附件：封面格式

行政院國家科學委員會補助專題研究計畫成果報告

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※ 多藥性大腸桿菌感染之臨床分析與 ※

※ 分子流行病學研究 ※

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計畫類別：☑個別型計畫　　☐整合型計畫

計畫編號：NSC90－2314－B－002－290

執行期間：90 年 08 月 01 日至 91 年 07 月 31 日

計畫主持人：陸坤泰 教授

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執行單位：國立台灣大學醫學院　檢驗醫學科

中 華 民 國 91 年 12 月 06 日

Bacteremia Due to Extended-Spectrum Cephalosporin-Resistant *Escherichia coli:* An Emphasis on the Correlation of Patients' Clinical Outcome and Resistant Mechanisms of the Isolates

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Abstract

Clinical features of 24 patients with bacteremia caused by extended-spectrum cephalosporin-resistant *Escherichia coli* seen from 1993 to 1998 at National Taiwan University Hospital were reviewed, and microbiological characteristics of the 24 isolates from these patients were analyzed. Among these isolates, eight, 20, and 12 isolates possessed the gene for bla_{SHV}, bla_{TEM}, and bla_{CTX}, respectively. Two isolates' resistance was determined to be AmpC β-lactamase by isoelectric focusing study. One isolate had unidentified β-lactamases. All bla_{TEM}-harboring isolates had TEM-1 except one belonging to TEM28. All isolates harboring bla_{SHV} and bla_{CTX} genes possessed SHV-5 and CYX-M-3, respectively. Genotyping by pulsed-field gel electrophoresis of these isolates and plasmid-digested profile of transconjugants could not document clonal spread or plasmid transfer among these isolates in the hospital. None except one isolate containing CTX-M-3 and two AmpC hyperproducers showed any synergistic effect when clavulanic acid was added with extended-spectrum cephalosporins. Among 22 patients with known drug history, 77.3% patients had received beta-lactam(s) treatment and only 45.5% had used extended-spectrum cephalosporin or aztreonam therapy. Among the 11 patients receiving imipenem-containing regimens, 10 were successfully treated except for one patient who died of concurrent fungal infection within 1 day. Five patients who received extended-spectrum cephalosporins or aztreonam-containing regimens died. Among five patients empirically treated with regimens containing a β-lactamase inhibitor combination, four failed to recover form the bacteremic episode. Three of seven patients who received treatment with cefametazole-containing regimens responded unsatisfactorily. In conclusion, extended-spectrum cephalosporin-resistant *E. coli* can occur in patients who have not experienced extended-spectrum cephalosporin

treatment when such a resistant pathogen is prevalent within a hospital. Infections due to extended-spectrum cephalosporin-resistant *E. coli* may cause fatality, and the susceptibility to inhibitor drug combinations and the cephamycin group antibiotics may not be reliable for treating these patients.

Introduction

With the increasing usage of the oxyimino-cephalosporins (including extended-spectrum cephalosporins and monobactams), various reports of the emergence of resistance to these drugs in *Klebsiella* spp. and *Escherichia coli* have reflected the discussion world-wide.[15] The TEM and SHV-type plasmid-mediated extended-spectrum β-lactamases (ESBLs) are the most frequently reported mechanisms for the emergence of this resistance in *K. pneumoniae* and *E. coli*.[8,12] Besides the TEM and SHV-derived β-lactamases, in different regions other variants, such as the CTX-type and AmpC-related Bush Group 1 β-lactamases, have also been implicated in resistance to oxyimino-cephalosporins.[4,31]

The oxyimino-cephalosporin-resistance of clinically-isolated *Enterobacteriaceae has* also been demonstrated in Taiwan, with the prevalence of resistant *K. pneumoniae* in hospitals reportedly ranging from 8.5% to 30.0% during 1997 and 1999.[10,33] Besides the SHV-5 and SHV-12 ESBLs,[10] a plasmid-mediated AmpC enzyme (CMY-8) has been identified as the mechanism of resistance for *K. pneumoniae* isolates in this country.[33] The existence of CTX-M-3, CMY-2, SHV-5, SHV-12, TEM-10 and unidentified enzymes in *E. coli* isolates from a southern-Taiwan hospital in 1999 suggests that the mechanisms responsible for oxyimino-cephalosporin resistance are complex,[32] however, most of the ESBL investigations in Taiwan were of

relatively short duration, with only the predominant β-lactamase(s) responsible for the resistance of oxyimino-cephalosporins studied. The prevalence and diversity of oxyimino-cephalosporin-resistant *E. coli* strains, and the proportion of ESBL-producing analogs, which cause such resistance in Taiwan have rarely been reported. It seems reasonable to suggest that longitudinal study elaborating the complexity and diversity of β-lactamases for third-generation cephalosporin-resistant *E. coli* isolates may be of particular interest. For this study at a Taiwan medical center, the prevalence for resistance to extended-spectrum *E. coli* β-lactams was reviewed from January 1993 to June 1998, with correlations determined for the mechanisms of such resistance, antimicrobial-susceptibility patterns, clonality, and plasmid typing for *E. coli* blood isolates, and the respective treatment outcomes for individual antimicrobial agents.

Materials and Methods

Bacterial strains and patient characteristics

Records dating from January 1, 1993 to June 31, 1998, which were stored in a computerized database at the National Taiwan University Hospital (NTUH), an 1900-bed primary and tertiary-care center, were searched for *E. coli* blood isolates completely or intermediately resistant to aztreonam or cefotaxime, as determined by the disc-diffusion method. [17] The demographics, procedures, prescribed antimicrobial agents, and outcomes for patients diagnosed with aztreonam or cefotaxime-resistant *E. coli* bacteremia were reviewed.

