封面式樣

計畫編號：DOH88-TD-1020

行政院衛生署八十八年度科技研究發展計畫

台灣地區具有 ESBL 的肺炎克雷伯氏桿菌的鑑定與應用分子生物學分型的流行病學分析

研 究 報 告

執行機構：台中榮總

計畫主持人：施智源

研究人員：劉有增，胡伯賢，林育蕙，張心玫

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「行政院衛生署八十八年度科技研究發展計畫」成果資料

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計畫名稱：台灣地區具有ESBL的肺炎克雷伯氏桿菌的鑑定

與應用分子生物學分型的流行病學分型

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				書面	電腦檔	無	備註
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資料讀我檔案

計畫名稱：台灣地區具有ESBL的肺炎克雷伯氏桿菌的鑑定與應用分子生物學分型的流行病學分析

計畫編號：DOH88-TD-1020

執行機構：台中榮總

計畫主持人：施智源

計畫主持人服務單位：感染科

主持人職稱：主治醫師

研究報告中文摘要：

自從1980年代發現質體 (plasmid) 帶有分解廣效性β-lactam酵素 (ESBL) 後, 世界各地具有ESBL的腸內菌已經變成很普遍. 在1990 年代, 具有ESBL的肺炎克雷伯氏桿菌的比率, 在法國約13%, 在英國約16%, 但是在法國, 葡萄牙, 和土耳其的某些醫院卻高達40%. 細菌的質體或轉移元素 (transposable elements) 可以帶有ESBL的基因. 帶有ESBL的質體會在細菌間經由接合而傳佈, 而轉移元素可以將ESBL的基因在質體間轉移.目前已經有不少有關細菌本身的繁衍和質體的接合而造成群突發. 爲了解台灣地區具有ESBL的肺炎克雷伯氏桿菌的流行病學, 我們收集臺灣地區81株具有ESBL的肺炎克雷伯氏桿菌,分析其最低抑菌濃度, 酵素等電點, 脈衝電泳分型. 最低抑菌濃度 (μg/ml) 和敏感比率 (%) 如下: ceftazidime > 512(30.9%), ceftazidime/clavulanate 1 (97.5%), ceftriaxone 256 (24.7%), ceftriaxone/clavulanate 0.25 (98.8%), flomoxef 0.5 (96.3%), moxalactam 4(97.5%), meropenem 0.06 (100%), ciprofloxacin 32 (85.2%), amikacin >512 (59.3%). 71 (87.6%) 株產生 SHV-5 β-lactamase (pI 8.2), 5 (6.2%)株產生 SHV-4 β-lactamase (pI 7.8). 這些肺炎克雷伯氏桿菌產生ESBL, 可導致對 oxyimino-β-lactam (如ceftazidime 和 ceftriaxone) 的抗藥性, 然而對β-lactamase inhibitor (如 clavulanate), cephamycins (如flomoxef 和 moxalactam), 和 carbapenem (如 meropenem) 仍然具有敏感性. 應用脈衝電泳分型來分析基因相關性, 並用UPGMA的方法來建立 dendrogram. 脈衝電泳分型把 24 (29.6%) 株分成7 群 (A 至 G). A群至 D 群分別從一個醫院收集而來, E群至 G 群分別從兩個醫院收集而來, 其他 57 (70.4%)株無基因相關性. 這些結果顯示台灣地區具有ESBL的肺炎克雷伯氏桿菌

已在醫院內和醫院間散佈. 無基因相關性的菌株可能由質體接合而獲得ESBL基因. 爲了控制ESBL的肺炎克雷伯氏桿菌的持續散佈, 對於具有ESBL的肺炎克雷伯氏桿菌的持續監測, 完善的抗生素的管制, 和嚴謹的院內感染管制系統, 是最重要的政策.

