［原　　著］

Phage set for methicillin-resistant *Staphylococcus aureus* (MRSA) strains combined with the international phage typing set and the phages from coagulase-negative staphylococci

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Phage typing has been a powerful tool for typing *Staphylococcus aureus*. However, when phage typing is applied to methicillin-resistant strains of *S. aureus* (MRSA), many strains remain non-typable (NT). In the previous report, most clinical isolates of methicillin-sensitive *S. aureus* (MSSA) could be grouped by a coagulase-negative staphylococci (CNS) phage set, as so-called type II. We tested 16 CNS phages for typing 158 strains of MRSA and the 22 phage propagating *S. aureus* strains. We chose four phages (Ph10, Ph12, U14, H96), because of their good discriminatory power, and added them to the international phage typing set (IPS) for *S. aureus*. These phages from CNS were classified as miscellaneous CNS group. The results obtained by this new combined phage set showed higher typeability and more discriminatory power than that by the IPS alone.

Six MRSA isolates from an outbreak in a surgical ward were NT by the IPS. However, with these additional CNS phages, three isolates became typable as phage pattern Ph10/Ph12/U14 while others remained NT. These six strains were further analyzed according to their restriction fragment length polymorphism by pulsed-field gel electrophoresis (PFGE) using the restriction enzyme *Sma*I. PFGE patterns of these strains fell into two groups, which coincided with those by typing with the new combined phage set.

Key words: methicillin-resistant *Staphylococcus aureus* (MRSA), bacteriophage typing

Methicillin-resistant strains of *Staphylococcus aureus* (MRSA) caused hospital infections throughout the world. The spread of MRSA in hospitals has been the major problem in Japan. A lot of investigations of MRSA by phage typing have been reported since MRSA was first identified in 1961[1]. The international phage typing set (IPS) has been used for phage typing of MRSA, but many strains (33～75%) are non-typable (NT) and even the typable ones give only restricted number of phage reactions with the available phages[2,3]. Various methods of typing MRSA strains have been used for epidemiological studies and compared with phage typing[4~10]. Some methods have enhanced the discrimination of MRSA typing. Tenover et al. reported that ultimately a combination of two methods might be the most effective one in epidemiological studies of MRSA[4]. One method, that would be sensitive enough to include all potential patients or sources, may be used for screening isolates in an early phase of an outbreak, and the other method for differentiating strains in detail may be used later.

Hoshina[11] have reported that *S. aureus* JCM 2413 is lysed by Pulverer's phages (Ph5, Ph9, Ph10, Ph12, Ph13, U4, U14, U16, U46) and Verhoef's phage (48(240)), which were isolated from coagulase-negative staphylococci (CNS). We added four phages isolated from CNS to the IPS. In this paper, we report an

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increased typeability of phage typing for MRSA strains with additional four supplementary phages in IPS.

Ⅰ. Materials and methods

1. Bacterial strains

A total of 158 MRSA isolates and 112 methicillin-sensitive *S. aureus* (MSSA) isolates were collected at the clinical laboratory of the Jikei University Hospital in Tokyo, Japan, between January to October in 1992. Additional 156 MRSA strains isolated in 1989～1991 at the Nagoya University Hospital were kindly provided by Dr. Satoshi Ichiyama. Thirty MRSA strains isolated in 1999 were kindly provided by Dr. Itaru Furuta of the Kinki University Hospital. Only one MRSA isolate from each patient was included.

Eleven clinically isolated MRSA were collected for clinical and epidemiological purposes from six patients at a surgical ward of the Jikei University Hospital in August 1994.

Twenty two propagating strains of phages of the IPS[12] were used to determine the host ranges and the activity spectrum of phages.

2. Susceptibility testing

Methicillin resistance of *S. aureus* was determined by MRSA screening agar containing 6μg /ml oxacillin (Becton Dickinson Company Co., Ltd., U.S.A.). Confirmation for methicillin resistance was performed by determining of the MIC of oxacillin by the standard agar dilution method recommended by the Japan Society for Chemotherapy[13].

