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Original Article

Clonal spread of *Klebsiella pneumoniae* producing CMY-2 AmpC-type β-lactamase in surgical intensive care units

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Background and purpose: CMY-2, an AmpC-type β-lactamase, has become more prevalent in *Klebsiella pneumoniae* isolates at the National Cheng Kung University Hospital, Tainan, Taiwan. This retrospective study investigated the epidemiological characteristics of CMY-2–producing strains of *K. pneumoniae*.

Methods: Molecular experiments were performed to describe the bla_{CMY-2} gene carriage rate and the trend of *K. pneumoniae* isolates with resistance to cefoxitin between January 2001 and June 2003. The epidemiological correlation between patients with bla_{CMY-2}–positive *K. pneumoniae* was further analyzed and compared by chart review and pulsed-field gel electrophoresis (PFGE).

Results: Of 285 cefoxitin-resistant *K. pneumoniae* isolates, 69 (24.2%) carried a bla_{CMY-2} sequence. The first bla_{CMY-2}–positive isolate of each patient was genotyped by PFGE; 49 (89.1%) of 55 were genetically related. Forty five isolates (91.8%) were noted between January and September 2002. Eighteen isolates were from patients in 2 surgical intensive care units (SICUs), and 23 were from patients with prior SICU stays. Compared with patients with non-epidemic CMY-2–producing isolates, patients with the epidemic clone had a shorter duration of hospital stay ($p = 0.007$) and SICU stay ($p = 0.01$) before isolation. Recent surgery was independently associated with acquisition of epidemic CMY-2–producing *K. pneumoniae*.

Conclusions: An unrecognized clonal spread of *K. pneumoniae* producing CMY-2 AmpC-type β-lactamase in SICUs was found. Cross-transmission of the epidemic clone, suggested by a shorter hospital stay before isolation of the bacterium and the association with recent surgery, highlights the importance of surveillance to recognize an epidemic and initiate control measures.

Key words: AmpC beta-lactamases; Cross infection; Disease outbreaks; Intensive care units; *Klebsiella pneumoniae*

Introduction

The emergence of plasmid-mediated AmpC-type β-lactamases in *Enterobacteriaceae* has become a global problem [1]. The expression of AmpC enzymes can confer resistance to extended-spectrum cephalosporins and cephamycins [1]. Among these enzymes, CMY-2 is the most prevalent and is widely distributed around the world [1-5]. Standard guidelines for the detection of AmpC-producing isolates remain unavailable for routine microbiology laboratories. Failure to detect these enzymes may be responsible for failure to implement appropriate control measures to prevent the rapid dissemination of pathogens and for the consequences of therapeutic failure among patients who receive inappropriate antibiotics [1,6].

Several plasmid-mediated AmpC β-lactamases have been detected in *Klebsiella pneumoniae* and

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Escherichia coli in Taiwan [5,7-9]. Among these β-lactamases, CMY-2 has become more common than extended-spectrum β-lactamases (ESBLs) in *E. coli*, but was rarely found in *K. pneumoniae* before 2001 [7,8]. Recently, the number of CMY-2–producing *K. pneumoniae* isolates has increased at the National Cheng Kung University Hospital (NCKUH), Tainan, Taiwan [9]. Therefore, a retrospective study to analyze the epidemiological characteristics of CMY-2–producing *K. pneumoniae* colonizations or infections was performed, and the molecular epidemiology of these strains was investigated.

Methods

Setting

NCKUH is a tertiary-care teaching hospital with approximately 1000 beds in southern Taiwan. The hospital provides acute medical and surgical care, as well as obstetric and pediatric services, with about 40,000 admissions per year. The hospital has 7 intensive care units (ICUs), including two 13-bed surgical ICUs (SICUs), designated SICU-1 and SICU-2. The patients in each SICU are cared for in a single room, and those in other ICUs in single-bed rooms.

