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STUDIES ON BACTERIOPHAGE TYPING OF STAPHYLOCOCCI ISOLATED FROM BOVINE MILK

I. TYPING BY MEANS OF 20 PHAGES OF THE INTERNATIONAL SERIES

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INTRODUCTION

Bacteriophage typing of staphylococci has been studied by many workers for the last 20 years or more, and has been widely used in recent years as an aid in the identification of strains of this organism. In 1935 BURNET and LUSH presented evidence to show that certain strains of *Staphylococcus aureus* could be grouped according to their susceptibilities to a series of phages. Subsequently, WILLIAMS and TIMMINS used 4 phages isolated by BURNET and LUSH and distinguished 6 types among strains of staphylococci which had been obtained in cultures from patients with osteomyelitis. When it had been confirmed that the phage typing was a valuable tool for the identification of the typhoid bacilli, FISK[12,13,14] made the first systematic investigation on the applicability of phage typing for epidemiological studies of staphylococci. He investigated forty-three strains for lysogenicity by cross-culture method and revealed nineteen strains to be lysogenic. Moreover, he showed that the phages obtained from these lysogenic strains exhibited selective activities on coagulase-positive strains of staphylococci. WILSON and ATKINSON extended the work of FISK to permit the differentiation of staphylococci in a way similar to that devised by CRAIGIE and his co-workers[8,9] for the bacteriophage typing of typhoid bacilli, using a "test dilution" of each staphylococcal phage. Subsequent to this, a number of studies[2,6,19,20,24,29,41,42] were carried out on the phage typing of staphylococci for practical use in various parts of the world. Above all, in the Public Health Laboratory Service at Colindale in London, the typing method of WILSON and ATKINSON was modified in various ways, under the direction of Dr. V. D. ALLISON, from 1946 till 1948. Then, WILLIAMS and RIPPON took over this work and established the method which is generally adopted by those at present engaged in phage typing staphylococci.

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However, most papers published hitherto deal with strains of human origin and not those of animal origin.

In Japan, the phage typing of staphylococci has been started by a number of laboratories in this country since 1955, employing the 20 phages of the International Series which were supplied from the Staphylococcus Reference Laboratory, Colindale, London. Up to the present time, several reports[16,17,37,38,39,47] concerning cultures of human origin have been published.

Therefore, the present study was initiated in an attempt to determine whether the phages of the International Series could be utilized in typing staphylococci isolated from milk samples in this country, comparing with the series of reports by many workers on those which originated from humans.

MATERIALS AND METHODS

Strains examined A total number of 375 strains of coagulase-positive staphylococci which were isolated from bovine milk were employed in the present study. Their histories are as follows.

(a) Two hundred and two strains were isolated by HIRATO et al. from milk samples of cows which were examined for mastitis in four different areas of Hokkaido, in 1955.

(b) Seventy strains were collected by the present author from milk samples of cows, a part of which were obtained in dairy farms in the vicinity of Sapporo, Hokkaido and the remainder were from animals slaughtered at the Sapporo slaughterhouse, in 1958.

(c) Sixty-seven strains were kindly supplied by Dr. Y. OCHI of the University of Tokyo. Twenty-three of them were obtained from dairy farms around the metropolis of Tokyo while the remaining 44 were isolated from the milk of cows sent to the Shibaura slaughterhouse, Tokyo.

(d) Thirty-six strains were kindly supplied by Dr. Y. TAJIMA of the Institute for Infectious Disease, Tokyo University. These were obtained mainly from the milk of cows slaughtered at the Shibaura slaughterhouse, Tokyo.

Following isolation from blood agar plates, these strains were maintained in stock at room temperature on ordinary agar slants, transfers being made at 2 or 3 months intervals. Before use, transfers were made to fresh agar plates and then to broth.

Bacteriophages employed The following 20 phages of the International Series which were supplied by the National Institute of Animal Health in Tokyo were used for phage typing. Their details are shown in table 1; their grouping is as follows:

Group I : 29, 52, 52A, 79
Group II : 3A, 3B, 3C, 55, 71
Group III: 6, 7, 42E, 47, 53, 54, 70, 73, 75, 77
Group IV: 42D

Methods of typing The technique followed was that of WILLIAMS and RIPPON and that described in the pamphlet published in the Staphylococcus Reference Laboratory, Colindale, London, but for details, reports [6,15,20,35,37,41,42] of some other workers were consulted.

TABLE 1. *Phages of the International Series*

PHAGE NO.	PROPAGATING STRAINS	SEROLOGICAL GROUP
29	29	B
52A	52A	B
52	52	B
79	925	B
3C	3C	A
3B	3B	A
3A	3A	A
55	55	B
71	71	B
70	70	A
42E	42E	A
6	6	A
7	7	A
73	73	A
47	47	A
54	54	A
75	75/76	A
53	53	B
77	77	F
42D	42D	F

a) Culture medium Nutrient agar was used. Its composition was as follows:

Beef extract (manufactured by Mikuni Chem. Co. in Japan)	10 g
Polypeptone	10
Sodium chloride	2
Powdered agar (manufactured by Wakō Chem. Co. in Japan)	12
Distilled water	1000 ml

The beef extract, pepton, agar and salt were dissolved in the distilled water, the pH was brought to 7.4 with 10% sodium carbonate, after which the medium was bottled and autoclaved at 15 lbs for 20 minutes.