3. Bacteriophage typing

We performed bacteriophage typing by the standard methods with slight modifications[14]. The routine test dilution (RTD) phage was determined by two fold dilution of the phage solution instead of ten-time serial dilution of the the standard method, and 4μl of RTD solution were dropped onto the bacterial lawn with Micro pipette (8-channel) (Sumitomo Bakelite Co., Ltd., Tokyo). Bacterial cells were inoculated into nutrient broth (Oxoid No.2) at about 10^7 CFU/ml, which corresponds to MacFarland No. 0.5. For the phage typing 9 centimeter typing agar plates (2% of Oxoid Nutrient broth No. 2, 0.75% of Oxoid Agar No. 1, 0.5% of NaCl (Nacalai tesque), 4 mM $CaCl_2$ (Nacalai tesque)) were flooded with 2 ml of bacterial suspension and the excess fluid was pipetted off. The plates were dried with their lids removed for 30 min. at room temperature. After phage inoculation, the plates were incubated at 30℃ for 18 h. Twenty three of *S. aureus* typing phages[14] and 16 phages of CNS[11] were used. The lysis was described in the following scales. +++ =confluent lysis, +++' = confluent lysis with second growth, ++= semi confluent lysis, +=more than 10 plaques, +/- = less than 10 plaques; when the number of plaques is 10 or less it is recorded by number (e.g.± 5). More than one plus (+) reaction was recorded as positive result. The reproducibility of phage typing was calculated by repeating all 158 MRSA strains. The second test was carried out 2 months after the initial test.

4. Numerical analysis

The Jaccard coefficient (Sj) was used as the basis of the comparisons of two phages selection: Sj=a/(a+b+c), where a was the number of positive matches, b and c were the numbers obtained from dissimilar test[15].

The discriminatory power (D) of a typing method is its ability to distinguish between unrelated strains.

This index is given by the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

where N is the total number of strains in the sample population, (s) is the total number of lysis pattern described, and (n_j) is the number of strains belonging to the (j) th lysis pattern[16].

5. Pulsed-field gel electrophoresis (PFGE)

Chromosomal DNA from *S. aureus* was prepared in agarose blocks and was cleaved with *Sma* I by the method of Prevost et al.[17]. The samples were run on 1% agarose gel in 0.5X TBE buffer on a CHEF-DRII PFGE system (Bio-Rad). Electrophoresis was carried out at 150 V at 13℃, in a 25- to 50-s pulse time gradient for 20 hours and then a 5- to 20-s gradient for 21 hours. A Lambda concatemer (Bio-Rad) and the yeast chromosome (Bio-Rad) were used as molecular size markers.

Ⅱ. Results

The 16 phages of CNS typing set[11] were screened at RTD on the 22 propagating strains of the IPS (Table 1), and on the 158 MRSA strains at the Jikei University Hospital (Table 2). Lysis by six polyvalent phages (Ph5,

Table 1 Activity spectrum of 16 CNS phages in RTD on the propageting strains for the international phage set for *S. aureus*

Group	propagating strain	The phages of the coagulase-negative staphylococci (CNS) phage set														
		Ph5	Ph10	Ph12	Ph13	U14	48(240)	H96	29	95	68	71(382)	48(407)	TU	Ph15	U20	71(240)
I	29	+++	++		+	+	++										
	52	++	+++	+	++		+										
	52A/79	++	+++	++	+	+	++										
	80	+++	+++	+++	+++’	++	+++										
II	3A	+++	++	+++’	+++	++	++										
	3C	+++	+++	+++	+++	++	+++										
	55	+++	+++	+++	+++	+++	+++										
	71	+++	+++	+++	+++	+++’	+++										
III	6	++	+	+	++	+	+										
	42E	+++	+++	+	+++	++	++										
	47		+														
	53	++	+++	++		+	++										
	54	+++	+++’	++	++	+	+++										
	75	+++	+++	+++	+++	++	+++										
	77																
	83A																
	84																
	85																
V	94	+					+										
	96																
Misc.*	81	+++	++	+++	+++	+++	+++										
	953																

*Miscellaneous

Table 2 Lytic reactions of the 16 phages of the CNS typing set on 158 MRSA strains

	phages of the CNS typing set															
	Ph5	Ph10	Ph12	Ph13	U14	48(240)	H96	29	95	68	71(382)	48(407)	TU	Ph15	U20	71(240)
Number of strains (and percentage)	126 (79.7)	126 (79.7)	124 (78.4)	124 (78.4)	124 (78.4)	124 (78.4)	4 (2.5)	0	0	0	0	0	0	0	0	0

The reactivity of the phages is based on their reactions on 158 MRSA strains from the Jikei University Hospital.

