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**STUDIES ON BACTERIOPHAGE TYPING OF
STAPHYLOCOCCI ISOLATED FROM BOVINE MILK
III. TYPING BY MEANS OF A NEW PHAGE SET**

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In the previous reports of this series^{3,4}, it was suggested that some of the phages carried by lysogenic strains of staphylococci originated from bovine milk samples might be more suitable than the International phages for the typing of staphylococci of the same origin. The present study was undertaken with the purpose of arranging them into a phage set and to examine their suitability in routine typing of milk strains.

MATERIALS AND METHODS

Strains employed Three collections of coagulase-positive staphylococcal strains were employed.

(1) The first collection included 442 strains which were isolated from bovine milk. Their histories and results of typing by the International phages were fully described in the previous report³.

(2) One hundred and thirty-two strains, a part of which were kindly supplied by Dr. Y. TAJIMA of the Institute for Infectious Diseases, Tokyo University, were obtained from human beings including some patients with suppurative illness of staphylococcal etiology in 5 different hospitals.

(3) The third collection consisted of 110 strains isolated from the nasal cavities, the tonsils and intestinal contents of 3 species of animals viz., sheep, pig and horse, which were slaughtered at the Sapporo slaughter house, Sapporo.

The first collection was employed throughout the present investigation and the use of the latter two collections is dealt with only in the last section of this study.

Bacteriophage employed Seventy-seven phages, which were isolated from 77 lysogenic milk strains of staphylococci including 74 phage carriers detected at the time of the previous report⁴, were used in this experiment.

Preparation of crude phage suspension from lysogenic strains Crude phage suspensions were prepared from lysogenic strains by irradiation with ultraviolet light (UV). Each of the lysogenic strains was incubated in broth for 6 hours at 37°C and 0.5 ml of this culture was

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poured into a petri dish. This was irradiated with UV, diluted ten times with broth and incubated overnight at 37°C. Supernatant of the culture by centrifugation was a crude phage suspension.

Details of the technics were given in the previous report⁴).

Purification of phages The crude phage suspension was plated with the propagating strain which had been selected from strains susceptible to the crude phage; an isolated plaque was fished and transferred into about 0.5 ml of broth. Several passages, usually 3~4 times, were continued in this manner. One of the plaques which appeared in last plating was suspended in 2 ml of broth and the suspension was centrifuged for 40~60 minutes at 3,000 rpm. The phage in the supernatant fluid was then propagated, titrated and stocked for typing according to the previously described method³).

Phage typing The technical procedures and the interpretation of results used in this work were fully described in report I of this series³).

Each strain of staphylococci was grown for 6~18 hours in nutrient broth and 0.2 ml of this culture was spread over the surface of an agar plate. The phages were then applied to the culture with a tuberculin syringe. In this case, phage concentration of $10 \times \text{R.T.D.}$ was used for routine typing. Reading was made after overnight incubation at 37°C. Readings were recorded according to the degree of lysis as follows: confluent lysis with or without secondary growth, CL; more than 50 plaques, ++; 20~50 plaques, +; less than 20 plaques, \pm .

The strains which were lysed strongly (++ or CL) were regarded as typable.

Cross-resistance tests These were carried out by the method of BAIL, which was modified by SMITH⁷).

(1) Production of phage-resistant strains: One loopful of a 6 hours broth culture of a propagating strain was spread evenly over a small area (4~5 cm²) of the surface of an agar plate. When the culture had been absorbed into the agar, a drop of an undiluted phage which was strongly active on the propagating strain was spotted on the culture. Then the plate was incubated overnight at 37°C. Secondary growth appeared within a zone of complete lysis. A part of this secondary growth was inoculated into broth, and incubated overnight at 37°C. After a drop of this culture had been spread on an agar plate, a single colony was picked up and several times transferred serially on agar plates. A single colony from the final plating was picked up, placed in broth and incubated. This culture was then tested on agar plate carrying the phage and found to be resistant.

(2) Cross-resistance tests: Resistant strains were prepared from one propagating strain by the action of several phages. Undiluted solutions of these phages were then applied to each of the resistant strains on agar plates. When a lytic action was not observed on a resistant strain, the phage which was applied to the resistant strain was regarded as identical with the phage used for the preparation of the strain.

