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計畫名稱：Methicillin 抗藥性金黃色葡萄球菌之分子流行病學研究分析及對 fluoroquinolone 抗藥性機轉研究

（中、英文）：Molecular epidemiology and mechanism of fluoroquinolone resistance of methicillin-resistant *Staphylococcus aureus*

計畫類別：☑個別型計畫　□整合型計畫

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（請打✓）　□一年後可對外提供參考

□兩年後可對外提供參考

（必要時，本會得展延發表時限）

執行單位：台大醫學院內科

中　華　民　國　90　年　10　月　31　日

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become an important nosocomial pathogen and has increased frequency in the past 20 years. MRSA is highly transmissible between wards, hospitals, and cities. In most major hospitals in Taiwan, MRSA accounts for more than 60% of the *S. aureus* isolates. Whether there is a predominant strain, which is spread over the whole of Taiwan has not yet been studied. Pulsed-field gel electrophoresis (PFGE) had been shown to be a good method of epidemiological analysis. We collected 208 sequential clinical isolates of MRSA from 22 hospitals: seven in northern Taiwan, seven in western Taiwan, five in southern Taiwan, and three in eastern Taiwan during a three-month period in 1998. Using pulsed-field gel electrophoresis, 112 MRSA isolates were shown to belong to one major type, — type C, and this type was shown to have spread widely across all of Taiwan. Ninety-six isolates belonged to 20 other minor types. Most MRSA isolates of this major type were multi-drug resistant and only susceptible to vancomycin and rifampin. We concluded that the high prevalence of MRSA in Taiwan was partly due to the spreading of a predominant strain and most of them were multi-drug resistant. This might imply that more effort should be made to control the spread of MRSA in Taiwan.

Key words: methicillin-resistant *Staphylococcus aureus*, epidemiology, pulsed-field

gel electrophoresis

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first documented in Taiwan in the early 1980s (Chang SC, et al. 1988). In 1990s, the incidence of nosocomial MRSA infections increased in Taiwan (Lee SC, et al. 1996; Chang SC, et al. 1997), especially in teaching hospitals. Because MRSA is highly transmissible (Cookson BD, et al. 1988), the increase in prevalence of MRSA infections might result from the spread of individual strains (Salmenlinna S, et al. 2000).

Precise typing methods to monitor the epidemiology of MRSA infections has become of primary importance for the identification of MRSA clones present in a hospital and for reducing their spread (Prevost G, et al. 1992; Boyce JM, et al. 1993). Pulsed-field gel electrophoresis (PFGE) has been shown to be a good method with high reproducibility and resolving power (Bannerman TL, et al. 1995; Tenover FC, et al. 1995; Walker J, et al. 1999).

Our hospital, National Taiwan University Hospital (NTUH), is a major teaching hospital with 2000 beds located in northern Taiwan. Nosocomial MRSA infections at NTUH had increased rapidly since the 1990s, from 1.5 episodes per 1,000 discharges in 1990 to 3.9 episodes per 1,000 discharges in 1997. Also, the rate of MRSA isolates over total isolates of *S. aureus* had increased from 26.7% in 1990 to 70.9% in 1997 (Chang SC, et al. 1997; National Taiwan University Hospital, 1998).

The same situation has been observed in many other hospitals in Taiwan (Lee SC, et al. 1996; Ho M, et al. 1999). Based on our previous study using PFGE as the typing method, there was a predominant MRSA clone spreading at our hospital and this clone could change from year to year (Chen ML, et al. 1999). Whether there is also a predominant MRSA strain spreading across other hospitals in Taiwan thus is both an interesting and important question. In order to understand the relationships of MRSA isolates from different hospitals in Taiwan, we analyzed the MRSA isolates collected from 22 hospitals located in various parts of Taiwan by antibiogram and PFGE.

2. Materials and Methods

2.1. Bacterial isolates

The bacterial isolates included in this study comprised 208 MRSA isolates collected from 22 hospitals during the period from October 1998 to December 1998. These MRSA isolates were isolated from various clinical specimens, including blood, sputum, pleural fluid, urine, wound pus, ascites, and catheter tip, of patients with MRSA infections. No duplicate isolates from a single patient were included. For the 22 hospitals, seven were located in northern Taiwan, seven in western Taiwan, five in southern Taiwan, and three in eastern Taiwan. Six of them are medical centers, and 15 are regional hospitals, and one is a local hospital.