Definitions

Nosocomial infection was defined according to the 1988 Centers for Disease Control (CDC) definition.[5] Treatment failure was defined where no clinical improvement was noted after more than two days of antimicrobial therapy.

Antimicrobial-susceptibility testing

The antimicrobial susceptibility of these isolates and the transconjugated analogs were determined concomitantly using the disk-diffusion and agar-dilution methods, as described in National Committee for Clinical Laboratory Standards (NCCLS) documents.[17,18] For susceptibility testing using agar dilution, the following antimicrobial agents were obtained as standard reference powders of known potency for laboratory use: amoxicillin, ampicillin, cephalothin (Sigma Chemical Co.; St. Louis, MO, USA); clavulanic acid (SmithKline Beecham; Brockhans Park, Surry, UK); piperacillin and tazobactam (Lederle Laboratories; Pearl River, NY, USA); cefmetazole (Upjohn Co.; Kalamazoo, MI, USA); imipenem (Merck Sharp & Dohme; West Point, PA, USA); cefotaxime, gentamicin and cefpirome (Hoechst Marion Roussel; Frankfurt, Germany); ceftazidime (Glaxo Group Research Limited; Greenford); cefepime, amikacin and aztreonam (Bristol-Muers-Squibb Laboratories; Princeton, NJ, USA); meropenem (ICI Pharmaceutical Inc.; Cheshire, UK); and ciprofloxacin (Bayer Co.; Leverkusen, Germany). All drugs were incorporated into Mueller-Hinton agar (Becton Diuckson Microbiology Systems; Sparks, MD) serially in two-fold concentration increments from 0.03 to 128 μg/ml. The broth-microdilution method with the Mueller-Hinton product (TREK Diagnostic System Ltd; West Sussex, UK) was used to determine the minimum inhibitory concentration (MIC) for the

following agents: ampicillin, cephalothin, amoxicillin/clavulanic acid, cefoxitin, tetracycline, cefpodoxime, cefpirome, cefotaxime, ceftazidime, ceftizoxime, amikacin, aztreonam, and ciprofloxacin, for serial two-fold concentration increments from 0.025 to 64 μg/ml, which were performed to assure the accuracy of the MIC testing. Two control strains, *E. coli* ATCC 35218 and 25922, were included for each set of tests. The plates were incubated in ambient air at 35 ^{0}C for 16 to 18 h. The MIC for each antimicrobial agent was defined as the lowest concentration which inhibited visible growth of the organism, with fine, barely visible hazes or single colonies disregarded.

Screening tests for ESBLs

Mueller-Hinton agar (BBL Microbiology Systems) and the following antimicrobial disks were included for double-disk synergy testing: cefotaxime (30 μg), ceftazidime (30 μg), aztreonam (30 μg), cefepime (30 μg), and amoxicillin/clavulanic acid (20 μg of amoxicillin plus 10 μg of clavulanate). All disks were purchased from Becton Dickson Microbiology System (Cockeysville, Md). Procedure and interpretation of the double-disk synergy test were performed as previously described.[21] The Etest ESBL screen (AB Biodisk, Solna, Sweden), which is based on the recognition of a reduction in ceftazidime MIC in the presence of clavulanic acid, was performed according to the manufacturer's instructions.

Isoelectric focusing

Cells were harvested from the brain-heart infusion broth culture at 20 h by centrifugation, and the pellet resuspended in 1 ml of phosphate buffer (0.05 M, pH 7). Enzymes were released by two cycles of freezing (at -70 ^{0}C) and thawing at room temperature, followed by sonication for 5 min in ice-cold water. Isoelectric focusing was performed in

ampholine gel (pH 3.0-10.0; Pharmacia). Preparations from standard strains known to harbor TEM-1, SHV-1 and SHV-5 were used as standards. After isoeclectric focusing, β-lactamases were detected by spreading nitrocefin (50 μg/ml) on the gel surface [14].

PCR amplification for *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$.

Oligonucleotide primers used for PCR assay were as follows: 5'-ATAAAATTCTTGAAGACGAAA (primer A); 5'-GACAGTTACCAATGCTTAATCA (primer B); 5'-GGGTAATTCTTATTTGTCGC (primer C); 5-CAGAATTCGCTTAGCGTTGCCAGT (primer D); 5'-CGCTTTGCGATGTGCAG (primer E); and 5'-ACCGCGATATCGTTGGT (primer F). Oligonucleotides were synthesized using GIBCO (New York). Primers A and B are known to be specific for *bla*$_{TEM}$.[13] Primers C and D are known to be specific for *bla*$_{SHV}$.[22] Primers E and F are known to be specific for *bla*$_{CTX-M}$.[2] Reactions were performed in a DNA Thermal Cycler (Bio-Rad; Hercules, CA) in 50 μl mixtures containing 2.5 U *Taq* polymerase (Promega; Madison WI), 1X buffer consisting of 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01 μg gelatin, 200 μM each of deoxynucleoside triphosphate, and 2 μM each of oligonucleotide primer. Thirty-five cycles were performed for each reaction with the following temperature profile: 94 °C, 1 min; 58 °C, 1 min; and 72 °C, 1 min.

For direct DNA sequencing, PCR products were purified using the MicroSpin S-300 HR PCR purification columns (Pharmacia; Uppsala, Sweden). Sequencing reactions were performed according to the method of Sanger et al., [25] and using consecutive primers specific for the *bla*$_{CTX-M}$, *bla*$_{TEM}$ and *bla*$_{SHV}$ gene.[2,13,22] An automatic sequencer (377, ABI Prism, PerkinElmer; Norwalk, CT, USA) was used .