中文關鍵詞：（至少三個）ESBL, 肺炎克雷伯氏桿菌, 分型

英文關鍵詞：（至少三個）ESBL, Klebiella pneumoniae, typing

※磁片檔案說明

檔案名稱	檔案性質	使用編輯軟體（請說明使用軟體）
LIST.DOC	交付項目一覽表	WORD 97
README.DOC	讀我檔案	WORD 97
REPORT.DOC	成果報告	WORD 97
PUBLIC.DOC	著作一覽表	WORD 97
RESULT.DOC	重要研究成果	WORD 97
POSITION.DOC	職級分析表	WORD 97
DEGREE.DOC	學歷分析表	WORD 97
DATA.XLS	數據	EXCEL 97

※連絡方式

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八十八年度計畫重要研究成果

計畫名稱：台灣地區具有ESBL的肺炎克雷伯氏桿菌的鑑定

與應用分子生物學分型的流行病學分析

主 持 人：施智源　　計畫編號：DOH88-TD-1020

1.計畫之新發現或新發明

台灣地區肺炎克雷伯氏桿菌的廣效性β-LACTAM分解酵素 (ESBL)87.6%是 SHV-5. 散佈的機轉主要是帶有ESBL質體的轉移和菌種本身的繁衍, 因而造成醫院內和醫院間的交互感染.

2.計畫對民眾具教育宣導之成果

抗生素的使用導致抗藥性菌種的增加, 必須加強院內感染控制和抗生素管制, 以免抗藥性菌種的散佈

3.計畫對醫藥衛生政策之具體建議

對於具有ESBL的肺炎克雷伯氏桿菌的持續監測, 完善的抗生素的管制, 和嚴謹的院內感染管制系統, 是控制ESBL的肺炎克雷伯氏桿菌比率最重要的政策. 因此我們建議衛生署

1.提供具ESBL的肺炎克雷伯氏桿菌的研究經費, 讓感染科專家繼續監測.

2.加重醫院評鑑對於抗生素管制的評分, 並評估實效.

3.健保局對於抗生素應嚴格管制, 抗生素的審核由感染科專科醫師負責, 以免臨床醫師濫用.

4.加重醫院評鑑對於群突發管制實例的評分, 以免院內感染評鑑淪爲紙上作業

Identification and Molecular Epidemiology of Extended-Spectrum β-Lactamase-Producing Klebsiella pneumoniae Isolates in Taiwan

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Molecular Epidemiology of ESBL-producing *Klebsiella pneumoniae* isolates in Taiwan

臺中榮總感染科

施智源 林育蕙 胡伯賢 劉有增

Since the discovery of the extended-spectrum β-lactamase (ESBL) encoded in transferable plasmid in 1980s, ESBL-producing *Enterobacteriaceae* have become common worldwide. In recent surveys, the frequency of ESBL in nosocomial *Klebsiella pneumoniae* isolates was estimated to be about 13% in France, 16% in England, and over 40% in some hospitals in France, Portugal, and Turkey. ESBL genes are usually carried by plasmids or transposable elements. ESBL genes on transposable elements can be translocated among different plasmids. Outbreaks have been reported to result from spread of epidemic clones and plasmid transfer. Eighty-one ESBL-producing *K. pneumoniae* isolates from 10 Taiwanese hospitals were analyzed for their MICs, values of isoelectric points, and PFGE typing. The MIC_{90}s (μg/ml) and susceptible percentages (%) were ceftazidime >512 (30.9%), ceftazidime/clavulanate 1 (97.5%), ceftriaxone 256 (24.7%), ceftriaxone/clavulanate 0.25 (98.8%), flomoxef 0.5 (96.3%), moxalactam 4 (97.5%), meropenem 0.06 (100%), ciprofloxacin 32 (85.2%), and amikacin >512 (59.3%). Seventy-one (87.6%) isolates produced an SHV-5 β-lactamase (pI 8.2), 5 (6.2%) isolates produced an SHV-4 β-lactamase (pI 7.8). These multi-resistant isolates produced the ESBLs which conferred resistance to oxyimino-β-lactams (e.g. cefotaxime and ceftazidime), but remained susceptible to β-lactamase inhibitors (e.g. clavulanate), cephamycins (e.g. flomoxef and moxalactam) and carbapenem (e.g. meropenem). The genetic relatedness was analyzed by PFGE patterns and a dendrogram was constructed by UPGMA method. PFGE typing grouped 24 (29.6%) isolates into seven clusters (A to G). Four clusters (A to D) each were recovered from a single hospital, while 3 clusters (E to G) each were recovered from two hospitals. The other 57 (70.4%) isolates were genetically unrelated. These results indicate that ESBL-producing *K. pneumoniae* can spread within a hospital and between hospitals. Genetically unrelated isolates may acquire the ESBL-genes via plasmid transfer. To prevent rapid spread of ESBL-producing K. pneumoniae, continuous surveillance of ESBL-producing strains, good antibiotic control programs and perfect nosocomial infection control system are the most important policies.

