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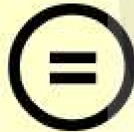

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A Thesis
For the Degree of Master of Veterinary Medicine

Isolation of bacteriophage for typing *Staphylococcus intermedius* isolated from dogs

Graduate School, Cheju National University
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2007. 2

Isolation of bacteriophage for typing *Staphylococcus intermedius* isolated from dogs

Hyun-ho Song
(Supervised by professor Won-geun Son)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Veterinary Medicine

2006. 12

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Department of Veterinary Medicine
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제주대

ABSTRACT

Isolation of bacteriophage for typing *Staphylococcus intermedius* isolated from dogs

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Staphylococcus intermedius, a coagulase-positive staphylococcal species, is a common canine pathogen and pus-forming bacterium in skin of dogs. Bacteriophage was isolated from 32 (71.1%) of 45 samples, including 32 dog skins and 13 feces, and some of these phage isolates showed same lytic pattern on *S. intermedius* isolates. Therefore, finally 20 different phages were used for typing 36 *S. intermedius* isolates, (12 nasal discharge, 11 bacterial dermatitis, 6 otitis externa, 2 cystitis, 1 pyometra and 4 isolates from nasal cavity of healthy dog) by the agar over layer method using nutrient medium. Primary phage tirers were ranging from 10^3 to 10^7 PFU/ml but the titers were expended ranging from 2 x 10^6 to 7 x 10^{11} PFU/ml to use as routine test dilution (RTD). Thirty-one (86.1%) of the 36 strains were typed either RTD or 100 X RTD into 25 phage patterns. Phage type (PT)-16 was the most common, but included only 4 *S. intermedius* isolates (11.1%). PT-8 and PT-16 have mainly participated in the respiratory disease, and PT-1 was associated with almost all kinds of diseases. Although, the bacterial lytic capability of 20 *S. intemedius* phage

(*SiP*) was various, but at least *SiP*-1, *SiP*-2, *SiP*-3, and *SiP*-4 lysed many *S. intermedius* isolates.

Key Words: *Staphylococcus intermedius*, Phage, Phage typing, Dog

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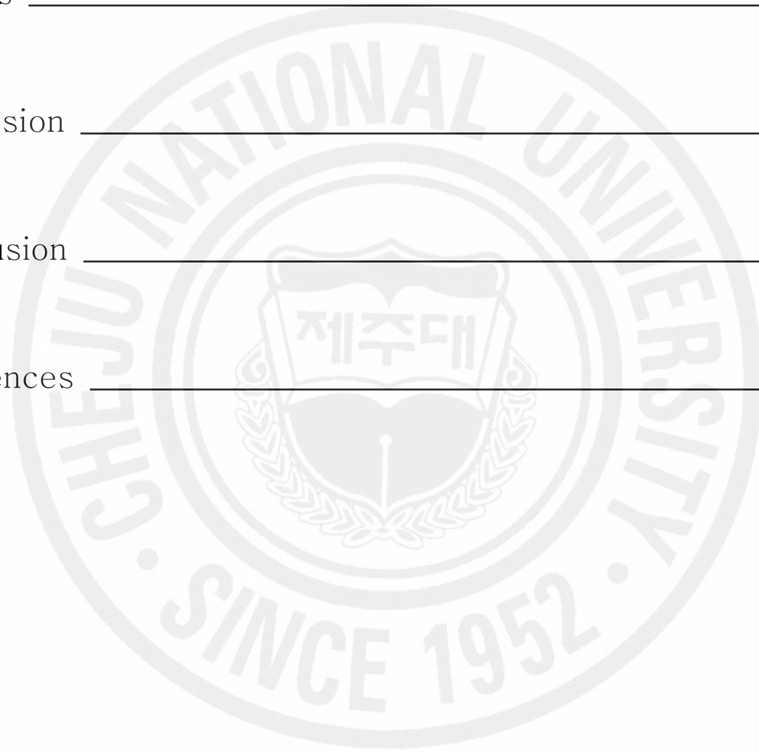


INTRODUCTION

Staphylococci are Gram-positive cocci, approximately 1 ㎛ in diameter, that tend to occur in irregular clusters resembling bunches of grapes. The name derives from the Greek words *staphyle* and *kokkos* for a ‘bunch of grapes’ and a ‘berry’ respectively. At least 30 *Staphylococcus* species are probably present in the mucous membranes and on other epithelial surfaces of all warm-blooded animals [5]. Most staphylococci are facultative anaerobes and catalase positive. They are non motile, oxidase negative and do not form spores. Two species, *S. aureus* subsp. *anaerobius* and *S. saccharolyticus* are anaerobic and catalase nagative. The coagulase positive *S. aureus* subsp. *aureus* and *S. intermedius*, and the coagulase variable *S. hyicus* are important pathogens of domestic animals. Coagulase production correlates with pathogenicity. Although coagulase negative staphylococci are usually of low virulence, some occasionally cause disease in animals and man [29].

S. aureus is most common pyogenic agents in humans and several animal species. Unlike other animals, *S. intermedius* is the leading pus-forming bacterium in dogs. *S. epidermidis* is universally present on skin and some mucous membranes, but it is rarely pathogenic. *S. hyicus*, which is found in several species, causes exudative epidermitis of swine and sometimes bovine mastitis. *S. schleiferi* subsp. *coagulans* is associated with otitis externa of dogs [21].

Increasing spread of polyresistant strains of *Staphylococcus* species is a problem of global extent [6]. For the control of the spread of these strains a number of epidemiologic typing methods are used: antimicrobial susceptibility testing (AST), biotyping, plasmid analysis, genomic restriction fragment length polymorphism analysis using pulsed-field gel

electrophoresis, and DNA hybridization [34, 40].

