agar held at 45 degrees C. in a water-bath. To this was added about 0.25 ml. homologous phage suspension. The contents of the tube were thoroughly mixed and immediately poured over the basal medium in the Petri dish. Two plates were treated in this manner, and one to which no phage was added was kept as a control. Incubation was at 30 degrees C. for 24 hours. Phage was harvested by transferring the soft agar layer with a sterile glass rod bent like a hockey stick into a sterile centrifuge tube. The base layer was then washed with 10 ml. broth. This broth with absorbed phage from the base layer was then added to the centrifuge tube containing the semi-solid layer and the whole thoroughly mixed in order to obtain a good suspension of the phage and centrifuged in a refrigerated centrifuge at 17,300 x g for 30 minutes. The supernatant was then removed with a sterile Pasteur pipette. Because it was impossible to prevent contamination of the walls of the tube with unlysed cells while the semi-solid layer was being transferred to the centrifuge tube,

it was found necessary to filter phage suspensions produced by this method. Millipore membrane filter type HA, pore size 0.45 μ, was used for filtering. The phage was then titered, and if the result was unsatisfactory, the procedure was repeated using this recently propagated phage at its R.T.D.* to propagate more phage.

The broth method of propagation was an adaptation of a method by Smith (17). It involved the addition of 0.3 ml. of an 18 hour broth culture to each of five tubes containing 10 ml. Trypticase Soy Broth and 0.5 ml. phage suspension to each of four of these tubes which were then thoroughly shaken. The fifth tube acted as a control. Incubation was at 37 degrees C. Visual observation of the extent of lysis compared to the amount of growth in the control tube was carried out, and if necessary, more cells were added. The process was continued until there was evidence of developing bacterial growth. The phage suspension was put into a sterile centrifuge tube, capped, and centrifuged under refrigeration at 17,300 x g for 30 minutes, so as to remove cell debris and unlysed cells. The phage supernatant was then removed with a sterile

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*The R.T.D. is the dilution that just fails to give confluent lysis.

Pasteur pipette, care being taken not to disturb the bottom portion. Propagation of phage from a lyophilized vial was carried out first by the plate method, and was followed by the broth method. The latter method yielded high titers (10^{-8}) for some phages, but was still laborious. Fig. 1 is an example of a high titer phage. Finally a slight modification of the method of Blair and Williams (4) was used.

Most of the 34 phages used in this study were propagated by the modification of Blair and Williams' method except phages 29, 42C, 44A, 52, 52A which were propagated by the plate method. By using this method a phage was propagated and prepared for titration within one day. Only one passage was necessary after preliminary treatment by the plate method before a high titer was obtained. In the soft-agar layer method the titer was reasonably good (10^{-6}) but was obtained after several passages when a lyophilized phage preparation was used. The method was considered too laborious for this study. All phages could be propagated by the plate method, but titers never exceeded 10^{-5}. Plates showing semi-confluent