台灣地區具有ESBL的肺炎克雷伯氏桿菌的鑑定與應用分子生物學分型的流行病學分析

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Since the discovery of the extended-spectrum β-lactamase (ESBL) encoded in transferable plasmid in 1980s (Kliebe et al. 1985; Sirot et al. 1987; Jarlier et al. 1988), ESBL-producing Enterobacteriaceae have become common worldwide (Bure et al. 1988; Legakis et al. 1995; Venezia et al. 1995; Gniadknowski et al. 1998; Nüesch-Inderbinen et al. 1997). In recent surveys, the frequency of ESBL in nosocomial K. pneumoniae isolates was estimated to be about 13% in France (Sirot et al. 1992), 16% in England (Liu et al. 1992), and over 40% in some hospitals in (Sirot et al. 1992), Portugal, and Turkey (Livermore and Yuan 1996).

ESBL genes are usually carried by plasmids (Kliebe et al. 1985; Sirot et al.1987). ESBL genes on transposable elements also can be translocated among different plasmids (Heritage et al. 1992; Sirot et al. 1991). Outbreaks have been reported to result from spread of epidemic clones (Nouvellon et al. 1994; French et al. 1996; Neuwirth et al. 1997) and plasmid transfer (Bingen, Prodinger, Legakis). In this paper, we use antimicrobial susceptibility test, isoelectric points of β-lactamases, and PFGE to identify the clones of ESBL-producing K. pneumoniae.

MATERIALS AND METHODS

Bacterial isolates. The 81 K. pneumoniae isolates were collected from 10 Taiwanese hospitals, including National Taiwan University Hospital (NTUH), Mackay Memorial Hospital (MMH), Tri-service General Hospital (TSH), Shinkong Hospital (SKH), and Chang Gung Children's Hospital (CGCH), each located in northern Taiwan; Taichung Veterans General Hospital (TCVGH), Chen-Ching Hospital (CCH), and Shalu Tungs' General Hospital (STH), each in central Taiwan; Kaohsiung Chang Gung Memorial Hospital (KSCGH), and Pingtung Christian Hospital (PCH), each in southern Taiwan. The isolates from TCVGH were consecutively collected during 1993 to 1996, while those from other hospitals were obtained randomly during during 1997 to January 1999.

Antimicrobial susceptibility. These isolates were then tested by broth microdilution with Mueller-Hinton broth as described in NCCLS standard M7-A4 (NCCLS, 1997b). Escherichia coli ATCC 25922 was used for quality control. The antimicrobial standard powders were kindly supplied by the following manufacturers: ceftazidime (Glaxo-Wellcome, Taiwan), ceftadime–clavulanic acid (4 μg/ml) (SmithKline Beecham, Surrey, UK), ceftriaxone (Roche, Basel, Switzerland), ceftriaxone-clavulanic acid (4 μg/ml), flomoxef (Shionogi, Osaka, Japan), moxalactam (Shionogi, Osaka, Japan), meropenem (ICI, Macclesfield, UK), ciprofloxacin (Bayer, Leverkusen, Germany), and amikacin (Bristol-Myers Squibb, Syracuse, NY).