Bacterial isolates

4119 *K. pneumoniae* isolates were prospectively collected from January 2001 to June 2003. Based on the database of the microbiology laboratory, 360 isolates with resistance to cefoxitin by the disk diffusion method were suspected of being AmpC producers [1]. Among the 360 cefoxitin-resistant isolates, 30 of 75 non-replicate isolates were confirmed to be CMY-2 producers between January 2001 and June 2002 [9]. The remaining 285 *K. pneumoniae* isolates were investigated for the carriage of bla_{CMY-2} in this study. When multiple isolates were recovered from a single patient, only the first bla_{CMY-2}–positive isolate from each patient was selected for molecular typing and clinical evaluation. Five bla_{CMY-2}–positive strains isolated before 2001 were also included for molecular typing and clinical analysis [7,9].

Detection of bla_{CMY-2}

bla_{CMY-2}–related genes were detected by polymerase chain reaction (PCR) and colony hybridization [10,11]. A fresh bacterial colony was suspended in 100 μL of sterile distilled water and boiled at 100°C for 10 min. After centrifugation, the supernatant was removed for PCR. The PCR assay for detecting bla_{CMY-2}–related genes was performed with primers ampC1 (5'-ATGATGAAAAAATCGTTATGC-3') and ampC2 (5'-TTGCAGCTTTTCAAGAATGCGC-3') under the PCR conditions, as described previously [9]. PCR products were purified with a commercial kit and the amplicons were sequenced on an ABI PRISM 310 automated sequencer (PE Applied Biosystems, Foster City, CA, USA). Sequence alignments and analyses were performed on-line using the Basic Local Alignment Search Tool (available at: www.ncbi.nlm.nih.gov/blast/). The PCR products were also used as templates to synthesize the digoxigenin-labeled bla_{CMY-2} probe. Colony hybridization with the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Mannheim, Germany) were performed, according to the manufacturer's instructions.

Pulsed-field gel electrophoresis analysis

Pulsed-field gel electrophoresis (PFGE) was performed with the CHEF-DR III apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's instructions. Chromosomal DNA was digested by *Xba*I (New England Biolabs, Beverly, MA, USA) and was separated on 1% agarose gels. A bacteriophage I DNA ladder (Gibco BRL, Gaithersburg, MD, USA) was used as a size marker. PFGE patterns were interpreted in accordance with the criteria of Tenover et al [12]. Isolates were considered identical if they had exactly the same electrophoretic pattern, and clonally related if they showed differences of ≤3 bands.

Isoelectric focusing

The expression of β-lactamases by bla_{CMY-2}–positive isolates was detected by isoelectric focusing (IEF). Crude β-lactamase extracts were prepared by using sonication, as described previously [13]. IEF was performed based on the method of Matthew et al [14] with an LKB Multiphor apparatus on prepared PAGplate gels (pH 3.5 to 9.5; Amersham Biosciences, Hong Kong, China), as described previously [5]. β-Lactamase activities were detected by overlaying the gel with 0.5 mM nitrocefin (Oxoid, Basingstoke, UK) in 0.1 M phosphate buffer, pH 7.0.

Susceptibility testing

Antimicrobial susceptibilities to gentamicin, tobramycin, amikacin, ciprofloxacin, levofloxacin, and trimethoprim-sulfamethoxazole were determined by

the standard disk diffusion method [15]. Minimal inhibitory concentrations of amoxicillin, amoxicillin-clavulanic acid, cefoxitin, cefotaxime, ceftazidime, cefepime, aztreonam, and imipenem were determined by the standard agar dilution tests, as described by the National Committee for Clinical Laboratory Standards (NCCLS) [16].

Data collection

Clinical data were collected from the medical records of patients with CMY-2–producing isolates. The data included demographic characteristics, primary reason for hospital admission, comorbid conditions, sites of infection, dates of previous and present admissions, and dates of ICU stay during present or prior admissions.

Statistical analysis

Characteristics of patients with the CMY-2–producing epidemic clone and patients with non-epidemic CMY-2–producing clones were compared. Dichotomous variables were analyzed by chi-squared test or Fisher's exact test, and continuous variables were analyzed with Student's *t* test. Variables with a *p* value of <0.25 in the univariate analysis were entered into a stepwise logistic regression model to determine factors independently associated with infection or colonization by the endemic clone. All statistical calculations were done using the Statistical Package for Social Sciences for Windows (Version 10.0; SPSS Inc., Chicago, IL, USA). All tests were 2-tailed, and a *p* value of <0.05 was considered significant.