Plasmid isolation and resistance transferal

Plasmid-profile analysis was performed using the alkaline extraction method.[9] Resistance transfer was carried out by conjugation, with a rifampicin-resistant strain of *E. coli* (JP-995) used as the recipient. Recipients and donors were separately inoculated into the brain-heart infusion broth (Oxoid; Basingstoke, Hampshire, England) and incubated at 37 °C for four hours, and then mixed at a 1:1 volume ratio for overnight incubation at 37 °C. An 0.01-ml volume of the overnight-broth mixture was then spread on a MacConkey agar plate containing rifampicin (100 μg/ml), and either ceftazdime (1 μg/ml) or aztreonam (2 μg/ml).

Plasmid DNA from the transconjugant was prepared as described previously.[1] Restriction-enzyme analysis of the transconjugant plasmids was performed according to the manufacturer's instructions. The restriction enzymes *Pst*I (Gibco, New York) was used, with λ-*Hin*dIII and *Eco*RI cleavage products used as molecular-weight markers.

Genomic fingerprinting using pulse-field gel electropheresis

Pulse-field gel electropheresis (PFGE) was performed as has been described previously.[1,27] A restriction enzyme, *Xba*I (Gibco, Taiwan), was used at the manufacturer's suggested temperature. Restriction fragments were separated using PFGE in 1% agarose gel (Bio-Rad; Mercules, CA) in 0.5X TBE buffer (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA, pH 8.0) using the Bio-Rad CHEF-DRII (Bio-Rad). The initial pulse time of 1 s was increased linearly to 35 s over 20 h at 200 V at 4 °C. Gels were then stained with ethidium bromide and photographed under ultraviolet light. To reveal polymorphism as determined from PFGE, each sample was analyzed using Molecular Analyst Fingerprinting, Fingerprinting Plus, and Fingerprinting DST

Software (Bio-Rad Laboratories; Richmond, CA). The grouping method was adopted to derive a dendrogram from the matrix via the Unweighted Pair Group Method using Arithmetic average (UPGMA) clustering technique after calculation of similarities using the Pearson correlation coefficient for every pair of organisms.

Results

Bacterial strains

As determined by the disc-diffusion method, the annual percentages for clinical *E. coli* isolates resistant to aztreonam or cefotaxime at NTUH, were 2.47, 3.72, 3.92, 4.92, and 6.67% from 1993 to 1997, respectively. Using this method, it was determined that 44 *E. coli* blood isolates were insensitive to cefotaxime or aztreonam from January 1993 to June 1998,[17] with 38 of these isolates recovered from stocks stored at –70 ^{0}C. Nine isolates were excluded because MICs to aztreonam, cefotaxime and ceftazidime were not elevated (≥2 μg/ml) after further determination.[18] Medical records for four infection cases were inadequate for analysis or missing, and 25 oxyimino-cephalosporin-resistant *E. coli* isolates, from 24 bacteremic episodes, were reviewed. Where two isolates from a patient had the same PFGE pattern (described below) and the same antibiogram and pI points, the two isolates were counted as one. In total, the results of 24 strains were analyzed for antimicrobial susceptibility and resistance mechanisms, with correlations derived for clinical results and microbiologic findings.

Molecular typing using the PFGE method

Of the 25 isolates, except for two (ec28 and ec39) from the same patient taken two days apart for which the same PFGE pattern was determined, all the other isolates had more than a three-band difference with the UPGMA clustering technique after

calculation of similarities using Pearson's correlation coefficient between every pair of organisms, indicating they are not related epidemiologically[30] (Figure not shown).

Patient characteristics

In total, medical records for 24 patients were analyzed (Table 1). Age distribution ranged from two months to 77 years, with nine (37.5%) patients younger than five years and seven (29.2%) over 65 years. The most common underlying disease was malignancy (16/24, 66.7%), with five patients diagnosed with bacteremia in a neutropenic state. Three patients had diabetes mellitus (DM) and three of liver cirrhosis. Except for two of the infections, most were acquired (91.7%) nosocomially. The only two community-acquired infections were malignancy cases where outpatient visits had been frequent during the previous two months. Three pediatric patients were excluded on the basis of ineligible APACHE III score. The mean (± standard deviation) Apache III scores for 21 patients was 38.5 ± 20.9 on admission, deteriorating to 46.5 ± 18.5 by the time the bacteremia was diagnosed, indicating the poor general condition of these patients after admission, with the physiological condition worsening for most during hospitalization. The infection foci were not identified for eight bacteremia patients, however, urinary tract infections were diagnosed for seven, with infections intra-abdominal for three, soft-tissue for three, and pneumonia for three. The day of blood-isolate recovery ranged from day 1-99 after admission (mean, 28.5; median, 15). Except for two patients with unknown history of antimicrobial-agent treatment, and two that had not received such treatment, most patients (20/22, 91%) had a history of use before the drug-resistant-*E. coli* bacteremia infection occurred. Of the 22 patients with known drug history, 17 (17/22, 77.3%) had received beta-lactam(s) treatment, and only 10 (10/22, 45.5%) had experienced extended-spectrum cephalosporins. When

patients had bacteremia, eight (33.3%) patients were not under antimicrobial therapy. Only 10 patients were receiving antimicrobial therapy containing beta-lactam(s), among which only four (16.7%) patients were receiving extended-spectrum cephalosporins. Total hospital stay was between 11 and 245 days (mean, 70.6; median, 49.5).

Antimicrobial susceptibility of clinical isolates.