RESULTS

A Selection of typing phages and their grouping

1. Selection of typing phages

Selection of typing phages was based on their host ranges.

In order to examine their host ranges in detail, seventy-seven phages of three concentrations, 1×R.T.D., 10×R.T.D. and 100×R.T.D., were applied on 169 strains of staphylococci, which had been picked out at random from the 442 milk strains to investigate for lysogenicity⁴⁾. At the same time, differentiation among phages of similar host range was examined by cross-resistance tests; the results indicated that these phages were not always identical.

In this way, 60 different phages were selected from the 77 phages.

The host ranges and degrees of lysis of the 60 phages were compared with one another and, finally, 16 phages (see table 1) which showed comparatively wider host ranges with less weak reactions were selected for typing of milk strains. They are called the "bovine phages" hereafter.

TABLE 1. *The Bovine Phages*

PHAGE NO.	PROPAGATING STRAIN
H98	Y14
260	Y11
264	Y140
316	289
365	Y139
BVM 16	78
T90	Y111
418	H161
257	H162
407	BVM 8
H30	83
H30a	Y29
H131	H166
Y97	444
883	94
S 32	Y98

2. Grouping of the bovine phages

Two experiments were carried out in order to classify the bovine phages according to their host ranges.

(a) Lytic spectra on the propagating strains: Lytic spectra of the bovine phages were made on the propagating strains with the results tabulated in figure 1.

It is apparent from the figure that the 16 phages are divided into 3 groups: the first group is made up of 7 phages (H98, 260, 264, 316, 365, BVM 16, T90), the second group 5 phages (418, 257, 407, H30, H30a) and the third group 4 phages (H131, Y97, 883, S32). Although some cross reactions are observed between the first and second groups by the use of phages of high concentration, the third group is clearly distinguishable from them.

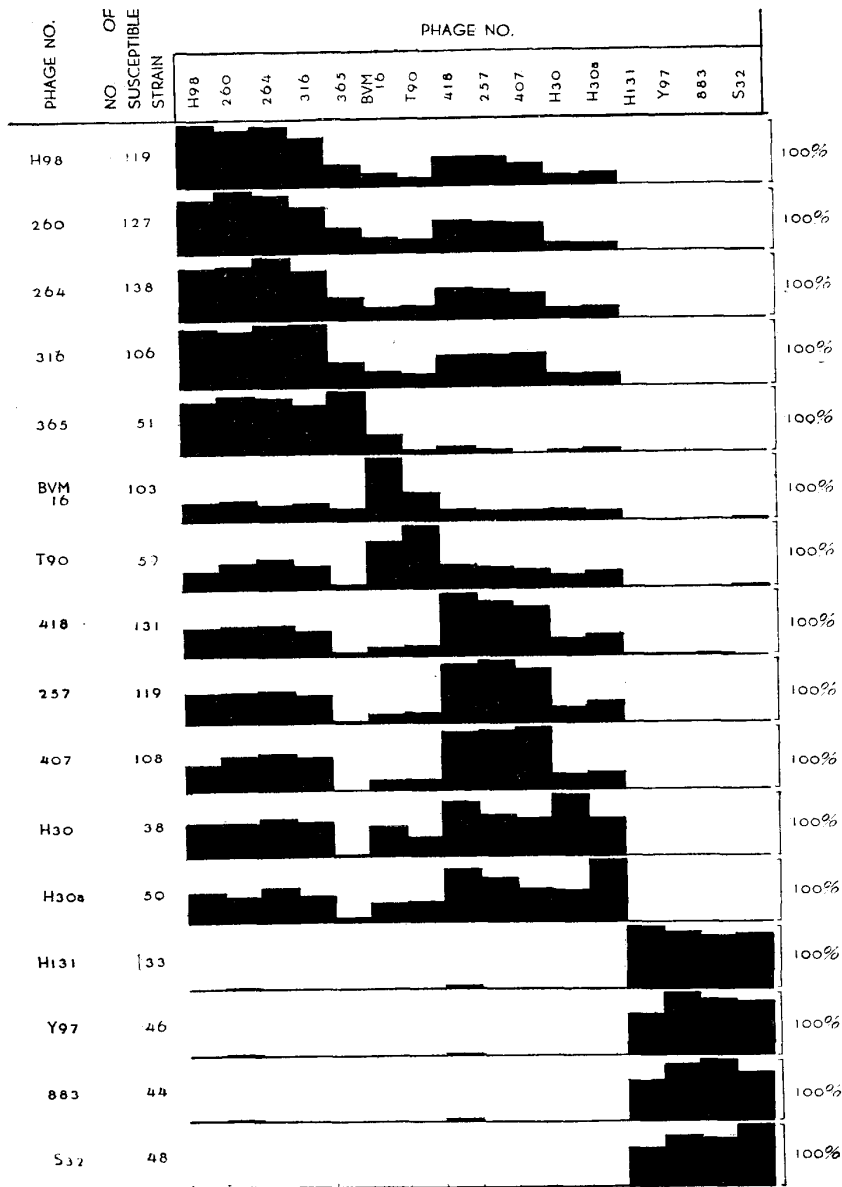
FIG. 1. *Lytic Spectra of the Bovine Phages on the Propagating Strains*

