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Spread of Erythromycin-, Tetracycline-, and Aminoglycoside-Resistant Genes in Methicillin-Resistant *Staphylococcus aureus* Clinical Isolates in a Kumamoto Hospital

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Various drug-resistance genes with different mechanisms have been identified in methicillin-resistant *Staphylococcus aureus* (MRSA). Knowing the prevalence of these drug-resistance genes is important for controlling of MRSA spread in hospitals.

In our previous paper (1), 24 MRSA clinical isolates obtained in October 2002 in a hospital with 550 beds in Kumamoto Prefecture were assessed by restriction fragment length polymorphisms (RFLP) of genomic DNA using pulsed-field gel electrophoresis (PFGE), plasmid DNA typing by

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Table 1. PFGE patterns of MRSA isolates; MICs of GM, EM, and TC from these isolates; and distribution of GM-, EM-, and TC-resistance genes among these isolates

No.	Lane No.[1)]	PFGE pattern[2)]	MIC (μg/ml) of			PCR products								Southern blot		
			GM	EM	TC	A[3)]	B	C	D	E	F	G	H	*acc6′-aph2″*	*ermA*	*tetM*
														plasmid/chromosome (kb)		
1245	1	A1	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1247	2	A1	≧16 (R)	≧8 (R)	2 (S)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1250	3	A1	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1251	4	A1	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1252	5	A1	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1255	6	A1	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1256	7	A1	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1258	8	A1	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1259	9	A1	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1261	10	A1	≦2 (S)	≧8 (R)	≧16 (R)	–	–	–	+	–	–	–	+	—/—	40/220, 580	40/290
1264	11	A1	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1246	12	A16	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1263	13	A25(M5)	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1257	14	A25(M5)	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1248	15	A6	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1268	16	AE	≧16 (R)	≦0.5 (S)	≦1 (S)	+	–	–	–	–	–	–	–	—/280	—/—	—/—
1249	17	AH2	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40, 200/110	40/220, 580	40/290
1253	18	A12(O5)	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	—/120	40/220, 580	40/340
1267	19	AJ	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	50, 280/—	40/220, 580	40/320
1262	20	AK	≦2 (S)	≧8 (R)	≧16 (R)	–	–	–	+	–	–	–	+	—/—	40/220, 580	40/290
1254	21	AL	≧16 (R)	≧8 (R)	≦1 (S)	+	–	–	+	–	–	–	–	50, 280/240	40/240, 530	—/—
1265	22	AM1	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40/130	40/260, 580	40/530
1260	23	AM2	≧16 (R)	≧8 (R)	≧16 (R)	+	–	–	+	–	–	–	+	40/180	40/260, 580	40/530
1266	24	AN	≧16 (R)	≧8 (R)	≦1 (S)	–	–	–	–	–	–	–	–	—/—	—/—	—/—

[1)]: Lane No. in electrophoresis shown in Fig. 1 and Fig. 2.
[2)]: The PFGE patterns was reported in reference 1.
[3)]: A: *aac6′-aph2″*, B: *aph*(*3′*)-*III*, C: *ant*(*4′*)-*I*, D: *ermA*, E: *ermB*, F: *ermC*, G: *tetK*, H: *tetM*.

using agarose gel electrophoresis, and antibiotic resistance. The same isolates were analyzed here by PCR and Southern blot to detect drug-resistance genes including gentamicin (GM)-resistant genes *aac6′-aph2″* and *aph*(*3′*)-*III*; kanamicin (KM)-resistant gene *ant*(*4′*)-*I*(2); erythromycin (EM)-resistant genes *ermA*, *ermB*, and *ermC*(3); and tetracycline (TC)-resistant genes *tetK* and *tetM*(4). The PCR results were evaluated based on the expected sizes of PCR products or confirmed by DNA sequencing.

Among the 24 MRSA isolates, 22 isolates, 23 isolates, and 20 isolates were resistant to GM, EM, and TC, respectively (Table 1). The majority of the isolates (18 of 24) were resistant to all of three antibiotics; five of the remaining isolates were resistant to two of the three, i.e., to EM and GM (Nos. 1247, 1254, and 1266), or to EM and TC (Nos. 1261 and 1262); and the last isolate (No. 1268) was resistant to GM. No isolate was sensitive to all three of the antibiotics. Isolate No. 1247 was sensitive to TC but showed an increase in MIC (2 μg/ml) compared with other TC-sensitive isolates (Nos. 1268, 1254, and 1266).