Results of the in vitro antimicrobial susceptibility tests are shown in Table 2. All *E. coli* isolates were resistant to ampicillin, with only two resistant to cefazolin and one to cefixime. The percentages of isolates susceptible to amoxicillin/clavulanic acid, cefoxitin and cefmetazole were 33.3, 45.8, and 75.0%, respectively. Not all the clinical isolates had MIC values against extended-spectrum β-lactams above the critical point suggested in NCCLS criteria[18]. Considering susceptibility to oxyimino-cephalosporins, a total of 79.2, 62.5, 87.5, 100, and 100% of the clinical isolates were susceptible to aztreonam, ceftazidime, cefotaxime and cefepime, cefpirome, respectively. Only 4.2% were susceptible to cefpodoxime. All these isolates were susceptible to pirpercillin/tazobactam, cefepime, and cefpirome; for some of the isolates, however, MICs were just at the upper limit of the susceptibility range. All were susceptible to imipenem and meropenem, with 22 (91.7%) isolates susceptible to amikacin, and only 54.2% to gentamicin. The percentages of isolates susceptible to ciprofloxacin, and moxifloxacin were 66.7 and 70.8%, respectively.

Screening test for ESBL, PCR amplification and sequencing of PCR products for *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$

Of the 24 isolates, 20, 8 and 12 were positive for *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX}$ PCR amplification, respectively. Nine isolates and their related transconjugants yielded

positive results from the Etest ESBL screening test (Table 3). It was demonstrated that the entire bla_{TEM} amino sequence, for all the *E. coli* isolates except one, was identical to the bla_{TEM-1} encoding *Tn*2 [7]. Substitutions at amino acid 164 (Arg→His) and 240 (Glu→Lys), which had an identical amino acid sequence to TEM-28, were determined for one isolate. For eight isolates positive for bla_{SHV} PCR amplification, an identical amino acid sequence to SHV-5 [16] was demonstrated for all, with all except one coexisting with TEM-1 β-lactamase. For 12 isolates positive for bla_{CTX} PCR amplification, all had identical amino acid sequencing to CTX-M-3.

Plasmid profiles, resistance transfer and restriction-enzyme-digestion profile of plasmids

Except for five isolates (Ec1, Ec8, Ec38, Ec41, and Ec44) with two to four plasmids, all the other 19 isolates had only one plasmid (Fig. 1). The size and number of plasmids for these multi-plasmid isolates were all different. The plasmids from a total of 15 isolates were transferrable using either ceftazidime or aztreonam selection. All the plasmids from the nine isolates carrying the TEM-28 or SHV-5 genes were transferable. For isolates containing TEM-1 and CTX-M-3, the transconjugants harbored both TEM-1 and CTX-M-3. Only one plasmid was transferred for each strain, with the resistant gene for each transconjugant located in a plasmid of >90 kb (Figure not shown). Restriction-enzyme digestion of the plasmids revealed different *Pst*I-digestion profiles for all 15 transconjugants. The result obtained from analysis of the digested plasmids indicated that plasmids of seven transconjugants which coexisted with TEM-1 and SHV-5 β-lactamase, and five which coexisted with TEM-1 and CTX-M-3 were also all different (Fig. 2).

Isoelectric focusing

Isoelectric focusing revealed that transconjugants possessing TEM-1 and SHV-5 β-lactamase have a pI point of 5.4 and 8.2, respectively. For the isolate with TEM-28, a pI of 6.1 was observed. All the isolates with CTX-M-3 had a pI point of 8.4. Besides the nine isolates that were TEM or SHV-type ESBL producers, there were six transconjugants with plasmids each carrying more than one β-lactamase, as identified from the different pI points (Table 3). Two isolates with a pI point greater than nine were referred to as AmpC-type β-lactamase producers. Three bands were determined for one isolate with pI points of 5.4, 8.0, and 8.7, indicating three beta-lactamses, however, only TEM-1 was identified.

Correlation for susceptibility characteristics and related resistance mechanism

Positive double-disk synergy tests were seen for TEM-28 and SHV-5-producing isolates, the isolate with unidentified beta-lactamase (Ec14), and two of 12 (16.7%) isolates containing CTX-M-3. The results of the ESBL Etest were positive only for the TEM-28 and SHV-5-producing isolates (Table 4). Isolates containing SHV-5 ESBL were susceptible to amoxicillin/clavulanate, and the only isolate with TEM-28 was intermediately resistant. By contrast, the isolates containing CTX-M-3 or the proposed AmpC–hyperproducing isolates to amoxicillin/clavulanate were categorized as not susceptible. Although, 77.8% of TEM-28 and SHV-5-producing isolates were susceptible to cefoxitin, only 25% containing CTX-M-3 were susceptible. With the addition of a fixed concentration (4 μg/ml) of clavulanic acid, a more than four-fold MIC decrease was observed for cefotaxime, ceftazidime, aztreonam and cefepime in 16, 16, 11, 15of the 24 isolates, respectively. Except for one isolate containing CTX-

M-3 and TEM-1, a synergistic effect was noted for all of the isolates (more than four-fold MIC decrease) when clavulanic acid was added to any of the four extended-spectrum β-lactams above. A two-fold decrease in concentration in an MIC for either cefotaxime or ceftazidime tested in combination with clavulanic acid versus its MIC when tested alone, as the NCCLS recommended as ESBL phenotypic confirmatory test, was seen for most isolates, except for six containing both CTX-M-3 and TEM-1.