Results

Prevalence of cefoxitin and ceftazidime resistance and *bla*$_{CMY-2}$

During the study period, the monthly number of *K. pneumoniae* isolates ranged from 85 to 205 (median, 128). The monthly incidence of cefoxitin resistance was <7% (range, 0.7-6.3%) in 2001, but >7% (range, 7.6-21.2%) after December 2001 (Fig. 1). The disk-diffusion ESBL confirmatory tests proposed by the NCCLS [10] were adopted as routine tests at NCKUH from 2000. The monthly incidence of non-ESBL–producing isolates with cefoxitin resistance and reduced susceptibilities to ceftazidime increased from January 2002, ranging from 4.3% to 13.1%, and decreased to <4% after January 2003. Of 285 cefoxitin-resistant isolates subjected to PCR and sequence analyses to detect *bla*$_{CMY-2}$, 69 (24.2%) were found to

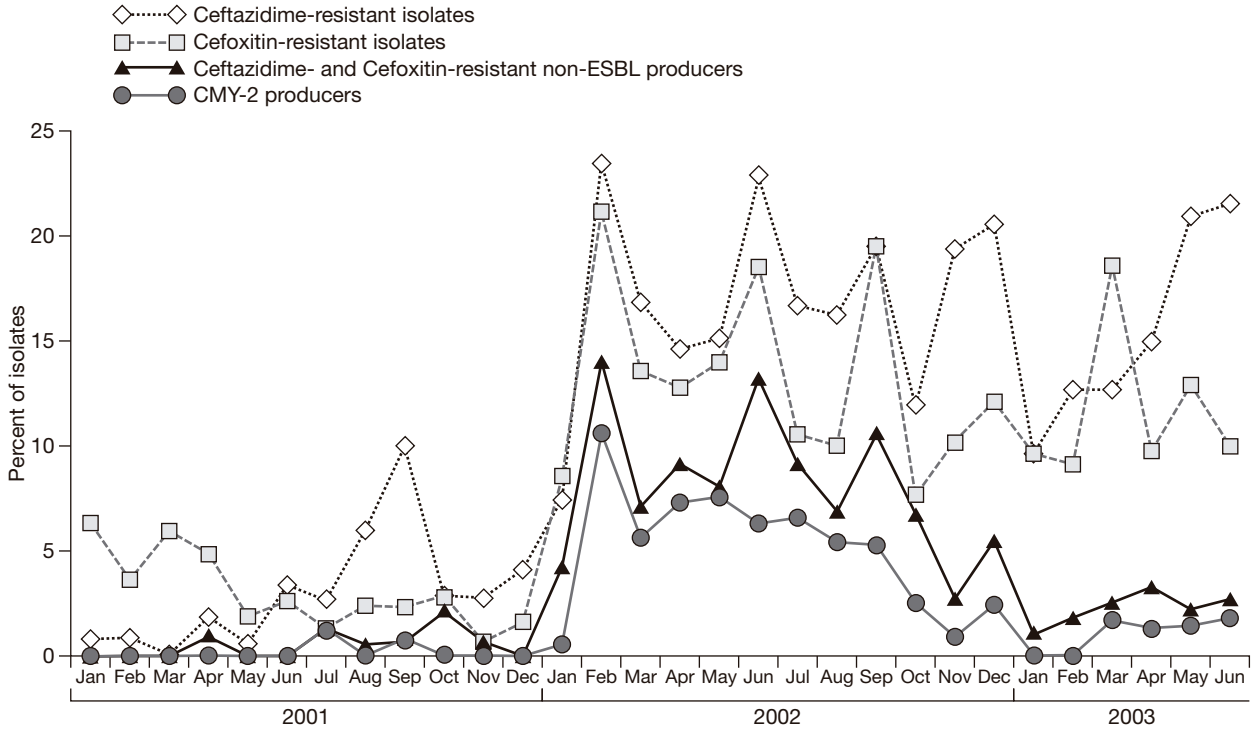

Fig. 1. Monthly incidences of ceftazidime-resistant isolates, cefoxitin-resistant isolates, ceftazidime- and cefoxitin-resistant non–extended-spectrum β-lactamase (ESBL)–producing isolates, and CMY-2–producing isolates of *Klebsiella pneumoniae* between January 2001 and June 2003.