Isoelectric focusing of β-lactamases. ESBLs were analyzed by isoelectric focusing (Matthew et al. 1975). The cells grown from nutrient broth were disrupted by sonication, and the cell debris was removed by centrifuge at 5,000 x g for 10 min. The extracted β-lactamases were loaded on the polyacrylamide gels containing Pharmalyte (Pharmacia). A constant power of 7 W supplied was applied at 4°C or 1 to 2 hours, until two drops of lysed blood met at their pI and focused

into a sharp band. The β-lactamases were stained by overlaying the gels with 0.5 mM nitrocefin in 0.1 M phosphate buffer (pH 7.0). The pI values of ESBLs were further confirmed by iodometric method. Mueller-Hinton agar, containing 0.6% ceftazidime and cefotaxime, 6% potassium iodide, and 0.6% iodine, was poured onto the isoelectric focusing gel. The bands corresponding to ESBLs produced clear halo in the black background of agar within 30 min, and the formation of halo was inhibited when the Muller-Hinton agar mixture was supplemented with 2 μg/ml of clavulanate.

Preparation of Plasmid DNA and plasmid fingerprinting. Plasmid DNA was prepared by alkaline lysis (Kado and Liu 1981) and the plasmid profiles were analyzed by electrophoresis on 0.7% agarose gel. For the fingerprinting analysis, about 5 μg of plasmid DNA was digested with 10 U of EcoRI restriction enzymes (GIBCO-BRL, Life Technologies, Gaithersburg, MD) at 37°C for 2 h. The DNA fragments were analysed in 1% agarose gel.

Transfer of resistance. Equal volumes (1 ml) of the donor and the recipient strains, Escherichia coli K-12 J53-2 (resistant to rifampin), (10^9 CFU/ml) grown in tryptic soy broth (Oxoid) were mixed and incubated for 18 h at 35 °C. Transconjugants were selected on MacConkey agar (Oxoid) supplemented with 64 μg/ml of rifampin to inhibit the growth of donor strains, and 2 μg/ml of ceftazidime to inhibit the growth of the recipient strain.

Sequencing of ESBL genes. SHV genes were amplified from plasmid DNA by PCR using the primers described previously (Rasheed et al. 1997). The gene

fragments were sequenced on both strands, on an ABI 310 Prism automated sequencer with BigDye terminators (PE Applied Biosystems).

PFGE. The chromosomal DNA was prepared as the protocol described previously (Shi et al. 1996). The DNA blocks were digested with 30 U of XbaI (GIBCO-BRL, Life Technologies, Gaithersburg, MD) at 37°C for 2 h. Restriction fragments of DNA were separated by PFGE with a contour-clamped homogeneous electric field CHEF-DRII apparatus (Bio-Rad Laboratories, Richmond, Calif.) through 1.2% SeaKem GTG agarose gel (FMC Bioproducts, Rockland, Maine). The fragmented DNA was run at the field strength of 6 V/cm for 24 h at 14°C, with the pulse time being increased from 5 to 40 s. A lambda ladder (Bio-Rad Laboratories) was used as the molecular size marker.

RESULTS

Antimicrobial susceptibility of isolates. The acitivity of the various antimicrobial agents against 81 isolates of K. pneumoniae is listed in Table 1. The minimal inhibitory concentrations for 90% (MIC_{90}s) of the 81 isolates and susceptible percentages were ceftazidime >512 (30.9%), ceftazidime/clavulanate 1 (97.5%), ceftriaxone 256 (24.7%), ceftriaxone/clavulanate 0.25 (98.8%), flomoxef 0.5 (96.3%), moxalactam 4 (97.5%), meropenem 0.06 (100%), ciprofloxacin 32 (85.2%), and amikacin >512 (59.3%).

pI values of isolates. Among the 81 ESBL-producing isolates, seventy-one (87.6%) isolates produced an SHV-5 β-lactamase (pI 8.2), 5 (6.2%) isolates produced an SHV-4 β-lactamase (pI 7.8).