Relationship of antibiotic usage, outcome and resistance mechanism

Seven patients (29.2%) died during the hospitalization period. Of the eleven patients receiving imipenem-containing therapy, ten were successfully treated. One patient expired from concurrent fungal infection within one day of treatment initiation. Five patients died during oxyimino-cephalosporin-containing therapies, and one during amphotericin B therapy. Only one individual was treated with ciprofloxacin, and the outcome was favorable for this case. Five patients were treated exclusively using regimens containing amoxicillin/clavulanate or ampicillin/sulbactam, prescribed empirically or according to the susceptibility results of agar-diffusion susceptibility, with only one successfully recovering form the bacteremic episode while being treated with the beta-lactam/inhibitor combination. Four patients were treated with cefmetazole exclusively and one recovered. Two cases of urinary tract infection (Ec41, Ec21) and one of biliary tract infection (Ec14) were cured using cefotiam-containing regimens (Table 1).

Discussion

Instead of using a short-term epidemiologic investigation, we examined all 24 blood isolates recovered from NTUH during a 6-year period in order to figure out the evolution of mechanisms of resistance of extended-spectrum cephalosporins[?] in

the hospital, and the clinical characteristics and treatment outcomes of infections due to these invasive and resistant strains. We did this even though the current NCCLS recommendations that clinical microbiology laboratories should report all ESBL-producing strains as resistant to all penicillins, cephalosporins, and aztreonam[19] had not yet been established. CTX-M-3 was identified for half of the resistant isolates, with the SHV-5 enzyme identified for one-third Two AmpC hyperproducers, one isolate with TEM-28, and another with an unidentified β-lactamase were also determined. No evidence of clonal spread or plasmid dissemination was detected in these blood isolates.

According to ESBL studies of *K. pneumoniae* isolates obtained from Taiwan ,[10,28,33] SHV-5 was prevalent in two hospitals located in northern and central Taiwan, however, the size of the plasmids conferring resistance was different comparing the two variants (36 kb and >90kb, respectively). Further, plasmids were identified as the main transmission mechanism for one hospital but not the other. In a study conducted at a hospital in southern Taiwan, SHV-12 was prevalent for *K. pneumoniae* whereas the transferable plasmid was diverse.[33] In the southern-Taiwan hospital, plasmid disseminated CTX-M-3 and CMY-2 were the main resistance enzymes determined for oxyimino-cephalosporins-resistant *E. coli* in 1999. [32] From the current retrospective analysis of NTUH blood isolates, it was determined that CTX-M-3, an Ambler Class A Bush Class 2be ESBL first identified in *E. coli* isolated in Europe in 1996, had existed in Taiwan since 1994. The NTUH plasmids carrying CTX-M-3 during the six-year study period were different. Apart from CTX-M-3, SHV-5 and TEM-28 producers, and AmpC hyperproducers caused oxyimino-cephalosporin-resistance in the *E. coli*. With respect to the AmpC-type enzymes, endemic spread was identified for CMY-8 in *K. pneumoniae* isolates, and CMY-2 in *E.*

coli isolates from a southern-Taiwan hospital.[33] Our investigation did not find and identify any CMY variants, however, with the two AmpC-hyperproducer *E. coli* isolates in this study having various pI points above 9.0, suggesting that there were various AmpC-type β-lactamases present in our hospital. The ESBL investigations outlined above indicate that the prevalent ESBL genes and mechanisms of transmission differ from hospital to hospital in this island nation. It also seems reasonable to suggest that the nationwide spread of ESBLs occurs mainly through the hospital environment, and is not related to community-transmission, although the emergence of oxyimino-cephalosporin-resistance was noted in many of Taiwan's hospitals almost simultaneously, [10,30,34] and episodes of infection involving oxyimino-cephalosporins-resistant *Enterobacteriaceae* were not considered predominately nosocomial [32].

In the current study, most (83.3%) of the *E. coli* strains carried a TEM-type β-lactamase, however, only one isolate from 1997 was a TEM-type ESBL (TEM-28) producer. To our knowledge, a TEM-10-producing *E. coli* [32] and a TEM-28-producing isolate are the only two TEM-derived ESBLs which have been identified in Taiwan. A TEM-28 with a two-point mutation (R164H, E240K) was first reported in the United States in 1996,[3] and was later discovered in a US hospital survey of ceftazidime-resistant strains in 1998.[34] It has generally been considered unlikely that two mutations would appear from spontaneous mutations of a β-lactamase gene. [20] Interestingly, our study discovered that the other TEM-derived ESBL reported in Taiwan, TEM-10, also possesses two-point mutations (R164S, E240K) at the same amino acid positions as TEM-28. No single-mutation TEM variant at E240K, the common mutation site of TEM-28 and TEM-10, however, has ever been reported. Although the possibility that the TEM-28-resistance gene may have spread from the US to Taiwan cannot be

excluded, the finding that the only two TEM-derived ESBLs both had two-point mutations and the lack of the singly mutated intermediates, TEM-29 (R164H) and TEM-12 (R164S), for TEM-28 and TEM-10 may be due to the limited scale of our single, hospital-wide survey. It is also possible that TEM variants carrying the E240K mutation did exist but were never reported because levels of the resistance phenotype were too low to be detectable. More-extensive nationwide surveillance may help to reveal why only two-point-mutation TEM-derived ESBLs have been determined at the time of writing, and the exact prevalence of TEM-derived ESBLs for Taiwan.

It has been reported that a ceftazidime/ceftazidime+clavulante MIC ratio greater than or equal to 16 is able to stratify the ESBL producers,[11] however, strong synergistic effects for ceftazidime/clavulanate and aztreonam/clavulanate are apparent for the TEM and SHV-derived ESBLs, but not so obvious for the other β-lactamase producer/s.Further, for non-TEM and SHV ESBLs the MIC ratios for ceftazidime/ceftazidime+clavulante and cefotaxime/cefotaxime+clavulanate may not meet the NCCLS ESBL confirmatory criteria for the six clinical isolates harboring CTX-M-3 and TEM-1 (Table 4).