Identification of *S. aureus* was based on colony morphology on trypticase soy agar supplemented with 5% sheep blood (BBL, Microbiology Systems, Cockeysville, MD, USA), Gram stain, and a positive Staphylase test (Oxoid Ltd, Basingstoke, England). The *S. aureus* isolates obtained were screened for methicillin resistance by disk diffusion method, using Mueller-Hinton agar (BBL, Microbiology Systems), 1 μg oxacillin disk and incubation for 24 hours at 35℃ (NCCLS, 2000). All isolates were frozen at –70℃.

2.2. Susceptibility test

The susceptibility of these isolates to various antibiotics was determined by

disk-diffusion method, as described by the National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 2000). The antimicrobial disks (BBL, Microbiology Systems) included penicillin G (10 units), oxacillin (1 μg), chloramphenicol (30 μg), gentamicin (10 μg), norfloxacin (5 μg), ofloxacin (5 μg), clindamycin (2 μg), erythromycin (15 μg), trimethoprim (1.25 μg)/sulfamethoxazole (23.75 μg), tetracycline (30 μg), vancomycin (30 μg), and rifampin (5 μg). Isolates with identical results from the susceptibility test were considered as having the same type antibiogram. This type was designated with a number, such as type 1.

2.3. PFGE

Genomic DNA was prepared in agarose plugs as described in our previous report (Chen ML, et al. 1999). In brief, the bacteria were suspended in 0.5 mL PIV buffer (1 mol/L NaCl, 0.01 mol/L Tris) and mixed with an equal volume of 1.6% low-melting agarose (Boehringer GmbH, Mannheim, Germany) in PIV buffer and allowed to solidify in plug molds. Then the bacteria were lysed by incubation of the agarose plugs at 37℃ for four hours with lysostaphin (50μg/mL) (Boehringer GmbH) in 1 mL EC buffer (6 mmol/L Tris, pH 8.0, 1 mol/L NaCl, 0.1 mol/L EDTA, pH 8.0, 0.2% sodium deoxycholate, 0.5% Sarkosyl). Next, the lysis buffer was replaced with 1 mL ESP buffer (0.5 mmol/L EDTA, pH 9.0, 0.1% Sarkosyl, 1 mg/mL proteinase K) and incubated overnight at 50℃. The agarose plugs were then washed three times with 10

mL of Tris EDTA (TE) buffer (10 mmol/L Tris-HCl, 0.1 mmol/L EDTA, pH 8.0) for 30 minutes at room temperature and transferred to a tube containing TE buffer and placed in a refrigerator at 4℃ until use.

For restriction endonuclease digestion, approximately 1 to 1.5 mm of a plug was cut and incubated overnight with 250 μL of restriction buffer containing 20U of *Sma* I (Biolab Laboratories, Beverly, MA, USA) at 25℃. After DNA digestion, the agarose plugs were incubated with 1 mL of TE buffer at 37℃ for one hour. The plugs were then inserted into 1% agarose gels (Bio-Rad Laboratories, Hercules, CA, USA) in 0.5x TBE buffer (0.089 mol/L Tris,, 0.089 mol/L Boric acid, 2 mmol/L EDTA), and restriction fragments were separated using a contour-clamped homogeneous electric field system (CHEF-DRII; Bio-Rad, Laboratories). Electrophoresis was performed at 200V for 24 hours with pulse times of 1 to 35 seconds at 4℃, and gels were stained with ethidium bromide and photographed under ultraviolet light.

2.4. Interpretation of PFGE patterns

The banding patterns were interpreted visually according to the following guidelines: 1) Isolates with banding patterns appearing identical in size and number of band were considered to represent the same type. This type was designated with one English capital letter, for example, type A. 2) Isolates with banding patterns that differed from the main pattern by up to three bands were considered to represent

subtypes within the same main type and were designated with different number (A1, A2, A3, etc). 3) Isolates with banding patterns that differed from the main pattern by four or more bands were considered to be of a different type and designated with other English capital letters (types B, C, D, etc) (Bannerman TL, et al. 1995; Tenover FC, et al. 1995). 4) The naming of the type and subtype with the English capital letters and numbers also followed the rule used in our previous study (Chen ML, et al. 1999).

