計畫編號：DOH97-DC-1203

行政院衛生署疾病管制局九十七年度科技研究發展計畫

院內感染多重抗藥菌分子特性及流行病學

Molecular characterization and epidemiology of multidrug resistant nosocomial bacteria in Taiwan

研 究 報 告

執行機構：財團法人國家衛生研究院
計畫主持人：楊采菱
研究人員：陳珮琛、黃怡雯、王惠瑩
執行期間：97 年 7 月 1 日至 97 年 12 月 31 日

＊本研究報告僅供參考，不代表衛生署疾病管制局意見＊

目　　錄

一、 摘要

（一）中文摘要

多重抗藥菌造成病人可因治療失敗，病情加重及住院日之加長，而增加許多醫療費用和病患死亡率。引起院內感染之細菌經常是抗藥性最高之多重抗藥菌。國家衛生研究院臨床研究組於 1998 年即開始進行「台灣微生物抗藥性監測計畫(Taiwan Surveillance of Antimicrobial Resistance, TSAR)」，每兩年進行一次，至今已完成五期，監測對象為多家醫院不同病人分離出的病原細菌(包含院感菌)。了解抗藥菌之抗藥機制及其流行病學，是探討抗藥菌傳播途徑及其抗藥性維持或增加原因之方法之一。比較不同年度之菌抗藥機制及分子流行病學，亦可增加對抗藥菌演變之了解。

此計畫使用 TSAR 第一至第五期(1998-2006)之院內感染細菌菌種，進行抗藥基因型及分子流行病學之研究，今年七月開始，研究對象為 TSAR I (1998)– V (2006)五期中對感具抗藥性的院感大腸桿菌及克雷白氏肺炎桿菌(extended spectrum β-lactam non-susceptible *Escherichia coli* and *Klebsiella pneumoniae*，簡稱 ESC-NS *E. coli* 及 ESC-NS *K. pneumoniae*)，調查那些是 ESBL，那些是 AmpC，及其 ESBL 及 AmpC 抗藥基因，並進行 PFGE 分析，以調查不同醫院菌種間之相關性。今年亦調查 TSAR V 所收集的抗甲氧苯青黴素金黃色葡萄球菌(Methicillin resistant *Staphylococcus aureus,* MRSA)中院感菌株，進行抗藥基因型及分子流行病學研究。

院感 *E. coli* 及 *K. pneumoniae* 菌之抗藥性明顯增加，包含對廣泛性乙內醯氨 cefotaxime (*E. coli*: 2000 年-6.1%, 2006 年-22.7%; *K. pneumoniae*: 2000 年-17.6% 2006 年-30.8%), cephamycin cefoxitin (*E. coli*: 2000 年-6.1%, 2006 年-33.3%; *K. pneumoniae*: 2000 年-5.9% 2006 年-32.3%) ($p < 0.01$)。總共對 100 株 ESC-NS *E. coli* 及 135 株 ESC-NS *K. pneumoniae* 進行 extended spectrum β-lactamase (ESBL)及 AmpC 表現型及抗藥基因(*bla*ESBL 及 *bla*AmpC)測試。產生 ESBL 之菌佔 1998 年院感 *E. coli* 菌之 8.6%，但佔 2006 年之 16.7%。同樣的，產生 ESBL 之菌佔 1998 年院感 *K. pneumoniae* 菌之 23.8%，但佔 2006 年之 31.3%。抗藥基因方面，44 株 *E. coli* 帶有 *bla*ESBL，5 株為 bla_{SHV} type (SHV-5-like), 34 株為 $bla_{CTX\text{-}M}$，5 株帶有 bla_{SHV} 及 $bla_{CTX\text{-}M}$ (12 株 CTX-M group I 及 27 株 CTX-M group IV)；共有 114 株 *K. pneumoniae* 帶有 *bla*ESBL，42 株為 $bla_{SHV\text{-}5,12\text{-}like}$, 57 株為 $bla_{CTX\text{-}M}$ type，14 株有 bla_{SHV} 及 $bla_{CTX\text{-}M}$ (35 株 CTX-M group I 及 36 株 CTX-M group IV)。一共有 68 株 E. coli 帶有 *bla*AmpC ，57 株是 $bla_{CMY\text{-}2}$, 1 株是 $bla_{CMY\text{-}4}$，1 株有 $bla_{DHA\text{-}1}$ 及 $bla_{CMY\text{-}2}$。同時帶有 *bla*ESBL 及 *bla*AmpC 的 *E. coli* 有 12 株。一共有 36 株 *K. pneumoniae* 帶有 *bla*AmpC，35 株是 $bla_{DHA\text{-}1}$，1 株為 $bla_{CMY\text{-}2}$，同時帶有 *bla*ESBL 及 *bla*AmpC 的 *K. pneumoniae* 有 21 株。

抗藥基因 *bla*ESBL 及 *bla*AmpC 所佔的比率在 ESC-NS 之 *E. coli* 及 *K. pneumoniae*

逐年改變。1998 年時, *bla*ESBL 佔 60% (6/10)之 ESC-NS *E. coli* 及 88.2% (15/17)之 ESC-NS *K. pneumoniae*，而 *bla*AmpC 則無發現。在 2006 年， *bla*ESBL 佔 26.9% (7/26)之 ESC-NS *E. coli* 及 38.7% (12/31)之 ESC-NS *K. pneumoniae*，*相對的 bla*AmpC 佔 73.1%之 ESC-NS *E. coli* 及 51.6%之 ESC-NS *K. pneumoniae*，同時帶有 *bla*ESBL 及 *bla*AmpC 之菌亦逐年增加。這些抗藥基因的增加及改變是院感 *E. coli* 及 *K. pneumoniae* 菌對廣泛性乙內醯氨及 cephamycin 抗藥性增加之原因。脈衝電泳法(pulsed field gel electrophoresis, PFGE)結果顯示，這些院感 ESC-NS *E. coli* 及 ESC-NS *K. pneumoniae* 大多菌的分子型皆不相同，只有少數幾株同年從同一家醫院來的菌是一樣的。這些結果顯示，這些抗藥菌的增加應該是經由抗藥基因在不同菌株間轉移而擴散。

相反的，MRSA 則大多屬於四個菌群，2006 之院感 MRSA 亦可分為四個主要群落(pulsotypes)，各佔了 51 株菌中 64.7% [pulsotype A (SCC*mec* III:ST239/241) (33 株)], 9.8% [pulsotype B(SCC*mec* IV:ST59，5 株)] , 2.0% [pulsotype C(SCC*mec* V:ST59 (1 株)，及 19.6% [pulsotype D(SCC*mec* II:ST5) (10 株)]。跟過去四期比較，pulsotype A 有明顯減少(從 1998 年之 93.7%，減少至 2006 之 64.7%, p <0.001)，同時 pulsotype D 從 2000 年之 2.6%，明顯增加至 2004 年之 20.4%，在 2006 年亦維持在 19.6%。因這些 pulsotype 之菌抗藥，及毒性皆有明顯差異，pulsotype A 對 ciprofloxacin (CIP)、gentamicin(GEN)、tetracycline 及 trimethoprim/sulfamethoxazole (SXT)幾乎全具抗性，pulsotype D 則全對 CIP 及 GEN 具抗性，而雖然 pulsotype B 及 C 之抗藥性較低(CIP/SXT 都無抗性)，但 pulsotype C 具 PVL 毒性因子，這些差異會影響疾病嚴重性及治療選擇。

此研究顯示，抗藥菌之防治與控制，需包含抗生素監控以減少抗藥基因在不同菌間之轉移，及感控措施之嚴謹實施以減少多重抗藥菌之擴散。此計劃比較由 TSAR 不同年度所收集的菌種，可以長期追蹤國內多重抗藥菌分子與臨床流行病學及抗藥基因之演變，協助建立多重抗藥菌之基因與流行病學資料，介此了解這些多重抗藥菌在國內不同醫院之分佈情況，做為協助於制定防止抗藥菌擴散及衍生之參考資料。此計畫並建立了 MRSA 分子流行病學之國際標準測試平台，可用於跟不同國家地區盛行之 MRSA 比對，以偵測 MRSA 之國際擴散。此計畫比較使用不同表現型方法測試台灣 *E. coli* 及 *K. pneumoniae* 具 ESBL 及 AmpC ß-lactamase 菌之資料，亦將提供給醫院微生物實驗室參考，以增加偵測這些多重抗藥菌之準確度。如果能適當控制院內感染之發生，不但能減少醫療費用，且可增進民眾之健康，對醫院、病人、和國家公衛機構都有降低經濟及心理負擔之貢獻。

關鍵詞：院內感染、抗甲氧苯青黴素金黃色葡萄球菌、廣泛性乙內醯氨抗藥性大腸桿菌、廣泛性乙內醯氨抗藥性克雷白氏肺炎桿菌

英文摘要

Background and Purpose

Bacterial pathogens causing nosocomial infections have the highest rates of antimicrobial resistance, and are resistant to multiple classes of antibiotics, which compromise treatment options and outcome. Strategies for the control of multidrug resistant bacteria require understanding of the mechanisms of resistance and local epidemiology within a region. This project utilized nosocomial isolates collected in the 5 rounds of the Taiwan Surveillance of Antimicrobial Resistance (TSAR) project. TSAR was implemented in 1998 and is conducted every 2 years. Between 21 to 44 hospitals participated in each round of TSAR. This year we studied nosocomial isolates of extended spectrum β-lactam non-susceptible *Escherichia coli* and *Klebsiella pneumoniae* from all 5 rounds of TSAR. Methicillin resistant *Staphylococcus aureus* (MRSA) from TSAR V (2006) were also studied and compared to those from previous years. Phenotypic and molecular characterizations were performed to investigate the mechanisms of resistance and determine clonal relatedness.

Methods

Nosocomial *E. coli* and *K. pneumoniae* having aztreonam, cefotaxime, or ceftazidime minimum inhibitory concentrations > 1 μ g/ml were subjected to extended spectrum β-lactamase (ESBL) confirmatory test and AmpC β-lactamase detection by modified Hodge test, Disk Potentiation test and Double Disc Synergy test. Multiplex PCR was used to detect the genes encoding ESBL (SHV, TEM, CTX-M type and others), and plasmid-mediated class C AmpC β-lactamases (DHA, CMY, and other types). The genes detected were confirmed by DNA sequencing. For MRSA, multiplex PCR was also used to determine the types of staphylococcus cassette chromosome *mec* (*SCCmec*), the element responsible for methicillin resistance and its mobilization. The presence of Panton-Valentine leukocidin (PVL) toxin genes in MRSA was determined by PCR. Multilocus sequence typing (MLST) (by PCR and DNA sequencing) was performed on selected representative pulsotype strains to determine their sequence type (ST). Pulsed Field Gel Electrophoresis (PFGE) was used to determine strain relatedness for all organisms and to look for clonal spread.

