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Bacteriophage Typing of Coagulase-Negative Staphylococci Isolated from Bovine Milk

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Abstract

The purpose of the present study is to determine whether the phages of the N.C.T.C. phage-set and Davidson's phage-set could be utilized in typing 417 coagulase-negative staphylococci from bovine milk and to device a new phage-set that would yield higher rate of typing than the two methods. The results are summarized as follows:

1) Rate of phage typing by the N.C.T.C. and Davidson's phage-set were 4.3% and 1.4%, respectively.

2) Only one phage was induced among 50 coagulase-negative staphylococci by irradiation with ultraviolet.

3) Sixty-five phages were isolated from sewage of dairy farms. Of these 9 phages were selected as typing phages and divided into 4 groups. We called these 9 phages "the provisional typing-set".

4) Relatively high typing rate, 54.2% (226 of 417 strains), attained by this typing-set proves that this typing-set is more readily applicable than above two phage-sets for coagulase-negative staphylococci from bovine milk.

Introduction

Coagulase-negative staphylococci (coag(-)sta) have frequently been isolated from abnormal milk of cow suffering from mastitis. It has been reported that coag(-)sta is one of pathogenic organisms responsible for causing bovine mastitis (BROWN *et al.*[1], FORBES[2], and KUME *et al.*[3]). Nevertheless, much attention has been paid into bacteriophage typing of coagulase-positive staphylococci (coag(+)sta) in the epizootiology of bovine mastitis.

In coag(-)sta of these studies, the N.C.T.C. phage-set was used for typing and rate of typing was reported to be less than 2% (TAKAGAKI *et al.*[4]). HOLMBERG[5] reported that 22.5% of the bovine coag(-)sta were typed by his bovine phage-set for coag(+)sta.

The purpose of the present study is to determine whether the phages of the N.C.T.C. phage-set and Davidson's phage-set[6] could be utilized in typing coag(-)sta from bovine milk and to device a new phage-set that would yield higher rate of typing than the two methods.

Materials and Methods

Strains. A total of 417 coag(-)sta from two different geographical area were used. Three hundred and fifty-six strains were isolated from bovine milk around Sakai area.

Sixty-one strains were isolated from bovine milk around Sapporo area.

Media. Nutrient broth and nutrient agar were used.

Typing phages. The N.C.T.C. phage-set and Davidson's phage-set were used.

Phage typing. Propagation of phages, titration of phage and methods of typing were followed after NAKAGAWA's descriptions[7]. Phage concentration of 100 RTD (Routine Test Dilution) was used. Reading was made after overnight incubation at 37°C. The lysis was scored in 4 degrees as follows; CL for confluent lysis with or without secondry growth, ++ for more than 50 plaques, + for 20-50 plaques, ± for less than 20 plaques.

Induction of phages by ultraviolet. This method was described by NAKAGAWA[8]. Namely, phages were induced from lysogenic strains under irradiation of ultraviolet light. Each stain of coag(-)sta was incubated in a broth at 37°C for 6 hr, irradiated at a distance of 80 cm for 80 seconds with a 10 watt ultraviolet lamp (wave length 2537 Å), and incubated at 37°C for 18 hr. The sample was then centrifuged at 3000 r.p.m. for 30 minutes, and the supernatant was filtered through 0.45μ pore size filter (Sartorius Membranfilter®). An agar plate was dried for 90-120 minutes at 37°C and then 0.2 ml of broth of 18 hr-culture of coag(-)sta was seed on its surface. When the culture was absorbed into the agar, 0.2 ml of the supernatant of the broth culture which had been irradiated with ultraviolet was spread over the plate by a grass-spreader, leaving a small margin as a control area to make sure whether coag(-)sta presents a spontaneous lysis. Then the plate was incubated at 37°C overnight. The presence of phage was shown by development of lysis, usually in the form of isolated plaque and less often as semi confluent lysis. An isolated plaque was picked and transferred into approximately 1.0 ml of broth and the suspension was centrifuged at 3000 r.p.m. for 30 minutes. The phage in the supernatant fluid was then propagated, titrated and stored for typing.