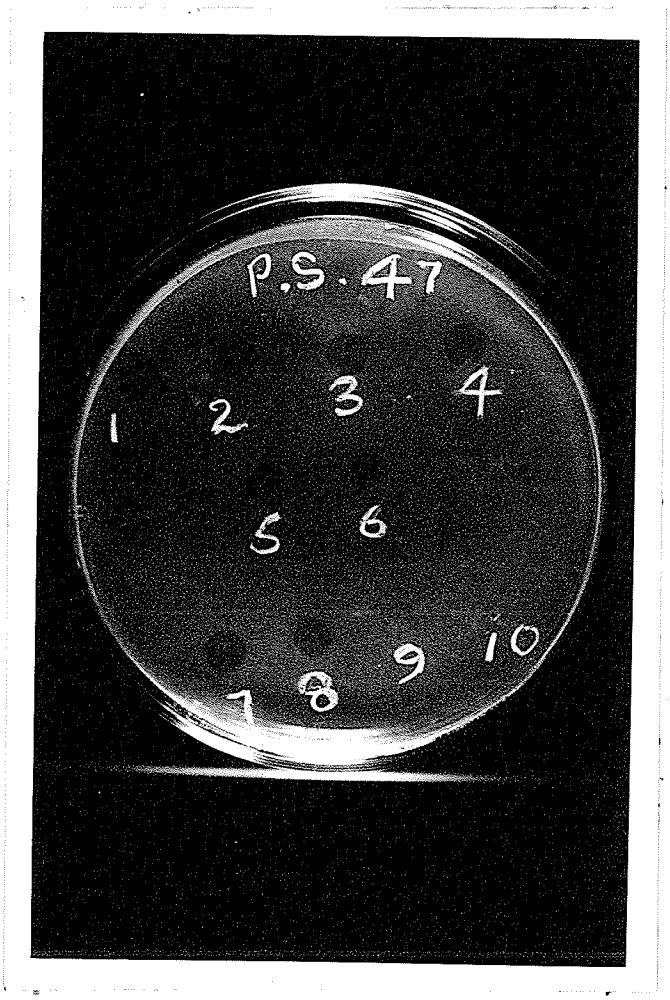

Fig. 1

A high-titer broth-propagated phage

A previously inoculated and dried agar plate of propagating strain 47 was spotted with serial 10-fold dilutions of homologous phage. The titer represented by the positive exponent is 10^{-8}.

lysis almost invariably gave higher titers than did those with confluent lysis.

PHAGE TITRATION

Serial 10-fold dilutions, 10^{-1} to 10^{-10}, of the phage preparation, either supernatant or filtrate, were made by diluting 0.5 ml. phage in 4.5 ml. Trypticase Soy Broth blanks. A single 1 ml. pipette was used for the series, each dilution of which was thoroughly mixed by trituration. Trypticase Soy Agar in 10 mm. Petri plates was dried at 37 degrees C. overnight, flooded with an 18-hour staphylococcal broth culture, and the excess broth removed with a sterile Pasteur pipette. The agar surface was allowed to dry for a few minutes and, using a 0.005 ml. loop, the appropriate phage dilution placed in the spot designated by the positive exponent of the dilution concerned. After drying for a few minutes, the plates were incubated in the inverted position either at 37 degrees C. for 4 hours, followed by overnight incubation at 22 degrees C., or at 30 degrees C. overnight. The plates were then read, and the R.T.D., which is defined as that dilution

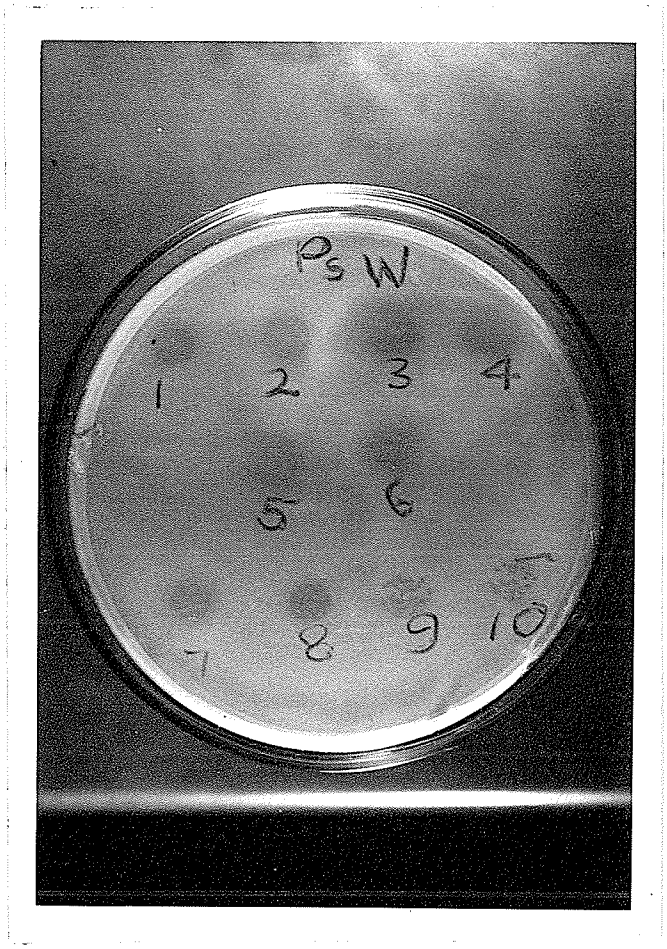

Fig. 2

Illustration of a phage titer

The Routine Test Dilution as determined by the dilution that just fails to give confluent lysis and represented by its positive exponent is 6.

that just fails to give confluent lysis (4) was determined. This is illustrated in Fig. 2. It was the dilution used in the identification of an unknown strain. The entire series of dilutions used for titration were stored at 4 degrees C., and the tubes representing the respective Routine Test Dilutions transferred to a rack set aside for this purpose. These test dilutions were found to retain their potency for as long as 12 weeks in some cases.