Results

Significant increase in resistance to β-lactams was observed in nosocomial *E. coli* and *K. pneumoniae* including resistance to cefotaxime (from 6.1% in 2000 to 22.7% in 2006 in *E. coli* and from 17.6% in 2000 to 30.8% in 2006 in *K. pneumoniae*), and cephamycin cefoxitin (from 6.1% in 2000 to 33.3% in 2006 in *E. coli*, from 5.9% in 2000 to 32.3% in 2006 in *K. pneumoniae*, $p < 0.01$). A total of 100 *E. coli* and 135 *K. pneumoniae* were subjected to phenotypic and molecular studies, of which 44 *E. coli* and 114 *K. pneumoniae* isolates were positive by ESBL-confirmatory test. The ESBL-positive rates increased from

8.6% in 1998 to 16.7% in 2006 (p >0.05) in *E. coli*, and from 23.8% in 1998 to 31.3% in 2006 (p >0.05) in *K. pneumoniae*.

Genes encoding ESBL (*bla*ESBL) were detected in 44 *E. coli* isolates, including 5 with bla_{SHV} type (SHV-5-like), 34 with bla_{CTX-M}, and 5 with both bla_{SHV} and bla_{CTX-M} (12 CTX-M group I and 27 CTX-M group IV). In *K. pneumoniae*, *bla*ESBL was detected in 114 isolates, including 42 with $bla_{SHV-5,12-like}$, 57 with bla_{CTX-M} type, and 14 isolates that carried both bla_{SHV} and bla_{CTX-M} (35 CTX-M group I and 36 CTX-M group IV). Genes encoding class C AmpC ß-lactamse (*bla*AmpC) was detected in 68 *E. coli* isolates, 57 of which were bla_{CMY-2}, 1 was bla_{CMY-4}, and 1 harbored both bla_{DHA-1} and bla_{CMY-2}. Co-carriage of *bla*ESBL and *bla*AmpC was found in 12 *E. coli* isolates. A total of 36 *K. pneumoniae* isolates were positive for *bla*AmpC (35 had bla_{DHA-1} and 1 had bla_{CMY-2}). Co-carriage of *bla*ESBL and *bla*AmpC was found in 21 *K. pneumoniae* isolates.

The proportions of isolates carrying genes encoding ESBL and AmpC in extended spectrum ß-lactam non-susceptible (ESC-NS) nosocomial *E. coli* and *K. pneumoniae* changed over the years. In 1998, isolates carrying *bla*ESBL comprised 60% (6/10) of ESC-NS *E. coli* and 88.2% (15/17) of ESC-NS *K. pneumoniae* and no AmpC was detected in either species. In 2006, isolates carrying *bla*ESBL comprised 26.9% (7/26) of ESC-NS *E. coli* and 38.7% (12/31) of ESC-NS *K. pneumoniae* while AmpC was detected in 73.1% of ESC-NS *E. coli* and 51.6% of ESC-NS *K. pneumoniae*. The increase in resistance to extended spectrum cephalosporins and cephamycin correlated with the increase in both ESBL producers and AmpC producers. No predominate clone or inter-hospital clonal dissemination was found based on PFGE of all ESC-NS *E. coli* and *K. pneumoniae* isolates. However, a few strains from the same hospital within the same year had identical PFGE patterns, indicating some intra-hospital spread may have occurred.

Among the 51 nosocomial MRSA isolates from 2006, 64.7% (33), 9.8% (5), 2.0% (1), and 19.6% (10) belong to pulsotypes A, B, C, and D, respectively. Compared to nosocomial MRSA isolates from the previous 4 rounds of TSAR (1998-2004), there was significant decrease in pulsotype A (SCC*mec* III:ST239/241) with concurrent emergence and increase of pulsotype D (SCC*mec* II:ST5). The proportion of pulsotype A MRSA decreased from 93.7% in 1998 to 64.7% in 2006 (p <0.001). Pulsotype D increased from 2.6% in 2000 to 20.4% in 2004, and remained at similar level in TSAR V. Both pulsotypes A and D are resistant to ciprofloxacin, but pulsotype D is susceptible to tetracycline and trimethoprim/sulfamethoxazole (SXT), both are common in different countries. Pulsotypes B (SCC*mec*:ST59) and C (SCC*mec* V:ST59) are not common in other countries but are the predominant community MRSA strain in Taiwan. They are susceptible to ciprofloxacin and

SXT but pulsotype C isolates carry the virulent PVL toxin gene so may cause more severe infections in susceptible hosts. The change in MRSA epidemiology may impact treatment strategies since these clonatypes have distinct resistance and toxin profiles.

Conclusions

The increase in resistance to extended spectrum β-lactams in *E. coli* and *K. pneumoniae* correlates with increasing ESBL and AmpC producers, which likely resulted form dissemination of resistance determinants between bacteria and not due to clonal spread. CTX-M type ESBL is prevalent and DHA-1 and CMY type AmpC are increasing. The increase in isolates with genes encoding both AmpC and ESBL is another concern since these genes are likely carried on transferable plasmids. In contrast, MRSA strains in Taiwan hospitals are highly clonal. Although the most multidrug resistant pulsotype A (SCC*mec* III:ST239/241) remained the predominant MRSA clonatype in nosocomial infections in Taiwan, another clone pulsotype D (SCC*mec* II:ST5) is increasing. In addition, community strain MRSA [pulsotype B (SCC*mec* IV:ST59) and pulsotype C (SCC*mec* V:ST59)] have also emerged in the hospitals. The risk factors, disease spectrum and treatment outcomes of different clonatypes warrant further studies since their resistance and toxin profiles differ. Strategies to control the rising antimicrobial resistance should include antibiotic stewardship to prevent polyclonal expansion of resistance determinants and enforcement of infection control measures to prevent the spread of resistant clones.

Key Words: Nosocomial infection, methicillin resistant *Staphylococcus aureus* (MRSA), extended-spectrum β-lactamase (ESBL), AmpC β-lactamase, extended-spectrum β-lactam resistant *Escherichia coli*, extended-spectrum β-lactam resistant *Klebsiella pneumoniae*

本文

(一) 前言 (Background)

對抗生素具抗藥性之細菌，尤其是多重抗藥菌，近年來在世界各國持續增加，台灣亦如此[2,11,12,17,36]，細菌抗藥性是一全球之公衛危機[18,25,26,31,34]。病人可因感染抗藥菌而治療無效或需使用昂貴之後線藥、及住院日之加長，而增加許多醫療費用和病患死亡率[16,19,29]，不只加深病人及其家屬之經濟及心理負擔與後遺症，也造成國家社會之經濟與公衛型態之負面影響。抗生素的使用是導致細菌在選擇性壓力(selective pressure)下產生抗藥性之主要原因，醫院之大量使用抗生素及後線抗生素使用之持續增加，使抗藥菌有機會在醫院環境中維持及繁殖，且可經一再突變或從其他細菌及不同菌種獲得抗藥基因，進而演變成多重抗藥性，所以造成院內感染的細菌多重抗藥性最高。進年來因醫療制度之改善，病人住院時之各種延續生命及維持器官機能的侵犯性醫療器材之使用(如：呼吸管及導管等)、及廣泛性抗生素之連續使用，都造成病人住院時被這些多重抗藥菌感染之機會。

了解抗藥菌之抗藥機制及其流行病學,是探討抗藥菌傳播途徑及其抗藥性維持或增加原因之方法之一，可協助防止抗藥菌之進一步擴散及衍生。比較不同年度所收集之菌種之抗藥機制及分子與臨床流行病學，亦可增加對抗藥菌演變之了解。國家衛生研究院臨床研究組於 1998 年即開始進行「台灣微生物抗藥性監測計畫(Taiwan Surveillance of Antimicrobial Resistance, TSAR)」,每兩年進行一次，監測對象為台灣北中南東地區之 21 至 44 家醫學中心及區域醫院由加護病房、普通病房及門診病人所分離出的病原細菌[11]，至今已完成五期。TSAR 資料顯示，台灣細菌之嚴重抗藥性不只是在醫院內，亦在于門診病人中[3,14,15,20]。但比較由不同醫院部門分離出的細菌之抗藥性，則以引起院內感染細菌的抗藥性最嚴重，舉例來說，TSAR V (2006)中抗甲氧苯青黴素金黃色葡萄球菌(Methicillin Resistant *Staphylococcus aureus*; 以下簡稱 MRSA)佔住院加護病房病人金黃色葡萄球之 73%，佔門急診病人中金黃色葡萄球菌之 48%，而引起院內感染之金黃色葡萄球菌中，MRSA 的比率亦高過 76%。

TSAR 之資料及國內之研究皆顯示，國內臨床最常見之院內感染細菌為：大腸桿菌(*Escherichia coli*)、克雷白氏肺炎桿菌(*Klebsiella pneumoniae*)、綠膿桿菌(*Pseudomonas aeruginosa*)、鮑氏不動桿菌 (*Acinetobacter baumannii*)、金黃色葡萄球菌(*Staphylococcus aureus*)、及腸球菌(*Enterococcus* spp.)。這些菌中，又以對廣泛性乙內醯氨具抗性之大腸桿菌及克雷白氏肺炎桿菌(extended spectrum β-lactam resistant *E. coli & K. pneumoniae*)、對 carbapenem (imipenem or meropenem)具抗性的綠膿桿菌及鮑氏不動桿菌(carbapenem resistant *P. aeruginosa* and *A. baumannii*)、對甲氧苯青黴素具抗性的金黃色葡萄球菌(MRSA)、及對萬古黴素具抗性之腸球菌(Vancomycin resistant *E. faecalis* 及 *E. faecium*)之多重抗藥性最嚴重[2,12,20]。

抗藥菌之抗藥機制包含抗生素目標之突變使抗生素與目標之親合力減低，或將抗生素排出或阻止抗生素進入細菌內，亦可經由抗藥基因轉錄之胺素可破解抗生素而造成抗生素無效。抗藥基因可位於細菌的染色體或質體(plasmid)上，而同一個質體上經常具有多重抗藥基因。另有些抗藥基因可存在轉位子(transposon)上，轉位子可在質體與染色體間互相跳躍來傳遞抗藥基因，另外一名為嵌入子(integron)的基因，則可讓外來的抗藥性基因嵌入質體或染色體內，這些基因是導致多重抗藥性菌之主要原因[13,18]。對廣泛性乙內醯氨感受性降低及具抗性之腸桿菌及克雷白氏肺炎桿菌(extended spectrum β-lactam resistant and reduced susceptible *E. coli* and *K. pneumoniae*)之主要抗藥機制為產生分解酵素，其中以質體誘導(plasmid-mediated)之廣泛性乙內醯氨酶(extended-spectrum β-lactamase; ESBL)及AmpC乙內醯氨酶(AmpC β-lactamase)最另人憂心，因這些抗藥基因較容易於不同細菌間傳遞。產生ESBL(ESBL producer)之菌大多在腸細菌科菌種(Enterobacteriaceae)，具ESBL 的大腸桿菌及克雷白氏肺炎桿菌(ESBL producing *E. coli & K. pneumoniae*)對所有青黴素類、頭孢子菌素類及aztreonam抗生素均具抗藥性，但會被乙內醯氨酶抑制劑(β-lactamase inhibitor)抑制，故對內醯氨加乙內醯氨酶抑制劑合併藥(β-lactam/β-lactamase inhibitor combination)無抗藥性[8,13,18,26]。