	PHAGE NO.																
	H98	260	264	316	365	BVM 16	T90	418	257	407	H30	H30a	H131	Y97	883	S32	
Propagating Strains	Y 14	CL*	CL	CL	CL	CL	++	--	--	--	--	--	--	--	--	--	--
		CL	CL	CL	CL	CL	+	--	--	--	--	--	--	--	--	--	--
		CL	CL	CL	+	CL	--	--	--	--	--	--	--	--	--	--	--
	Y 11	CL	CL	CL	CL	CL	CL	CL	--	--	--	--	--	--	--	--	--
		CL	CL	CL	CL	CL	CL	CL	--	--	--	--	--	--	--	--	--
		CL	CL	CL	CL	CL	+	+	--	--	--	--	--	--	--	--	--
	Y140	CL	CL	CL	CL	CL	++	--	--	--	--	--	--	--	--	--	--
		CL	CL	CL	CL	CL	+	--	--	--	--	--	--	--	--	--	--
		CL	CL	CL	+	+	±	--	--	--	--	--	--	--	--	--	--
	289	CL	CL	CL	CL	CL	+	--	--	--	--	--	--	--	--	--	--
		CL	CL	CL	CL	CL	±	--	--	--	--	--	--	--	--	--	--
		CL	CL	CL	CL	CL	--	--	--	--	--	--	--	--	--	--	--
Y139	CL	CL	CL	CL	CL	--	--	--	--	--	--	--	--	--	--	--	
	CL	CL	CL	CL	CL	--	--	--	--	--	--	--	--	--	--	--	
	CL	CL	CL	+	CL	--	--	--	--	--	--	--	--	--	--	--	
78	--	--	--	--	--	CL	--	--	--	--	--	--	--	--	--	--	
	--	--	--	--	--	CL	--	--	--	--	--	--	--	--	--	--	
	--	--	--	--	--	CL	--	--	--	--	--	--	--	--	--	--	
Y111	--	--	--	--	--	--	CL	--	--	--	--	--	--	--	--	--	
	--	--	--	--	--	--	CL	--	--	--	--	--	--	--	--	--	
	--	--	--	--	--	--	CL	--	--	--	--	--	--	--	--	--	
H161	±	+	±	±	--	--	--	CL	CL	CL	--	±	--	--	--	--	
	--	±	--	--	--	--	--	CL	CL	CL	--	--	--	--	--	--	
	--	--	--	--	--	--	--	CL	CL	+	--	--	--	--	--	--	
H162	±	±	±	±	--	--	--	CL	CL	CL	±	+	--	--	--	--	
	--	--	--	--	--	--	--	CL	CL	CL	--	±	--	--	--	--	
	--	--	--	--	--	--	--	CL	CL	+	--	--	--	--	--	--	
BVM 8	±	±	±	±	--	+	±	CL	CL	CL	+	CL	--	--	--	--	
	--	--	--	--	--	±	--	CL	CL	CL	±	CL	--	--	--	--	
	--	--	--	--	--	--	--	±	--	--	CL	CL	--	--	--	--	
83	--	--	--	--	--	--	--	+	--	--	CL	CL	--	--	--	--	
	--	--	--	--	--	--	--	±	--	--	CL	+	--	--	--	--	
	--	--	--	--	--	--	--	±	--	--	CL	+	--	--	--	--	
Y29	--	--	--	--	--	--	±	CL	CL	--	+	CL	--	--	--	--	
	--	--	--	--	--	--	--	CL	CL	--	±	CL	--	--	--	--	
	--	--	--	--	--	--	--	±	±	--	--	CL	--	--	--	--	
H166	--	--	--	--	--	--	--	--	--	--	--	--	CL	CL	CL	CL	
	--	--	--	--	--	--	--	--	--	--	--	--	CL	CL	CL	CL	
	--	--	--	--	--	--	--	--	--	--	--	--	CL	CL	CL	+	
444	--	--	--	--	--	--	--	--	--	--	--	--	CL	CL	CL	CL	
	--	--	--	--	--	--	--	--	--	--	--	--	CL	CL	CL	CL	
	--	--	--	--	--	--	--	--	--	--	--	--	CL	CL	CL	+	
94	--	--	--	--	--	--	--	--	--	--	--	--	--	CL	CL	CL	
	--	--	--	--	--	--	--	--	--	--	--	--	--	CL	CL	CL	
	--	--	--	--	--	--	--	--	--	--	--	--	--	+	CL	CL	
Y98	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	CL	
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	CL	
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	CL	