carry a bla_{CMY-2} sequence. The results of colony hybridization were consistent with the PCR results (data not shown). From January 2001 to June 2003, together with 30 bla_{CMY-2}-positive isolates identified previously [9], 99 isolates from 55 patients carried bla_{CMY-2}. The monthly incidence of bla_{CMY-2}–positive isolates was low between January 2001 and January 2002 (0-1.3%), increased between February and September 2002 (5.3-10.6%), and declined thereafter (0-2.5%) [Fig. 1]. The prevalence trend of non-ESBL–producing isolates with cefoxitin resistance and reduced susceptibilities to ceftazidime correlated with that of CMY-2 producers during the study period.

Pulsed-field gel electrophoresis

Sixty bla_{CMY-2}–positive isolates from different patients, including 55 isolates collected since 2001 and 5 control isolates identified before 2001 [7,9], were genotyped by PFGE. Eleven major PFGE patterns, designated patterns I to XI, were identified (Table 1 and Fig. 2). Forty nine PFGE pattern I isolates, accounting for 89% of 55 CMY-2–producing isolates, were further separated into 6 subtypes, i.e., subtypes I_a (n = 35), I_b (n = 2), I_c (n = 7), I_d (n = 3), I_e (n = 1), and I_f (n = 1). Pattern III was represented by 2 isolates, and each of the other 9 patterns were represented by a single isolate. All 49 clone I isolates were isolated in 2002 and 2003, but none of the 5 control isolates isolated prior to 2001 were clone I.

Isoelectric focusing

β-Lactamases expressed by bla_{CMY-2}–positive isolates have been previously determined in 35 of 60 non-replicate isolates [7,9]. The remaining 25 isolates were subjected to IEF and were found to express 2 β-lactamases with isoelectric points (pIs) of 9.0 and 7.6 on IEF gels. The pI of 9.0 for β-lactamase was consistent with the expression of CMY-2, and the pI of 7.6 for

Table 1. Resistance phenotypes and pulsed-field gel electrophoresis (PFGE) genotypes of 60 non-replicate CMY-2–producing *Klebsiella pneumoniae* isolates.

Resistance phenotypes[a]	PFGE pattern (no. of isolates)	No. of isolates (%)
Levofloxacin, trimethoprim-sulfamethoxazole	I_a (24), I_b (1), I_d (1), I_e (1), VI (1), IX (1)	29 (48.3)
Levofloxacin, gentamicin, tobramycin, trimethoprim-sulfamethoxazole	I_a (11), I_b (1), I_c (7), I_d (2), I_f (1), XI (1), X (1)	24 (40.0)
Gentamicin	III (2)	2 (3.3)
Trimethoprim-sulfamethoxazole	V (1), VII (1)	2 (3.3)
Gentamicin, levofloxacin, trimethoprim-sulfamethoxazole	VIII (1)	1 (1.7)
Gentamicin, tobramycin, trimethoprim-sulfamethoxazole	II (1)	1 (1.7)
Gentamicin, tobramycin	IV (1)	1 (1.7)

[a]Susceptibilities to β-lactam agents were determined by the agar dilution method, and those to non–β-lactam agents by the disk diffusion method. Intermediate category was included in the resistant category in this study. All isolates were resistant to amoxicillin-clavulanic acid, amoxicillin, cefoxitin, and extended-spectrum cephalosporins (ceftazidime, cefotaxime, or aztreonam).