AmpC β-lactamase 除了對不同頭孢子菌素類抗生素有抗藥性，對乙內醯氨酶抑制劑(β-lactamase inhibitor)亦具抗性，但不一定對所有後線頭孢子菌素類及aztreonam具抗性[12]。因為帶ESBL及AmpC β-lactamase之質體上大多同時帶有其他抗藥基因，故這些菌都是多重抗藥菌，導致醫生常以carbapenem 類最後線抗生素治療。但國內已有醫院發現對carbapenem有抗藥性的大腸桿菌[17]，這也是一需仔細監測的新興抗藥菌。我們最近與國衛院分子基因組合作也發現國內有同時具ESBL及AmpC兩種β-lactamase位於同一個質體之*K. pneumoniae* [4]，這個質體同時具有對兩種其他非β-lactam抗生素之抗藥基因。此外，因為AmpC的存在使得ESBL表現型之測試為假陰性，這對臨床診斷及用藥選擇會是一個危險。因為ESBL跟AmpC兩種β-lactamase之抗生素抗藥目標(substrate profile)會有差異，故會影響臨床用藥之選擇，所以正確的分別及鑑定出此兩群乙內醯氨酶，是協助臨床醫師用藥決定的一重要關鍵[13,26]。

金黃色葡萄球菌中對 methicillin 有抗藥性(methicillin-resistant *S. aureus*, MRSA)之產生是經金黃色葡萄球菌獲得一移動性的基因片段，稱為 staphylococcal cassette chromosome mec (SCC*mec*)，而對 methicillin 產生抗性[10]。MRSA 可製造一種對 β-lactam 類抗生素之親和性都降低的蛋白質，導致 MRSA 對 β-lactam 類抗生素幾乎全具抗性，包含所有青黴素類及頭孢子素類抗生素。同時，大多 MRSA 菌對不同非 β-lactam 類之抗生素亦具抗藥性，包含分子類(如紅黴素)、四環黴素、克林達黴素(clindamycin)、胺基醣苷類(如 gentamicin)、氟化恩氮類(如 ciprofloxacin)、及複方磺胺類[21]，而台灣之 MRSA 抗藥性更高[3]。治療嚴重 MRSA 感染病患的最常用的後線抗生素為萬古黴素(vancomycin)，但近年來日本、美國及其它國家已發現對 vancomycin

有具感受性降低及具抗藥性的菌[30,33]，台灣亦已有個案報告[5]。

1990 年代時，MRSA 在不同國家醫療機構引起住院病人院內感染之個案逐年增加，近年來許多國家也發現社區感染之個案遽增，且有群突發案例發生，如美式足球隊員、監獄犯人等群體感染之事件。但近年來醫院感染及社區感染個案之區分、定義、及其危險因子(risk factors)，包含病人與醫療機構之接觸史，逐漸模糊，故原 hospital-acquired 及 community-acquired 簡稱 H-MRSA 及 C-MRSA 已被 hospital-onset 及 community-onset 或 hospital-associated 及 community-associated 所取代。H-MRSA 及 C-MRSA 之表現型及基因型有許多相異之處。到目前為止，許多其他國家之 H-MRSA 多具 SCC*mec* types II 或 III，而 C-MRSA 則大多具 SCC*mec* type IV。C-MRSA 對上述之非 β-lactam 類抗生素，尤其是 ciprofloxacin 及 SXT，較無抗藥性。另 C-MRSA 跟 H-MRSA 明顯不同之處是其毒素因子，C-MRSA 大多帶有 PVL(Panton-Valentine leukocidin)毒素。PVL 是一種能破壞白血球之毒素，而被具有此 PVL 毒素 MRSA 感染之病狀，可從局部皮膚感染至嚴重疾病包含壞死性肺炎(necrotizing pneumonia)，甚至死亡。這些不同基因型之 MRSA 對非 β-lactam 抗生素之抗藥性亦明顯不同，故了解基因型的分佈可協助了解抗藥趨勢的改變。

此研究計畫為建立抗藥基因庫及抗藥菌分子與臨床流行病學系統之起使。此研究所使用之 TSAR 菌種及基因序列亦將逐年分批一份給疾病管制局做為研究資源之備份及基因序列資料庫之建立，以做為與將來抗藥細菌感染突發調查及研究、以探討及比較其演變及防治其擴散方策之依據。

(二) 材料與方法 (Materials and Methods)

■ **研究對象：**

此計畫為連續型計畫之第一年，計劃於今年七月開始，研究之院感多重抗藥菌為第一至第五期 TSAR (TSAR I – V)中對廣泛性乙內醯氨具抗藥性的大腸桿菌及克雷白氏肺炎桿菌(extended spectrum ß-lactam nonsusceptible *Escherichia coli* and *Klebsiella pneumoniae*)，並調查第五期 TSAR (TSAR V)的抗甲氧苯青黴素金黃色葡萄球菌(Methicillin resistant *Staphylococcus aureus,*簡稱 MRSA)。

■ **研究及分析方法：**

對廣泛性乙內醯氨具抗藥性的大腸桿菌及克雷白氏肺炎桿菌(簡稱 ESB-R *E. coli* 及 ESB-R *K. pneumoniae*)的調查，包含乙內醯氨酶表現型及 ESBL 及 AmpC ß-lactamase 基因之測試、並使用脈衝電泳法(Pulsed-Field Gel Electrophoresis，簡稱 PFGE)調查菌種間相關性。MRSA 之研究則包含 PFGE、多位基因序列分析法 (Multi Locus Sequence Typing, MLST)、抗 methicillin 基因夾 *SCCmec* (Staphylococcus Cassette Chromosome *mec*)型分類、及 Panton-Valentine leukocidin (PVL) toxin gene 測試，並與過去 HAI-MRSA 比對。實驗方法簡述如下：

I). 抗藥測試 (Antimicrobial susceptibility test). 抗藥測試除使用美國 Clinical and Laboratory Standards Institute (CLSI)之 microbroth dilution 方法[7]測試菌株對不同抗生素之最小抑制濃度(Minimum inhibitory concentration，簡稱 MIC)來判讀其抗敏性，亦加用 Etest 測試或確認其中部份菌對一些抗生素之抗敏性。

II). 乙內醯氨酶表現型測試(β-lactamase phenotypic detection).

大腸桿菌及克雷白氏肺炎桿菌中，如其 ceftazidime, cefotaxime,或 aztreonam 抗生素的 MIC 大於或等於 2 μg/ml，則為疑是 ESBL-producer，故先調查其是否會產生 ESBL 外(ESBL confirmatory test)。這些菌亦做 AmpC 表現型試驗，以檢測菌株是否有 class C β-lactamases 類酵素。

i. ESBL confirmatory test (產生 ESBL 菌之確定測試)[7]：將懷疑是 ESBL 之菌從培養 18-24 小時的培養皿挑取 3-5 個相同菌落至食鹽水，調整菌液濃度至 0.5 McFarland 標準，將菌落畫至 Mueller-Hinter agar plate 後，同時測定 ceftazidime (CAZ)，cefotaxime (CTX)及這兩種藥物加入 clavulanic acid 的最低抑菌濃度。如加入 clavulanic acid 的 CAZ 或 CTX 之 MIC 比沒加入 clavulanic acid 之 MIC 降低八倍以上，即確認是 ESBL producer。

ii. AmpC 乙內醯氨酶表現型測試(AmpC β-lactamase phenotypic detection)：

此部份使用三個不同的方法[8,13,35]，以比較其準確性及敏感性。

A. 雙紙錠協力測試法 (Double Disk Synergy Test, DDST): 將調至 0.5 McFarland 濃度的菌液。將菌液均勻塗佈在 Mueller-Hinton Agar (MHA)上，乾

燥後將含有 300 μg aminophenylboronic acid hemisulfate (APB)及 30 μg ceftazidime(CAZ)紙錠及 30 μg cefotaxime(CTX)紙錠以間距 18 毫釐的距離排成直線貼在 MHA 上。放入 35 C 培養箱中培養 16-18 小時後，觀察 CAZ 及 CTX 紙錠與含有 APB 紙錠相鄰處的形狀，若與 APB 紙錠相鄰處有延長的抑制圈，則為陽性反應，若與 APB 紙錠相鄰處無延長的抑制圈，則為陰性反應。

B. 抗生素誘發測試法(Disk Potentiation Test, DPT): 將調為 0.5 McFarland 濃度的菌液均勻塗佈在 Mueller-Hinton Agar (MHA)上，乾燥後將含有 300 μg APB 的 CAZ 紙錠及未含 APB 的 CAZ 紙錠以間距 30 毫釐的距離排成直線貼在 MHA 上，在 35 度 C 培養 16-18 小時後，比較含有 APB 的 CAZ 紙錠及沒含 30 μg 的 CAZ 紙錠之抑制圈大小，若含有 APB 的 CAZ 紙錠之抑制圈比不含 APB 的 CAZ 紙錠大(含)5 mm 則為陽性反應，反之則為陰性反應。

C. 修改之 Hodge 測試法(Modified Hodge Test)：將調為 0.5 McFarland 濃度的菌液均勻塗佈在 Mueller-Hinton Agar (MHA)上，乾燥後將含有 30 μg 的 cefoxitin 紙錠貼在 MHA 正中央的位置，再以取菌環沾取 2~3 個測試菌株的菌落由紙錠邊緣劃直線至培養皿邊緣。放入 35 度 C 培養 16-18 小時，觀察紙錠旁之生長抑制圈及測試菌株生長線處是否有抑制圈減少或扭曲的現象；若有則為陽性反應，無則為陰性反應。