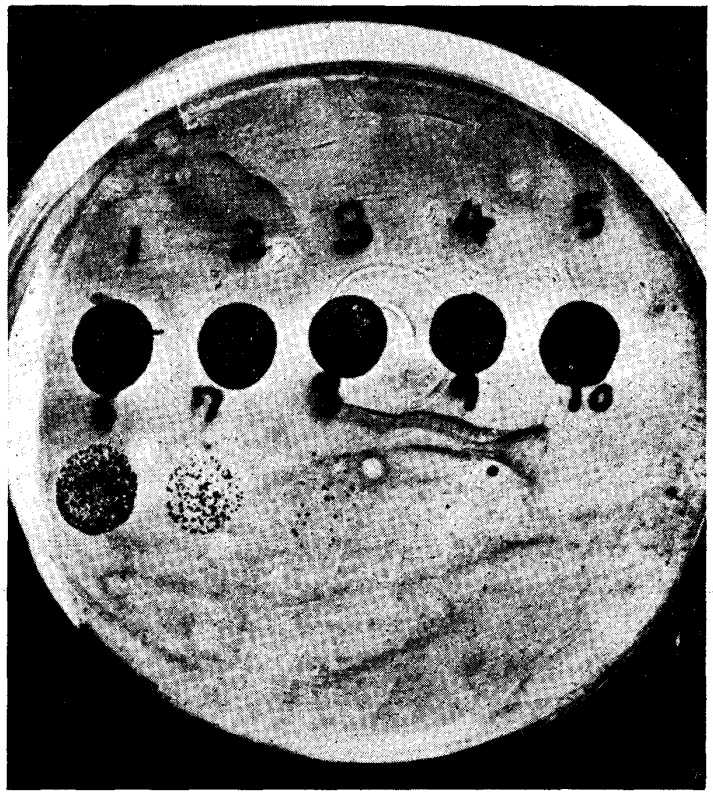
The medium was transparent and the composition was comparatively constant. The overgrowth of staphylococci seeded, which occasionally caused difficulty in discriminating phage plaques, was reduced. From the above stated points, this medium seemed more satisfactory for present purposes than the nutrient agar made from horse meat infusion.

b) Propagation of phage The phage was propagated on the surface of agar plates. The above medium was poured into a petri dish to a depth of about 5 mm. The plate was dried for 90~120 minutes at 37°C and then 0.2 ml of a 6- to 18-hour broth culture of the propagating strain was evenly seeded on its surface using a glass spreader. When the culture had been absorbed into the agar, the diluted phage (a phage concentration of

10～100×R.T.D. was usually employed) was spread over the plate, on which a small segment was left without being over spread. This segment served as a control area to examine whether the propagating strain showed good growth and presented spontaneous lysis. After incubation, when the unlysed control area showed good growth with no evidence of spontaneous lysis (any plates showing spontaneous lysis should be discarded), each plate was washed off with about 5 ml of nutrient broth ; the washings were pooled and centrifuged for 40～60 minutes at 3,000 r. p. m. After removal of the precipitate, the clear supernatant was sterilized with a piece of crystal thymol in place of Seitz filtration, because a considerable amount of phage particles was sometimes lost by adsorption on Seitz filter.

c) Titration of phage The culture medium was poured into a petri dish and dried at 37°C for about 5～6 hours. On the surface of the dried plate, 0.2 ml of a 6～18-hour broth culture of the propagating strain was seeded uniformly and the inoculum was allowed to dry for 5～10 minutes at room temperature. A series of tenfold dilutions of the phage in nutrient broth was prepared (ranging from 1 : 10 to 1 : 1,000,000), and one drop of each phage dilution was spotted on the propagating strain by means of a 1/4 needle attached to a tuberculin syringe at spaced intervals. The plate was inverted and incubated at 37°C overnight. An example of titration is shown in fig. 1. The highest dilution, which was

FIG. 1. *Titration of Phage by Means of Serial Tenfold Dilutions*



Notes: Numbers indicate the reciprocals of the log-dilutions.
The routine test dilution (R.T.D.) is 10^{-5}.

shown as 10^{-5} in the figure, just showing confluent lysis against the propagating strain was determined the routine test dilution (R.T.D.) or the critical test dilution (C.T.D.). Phage for routine use should have a minimum R.T.D. of 1/1,000.

When a satisfactory titre was obtained, the stock phage was stored at 2~5°C and retitred every week to make certain that the preparation was of suitable potency. Under these conditions, the phages showed no appreciable decrease in titre after they had been stored for a week. When the titre of the stock phage decreased more than one tenfold dilution, the phage was repropagated.