S. intermedius, a coagulase positive staphylococcus, is a common component of the skin and oral or nasal flora of normal dogs, horses, and other lower animals including some birds [27]. It is distinguishable from *S. aureus* by its slow fermentation of mannitol, a negative acetoin reaction, and a positive β-galactosidase reaction in the API Staph Ident system [44]. *S. intermedius* is also a common skin or wound pathogen in dogs [27] and an occasional pathogen in humans bitten by dogs [44].

Bacteriophage (phage) are obligate intracellular parasites that multiply inside bacteria by making use of some or all of the host biosynthetic machinery (i.e., viruses that infect bacteria.). There are many similarities between bacteriophages and animal cell viruses. Thus, bacteriophage can be viewed as model systems for animal cell viruses. In addition knowledge of the life cycle of bacteriophage is necessary to understand one of the mechanisms by which bacterial genes can be transferred from one bacterium to another. At one time it was thought that the use of bacteriophage might be an effective way to treat bacterial infections. In addition, bacteriophage are used in the diagnostic laboratory for the identification of pathogenic bacteria (phage typing). Although phage typing is not used in the routine clinical laboratory, it is used in reference laboratories for epidemiological purposes. Recently, new interest has developed in the possible use of bacteriophage for treatment of bacterial infections and in prophylaxis [11].

Phage typing was started to apply in England in 1940, widely spread, [6] and retains its significance until now[38, 51]. Typing of staphylococci is important in epidemiology, when it is needed to find the similarities and differences of the strains obtained from different sources, to determine epidemic strains of *Staphylococcus* species, and to evaluate the importance of different strains for animal infectious pathology[2].

The purpose of this study was to isolate phages of *S. intermedius* from

dog skin and fecal samples, and assess their potential use as typing phages. We also investigated typability of our cultures with our phages.

MATERIALS AND METHODS

Staphylococcus intermedius isolates

A total of 36 *S. intermedius* isolates were subjected to phage typing. The pathogens were isolated from dogs submitted to the Animal Hospital of the Cheju National University due to nasal discharge (n=12), bacterial dermatitis (n=11), otitis externa (n=6), cystitis (n=2), pyometra (n=1), during period from 2003 to 2006 and included 4 nasal cavity isolates of apparently healthy dogs (Table 1). All bacterial organisms were identified by API 20 Staph (Biomérieux, Marcy-l'Etoile, France) according to the manufacturer's protocol. The *S. intermedius* strains B1F8, YE9 and/or YA8 were used as the host for all phage propagation.

Table 1. *Staphylococcus intermedius* isolates used for phage typing

Source	No. of bacteria	Bacterial strains	Source	No. of bacteria	Bacterial strains
Nasal discharge	12	YB3	Bacterial dermatitis	11	YB8
		YD1			YC3
		YD4			B1E3
		YD8			B1F7
		YE2			B1G5
		YE6			B1H5
		YE8			B1H7
		YE10			B1F5
		YF2			B1F8
		YF9			B1G1
		YG1			B1G2
		YG2	Cystitis	2	B1I1
Otitis externa	6	YB4			B1I2
		YB5	Pyometra	1	YG5
		YB6	Normal nasal cavity	4	YA2
		YB7			YA3
		B1G7			YA4
		B1H2			YB1

Bacteriological media

All bacteriological media for bacterial culture and phage typing were purchased from Difco Ltd., MI, USA. Media using in this study are followed. Tryptic soy broth and agar were used for the activation of *S. intermedius* strains which have been stored at -80℃ deep freezer. Phage propagation and phage typing were conducted in nutrient broth and agar [23].

Sample collection for bacteriophage isolation

Samples were taken from 12 skins of experimental dogs in animal hospital, and 20 skins and 13 feces of stray-dogs in Jeju during December 2005 to March 2006. Skin samples were collected from abdominal sites using gauze (10 ㎝ × 10 ㎝) moisturized with sterile saline and fecal samples were collected directly from rectum using sterile wooden swab. All samples were transported to the laboratory and processed on the day of receipt.

Bacteriophage propagation from samples

To propagate phage from the samples, each colony *S. intermedius* strain B1F8, YE9 and YA8 activated onto nutrient agar were inoculated into 5 ㎖ nutrient broth and incubated at 37℃ for 18 to 24 h. And then each 200 ㎕ of bacterial cultures and 25 ㎖ of nutrient broth were added to 50 ㎖ Conical tube (SARSTEDT, Nümbrecht, Germany) containing skin

samples collected with gauze. In case of feces, 2 g of fecal sample and distilled water 30 ㎖ were added to 50 ㎖ Conical tube (SARSTEDT, Nümbrecht, Germany), and incubated at 37℃ for 30 min in shaking incubator (JEIO TECH, Seoul, Korea) at 200 rpm. Next, the cultures were centrifuged at 2,000 rpm for 30 min with centrifuge VS-5500 CFN (Vision, Seoul, Korea), 10 ㎖ supernatant fluids are transferred to 14 ㎖ polypropylene round-bottom tube (SPL, Seoul, Korea) and centrifuge at 3,500 rpm for 15 min. After that, 10 ㎖ supernatant fluids are transferred to 50 ㎖ Conical tube (SARSTEDT, Nümbrecht, Germany), containing 10 ㎖ of 2X Nutrient broth, and than each 200 ㎕ of bacterial cultures were inoculated. Those were incubated at 37℃ for 24 to 48 h in shaking incubator (JEIO TECH, Seoul, Korea) at 200 rpm to propagate unknown phages in samples.