III). 抗廣泛性乙內醯氨大腸桿菌及克雷白氏肺炎桿菌抗藥基因及分子流行病學研究。

i. 脈衝電泳法(Pulsed-Field Gel Electrophoresis, PFGE): 以膠體包埋法 (SeaKem Gold Agarose, Lonza)抽取菌株 DNA 後，用 *Xba*I (10 Units, New England BioLabs) 的限制酵素於 56 度 C 的水浴槽中作用 2 小時，再以初始轉換時間 (initial switch time)2.16 秒及最終轉換時間 (final switch time)54.17 秒，30~600 kbp 的範圍用 CHEF MAPPER 電泳系統 (Bio-Rad)跑電泳 19 小時。跑完後將膠體放入 1 μg/ml 的溴化乙苯非啶溶液 (ethidium bromide)中染色，再用紫外光檢測產物並存檔。存取之影像檔使用 BioNumerics 分析軟體分析電泳片段大小介於 33.3~668.9 kb 間的序列，並以 Dice (Optimization:1.0%, Tolerance: 1.0%)的條件製作 dendrogram 樹狀圖。菌株間之相似性亦參照 Tenover et al 之規則[32]。

ii. ESBL gene & AmpC gene detection：使用多對引子聚合酶連鎖反應法 (Multiplex PCR)參照文獻[1,22-25,28,29,34]，每一反應管中整體積為 50 μl，含有 20 mM Tris-HCl (pH 8.4)、50 mM KCl、0.2 mM dNTP、1.5 mM $MgCl_2$、不同引子之序列(Table 1 and Table 2)及濃度列於下表、1.25 U 的 Taq DNA 聚合酶及 2 μl 的測試菌株 DNA。聚合酶連鎖反應之流程如下：94°C-3 min，25 cycle 之 94°C-30 sec; 64°C-30 sec; 72°C-1 min，及 72°C-7 min。反應做完後以 5 μl 的反應物用 2% 膠體跑電泳，跑完後以 Ethidium bromide 染色，再用 UV 檢測是否有聚合酶反應之產物。

iii. 核酸定序：確認約 DNA 產物後，如需要則純化 PCR 產物進行核酸定序步驟。去氧核醣核酸序列分析 (DNA sequencing): 加入 PCR 反應溶液純化配後，置定

IV). MRSA 抗藥基因及分子流行病學研究

包含 PFGE, *SCCmec* typing, PVL toxin gene 測試，並由 PFGE 結果挑選主要脈衝電泳型別(pulsotype)進行 MLST 之序列型別(Sequence type, ST)，方法簡述如下：

i. MRSA 脈衝電泳法(Pulsed-Field Gel Electrophoresis, PFGE): 實驗根據美國 CDC 建立之標準操作[22]，使用 20 單位之限制酵素 *Sma*I 之反應溶液；經 DNA 分解 agarose plugs 放入 TE buffer 以電泳槽 CHEF-Mapper 跑膠質，電泳後以 0.5 μg/mL ethidium bromide 染色 30 分鐘，清洗後以紫外光照射顯像。用 BioNumerics 的 PFGE 分析專用軟體進行分型圖譜 dendrogram 電泳相似性（similarities）比對，亦參照 Tenover et al 之規則[32]。當這些菌株 DNA bands 之差異低於 6 條時，即被認為來自相關菌源，稱為 pulsotype (A to D)，而各 pulsotype 中如有少數 DNA banding 差別，則稱為 subtype (如：A1, A2 或 C1, C2 等)。

ii. MRSA DNA extraction: 將金黃色葡萄球菌懸浮於 1.5 ml 離心管的培養液中離心 10 分鐘，去除上清液後，以 180 μl 的 lysis buffer 懸浮菌體，並加入 5 μl 濃度為 5 mg/ml 之 lysostaphin，置於 37℃ 反應 30 分鐘。此後步驟採用 Qiagene DNA extraction kit 之 Gram-positive bacteria 方法(Qiagene DNAeasy Blood and Tissue Kit)。

iv. *SCCmec* typing: 使用 multiplex PCR [6]，測試 MRSA 之 *ccr* 及 *mec* 之型。PCR 之步驟基本如下：過夜培養之培養基(非選擇性培養基)上挖取約 $2x10^9$ cell 的細菌萃取 DNA，於 0.2 ml 的 PCR 專用薄壁離心管內加入(每一個檢體) ddH_2O、1X buffer、10 picomole 核酸引子對(primers)(不同抗藥基因各有其目標之 primer 序列(REF)、2.5 nM dNTP、0.2 U Taq polymerase、細菌 DNA，經過 30 cycles 的 denaturing, annealing, elongation，放 5 ul 之 PCR 反應溶液與 loading dye 於洋菜膠，電泳後用 EtBr 染色照相判斷結果。*SCCmec* 型之判斷是由以下列組合決定。

SCCmec type	*Ccr*	*mec*
Type I	Type-1 *ccr* (*ccrA1* and *B1*)	Class B *mec*
Type II	Type-2 *ccr* (*ccrA2* and *B2*)	Class A *mec*
Type III	Type-3 *ccr* (*ccrA3* and *B3*)	Class A *mec*
Type IV	Type-2 *ccr* (*ccrA2* and *B2*)	Class B *mec*
Type V	Type-5 *ccr* (*ccrC*)	Class C *mec*

iv. PVL toxin gene detection: Primer 及 PCR 參考文獻[21]。

v. 多位基因序列分析法(MLST): 抽取測試之金黃色葡萄球菌的DNA，每株菌分別進行下列 7 組引子(primer)之PCR：*arcC, aroE, glpF ,gmk, pta, tpi, yqiL*，PCR反應溶液純化配後，序列反應後，用組合序列之軟體先將forward及reverse端組合(如chromas、vectorNTI等)之後，進行核酸序列比對，將每株菌所得的 7 段序列貼上MLST網(http://saureus.mlst.net/sql/multiplelocus.asp)，以找出此株菌相對應的多位基因序列分型(Sequence type, ST)，進而與世界其他國家之MRSA之ST做比對，以便追蹤國內MRSA之演變[9]。

V). 資料分析：使用世界衛生組織之 Whonet 分析軟體，分析這些細菌對多種抗生素之抗藥性。並使用 Epi Info 6.04 軟體(CDC, Atlanta, GA)統計學比較其不同年度趨勢。

(三、四) Results and Discussion (結果 與 討論)

- **Extended-spectrum β-lactam non-susceptible *Escherichia coli* from nosocomial infections, 1998-2006.**

E. coli comprised 12.3% (325 isolates) of the nosocomial pathogens collected in the 5 rounds of TSAR between 1998 and 2006 (Table 3). Among these 325 isolates, 70, 33, 71, 85, and 66 were from TSAR I, II, III, IV, and V, respectively. Rates of antimicrobial resistance of these nosocomial isolates from different years are listed in Table 4. There was significant increase in resistance to β-lactams including first and second generation cephalosporins cefazolin (21.4% in 1998 to 48.5% in 2006, $p < 0.001$) and cefuroxime (15.7% in 1998 to 45.5% in 2006, $p < 0.001$), extended spectrum cephalosporins cefotaxime (6.1% in 2000 to 22.7% in 2006, NS) and ceftazidime (2.9% in 1998 to 18.2% in 2006, $p < 0.01$). There were also significant increases in resistance to β-lactam/β-lactamase inhibitors amoxicillin/clavulanic acid (17.1% in 1998 to 39.4% in 2006, $p < 0.001$), cephamycin cefoxitin (6.1% in 2000 to 33.3% in 2006, $p < 0.01$), and fluoroquinolone ciprofloxacin (22.1% in 1998 to 43.9% in 2006, $p < 0.01$). The increase in resistance to cephalosporins, and cephamycin indicated that in addition to increase in extended spectrum β-lactamase (ESBL) producers, AmpC producers likely increased.

Among the 325 *E. coli* isolates, 103 (31.6%) were possible ESBL-producers (MIC of aztreonam, cefotaxime or ceftazidime $\geq$ 2 ug/ml), including 10, 5, 22, 37, and 29 from TSAR I, II, III, IV, and V, respectively. The isolates were predominately from urine (61), with the rest from blood (19), respiratory tract (5), pus/abscess (12) and other specimen types (6). These isolates were subjected to further phenotypic ESBL confirmatory tests, AmpC phenotypic tests, and genotypic detection of ESBL and AmpC β-lactamases genes. A total of 44 isolates were confirmed to be ESBL-producers (13.5% of 325). The ESBL-positive rates increased from 8.6% in 1998 to 16.7% in 2006 ($p > 0.05$) (Table 5). This is consistent with the observation of increasing resistance to extended spectrum cephalosporins. Among these 44 confirmed ESBL-producers, 14 tested positive by cefotaxime only, 2 tested positive by ceftazidime only, and 28 tested positive by both agents. Among the *E. coli* isolates studied for the presence of *bla*ESBL genes, 5 harbored bla_{SHV} type (SHV-5-like), 34 had $bla_{CTX\text{-}M}$ and 5 isolates had both bla_{SHV} and $bla_{CTX\text{-}M}$ (12 CTX-M group I and 27 CTX-M group IV) (Table 6).

A total of 68 *E. coli* isolates also tested positive for AmpC by either one or more the 3 AmpC phenotypic methods, 10 of which were also positive by ESBL-confirmatory test. Among the 58 ESBL-confirmatory test negative/AmpC-positive isolates, 49

harbored bla_{CMY-2} AmpC, 1 harbored both bla_{DHA-1} and $bla_{CMY-2.}$ Of the other 10 phenotypic ESBL-positive/AmpC-positive isolates, AmpC bla_{CMY-2} was detected in 8, bla_{CMY-4} was detected in 1, the other blaAmpC negative isolate carried bla_{CTX-M} type ESBL. Co-carriage of *bla*ESBL and *bla*AmpC was found in 12 isolates.

■ Extended-spectrum β-lactam non-susceptible *Klebsiella pneumoniae* from nosocomial infections, 1998-2006

Among all the nosocomial isolates collected in the 5 rounds of TSAR between 1998 and 2006, 255 (9.7%) were *K. pneumoniae* (Table 3), including 63, 17, 49, 61, and 65 isolates from TSAR I, II, III, IV, and V, respectively. Rates of antimicrobial resistance of these nosocomial isolates from different years are listed in Table 7. There was significant increase in resistance to nearly all antimicrobials including first and second generation cephalosporins cefazolin (28.6% in 1998 to 52.3% in 2006, $p < 0.01$) and cefuroxime (28.6% in 1998 to 50.8% in 2006, $p < 0.01$), extended spectrum cephalosporins cefotaxime (17.6% in 2000 to 30.8% in 2006, $p < 0.05$) and ceftazidime (11.1% in 1998 to 32.3% in 2006, $p < 0.01$). There were also significant increases in resistance to monobactam aztreonam (15.9% in 1998 to 32.3% in 2006, $p < 0.05$), β-lactam/β-lactamase inhibitors amoxicillin/clavulanic acid (12.7% in 1998 to 40% in 2006, $p < 0.001$), cephamycin cefoxitin (5.9% in 2000 to 32.3% in 2006, $p < 0.01$), and fluoroquinolone ciprofloxacin (14.3% in 1998 to 44.6% in 2006, $p < 0.01$), again indicating the likelihood of increase in extended spectrum β-lactamase (ESBL) producers as well as AmpC producers likely increased.

Among the 255 nosocomial *K. pneumoniae* isolates, 90 (35.3%) were possible ESBL-producers MIC of aztreonam, cefotaxime or ceftazidime $\geq$ 2 ug/ml), including 10, 5, 22, 37, and 29 from TSAR I, II, III, IV, and V, respectively. Due to special collection of ESBL producing *K. pneumoniae* in TSAR II and III, there were additional 45 nosocomial isolates for further characterization. Of these 135 studied, 63, 26, 25, and 14 were from urine, blood, respiratory, and pus/abscess specimens, respectively. Among these possible ESBLs, 114 were confirmed to be ESBL-producers. The ESBL-positive rates increased from 23.8% in 1998 to 31.3% in 2006 ($p > 0.05$) (Table 7). Among these 114 ESBL-confirmatory test positive isolates, 31 tested positive by cefotaxime only, 7 tested positive by ceftazidime only, and 76 tested positive by both agents. Among the 114 isolates, 42 harbored SHV-5,12-like *bla*ESBL, 57 harbored CTX-M type *bla*ESBL bla_{CTX-M} and 14 isolates that carried both bla_{SHV} and bla_{CTX-M} (35 CTX-M group I and 36 CTX-M group IV) (Table 8).