3. Results

PFGE analyses of the 208 MRSA isolates revealed one predominant type (type C), which comprised 112 isolates and was found in every geographic region of Taiwan. There were 20 other types and each of them consisted of far fewer isolates (Table 1). Among the isolates of PFGE type C, PFGE subtype C1, with 81 isolates in total, was the most common one all over Taiwan, not only in all four geographic regions, but also in nearly every hospital enrolled in this study. Other than type C, three other types (type D, G and J) were also wide spread over the different regions of Taiwan (Figure 1). However, the number of isolates of type D, G and J was much less than that of type C. The distribution was similar when medical centers and regional hospitals were compared (Figure 2). Therefore, of the 208 isolates, there were 167 isolates in total belonging to four types, which were widely distributed in Taiwan.

The susceptibility test of all 208 MRSA isolates revealed that they were usually multi-drug resistant strains. Less than two percents (only three isolates) of these isolates were susceptible to penicillin, 0.5% susceptible to erythromycin, 5.8% susceptible to clindamycin, 6.7% susceptible to tetracycline, 16.8% susceptible to gentamicin, 26.4% susceptible to ofloxacin and norfloxacin, 29.4% susceptible to trimethoprim/sulfamethoxazole, 33.2% susceptible to chloramphenicol, 95.2% susceptible to rifampin, and 100% susceptible to vancomycin. By the antibiogram,

they could be divided into 28 different types. Antibiogram type 5, which was only susceptible to vancomycin and rifampin, was the most common type. This type comprised 83 isolates. Five isolates belonged to antibiogram type 4, which was the most resistant type and only susceptible to vancomycin. The relationship between antibiograms and PFGE patterns is shown in Table 2.

The results of the susceptibility test for PFGE type C isolates are shown in Table 3 in details. All isolates were multiple-drug resistant and most of them belonged to the antibiogram type 5. None of them were susceptible to penicillin, norfloxacin, ofloxacin, erythromycin, and tetracycline; 0.9% of them were susceptible to gentamicin, 0.9% susceptible to trimethoprim/ sulfamethoxazole, 4.5% susceptible to clindamycin, 29.5% susceptible to chloramphenicol, and 97.3% susceptible to rifampin.

4. Discussion

Our present study demonstrated that MRSA isolates in Taiwan belonged to multiple clones as determined by PFGE typing method. However, it was also found that there was a predominant clone, which was typed as PFGE type C, spreading all over Taiwan and isolates belonging to this clone were multi-drug resistant with the majority of them being only susceptible to vancomycin and rifampin. Among the isolates of PFGE type C, subtype C1 was the most common one no matter where the hospital was located and no matter what kind of the hospital it was (Figure 2, 3). This meant that there was a predominant MRSA clone spreading across hospitals in Taiwan.

There are many phenotypic and genotypic methods, which could be used for epidemiological differentiation of MRSA isolates (Mulligan ME, et al. 1991; Waller TMA, 2000). At present, antibiotyping is the simplest one; however, its results are, in general, unsatisfactory (Waldvogel RA, 2000; Waller TMA, 2000). PFGE has been proved to be a good method with high reproducibility and resolving power for many different bacteria, including MRSA (Bannerman TL, et al. 1995; Tenover FC, et al. 1995; Walker J, et al. 1999; Waller TMA, 2000). Therefore, we used PFGE as the typing method in this study.

Previous studies had proved that MRSA can be spread interhospitally (Boyce

JM, 1990; Dominguez MA, et al. 1994; Cox RA, et al. 1995; Wildemauwe C, et al. 1996; Roman RS, et al. 1997; Van Belkum A, et al. 1997; Salmenlinna S, et al. 2000) or even across the country (Townsend SE, et al. 1987; Schito G, et al. 1988; Santos Sanches I, et al. 1995; De Lencastre H, et al. 1997). In our study, we found that the PFGE type C, D, G, and J were widely distributed all over Taiwan, which may be due to interhospital spreading. Taiwan is a small and isolated island. Patients like to visit different hospitals and the transfer of patients from one hospital to another hospital is very common, even between hospitals in different geographic region. Poor adherence to infection control precautions by health care workers is also common in many hospitals. We believe that the above three phenomena may result in the current high prevalence of MRSA infection in Taiwan.

Another important finding in our present study is that most MRSA isolates in Taiwan are multi-drug resistant and those of PFGE type C were especially highly resistant. As shown in Table 3, 75 of the 112 isolates of PFGE type C were susceptible to vancomycin and rifampin only, and 26 were susceptible to chloramphenicol, vancomycin, and rifampin only. Compared to the other isolates with different PFGE patterns, type C was much more resistant, especially to gentamicin, new flouroquinolones and trimethoprim/sulfamethoxazole (Table 4). Some previous studies demonstrated high rate of antibiotics use (Chang SC, et al. 1998; McDonald

LC, et al. 2001) and an alarmingly high level resistance in different bacteria to antibiotics in Taiwan (Chen ML, et al. 1999; Ho M, et al. 1999; Chang SC, et al. 2000), which might imply the presence of a strong selective pressure, caused by over use of antibiotics, on common pathogenic bacteria. This might explain why the most resistant clone has become the predominant and the most widespread one.