Ph10, Ph12, Ph13, U14, 48(240)) was observed. A phage H96 was a sporadic phage, which did not lyse the propagating strains of the IPS (Table 1), and lysed 4 of the 158 (2.5 %) MRSA clinical strains (Table 2). To select the additional phages, the similarities between the seven candidate phages (Ph5, Ph10, Ph12, Ph13, U14, 48(240) and H96) were determined by the Jaccard coefficient (Sj) (Table 3). Four phages (Ph12, Ph13, U14, 48(240)) and one pair of phages (Ph5 and Ph10) had 100% similarity respectively (Table 3). Although phages ph12 and U14 had 100% similarity against the 158 MRSA strains, the activities of these phages against the propagating strains 29 and 52 were different each other (Table 1). Finally four phages, Ph10, Ph12, U14, H96 were chosen for their typeability and distinctiveness, and were added to the IPS since they were considered to be useful for subsequent typing. The final set comprised 27 phages, the addition of four CNS phages (Ph10, Ph12, U14, H96) to IPS (23phages), called the new phage set. *S. aureus* phages in the IPS indicated a narrow lytic pattern to their propagating strains. In

Table 3 Similarity among the 7 CNS phages (Ph5, Ph10, Ph12, Ph13, U14, 48(240), H96)*

Phage	Similarity with phage:						
	Ph5	Ph10	Ph12	Ph13	U14	48(240)	H96
Ph5							
Ph10	100						
Ph12	98.4	98.4					
Ph13	98.4	98.4	100				
U14	98.4	98.4	100	100			
48/240	98.4	98.4	100	100	100		
H96	3.2	3.2	3.2	3.2	3.2	3.2	

*The data are based on S_j. The reactivity of the phages is based on their reaction on 158 MRSA strains.

contrast, the CNS phages showed broad spectrum of lytic patterns (Table 1). We propose amended criteria for MRSA grouping to add a new group of miscellaneous CNS (MCNS). Distribution and number of lysis patterns of the MRSA of three hospitals in differnt areas of Japan, (i.e. Osaka (The Kinki University Hospital), Nagoya (The Nagoya University Hospital) and Tokyo (The Jikei University Hospital)) are shown in table 4. Those of the 112 MSSA strains of Tokyo (The Jikei University Hospital) are shown in Table 5. 72.3% of the MSSA strains were typable by the

Table 4 Discrimination of MRSA strains

Group	The Jikei University Hospital(n=158)				The Nagoya University Hospital(n=156)				The Kinki University Hospital(n=30)			
	International phage set		*New phage set		International phage set		*New phage set		International phage set		*New phage set	
	No. of lysis pattern	No. of strain	No. of lysis pattern	No. of strain	No. of lysis pattern	No. of strain	No. of lysis pattern	No. of strain	No. of lysis pattern	No. of strain	No. of lysis pattern	No. of strain
I	4	10	5	10	5	6	5	6	1	1	1	1
Ⅱ	0	0	0	0	0	0	0	0	0	0	0	0
Ⅲ	7	49	8	49	22	27	24	27	3	4	3	4
I, Ⅱ, I	0	0	0	0	1	1	1	1	0	0	0	0
I, Ⅲ	22	26	23	26	19	21	20	21	0	0	0	0
I, V	0	0	0	0	1	1	1	1	0	0	0	0
Misc.	0	0	0	0	1	1	1	1	0	0	0	0
MCNS	-	-	2	47	-	-	4	78	-	-	2	20
NT	1	73	1	26	1	99	1	21	1	25	1	5
Total	34	158	39	158	50	156	57	156	5	30	7	30

NT=non-typable.
Lytic group I, Ⅱ, Ⅲ, V, and Misc are the international phage set.
I=29,52,52A,79,80
Ⅱ=3A,3C,55,71
Ⅲ=6,42E,47,53,54,75,77,83A,84,85
V=94,96
Misc.=81,95
Lytic group MCNS, addittional phages, Ph10, Ph12, U14, H96 University Hospital.

* The addition of 4 CNS phages (Ph10, Ph12, U14, H96) to international phage set.