A total of 36 of the *K. pneumoniae* isolates also tested positive for AmpC by either one or more of the 3 AmpC phenotypic methods, 20 of which were also positive by ESBL-confirmatory test. All of the 20 ESBL-confirmatory test negative/AmpC-positive isolates harbored the bla_{DHA-1} AmpC. Of the other 17 ESBL-confirmatory test negative/AmpC-positive isolates, 16 also carried bla_{DHA-1}. AmpC bla_{CMY-2} was detected only in 1 *K. pneumoniae* (Table 8).

- **Pulsed filed gel electrophoresis of extended spectrum β-lactam non-susceptible nosocomial *E. coli* and *K. pneumoniae*.**

PFGE was performed on XbaI digested chromosomal DNA of all 100 *E. coli* and 133 *K. pneumoniae* isolates studied for resistance mechanisms described above to determine if a particular clone predominated or if there were inter-hospital clonal dissemination. 2 *E. coli* isolates (2006HLH110 and 2006HLH180) (Fig 1A) and 2 pairs of *K. pneumoniae* isolates (2002SCM148 and 2002SCM157; 2002CHC216 and 2002CHC217) (Fig 2) from the same hospital within the same year had identical PFGE patterns, indicating some intra-hospital spread may have occurred. All other isolates had PFGE patterns distinct from each other, indicating that the increase in ESBL and AmpC-producing *E. coli* and *K. pneumoniae* is likely not due to clonal spread since no predominate clone or inter-hospital clonal dissemination was found based on PFGE.

- **Discussion on ESC-NS *E. coli* and *K. pneumoniae***

The proportions of isolates carrying genes encoding ESBL and AmpC in extended spectrum ß-lactam non-susceptible (ESC-NS) nosocomial *E. coli* and *K. pneumoniae* changed over the years (Fig. 3). In 1998, isolates carrying *bla*ESBL comprised 60% (6/10) of ESC-NS *E. coli* and 88.2% (15/17) of ESC-NS *K. pneumoniae* and no AmpC was detected in either species. In 2006, isolates carrying *bla*ESBL comprised 26.9% (7/26) of ESC-NS *E. coli* and 38.7% (12/31) of ESC-NS *K. pneumoniae* while AmpC was detected in 73.1% of ESC-NS *E. coli* and 51.6% of ESC-NS *K. pneumoniae*. The increase in resistance to extended spectrum cephalosporins and cephamycin correlated with the increase in both ESBL producers and AmpC producers. Concurrent to the decrease in the proportions of isolates carrying only *bla*ESBL, there was increase in isolates with blaAmpC and in isolates carrying both *bla*ESBL and *bla*AmpC.

Of noteworthy is that even though bla_{CTX-M} type is the predominate ESBL carried in both *E. coli* and *K. pneumoniae* isolates, their AmpC profiles differed. All 59 AmpC-positive *E. coli* isolates carried *bla*$_{CMY}$-type AmpC, except one that also carried

bla$_{DHA-1}$ AmpC. In contrast, 36 of 37 AmpC-carrying *K. pneumoniae* carried *bla*$_{DHA-1}$ AmpC, and only one isolate carried *bla*$_{CMY}$-type AmpC. This study of nosocomial isolates from multiple hospitals in different years confirmed that CTX-M extended-spectrum β-lactamases (ESBLs) is endemic in Taiwan and plasmid-mediated AmpC β-lactamases are increasing in Taiwan hospitals.

In an earlier study of *E. coli* and *K. pneumoniae* from 7 medical centers in 2003, Yan et al found identical or similar restriction patterns of conjugative plasmids from CMY-2 producing *E. coli*, and those from CTX-M producing *E. coli* and *K. pneumoniae* [36]. Recently, a multidrug resistant conjugative plasmid carrying genes encoding CMY-type AmpC and CTX-M ESBL has also been reported by Chen et al [4]. The present study found that the genes coding extended spectrum β-lactamses (ESBL) mostly belong to the SHV-5- like and CTX-M group I and group IV *bla*ESBLs, and CMY-2 (in *E. coli*) and DHA-1 (in *K. pneumoniae*) *bla*AmpC. PFGE of chromosomal DNA also found diversed heterogeneity among these extended spectrum β-lactam nonsusceptible *E. coli* and *K. pneumoniae* nosocomial strains. These results indicate that transfer of plasmids carrying resistance *bla*ESBL and *bla*AmpC genes likely account for the increasing ESBL and AmpC producers in these 2 species.

■ **MRSA from nosocomial infections, 2006**

Among the 694 *S. aureus* collected from 25 hospitals in TSAR V (2006), 482 were from inpatients, including 67 (13.9% of inpatient isolates) from hospital-acquired infections (HAI), of which 51 isolates were methicillin resistant (76.1%). Among these 51 HAI-MRSA, 19, 10, 16 were from blood, respiratory, and wound specimens, and 5 were from catheters, and the other one was from pleural fluid. Based on pulsed field gel electrophoresis (PFGE) results, these 51 isolates can be grouped into 4 main distinct pulsotypes (clonotypes), with 64.7% (33), 9.8% (5), 2.0% (1), and 19.6% (10) of the isolates belonging to pulsotypes A, B, C, and D, respectively (Table 9). Together these 4 main pulsotypes comprised 96.1% of the nosocomial MRSA in TSAR V. Isolates assigned within the same main pulsotype (A, B, C or D) are either indistinguishable from each other, closely related or possibly related to each other (Figure 4). Isolates within the same pulsotype also share other characteristics, such as SCCmec, MLST sequence type, and antimicrobial resistance profiles (Table 10).

Isolates of the same pulsotype, SCC*mec*, and sequence type clonal complex are considered the same clonatype. Pulsotype A (SCC*mec* III:ST239 or 241) belongs to the Hungarian/Brazilian lineage and is more prevalent in Europe, while pulsotype D

(SCCmec II:ST5) belongs to the New York/Japan lineage and is more prevalent in USA and Japan. Both pulsotype A and D are resistant to ciprofloxacin, but pulsotype D is susceptible to trimethoprim/sulfamethoxazole (SXT) and tetracycline. Pulsotype B (SCC*mec*:ST59) and pulsotype C(SCC*mec* V:ST59) are the predominant community strain MRSA found in Taiwan [3] but are not common in other countries, and they are usually susceptible to ciprofloxacin and trimethoprim/sulfamethoxazole. Pulsotype C isolates also carry the virulent PVL toxin gene so may cause more severe infections in susceptible hosts. The change in MRSA epidemiology is intriguing and may impact treatment strategies since these clonatypes have distinct resistance profiles.

The distribution of these 4 pulsotypes in nosocomial MRSA isolates from the previous 4 rounds of TSAR (1998-2004) was compared with those from TSAR V (2006) (Table 9). There was significant decrease in pulsotype A with concurrent emergence and increase of pulsotype D. The proportion of pulsotype A MRSA decreased from 93.7% in 1998 to 68.4% in 2002 to 64.7% in 2006 (p <0.001). Of noteworthy is that there was no pulsotype D in 2000, but it emerged in 2000 (1 isolate, 2.6%), and further increased to 20.4% in 2004, and this clonatype accounted for 19.6% of nosocomial MRSA in TSAR V. The increase of pulsotype B and C in causing nosocomial infections is also worrisome since this indicates that they are able to survive in the hospital environment, where they may acquire additional resistance. The reason for the changing epidemiology of MRSA clonotypes in Taiwan hospitals is unknown at the present time. Changes in antimicrobial use, possession of different virulence factors (such as PVL toxin in pulsotype C) and biological fitness are some possible reasons. However, further studies are needed to identify the factors affecting MRSA epidemiology in Taiwan.

(五)、Conclusions and Suggestions (結論與建議)

These results indicate that the increase in resistance to extended spectrum β-lactams in *E. coli* and *K. pneumoniae* is likely due to transfer of resistance determinants between bacterial strains and clonal spread may play a role within a hospital. The most common extended spectrum β-lactamase (ESBL) gene is $bla_{CTX\text{-}M}$ type, followed by bla_{SHV}- type. The most common AmpC β-lactamase genes are $bla_{DHA\text{-}1}$ in *K. pneumoniae* and bla_{CMY} type in *E. coli*. The increase in isolates with genes encoding plasmid-mediated AmpC enzymes and of isolates possessing both ESBL and AmpC enzymes is a concern since these genes are likely carried on transferable plasmids. Strict control and monitoring of antimicrobial use is a must to prevent the further emergence and dissemination of transferable resistance elements.

In contrast, MRSA strains in Taiwan hospitals are highly clonal. The predominant MRSA clone causing nosocomial infections in Taiwan hospitals continues to be the most multidrug resistant pulsotype A (SCC*mec* III:ST239/241). However, another clone pulsotype D (SCC*mec* II:ST5) is increasing. In addition, community strain MRSA [pulsotype B (SCC*mec* IV:ST59) and pulsotype C (SCC*mec* V:ST5)] have also migrated into the hospital and may have established a niche in the hospital environment. The risk factors, disease spectrum and treatment outcomes of these MRSA infections warrants further study since their resistance and toxin profiles differ and may affect treatment outcome. Since MRSA carriage precedes infection and is a hidden reservoir for transmitting MRSA to other patients, active surveillance for the carriage of MRSA on high-risk patient groups to identify MRSA carriers and implement appropriate contact precaution and infection control measures may help to reduce the further spread of this multidrug resistant organism in our hospitals.

In conclusion, strategies for the control of rising antimicrobial resistance should include antibiotic stewardship and enforcement of infection control policy. Decrease in selective pressure can prevent polyclonal expansion of resistance determinants and adequate infection control measures can prevent the spread of resistant clones.

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(七) 圖表 Tables and Figures.

Table 1. Primers for extended spectrum β-lactamase genes detection.