It was always ascertained that the phages showed no variation in their host ranges by determining the lytic spectra on the set of 20 propagating strains before the use of phages for typing of unknown strains, employing the phages at their 1×R.T.D., 10×R.T.D. and 100×R.T.D. (Fig. 2).

d) Phage typing of unknown strains In the phage typing of unknown strains of staphylococci a phage concentration of ten times greater strength than the routine test dilution (i.e. 10×R.T.D.) was employed.

The technique used was as follows. Into a petri dish the culture medium was poured and etched on the back with a grid of 20 squares, after drying at 37°C for about 6 hours. Each strain of staphylococci to be typed was incubated in the broth for 6~18 hours and 0.2 ml of this culture was used to seed on the surface of the plate. The plate was left at room temperature for about 10 minutes, to permit drying of the inoculated surface. Then a drop of each of the 20 phages at their 10×R.T.D. was deposited in the center of a square with a 1/4 needle attached to a tuberculin syringe and dried at room temperature. Readings were made following overnight incubation at 37°C.

When the strains to be tested begin to grow, their sensitivities to phages turn to poor. Therefore, care must be taken to finish the whole process from the seeding of unknown strains on the agar plate to the dropping of phages within 90 minutes.

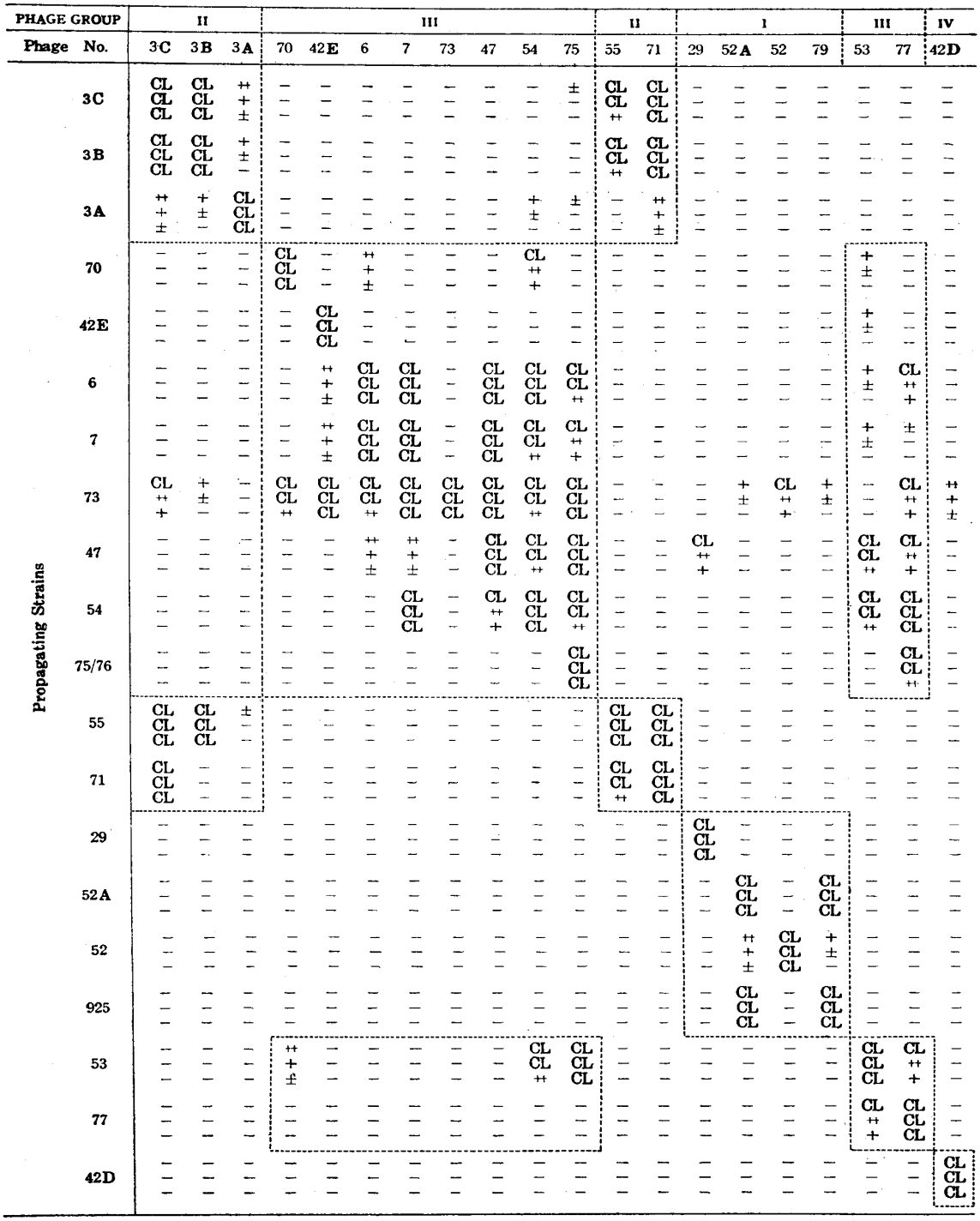
e) Recording of results The degrees of lysis were recorded according to the following signs which have been adopted in the Staphylococcus Reference Laboratory, Colindale, London, except that both ⧺ and ++ were included in the symbol of CL (confluent lysis).