Isolation of phage from sewage. Sewage sample were collected from 20 dairy farms in Sakai. Each sample was centrifuged at 7000 r.p.m. for 30 minutes, and the supernatant was adjusted to pH 7.0 ± 0.2, and filtered through 0.45μ pore size filter. And its supernatant and a nutrient broth of twice density were mixed. Its mixture fluid was divided into 93 test-tubes. And 93 coag(-)sta that selected at random from 417 strains were inoculated into 93 tubes and incubated at 37°C overnight. The broth culture was centrifuged at 3000 r.p.m. for 30 minutes and the supernatant was filtered through 0.45μ pore size filter. A 0.2 ml of broth of coag(-)sta was seed on an agar plate, and each supernatant was spread over the plate by a grass-spreader, and incubated at 37°C overnight. When presence of plaque was shown, the phage was isolated by the same method described above.

Selection of typing phages. In order to examine lytic spectra of isolated phage, these phages were applied at three degrees concentration (1, 10, and 100 RTD). On 93 coag(-)sta, phages of wide lytic spectra were selected. On this examination, phages of the same lytic spectrum belonged to the same group. And it was also examined on lytic patterns of 93 coag(-)sta to each of selected phages, that is, according to whether or not the bacterial strains sensitive to one typing-phage were sensitive to the another.

Results

Typing by the N.C.T.C. phage-set and Davidson's phage-set

Results of phage typing of 417 coag(-)sta examined by the N.C.T.C. phage-set are summarized in Table 1. A total of 18 strains (4.3%) were typed. By Davidson's phage-

set, at total of 6 strains (1.4%) were typed (Table 2).

Table 1. Phage-typing by the N.C.T.C. phage-set of coagulase-negative staphylococci from two different geographical area.

Phage type	Sakai	Sapporo	Total
I	2	0	2
III	10	0	10
I. III	2	0	2
I. II. III	3	0	3
Miscellaneous	1	0	1
Typable strains	18	0	18
Untypable strains	338	61	399
Total	356	61	417

Table 2. Phage-typing by Davidson's phage-set of coagulase-negative staphylococci from two different geographical area.

Phage type	Sakai	Sapporo	Total
II	1	0	1
III	1	0	1
I. III	1	0	1
II. III	1	0	1
II. IV	1	1	2
Typable strains	5	1	6
Untypable strains	351	60	411
Total	356	61	417

Induction of phage by ultraviolet

By irradiation with ultraviolet, only one phage was induced among 50 coag(-)sta that selected at random from 417 strains. This induced phage that lysed one out of 93 strains randomly selected from 417 strains had very narrow lytic spectra.

Isolation of phage from sewage

Sixty-five phages were isolated from sewage of dairy farms in Sakai. And only one phage was selected from phages which lysed the same strain. By removing unselected phages, 30 out of these 65 phages were selected. When phages were isolated, coag(-)sta lysed by those phages were determined on their propagating strains. These 30 phages and their propagating strains were used for the study.

Selection of typing phages and grouping

The host ranges of 30 phages which had been propagated to 100 RTD were examined with 93 strains. Based on their host ranges, 9 phages which lysed 5 or more of the 93 strains and shown confluent lysis at the density of 1000 fold dilution were finally selected. Nine phages and their propagating strains are shown in Table 3.

The nine phages were divided into groups on the basis of the host range. For this purpose, their lytic spectra at 1, 10, and 100 RTD were determined by 9 phages and 9 propagating strains. As shown in Fig. 1., nine phages are divided into 4 groups where cross reaction among the 4 groups were scarcely noted. The first group was consisted of 5 phages (p-2, p-4, p-16, p-19, and p-20), the second group of 2 phages (p-9 and p-37), the third (p-82) and the fourth (p-85) group of one phage each.

The 9 phages were also divided into 4 groups from the results of lytic patterns of

Table 3. The provisional phages and their propagating strains.