Empirical change of antibiotics for suspected ESBL producers is a prominent clinical option when patients receiving oxyimino-cephalosporin treatment fail to improve. In a case-control study, the type of oxyimino-cephalosporins taken in the month preceding bacteremia appear to be a significant risk factor for bacteremia due to ESBL producers .[26] It is worth noting, however, that only 35.5% of the patients in one study[26] and 45.5% in the current investigation had been treated with oxyimino-cephalosporins. Our data reveal that oxyimino-cephalosporin-resistant *E. coli* blood-stream infection may occur in patients not receiving oxyimino-cephalosporins, or even those not currently on antimicrobial therapy but who have taken antibiotics recently,

with most of the latter treated with β-lactams. Hyperproduction of the TEM-1 enzyme may be responsible for resistance to amoxicillin and clavulanate combinations,[29] and both ceftazidime and inhibitor-drug-combination resistance may be attributable to ESBL in combination with this classic beta lactamase.[23] Thus, the high prevalence of plasmids harboring TEM-1 and the ESBLs in the *E. coli* isolates, and the similar findings for *K. pneumoniae,* [28] at our hospital suggests that inhibitor-drug combinations are not a good choice for empirical utilization of TEM and SHV-variant producers. The susceptibility of CTX-M-3 to amoxicillin/clavulanate has not been reported previously.[6,31,32] For this study, isolates with CTX-M-3, alone or in combination with TEM-1, were intermediately susceptible or resistant to amoxicillin/clavulanate (Table 4), which suggests amoxicillin/clavulanate is not a good candidate treatment where CTX-M-3 is prevalent in oxyimino-cephalosporin resistant *E. coli* isolates from a hospital environment. The poor treatment response to inhibitor-drug combinations demonstrated in this study also supports the microbiological viewpoint outlined above.

In animal models, imipenem and combinations of cefperazone and sulbactam have been more effective than oxyimino-cephalosporin monotherapy for ESBLs producers.[24] In light of the recommendation to avoid oxyimino-cephalosporins as single agents for treatment of serious infections due to these organisms,[24] and given the fact that five of our patients expired while on oxyimino-cephalosporin therapy, it seems reasonable to suggest that the clinical response to combinations of oxyimino-cephalosporin and β-lactamase inhibitor needs further investigation. Additionally, investigation appears necessary to confirm the lack of efficacy, as has been determined in this study, for combinations of oxyimino-cephalosporin and β-lactamase inhibitor

where ESBL-producing strains have been confirmed, given the prevalence of resistant strains with multiple beta-lactamases.

Imipenem showed the most promising clinical response for patients with oxyiminocephalosporin-resistant *E. coli* bacteremia in this study. More than half of the patients using a β-lactamase inhibitor combination or cefmetazole according to the susceptibility test by the disc agar method experienced clinical failure. Larger-scale clinical investigation is needed to elucidate the role of the various antimicrobial agents for treatment of oxyimino-cephalosporin-resistant *Enterobacteriaceae* infection.

In summary, a high diversity of oxyiminocephalsporin-resistant *E. coli* was seen among these blood isolates. The appearance of various β-lactamases, alone or in combination, might have resulted in one-fourth of isolates not being confirmed to have ESBL by the NCCLS ESBL confirmatory test[19]. The classic phenotype of susceptibility to amoxicillin/clavulanate and the ESBL Etest might fail to identify CTX-M-3 producers as Ambler class A ESBLs. Our clinical observations have alerted us to the possibility that oxyiminocephalosporin-resistant *E. coli* can occur in patients not using oxyiminocephalosporin when such a resistant pathogen is prevalent within the hospital. Infections due to oxyiminocephalosporin-resistant *E. coli* may cause death, and the susceptibility to inhibitor drug combinations and cefamycin-group antibiotics may not be reliable to treat these patients.

Acknowledgements

This work was supported by a grant from the National Health Research Institutes, and the National Science Council (NSC89-2314-B002-104), Taiwan.

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Table 1: Clinical characteristics, β-lactamases, experienced antibiotics and treatment outcome of 24 patients.

Isolate No., isolation year/month	β-lactamase type	Underlying disease	Infection focus	Previous antibiotics[a]	Antibiotics when bacteremia occurred	Response to antimicrobial agents[b]
Ec38, 1995/11	TEM-1, AmpC	AML	Primary bacteremia	cephalexin, piperacillin, amikacin, vancomycin, ceftazidime, ciprofloxacin	ciprofloxacin	Cured with imipenem
Ec44, 1998/01	AmpC	Gastric cancer	Intra-abdominal Infection	cefmetazone, ceftazidime, amikacin, cefotaxime, metronidazole	ciprofloxacin	Death, received less than 1 day of cefotaxime + metronidazole
Ec4, 1998/03	TEM1, CTX-M3	Bedridden, CAD, previous CVA, adrenal insufficiency	Primary bacteremia	cefoxitin,ceftazidime, gentamicin, fluconazole, ciprofloxacin, imipenem, amphotericin B	Amphotericin B	Failure with amphotericin B treatment, died 2 days later
Ec20, 1997/07	CTX-M3	Liver cirrhosis	peritonitis	cefotaxime, norfloxacin	norfloxacin	No improvement after 4 days of ceftriaxone , cured with imipenem
Ec15, 1997/04	TEM1, CTX-M3	Lung cancer with liver and bone metastasis	Pneumonia	cefoxitin, gentamicin, clindamycin, ceftazidime, tobramycin	clindamycin, ceftazidime, tobramycin	Failure with clindamycin + aztreonam treatment, died 23 days later

