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臺灣methicillin抗藥性金黃色葡萄球菌抗藥性基因之分型研究(1/2)

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研究計畫中文摘要及關鍵詞

methicillin 抗藥性金黃色葡萄球（methicillin-resistant *Staphylococcus aureus*）菌自 1981 年於台灣出現後，在 1990 年代迅速增加，目前已經成為引起台灣各醫院院內感染的重要致病菌之一。再加上可資用以治療 methicillin 抗藥性金黃色葡萄球菌感染的用藥有限，methicillin 抗藥性金黃色葡萄球菌感染對臨床醫學造成了莫大的衝擊。因之，對 methicillin 抗藥性金黃色葡萄球菌的 methicillin 抗藥性機轉的研究，一直是臨床上十分重要的課題。

在過去 20 多年來對 methicillin 抗藥性金黃色葡萄球菌之抗藥性機轉的研究中，發現其 methicillin 抗藥性主要源自於 *mecA* gene 的存在。除了 *mecA* gene 之外，尚有 *mecI* 及 *mecR1* 等調節基因，以此共同組成 *mecA gene* complex。研究中更發現 *mecA gene* complex 廣泛的存在 methicillin 抗藥性金黃色葡萄球菌及凝固酶陰性的葡萄球菌（coagulase-negative Staphylococci）中；由此，抗藥性基因——*mecA* gene complex 可在不同種的葡萄球菌中轉移的可能性，應該是存在的。再進一步針對 methicillin 抗藥性金黃色葡萄球菌的 mecA gene complex 鄰近的染色體做研究，日本學者 Hiramatsu 等人發現，methicillin 抗藥性金黃色葡萄球菌的染色體基因上帶有一段 methicillin 感受性金黃色葡萄球菌所沒有的染色體序列，命名為 SCC*mec* 元件 (Staphylococcal cassette chromosome *mec* element)。SCC*mec* 元件中，除了 *mecA* gene complex 外，另有兩個命名為 *ccrA* (cassette chromosome recombinase A)和 *ccrB*(cassette chromosome recombinase B）的基因，負責將整個 SCC*mec* 元件精準的插入及移出金黃色葡萄球菌的染色體。藉由對 *mecA* gene complex 及 *ccr* gene 的分型，目前已知世界上的 methicillin 抗藥性金黃色葡萄球菌的 SCC*mec* 元件，可分作四個主要的型態。

台灣過去對 methicillin 抗藥性金黃色葡萄球菌的研究，鮮有針對抗藥性基因的部份。本研究希望藉由對台灣 methicillin 抗藥性金黃色葡萄球菌 *mecA* gene complex 及 *ccr* genes 的研究，能釐清台灣本土 methicillin 抗藥性金黃色葡萄球菌其 SCC*mec* 元件的組態為何；除了能用以和國外報告相比對以明白台灣 methicillin 抗藥性金黃色葡萄球菌 methicillin 抗藥性之種源外，對本土 MRSA 菌株的抗藥性機轉，也將有更清楚的認識。

關鍵詞：methicillin 抗藥性金黃色葡萄球、*mecA gene* complex、*ccr* complex、SCC*mec* 元件

研究計畫英文摘要及關鍵詞

The first clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) in Taiwan was found in 1981. During the past decades, the nosocomial MRSA infection rate increased rapidly and MRSA became one of the most important nosocomial pathogens in Taiwan. Given the fact that only few antibiotics can be used to treat MRSA infections, MRSA had become a major problem for current medicine. Thus the mechanism of methicillin resistance carried by MRSA is also an important clinical issue.

In the previous studies on methicillin resistance of MRSA, it has been found that the methicillin resistance of MRSA is carried by a chromosome gene, *mecA* gene. The *mecA* gene and its regulator genes, *mecI* and *mecR1*, consists of the *mecA* gene complex. The *mecA* gene complex is widely distributed among *S. aureus* species as well as among other staphylococcal species collectively called coagulase-negative staphylococci. Therefore, it has been speculated that *mec* may be freely transmissible among staphylococcal species. In the 1980s, direct chromosome analysis of MRSA strains revealed that a substantial length of the chromosomal DNA segment (greater than 30 kb) carrying *mec* has no allelic equivalence in methicillin-susceptible *S. aureus* (MSSA) strains. According to the studies of Hiramatsu et al, the “mec DNA” is now formally renamed as “SCC*mec* (Staphylococcal cassette chromosome *mec*) element” and the SCC*mec* element is almost universally found in all MRSA isolates. The most important components in SCC*mec* element are the *ccr* (cassette chromosome recombinase) genes, *ccrA* and *ccrB*, and *mecA* gene complex. The *mecA* gene complex can be classified into four types, type A, B, C, D. The *ccr* genes can be classified into to three types. The function of *ccr* genes is to precisely excise and insert the SCC*mec* element in way of both site and orientation specifically. Based on the structures and combinations of *ccr* genes and *mecA* gene complex, the SCC*mec* gene element in MRSA can be classified into four types