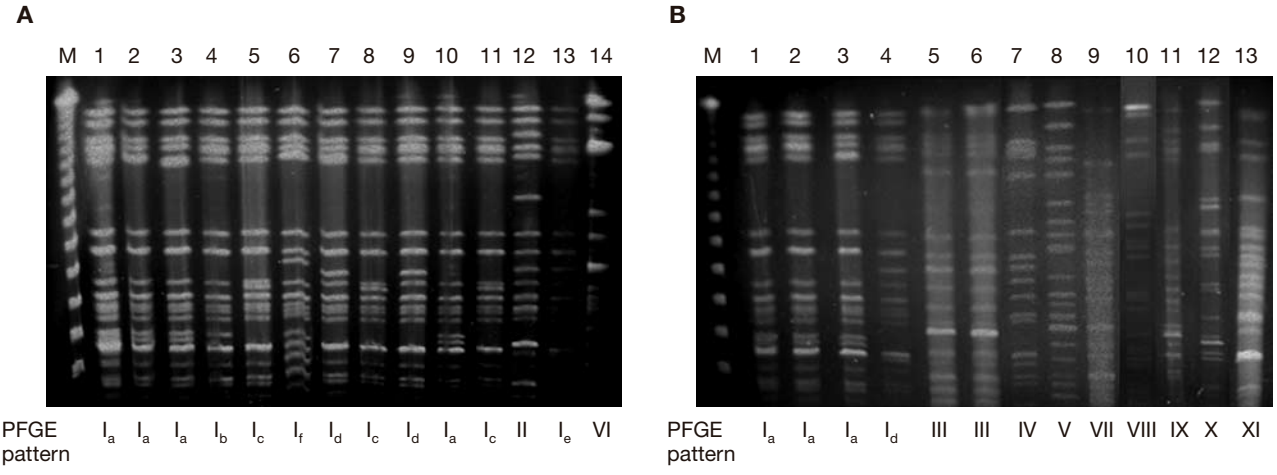

Fig. 2. (A-B) Eleven pulsed-field gel electrophoresis (PFGE) patterns of CMY-2–producing *Klebsiella pneumoniae* after digestion with *Xba*I. Lane M, bacteriophage I DNA ladder.

β-lactamase was consistent with the intrinsic SHV-1 enzyme of *K. pneumoniae* [17].

Susceptibility testing

Sixty non-replicate CMY-2–producing isolates demonstrated resistance to amoxicillin (>256 μg/mL), amoxicillin-clavulanic acid (64-128 μg/mL), cefoxitin (128->256 μg/mL), and ceftazidime (64->256 μg/mL), decreased susceptibilities to cefotaxime (16-128 μg/mL) and aztreonam (16-64 μg/mL), and susceptibility to cefepime (0.13-0.50 μg/mL) and imipenem (0.25-1.00 μg/mL). The resistant phenotypes of susceptibilities to non–β-lactam agents determined by the disk-diffusion method are summarized in Table 1. All isolates were susceptible to amikacin.

Epidemiologic investigation

Clinical characteristics of patients with epidemic clone I CMY-2–producing isolates (clone I patients) and those with non–clone I isolates (non–clone I patients) were compared. Patients in the 2 groups were similar in terms of sex, age, and focus of infection or colonization (Table 2). More non–clone I patients (3/11; 27.3%) than clone I patients (2/49; 4.1%) were admitted to hospital due to infections caused by CMY-2–producing *K. pneumoniae* (p = 0.04). In the univariate analysis of comorbid illness or conditions, renal insufficiency (serum creatinine level, >2.5 mg/dL) was significantly associated with the acquisition of non–clone I isolates ($p = 0.010$), and surgery within the previous 1 month was significantly associated with the clone I isolates ($p = 0.017$). The categorical factors with a p value of <0.25 in the univariate analysis were assessed by multivariate analysis with a stepwise logistic regression model. Only recent surgery was significantly associated with the acquisition of clone I isolates (odds ratio, 8.3; 95% confidence interval, 1.2-40.2; $p = 0.009$).