The results of PCR are shown in Table 1. Among the 24 MRSA isolates, 21 were PCR-positive for *aac6′-aph2″*, 22 were positive for *ermA*, and 21 were positive for *tetM*. None of the isolates was positive for *aph*(*3′*)-*III*, *ant*(*4′*)-*I*, *ermB*, *ermC* or *tetK*. The majority of the isolates (19 of 24) were positive for three genes: *aac6′-aph2″*, *ermA*, and *tetM*. Two isolates (Nos. 1261 and 1262) were positive for the two genes *ermA* and *tetM*. Isolate No. 1254 was positive for the two genes *ermA* and *aac6′-aph2″*. Isolate No.1268 was positive for the gene *aac6′-aph2″*. Isolate No.1266 was negative for all genes tested. The existence of *aac6′-aph2″*, *ermA*, and *tetM* was consistent with the susceptibility to GM, EM, and TC, respectively, in all MRSA isolates excepting two (Nos. 1247 and 1266). That is, all the isolates resistant to GM, EM, and TC, had *aac6′-aph2″*, *ermA*, and *tetM*, respectively. Isolates Nos. 1261 and 1262, resistant to EM and TC but sensitive to GM, had *ermA* and *tetM* but not *aac6′-aph2″*. Isolate No. 1254, resistant to GM and EM but sensitive to TC, had *ermA* and *aac6′-aph2″* but not *tetM*. Isolate No.1268, resistant to GM but sensitive to EM and TC, had *aac6′-aph2″* but not *ermA* or *tetM*.

There were exceptional isolates in which the existence of the drug-resistance genes was not consistent with the phenotype. Isolate No. 1247, resistant to GM and EM but sensitive to TC (with relatively higher MIC, as above described), had all these three genes. Other TC genes might be affecting the susceptibility to TC, or the detected TC resistance gene was non-functional due to mutation. Isolate No.1266, resistant GM and EM but sensitive to TC, did not have any of the genes tested, indicating that there are other GM- and EM-resistant genes.

To determine whether the drug-resistant genes *aac6′-aph2″*, *ermA*, and *tetM* existed on the plasmid DNA or genomic DNA of these MRSA isolates, Southern blotting was carried out (Fig. 1). The GM-resistance gene *aac6′-aph2″* was detected on four different-sized plasmids (40 kb, 50 kb, 200 kb, and 280 kb), as well as in a 15 kb *Sma*I-digest chromosome fragment derived from (Fig. 1B and Table 1). The majority of the