Table 5 Discrimination of MSSA strains

Group	The Jikei university Hospital(n=112)			
	International phage set		*New phage set	
	No. of lysis pattern	No. of strain	No. of lysis pattern	No. of strain
I	11	12	12	12
Ⅱ	5	14	6	14
Ⅲ	9	10	9	10
V	2	6	2	6
I, Ⅱ, Ⅲ	0	0	0	0
I, Ⅲ	28	31	28	31
I, V	0	0	0	0
Misc.	1	8	2	8
MCNS	-	-	5	25
NT	1	31	1	6
Total	57	112	65	112

Group names are the same as those in Table 4

* The addition of 4 CNS phages (Ph10, Ph12, U14, H96) to international phage set.

IPS. On the other hand the typeability of MRSA strains from each hospital was lower than that of the MSSA strains (Table 6). By the IPS, only 53.8%, 36.5% and 16.7% of the MRSA isolates from the Jikei University Hospital, the Nagoya University Hospital, and the Kinki University were typable, respectively. By using the new phage set, however, Typeability were raised to 83.4%, 86.5% and 83.3% in each hospital. Although there was a few fluctuation of number of plaques between initial and second test, all strains gave identical results (data not shown).

We applied phage typing to an epidemiological analysis of a nosocomial infection by using our new phage set. In a case of an outbreak in August 1994, 11 MRSA strains were isolated from 6 patients in a surgical ward. Six isolated strains from 4 patients were NT by the IPS. Among these NT strains, three strains became typable as MCNS (Ph10/Ph12/U14) by using

Table 6 Typabilities and discriminatory power of the international phage set and the new phage set

	MSSA		The Jikei University Hospital(n=158)		The Nagoya University Hospital(n=156)		The Kinki University Hospital(n=30)	
	International phage set	*New phage set	International phage set	*New phage set	International phage set	*New phage set	International phage set	*New phage set
Typability (%)	72.3%	94.6%	53.8%	83.4%	36.5%	86.5%	16.7%	83.3%
Discriminatory power	0.91	0.97	0.76	0.86	0.59	0.84	0.62	0.66

* The addition of 4 CNS phages (Ph10, Ph12, U14, H96) to international phage set.

Table 7 Distribution of MRSA isolates by phage typing of the IPS and using the new phage set

Patient no.	Isotate no.	Source	Date of isolation	International phage set	*New phage set
A	1	sputum	8/10/94	NT	MCNS(Ph10/Ph12/U14)
	2	sputum	8/11/94	Ⅲ(85)	Ⅲ(85/Ph10/Ph12/U14)
B	3	throat	8/10/94	NT	MCNS(Ph10/Ph12/U14)
	4	sputum	8/15/94	NT	MCNS(Ph10/Ph12/U14)
C	5	throat	8/2/94	NT	NT
D	6	throat	8/11/94	Ⅲ(54)	Ⅲ(54/Ph10/Ph12/U14)
	7	pus	8/15/94	Ⅲ(54)	Ⅲ(54/Ph10/Ph12/U14)
	8	sputum	8/15/94	Ⅲ(54)	Ⅲ(54/Ph10/Ph12/U14)
E	9	sputum	8/23/94	NT	NT
	10	sputum	8/30/94	NT	NT
F	11	sputum	8/29/94	I(29)	I(29/Ph10/Ph12/U14)

A total of 11 clinical isolates of MRSA were collected from 6 patients admitted to a surgical ward.
Non-typable strain (strains no. 1,3,5,9) by IPS were analyzed by PFGE, see Fig1.

*The addition of 4 CNS phages (Ph10, Ph12, U14, H96) to international phage set.

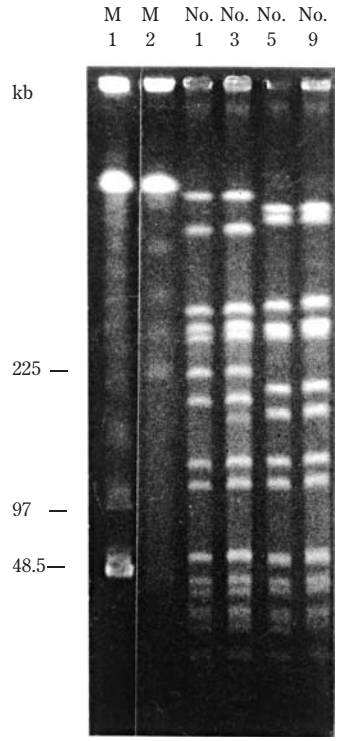

Fig. 1 Pulsed-field gel electrophoresis of *Sma*I-digested chromosomal DNA from 4 MRSA strains, which were non-typable by the IPS (strains nos. 1, 3, 5, 9). For details of the strains, see Table 7. Lanes M1 and M2, molecular size marker (M1: lambda concatemer, M2: Yeast chromosomes).