Target(s)	Primer	Sequence (5′ to 3 ′)	Expected amplicon size (bp)	Nucleotide positions	PCR working concentration
*bla*CTX-M	CTX-M-U1	ATGTGCAGYACCAGTAARGTKATGGC	593		0.4 M
*bla*CTX-M	CTX-M-U2	TGGGTRAARTARGTSACCAGAAYCAGCGG			0.4 M
*bla*PER	PER-1F	ATGAATGTCATTATAAAAGCT	926	1-20	1 M
*bla*PER	PER-1R	TTAATTTGGGCTTAGGG		927-911	1 M
*bla*PER	PER-2F	ATGAATGTCATCACAAAATG	926	278-997	1 M
*bla*PER	PER-2R	TCAATCCGGACTCACT		1204-1189	1 M
*bla*SHV	SHV.SE	ATGCGTTATATTCGCCTGTG	747		0.4 M
*bla*SHV	SHV.AS	TGCTTTGTTATTCGGGCCAA			0.4 M
*bla*TEM	TEM-164.SE	TCGCCGCATACACTATTCTCAGAATGA	445		0.4 M
*bla*TEM	TEM-165.AS	ACGCTCACCGGCTCCAGATTTAT			0.4 M
*bla*VEB	VEB-1F	GATAGGAGTACAGACATATG	914	12-31	1 M
*bla*VEB	VEB-1R	TTTATTCAAATAGTAATTCCACG		925-903	1 M
*bla*VEB	VEB-2F	CGACTTCCATTTCCCGATGC	642	343-362	1 M
*bla*VEB	VEB-2R	GGACTCTGCAACAAATACGC		985-966	1 M

Table 2. Primers used for detection of AmpC β-lactamase genes in multiplex PCR.

Target(s)	Primer	Sequence (5′ to 3 ′, as synthesized)	Expected amplicon size (bp)	Nucleotide positions	PCR working concentration
MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	MOXMF	GCTGCTCAAGGAGCACAGGAT	520	358-378	0.6 M
	MOXMR	CACATTGACATAGGTGTGGTGC		877-856	0.6 M
LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITMF	TGGCCAGAACTGACAGGCAAA	462	478-498	0.6 M
	CITMR	TTTCTCCTGAACGTGGCTGGC		939-919	0.6 M
DHA-1, DHA-2	DHAMF	AACTTTCACAGGTGTGCTGGG T	405	1244-1265	0.6 M
	DHAMR	CCGTACGCATACTGGCTTTGC		1648-1628	0.6 M
ACC	ACCMF	AACAGCCTCAGCAGCCGGTTA	346	861-881	0.5 M
	ACCMR	TTCGCCGCAATCATCCCTAGC		1206-1186	0.5 M
MIR-1T ACT-1	EBCMF	TCGGTAAAGCCGATGTTGCGG	302	1115-1135	0.5 M
	EBCMR	CTTCCACTGCGGCTGCCAGTT		1416-1396	0.5 M
FOX-1 to FOX-5b	FOXMF	AACATGGGGTATCAGGGAGATG	190	1475-1496	0.4 M
	FOXMR	CAAAGCGCGTAACCGGATTGG		1664-1644	0.4 M

Table 3. Top 10 species of nosocomial pathogens in TSAR collection

Organism	TSAR I (1998)		TSAR II (2000)		TSAR III (2002)		TSAR IV (2004)		TSAR V (2006)		TSAR I to IV	
	N	%	N	%	N	%	N	%	N	%	N	%
P. aeruginosa	141	16.7	24	9.4	75	14.2	55	10.7	85	16.6	380	14.3
S. aureus	119	14.1	50	19.7	56	10.6	78	15.2	70	13.7	373	14.1
E. coli	70	8.3	33	13.0	71	13.4	85	16.6	66	12.9	325	12.3
A. baumannii	102	12.1	13	5.1	50	9.5	41	8.0	68	13.3	274	10.3
K. pneumoniae	63	7.5	17	6.7	49	9.3	61	11.9	67	13.1	257	9.7
E. faecalis	21	2.5	22	8.7	28	5.3	31	6.1	23	4.5	125	4.7
S. marcescens	43	5.1	25	9.8	20	3.8	8	1.6	11	2.1	107	4.0
E. cloacae	30	3.6	10	3.9	16	3.0	17	3.3	18	3.5	91	3.4
P. mirabilis	28	3.3	1	0.4	12	2.3	17	3.3	21	4.1	79	3.0
E. faecium	2	0.2	9	3.5	12	2.3	10	2.0	17	3.3	50	1.9

Table 4. Comparison of antimicrobial resistance rates (%) in nosocomial *Escherichia coli* from different years

Antimicrobial agent	TSAR I (1998)	TSAR II (2000)	TSAR III (2002)	TSAR IV (2004)	TSAR V (2006)	p [b]
	N = 70	N = 33	N = 71	N = 85	N = 66	
Amikacin	2.9	3	9.9	7.1	6.1	NS
Amoxicillin/CA[a]	17.1	6.1	28.2	37.6	39.4	<0.001
Ampicillin	85.7	75.8	91.5	89.4	84.8	NS
Aztreonam	4.3	12.1	8.5	12.9	15.2	NS
Cephalosporin						
Cefazolin	21.4	18.2	42.3	47.1	48.5	<0.001
Cefuroxime	15.7	15.2	35.2	44.7	45.5	<0.001
Cefotaxime	NT	6.1	14.1	15.3	22.7	NS
Ceftazidime	2.9	9.1	8.5	22.4	18.2	<0.01
Cefepime	NT	3	7	12.9	12.1	NS
Cefoxitin	NT	6.1	22.5	35.3	33.3	<0.01
Ciprofloxacin	22.1	27.3	46.5	41.2	43.9	<0.01
Gentamicin	52.9	57.6	60.6	55.3	45.5	NS
Imipenem	0	0	0	1.2	0	NS
Trimeth./Sulfa.	75.7	78.8	83.1	68.2	60.6	<0.05

[a] CA, Clavulanate acid.

[b] p value for trend analysis; NS, not significant (p >0.05).

Table 5. Proportions (%) of extended spectrum β-lactamase (ESBL) confirmatory test positive isolates in nosocomial *E. coli* and *K. pneumoniae* from different years[a]

TSAR (Year)	% (# positive/# tested)	
	E. coli	*K. pneumoniae*
TSAR I (1998)	8.6 (6/70)	23.8 (15/63)
TSAR II (2000)	9.1 (3/33)	* (14/27)
TSAR III (2002)	16.9 (12/71)	* (42/84)
TSAR IV (2004)	14.1 (12/85)	36.1 (22/61)
TSAR V (2006)	16.7 (11/66)	31.3 (21/67)

[a] ESBL Confirmatory test was performed following the guidelines of Clinical and Laboratory Standards Institute (CLSI) [REF].

* In TSAR II and III, additional ESBL-producing *K. pneumoniae* were collected under a special collection. Most of these ESBL-producers turn out to be from nosocomial infections and are included in this study. But the ESBL-positive rates in these 2 rounds of TSAR cannot be calculated.

Table 6. Distribution of extended spectrum ß-lactamase (ESBL) and AmpC ß-lactamase in nosocomial *Escherichia coli*

Phenotypic detection of:				ESBL genes detected[a]				AmpC genes detected[b]			
ESBL		AmpC	N	*bla*$_{SHV}$ type	*bla*$_{CTX-M}$ type	Both types	None	*bla*$_{DHA}$ type	*bla*$_{CMY}$ type	Both types	None
Ceftazidime	**Cefotaxime**										
Neg	Pos	Pos	5	1	3	1	0	0	4	0	1
Pos	Neg	Pos	2	0	0	0	2	0	2	0	0
Pos	Pos	Pos	3	0	1	2	0	0	3	0	0
Neg	Pos	Neg	6	0	5	1	0	0	0	0	6
Pos	Neg	Neg	0	0	0	0	0	0	0	0	0
Pos	Pos	Neg	25	2	22	1	0	0	0	0	25
Neg	Neg	Pos	58	2	3	0	53	0	49	1	8
Neg	Neg	Neg	1	0	0	0	1	0	0	0	1
Total			100	5	34	5	56	0	58	1	41

[a] *bla*$_{SHV:}$ 8 SHV-5-like, the other 2 are pending further analysis; *bla*$_{CTX-M}$ (12 CTX-M group I and 27 CTX-M group IV).

[b] *bla*$_{CMY}$ AmpC were all *bla*$_{CMY-2}$ except one was bla $_{CMY-4}$, *bla*$_{DHA}$ were DHA-1

Table 7. Comparison of antimicrobial resistance rates (%) in nosocomial *K. pneumoniae* from different years

Antimicrobial agent	TSAR I (1998)	TSAR II (2000)	TSAR III (2002)	TSAR IV (2004)	TSAR V (2006)	p [b]
	N = 63	N = 17	N = 49	N = 61	N = 65	
Amikacin	17.5	11.8	4.1	19.7	26.2	<0.05
Amoxicillin/CA[a]	12.7	11.8	12.2	31.1	40	<0.001
Aztreonam	15.9	11.8	10.2	27.9	32.3	<0.05
Cephalosporin						
Cefazolin	28.6	29.4	24.5	47.5	52.3	<0.01
Cefuroxime	28.6	23.5	16.3	45.9	50.8	<0.001
Cefotaxime	NT	17.6	8.2	29.5	30.8	<0.05
Ceftazidime	11.1	11.8	8.2	19.7	32.3	<0.01
Cefepime	NT	11.8	2	24.6	23.1	<0.01
Cefoxitin	NT	5.9	8.2	27.9	32.3	<0.01
Ciprofloxacin	14.3	23.5	14.3	39.3	44.6	<0.01
Gentamicin	31.7	35.3	20.4	39.3	40	NS
Imipenem	0	0	0	1.6	0	NS
Trimeth./Sulfa.	46	52.9	24.5	50.8	53.8	<0.05

[a] CA, Clavulanate acid.

[b] p value for trend analysis; NS, not significant (p >0.05).

Table 8. Distribution of extended spectrum ß-lactamase (ESBL) and AmpC ß-lactamase in nosocomial *K. pneumoniae*

Phenotypic detection of:				ESBL genes detected				AmpC genes detected			
ESBL		AmpC	N	*bla*$_{SHV}$ type	*bla*$_{CTX-M}$ type	Both types	None	*bla*$_{DHA}$ type	*bla*$_{CMY}$ type	Both types	None
Ceftazidime	**Cefotaxime**										
Neg	Pos	Pos	13	1	12	0	0	13	0	0	0
Pos	Neg	Pos	3	1	2	0	0	0	0	0	3
Pos	Pos	Pos	4	3	0	1	0	4	0	0	0
Neg	Pos	Neg	18	1	13	3	1	1	0	0	17
Pos	Neg	Neg	4	2	0	0	2	1	0	0	3
Pos	Pos	Neg	72	30	30	10	2	1	0	0	71
Neg	Neg	Pos	17	0	0	0	17	16	1	0	0
Neg	Neg	Neg	4	4	0	0	0	0	0	0	4
Total			135	42	57	14	22	36	1	0	98

[a] *bla*$_{SHV:}$ all SHV-5,12-like, *bla*$_{CTX-M}$ (35 CTX-M group I and 36 CTX-M group IV).