* The upper, middle and low lines represented spectra by the phages at 100×R.T.D., at 10×R.T.D. and at 1×R.T.D. respectively.

(b) Combinations of phage reactions: Staphylococcal phages forming patterns are not randomly assorted; some phages tend to attack one strain. To analyze quantitatively this association of the bovine phages, the 16 phages of 10×R.T.D. were applied to 339 milk strains which were sensitive to one or more of the phages. The results of this test are set out in the form of a block diagram in figure 2. Each block indicates the frequency with which two phages lysed together one staphylococcal strain.

FIG. 2. Association of Lytic Reactions in Phage Patterns



It is notable from the figure that each of the 3 groups distinguished above is also discriminated from the others in this experiment. Especially, the phages of the third group are set off from members of the other groups. However, the phages of the first and the

second groups do not stand out distinctly from each other; some phage patterns were found in combinations with phages of these two groups. For example, out of 119 strains which were sensitive to phage H98 of the first group, 53.8%, 52.1%, 38.7%, 18.5% and 21.0% were also lysed by phages 418, 257, 407, H30 and H30a of the second group respectively.

From the two results above mentioned, the bovine phages were classified as listed in table 2. Twelve phages of the first and second groups were included into one group which

TABLE 2. *Classification of the Bovine Phages*

GROUP	TYPE	PHAGE NO.	PROPAGATING STRAIN
A	A ₁	H98	Y 14
		260	Y 11
		264	Y140
		316	289
		365	Y139
		BVM16	78
		T90	Y111
	A ₂	418	H161
		257	H162
		407	BVM 8
		H30	83
		H30a	Y29
B		H131	H166
		Y97	444
		883	94
		S 32	Y98

is called group A; four phages of the third group were separated from those of group A and named group B. The phages of group A were divided further into two types, type A₁ and type A₂. Type A₁ consists of 7 phages and type A₂ 5 phages. Although phages BVM16 and T90 were considerably peculiar in the association of phage reaction, they were included in type A₁ for the present depending on their lytic spectra on the propagating strains.

B Practical use of the bovine phages

In this section are described examinations whether or not the bovine phages were suitable for typing of staphylococci originated from bovine milk, referring to the typing results secured by use of the International phages which have been published in report I of this series³).

1. Phage typing of milk strains employed

Four hundred and forty-two strains of coagulase-positive staphylococci isolated from milk samples were tested with the bovine phages. The results are given in table 3. Three hundred

TABLE 3. *Typing of Milk Strains by means of the Bovine Phages*

DISTRICT	NO. OF STRAINS	TYPABLE STRAINS					UNTYPABLE STRAINS
		A ₁	A ₂	A ₁ +A ₂	B	A+B	
Hokkaido	339	96	44	57	54	1	87
Tokyo	103	28	9	22	6	0	36
Total	442	124	53	79	62	1	123

and nineteen strains (72.2%) showed strong lysis with one or more of the phages. A large number of the typable strains were classified into group A; specifically, 124 strains were lysed by phages of type A₁, 53 strains by phages of type A₂ and 79 strains by both of the phages. All the remaining strains except one belonged to group B. It is noteworthy that no more than one strain was strongly lysed by phages of both groups A and B.

