Bacteriophage Typing of Canine Staphylococci

II. Isolation of Phage from Lysogenic Strains and Establishment of a New Phage Set

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Abstract. An attempt was made to isolate phages from canine staphylococci, so that phage typing of these organisms might be done. Of 150 coagulase-positive strains, 42 (28%) were proved to be lysogenic. All of them, except seven, were found to carry phages of different host ranges. Of the phages detected, a few propagated to a titer of 100 RTD, and considerably many disappeared during passages for propagation. Multi-lysogenicity was demonstrated in four strains which produced two or three different phages. Based on host ranges, 16 phages were selected for typing of canine staphylococci and called "canine phages". They were divided into 7 groups according to their host ranges. Of them, five phages belonged to group I, six to group II, and one each to groups III to VII. Group II was further divided into sub-groups IIa and IIb, to which belonged three phages each.

In the previous study [15], the N.C.T.C. and bovine phages were found to be useless for typing of canine staphylococci and the establishment of a new phage set was stressed to be necessary for this purpose.

To obtain new phages, two methods are usually used; one of them is to adapt some of the existing phages to bacterial strains which are lysed weakly by the phages, and the other to select some phages from among those newly isolated from lysogenic strains. The first method seems to be impracticable, because none of the white-pigment producers which are predominant among canine staphylococci show any susceptibility to the existing N.C.T.C. or bovine phages [15]. Therefore, it is considered that isolation of new phages from lysogenic strains may be the only way to enable typing of canine staphylococci.

The present study was performed to isolate phages from lysogenic strains of canine staphylococci and to establish a new phage set for typing.

Materials and Methods

Strains used: A total of 185 coagulase-positive strains of staphylococci were selected at random from the 221 strains which had been isolated from dogs and used in the previous study [15]. Of them, 150 strains were tested for lysogenicity.

Induction of phages: Phages were induced by irradiating lysogenic strains with ultraviolet ray. Five ml of 6-hour culture of the strain to be tested was poured into a petri dish and exposed to ultraviolet ray irradiation (Mazda's low ozone germicidal lamp; wavelength, 2537 Å) for 80 seconds at a distance of 80 cm. The exposed culture was diluted tenfold with broth, incubated at 37°C overnight, and then centrifuged at 3,000 rpm for 30 to 40 minutes. The resultant supernatant with a small piece of crystal thymol added was stored at 2-5°C and submitted to checking phages within 5 days. The exposure to ultraviolet ray irradiation and the

overnight incubation were carried out under interception of visible light, because the induction effect was reversible by irradiation with visible light [10, 16].

Test for lysogenicity: A 6-hour broth culture of an indicator strain was spread over agar medium in a petri dish and left for 4–5 minutes for drying. Then, 0.2 ml of the supernatant to be tested for the presence of phages was inoculated onto the plate, but a small area was left uninoculated to check the spontaneous lysis of the indicator strain. After overnight incubation at 37°C, the culture was observed for plaque formation. When plaques were formed in very small number or unclear, some of them were picked up with a loop and suspended in 0.5 ml of broth. The presence of phages was reexamined by the same procedure as mentioned above.

Isolation of phages: Phages were isolated from the crude phage suspensions of lysogenic strains exposed to ultraviolet ray irradiation. Of the indicator strains lysed by an induced phage, the most susceptible one was selected as the propagating strain of the phage. For purification of phages, an isolated plaque formed on the propagating strain seeded on an agar plate was picked up with the tip of a platinum loop and suspended in 0.5 ml

of broth. A purified phage suspension was obtained after repeating twice the same procedure.

Cross resistance test: In order to examine the mutual identity of isolated phages, the cross resistance test was performed by the method described by Smith [13], which was a modification of Bail's method [1].

Propagation, titration and typing of phages: The methods used were described already in detail in the previous report [15].