⧺ Confluent lysis with no secondary growth
++ Confluent lysis with secondary growth
++ More than 50 plaques; these plaques were fused with each other and formed the so-called semi-confluent lysis
\+ 20~50 plaques
± Less than 20 plaques

The strains which were lysed strongly (i.e. ++ lysis or more) were recognized to be typable.

The strains which were strongly lysed by the phages of group I were represented as strains of type I, similarly those lysed by phages of groups II, III or IV were called the strains of types II, III or IV respectively. Furthermore, the strains typed by phages belonging to different groups were named as strains of mixed type.

FIG. 2. *Lytic Spectra of the Phages of International Series*



Notes: The upper, middle and lowest lines represent the spectra of the phages at their 100×R.T.D., 10×R.T.D. and 1×R.T.D., respectively.

RESULTS

1. Phage Typing of Strains Employed

Three hundred and seventy-five strains of coagulase-positive staphylococci isolated from milk samples were tested with the phages of the International Series. The results are listed in table 2. One hundred and seventy-one strains were typable with one or more of the phages. This represents 45.6 per cent of those tested which percentage is lower than that previously reported[6,16,21,28,37,38,44] (50~80 per cent) on the staphylococci of human origin. Staphylococci of type III was the predominant one; it supplied about 36 per cent of the 171 typable strains. This may be explained in part by the use of more phages than other groups. Strains belonging to type IV were 33.3 per cent of the typable ones, while type I and type II were less than 10 per cent. In addition, six mixed phage types were recognized. These were I+II, I+III, I+IV, II+III, III+IV and I+II+III+IV. Among these mixed types, the strains which were combined with type II were comparatively rare. The distribution of typable strains was considerably different from that of typable strains of staphylococci from humans[16,17,20,28,37,38,40,44,47]. It is noteworthy that a considerable number of strains from bovine milk were type IV which was rarely encountered in the strains of human origin and that the strains of types I and II from bovine milk were less than those of human origin.

TABLE 2. *Results of Typing by the Phages of the International Series*

PHAGE TYPE	NUMBER OF STRAINS	
I	16	
II	14	
III	62 (36.3)	
IV	57 (33.3)	
I + II	1	171 (45.6)
I + III	6	
I + IV	5	
II + III	1	
III + IV	8	
I + II + III + IV	1	
Untypable		204 (54.4)
Total		375

( ): per cent

To study the relative frequency with which staphylococci were lysed by the different phages, 258 strains which were sensitive to anyone of the twenty typing phages were subjected. The number of strains giving the lytic reactions of CL, ++, + or ± with

each of the twenty typing phages was tabulated in table 3, regardless of other phages that attacked the strain. A great number of strains were sensitive to phages 42E, 6, 47, 75 or 42D, of which the former 4 belong to group III and the last one to group IV. The number of strains lysed by these phages was: 98 by 42E; 78 by 6; 119 by 47; 78 by 75 and 98 by 42D. The fact that the frequency of lysis by phage 42D was very high is particularly worthy of notice as compared with the strains of human origin[37,38,44].

TABLE 3. *Frequency of Lysis by the Twenty Typing Phages*

PHAGE		NO. OF STRAINS LYSED				TOTAL
Group	No.	CL	++	+	±	
I	29	11	10	20	15	56
	52A	7	10	8	6	31
	52	10	3	8	13	34
	79	4	9	4	11	28
II	3C	4	7	4	7	22
	3B	4	3	9	8	24
	3A	0	0	5	4	9
	55	3	0	2	2	7
	71	2	2	7	8	19
III	70	0	3	5	4	12
	42E	6	21	24	47	98
	6	7	17	21	33	78
	7	7	8	3	17	35
	73	3	4	11	30	48
	47	10	21	48	40	119
	54	4	3	20	33	60
	75	9	22	19	28	78
	53	4	6	11	7	28
	77	3	2	5	16	26
IV	42D	54	17	14	13	98

Viewed from the degree of lysis, it was found that phage 42D tended to produce strong lysis in most strains, whereas the phages of group III tended to produce generally weak reactions. The phages belonging to groups I and II showed strong and weak reactions in almost equal numbers. On the whole, the strains obtained from bovine milk showed weak reactions more often than those of human origin[37].

2. Combination of Phage Reactions

Certain phages tend to appear together in patterns and, on the basis of such associations, the phages of the International Series were distinguished into four phage groups; they are known as the I, II, III and IV groups respectively. It is necessary to determine

whether this grouping of the phages can be applied to the classification of staphylococci isolated from bovine milk. The author attempted to define the combinations of reactions observed in the set of 258 strains which were sensitive to any one of the 20 typing phages in use. In figs. 3~6 the results of this enumeration are set out in the form of a block diagram. Each block indicates the frequency with which two phages were observed together lysing one strain.