In Taiwan, previous studies rarely deal with the genetics of methicillin resistance of MRSA. Our study will focus on the genetic classification of *mecA* gene complex and *ccr* complex of MRSA isolated in Taiwan. Despite further determining the types of SCC*mec* element of MRSA in Taiwan to compare with those in other country and then clarify the its phylogenic source, the mechanism of methicillin resistance of MRSA in Taiwan will be also more illuminated at the same time.

Key Words: methicillin-resistant *Staphylococcus aureus*, MRSA, *mecA* gene complex, *ccr* complex, SCC*mec* element

研究計畫之前言、目的、及文獻探討

The first report of methicillin-resistant *Staphylococcus aureus* (MRSA) in the world is by Dr. Jevons in 1961 (1). And the first isolate of MRSA in Taiwan is found in 1981 (2). Thereafter, the rates of nosocomial MRSA infections increased rapidly in Taiwan and most hospitals in Taiwan now have a high incidence of nosocomial MRSA infections (3, 4). The rates of MRSA over all nosocomial isolates of *S. aureus* in some Taiwan hospitals has already exceeded 80% in 1998 (5), which was much higher than that reported by the National Nosocomial Infection Surveillance System (NNIS) (6). Our previous studies have proved that the reasons leading to rapid increased of MRSA infection in Taiwan include overuse of antibiotics, poor adherence to isolation precaution of health care worker, and introduction of endemic strain (7-10). MRSA thus has become a major pathogen in Taiwan. In addition, because of its resistance, limited choice of drug to treat MRSA infection is another important clinical problem. However, there is still no detailed study on the genetic mechanism of methicillin resistance of MRSA in Taiwan. Understanding the detailed genetics of methicillin resistance of MRSA may be helpful to overcome this resistance in the future.

The genetic coding for methicillin resistance in *S. aureus* has been proven to be *mecA* gene (11). The expression of *mecA* gene results in a specific penicillin-binding protein, PBP2', that has a decreased binding affinity to β-lactam antibiotics and thus leading to methicillin resistance. The expression of *mecA* gene is regulated by two adjacent regulatory gene, *mecI* and *mecR1*. The *mecA*, *mecI*, and *mecR1* genes consist of the *mecA* gene complex (12). The *mecA* gene complex is widely distributed among *S. aureus* species as well as among other staphylococcal species collectively called coagulase-negative staphylococci (13-15). Therefore, it has been speculated that *mec* may be freely transmissible among staphylococcal species. In the 1980s, direct chromosome analysis of MRSA strains revealed that a substantial length of the chromosomal DNA segment (greater than 30 kb) carrying *mec* has no allelic equivalence in methicillin-susceptible *S. aureus* (MSSA) strains; the segment was called "additional DNA" or "*mec* DNA" (16, 17). The size, structure, and biological properties of *mec* DNA had long remained unclear (18).

The most striking findings in recent studies on MRSA are the existence of SCC*mec* gene element (19). According to the studies of Hiramatsu et al, the "mec DNA" is now formally renamed as "SCC*mec* (Staphylococcal cassette chromosome *mec*) element" and the SCC*mec* element is almost universally found in all MRSA isolates (20). The most important components in SCC*mec* element are the *ccr* (cassette chromosome recombinase), *ccrA* and *ccrB*, genes and *mecA* gene

complex (19, 20). The *mecA* gene complex can be classified into four types, type A, B, C, D. The *ccr* genes can be classified into to three types. The function of *ccr* genes is to precisely excise and insert the SCC*mec* element in way of both site and orientation specifically (19). Based on the structures and combinations of *ccr* genes and *mecA* gene complex, the SCC*mec* gene element in MRSA can be classified into four types (20, 21). Type III SCC*mec* gene carries more drug-resistant determinants than any other types. According to the study, conducted by Ito et al, on the analysis of SCC*mec* element of 38 major hospital-acquired MRSA strains isolated worldwide, SCC*mec* elements of MRSA isolates from Europe belong to type I and III, those from northern America belong to type II, most of those from Japan belong to type II, those from Australia and southeastern Asia belong to type III, and those from south Africa belong to type I (20). Type IV SCCmec element is so far only found in community-acquired MRSA isolates (21).