Confirmation of bacteriophages from samples propagated

After propagation of unknown phage from samples, the cultures were centrifuged at 3,000 rpm for 30 min. The supernatant fluids were filtered through a 0.22 ㎛ membrane filter (Millipore, MA, USA), added 1 drop of chloroform to inhibit bacterial growth and stored at refrigerator before using.

To confirm the presence and absence of phage in samples incubated with *S. intermedius* strains, the organisms were prepared onto nutrient agar at 37℃ overnight, and the suspension density of the organisms was adjusted with a 0.5 McFarland standard with 0.85% sterile saline. The adjusted bacterial suspension was inoculated onto the nutrient agar with sterile swab, 10 ㎕ of phage solution propagated from samples was placed onto the medium, the plates were kept at room temperature for 10

min to allow the drop to dry, and were incubated at 37℃ for 24 h. The plate with bacterial lysis zone or plaque was considered to be phage in the samples.

Phage titeration

Tenfold serial dilutions (10^{-1} to 10^{-10}) of the phage suspension were prepared in 0.85% saline and 0.1 mℓ of each dilution poured on 0.5 mℓ bacterial cultures. Those were incubated at 37℃ for 20 min to be reacted bacteria and phage. These bacteria-phage solutions (0.6 mℓ) were mixed with 4 mℓ semisolid agar. The mixtures were poured on seeded solid agar lawns of each of the single strains. The lawns were prepared by inoculating 0.1 mℓ of bacterial culture into a plate and bacterial cultures were used to make a 0.5 McFarland standard. Next, the mixtures cooled (50℃) semisolid agar which was poured over a plate containing the hard basal layer [23].

Purification of bacteriophage

Each phage was purified by two transfers of single plaques [20]. A plaque was picked for long-term storage from the one of the least-crowded plate. Using either the large or small end of a blue tip (depending on the size of the plaque and the space around it), the plaque were pierced with the agar surrounding it, placed into the 1mℓ of brain-heart infusion (DIFCO, MI, USA), and incubated at 37℃ for 48 h. After that, the culture centrifuged at 8,000 rpm for 10 min with centrifuge MICRO 17TR (Hanil, Inchun, Korea) and filtered through a 0.22 ㎛ membrane filter (Millipore, MA, USA) and 1 drop of chloroform was added. Next, purified liquids stored in the refrigerator (4℃) before using

[3].

Phage typing

The routine test dilution (RTD) of each phage suspension was determined prior to used in the typing procedure [12]. The RTD was defined as the highest dilution that just failed to give confluent or complete lysis. *S. intermedius* strain B1F8 was used as the host for determining the RTD for each phage. Phage typing was performed by initial concentration of phages-1TD (test dilution-10^{-6}) and the strains, untypable by this concentration, were typed repeatedly diluting bacteriophage 100TD (test dilution-10^{-4}). A single colony from nutrient agar was inoculated into nutrient broth and incubated under stationary conditions for 18 h at 37℃. The young broth culture (0.1 ㎖) was inoculated onto a nutrient agar plate with sterilized swab. The plates were then allowed to dry at room temperature. Small drops (0.01 ㎖) of phage lysates at the RTD or 100 X RTD were then applied to the plates with a micropipette without touching the agar. The plates were kept at room temperature for at least 10 min to allow the drop to dry and incubated at 37℃ for 18 h and were examined for lysis. Complete lysis or confluent lysis (more than 20 plaques) within 10 ㎕ spot was recorded as +; a ± mark consisted of an area that was approximately weak lysed or severe clear spot (less than 20 plaques). A - mark was no plaques or opaque spot [29].

RESULTS

Isolation rates of *S. intermedius* phage

A total of 32 phages for *S. intermedius* (71.1%) were isolated from 45 samples, which of them, 23 (71.8%) and 9 (69.2%) phages were from 32 skin and 13 fecal samples, respectively (Table 2).

Table 2. Isolation rates of bacteriophage from skin and fece of dogs

Samples	No. of samples	No. of bacteriophage isolated	%
Skins	32	23	71.8%
Feces	13	9	69.2%
Total	45	32	71.1%

Plaque morphology of bacteriophage

Phages originated from both canine skins and canine feces produced similar plaque morphology. It was clear and round, but small and large plaques were simultaneously observed in all cases. Large phage plaque is same as small one in the plaque test after purification (Fig. 1).

Fig. 1. Morphology of plaques isolated *Staphylococcus intermedius* phages

Titeration of bacteriophages

Phage titers were variable ranging from 10^3 to 10^7 PFU/ml in primarily propagation of phage (Data not shown). Because these phage titers were too low to use for phage typing, all phages were propagated more than 1×10^6 PFU/ml to use as the RTD after purification of each phage plaque (Fig. 2 and Table 3). Original phage name was designated by sample name, such as CS-1 phage was from canine skin sample #1 and CF-1 was from canine fecal sample #1. After phage typing, each phage was renamed as *S. intermedius* phage (*SiP*) according to bacterial lysis patterns, indicating that phage

showing same bacterial lysis patterns was considered as same phage. Therefore, 20 of 32 phage isolates were selected to use in phage typing of *S. intermedius*.

Fig. 2. Representative titer of bacteriophage to *Staphylococcus intermedius* strain B1F8 which was determined by agar overlay method. A to G showed phage plaques which were produced in neat to X 10^{-6} the dilution concentration. This phage produced 1 X 10^{9} PFU/ml.

Phage typing

Twenty phage solutions were prepared from the preliminary typing experiments. Lytic activity was observed on 31 of 36 (86.2%) *S. intermedius* isolates, yielding 25 lytic patterns with individual strains susceptible to one

or more phage. Most of phage types had just one *S. intermedius* isolates, but phage type (PT)-16, PT-8, PT-15, and PT-21 was included 4 (11.1%), 3 (8.3%), 2 (5.5%), and 2 (5.5%) *S. intermedius* isolates (11.1%).