Many previous reports demonstrated that once an endemic strain of MRSA is introduced into a hospital, it is very difficult to eradicate it (Cohen SH, et al. 1991; Boyce JM, et al. 1992; Kauffman C, et al. 1993; Adeyemi-Doro FA, et al. 1997) and result in an increased incidence of nosocomial MRSA infections (Salmenlinna S, et al. 2000). In our previous study, we found PFGE type C strain appeared in our hospital (NTUH) in 1994, increased rapidly thereafter, and became the predominant clone in our hospital in 1997 (Chen ML, et al. 1999). Our present study found that type C is also the predominant clone in all analyzed hospitals in Taiwan. This may imply that type C MRSA clone has spread rapidly all over Taiwan in recent years and this may lead to a continuing increase in nosocomial MRSA infections in Taiwan. Given that rates of MRSA in some Taiwan hospitals have already exceeded 80% of their *S. aureus* isolates in 1998 (Ho M, et al. 1999), the above possibility is much more worrisome.

In conclusion, our study demonstrated that there is a predominant MRSA clone,

which is widely spreading across Taiwan. This might partly explain the high prevalence of MRSA and lead to a continuing increase in MRSA infections in Taiwan. As this predominant clone is multi-drug resistant, we must pay much more attention and effort to the prevention and treatment of MRSA infections and carefully consider the use of antibiotics in Taiwan in the future.

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Table 1. PFGE patterns of 208 MRSA isolates

Type (number of isolates)	Subtypes (number of isolates)
B (3)	B1 (1), B2 (1), B5 (1)
C (112)	C1 (81), C2 (18), C3 (3), C4 (1), C5 (2), C8 (1), C9 (3), C10 (1), C11 (1), C12 (1)
D (23)	D1 (17), D3 (6)
G (6)	G1 (2), G2 (1), G3 (1), G4 (1), G5 (1)
H (13)	H1 (7), H2 (1), H3 (1), H4 (3), H5 (1)
J (26)	J1 (18), J2 (3), J3 (2), J4 (3)
L (4)	L1 (3), L2 (1)
Others* (21)	

* Including one isolate of type A, four of type K, two of type M, three of type N, two of type O, one each of type P, Q, R, S, T, U, V, W, X.

Table 2. Relationship between types of antibiograms and PFGE patterns of 208 MRSA isolates

Antibiogram type (number of isolates)	PFGE patterns (number of isolates)
4 (5)	C1 (2), P (1), K (1), U (1)
5 (83)	C1 (62), C2 (6), C3 (2), C4 (1), C8 (1), C9 (2), C10 (1), J1 (3), J3 (1), J4 (1), K (3)
8 (11)	H1 (4), H2 (1), H4 (1), M (2), N (2), S (1)
9 (4)	H1 (2), H4 (1), H5 (1)
12 (18)	D1 (7), D3 (6), G1 (2), G2 (1), H1 (1), N (1)
13 (4)	D1 (3), G4 (1)
15 (2)	L1 (1), V (1)
16 (3)	B1 (1), B5 (1), C2 (1)
17 (47)	C1 (14), C2 (11), C9 (1), J1 (15), J2 (3), J3 (1), J4 (2)
18 (4)	C2 (1), L1 (2), L2 (1)
19 (4)	B2 (1), C1 (2), C12 (1)
22 (4)	D1 (2), H4 (1), Q (1)
24 (2)	R (1), X (1)
Others* (17)	

*Including one isolate (PFGE D1) of type 1, one (G2) of type 2, one (C1) of type 3, one (A) of type 6, one (C11) of type 7, one (G3) of type 10, one (C5) of type 11, one (W) of type 14, one (H3) of type 20, one (C5) of type 21, one (T) of type 23, one (D1) of type 25, two (O) of type 26, two (D1) of type 27, and one (D1) of type 28.