There is still no published report about the detailed genetics of methicillin resistance of MRSA isolates in Taiwan. The type of SCCmec elements of MRSA isolated in Taiwan is also obscure. Our study is designed to illuminate the *mecA* complex (in the first year), and *ccr* genes (in the second year) of MRSA isolates in Taiwan as well as to determine the types of *SCCmec* elements in Taiwan and compare these results with MRSA isolates in other country. In addition, there are some sporadic cases of community-acquired MRSA infections. By typing the SCC*mec* gene element of MRSA isolates isolated from patients with nosocomial MRSA infections and those with community-acquired MRSA infections, whether the methicillin resistance comes from the same source between these isolates or not can also be determined.

研究方法

Definitions and MRSA isolates:

Patients with community-acquired MRSA septicemia is defined as that patients without history of hospitalization within prior 30 days develop signs and symptoms of sepsis before admission and their blood cultures taken within 48 hours after hospitalization yield MRSA. Patients with nosocomial MRSA septicemia is defined as that patients develop signs and symptoms of sepsis three more days after admission and their blood cultures yield MRSA. Based on our previous study, there are seven major types of nosocomial MRSA isolates determined by pulsed-field gel electrophoresis (PFGE) in Taiwan (10). Seven type-specific nosocomial MRSA strains, determined in our previous study, and at least 30 community-acquired MRSA isolates collected from our hospital, National Taiwan University Hospital, during January 2003 to December 2003 (estimated according to the database of National Taiwan University Hospital in year 2002), are preserved for the following studies.

Study in the first year: Genetic organization of *mecA* gene complex

Determination of minimum inhibitory concentration (MIC):

All isolates will be tested for their MIC levels of oxacillin, gentamicin, clindamycin, erythromycin, ofloxacin, levofloxacin, chloramphenicol, tetracycline, rifampin, trimethoprim/sulfamethoxazole, vancomycin, teicoplanin, and linezolide using agar dilution method proposed by NCCLS (22).

PFGE:

All isolates will be typed first by PFGE to determine whether those community-acquired isolates belonged to the same molecular types of nosocomial isolates or not. The methods used for undergoing PFGE will be as those described in our previous study (9). The interpretation of PFGE patterns will be according to the principals proposed previously (23, 24). Once a community-acquired isolate is proved to belong to the same type as a nosocomial isolate, it will not be used for further molecular study. All results will be double checked.

PCR and nuleotide sequencing for the analysis of mecA complex:

The chromosomal DNA will be prepared by means of the method described by Hiramatsu et al and Matsuhashi et al (25, 26). PCR amplification will be performed using 1 unit AmpliTaq (Perkin-Elmer Cetus,

Foster City, Calif.) in 50 μl of reaction mixture (10mM Tris-HCl [pH 8.3], 50 mM KCl, 0.001% [wt/vol] gelatin, 50% [vol/vol] glycerol, 1.5 mM $MgCl_2$, 200 mM each deoxynucleoside triphosphatge, 1.0 mM each primer, and template DNA). The reaction will be carried out by using a Gene Amp PCR system 9600 (Perkin-Elmer). Thermal cycling will be set at 30 cycles (30 s for denaturation at 94℃, 1 min for annealing at 50℃, and 2 min for elongation at 72℃)

Long-range PCR amplification will be performed using 2.6 U of Expand high-fidelity PCR system enzyme mix as recommended by the manufacturer (Boehringer Mannheim Biochemica, Mannheim, Germany). A 5-μl portion of the reaction volume was subjected to electrophoresis in a 0.8% agarose gel containing 1 μl of ethidium bromide per ml to detect the amplified DNA fragment. All PCR products will be further sequenced using a 377 automated fluorescent DNA sequencing system (Perkin-Elmer, Foster City, Calif.) to compare the nucleotide homology with the published sequence in GenBank. The PCR and sequencing results will also be double-checked.