THE LYTIC SPECTRUM OF A PHAGE

This was determined by testing the phage against its propagating strain and a set of standard strains (4). The 16 test strains used differentiated phages with similar lytic spectra. The procedure involved the use of each phage to be used in typing at a dilution dependent upon the R.T.D. The phage was spotted on each of the test strains forming a lawn on Trypticase Soy Agar in 9 cm. plates, and was incubated at the same time and temperature as was used for routine typing. Subsequently it was titrated on those strains showing lysis or inhibition. A dilution giving a minimal +2 reaction on the various test

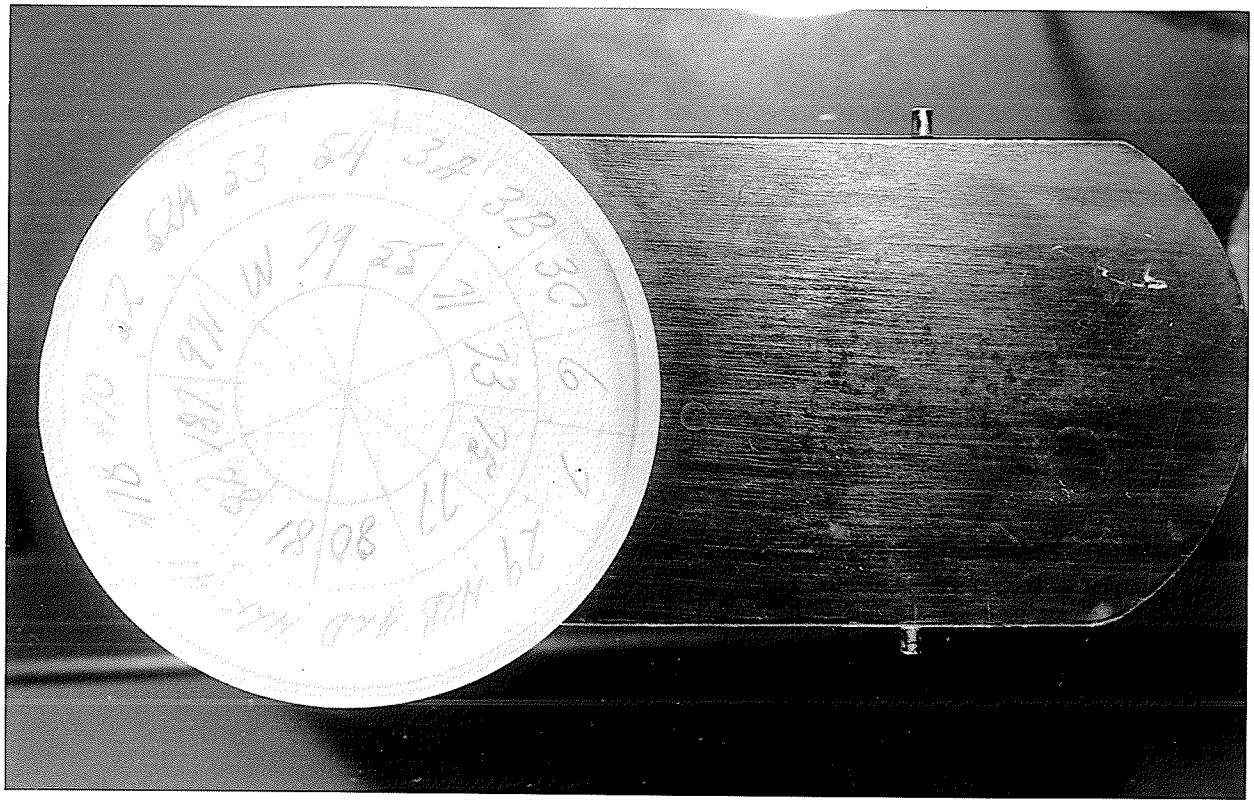

Fig. 3

Spotting of isolates with phages

A previously inoculated and dried agar plate was placed in a fixed position on a circular piece of cardboard sectioned as shown; and the different phages spotted on the sections designated on the cardboard.