Table 3. Bacteriophage titer and name

Rename of phage	Original phage name	PFU/ml	Rename of phage	Original phage name	PFU/ml
*Si*P-1	CS-24	4×10^{10}	*Si*P-12	CS-16	1×10^{7}
*Si*P-2	CF-13	1×10^{7}	*Si*P-12	CS-18	1×10^{6}
*Si*P-3	CS-23	1×10^{11}	*Si*P-13	CF-11	3×10^{6}
*Si*P-4	CS-7	6×10^{6}	*Si*P-14	CS-10	2×10^{6}
*Si*P-4	CS-15	1×10^{6}	*Si*P-15	CF-2	2×10^{10}
*Si*P-5	CS-11	5×10^{8}	*Si*P-16	CF-12	7×10^{8}
*Si*P-6	CS-32	2×10^{11}	*Si*P-16	CS-17	4×10^{9}
*Si*P-7	CS-6	3×10^{7}	*Si*P-16	CS-19	6×10^{7}
*Si*P-8	CS-8	2×10^{7}	*Si*P-16	CS-20	4×10^{7}
*Si*P-9	CS-13	9×10^{9}	*Si*P-17	CS-1	2×10^{8}
*Si*P-10	CS-26	3×10^{11}	*Si*P-17	CS-2	1×10^{8}
*Si*P-11	CS-22	9×10^{10}	*Si*P-17	CS-12	9×10^{8}
*Si*P-12	CF-1	5×10^{6}	*Si*P-18	CS-14	1×10^{9}
*Si*P-12	CF-9	2×10^{6}	*Si*P-19	CS-5	7×10^{8}
*Si*P-12	CF-10	2×10^{7}	*Si*P-19	CF-3	2×10^{9}
*Si*P-12	CS-9	9×10^{6}	*Si*P-20	CF-4	7×10^{11}

Table 4. Definition of the types in the phage typing system.

Phage type	Reaction with phage (n)																				No. of isolates (%)
	SiP-1	*SiP*-2	*SiP*-3	*SiP*-4 (2)	*SiP*-5	*SiP*-6	*SiP*-7	*SiP*-8	*SiP*-9	*SiP*-10	*SiP*-11	*SiP*-12 (6)	*SiP*-13	*SiP*-14	*SiP*-15	*SiP*-16 (4)	*SiP*-17 (3)	*SiP*-18	*SiP*-19 (2)	*SiP*-20	
1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	5 (13.8)
2	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	1 (2.7)
3	−	±	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	1 (2.7)
4	+	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	1 (2.7)
5	+	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	1 (2.7)
6	+	±	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	1 (2.7)
7	−	±	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	1 (2.7)
8	+	±	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	3 (8.3)
9	−	±	−	±	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	1 (2.7)
10	−	±	−	±	±	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	1 (2.7)
11	−	±	±	±	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	1 (2.7)
12	+	−	+	−	−	±	−	−	±	±	−	−	−	−	−	−	−	−	−	−	1 (2.7)
13	−	±	−	±	±	±	−	±	−	−	−	−	−	−	−	−	−	−	−	−	1 (2.7)
14	+	±	+	±	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	1 (2.7)
15	+	±	+	±	−	±	−	−	+	±	−	−	−	−	−	−	−	−	−	−	2 (5.5)
16	+	±	+	±	−	+	−	−	±	+	+	−	−	−	−	−	−	−	−	−	4 (11.1)
17	−	±	−	+	−	−	+	+	±	−	−	+	−	−	−	−	−	−	−	−	1 (2.7)
18	+	±	±	+	−	−	±	+	−	−	±	+	−	−	−	−	−	−	−	−	1 (2.7)
19	−	+	−	+	−	−	±	+	−	−	−	±	±	±	±	−	−	−	−	−	1 (2.7)
20	±	+	±	+	−	±	±	+	±	−	−	±	+	+	−	−	−	−	−	−	1 (2.7)
21	−	+	−	+	+	−	+	+	−	−	−	+	+	−	−	+	+	−	−	−	2 (5.5)
22	±	±	+	±	±	±	±	±	−	±	±	+	+	+	+	±	−	±	−	−	1 (2.7)
23	−	+	±	+	+	−	+	+	−	−	−	+	+	+	+	±	±	+	±	−	1 (2.7)
24	+	+	+	+	±	+	+	+	−	±	+	+	+	+	+	+	±	+	−	±	1 (2.7)
25	±	+	±	+	±	±	+	+	±	±	±	+	+	+	+	+	±	+	+	±	1 (2.7)

+, more than 20 plaques, semiconfluent lysis, confluent lysis, i.e., strong lytic reaction; ±, less than 20 plaques, i.e., weak lytic reaction; −, no plaque; (n), no. of phages with same lysis patterns

Distribution of phage types in different samples

Distribution of phage types was various according to the origin sites of samples (Table 5). It is difficult to compare phage type with a specific disease, however the most of *S. intermedius* isolates had various phage types. Nevertheless, the isolates showing same phage type, such as PT-8 and PT-16 have participated in respiratory disease, and PT-1 was associated with almost all of lesions.