Results

1. Detection of lysogenic strains

Of the 150 strains examined, 42 lysogenic strains (28%) and 43 indicator strains were detected. The relationship between the lysogenic and indicator strains is shown in Table 1. The phages released from the lysogenic strains varied in host range. Some of them lysed 11 indicator strains, but others did only one. It is interesting to note that the phages from 5 lysogenic strains (strains 77, 201, 443, 449 and 471) lysed their parent strains from which they

Table 1. Lysogenic strains and their indicator strains of staphylococci of canine origin

Strain No.	Lysogenic strain	Indicator strain	Strain No.	Lysogenic strain	Indicator strain
1	20	43, 213, 249, 463	13	327	201, 305, 492
2	133	80, 97, 103, 105, 115	14	365	213, 249, 366, 463
3	135	80, 97, 103, 105, 115	15	422	201, 213, 249, 347, 348, 463
4	190	201, 313, 422, 423, 480, 492	16	423	347, 348
5	192	201, 313, 422, 423, 480, 492	17	427	443, 463, 464, 492
6	201	190, 191, 201	18	443	213, 249, 366, 443, 463, 492
7	214	213, 249, 342, 387, 440, 443 463, 464	19	446	449
8	215	211, 446, 449, 492, 523	20	449	446, 449
9	248	213, 249	21	468	492
10	305	313, 492	22	471	440, 443, 463, 464, 471, 492 201, 213, 249, 309, 342
11	313	309, 492	23	480	347, 348
12	318	319	24	626	347, 348
25	26	77, 80, 96	34	228	217, 218
26	28	259	35	245	259, 269, 282
27	74	77, 96	36	260	259, 269, 282
28	77	77, 96	37	268	245
29	114	103	38	329	366
30	131	103	39	395	364
31	183	43	40	420	387, 440, 492, 523
32	216	217, 218	41	441	523
33	227	218	42	462	443

Table 2. Lytic spectra of multi-phages from 4 lysogenic strains

Indicator strain	Phage								
	20	20a	443	443a	422a	422b,c	422d	471	471b
249	CL*	—	CL	—	CL	CL	—	CL	—
	‡‡*	—	CL	—	‡‡	CL	—	‡‡	—
	‡‡*	—	‡‡	—	‡‡	‡‡	—	‡‡	—
213	CL	—	CL	—	‡‡	CL	—	CL	—
	‡‡	—	CL	—	‡‡	‡‡	—	CL	—
	‡‡	—	CL	—	+	‡‡	—	‡‡	—
463	+	—	CL	—	CL	‡‡	—	CL	—
	±	—	‡‡	—	CL	+	—	‡‡	—
	—	—	‡‡	—	‡‡	±	—	‡‡	—
201	—	—	—	—	‡‡	‡‡	—	‡‡	—
	—	—	—	—	‡‡	+	—	‡‡	—
	—	—	—	—	+	+	—	+	—
342	—	—	—	—	—	—	—	‡‡	—
	—	—	—	—	—	—	—	+	—
	—	—	—	—	—	—	—	±	—
43	—	CL	—	—	—	—	—	—	—
	—	CL	—	—	—	—	—	—	—
	—	CL	—	—	—	—	—	—	—
366	—	—	CL	—	—	—	—	—	—
	—	—	‡‡	—	—	—	—	—	—
	—	—	‡‡	—	—	—	—	—	—
492	—	—	—	CL	—	—	—	—	CL
	—	—	—	CL	—	—	—	—	CL
	—	—	—	‡‡	—	—	—	—	‡‡
347	—	—	—	—	—	—	CL	—	—
	—	—	—	—	—	—	CL	—	—
	—	—	—	—	—	—	CL	—	—
348	—	—	—	—	—	—	CL	—	—
	—	—	—	—	—	—	CL	—	—
	—	—	—	—	—	—	CL	—	—

Remarks.

CL: Confluent lysis. ‡‡: Semi-confluent lysis.