strains was compared with the dilution giving approximately the same reaction on the homologous propagating strain and recorded according to the method of Blair and Williams (4). A tolerance of ±1 from the norm was permitted. A phage differing from this norm was rejected. Before doing so, however, the staphylococcal indicator strains were tested for variations.

PHAGE TYPING

The method of typing was that of Blair and Williams (4). A freshly made 9 cm. Trypticase Soy Agar plate, previously dried at 37 degrees C. overnight was flooded with a broth culture of the unknown previously incubated at 37 degrees C. for 4 hours. Excess broth was removed with a sterile Pasteur pipette, and the agar surface allowed to dry by raising the lid slightly. The dried plate was placed on a circular strip of cardboard containing the respective phage numbers arranged in such a position that two guiding marks on the plate corresponded to two points on the cardboard. The whole was then placed on a freely rotating circular stand as shown in Fig. 3 and a 0.005 ml.

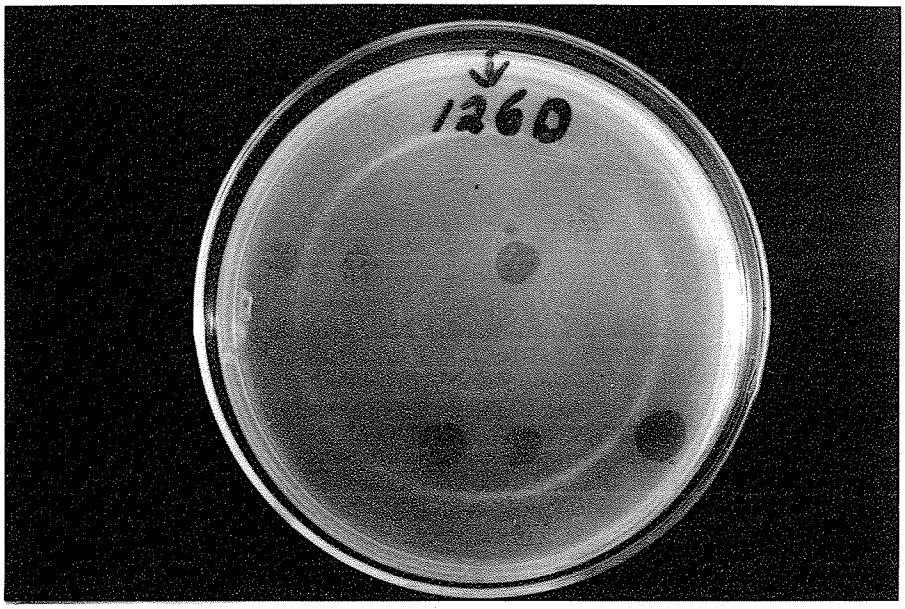

Fig. 4

Phage pattern of an isolate

A previously inoculated and dried agar plate of isolate 126D was spotted with the test phages. Test zones showing lysis were recorded as follows:

confluent lysis	+3
confluent lysis - 50 plaques	+2
50-20 plaques	+1
less than 20 plaques	±

loopful of phage suspension at the R.T.D. was spotted on its corresponding site. After all the phages were spotted, the plates were allowed to dry for a few minutes then incubated in the inverted position at 37 degrees C., for 4 hours, followed by 22 degrees C. overnight. Examination was made on a darkfield Quebec Colony counter, and the lysed areas matched with the card, lysis being recorded as follows:

+3: confluent lysis

+2: 50 or more plaques

+ : 20-50 plaques

± : less than 20 plaques or occasional weak reaction.