Phage NO.	Propagating strain NO.
p-2	2
p-4	4
p-9	9
p-16	16
p-19	19
p-20	20
p-37	37
p-82	82
p-85	85

Propagating strain NO.	Phage NO.								
	p-2	p-4	p-16	p-19	p-20	p-37	p-9	p-82	p-85
2	CL a)	-	-	-	-	-	-	-	-
	CL b)	-	-	-	-	-	-	-	-
	CL c)	-	-	-	-	-	-	-	-
4	-	CL	-	-	-	-	-	-	-
	-	CL	-	-	-	-	-	-	-
	-	CL	-	-	-	-	-	-	-
16	++	CL	CL	CL	CL	-	-	-	-
	++	CL	CL	CL	CL	-	-	-	-
	CL	CL	CL	CL	CL	-	-	-	-
19	+	+	CL	CL	CL	-	-	-	-
	+	++	CL	CL	CL	+	-	-	-
	+	CL	CL	CL	CL	++	-	-	-
20	+	CL	CL	CL	CL	-	-	-	-
	+	CL	CL	CL	CL	+	-	-	-
	CL	CL	CL	CL	CL	++	-	-	-
37	-	-	-	-	-	CL	CL	-	-
	-	-	-	-	-	CL	CL	-	-
	-	-	-	-	-	CL	CL	-	-
9	-	-	-	-	-	-	CL	-	-
	-	-	-	-	-	+	CL	-	-
	-	-	-	-	-	++	CL	-	-
82	-	-	-	-	-	-	-	CL	-
	-	-	-	-	-	-	-	CL	-
	-	-	-	-	-	-	-	CL	-
85	-	-	-	-	-	-	-	-	CL
	-	-	-	-	-	-	-	-	CL
	-	-	-	-	-	-	-	-	CL

Fig. 1. Lytic spectra of the provisional typing-phages on their propagating strains.

Remarks : In each of propagating strain, a), b), and c), respectively represent the lytic spectra of the phage at 1, 10, and 100 RTD.

coag(-)sta to each of 9 phages. The results of this test are shown in Fig. 2. Though the phages of the third and the fourth groups were not distinctly separated from each other, 9 phages were divided into 4 groups. We called these 9 phages "the provisional typing-set".