‡‡: More than 50 plaques. +: 20 to 50 plaques.

±: Less than 20 plaques. —: No plaque.

*: Upper level, 100 RTD; Middle level, 10 RTD; Lower level, 1 RTD.

had been released. The reason for this phenomenon is unknown. The phages derived from lysogenic strains 133 and 135; 190 and 192; 423, 480 and 626; 74 and 77; 114 and 131; 216 and 228; and 245 and 260 lysed the same indicators. Accordingly, it was found that 35 lysogenic strains carried phages which lysed different indicator strains.

2. Isolation of phages

The phages produced by 19 of 42 lysogenic strains failed to be transferred serially on their propagating strains. Thus, 28 phage strains were obtained from 23 lysogenic strains. Of them, 11 strains were propagated to 100 RTD or a titer high enough for practical use after 8 to 10 passages on their propagating strains. The re-

Table 3. An example of cross-resistance test

Resistant strain	Phage
	422a
	— *
422a-R	— *
	— *
	CL
422c-R	++
	+

Remarks.

*: Upper level, 100 RTD; Middle level, 10 RTD; Lower level, 1 RTD.

maintaining 17 phages did not reach 100 RTD even after 10 to 20 passages on their propagating strains.

On the other hand, multi-phages were obtained from 4 lysogenic strains, Nos. 20, 443, 442 and 471. Therefore, in order to examine the identity of phages from the same lysogenic strain, the lytic spectra of the phages were compared using selected indicator strains. The results obtained are shown in Table 2. Although the phages derived from strains 20, 443 and 471 appeared to be different from one another, three of four phages from strain 422 had a similar spectrum. Especially, the spectra of phages 422b and 422c were quite the same. They were shown to be identical.

Next, the cross-resistance test was performed between phages 422a and 422c, using phage 422a and two resistant strains, 422a-R and 422c-R, which were derived from lysogenic strain 42 and which were proved to be resistant to phages 422a and 422c, respectively. As shown in Table 3, strain 422c-R was lysed by phage 422a, indicating that phages 422a and 422c were different from each other. It was proved from the results mentioned above that each of the lysogenic strains had produced at least 2 or 3 different phages.

3. Establishment of a phage set for typing

Table 4. Canine phages

Phage No.	Propagating strain
443	463
471	463
214a	463
422b	213
248	249
190	201
327	305
305	313
449	446
446	449
215a	449
626	347
313	309
133	80
318	319
20a	43

The host ranges of 17 phages which had been propagated to 100 RTD were examined with 185 strains of canine origin as indicators. Based on their host ranges, all of them, except one, listed in Table 4 with their propagating strains were regarded to be valuable for typing. They are referred to as "canine phages" hereafter.

The 16 canine phages were divided into groups on the basis of the host range. For this purpose, their lytic spectra at 1 and 100 RTD were made by use of 15 test strains, including 13 propagating strains. As shown in Fig. 1, the 16 phages were divided into eight distinct groups, among which no cross reactions were seen. The 1st group consisted of 5 phages (214a, 248, 422b, 443 and 471), the 2nd group of 3 phages (190, 305 and 327), the 3rd group of 3 phages (215a, 449 and 446), and the 4th to 8th groups of one phage each.

Analysis of correlation between two phages was made by examining a frequency at which these phages lysed one staphylococcal strain simultaneously. The results obtained are shown in Fig. 2 in the form of a block diagram. The height of a block indicates the percentage of simultaneous