2. Reproducibility of typing results

As the phage concentrations used in routine typing were decided by tenfold dilutions of undiluted phages, a given concentration of phages should lie between two of the tenfold levels. Therefore, it must be needful in connection with reproducibility of typing results to examine the influence of phage concentration on such results.

This problem was analyzed in the following experiments, in which the bovine phages of 3 concentrations (1×R.T.D., 10×R.T.D. & 100×R.T.D.) were applied to 442 milk strains.

(a) Uniformity of group and type: Influence of phage concentrations on groups or types of the milk strains was investigated in this experiment. Classification of the strains by the phages at 10×R.T.D. is given in the center column of table 4, and the influence of a diluted (1×R.T.D.) or concentrated (100×R.T.D.) inoculum of phages is compared with the

TABLE 4. *Comparison of Results of Typing with 3 Phage Concentrations*

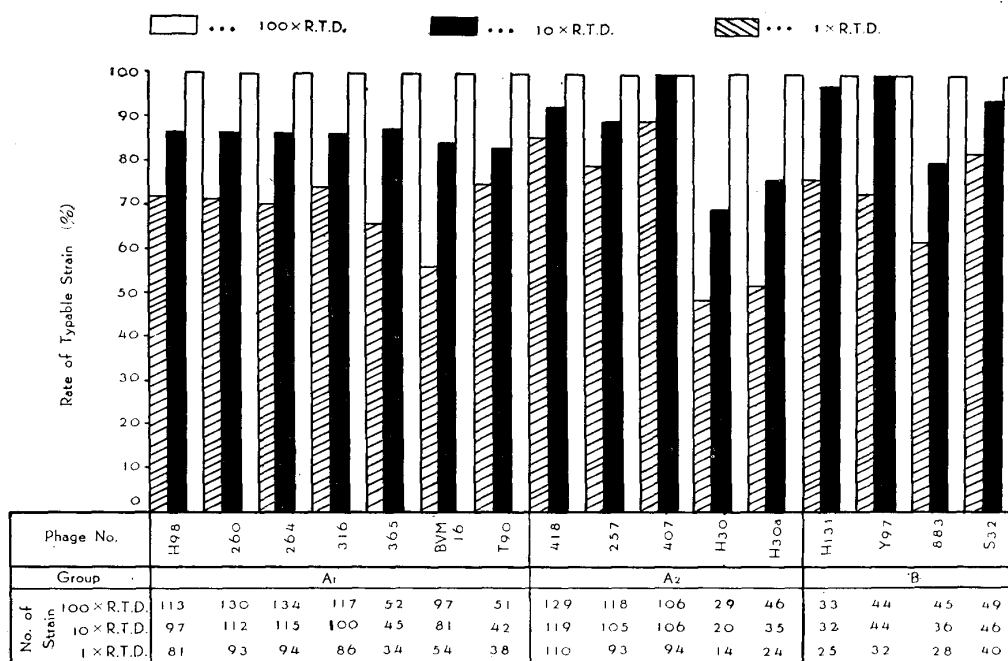
Untypable	PHAGE CONCENTRATION											Untypable
	1×R.T.D.			10×R.T.D.				100×R.T.D.				
B	A+B	A ₁ +A ₂	A ₂	A ₁	No. of Strain	A ₁	A ₂	A ₁ +A ₂	A+B	B	Untypable	
18	106	A ₁ 124	120	.	4	.	.	.
6	.	.	.	47	.	A ₂ 53	.	46	7	.	.	.
2	.	.	58	14	5	A ₁ +A ₂ 79	.	.	79	.	.	.
.	1	A+B 1	.	.	.	1	.	.
8	54	B 62	62	.
123	Untypable 123	9	2	2	.	.	110

result obtained using the $10\times R.T.D.$ in the left-hand or right-hand portions of the table respectively. In all columns, strains showing no strong reaction ($++$ & CL) were classed in the untypable strains.