Analysis of relatedness of isolates. The genetic relatedness was analyzed by PFGE patterns and a dendrogram (Figure 1) was constructed by UPGMA method. PFGE typing grouped 24 (29.6%) isolates into seven clusters (A to G). The largest cluster (cluster A) included 10 isolates (isolates TCVGH49 to TCVGH58) which had a similarity of 81.8 ± 2.9%. Cluster B contained three isolates (MMH6 to MMH8) which ahd a similarity of 91.9 ± 0.0%, Cluster C contained 3 isolates (TSGH 2 to TSGH 4) which had a similarity of 94.2 ± 2.8%. Cluster D included 2 isolates (CGCH4 and CGCH6) with the identical PFGE pattern. Cluster E included 2 isolates (KSCGH 2 and SKH1) which had a similarity of 92.9 ± 0.0%. Cluster F contained 2 isolates (KSCGH4 and SKH4) which had a similarity of 81.2 ± 0.0%. The other 57 (70.4%) isolates were genetically unrelated.

Plasmid profiles. Ten isolates of cluster A from TCVGH had the same plasmid profiles. The other stains have a common plasmid with the size about 11 kb.

Sequence of ESBL-genes. DNA sequence analysis demonstrated that the SHV genes encoding the pI 8.2 β-lactamases were SHV-5 and its variants. The SHV genes mediated the pI 7.8 β-lactamases were SHV-4. We have to do more sequence reactions to identify the SHV-5 variants.

DISCUSSION

ESBL producer are prone to be reported as susceptible to cephalosporins, as MICs often remains below the classical (e.g. NCCLS) breakpoints of 8-16 μg/ml (Jacoby and Carreras 1990; Katsanis et al. 1994). Even low-level (MIC 1-2 μg/ml) ESBL-mediated resistance is associated with clinical failure (Brun-Buisson et al. 1987). In this study, 30.9% and 24.7% of these 81 ESBL-producers were reported

as susceptible to ceftazidime and ceftriaxone respectively. These strains showed MICs above the normal susceptible population but below the standard breakpoints for ceftriaxone and ceftazidime. Confirmatory testing requires use of ceftazidime and ceftriaxone, alone and in combination with clavulanic acid. These multi-resistant isolates produced the ESBLs which conferred resistance to oxyimino-β-lactams (e.g. cefotaxime and ceftazidime), but remained susceptible to β-lactamase inhibitors (e.g. clavulanate), cephamycins (e.g. flomoxef and moxalactam) and carbapenem (e.g. meropenem). Only 59.3% of isolates were susceptible to amikacin. These plasmids have also frequently been found to carry genes responsible for resistance to other antibiotics, and this has resulted in the growing prevalence of multidrug-resistant organisms (Legakis et al. 1995; Sirot et al. 1991).

PFGE typing grouped 24 (29.6%) isolates into seven clusters (A to G). Four clusters (A to D) each were recovered from a single hospital, while 3 clusters (E to G) each were recovered from two different hospitals. The other 57 (70.4%) isolates were genetically unrelated. Ten isolates of cluster A were isolated from TCVGH over a short period of 1 month. Among these 10 isolates, three (TCVGH 51, 52 and 56) were collected from a intensive care unit, the other 7 isolates each were recovered from different wards. The PFGE patterns of this cluster were so similar that they should have originated from a common ancestor. This ancestral strain could have acquired an SHV-5 gene and became a successful clone under the selective pressure of antibiotics. This SHV-producing clone spread to other wards by patient transfer or some unidentified sources. ESBL-producing strains can be maintained over prolonged periods of time in hospitals and can cause clonal outbreaks (Gouby et al. 1994; Rice et al. 1996). They can be transferred between different wards, as well as between different hospitals or health care institutions

and even, with the case of international travel, between different countries (Bradford et al.1994; Gouby et al. 1994; Rice et al. 1996; Shannon et al. 1990).