Table 3. The detailed antibiogram of PFGE type C

Antibiogram type	Antibiotics susceptibility												PFGE subtype (number of isolates)
	Pen	Oxa	Chl	Gen	Nor	Ofl	Cli	Ery	Sxt	Tet	Van	Rif	
3	R	R	R	R	R	I	S	R	R	R	S	S	C1 (1)
4	R	R	R	R	R	R	R	R	R	R	S	R	C1 (2)
5	R	R	R	R	R	R	R	R	R	R	S	S	C1 (62), C2 (6), C3 (2), C4 (1), C8 (1), C9 (2), C10 (1)
7	R	R	R	R	R	R	S	R	R	R	S	S	C11 (1)
11	R	R	R	S	R	R	R	R	R	R	S	S	C5 (1)
16	R	R	S	R	R	R	R	R	R	R	S	R	C2 (1)
17	R	R	S	R	R	R	R	R	R	R	S	S	C1 (14), C2 (11), C9 (1)
18	R	R	S	R	R	R	R	R	S	R	S	S	C3 (1)
19	R	R	S	R	R	R	S	R	R	R	S	S	C1 (2), C12 (1)
21	R	R	S	S	R	R	R	R	R	R	S	S	C5 (1)

Table 3. Continued

Abbreviation: Pen, penicillin G; Oxa, oxacillin; Chl, chloramphenicol; Gen, gentamicin; Nor, norfloxacin; Ofl, ofloxacin; Cli, clindamycin; Ery, erythrmomycin; Sxt, trimethoprim/sulfamethoxazole; Tet, tetracycline; Van, vancomycin; Rif, rifampin; R, resistant; S, susceptible; I, intermediately susceptible.

Table 4. The comparison of susceptible rates to 12 different antibiotics between PFGE type C and others.

PFGE type	Antibiotics susceptibility rate											
	Pen	Oxa	Chl	Gen	Nor	Ofl	Cli	Ery	Sxt	Tet	Van	Rif
C	0	0	29.46%	0.9%	0	0	4.46%	0	0.9%	0	100%	97.32%
Others	3.13%	0	37.5%	35.42%	57.29%	57.29%	7.29%	1.04%	62.5%	14.58%	100%	92.71%

Abbreviation: Pen, penicillin G; Oxa, oxacillin; Chl, chloramphenicol; Gen, gentamicin; Nor, norfloxacin; Ofl, ofloxacin; Cli, clindamycin; Ery, erythrmomycin; Sxt, trimethoprim/sulfamethoxazole; Tet, tetracycline; Van, vancomycin; Rif, rifampin.

Figure 1.