[b] bla$_{DHA}$ were all DHA-1, *bla*$_{CMY}$ AmpC was bla$_{CMY-2}$,

Table 9. Distribution of major pulsotypes in MRSA causing hospital acquired infections in different years in Taiwan

Pulsotype[a]	Number of isolates (%) with pulsotype in:					P^b
	1998 (n = 95)	2000 (n = 39)	2002 (n = 38)	2004 (n = 54)	2006 (n = 51)	
A	89 (93.7)	31 (79.5)	26 (68.4)	31 (57.4)	33 (64.7)	<0.001
B	3 (3.15)	4 (10.3)	7 (18.4)	5 (9.3)	5 (9.8)	0.08
C	0	2 (5.1)	1 (2.6)	6 (11.1)	1 (2.0)	0.01
D	0	1 (2.6)	3 (7.9)	11 (20.4)	10 (19.6)	<0.001
Not A-D	3 (3.15)	0	1 (2.6)	1 (1.8)	2 (3.9)	Not done

[a] Pulsotype designation was based on 80% similarity of *Sma*I digested chromosomal DNA pattern

[b] *p* value for trend analysis.

Table 10. Comparison of characteristics of the main pulsotypes of MRSA causing hospital acquired infections in Taiwan from 1998-2006

Characteristics	Pulsotype			
	A (n=210)	B (n=24)	C (n=10)	D (n=25)
Antimicrobial resistance profile:				
n (%) resistant to				
Chloramphenicol	47 (22.4)	22 (91.7)	10 (100)	2 (8.0)
Ciprofloxacin	209 (99.5)	0	0	25 (100)
Erythromycin	210 (100)	24 (100)	10 (100)	25 (100)
Gentamicin	209 (99.5)	18 (75.0)	1 (10.0)	25 (100)
Tetracycline	209 (99.5)	15 (62.5)	6 (60.0)	0
SXT[a]	207 (98.6)	0	0	1 (4.0)
Molecular typing[b]:				
SCC*mec* type	III	IV	V	II
MLST performed (n)	27	9	2	12
Sequence type (n)	ST239 (19), ST241 (6), ST900 (1) ST239-like (1)	ST59 (9)	ST59 (2)	ST5 (10), ST5-like (2)
pvl detection	-	-	+	-

[a] SXT, trimethoprim/sulfamethoxazole

[b] Molecular typing included: SCC*mec* typing and Panton-Valentine leukocidin gene (*pvl*) detection, which were done on all isolates. MLST was used to determine the sequence type (ST), which was performed on selected isolates from each representative pulsotype.

Figure 1A and 1B. Dendrograms of *Xba*I digested chromosomal DNA of extended spectrum β-lactam non-susceptible nosocomial *E. coli* in Taiwan (only selected strains are shown)

Fig 1A.

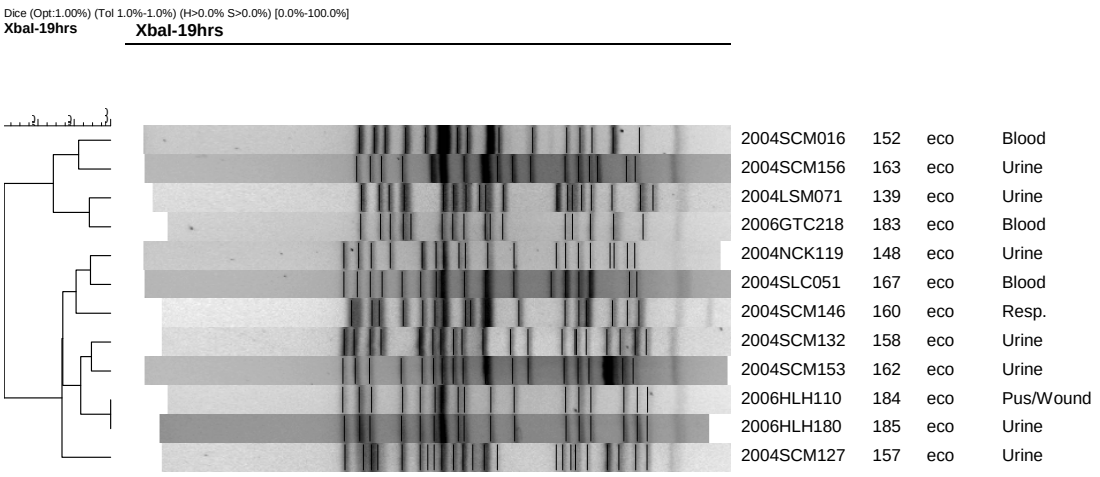


Fig 1B.

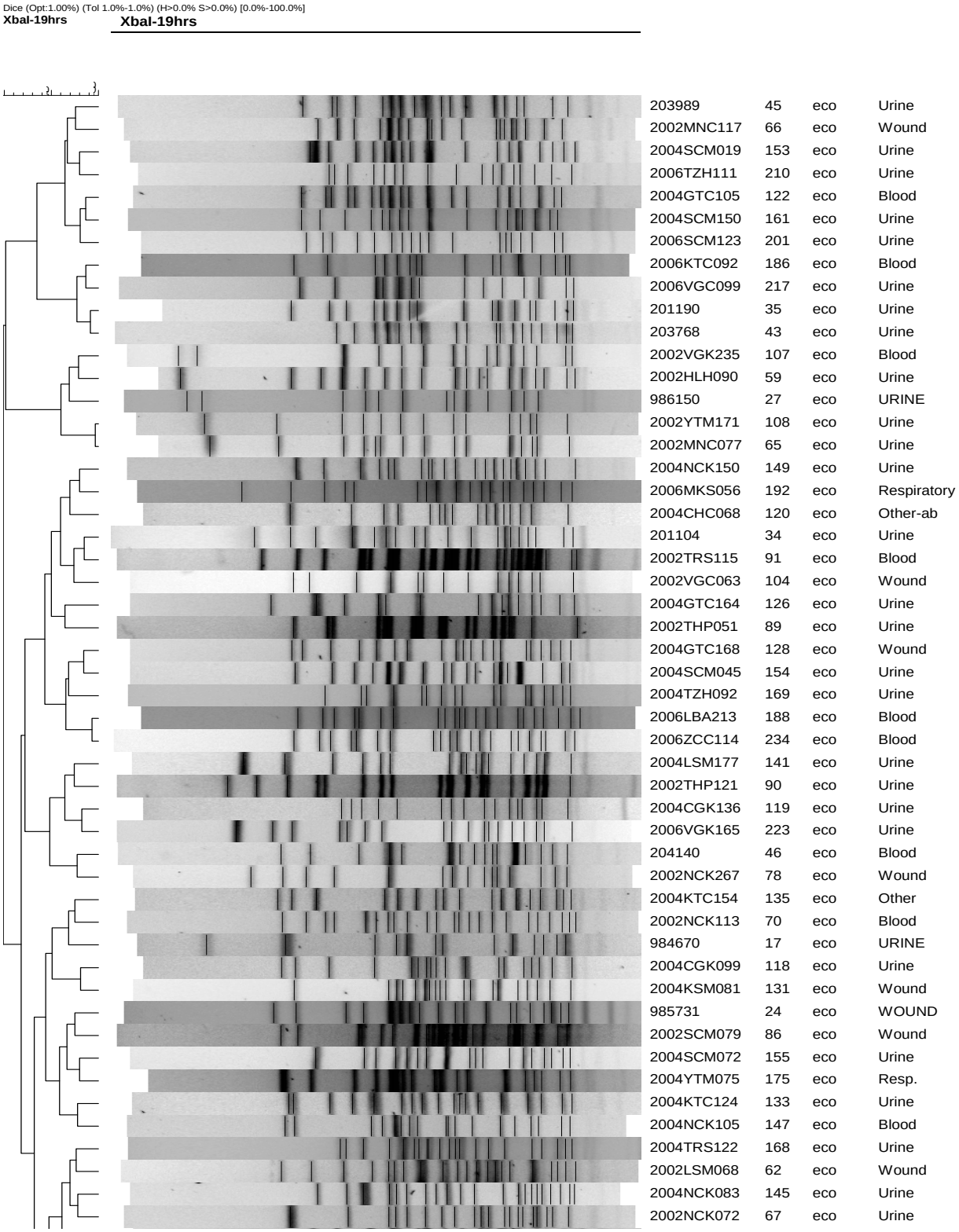

Dice (Opt:1.00%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
XbaI-19hrs
XbaI-19hrs
203989 45 eco Urine
2002MNC117 66 eco Wound
2004SCM019 153 eco Urine
2006TZH111 210 eco Urine
2004GTC105 122 eco Blood
2004SCM150 161 eco Urine
2006SCM123 201 eco Urine
2006KTC092 186 eco Blood
2006VGC099 217 eco Urine
201190 35 eco Urine
203768 43 eco Urine
2002VGK235 107 eco Blood
2002HLH090 59 eco Urine
986150 27 eco URINE
2002YTM171 108 eco Urine
2002MNC077 65 eco Urine
2004NCK150 149 eco Urine
2006MKS056 192 eco Respiratory
2004CHC068 120 eco Other-ab
201104 34 eco Urine
2002TRS115 91 eco Blood
2002VGC063 104 eco Wound
2004GTC164 126 eco Urine
2002THP051 89 eco Urine
2004GTC168 128 eco Wound
2004SCM045 154 eco Urine
2004TZH092 169 eco Urine
2006LBA213 188 eco Blood
2006ZCC114 234 eco Blood
2004LSM177 141 eco Urine
2002THP121 90 eco Urine
2004CGK136 119 eco Urine
2006VGK165 223 eco Urine
204140 46 eco Blood
2002NCK267 78 eco Wound
2004KTC154 135 eco Other
2002NCK113 70 eco Blood
984670 17 eco URINE
2004CGK099 118 eco Urine
2004KSM081 131 eco Wound
985731 24 eco WOUND
2002SCM079 86 eco Wound
2004SCM072 155 eco Urine
2004YTM075 175 eco Resp.
2004KTC124 133 eco Urine
2004NCK105 147 eco Blood
2004TRS122 168 eco Urine
2002LSM068 62 eco Wound
2004NCK083 145 eco Urine
2002NCK072 67 eco Urine

Figure. 2. Dendrogram of *Xba*I digested chromosomal DNA of extended spectrum β-lactam non-susceptible nosocomial *K. pneumoniae* in Taiwan (Not all 135 strains are shown).