FIG. 3. *Frequency of Pairing of Reactions in Phage Patterns*

GROUP I

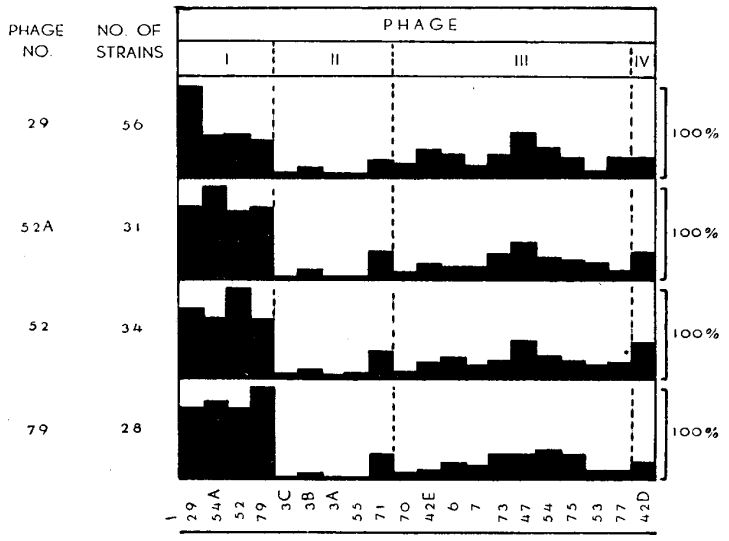
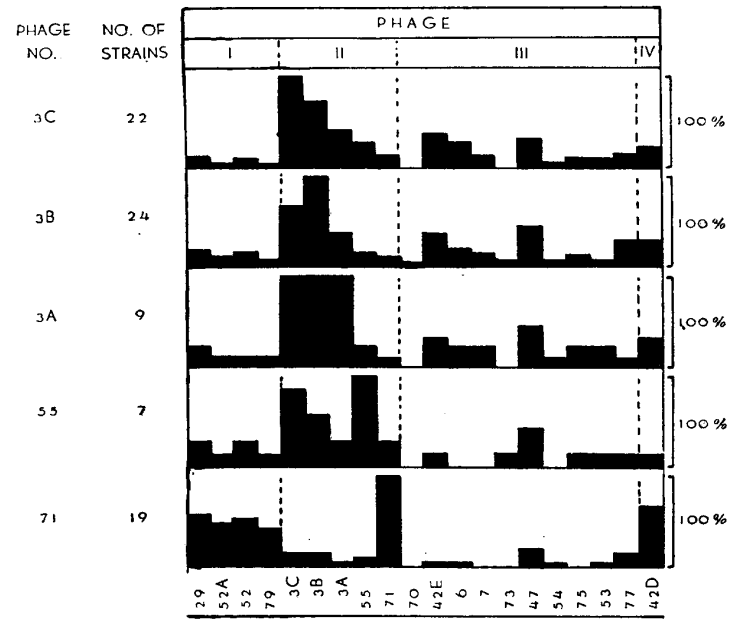