Forty four (89.8%) of 49 clone I patients and 8 (72.7%) of 11 non–clone I patients first acquired CMY-2–producers after hospital stay of more than 48 h (Table 3). However, the hospital stays before isolation of CMY-2–producing isolates were shorter in clone I patients than in non–clone I patients ($p = 0.007$). Of patients admitted to hospital for 48 h or less before isolation of

Table 2. Demographic and clinical characteristics of patients with clone I and non–clone I isolates of CMY-2–producing *Klebsiella pneumoniae*.

Characteristic	Clone I (n = 49) No. (%)	Non–clone I (n = 11) No. (%)	*p*
Male	31 (63.3)	7 (63.6)	0.982
Mean age (years)	59.5	62.7	0.649
Comorbid conditions			
Surgery in previous 1 month	30 (61.2)	2 (18.2)	0.017
Hypertension	17 (34.7)	2 (18.2)	0.476
Diabetes mellitus	16 (32.7)	5 (45.5)	0.421
Trauma	10 (20.4)	0 (0)	0.099
Previous stroke	10 (20.4)	2 (18.2)	1.000
Renal insufficiency (serum creatinine, >2.5 mg/dL)	6 (12.2)	5 (45.5)	0.010
Hepatobiliary disease	6 (12.2)	1 (9.1)	1.000
Malignancy	5 (10.2)	3 (27.3)	0.154
Autoimmune disorder	3 (6.1)	0 (0)	1.000
Congestive heart failure	2 (4.1)	1 (9.1)	0.462
Invasive treatment			
Mechanical ventilator	22 (44.9)	4 (36.4)	0.606
Urinary catheter	32 (65.3)	7 (63.6)	1.000
Central venous catheter	28 (57.1)	5 (45.5)	0.481
Focus of infection[a]			
Bacteremia	11 (22.4)	3 (27.3)	0.707
Urinary tract infection	13 (26.5)	3 (27.3)	1.000
Postoperative wound infection	8 (16.3)	3 (27.3)	0.407
Intra-abdominal infection	2 (4.1)	0 (0)	1.000
Pneumonia	4 (8.2)	0 (0)	1.000
Epidural or brain abscess	2 (4.1)	0 (0)	1.000
Colonization	19 (38.8)	3 (27.3)	0.731

[a]Some patients had more than 1 infectious foci.

Table 3. Hospital admission history of patients with clone I and non–clone I CMY-2–producing *Klebsiella pneumoniae* isolates.

History	Clone I (n = 49) No. (%)	Non–clone I (n = 11) No. (%)	*p*
Hospital stay >48 h before isolation	44 (89.8)	8 (72.7)	0.154
Mean duration of hospital stay before isolation (days)	28.4	63.6	0.007
Hospital stay ≤48 h before isolation	5 (10.2)	3 (27.3)	0.154
Prior hospital stay (no. of patients)	4	3	1.000
Mean duration between prior discharge and isolation (days)	73.5	910.7	0.110
Transfer from another hospital (no. of patients)	1	0	1.000
SICU stay[a]	44 (89.8)	7 (63.6)	0.050
SICU stay at the time of isolation	18 (36.7)	1 (9.1)	0.148
Mean duration of SICU stay before isolation (days)	10.1	7	0.572
Prior SICU stay	26 (53.1)	6 (54.5)	0.929
Mean duration between discharge from SICU and isolation (days)	30.5	124.0	0.010
Mean duration of prior SICU stay (days)	14	13.8	0.975
Other ICU stay[b]	9 (18.4)	3 (27.3)	0.679

[a]Includes patients with CMY-2–producing *Klebsiella pneumoniae* isolates at SICUs, and with CMY-2–producing *Klebsiella pneumoniae* isolates and a history of SICU stay.

[b]Includes patients with CMY-2–producing *Klebsiella pneumoniae* isolates at other ICUs, and with CMY-2–producing *Klebsiella pneumoniae* isolates and a history of stay at other ICUs.

Abbreviation: SICU = surgical intensive care unit.