It is apparent from the table that strains of group A were distinguished clearly from members of group B regardless of the phage concentrations and vice versa. For example, none of the 257 strains classified into group A using the phages at $10\times R.T.D.$ was typed by the phages of group B concentrated even to $100\times R.T.D.$, though some of them changed to untypable as the result of use of the diluted phages. Such a clear-cut segregation was also observed among the 3 types of group A, though it was not so strict.

(b) Uniformity of phage pattern: Variance in bacteriophage pattern introduced by different phage concentrations was analyzed in figure 3, in which the proportion of strains showing strong reaction ($++$ or CL) with $1\times R.T.D.$ or $10\times R.T.D.$ of phage to those with $100\times R.T.D.$ was presented in percentages separately by individual phage.

FIG. 3. *The Influence of Phage Concentration on the Number of Strains Typable by Individual Phage*



As shown by the figure, in all of the bovine phages except two (H30 & H30a), more than 80 per cent of the strains strongly lysed by a phage at $100\times R.T.D.$ was also typed with $10\times R.T.D.$ of the phage. This means that the number of strains typable by the bovine phages is not markedly affected by differences in phage concentration and, therefore, lytic patterns introduced by the phages are reproducible.

3. Relationship between the bovine phages and the International phages

Results of typing the 442 milk strains which were obtained using the bovine phages and the International phages were compared in table 5 in order to clarify the relation between

TABLE 5. *Relationship between the Bovine Phages and the International Phages*

GROUP	TYPE	NO. OF STRAINS	TYPING RESULTS BY INTERNATIONAL PHAGES						
			I	II	III	IV	Mixed	Untypable	
Typing Results by Bovine Phages	A	A ₁	124	5	•	44	3	4	68
		A ₂	53	1	•	2	35	3	12
		A ₁ +A ₂	79	3	1	14	30	11	20
	B	62	•	•	•	•	2	60	
	A+B	1	•	•	•	•	•	1	
Untypable	123	13	13	5	4	2	86		
Total		442	22	14	65	72	22	247	

the two phage sets. Most of the strains classified into types III and IV by the International phages were included into group A by the bovine phages; special correlations were observed between type III and type A₁ and between type IV and type A₂. In contrast with this, almost all of the strains belonging to group B according to the bovine phages were untypable by the phages of the International Series. This means that no phage of group B has connection with the International phages. Furthermore, it is an additional noticeable fact that most of the type II strains and a half of the type I strains were not typable by the bovine phages.

4. Phage typing of staphylococci isolated from humans and several species of animals.

Some staphylococcal strains isolated from human beings, sheep, pig and horse were tested with the bovine phages, and the results were compared with those of milk strains in table 6. In addition, their results of typing by the 20 phages of the International Series were included in the table.

No association between ability to produce reaction and the source of the organisms was

TABLE 6. *Typing of Staphylococcal Strains from Human and Several Species of Animals*

SOURCE	NO. OF STRAIN TESTED	INTERNATIONAL PHAGES						BOVINE PHAGES					
		No. of Typable Strains						No. of Typable Strains					
		I	II	III	IV	Mixed	Per cent	A ₁	A ₂	A ₁ +A ₂	B	A+B	Per cent
Human	132	16	6	21	•	6	37.1	3	4	•	2	•	6.8
Sheep	10	•	3	•	•	2	50.0	1	•	•	•	•	10.0
Pig	26	3	2	5	1	•	42.3	•	2	3	1	•	23.1
Horse	74	1	•	6	•	2	12.2	3	•	3	•	•	8.1
Bovine Milk	442	22	14	65	72	22	44.1	124	53	79	62	1	72.2

observed in the International phages. However, the reacting ability of the bovine phages seemed to be limited to strains isolated from bovine milk.

Further experiments will be necessary before a conclusion based upon these observations can be reached.

DISCUSSION

In the previous reports^{3,4}, the opinion was expressed that the International phages are not fully suitable for the typing of staphylococcal strains isolated from bovine milk samples in Japan. Four important reasons were stated for this conclusion; namely (1) many untypable strains were detected, (2) results of typing were often not reproducible, (3) a clear-cut segregation among groups was not observed, (4) all of type IV strains which occupied a considerable proportion of milk strains were not differentiated from one another, because no more than one phage was included in group IV.