FIG. 4. *Frequency of Pairing of Reactions in Phage Patterns*

GROUP II

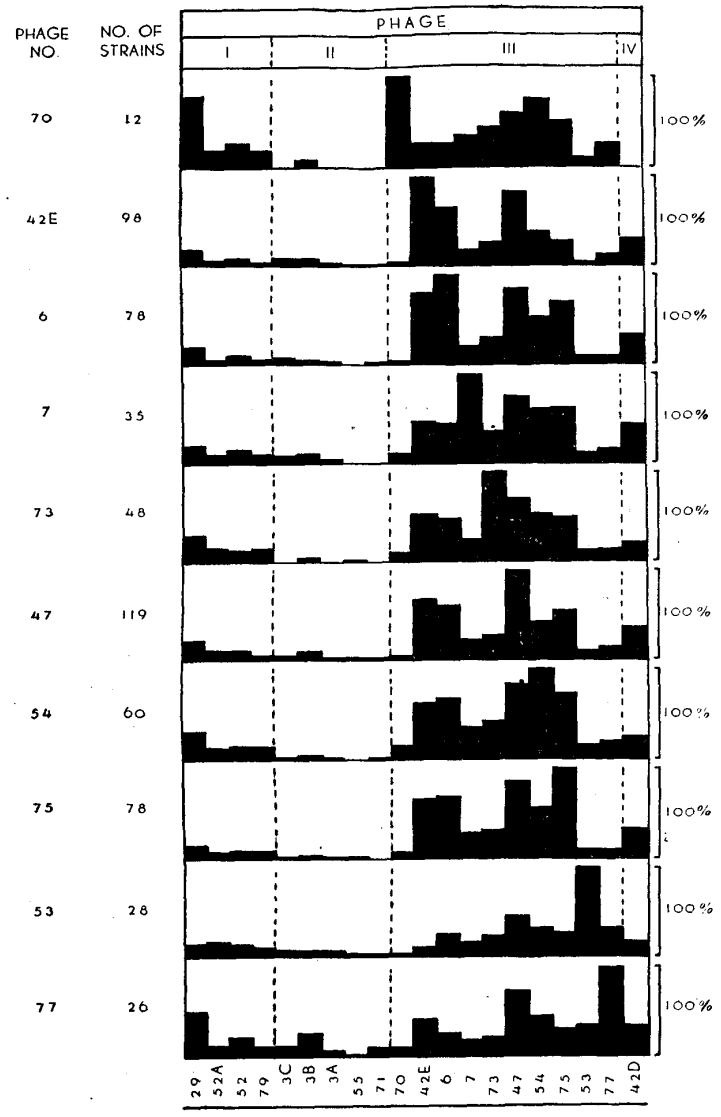


The frequency of association of reactions of group I phages with other phages is shown in fig. 3. These 4 phages reacted principally with each other, particularly, phages 52A and 79 were most closely associated; that is, of all the attacks by phage 52A, 77.4% were in combination with 79 and of the 79 attacks, 85.7% were in combination with 52A. However, these phages of group I did not stand out distinctly and were found occasionally in combination with phage of group IV and more frequently in combination with the some of the phages of group III.

Among group II of phages, fig. 4, there was a frequent association with one another except in the cases of phages 55 and 71. For example, all strains lysed by 3A were also lysed by 3B and 3C. In contrast, the associations of phages 55 and 71 of group II seemed

FIG. 5. *Frequency of Pairing of Reactions in Phage Patterns*

GROUP III

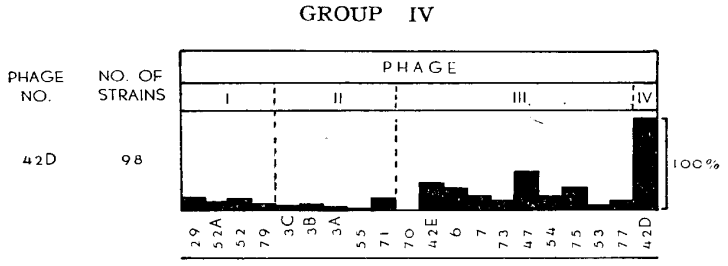


to differ from those of the above 3 phages. For instance, out of 19 strains lysed by phage 71, fourteen failed to react with other phages of this group. Both WILLIAMS and RIPPON and JACKSON et al.,[20] employing staphylococci of human origin, observed that the associations of group II phages were restricted to other members of the group. However, in the present study, the phages of group II as well as of group I were frequently found in combination with phages outside group II and many patterns were produced by association with the phages within groups III and IV.

It can be seen from the results presented in fig. 5 that there were two groups among the group III phages, the one comprising phages 42E, 6, 7, 73, 47, 54, and 75, and the other comprising phages 53 and 77. The association of phage 70 was different to some extent from that of the above two groups. Further, it is noteworthy that the group III phages, except 70, were associated occasionally with phage 42D (group IV). For example, 15 or 42.9 per cent of the 35 strains which were susceptible to phage 7 and 42 or 35.3 per cent of the 119 strains which were susceptible to phage 47 were also lysed by phage 42D.

The association of phage reaction of group IV phage is shown in fig. 6. It is apparent that there is a considerable overlapping between groups III and IV ; certain characteristic patterns are evident. These are 42E/6/47/75/42D and 42E/6/7/47/54/75/42D.

FIG. 6. *Frequency of Pairing of Reactions in Phage Patterns*

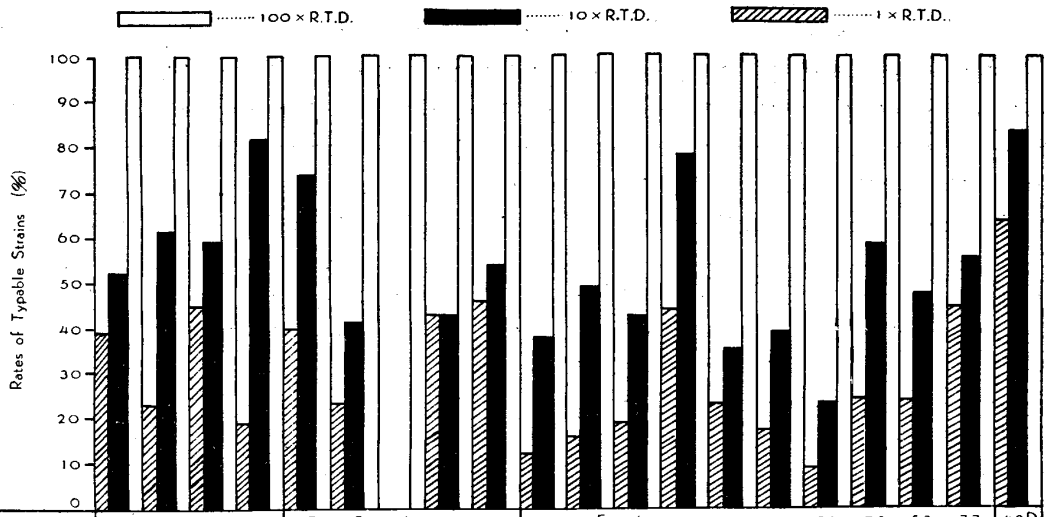


3. The Uniformity of Results

As the phage concentrations used in the typing of unknown strains were determined by tenfold dilutions of the undiluted phages, a given concentration of phages should lie between two of the tenfold levels; in the present study, as phages at 10×R.T.D. were employed in the typing, these two levels mean the 10×R.T.D. which was calculated logically and the nearest to 100×R.T.D. Therefore, it is needful to compare number of typable strains by various concentrations of phages to decide whether the same results are obtained every time from a set of unknown strains. In the present study, an investigation was carried out by the following method.