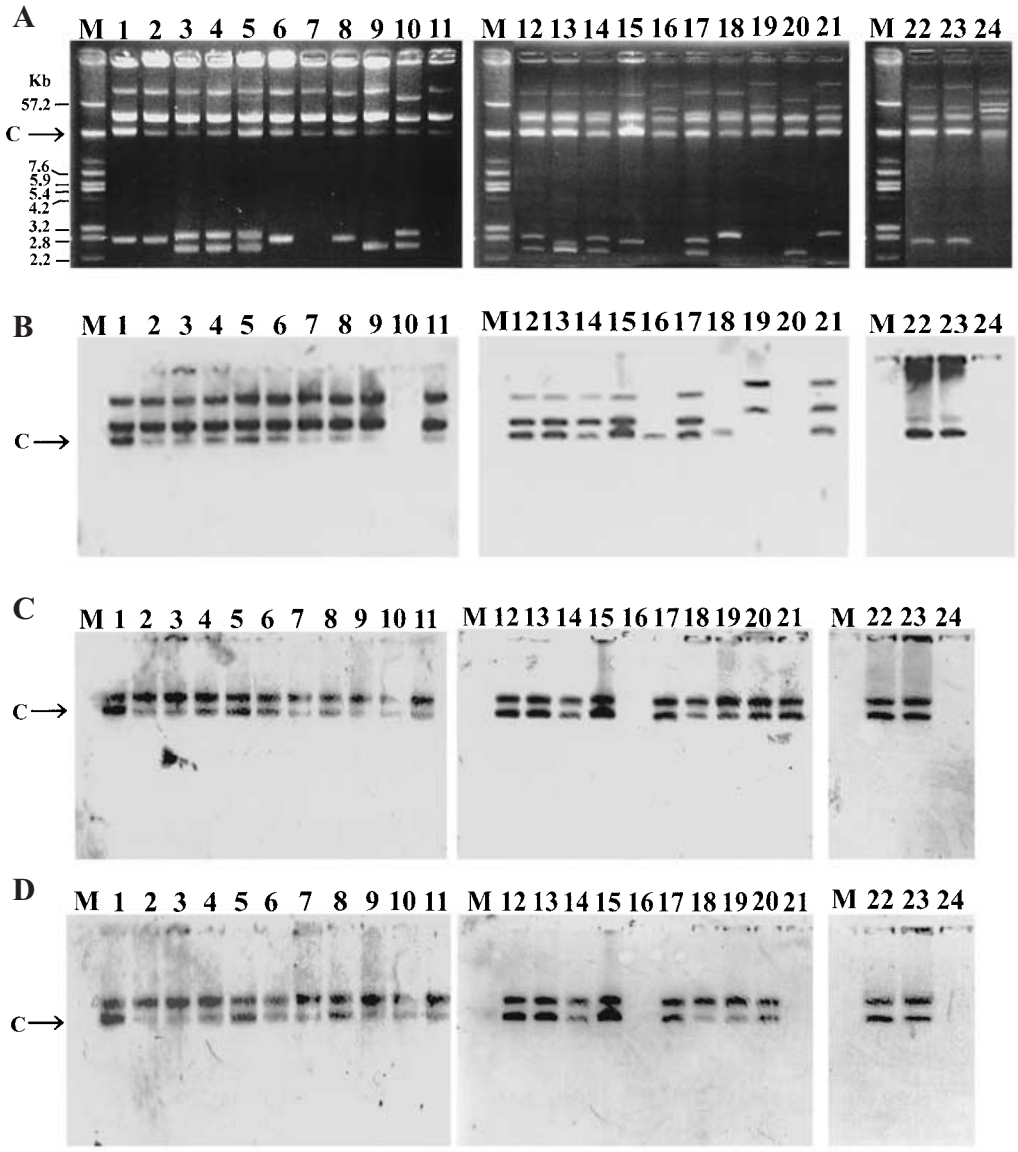

Fig. 1. Agarose gel electrophoresis of plasmid DNA from MRSA isolates (A) and Sourthern blotting hyblidized with *aac6′-aph2″* (B), *ermA* (C), and *tetM* (D).
M: marker plasmids derived from *E. coli* V517. C: DNA fragments derived from genomes. Lanes 1 to 24: MRSA isolates Nos. were listed in Table 1.

isolates had *aac6′-aph2″* on the 40 kb and 200 kb plasmids and on the chromosome. Isolates No. 1254 (lane 21) had *aac6′-aph2″* on the 50 kb and 280 kb plasmids and also on the chromosome. Isolate No.1267 (lane 19) had *aac6′-aph2″* on the 50 kb and 280 kb plasmids. Isolates Nos. 1265 and 1260 (lanes 22 and 23) had *aac6′-aph2″* on the 40 kb plasmid. Isolates Nos.1268 and 1253 (lanes 16 and 18, respectively) had *aac6′-aph2″* on the chromosome but not on the plasmid. The *aac6′-aph2″* gene was not detected in isolates Nos. 1261, 1262, and 1266 (lanes 10, 20, and 24, respectively). The majority of the isolates had *ermA* and *tetM* on the 40 kb plasmid and on the chromosome (Fig. 1C and D). Isolate No. 1254 (lane 21) had *ermA* on both the plasmid and the chromosome but had no *tetM*. Isolates Nos. 1268 and 1266 had neither *ermA* nor *tetM*.

To locate the drug-resistant genes on the chromosome, Southern blotting was done after separation of *Sma*I digests of the genomic DNA by PFGE (Fig. 2). The *aac6′-aph2″* was detected on 110 Kb of the *Sma*I digest in the majority of the isolates with the PFGE pattern A (A1, A16, A25[M5], and A6). Notably, *aac6′-aph2″* was not detected in isolate No. 1261 (lane 10) even though it was detected in other isolates with the same PFGE pattern. In isolates with other PFGE patterns, *aac6′-aph2″* was detected on variously sized *Sma*I digests (100 Kb, 120 Kb, 130 Kb, 180 Kb, 240 Kb, and 280 Kb). The *ermA* was detected on both the 220 Kb and 580 Kb *Sma*I digests in all isolates with the PFGE pattern A (lanes 1-15) and in isolates Nos. 1249 (lane 17), 1267 (lane 19), and 1262 (lane 20), respectively, with PFGE patterns AH2, AJ, and AK (Fig. 2C and Table 1). The *ermA* was also detected on the 230 Kb and 580 Kb *Sma*I fragments in isolates No. 1253 (lane 18), on the 240 Kb and 530 Kb fragments in No. 1254 (lane 21), and on the 260 Kb and 580 Kb fragments in Nos. 1265 (lane 22) and 1260 (lane 23). The *tetM* was detected on the 290 Kb *Sma*I fragment in all isolates with the PFGE pattern A (lanes 1 - 15), in isolates No. 1249 with the PFGE pattern AH2 (lane 17), and in isolate No. 1262 with PFGE pattern AK (lane 20). In isolates with other PFGE