Ec10, 1998/06	TEM1, CTX-M3	Neuroblastoma	Wound infection	cefazolin, cephalexin	cephalexin	Cured with cefmetazole + amikacin
Ec9, 1998/04	TEM1, CTX-M3	Burn	Primary bacteremia	cefazolin, ampicillin/sulbactam	cefazolin, ampicillin/sulbactam	Cured with ampicillin/sulbactam + gentamicin
Ec18, 1997/09	TEM1, CTX-M3	Lymphoma Hypertention.	Primary bacteremia	nil	nil	Continued fevers with ampicillin/sulbactam + gentamicin for 2 days and then cefotaxime + tobramycin for 5 days, Cured with imipenem
Ec25, 1997/12	TEM1, CTX-M3	DM, Prostate cancer, Colon cancer	UTI	cefazolin, gentamicin, cefoxitin	nil	Failure with ceftazidime + tobramycin therapy
Ec37, 1995/09	TEM1, CTX-M3	Colon cancer, DM	Primary bacteremia	ceftriaxone, ciprofloxacin, cefmetazole	nil	Failure with ampicillin/sulbactam + ceftazidime + metronidazole therapy
Ec41, 1996/01	TEM1, CTX-M3	Bladder cancer	UTI	trimthoprim/sulfamethoxazole	nil	Cured with Cefotiam
Ec26, 1996/10	TEM1, CTX-M3	Bladder cancer	UTI	pipemidic acid	cefotiam	Cured with cefotiam + ciprofloxacin
Ec36, 1994/07	TEM1, CTX-M3	Liver cirrhosis	Fascitis	unknown	ampicillin/sulbactam	Continued fevers with ampicillin/sulbactam + ceftriaxone therapy for 3 days, Cured with imipenem
Ec11,	CTX-M3	ASD, PS, corpus	Pneumonia	cefuroxime, netilmicin,	ampicillin/sulba	Cured with imipenem

1998/06		callosum agnesis, laryngomalacia, undescending testis		cefazolin,gentamicin , ampicillin/sulbactam	ctam, gentamicin	
Ec23, 1997/12	TEM-1, SHV-5	Infectious mononucleosis	UTI	ampicillin, cefotaxime, ceftriaxone	ceftriaxone	Continued fevers with cefmetazole + amikacin therapy for 3 days, Cured with imipenem
Ec32, 1993/10	TEM-1, SHV-5	ALL	Wound infection	cefazolin, piperacillin, amikacin, ceftazidime, amphotericin B, vancomycin, penicillin, azactam, itraconazole, metronidazole	cefazolin, amikacin, metronidazole, amphotericin B	vancomycin, imipenem, amphotericin B used, died within 1 day
Ec28, 1997/08	TEM-1, SHV-5	ALL	Primary bacteremia	ceftazidime,cefazolin, amikacin	ceftazidime	Cured with imipenem + gentamicin
Ec1, 1998/02	SHV-5	Lymphoma, DM, hypertension	Pneumonia	nil	nil	Failure with amoxicillin/clavulanate + gentamicin for 4 days, then ceftazidime + tobramycin + trimethoprim/sulfamethoxazole for 3 days
Ec8, 1998/04	TEM-1, SHV-5	TOF, renal agenesis, anorectal anomaly	UTI	imipenem	nil	Cured with cefmetazole
Ec34, 1993/12	TEM-1, SHV-5	ALL	UTI	cefazolin, piperacillin, amikacin	nil	Continued fevers with cefazolin + piperacillin + amikacin for 3 days, cured with imipenem + amikacin

Ec40, 1996/05	TEM-1, SHV-5	AML	Primary bacteremia	cefazolin, piperacillin, amikacin	nil	Continued fevers with cefmetazole + gentamicin for 4 days, cured with imipenem + vancomycin
Ec13, 1997/12	TEM-1, SHV-5	AML, chronic liver disease	Primary bacteremia	ticarcillin/clavulanate, tobramycin, ceftazidime, amikacin, metronidazole, piperacillin	piperacillin, tobramycin	Cured with imipenem
Ec21, 1997/06	TEM28	Systemic sclerosis, CHF, pulmonary hypertension, uremia	UTI	amphotericin B, clindamycin	amphotericin B, clindamycin	Cured with clindamycin + cefotiam
Ec14, 1997/09	TEM-1, unknown	Cholangiocarcimoma, Calori's disease, Liver cirrhosis	Biliary tract infection	unknown	unknown	Cured with cefotiam + tobramycin

a. antimicrobial agents used during the same hospitalization course or three weeks before the bacteremia occurred.

b. The antibiotics after bacteremia include empirical antibiotics use and adjusted antibiotics after the culture report and disc diffusion susceptibility testing results available. The timing of changing antibiotics when adjusting antibiotics on the day of bacteremia is presented as "within 1 day".

ASD: atrial septal defect. AML: acute myeloid leukemia, ALL: acute lymphoid leukemia. CAD: coronary artery disease. CHF: congestive heart failure. CVA: cerebral vascular accident. DM: Diabetes Mellitus. PS: pulmonary stenosis. TOF: tetralogy of Fallot. UTI: urinary tract infection.

Table 2. In vitro susceptibilities of 24 *E. coli* isolates determined by the agar dilution method.