Consulting these issues introduced from results of typing by the International phages, the valuation of a new phage set called the bovine phages will be discussed on the typing of milk strains.

Out of the 442 milk strains tested by the bovine phages, 319 strains (72.2%) were typable. This is far higher than 44.1 per cent obtained by use of the International phages³, but not a satisfactory percentage. Accordingly, additional phages will be needed in order to increase the number of typable strains. The distribution of the typable strains among phage groups indicated that a great majority of them belong to group A. Such an unbalanced distribution of milk strains was also observed in results of typing reported by some previous workers^{2,6,8,9}. The disparity in number of phages among the groups may have some connection with this. Therefore, it is desirable that typing phages belonging to each of the groups be arranged to equal number.

An important factor concerning the reproducibility of results of typing is a difference of phage concentrations which lies between two of tenfold dilution levels, because the phage concentration used in routine typing is determined by tenfold dilutions of an undiluted phage suspension. The present author has demonstrated already the fact that the results of typing of milk strains obtained using the International phages were influenced considerably by the difference of phage concentrations, representing that in most of the phages, about a half of milk strains typable with $100 \times$ R.T.D. of a phage were changed to untypable by use of the phage diluted to $10 \times$ R.T.D.³ Such an influence of phage concentrations was not observed in the results obtained by use of the bovine phages (Table 4 & Figure 3). Therefore, it is apparent that the results of typing of milk strains produced by the bovine phages are far more reproducible than those by

the International phages.

When the International phages were used for typing of milk strains, the main four groups arranged in that phage set did not stand out distinctly from one another, and strains regarded as belonging to a border-line type between two types were frequently encountered³⁾. However, the milk strains typed by the bovine phages were classified into two groups (A & B) which were clearly distinct from each other regardless of the degree of lysis and concentration of phage. The strains of group A were subdivided into three types (A_1 , A_2 & A_1+A_2). Although cross reactions were frequently observed among the three types, their differentiations were definite and the type to which a given strain had been decided was rarely revised even as a result of the use of different phage concentrations.

It was apparent from the relationship between the bovine phages and the International phages that milk strains of types III and IV which were differentiated by the International phages were included together into the same group by the bovine phages. This is considered to be a natural result derived from the fact that phages of groups III and IV showed frequently lytic actions on a strain³⁾. On the other hand, it was noteworthy that almost all of type II strains and about a half of type I strains (by the International phages) were not typed by the bovine phages. Therefore, some new phages which are available for these strains should be added to the set of bovine phages in the future, even though these strains occupied a small portion of milk strains.

SMITH⁷⁾ stated that the strains of type 42D (type IV) as classified by the International phages were not always identical but classified into a number of types by use of additional phages. This statement was confirmed in the present study. Namely, milk strains classified into type IV using the International phages were divided generally into two types, one of which was type A_2 and the other type A_1+A_2 , showing some distinct lytic patterns in each type.

Recently, it was reported by OCHI and SHIMIZU that some biological properties of staphylococcal strains, such as β hemolysin, lipase and yellow pigment productions, Voges-Proskauer and methyl red reactions and pellicle formation, were characteristic according to the species of animals from which the strains were isolated. This relation perhaps also exists in phage susceptibilities of staphylococcal strains. This supposition was based on the fact that host ranges of the bovine phages were limited to milk strains (Table 6). Therefore, for the typing of staphylococcal strains which have originated from different species of animals, several phage sets which should be arranged by the species of animals may be necessary.

It is apparent from the present discussion that the bovine phages were far serviceable than the International phages for the typing of staphylococcal strains isolated from bovine milk samples in Japan.

SUMMARY

A new phage set was proposed for the typing of milk strains and its suitability was examined. Conclusions are summarized as follows:

1. Out of 77 phages isolated from lysogenic milk strains of staphylococci, 16 were selected for the typing of milk strains and were named the "bovine phages".

2. These bovine phages were classified into two groups according to their host ranges; one was called group A and the other group B. The phages of group A were subdivided into types A₁ and A₂.

3. It was proved that the bovine phages were more suitable than the International phages for the typing of staphylococcal strains isolated from bovine milk in Japan.

4. The relationship between the bovine phages and the International phages was shown.

5. The reacting ability of the bovine phages seemed to be limited to milk strains.

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