Table 5. Phage types of 20 phages by isolate source of *Satphylococcus intermedius*

<table>
<tr><th>Source</th><th>Bacterial stains</th><th>Phage type</th><th>Source</th><th>Bacterial stains</th><th>Phage type</th></tr>
<tr><td rowspan="12">Nasal discharge</td><td>YE8</td><td>PT-1</td><td rowspan="11">Bacterial dermatitis</td><td>B1E3</td><td>PT-1</td></tr>
<tr><td>YG2</td><td>PT-1</td><td>B1G5</td><td>PT-10</td></tr>
<tr><td>YD1</td><td>PT-3</td><td>B1F7</td><td>PT-12</td></tr>
<tr><td>YD8</td><td>PT-4</td><td>YB8</td><td>PT-16</td></tr>
<tr><td>YE2</td><td>PT-8</td><td>YC3</td><td>PT-18</td></tr>
<tr><td>YE6</td><td>PT-8</td><td>B1H5</td><td>PT-24</td></tr>
<tr><td>YF2</td><td>PT-8</td><td>B1H7</td><td>PT-23</td></tr>
<tr><td>YB3</td><td>PT-15</td><td>B1F5</td><td>PT-2</td></tr>
<tr><td>YG1</td><td>PT-16</td><td>B1G1</td><td>PT-15</td></tr>
<tr><td>YE10</td><td>PT-16</td><td>B1G2</td><td>PT-19</td></tr>
<tr><td>YF9</td><td>PT-16</td><td>B1F8</td><td>PT-25</td></tr>
<tr><td>YD4</td><td>PT-17</td><td rowspan="2">Cystitis</td><td>B1I2</td><td>PT-1</td></tr>
<tr><td rowspan="6">Otitis externa</td><td>YB7</td><td>PT-1</td><td>B1I1</td><td>PT-22</td></tr>
<tr><td>YB5</td><td>PT-6</td><td>Pyometra</td><td>YG5</td><td>PT-14</td></tr>
<tr><td>YB6</td><td>PT-7</td><td rowspan="4">Normal nasal cavity</td><td>YA2</td><td>PT-5</td></tr>
<tr><td>B1H2</td><td>PT-9</td><td>YB1</td><td>PT-13</td></tr>
<tr><td>B1G7</td><td>PT-11</td><td>YA3</td><td>PT-21</td></tr>
<tr><td>YB4</td><td>PT-20</td><td>YA4</td><td>PT-21</td></tr>
</table>

DISCUSSION

Since the discovery of bacteriophages made independently by Twort in England and D' Herelle in France in 1916-1917, a number of bacterial viruses (Bacteriophage) have been adopted for use in current diagnostic methodology [33].

Bacteria have been identified as the main cause for disease outbreaks [20]. Defining an effective and preventive treatment involves a characterization of the disease outbreak by identifying its pathogens. The identification of pathogens in a bacteria level or bacteria-species level is not satisfactory for epidemiological and clinical concerns, in particular due to increasing bacterial adaptation to human environments including resistance of bacteria to antimicrobial agents. Therefore, a bacterial type diagnosis is required, i.e., the identification of pathogens below the species level [7, 20, 36, 46]. This yields information for controlling the disease. Subgrouping of bacterial species to types (bacterial types) are used for many important pathogenic bacteria such as the *Salmonella* species and the *Staphylococcus* species. The *Staphylococcus* species are a major cause of community-acquired infections as well as farm animals' diseases such as mastitis of lactating cows [4, 7, 20].

Phage typing is a method for determining the species reactivity to a set of selected bacteriophages (phages) [20], hence, to define its type. A phage is a bacterial virus activated by specific bacterial surface constituents of the checked species. The phage receptor binds to a matching bacterial surface component, invades and multiplies in the bacterial host. When a phage infects a layer of bacterial cells, a zone of lysis produces a plaque, viewed as a clear area in the bacterial lawn, such as the full circles (spots). These represent positive reactions to

different phages. When the phage receptor does not recognize any of the tested bacterial surface constituents, no plaque is formed and it is defined as a negative reaction. In this case, no surface change is visible. The molecules from each phage strain, involved in interactions such as described, are specific for bacterial types and are known to correlate with important epidemiological factors. For this reason, bacteriophage typing has been a useful epidemiological tool and has been utilized in typing *Salmonella*, *Shigella sonnei*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, and *S. aureus* [9, 43, 54]. Since bacteriophage typing is not a routine procedure, its use should be restricted to the analyses of those specimens collected during overt epidemiological outbreaks. The use of this technique in tracing the source and routes of spread of hospital-acquired infections has been most effective [33].

Recently, a new approach for phage production and phage typing has been developed by Spring Diagnostics. Spring Diagnostics arrays are the visual input to the proposed system. The images are scanned using a printhead, Powerlook2 model, with a transparency adaptor. The Petri-dishes seen in the images contain a surface of *Staphylococcus* species. Reactions to different phages are present on the surface of the dish. The reactions are organized in a fixed array and known order. An image group contains a set of images. A given database consists of image groups, each group representing a particular *Staphylococcus* species bacterial type. A significant variability between the scanned images and irregularities in each image exist within a given database. Image contrast and dynamic range are considerably different across the image group. Reaction shapes and sizes are irregular, both within an image as well as across the images. Reactions are not positioned in a uniform layout. Finally, the background, i.e., the dish surface, also exhibits nonuniformity due to inevitable differences in experimental conditions, and variability in the pigmentation of bacterial isolates [42, 47, 50]. The

production technology enables a much larger quantity of typing phages than the present international sets that are used. A phage typing experiment consists of placing the phages on a monolayer of pure bacterial culture by using a printhead. The distinction between positive and negative reactions is defined by parameters of brightness level, size of reaction, and graininess level. Significant expertise to perform and to interpret the results still yields ambiguous results of typing information. Large variability exists in the decision making process and the analysis is time-consuming [46].