Three levels of phage concentrations, 1×R.T.D., 10×R.T.D. and 100×R.T.D., were employed, and the rates of typable strains (++ or more) at each concentration of 1×R.T.D. and 10×R.T.D. to at 100×R.T.D. were calculated separately as summarized in fig. 7. In phages 79, 3C, 7 and 42D, the differences in number of typable strains among the 3 phage concentrations were not so great. For example, in the case of phage 42D, out of 85 strains typed by 100×R.T.D., seventy-one (83.5%) were typed by means of 10×R.T.D. and fifty-four (63.5%) by 1×R.T.D., respectively. In many phages represented by 3B, 3A, 70 and 54, however, the numbers of typable strains varied considerably according

FIG. 7. *Rates of Typable Strains according to Concentrations of Phages*



Phage no.		29	52A	52	79	3C	3B	3A	55	71	70	42E	6	7	73	47	54	75	53	77	42D
Phage group		I				II					III										IV
No. of Strains	100 × R.T.D.	42	26	22	16	15	17	6	7	13	8	57	58	18	17	81	34	53	21	9	85
	10 × R.T.D	22	16	13	13	11	7	0	3	7	3	28	25	14	6	32	8	31	10	5	71
	1 × R.T.D.	10	6	10	3	6	4	0	3	6	1	9	11	8	4	14	3	13	5	4	54

to the phage concentrations. Particularly, in the case of phage 3A, none of 6 strains typed by 100 ×R.T.D. was typed by 10 ×R.T.D. Such a remarkable reduction in number of typable strains was more frequently observed in phages of group III than in those of other groups.

DISCUSSION

In recent years, bacteriophage typing of staphylococci of human origin has been studied and widely used in tracing the origin and spread of certain staphylococcal infections by many investigators. Attempts have been made to type strains of staphylococci from bovine milk by several workers[4,10,11,25,30,31,34] using the phages of the International Series or those isolated by these workers.

In England, SMITH[34] found that a total of 93 per cent of 1,016 strains of staphylococci isolated from milk samples were typable by one or more phages and that phage type 42D (type IV) was the most common among the cultures. In that country, similar result was reported by EDWARDS and RIPPON with cultures isolated from bovine milk. THATCHER and SIMMON in Canada found that the phage type 42D (type IV) was the predominant type among 100 cultures isolated from butter and cheese. However, it was stated by SETO and WILSON that 66.7 per cent of 102 strains which were incriminated in bovine mastitis in America were typed; most of these typable strains fell into the miscellaneous type and type IV, the remaining cultures fell into type III. Furthermore in Canada,

BARNUM observed that strains which were sensitive to phages of group III were common in staphylococci of bovine origin. The observations of these authors, though the distribution of staphylococci among the phage types varied considerably according to countries or regions, would indicate that, in general, the main phage types among strains of bovine origin were type III, IV or miscellaneous type whilst strains classified into type I or II were found infrequently. The data obtained in the present study (table 2) may be compared with the series of above mentioned reports. The number of strains typable by the phages in this study was less than that reported in the other countries. This may be explained in part by the fact that only the phages of the International Series were used in this experiment and in part by the difference of phage concentrations at which they were used for typing; however, the most part of 171 typable strains belonged to phage type III or IV, showing a similarity to the results published by other investigators. However, attention must be paid to the following: types III and IV were not so distinctly differentiated, but some strains which were regarded as a border-line type between types III and IV existed in considerable number (Figs. 5 and 6).

These facts will suggest that there exist some dissimilarities between the strains of human origin and those from bovine milk in their phage susceptibilities; namely, the cultures from bovine milk had large numbers of type IV strains which were scarcely encountered in the strains of human origin and had small numbers of types I and II strains which abounded comparatively in those from humans; in addition, the cultures from bovine milk had considerable number of cultures regarded as a borderline type between III and IV, unlike the isolates from human.

Accordingly, as shown in the present study, the utilization of the phages of the International Series, which was arranged originally for the typing of staphylococci of human origin, carry the following defects for the typing of strains isolated from bovine milk in this country.

1. More than half of the strains submitted for typing were untypable.
2. The results obtained were often inconstant. This was explicable mainly by the following reason. The phage concentration used in typing, calculating by tenfold dilutions of undiluted phages, fluctuates between two of the tenfold levels according to preparation. Therefore, the fact that the numbers of typable strains varied significantly according to the phage concentrations reveals that the results obtained from the same strain are sometimes irregular. It may be an important reason for these phenomena that the phages of the International Series released from staphylococci of human origin and propagated on staphylococci of the same origin were employed for the typing of the cultures from bovine milk.