CONCLUSIONS AND SUGGESTIONS

This study demonstrates that ESBL-producing K. pneumoniae are widespread in Taiwanese hospitals. The evolution of resistance included intra- and inter-hospital spread of ESBL-producing clones, and plasmid transfer among genetically unrelated K. pneumoniae strains. To prevent rapid spread of ESBL-producing K. pneumoniae, continuous surveillance of ESBL-producing strains, good antibiotic control programs and perfect nosocomial infection control system are the most important policies.

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Table 1. Antimicrobial susceptibility of 81 ESBL-producing K. pneumoniae isolates

Antibiotic	MIC (μg/ml)			
	Range	50%	90%	Susceptible (%)
Ceftazidime	0.5->512	64	>512	30.9
Ceftazidime/CLA[a]	0.06->512	0.25	1	97.5
Ceftriaxone	4->512	32	256	24.7
Ceftriaxone/CLA	<0.01-32	0.06	0.25	98.8
Flomoxef	0.03-64	0.06	0.5	96.3
Moxalactam	0.125-128	0.5	4	97.5
Meropenem	<0.01-2	0.03	0.06	100
Ciprofloxacin	0.01-256	0.03	32	85.2
Amikacin	0.5->512	16	>512	59.3

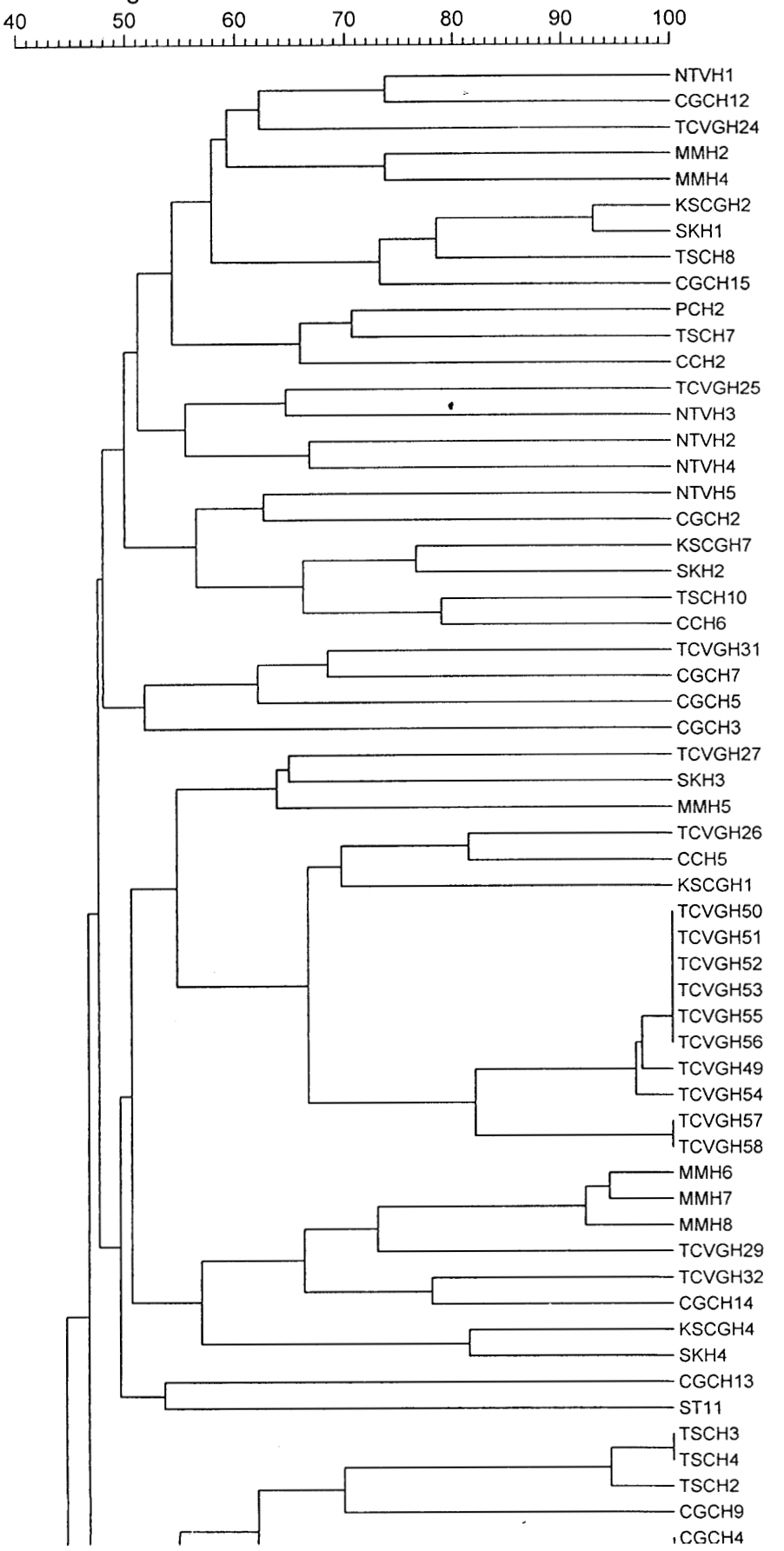
[a]CLA, clavulanic acid.