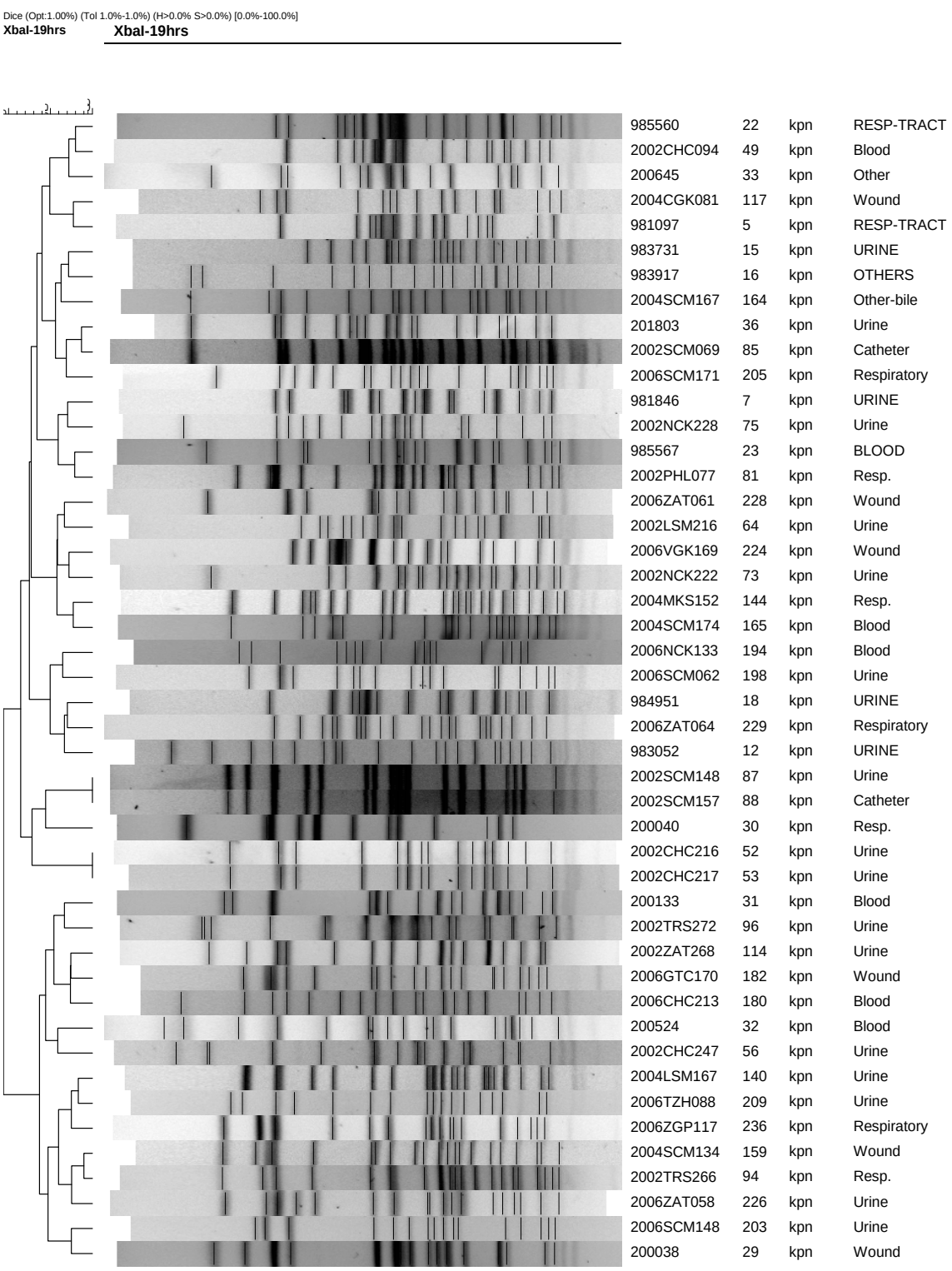
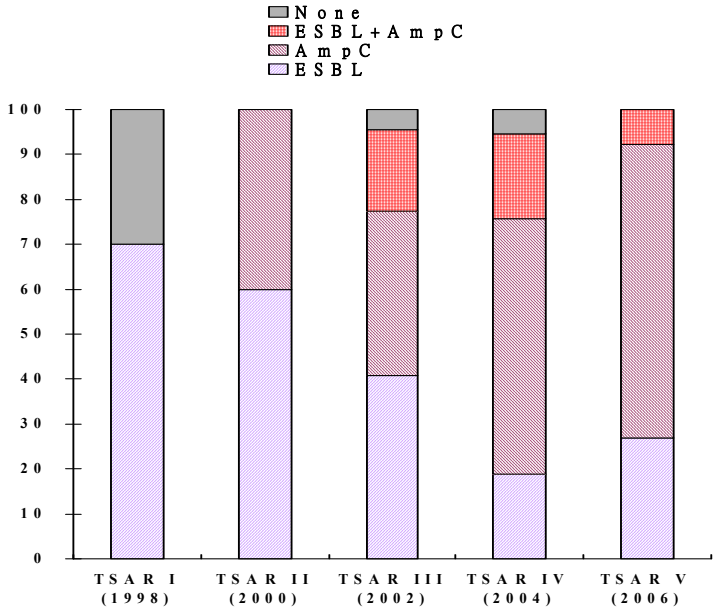


Figure 3. Changes in proportions of ESBL and AmpC positive isolates in extended spectrum ß-lactam non-susceptible *E. coli* (top graph) and *K. pneumoniae* (bottom graph)

A. *Escherichia coli*



B. *Klebsiella pneumoniae*

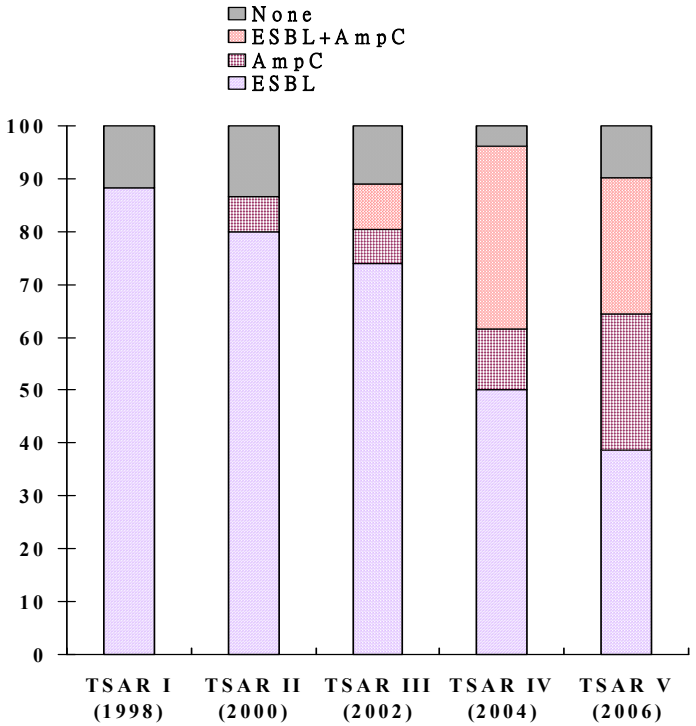


Figure 4. Dendrogram of *Sma*I digested pulsed field gel electrophoresis (PFGE) patterns of 51 nosocomial MRSA isolates from 2006. If more than one isolate is found in a subtype, only 1 isolate is shown. SCC*mec* type, presence of PVL toxin gene, sequence type (ST), and resistance profile (cip, ciprofloxacin; gen, gentamicin; sxt, trimethoprim/sulfa; tcy, tetracycline) are also shown.

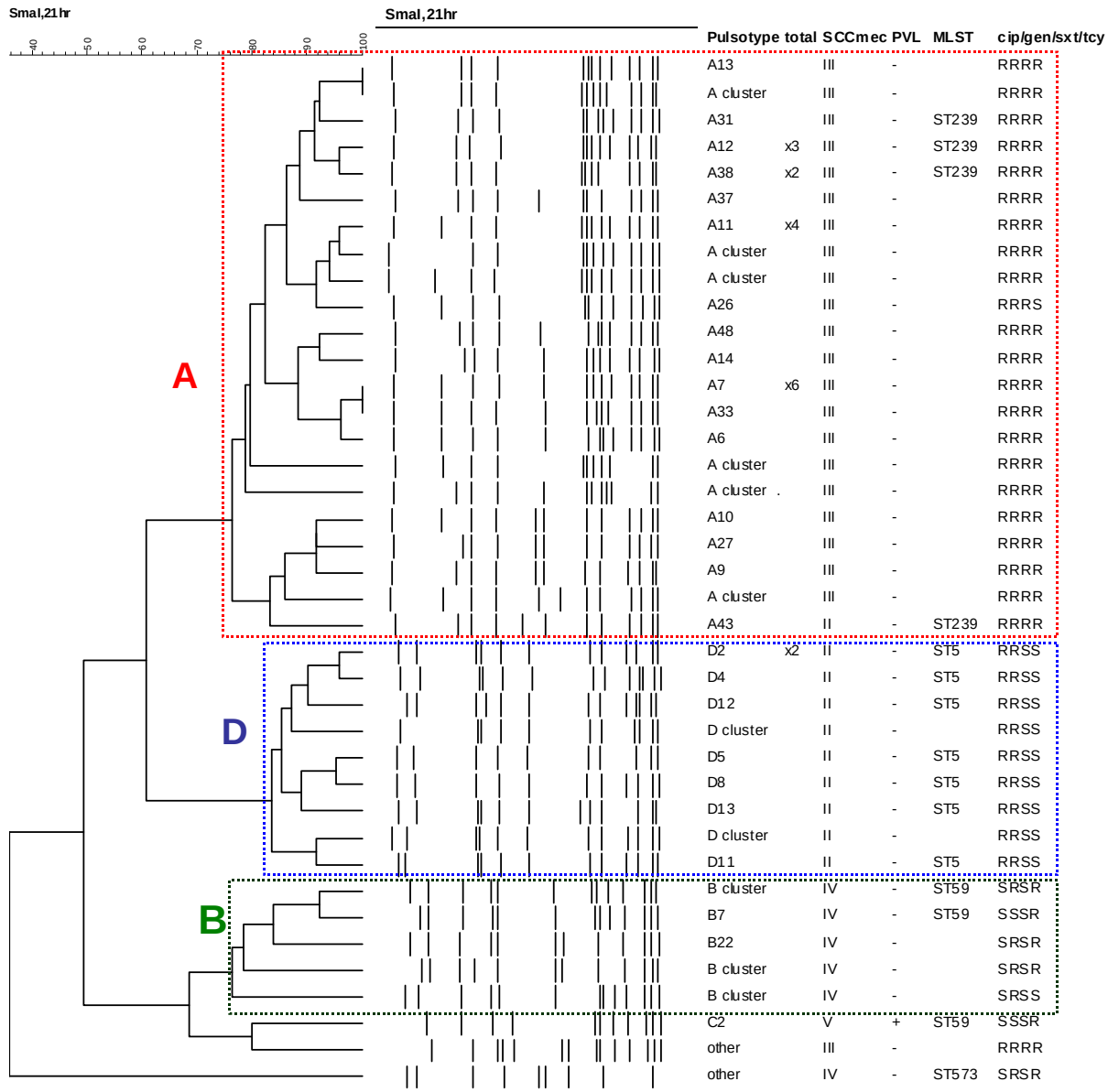


Fig 5. Dendrogram of *Sma*I digested pulsed field gel electrophoresis (PFGE) patterns of 51 nosocomial MRSA isolates from TSAR I (1998) – TSAR V (2006). Only 1 isolates of a pulsotype is shown. For pulsotype A, only subtypes with 2 or more isolates are shown. SCC*mec*, sequence type (ST), and resistance profile (cip, ciprofloxacin; gen, gentamicin; sxt, trimethoprim/sulfa; tcy, tetracycline) are also shown.