In addition bacteriophages have been used to prevent and treat various bacterial infections. Although phage therapy has been historically asssocited with the use of bacteriophages in human medicine, phages also have been extensively used in veterinary medicine and in various agricultural settings. Many studies review the past and current use of phages to prevent and treat naturally occurring and experimentally induced infections of animals. In addition they discuss the potential applications of phage therapy in various agricultural settings, including the potential value of bacteriophages for improving the safety of foods and preventing foodborn diseases of bacterial etiology, and their potential to reduce the use of antibiotics in livestock. The first-known therapeutic use of phages in veterinary medicine is associated with Felix d'Herelle, the co-discoverer of bacteriophages. Early attempts at phage treatment of experimentally induced staphylococcal and streptococcal septicaemias in rabbits and mice were repored to be unsuccessful by several investigator [12, 31], including Giorgia Eliava [19]. Also, many attempts to treat experimental plaque in rabbits, guinea pigs, rats, and mice failed to influence the course of the disease [14, 15, 16, 35].

More research is likely to provide much needed information in that regard, and to generate critical data needed for the optimal design and

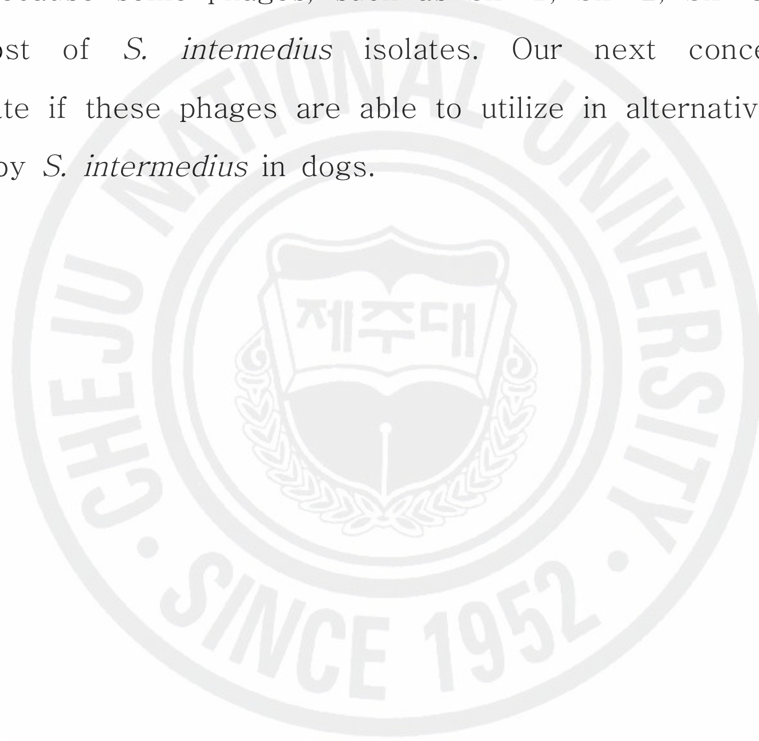
implementation of phage-mediated prophylaxis and therapy of *staphylococcs* infection in various animals [17, 18].

In this study, 36 cultures confirmed as *S. intermedius* represented isolates from a variety of clinical sources. It was established that 5 (14 %) of these strains were nontypable. Consideration was given to two concepts that might be employed singly or in combination to reduce the percentage of nontypable strains. First, an expansion of phage typing sets might be considered, i.e., incorporation of available phages isolated from canine sources [10]. Second, it is conceivable that some of the nontypable strains might be susceptible to phages originated from sites other than samples used in this study [11]. This latter technique has been employed with increasing frequency, and investigators have noted a variety of patterns displayed by staphylococci [30]. However. those phages isolated in this study used in conjunction with the currently accepted basic set of typing phages and may be the additional tools necessary in the epidemiological investigation of presently refractile *staphylococcus* strains. Phage typing systems for canine staphylococci similar to *S. intermedius* have been described previously by American [27] and French [22] investigators. Early phage sets consisted of four to five phage and were capable of typing 67 to 74% of canine isolates [22, 27], whereas traditional bovine and human phage sets were capable of typing less than 10% of canine staphylococci [53]. Chinese and Japanese investigators have also described phage sets derived from presumptive *S. intermedius* isolates (i.e., *S. aureus* biotyoes E and F) utilizing eight to nine phages [41, 52, 53]. Although Shimizu and Kato [41] were able to classify 72.4% of their isolates in 13 lytic patterns, Wang [52, 53] was able to type only 10% of isolates in 12 lytic patterns.

In this evaluation of *S. intermedius*-derived lytic bacteriophages, 31 (86.0%) of 36 of *S. intermedius* isolates from various sources displayed

lytic patterns. Although PT-1 was observed more frequently regardless of bacterial sources, most of phage types of *S. intermedius* isolates were specific to the disease and 4 *S. intermedius* isolates from normal dog was also yielded different phage types. These results indicate that *S. intermedius* may be a unique subset depending on the canine disease, suggesting an associated pathogenicity with certain phage types.

In this study, we have not examined in detail the phage therapy on canine diseases. However, phage typing showed the potential of phage therapy because some phages, such as *Si*P-1, *Si*P-2, *Si*P-3, and *Si*P-4, lysed most of *S. intemedius* isolates. Our next concerns are to demonstrate if these phages are able to utilize in alternative therapy on infection by *S. intermedius* in dogs.


CHEJU NATIONAL UNIVERSITY · SINCE 1952 · 제주대

CONCLUSION

We tried to isolate phages from well-charaterized clinical and healthy isolates of *Staphylococcus intermedius* and assess their potential use as typing phages.