Fig. 1. Lytic spectra of canine phage on indicator strains

	Phage No.															
	443	471	214	422	248	190	327	305	449	446	215	626	313	133	318	20
	a										a				a	
249	CL	CL	CL	CL	CL	—	—	—	—	—	—	—	—	—	—	—
	CL	CL	CL	+++	CL	—	—	—	—	—	—	—	—	—	—	—
	CL	CL	CL	++	CL	—	—	—	—	—	—	—	—	—	—	—
213	CL	CL	CL	CL	CL	—	—	—	—	—	—	—	—	—	—	—
	CL	CL	+++	CL	+++	—	—	—	—	—	—	—	—	—	—	—
	CL	+++	++	CL	++	—	—	—	—	—	—	—	—	—	—	—
463	CL	CL	CL	±	—	—	—	—	—	—	—	—	—	—	—	—
	CL	CL	CL	±	—	—	—	—	—	—	—	—	—	—	—	—
	CL	+++	CL	—	—	—	—	—	—	—	—	—	—	—	—	—
366	CL	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	+++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
492	—	—	—	—	—	CL	++	CL	—	—	—	—	±	—	—	—
	—	—	—	—	—	CL	+	++	—	—	—	—	±	—	—	—
	—	—	—	—	—	+++	±	+	—	—	—	—	—	—	—	—
313	—	—	—	—	—	CL	—	CL	—	—	—	—	—	—	—	—
	—	—	—	—	—	CL	—	CL	—	—	—	—	—	—	—	—
	—	—	—	—	—	+++	—	+++	—	—	—	—	—	—	—	—
201	—	++	—	—	—	CL	++	—	—	—	—	—	—	—	—	—
	—	+	—	—	—	CL	+	—	—	—	—	—	—	—	—	—
	—	±	—	—	—	+++	±	—	—	—	—	—	—	—	—	—
305	—	—	—	—	—	—	CL	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	+++	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	++	—	—	—	—	—	—	—	—	—
449	—	—	—	—	—	—	—	—	+++	CL	CL	—	—	—	—	—
	—	—	—	—	—	—	—	—	++	CL	CL	—	—	—	—	—
	—	—	—	—	—	—	—	—	+	++	CL	—	—	—	—	—
446	—	—	—	—	—	—	—	—	CL	—	++	—	—	—	—	—
	—	—	—	—	—	—	—	—	CL	—	+	—	—	—	—	—
	—	—	—	—	—	—	—	—	CL	—	±	—	—	—	—	—
347	—	—	—	—	—	—	—	—	—	—	—	CL	—	—	—	—
	—	—	—	—	—	—	—	—	—	—	—	CL	—	—	—	—
	—	—	—	—	—	—	—	—	—	—	—	CL	—	—	—	—
309	—	—	—	—	—	—	—	—	—	—	—	—	CL	—	—	—
	—	—	—	—	—	—	—	—	—	—	—	—	CL	—	—	—
	—	—	—	—	—	—	—	—	—	—	—	—	CL	—	—	—
80	—	—	—	—	—	—	—	—	—	—	—	—	—	CL	—	—
	—	—	—	—	—	—	—	—	—	—	—	—	—	CL	—	—
	—	—	—	—	—	—	—	—	—	—	—	—	—	CL	—	—
319	—	—	—	—	—	—	—	—	—	—	—	—	—	—	CL	—
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	CL	—
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	CL	—
43	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	CL
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	CL
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	CL

Remarks.

The upper, middle and lower levels present the lytic spectra of the phage at 100, 10 and 1 RTD, respectively.

lysis with other phages. Eight groups of phages differentiated in the lytic spectra were also discriminated in this figure, showing low frequencies of cross reactions among the groups, except the 2nd and the 3rd. Although these two groups could be dis-

tinguished from each other, cross reaction were observed between them more frequently than between any other two groups. For example, of seven strains lysed by phage 327 of the 2nd group, three were not only lysed by phage 305 of the same group, but

Fig. 2. Association of lytic reaction in phage patterns

Remarks.

Lytic reactions of $\pm$ and stronger were all regarded as positive.

also by phages 215a and 449 of the 3rd group. On the other hand, a considerable number of strains susceptible to the 3rd-group phages were also lysed by the 2nd-group phages. Accordingly, it seemed unnatural to consider the two groups independent from each other. These groups should be regarded basically as one and the same group.