Antibiotics	Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Percentages of susceptible strains(%)
ATM	0.06 - 64	4	32	79.2
ATM+CLA	≤0.03 - 16	0.25	8	-
CAZ	0.5- ≥256	4	64	62.5
CAZ+CLA	≤0.03 - 16	0.25	8	-
CTX	0.125 – 16	2	16	87.5
CTX+CLA	≤0.03 - 16	≤0.03	4	-
FEP	≤0.03 - 32	0.25	8	100
FEP+CLA	≤0.03 – 0.12	≤0.03	0.12	-
AMP	≥256	≥256	≥256	0
AMX+CLA	1- 32	16	32	33.3
CFZ	8 - ≥256	64	≥256	8.3
cefixime	0.5 - >32	32	>32	4.2
cefpodoxime	<0.5 - >32	32	>32	4.2
FOX	1 - >32	16	>32	45.8
cefpirome	0.06-8	0.12	8	
GM	0.06 - ≥256	4	≥256	54.2
TC	1 - >32	>32	>32	12.5
CMZ	0.25 - 64	4	32	75
AMK	0.25 - ≥256	1	128	91.7
MPM	≤0.03	≤0.03	≤0.03	100
IPM	≤0.03 - 0.5	0.12	0.12	100
moxifloxacin	≤0.03 - 64	0.06	64	70.8
CIP	≤0.03 - 128	≤0.03	64	66.7
PIP +TZB	0.5/4 - 16/4	4/4	16/4	100

Footnote: ATM: aztreonam, CAZ: ceftazidime, CTX: cefotaxime, FEP: Cefepime, AMP: ampicillin, AMX: amoxicillin, CFZ: cefazolin, CMZ: cefmetazole, AMK: amikacin, IPM: imipenem, CIP: ciprofloxacin, PIP: piperacillin. CLA: clavulanic acid with fixed concentration 4 μg/ml when tested with ceftazidime, cefotaxime, aztreonam and cefepime and with ratio 2:1 when tested with amoxicillin; TZB: tazobactam with fixed concentration 4 μg/ml.

Table 3. Molecular characterizations of transconjugcants

Transconjugcant	TEM	SHV	CTX	pI	ESBL Etest
Ec1	-	SHV-5	-	8.2	+
Ec8	TEM-1	SHV-5	-	5.4, 8.2	+
Ec9	TEM-1	-	CTX-M-3	5.4, 8.4	-
Ec10	TEM-1	-	CTX-M-3	5.4, 8.4	+
Ec11	-	-	CTX-M-3	8.4	-
Ec13	TEM-1	SHV-5	-	5.4, 8.2	+
Ec15	TEM-1	-	CTX-M-3	5.4, 8.4	-
Ec18	TEM1	-	CTX-M-3	5.4, 8.2, 8.7	-
Ec21	TEM-28	-		6.1	+
Ec23	TEM-1	SHV-5		5.4, 8.2	+
Ec25	TEM1	-	CTX-M-3	5.4, 8.4	-
Ec28	TEM-1	SHV-5		5.4, 8.2	+
Ec32	TEM-1	SHV-5		5.4, 8.2	+
Ec34	TEM-1	SHV-5		5.4, 8.2	+
Ec40	TEM-1	SHV-5		5.4, 8.2	+

Table 4: The susceptibility patterns to β-lactams and combinations of β-lactam/β-lactam inhibitors of isolates harboring different β-lactamases.

Isolate No.	ESBL Etest	β-lactamase	pI point	MIC Range								
				AMX/ CLA	FOX	CMZ	CAZ	CAZ+ CLA	CAZ/C AZ+CL A	CTX	CTX+ CLA	CTX/C TX+CL A
7	+	TEM-1, SHV-5	5.4, 8.2	4/2 – 8/4	4-16	0.5-1	4->128	<0.03-0.12	256->4096	0.12-8	<0.03-0.06	2->256
1	+	SHV-5	8.2	1/0.5	1	0.25	2	<0.03	>64	1	<0.03	>64
1	+	TEM28	6.1	16/8	32	8	32	0.25	128	1	0.25	4
10	-	TEM1, CTX-M3	5.4, 8.4	16/8-32/16[b]	2-64[d]	1 - 32	0.5-16	0.06-16	1-8	1-16	0.06-16	1-128
2	-	CTX-M3	8.4	16/8, 32/16	8, 32	1, 32	1, 32	0.12, 2	8, 16	4, 16	0.06, 4	1, 256
1	-	AmpC[a]	>9	32/16	16	16	16	8	2	2	0.25	8
1	-	AmpC[a], TEM-1	5.4, >9	32/16	32	2	2	0.12	16	2	0.06	32
1	-	TEM-1, unidentified	5.4, 8.0, 8.7	32/16	32	16	4	0.5	8	4	4	1

a. AmpC indicates proposed AmpC hyperproducer b. Only 2 isolates whose MICs of amoxicillin/clavulanate were 16/8. c. The ratio of MIC of Aztreonam/ aztreonam+clavulanate was 1 in only one isolate, the ratios of the others were 128 - >2048. d. Except one isolate's MIC of cefoxitin was 2 and two isolates's MICs were 8, all the other 7 isolates's MICs of cefoxitin were 32 or more than 32 that indicates resistance to cefoxitin.

Footnote: AMX: amoxicillin, FOX: cefoxitin, CMZ: cefmetazole, CAZ: ceftazidime, CTX: cefotaxime, ATM: aztreonam, FEP: cefepime, CPR: cefpirome. CLA: clavulanic acid with fixed concentration 4 μg/ml when tested with ceftazidime, cefotaxime, and aztreonam and with ratio 2:1 when tested with amoxicillin.

Legends of Figures.

Figure 1. The number and size of plamids form the oxyiminocephalosporin-resistant *E. coli* isolates. *Eco*RI + *Hin*dII digested λ DNA ladder.

Figure 2. *Pst*I digestion profiles of plasmids from transconjugants of *E. coli*. Lane M: *Eco*RI + *Hin*dII digested λ DNA ladder.