1. Bacteriophage isolated from dogs 23 of 32 (71.8%) canine skins and 9 of 13 canine feces samples (69.2%) were isolated by agar over-layer method.

2. Phage titer was observed in $1 \times 10^{8} \sim 3 \times 10^{11}$ PFU/ml in canine skins and $2 \times 10^{6} \sim 7 \times 10^{11}$ PFU/ml in canine feces.

3. Isolated bacteria was observed on 25 lytic patterns with individual strains susceptible to one or more phage. Phage type (PT)-16 was the most common and but included only 4 *S. intermedius* isolates (11.1%).

4. Phage type (PT)-8 and PT-16 have mainly participated in the respiratory disease, and PT-1 was associated with almost all kinds of diseases.

REFERENCES

1. Allaker, R. P., Lamport, A. I., Lloyd, D. H. and Noble, W. C. Microbiol. Ecol. Health. Dis. 1991. 4:169-173.

2. Akatov, A.K., Zujeva, V.C. Vnutrividovoje diferencirovanije stafilokokov: stafilokoki. (Differentiation staphylococci inside of strains: staphyllococci.) 1983. p. 53-100

3. Antje, W., Stefan, S.B., Hans, R.G., Thorsten, B., Gerard, M. and Christian, S. Bacteriophage Diversity in the North Sea. Appl. Environ. Microbial. 1998. 64:4218-4133

4. Arbit R. D. Laboratory procedures for the epidemiologic analysis of microorganism in manual of clinical microbiology. 7th ed. Washington, DC: Amer. Soc. Microbiol. 1999. 7:116-137

5. Asako, S., Akira, S., Junichi, K., Yoshihisa, W., Toshikatsu, H., and Shigenobu, O. Characteristics of *Staphylococcus intermedius* Isolates from Diseased and Healthy Dogs. J. Vet. Med. Sci. 2005. 67(1):103-106

6. Ayliffe, G.A.J. The progressive intercontinental spread of methicillin-resistant *Staphylococcus aureus*. Clin Infect Dis. 1997. 24:79-9

7. Baron S. Medical Microbiogy, 4th ed. Galveston, TX: Univ. Texas 1996. Med. Branch, Sch. Med.,

8. Biberstein, E. L., Jang, S. S. and Hirsh D. Species distribution of

coagulase-positive staphylococci in animals. J. Clin. Microbiol. 1984. **19**: 610-615

9. **Blair, J. E., and R. E. Wilians.** Phage typing of staphylococci. Bull. W.H.O. 1961. **24**:771-784

10. **Blouse, L. E., Mauney, C. U., Marraro, R. V. and Dupuy, H. J.** Apparent spontaneous induction of *Staphylococcus aureus* isolated from clinical source. Appl. Microbiol. 1972. **23**:1023-1024.

11. **Blouse**, L. E., **and Meekins.**, W. E. Isolation and use of experimental phages for typing *Staphylococcus aureus* isolates from sentry dogs. Am. J. Vet. Res. 1968. **29**:1817-1822

12. **Clark**, P.F. **and Clark**, A.S., Bacteriophage active against virulent hemolytic streptococcus. Proc Soc Exper Biol & Med. 1927. **24**: 635-639

13. **Cole**, L. K., **Kwochka**, K. W., **Kowalski**, J. J. **and Hillier, A.** Microbial flora and antimicrobial susceptibility patterns of isolated pathogens from the horizontal ear canal and middle ear in dogs with otitis media. J. Am. Vet. Med. Assoc. 1998. **212**: 534-538

14. **Colvin, M.G.,** Relationship of bacteriophage to natural and experimental diseases of laboratory animals, with special reference to lymphadenitis of guinea pigs. J. Infect Dis. 1932. **51**: 17-29

15. **Compton, A.,** Immunization in experimental plaque by subcutaneous inoculation with bacteriophage. Comparison of plain and formaldehyde-treated phage-lysed plaque vaccine. J Infect Dis. 1930. **46**:

152-160

16. Compton, A., Sensitization and immunization with bacteriophage in experimental plaque. J Infect Dis. 1928. 43: 448-457

17. d'Herelle, F., Le baceriophage: son role dans limmunite. Masson et Cie, Paris, 1921

18. d'Herelle, F., The bacteriophage and Behavior. Williams and Wilkins, Baltimore, Maryland, 1926

19. Eliava, G., Au sujet de ladsorption de bacteriophage par les leucocytes. *Comp rend Soc de biol.* 1930.105: 829-831

20. Emori T. G. and Gaynes R. P. An overview of nosocomial infections, including the role of the microbiology laboratory. Clin. Microbiol. Rev. 1993. 6:428-442

21. Ernst, L.B., Dwight, C.H. Veterinary Microbiology, Blackwell Publishing Professional, New York, 1999. p.115.

22. Fouace, J., and J. Guilhon. Etude bacteriologique de souches de staphylocoques d'origine canine. Essai de lysotypie au moyen de nouveaux phage. Ann. Inst. Pasteur 1966. 111:334-344

23. Gary, D.O., David, A.T., Kathleen, S., Nancy, A., J., I.M., Russell, T.G. and Seymour, F. Phage Typing of *Staphylococcus intermedius*. J. Clin. Microbiol. 1991. 1:180-184

24. **Gelderblom, H.R., Renz, H., Özel, M.** Negative staining in diagnostic virology. Micron Microsc Acta. 1991. **22**:435–447.

25. **Gelderblom, H.R., Bauer, H., Frank, H., Wigand, R.** The structure of group II adenovirus. J Gen Virol. 1967. **1**:553–560.