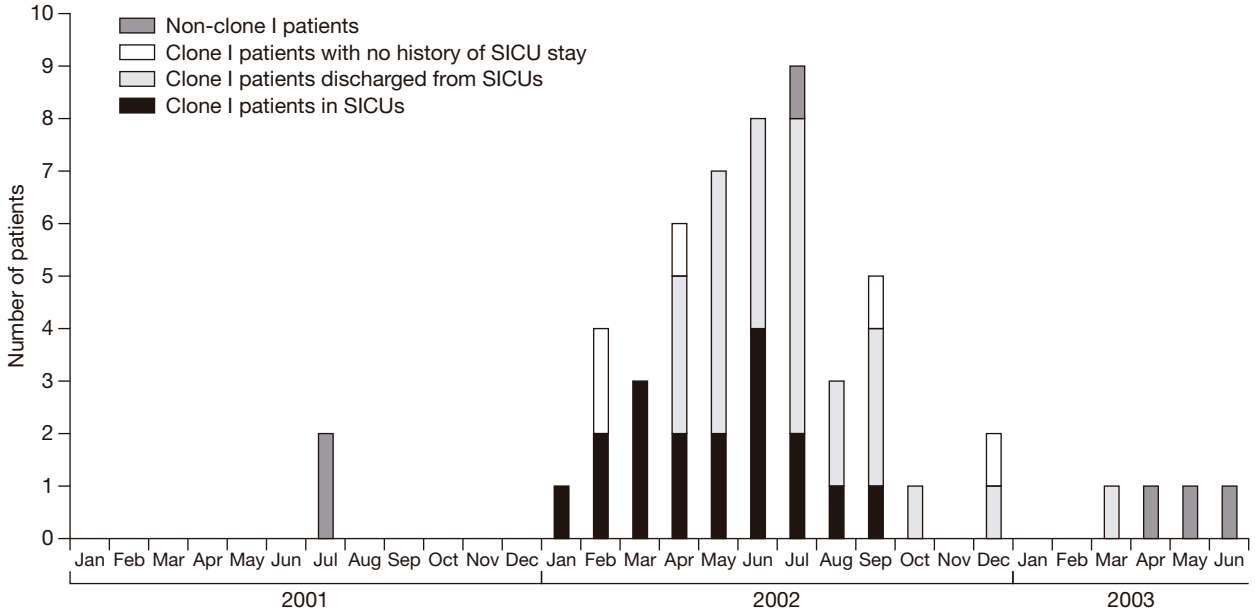

Fig. 3. The monthly distribution of 55 patients colonized or infected with CMY-2–producing *Klebsiella pneumoniae* from January 2001 through June 2003.

Abbreviation: SICU = surgical intensive care unit.

CMY-2–producing isolates, 4 of 5 clone I patients and all 3 non–clone I patients had been recently admitted to hospital. Forty four (89.8%) of 49 clone I isolates were obtained from patients in SICUs or those who had been admitted to an SICU; in contrast, only 7 (63.6%) of 11 non–clone I isolates were from patients in SICUs or those who had been admitted to an SICU. Eighteen CMY-2–producing clone I isolates were from 15 patients in SICU 2 and 3 patients in SICU 1 between January and September 2002 (Fig. 3). The other 26 clone I isolates were found in patients in 8 general wards between April 2002 and March 2003, and 25 of the isolates were from patients who had stayed in an SICU between January and September 2002. The duration between the shift from the SICUs and isolation of CMY-2–producing isolates was short-

er among clone I patients (range, 1-248 days; median, 22 days) than among non–clone I patients (range, 5-417 days; median, 65 days) [$p = 0.01$].

Five of 49 clone I patients had never been admitted to an SICU before the isolation of CMY-producing isolates. One patient was transferred from a district hospital, and the other 4 patients were admitted between February and October 2002 to 4 general wards in which clone I isolates had been obtained from patients transferred out of the SICUs. Two clone III isolates were obtained from 2 patients in 2 wards, but they had stayed in the medical ICU before the isolation.