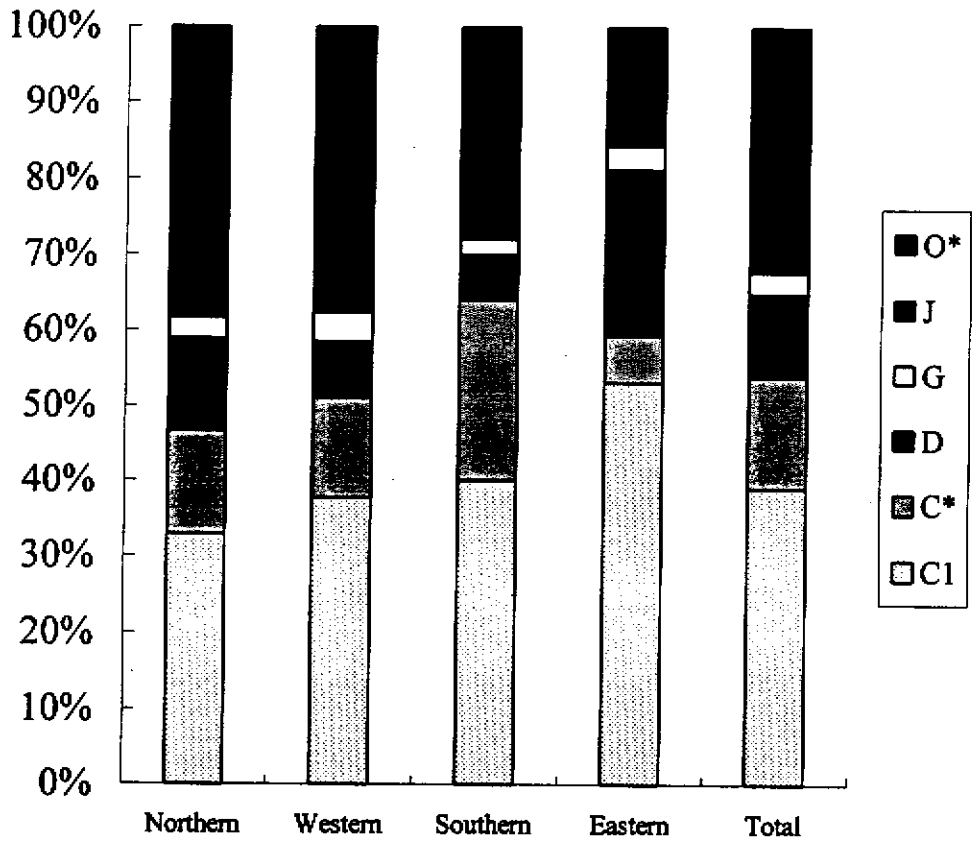
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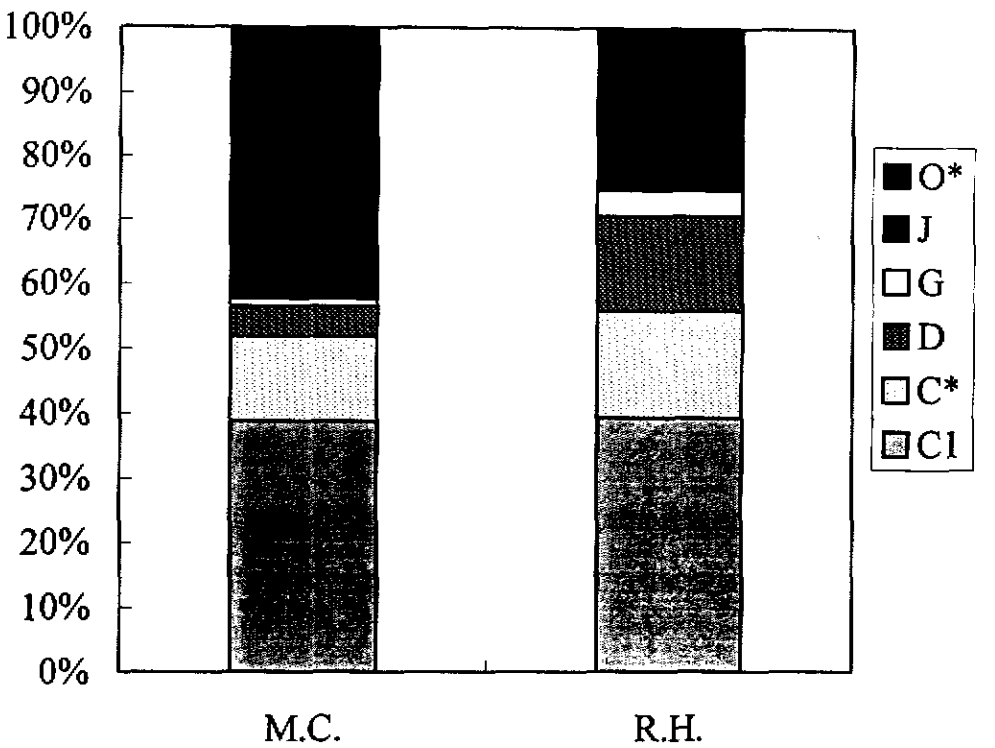
Figure 2



Legends of Figure 1

Figure 1. The distribution of PFGE patterns of MRSA isolates in the four geographic regions of Taiwan. MRSA isolates belonging to PFGE type C, D, G, and J could be found in every geographic region of Taiwan and type C compromised the most isolates in every region. Among type C, subtype C1 was the most common one in every region. The total number of isolates collected from northern Taiwan was 73, from western Taiwan was 53, from southern Taiwan was 50, and from eastern Taiwan was 32. Abbreviation: northern, northern Taiwan; western, western Taiwan; southern, southern Taiwan; eastern, eastern Taiwan; C1, PFGE subtype C1; C*: PFGE type C other than subtype C1; D, PFGE type D; G, PFGE type G; J, PFGE type J; O*, other PFGE types.

Legends of Figure 2

Figure 2. The distribution of PFGE patterns of MRSA isolates collected from six medical centers and 15 region hospitals of Taiwan. Both in medical centers or regional hospitals, PFGE type C still compromised the most isolates. Among the PFGE type C, subtype C1 was also the most common one. The number of total isolates from medical centers was 85, from regional hospitals was 122. Abbreviation: M.C., medical center; R.H., regional hospital; C1, PFGE subtype C1; C*, PFGE type C other than subtype C1; D, PFGE type D; G, PFGE type G; J, PFGE type J; O*, other PFGE types.