26. **Greene, R. T. and Lammler, C. H.** J. vet. Med. B 1993. **40**: 206-214

27. **Hajek, V.** *Staphylococcus intermedius*, a new species isolated from animals. Int. J. Syst. Bacteriol. 1976. **26**: 401-408

28. **Hazel, M.A. and Kate W.** Reaction Difference Rule for phage Typing of *Staphylococcus aureus* at 100 Times the Routine Test Dilution. J. Clin. Microbiol. 2001. **40**:292-293

29. **Henry, W.T., JR., and Joseph, T.P.** Phage Typing of *Staphylococcus epidermidis*. J. Clin. Microbiol. 1976. **3**:519-523

30. **Klatersky, J., Beumer, J. and Daneau, D.** Bacteriophage types and antibiotic susceptibility of *Staphylococcus aureus*. Appl. Microbiol. 1971. **22**:1000-1007

31. **Krueger, A.P., Lich, R. and Schulze, K.R.,** Bacteriophage in experimental staphylococcal septicemia. Proc Soc Exper Biol & Med. 1932. **30**: 73-75

32. **Lloyd, D. H., Allaker, R. P. and pattinson, A.** Vet. Dermatol. 1991. **2**: 161-164.

33. Marraro, R. V. and Mitchell J. L. Experiences and Observations with the Typing of *Saphylococcus aureus* Phage 94. J Clin Microbiol. 1974. p. 180-184

34. Murchan, S., Trzcinski, K., Skoczynska, A., Van Leeuwen W., Van BElkum, A., Pietuszko, S., et al. Spread of old and new clones of epidemic methicillin-resistant *Staphylococcus aureus* in poland. Clin Microbiol Infect. 1998. 4:481-90.

35. Naidu, B.P.B and Avari, C.R., Bacteriophage the treatment of plaque. Ind Jour Med Res. 1932. 19: 737-748

36. Patrick A. and Grimont D. Taxonomy and classification of bacteria in manual of clinical microbiology. 7th ed. Washington, DC: Amer. Soc. Microbiol. 1999. 14:249-261

37. Phillips, W. E. Jr. and Kloos, W. E. Identification of coagulase-positive *Staphylococcus intermedius* and *Staphylococcus hyicus* subsp. *hyicus* isolates from veterinary clinical specimens. J. Clin. Microbiol. 1981. 14: 671-673

38. Pitt, T.L. Bacterial typing system: the wayahead. J Med Microbiol. 1994. 40:1-2

39. Quinn, P.J., Markey, B.K., Cater, M.E., Donnelly, W.J.C., Leonard. Veterinary Microbiology and Microbial Disease, Blackwell Publishing Professional, New York, 2002. p.43

40. Saulnier, P., Bourneux, C., Prevost, G., Andremont, A. Random amplified polymorphic DNA assay in less discriminatory than pulsed field gel electrophoresis for typing strains of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol. 1993. **31**:798-803.

41. Shimizu, A., and Kato, E. Bacteriophage typing of *S. aureus* isolated from dogs in Japan. Jpn. J. Vet. Sci. 1979. **41**:405-408.

42. Spring Diagnostics. [online]. Available: http://www.itek.co.il/spring

43. Subcommittee on phage-typing of Staphylococci of the International Committee on Nomenclature of Bacteria, Moscow, July 1996. 1967. Report. Int. J. Syst. Bacteriol. 17:113-125

44. Talan D. A., Goldstein E. J. C., Staatz D. and Overturf G. D. *Staphylococcus intermedius*: clinical presentation of a new humen dog bite pathogen. Ann. Emerg. Med. 18:410-412

45. Talan, D. A., Staatz. A. Staatz, and Overturf G. D. Frequency of *Staphylococcus intermedius* as human nasopharyngeal flora. J. Clin. Microbial. 1989. 27:2393.

46. Tenover F. C., Arbeit R. D. and Goering R. V. How to select and interpret molecular strain typing methods for epidemiological studied of bacterial infections: A review for healthcare epidemiologists. Infection Contr. Hospital Epidemiol. 1997. 18:426-439

47. Teper G., Ziv G. and Skutelski E. Flow cytometry analysis of *S. aureus*-Bacteriophage interaction. proc. 3rd Int. Mastitis Sem. 1995. 1:8

48. **Topley, W.W.C. and Wilson, J.,** Further observations of the role of the Twort-d'Herelle phenomenon in the epidemic spread of murine typhoid. J Hyg. 1925. **24**:295-300

49. **Topley, W.W.C. and Wilson, J. and Lewis, E.R.,** Role of Twort-d'Herelle phenomenon in epidemics of mouse typhoid. J hyg. 1925. **24**: 17-36

50. US PCT Patent application no. PCT/IL00/00 366.

51. **Van Belkum, A., Bax, R., Peebooms, P., Goessens,** W.H.V., **Leeuwen, N., Quint,** W.G.V. Comparison of phage typing and DNA fingerprinting by polymerase chain reaction for discrimination of methicillin-resistant *Staphylococcus aureus* strains. J Clin Microbiol. 1993. **31**:798-803

52. **Wang C. T.** Bacteriophage typing of canine staphylococci. I. Typing by use of the international phage sets for human and bovine staphylococci. Jpn. J. Vet. Sci. 1979. **40**:401-405

53. **Wang, C. T.** Bacteriophage typing of canine staphylococci. II. Phage typing by use of canine phage with special reference to the epizootiology of staphylococcus. Jpn. J. Vet. Sci. 1978. **40**:633-643

54. **Wentworth, B. B.** Bacteriophage typing of the staphylococci. Rev. 1963. **27**:253-272

55. **Wrigley, N. G.** The lattice spacing catalase as an internal standard of length in electron microscopy. J Ultrastruct Res. 1968. **24**:454-464.

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