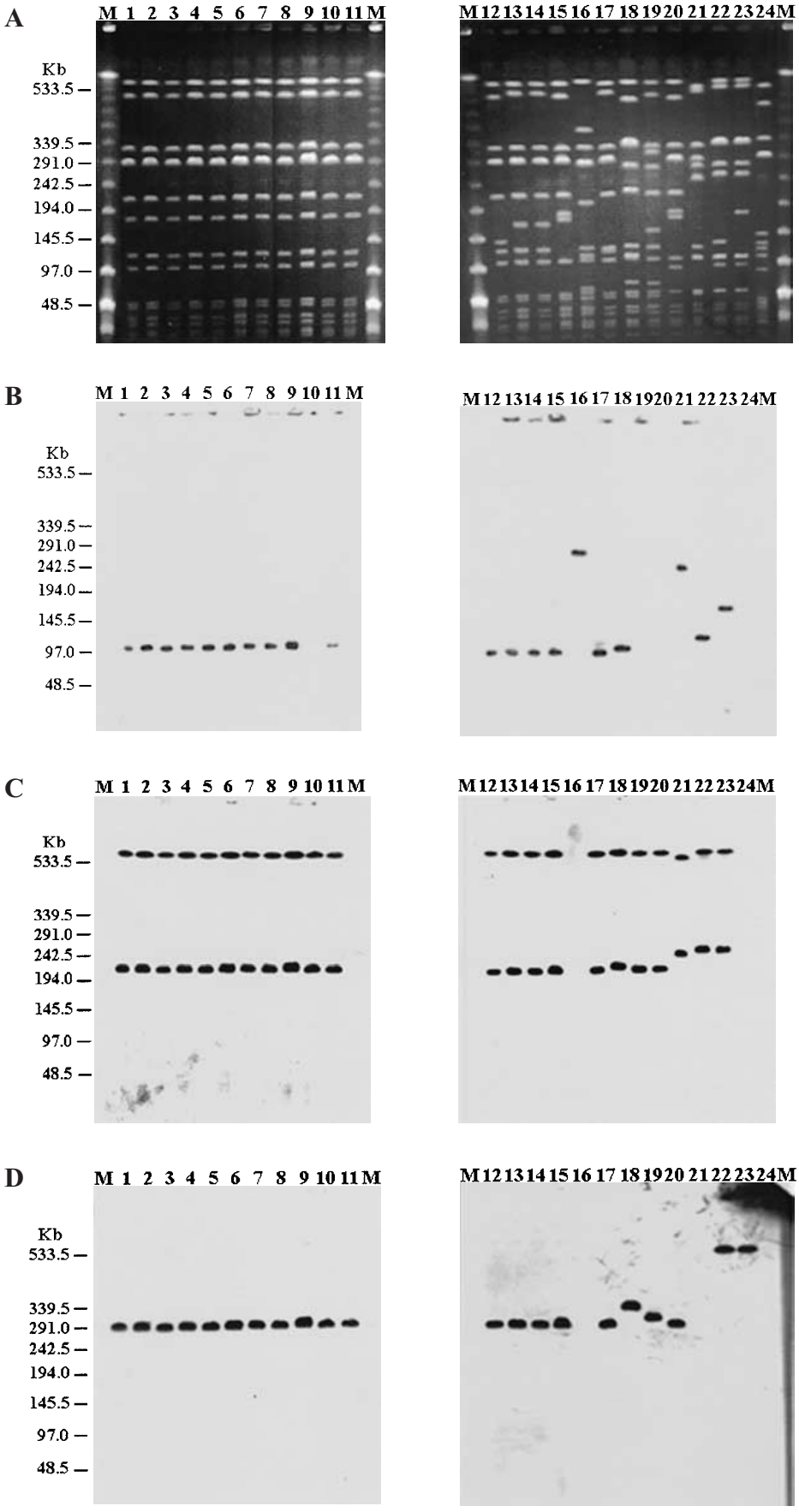

Fig. 2. Pulsed-field gel electrophoresis of *Sma*I-digested genomic DNA from MRSA isolates (A) and Sourthern blotting hyblidized with *aac6′-aph2″*(B), *ermA* (C), and *tetM* (D).
M: low range PFG Marker. Lanes 1 to 24: MRSA isolates Nos. were listed in Table 1.

patterns, the *tetM* was detected on different-sized *Sma*I fragments of 320 Kb, 340 Kb, and 530 Kb.

Based on the PFGE patterns, 14 MRSA isolates among the 24 isolates belonged to one group. Among these isolates, 13 were resistant to GM, EM, and TC. All of them had a multidrug resistant 40 kb plasmid harboring *aac6′-aph2″*, *ermA*, and *tetM*, and a large plasmid of 200 kb with *aac6′-aph2″*. They had *aac6′-aph2″* and *tetM* each on at least one chromosome site, and *ermA* on at least two chromosomal sites.

The above molecular analysis of the drug-resistance genes clearly indicates the clonal expansion of MRSA and confirms the data obtained with RFLP, although our previous antibiogram data appears to have given results less convincing than those of RFLP.

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