Only lysis of +2 or more was considered significant in determining the lytic pattern of the isolate, however, all reactions were recorded. Phage reactions of an isolate are found in Fig. 4. If significant reactions occurred with phages 6, 7, 29, the result was recorded as 6/7/29. If in addition, weak reactions occurred with other phages, the result was recorded as 6/7/29+ where + indicates weak reactions with other phages. On the other hand if no conclusive result was obtained with phages at the R.T.D.,

the isolate was tested with phages 1000 x the R.T.D.; in no case however, with undiluted phages.

The terminology used in reporting results was as follows: a culture sensitive only to one phage was designated as a phage type; a culture sensitive to more than one phage was designated as belonging to a phage pattern. As an aid in determining the phage pattern of an isolate, consideration was given not only to the lytic pattern, but also to the animal from which it was obtained. This was done because it was observed that at one time an isolate gave significant reactions with certain phages, and at a later date, another isolate from the same quarter of the same animal either did not react with one or other phage to which the first isolate was susceptible, or was significantly lysed by a phage strain to which it was formerly insensitive or had shown only a weak lysis. If in considering the phage pattern of the first isolate it was found to be 6/7/47/53+, where + denoted weak reactions with phages 3A/29/42D, and at a subsequent date an isolate from the same source reacted as 3A/6/7/29+, weak reactions being with phages 47/53/42D, the two isolates belong to

TABLE 1

Grouping of Isolates According to Phage Pattern

Isolate number	Phage Reactions S3	S2	81	80	47C	42E	42C	6	Phage Pattern	Group*
151D	+3		+2			±	+2	+2	S3/81/80/42C/6+	A
151A	+3		+2			±	+2		S3/81/80/42C+	
128C'	+3		±			±	+2	±	S3/80/42C+	
126D	+3		+2	+2		+2	+3	+2	S3/81/80/42E/42C/6+	
86D''	±	+3	+2	+1	±		+2	+2	S2/81/42C/6+	B
86D'		+3	±	±		±		±	S2+	
126B'	+3	+2	+1	+1		±	+2	+1	S3/S2/42C+	C

*The term "Group" as used here is arbitrary.

the same phage pattern. If, however, the reaction was 6/52/77+, weak reactions in this case being with phages 3A/7/47, then the second isolate undoubtedly represents a different strain. Examples of phage-pattern groupings of isolates are shown in Table 1.

RESULTS

RESULTS

Phage Titers Obtained by Broth Propagation

The phages studied by the broth method were 3A, 3B, 6, 7, 42B, 42D, 42E, 47, 53, 54, 55, 71, 73, 75, 77, 79, 80, 81, 82, 83, 187, W, S2, S3, S4, S5, S6. The titer of a phage after propagation was not predictable even with visual observation of the extent of lysis in the propagation flask. Some strains however, after titering yielded an R.T.D. of 10^{-6} to 10^{-8}. Table 2 shows the concentration and stability of phages propagated by this method. It may be noted that these phages usually gave high titers. Even though the titres in most cases when subsequently propagated were not exactly the same, they were comparable. Stability of these phages was determined by titering at weekly intervals for nine weeks. Some phages, e.g. 42D, 42E, 73 remained constant for as long as 7 weeks at 4 degrees C. On the other hand, others, e.g. 54, 187 dropped

TABLE 2

Concentration and Stability of Phages Propagated by the Broth Method

Phage Number	Initial R.T.D.	Stored at 4 degrees C.	Weeks Stored
3A	10^{-5}	10^{-4}	7
3B	10^{-6}	10^{-2}	7
3C	10^{-5}	10^{-3}	7
6	10^{-4}	10^{-2}	5
7	10^{-4}	10^{-3}	5
42B	10^{-4}	10^{-4}	7
42D	10^{-4}	10^{-4}	7
42E	10^{-3}	10^{-3}	7
47	10^{-8}	10^{-4}	12
47C	10^{-6}	10^{-4}	12
53	10^{-3}	10^{-3}	7
54	10^{-3}	10^{-2}	1
55	10^{-5}	10^{-3}	7
71	10^{-4}	10^{-3}	12
73	10^{-3}	10^{-3}	12
75	10^{-3}	10^{-3}	7
77	10^{-3}	-	-
79	10^{-3}	10^{-3}	7
80	10^{-3}	10^{-3}	7
81	10^{-3}	10^{-1}	1
82	10^{-3}	10^{-2}	6
83	10^{-5}	10^{-4}	3
187	10^{-4}	10^{-2}	1
W	10^{-6}	10^{-4}	12
S2	10^{-8}	10^{-3}	12
S3	10^{-4}	10^{-3}	12
S4	10^{-5}	10^{-4}	12
S5	10^{-8}	10^{-3}	15
S6	10^{-5}	10^{-4}	9

significantly at one week.