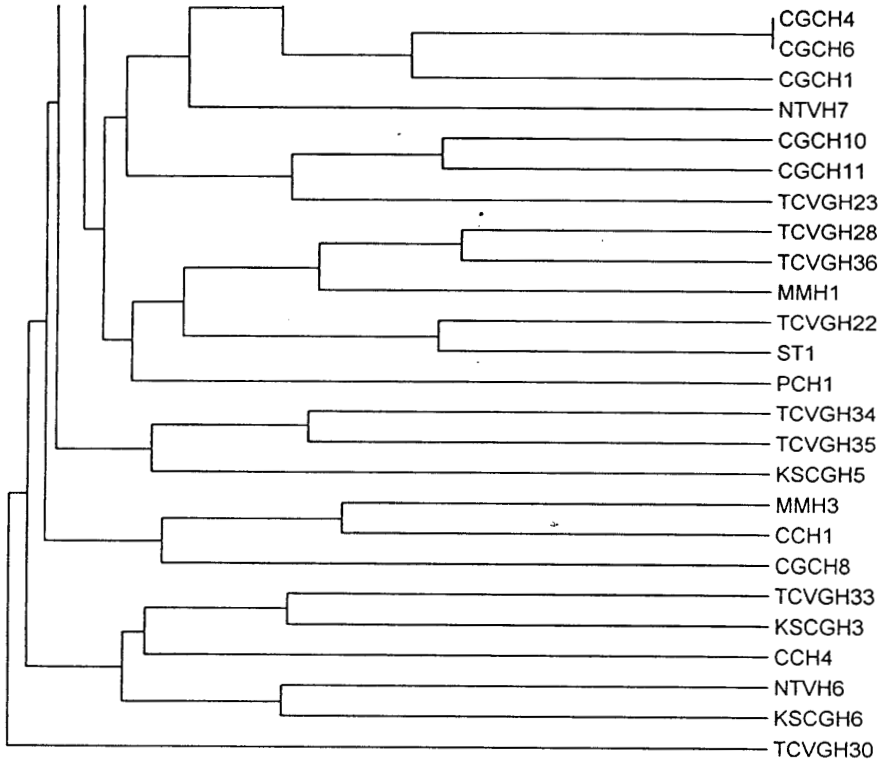
GelCompar

Department of Internal Medicine
Veteran General Hospital Taichung Branch

JOB:
Tree KPESBL81
10-8-1999 4:25
sn

List: KPESBL81
Entries: 81
Correlation: Bands, Dice (Max. tol. 1.2%, Min. surf. 0.2%)
Zones: [1-400]
Clustering: UPGMA