3. Although a considerable number of strains from bovine milk were classified into type IV, no more than one phage (42D) belonged to group IV in the phages of this set. Therefore, at present, the cultures susceptible only to phage 42D cannot be refused classification as identical. However, it has been proved by SMITH[32] that strains regarded as 42D type were not always identical and that they classified into a number of types by use of additional phages. As shown in the present study, SMITH's statement is endorsed by the existence of many type IV strains which were weakly lysed by phages of group III.

4. It is apparent from the association of phage reactions shown in figs. 3 to 6 that the four main phage groups distinguished in the phages of the International Series are not appropriate for application to the classification of staphylococci originated from bovine milk. Cross reactions by phages belonging to different groups were frequently observed in the cultures originated from bovine milk, and these reactions must be a great defect from the viewpoint of classification, even if they do not act as a fault in differentiation according to phage patterns.

Bearing in mind the defects referred to above, the present worker will next discuss how to design a typing method which will be more suitable to milk strains of staphylococci.

For the selection of typing phages, three procedures are available. The first is to adopt phages which are released by the lysogenic strains from bovine milk. SMITH[32] found, in his study on lysogenic staphylococci, that when lytic action was noted between two strains, these two were closely related to each other; MCLEAN reported an ovine phage which lysed only strains of ovine origin. These findings will suggest that this way for selecting phages is apposite. The second is to use the phages of the International Series adapted to milk strains. In this case, the following points must be remembered. As the host ranges of adapted phages will be varied from the host ranges of their parent ones by the phenomenon called "Host Controlled Variation"[5,22], the phage group distinguished in the set of International phages may not be always carried on in the adapted phages. And the phages to be adapted may be exchanged in the process of adaptation for the phage releasing from the propagating strain when it is lysogenic[27]. Even if these points are negligible, it is doubtful whether a sufficient increase in number of typable strains is secured, because no more than one phage is classified as group IV among the phages of the International Series, although a considerable number of type IV strains spread into bovine milk. The third is to make up an entirely new set of typing phages, selecting some suitable ones by the above ways.

On the other hand, for improvement of bacteriophage typing of staphylococci, the selection of propagating strain of typing phage is, of course, no less important

than that of typing phage. It is well known that the host ranges of phages are affected by propagating strains, and, in addition, the phage concentration used in typing is determined according to the degree of lysis on the propagating strain. Therefore, in order to type the greatest possible number of staphylococci and to obtain stability in the results, it is desirable that propagating strains to be selected resemble in phage susceptibilities the strains to be tested. From this point of view, the propagating strains of phages used in typing of the cultures from bovine milk had better be picked out from those of the same origin.

In bacteriophage typing, it may be an additional important item to know whether the strains to be typed are lysogenic, being connected with interference effects owing to lysogenicity. By means of cross-resistance tests carried out according to BAIL's technique, SMITH[32,33] concluded that the carriage of a phage by a given strain precluded its lysis by a typing phage. On the contrary, ROUNTREE[36] published statements that the majority of the phages carried by lysogenic strains were distinguished from the typing phages and the possession of lysogenicity does not render the staphylococci resistant to the lytic action of bacteriophages.

In the next report, the lysogenicity of staphylococci isolated from bovine milk will be discussed to bring light on the subjects discussed above.

SUMMARY

An investigation was carried out on the bacteriophage typing of 375 strains of coagulase positive staphylococci isolated from bovine milk in this country by the use of the phages of the International Series; further, a comparison was made with the series of findings previously reported on staphylococci of human origin by many workers. Furthermore, it was discussed whether these phages could be applied to the classification of the cultures which were originated from bovine milk. The results are summarized as follows:

1. Out of the 375 strains above mentioned, 171 (45.6%) were typed by one or more of the twenty phages used; these typable strains belonged predominantly to type III (62 strains) and to type IV (57 strains).

The relative frequency with which the staphylococci were lysed by the different phages showed that the greater portion of strains were sensitive to phages 42E, 6, 47, 75 or 42D.

2. It was concluded that there existed some dissimilarities between the strains of human origin and the milk strains in their phage susceptibilities. This conclusion is based on the following facts: a considerable number of strains isolated from bovine milk were those of type IV which were rarely encountered in the cultures of human origin, whereas types I and II were rare in number.

Furthermore, the type III strains from bovine milk were often sensitive to the phage of group IV, though their reactions were weak.

3. The phages of the International Series are proven to be unsuitable for the typing of staphylococci from bovine milk in Japan. The important reasons for this were as follows: (a) more than half of the milk strains submitted to typing were untypable, and, in addition, the results obtained were often inconstant, (b) although a considerable number of the milk strains were classified into type IV, no more than one phage belongs to group IV in the phages of the International Series, (c) the main four phage groups distinguished in this phage set were not suitable for the classification of the milk strains of staphylococci, and the strains regarded as a border-line type between two types were frequently observed.

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