Discussion

This report describes the epidemiological characteristics of patients with CMY-2–producing *K. pneumoniae* in a university hospital. Predominance of an epidemic clone, designated clone I, was found. Clone I isolates were recovered from 18 patients in SICUs between January and September 2002, indicating the occurrence of a clonal spread during this 9-month period. The finding that clone I patients were in hospital for a shorter duration before isolation than non–clone I patients is suggestive of cross-transmission of clone I. Moreover, the beds in 2 SICUs were arranged in large rooms without individual cubicles or physical barriers between the beds; such an arrangement may play a role of spread of the clone among patients in the units. Recent surgery was the only risk factor for acquisition of the clone I isolates by multivariate analysis. Therefore, patients in SICUs with recent surgery, poor physical condition, and invasive devices have an increased risk for cross-contamination by the epidemic clone.

Clone I isolates were found in 26 patients from SICUs, but was rarely found during periods other than the dissemination period — between January and September 2002. Moreover, the duration between moving from the SICU and the isolation of CMY-2–producing isolates was shorter for clone I patients than for non–clone I patients. These data suggest that these patients were colonized by CMY-2–producing *K. pneumoniae* during their stay in the SICU.

Although 8 patients had been admitted to hospital for less than 48 h before the isolation of CMY-2–producing isolates, 4 of 5 patients with clone I isolates had recently been admitted to hospital, and had stayed in the SICUs for some time during the outbreak period — from February 2002 to September 2002. These findings suggest that CMY-2–producing *K. pneumoniae* can colonize patients for several months. Surveillance for multidrug-resistant Gram-negative pathogens and early detection of colonized patients are considered to be effective infection control strategies to attenuate the transmission of ESBL- or AmpC-producing *Enterobacteriaceae* [18-20]. The results of this study reinforce the concept that patients with a prior admission to the outbreak unit should be surveyed to determine the risk of occult carriage of the epidemic clone.

The small number of patients with CMY-2–producing *K. pneumoniae* per month in the SICUs (range, 1 to 4 patients), and the presence of CMY-2–producing isolates in several different wards might be the reason for the lack of awareness of clonal spread by the health care personnel. Review of the computer data from the microbiology laboratory showed that CMY-2–producing isolates would cause a negative result in the NCCLS ESBL confirmatory tests. Also, the trend for an increasing incidence of non-ESBL–producing isolates with cefoxitin resistance and reduced susceptibilities to ceftazidime was consistent with the occurrence of the outbreak (Fig. 1). However, the clinical microbiology laboratory has the earliest opportunity to detect a specific antimicrobial resistance phenotype emerging in hospital settings.

Since this study was a retrospective analysis, the source of the epidemic CMY-2–producing clone and the precise mode of transmission could not be confirmed. How the epidemic clone was introduced into the SICUs was also unclear. The first isolate of the epidemic clone during the study period was from a patient who underwent emergency surgery at admission and was admitted to SICU 2 immediately after surgery. The isolate was recovered from his sputum after 14 days of SICU stay. The finding suggests the presence of the epidemic clone in the SICU before the outbreak.

Although the clonal spread was not recognized early and lasted for approximately 9 months, it was finally terminated. Tracing the surveillance data of nosocomial infections between 2000 and 2002, there has been an outbreak of *Acinetobacter baumannii* bacteremia in the SICU 2 in the fourth quarter of 2000, followed by increasing *A. baumannii* colonizations and infections in the SICUs between 2001 and 2002. Three major infection control measures of environmental decontamination with 1000 ppm sodium hypochlorite, contact isolation for patients colonized or infected with multidrug-resistant bacteria, and reinforcement

of the sterile precautions with gowns and gloves while implanting central venous catheters were implemented to halt the *A. baumannii* epidemic. It is not known how much these interventions contributed to the termination of this unrecognized clonal spread of CMY-2–producing isolates. However, it is important to note that ICUs, where antimicrobial prescription is common and aims for extended-spectrum cover, often serve as important reservoirs for multidrug-resistant bacteria [21,22]. Surveillance for antimicrobial resistance in such clinical settings should be seriously considered. In the instances of increasing prevalence of clinical isolates with specific resistant phenotypes, appropriate antibiotic control and infection control measures should be initiated to prevent their spread.

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