Phage Types of Staphylococci Isolated from Milk

During this study 883 milk samples were used from which 72 coagulase-positive and 71 coagulase-negative isolates were obtained. Of the coagulase-positive isolates 68 or 95.8% were identified using phages capable of typing staphylcocci of human origin, and as well phages adapted on bovine staphylococci. Each phage was at its Routine Test Dilution. Three isolates or 4.2% were unidentifiable by either the R.T.D. or 1000 x R.T.D. of the phages used in this study. One isolate became lysogenic after coagulase testing was done and was discarded. With the phages from human staphylococci, it was possible to type 31 of the 68 identified isolates (45.6%). Most of the isolates reacted with phages of different groups, and as a result, could not be placed in one of the broad groups recommended by the International Committee on Phage typing of Staphylococci (4). Each isolate that could be considered a phage type belonged to the phage group termed in this study "additional phages". Phage 42C,

a member of this group, typed 13 or 31 cultures (42%), and formed patterns with another 15 of the 31 (48.4%). Also phage 47C typed one of 31 cultures (3.2%) and formed additional patterns with 2 of 31 (6.4%). Phage 80, a Group I phage, formed patterns with 12 of 31 cultures (39%), likewise did phage 81; and vice versa.

With the phages adapted to bovine staphylococci all typed isolates (100%) were identifiable. The predominant reaction was phage type/pattern S3. This accounted for 80.5% of the identified isolates, 48.2% of which were of phage type S3. One strain gave a pattern S3/S6. The next predominant reaction was phage type/pattern S2, which accounted for 13.9% of the identified isolates. Of these, 50% were of phage type S2.

In considering the results obtained with phages of bovine origin, it should be remembered that phage S1 was not used, consequently it is possible that the phage patterns could be different from those obtained. Detailed results are in Table 3.

Of the 71 coagulase-negative isolates, attempts were made to identify 16. None reacted with any of the

TABLE 3

Phage and Biochemical Reactions of Coagulase-positive Staphylococci of Bovine Origin

Isolate No.	Animal Phage Reaction	Human Phage Reaction	Hemolysis	Mannitol Fermentation
8A	S3/S6	-	α	+
8B	S3	-	α	+
14B	S2	-	β	+
14BB	S2	-	β	-
21B	S3	-	β	+
21D	S3	-	β	+
24A'	S3	-	α	+
24A''	S3	-	α	+
24B	S3	-	α	+
24C	S3	-	β	+
24DD'	S3	-	α	+
24DD''	S3	-	α	+
33A	S3	-	α	-
38B	S2	-	β	-
48C	S3	-	β	+
50B	S3	-	β	+
50C	S3	-	β	-
50D	S3	-	β	+
63B	-	-	β	-
70B	S3	-	β	+
70D	S3	-	β	+
74C	S3	-	β	+
74D	S3	-	α	+
83B	S3	47C/80+	α	+
83D	S3	6/42C/80/81+	α	+
86D'	S2	-	β	+
86D''	S2+	6/42C/81+	β	+
99A	S3	-	α	+
99A'	S3	47C+	α	+

Table 3 (cont'd)