PCR primers and detection of mecA gene complex:

The primers will be used for the detection of mecA gene complex include:

mA2 (5'-AACGTTGTAACCACCCCAAGA-3'),
mA4 (5'-AGTGTATGATGAGCTATGAGA-3'),
mA5 (5'- CGCTCAGGAAATTTGTTGTGC-3'),
mA6 (5'-TATACCAAACCCGACAAC-3'),
iS1 (5'-ACATTAGATATTTGGTTGCGT-3'),
iS3 (5'-TCGGATGCTATCATTAAGCAT-3'),
iS4 (5'-ACAATCTGTATTCTCAGGTCGT-3'),
mI-1 (5'-AATGGCGAAAAAGCACAACA-3'),
mI-2 (5'-GACTTGATTGTTTCCTCTGTT-3'),
mcR2 (5'-CGCTCAGAAATTTGTTGTGC-3'), and
mcR3 (5'-ATACTCCACGTTAATTCCATT-3') (27).

Technical detection of the class A *mecA* gene complex will be based on the positive PCR test results for both sets of primers, mI-1 plus mI-2 and mcR2 plus mcR3. The class B *mecA* gene complex will be detected by long-range PCR using two sets of primers, iS-3 plus mA5 and iS-4 plus mA5. Detection of the Class C *mecA* gene complex will be based on the negative PCR test results for two sets of primer, mI-1 plus mI-2 and mcR2 plus mcR3,

as well as a positive long-range PCR test result for one set of primers, iS-1 plus mA6. Detection of the Class D *mecA* gene complex will be based on the negative PCR test results for two sets of primer, mI-1 plus mI-2 and mcR2 plus mcR3, as well as a negative long-range PCR test result for one set of primers, iS-1 plus mA6 (27, 28).

結果

MRSA isolates

Based on our previous study using pulsed-field gel electrophoresis (PFGE) for molecular typing, there are seven major PFGE types (type C, G, J, K, R, U, and X, Figure 1) of nosocomial MRSA allover Taiwan. We randomly selected one MRSA isolate of each major type (seven MRSA isolates in total) to undergo further molecular studies. In addition, there are 55 MRSA isolates causing community-acquired MRSA infection from January 2003 to December 2003. These 55 MRSA isolates are also enrolled for PFGE typing.

Determination of minimum inhibitory concentration (MICs) of various antibiotics:

For the seven nosocomial MRSA isolates, the MICs level of various antibiotics are described in details in Table 1. For the 55 community-acquired MRSA isolates, the MIC_{50}s and MIC_{90}s of various antibiotics are described in details in Table 2.

PFGE molecular typing:

Using PFGE molecular typing, we find the 55 community-acquired MRSA isolates belong to the 11 PFGE types (type C, G, J, K, R, U, X, D, H, S, and A, Figure 2), which all have been already found in nosocomial MRSA isolates in our previous study. However, 34 of the 55 community-acquired MRSA isolates belong to other four minor PFGE types (type D, H, S, and A) and not to the seven major PFGE types. We also randomly select one MRSA isolate of each minor types (four isolates in total) to undergo analysis of their *mecA* gene complex. Therefore, 11 isolates in total are handled to determine types of their *mecA* gene complex.

Analysis of *mecA* complex:

Class A *mecA* gene complex, determined by positive polymerase chain reaction (PCR) test results for both sets of primers, mI-1 plus mI-2 and mcR2 plus mcR3, is found in MRSA isolates of PFGE type C, J, K, and U. Class B mecA gene complex, determined by long-range PCR using two sets primers, iS-3 plus mA5 and iS-4 plus mA5, is found in MRSA isolates of type G, R, X, D, H, S, and A. No class C or class D *mecA* gene complex is found in our MRSA isolates.

Table 1. The MIC level of various antibiotics of seven nosocomial MRSA isolates

PFGE	Ofloxacin	Rifampin	Levofloxacin	Tetracyclin	Vancomycin	Erythromycin	SXT	Linezolid	Gentamicin	Clindamycin	Oxacillin
K1	128	<0.03	8	128	1	>128	>128	1	16	>128	>128
J5	128	1	2	16	0.5	4	>128	1	16	0.06	128
U1	2	1	0.125	128	0.5	4	128	1	0.5	0.06	128
C1	128	<0.03	8	128	4	>128	>128	1	>128	>128	>128
G1	4	<0.03	0.25	16	0.125	128	4	1	0.5	>128	4
R1	2	<0.03	0.125	0.125	0.5	128	2	0.5	1	0.125	2
X1	4	<0.03	0.125	0.125	1	128	4	0.5	1	0.06	2