the additional phages (Table 7). These six non-typable strains by the IPS were analyzed by PFGE. The PFGE patterns of the six strains by *Sma* I digestion were divided into two groups. In the first group, two NT strains nos.5 and 9 by the new phage set from patients C and E respectively presented the identical PFGE pattern (Fig.1). The PFGE patterns of the other strain nos.10 from patient E was also identical to that of nos.5 and 9 (data not shown). In the second group, including MCNS strains nos. 1 and 3 from patients A and B respectively, showed similar PFGE patterns that differed in only one 160kb band from each other (Fig. 1). The PFGE patterns of strain nos. 3 and 4 from patient B were identical (data not shown).

Ⅲ. Discussion

Phage typing has been used as a traditional epidemiological typing method for *S. aureus*. Many epidemiological investigations of MRSA by phage typing have been reported world wide[2,3,18] since MRSA was first identified in England[1]. However, most MRSA strains are NT by phages of the IPS[2, 3, 18]. Some investigators introduced a set of phages especially for typing for MRSA to distinguish these NT strains of MRSA[19～21].

In this paper, we supplemented the IPS with phages isolated from CNS strains, so that we succeeded to get a higher typeability of MRSA. The discriminatory power of the combined phage set was higher than that of the IPS alone. However in the case of the Kinki University, the new phage set did not show higher discrimination ability (Table 6), because that there are three possible reasons. One is the isolates were really same origin of source, the other is number of studied strain was relatively small, third is the additional

phages did not work well. The modifications in the method of phage typing, that include the exact determination of RTD by two-fold dilution and the dropping of the exact volume (4μl) of phage, worked quite well. Therefore the reproducibility test was also quite reliable. Even by using additional phages from *S. aureus* enhancement of discrimination ability for typing MRSA is more difficult than that for typing MSSA. This may be due to that the most strains of MRSA are derived from a relatively few clones[22,23]. The analysis of the evolutionary origins of MRSA by multilocus sequence typing (MLST) demonstrates the limited number of major epidemic MRSA genotypes[23]. MLST combined with the staphylococcal cassette chromosome *mec* (SCCmec) typing show that isolates with the same sequence type but with different SCCmec type, presumably have arisen by independent acquisitions of the *mec* gene[23]. The results of the phage typing may be determined by the origin of MSSA strains, from which MRSA derives by *mec* acquisition, and conserved thereafter. For this reason, the phage typing is a suited way to long-term epidemiological studies.

When we performed the epidemiological survey from a surgical ward, we used phage typing for screening the strains at first. Then, we used the restriction enzyme analysis for more detailed discrimination of those strains showing the same phage pattern. Three isolates (from patients C and E), which were not lysed by any of the IPS plus additional CNS phages, showed the same PFGE pattern. These isolates were considered to be originated from the same strain. The other 3 isolates (from patients A and B) with phage pattern Ph10/Ph12/U14 had similar PFGE patterns except one band. Tenover[24] has suggested that a single genetic event can result in two to three band differences. Based on this notion, these isolates are considered to be closely related to each other.

We believe that phage typing with the combined phage set is a suitable method for the first step of typing MRSA. PFGE alone cannot be applied as a standard method for a long-range typing because multiple genetic evens which occur in a long period result in too much band difference accumulation for estimating relatedness of each other strain.

Important criteria for typing systems are typeability, reproducibility, and discriminatory power. In an addition ideal typing system would be rapid, inexpensive, technically simple, and readily available[5]. Phage typing is rapid, inexpensive, and technically simple. It will be a complementary typing method of *S. aureus* to MLST and PFGE, especially of MRSA when the combined phage set is used. The fully automated system of phage typing remains to be developed in the future.

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