Isolate No.	Animal Phage Reaction	Human Phage Reaction	Hemolysis	Mannitol Fermentation
99C	S3	6/42C/81+	β	+
99C'	S3	42C/80/81+	β	+
99D	S3	6/42C/81+	α	+
99D'	S3	6/42C/80/81+	α	+
110A	S3	6/42C/80/81+	α	+
110A'	S3	6/47C/80+	α	+
110C	S3	-	α	+
110C'	-	-	α	+
112B	S2	-	β	+
112B'	S2	-	β	+
122B	S3+	-	β	+
122B'	S2/S3	-	β	+
122D	S3	42C+	β	+
122D'	S3	42C+	β	+
124A	S3	-	α	+
124C	S3	42C+	β	+
124C'	S3	-	β	+
126B	S2/S3	42C+	β	+
126B'	S2/S3+	42C+	β	+
126D	S3	6/42C/42E/80/81+	β	+
127C	S3	6/42C/80+	β	+
128A	S3	42C/80+	α	+
128A'	S3	42C+	α	+
128C	S3	42C+	α	+
128C'	S3	42C/80+	α	+
151A	S3	42C/80/81+	α	+
151C	S3	42C+	α	+
151C'	-	-	α	+
151D	S3	6/42C/80/81+	α	+
164B	S2	42C/81+	α	+
164B'	S2+	42C/81+	α	+
172B	S3	-	β	+
172D	S3	-	β	+
186A	S3	-	α	+

Table 3 (cont'd)

Isolate No.	Animal Phage Reaction	Human Phage Reaction	Hemolysis	Mannitol Fermentation
186C	S3	42C	α	+
186D	S3	42C	α	+
188B	S2+	42C+	β	+
198B	S2/S3+	42C+	β	+
198D	S3	42C+	β	+
201C	S3	-	β	+
211A	S3	-	α	+
211D	S3	-	α	+

phages at either of the two concentrations used.

Mannitol Reactions

Sixty-nine of seventy-one coagulase-positive isolates were mannitol-positive; two were mannitol-negative, one of these not reacting with any of the phages. Sixteen coagulase-negative isolates were also mannitol-negative.

Hemolysis Reactions

Of seventy-one coagulase-positive isolates streaked on sheep blood agar, thirty-six (50.7%) were β-hemolytic and thirty-five (49.3%) α-hemolytic. Of the three unidentified isolates one was β-hemolytic and two α-hemolytic. No attempt was made to determine other possible types of lysins.

Immunization Treatments

As regards immunization, each of four animals producing milk known to contain coagulase-positive staphylococci when treated with two doses of antigen produced milk free from these bacteria on the third, fourth and fifth weeks, following the last injection. The details are

TABLE 4

Effect of Antigen Treatment on Animals Known to Carry Coagulase-positive Staphylococci - Two Injections at Weekly Intervals

Animals Tested	Presence of Staphylococci at				
	1 wk	2 wk	3 wk	4 wk	5 wk
Glen B. Kay	+	+	-	-	-
B.S. 49-58	+	+	-	-	-
B.S. 1-60	+	+	-	-	-
Jewel R. Jemima	+	+	-	-	-

presented in Table 4.

Another animal known to harbour these organisms when treated with a mixture of equal parts of antigen and toxoid, likewise, produced milk free from these bacteria; but the effect was not evident until the fourth week.

Each of four animals producing milk known not to contain coagulase-positive staphylococci when treated with antigen as above produced milk free from these bacteria throughout. This group served as one control. Two other controls were used. Two untreated animals, positive carriers of staphylococci, produced these organisms at each sampling; while four untreated animals, which did not carry these organisms produced milk free from them during the entire testing period.

DISCUSSION

DISCUSSION

The results obtained from mannitol and hemolysis tests have shown only what is an accepted fact viz. that biochemical reactions, the basis for which depends upon the metabolic qualities of the organism, only serve to segregate broad groups: so broad in fact that the only information to be derived from them is that a certain group of isolates probably contains pathogens depending upon the source of the isolates and a knowledge of the epidemic. Most of the isolates tested for hemolysis produced β-lysin, showing that they were of animal origin; those isolates producing α-lysin were of human origin and could have been transferred to the animals by human carriers, or dust.

Because of limitations in current propagation techniques, a preliminary study was made by the broth method. Yields were better than those obtained either by the laborious semi-solid agar technique or by the plate method.

Calcium has been reported by some workers, including

Ghitter and Wolfson (10), as being necessary for phage lysis. Using freshly made Trypticase soy broth and agar (B.B.L.) no special requirement for calcium was necessary.

It is unfortunate that three members of the Group I phages viz. 29, 52, 52A could not be propagated by this method. A possible solution to this problem might be a knowledge of the genetic constitution of these strains. This might enable modifications to be made to current broth propagation methods.