Based on the results of the two experi-

ments mentioned above, the 16 canine phages were divided into groups, as shown in Table 5. Of them, five were classified into group I, six into group II, and one each into groups III to VII. Group II was further divided into sub-groups IIa and IIb of 3 phages each.

4. Grouping of other phages isolated

As already mentioned in the section "Isolation of phages", 28 phages were iso-

Table 5. Grouping of canine phages

Group	Phage No.	Propagating strain
I	443	463
	471	463
	214a	463
	422b	213
	248	249
II	IIa	190
		201
		327
	IIb	305
		313
		449
III	626	446
		449
		215a
IV	313	449
V	133	347
VI	318	309
VII	20a	80
		319
		43

lated from 23 lysogenic strains, and 16 of them selected for a canine phage set. Of the remaining 12 phages, eight were proved to be different from one another by their host ranges and the cross-resistance test. An attempt was made to classify these eight phages according to the grouping system of the canine phage set, using 20 indicator strains of strictly definite phage groups, including 16 propagating strains of the canine phages. As a result, four phages were classified into group I, one was into group IIa, and three were into group III. None of the phages showed a host range extending over two or more groups. These results indicated that the grouping system of the canine phage set was also applicable to other canine phages.

Discussion

The presence of free phages in the filtrate of staphylococcal broth culture was demonstrated by Callow [4], Burnet and Luch [2] and Rountree [12]. For the detection of phages, Hatano and Iwasaki [7] used the supernatant of untreated staphylococcal

broth culture. Their method sometimes resulted in failure of detecting phages when the bacterial growth was slow or poor. Therefore, in the present study, a release of phages from lysogenic strains was provoked by irradiation of ultraviolet ray [5, 9, 11, 14]. As a result, the presence of phages was demonstrated in 42 (28%) of 150 strains of canine staphylococci. This rate was lower than that, or 37.5%, reported by Jaekel et al. [8] who examined 154 staphylococcal strains isolated from lesions and normal sites of dogs. It was higher than that, or 7.7% reported by Blouse and Meekins [3] who observed 155 strains from diseased and healthy dogs.

The detection rate of lysogenic strains depends not only on the method of test, but also on the number and variety of indicator strains used. According to other previous investigators, the rate of lysogenic strains among canine staphylococci was generally lower than that among human [6, 7] or bovine [11] staphylococci, which was proved to be 40–50%. These facts suggest that staphylococcal strains of genetically similar characteristics may be distributed among dogs.

Of 47 phages derived from canine staphylococci, as many as 19 disappeared during passages on their propagating strains. In addition, 17 phages were not propagated to 100 RTD after repeated passages on propagating strains. Such an instability was also found in phages from canine staphylococci by Fouace and Guilhan [5] and Jaekel et al. [8], but rarely reported in phages from human and bovine staphylococci [6, 7, 11]. These phenomena may be caused by the instability of propagating strains used and/or the small burst size of phages from lysogenic strains.

One of the purposes of the present study was to establish a phage set for typing of

canine staphylococci. Sixteen phages called "canine phages" were selected for this purpose on the basis of the host range and readiness of propagation. Some of them were isolated from lysogenic strains which had shown lytic activity on as many as 6 to 11 indicator strains. Therefore, it is expected that more strains of canine staphylococci will be typed by the canine phages than by the existing N.C.T.C. or bovine phages. The usefulness of the canine phages will be examined in the next study.

Two methods can be used for grouping of phages. One of them is based on the serological characteristics of phages and the other on the host range. The latter is more practical for typing phages. Therefore, the canine phages were divided into 7 groups and 2 sub-groups according to the host range. Although this grouping system was not directly compared with that of the N.C.T.C. or bovine phages, no correlation might exist between the former and the latter 2 phage sets, because a small number of strains of canine origin was lysed by the N.C.T.C. and bovine phages [15].

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