SXT, trimethoprim/sulfamethoxazole

Table 2. The MIC_{50}s and MIC_{90}s of various antibiotics of 55 community-acquired MRSA isolates

	Ofloxacin	Rifampin	Levofloxacin	Tetracyclin	Vancomycin	Erythromycin	SXT	Linezolid	Gentamicin	Clindamycin	Oxacillin
MIC_{90}	128	<0.03	8	128	1	>128	>128	1	16	>128	>128
MIC_{50}	4	<0.03	0.125	16	0.5	128	4	1	0.5	>128	4

Figure 1. PFGE types of seven nosocomial MRSA isolate

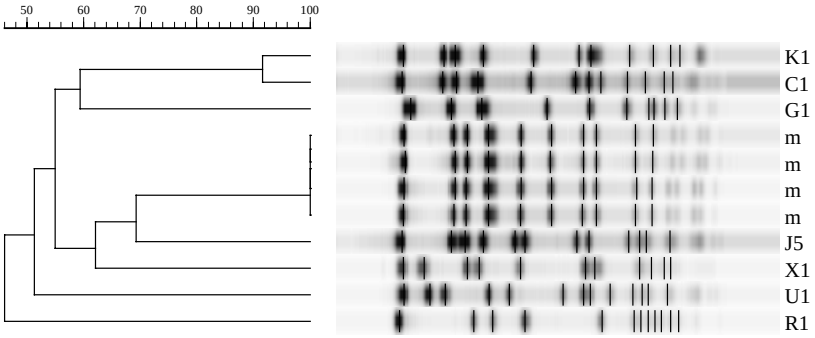
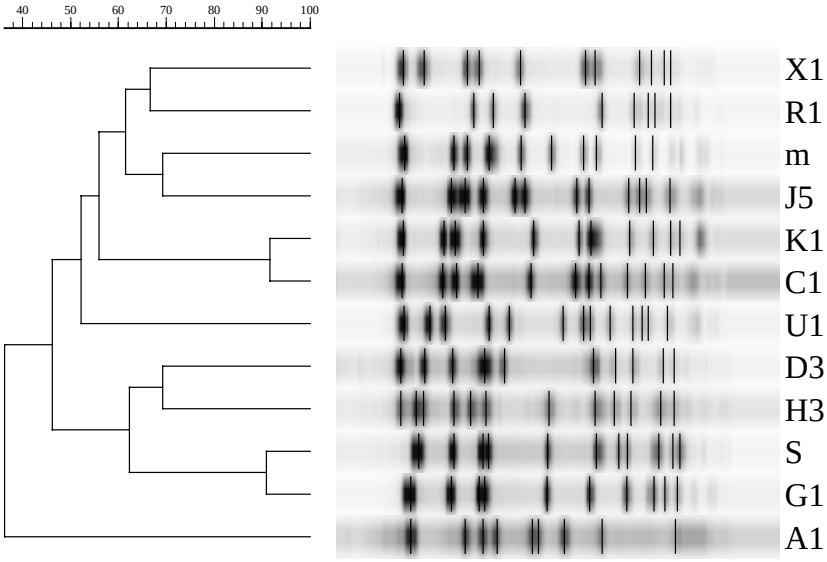


Figure 2. The 11 PFGE types of community-acquired MRSA isolates



討論

MRSA isolates of PFGE type C comprise about half of the all nosocomial MRSA isolates allover Taiwan. PFGE type C is the predominant type in Taiwan. Our study finds that class A *mecA* gene complex is the major *mecA* gene complex in Taiwan. As previous studies, no class C or D *mecA* gene complex is found in our MRSA isolates. All MRSA isolates tested in our study are susceptible to rifampin, vancomycin, and linezolid. In addition, almost During the following year, we will determine the *ccr* gene types of the 11 MRSA isolates. After the availability of *ccr* gene and *mecA* gene complex typing, we can further determine the types of *SCCmec* element of the 11 MRSA isolates.

計劃成果自評：

All the objects listed in our planning have been achieved. Our study demonstrates the distribution of *mecA* gene complex in MRSA in Taiwan. This will be helpful for further discovering the whole picture of resistant mechanism of MRSA isolates in Taiwan. And after the results of typing of *ccr* gene complex become available, we think it is suitable to publish our findings in literatures cited by SCI.

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