Using phages capable of typing Staphylococci of human origin, it was difficult to decide whether a phage group was predominant. The isolates reacted with phages from the various groups. However, the most common phages were 80 and 8I. Phage 8I was found by Barnum (1) to be quite common among staphylococcal strains isolated from cases of bovine mastitis in Canada. Staphylococci reacting with this phage were lysed by other phages also, especially 42C, 6, and 47C. Of interest is the fact that phage 42D did not lyse any of the isolates in the present study. This phage was reported by Thatcher and Simon (19) to be the predominant type among cultures isolated from butter

and cheese. This probably can be explained to be due to a change in phage types resulting from immune reactions.

At first glance, the fact that phages 80 and 81 lysed the same number of isolates may be considered coincidental; however, further consideration should dispel this idea. Firstly, these two are considered by some workers as a complex "type 80/81", even though they are classified separately. However, because of the lytic patterns obtained by Wallmark and Finland (20) and because of the frequency with which they occurred in this study, a reconsideration of their present taxonomic status should prove important.

The finding that the four phage groups of the basic set of phages was of little value in the differentiation of the isolates concurs with that of other workers (5, 8, 21). Also in agreement is the fact that phages not listed in the basic set were more valuable in the identification of these isolates than those in it.

Using phages of animal origin, it was shown that these exhibited considerable specificity and were necessary for identifying a larger number of isolates than were the

human phages. (In considering the results obtained, it should be recalled that animal phage S1 was not used). It may be hoped that with continued investigations, phages will be found which exhibit absolute specificity, i.e. they will react only with a particular strain.

Table 3 reveals also that in one case, two different strains were isolated from the same quarter; for example isolates 86D' and 86D''. The former reacted with phage S2 alone; on the other hand, isolate 86D'' was lysed by S2, and gave weak reactions with S3 and S5. It could be identified also by phages of human origin, giving the reaction 6/42C/8I +. These two strains must be considered different. This occurrence is not unique, for Coles (6) also had a similar experience. This change in phage characteristics may be explained by two different strains. On reviewing the isolates obtained from this animal, it was found that no staphylococci were obtained from that quarter previously. Therefore the infection was either recent, or the numbers present were so few that they were undetected. Five weeks further testing did not yield further isolates

from this quarter. Even though it is known that Tellurite-Glycine Agar is inhibitory to some strains of coagulase-positive staphylococci, control plates of mammalian-blood-agar did not yield isolates either; added support for the reasons given earlier for the presence of these two strains.

Investigations by Seto and Wilson (18) showed that a common phage pattern existed when only a few isolates were examined from a herd. From the number of isolates examined in this study, the results clearly indicate the presence of a phage pattern. This is supported by the results of other workers (6, 8), thus dispelling the idea that the number of animals in the herd is significant in determining phage pattern. Seto and Wilson (18) also found that cultures lysed by phage S2, were lysed by phage 44A. In this study however, S2-sensitive cultures were commonly lysed by phages 42C, 80 and 81.

Finally, the phages used in this investigation lysed 95.8% of all cultures tested. This is higher than the percentage identified by Seto and Wilson (18), using the same set of phages. With the bovine adapted phages they

identified 94.7% of the identifiable isolates; whereas in this work 100% were identified.

Results obtained from immunization treatments using antigen were encouraging, thus indicating that this antigen may be used to remove coagulase-positive staphylococci from a cow's udder. Because none of the animals tested showed symptoms of mastitis, and considering that it is possible to have carriers of certain pathogens, it is difficult to anticipate whether this antigen would be equally effective in the control of bovine mastitis caused by staphylococci. Again, it should be pointed out that no attempt was made to determine relative antibody titers during this study. Because of this, one may suspect that removal of the bacteria could have been caused by other factors; however, because the controls were unchanged, and because the four treated animals responded in the same way, it may be concluded that the effect was due to the antigen.

The animal treated with antigen-toxoid mixture also showed removal of coagulase-positive staphylococci. This may have been due to the effectiveness of the antigen alone. It took one week longer for the effect to occur; but in

this animal, antigen represented only one half that given to the other animals.

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