CGCH4
CGCH6
CGCH1
NTVH7
CGCH10
CGCH11
TCVGH23
TCVGH28
TCVGH36
MMH1
TCVGH22
ST1
PCH1
TCVGH34
TCVGH35
KSCGH5
MMH3
CCH1
CGCH8
TCVGH33
KSCGH3
CCH4
NTVH6
KSCGH6
TCVGH30

八十八年度計畫著作一覽表

計畫名稱：台灣地區具有ESBL的肺炎克雷伯氏桿菌的鑑定與應用分子生物學分型的流行病學分析

主 持 人：施智源 計畫編號：DOH88-TD- 1020

列出貴計畫於本年度中所有計畫產出於下表，包含已發表或已被接受發表之文獻、已取得或被接受之專利、擬投稿之手稿（manuscript）以及專著等。「計畫產出名稱」欄位請依「臺灣醫誌」參考文獻方式撰寫；「產出形式」欄位則填寫該產出爲期刊、專利、手稿或專著等，舉例如下：

序號	計畫產出名稱	產出形式	SCI*
1	Tseng CY, Liu YF, Wu WL, Lau YJ, Hu BS, **Shi ZY**, Lin YH. Comparison of detectiion of extended-spectrum β-lactamases by agar dilution method, E-test ESBL screen and double disk test. J Microbiol Immunol Infect 1998, 31:90-4	期刊	
2			
3			
4			
5			
6			

＊SCI: Science Citation Index，若發表之期刊爲SCI所包含者，請打「∨」。

參與八十八年度計畫研究人力之職級分析表

計畫名稱：台灣地區具有ESBL的肺炎克雷伯氏桿菌的鑑定與應用分子生物學分型的流行病學分析

主 持 人：施智源　　計畫編號：DOH88-TD- 1020

職級	所含職級類別	參與人次
第一級	研究員、教授、主治醫師	4人
第二級	副研究員、副教授、總醫師	人
第三級	助理研究員、講師、住院醫師	人
第四級	研究助理、助教、實習醫師	1人
第五級	技術人員	人
第六級	支援人員	人
合 計		5人

〔註〕

第一級：研究員、教授、主治醫師、簡任技正，若非以上職稱則相當於博士滿三年、碩士滿六年、或學士滿九年之研究經驗者。

第二級：副研究員、副教授、助研究員、助教授、總醫師、薦任技正，若非以上職稱則相當於博士、碩士滿三年、學士滿六年以上之研究經驗者。

第三級：助理研究員、講師、住院醫師、技士，若非以上職稱則相當於碩士或學士滿三年以上之研究經驗者。

第四級：研究助理、助教、實習醫師，若非以上職稱則相當於學士或專科畢業目前從式研究發展，經驗未滿三年者。

第五級：指目前在研究人員之監督下從事與研究發展有關之技術性工作，且具備下列資格之一者屬之：具初（國）中、高中（職）、大專以上畢業者或專科畢業目前從式研究發展，經驗未滿三年者。

第六級：指在研究發展執行部門參與研究發展有關之事務性及雜項工作者，如人事、會計、祕書、事務人員及維修、電機人員等。

參與八十八年度計畫研究人力之學歷分析表

計畫名稱：台灣地區具有ESBL的肺炎克雷伯氏桿菌的鑑定與應用分子生物學分型的流行病學分析

主 持 人：施智源 計畫編號：DOH88-TD-1020

類別	學 歷 別	參與人次
1	博 士	人
2	碩 士	人
3	學 士	4人
4	專 科	1人
5	博士班研究生	人
6	碩士班研究生	人
7	其 他	人
	合 計	5人