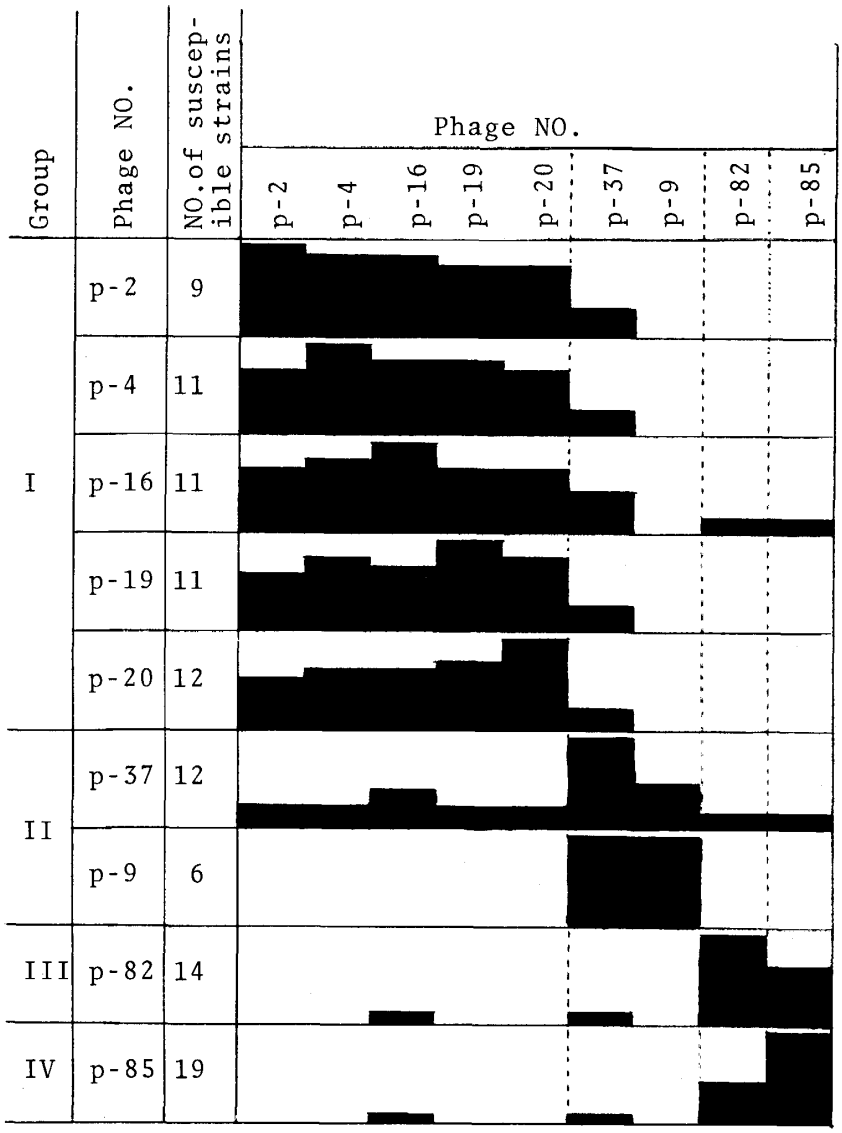

Fig. 2. Lytic patterns of coagulase-negative staphylococci to each of 9 phages under the provisional typing-set.

Remark : ■ 100 %

Typing by the provisional typing-set

Results of phage typing of 417 coag(-)sta examined are shown in Table 4. A total of 226 strains (54.2%) were typed with one or more of the phages at 100 RTD. One hundred ninety-two out of 356 coag(-)sta isolated from Sakai were typed. Of 192

Table 4. Phage-typing by the provisional typing-set of coagulase-negative staphylococci from two different geographical area.

Phage type	Sakai	Sapporo	Total
I	49	9	58
II	22	8	30
III	13	0	13
IV	45	12	57
I. II	15	0	15
I. III	3	1	4
I. IV	8	0	8
I. II. IV	4	0	4
I. III. IV	4	3	7
I. II. III. IV	5	0	5
II. IV	1	1	2
III. IV	23	0	23
Typable strains	192	34	226
Untypable strains	164	27	191
Total	356	61	417

typable strains, 49 (25.2%) belonged to type I, 45 (23.4%) to type IV, 23 (12.0%) to type III. IV and 22 (11.5%) to type II.

Thirty-four (55.7%) of 61 coag(-)sta isolated from Sapporo were typed. Of these, 12 (35.3%) belonged to type IV, 9 (26.5%) to type I and 8 (23.5%) to type II.

Discussion

NAKAGAWA[8] and LORBACHER *et al.*[9] reported that many phages were induced from coag(+)sta by irradiation of ultraviolet light. On the other hand, KARSKA *et al.*[10] indicated that few phages were induced from coag(-)sta by same method. Similar result was obtained in the present study. These findings indicate difficulties in inducing phages from coag(-)sta at higher rate. Many typing phages of coag(-)sta were isolated from sewage of dairy farms. These phages are useful to type coag(-)sta, but each phage of the conventional phage-sets; e.g. the N.C.T.C. phage-set, Davidson's phage-set, and others; was induced from lysogenic strains. Recently, it was reported that many phages were induced from coag(-)sta by a treatment with mitomicin c (VERHOEF *et al.*[11]) and by Fisk cross culture technique (ROSE *et al.*[12]). Therefore, these methods can be applied for inducing the phage from lysogenic strain.

In Fig. 1. cross reaction was shown between first group and second group and in Fig. 2. among 4 groups. Since cross reactions at these extent was also seen on the con-conventional phage-sets, the provisional phage-set were similarly divided into 4 groups.

The phage typing of coag(-)sta isolated from bovine milk have rarely been conducted. HOLMBERG[5] reported low phage typing-rate of coag(-)sta from cow by using his bovine-set. TAKAGAKI *et al.*[4] reported low phage typing-rate of coag(-)sta from bovine milk by using the N.C.T.C. phage-set. Similary, in this study low rates of phage typing of coag-(-)sta from bovine milk by the N.C.T.C. phage-set and Davidson's phage-set were obtained. On the other hand, the phage typing-rate by the present "provisional typing-set" was relatively high. This clearly indicated that the provisional typing-set is more readily

applicable than the N.C.T.C. phage-set, Davidson's phage-set and Holmberg's phage-set for coag(-)sta from bovine milk.

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