行政院國家科學委員會專題研究計畫成果報告

計畫名稱：台灣地區帶有 Extended-spectrum Beta-lactamase (ESBL)之 E. coli 菌血症菌株的研究分析

(中、英文) Analysis of Bacteremic E. coli Carrying Extended-spectrum Beta-lactamase in Taiwan

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Part One:

Bacteremia Due to Extended Spectrum Beta-lactamase Producing *Escherichia coli* and *Klebsiella pneumoniae* in a Pediatric Oncology Ward：Clinical Features and Identification of Different Plasmids Carrying both SHV-5 and TEM-1 Genes

Abstract

Thirteen patients who had 16 episodes of bacteremia were observed between 1993 and 1997 in a pediatric oncology ward with a high background isolation rate of cefotaxime or aztreonam resistant Gram negative bacteria. Four blood isolates were *Escherichia coli,* and 12 were *Klebsiella pneumoniae* harboring extended spectrum β-lactamases (ESBL). All episodes of bacteremia were nosocomial and occurred in neutropenic patients except one, and all patients were treated with piperacillin or ceftazidime with amikacin and cefazolin prior to onset of bacteremia. Nine of 13 patients were receiving extended spectrum β-lactam treatment when ESBL producer bacteremias occurred. Molecular studies revealed that four *K. pneumoniae* SHV-2 producing isolates from 1994 were of the same clone. Other ESBL producers, including six carrying both TEM-1 and SHV-5, five carrying SHV-5 and one carrying SHV-2 alone, were unrelated. In conclusion, SHV-5 was present in 11 of the 16 isolates, and co-existed with TEM-1 in 6 isolates. Acquisition of resistant genes probably occurred under antibiotic selection pressure. This study highlights the importance of routinely checking for and detecting ESBL producers. Effective therapy for ESBL producers should be considered early for children with malignancies and neutropenia who are septic despite extended-spectrum β-lactam containing regimen in a clinical setting of increased incidence of ESBL producing bacteria.

Introduction

Neutropenic patients are at high risk for various infectious diseases even if cultures are not positive. Extended-spectrum β-lactam monotherapy or in combination with an aminoglycoside is generally accepted as the empirical regimen for febrile neutropenia following chemotherapy for malignancy (6, 7, 17, 27, 40). However, the choice of empirical antimicrobial therapy should be evaluated periodically to prevent treatment failure due to antimicrobial resistance. The combination therapy of anti-pseudomonal β-lactam antibiotic such as piperacillin or ceftazidime with an aminoglycoside (amikacin usually) had been extensively used to treat patients with malignancies and infectious diseases in the pediatric oncology ward at NTUH in the recent ten years. Such combination therapy was particularly used in febrile neutropenic patients. However, recent reports of treatment failure due to extended-spectrum β-lactamase (ESBL)-producing bacteria were of great concern to clinical practice (3, 21). High incidences of infections caused by ESBL-producing bacteria in the intensive care units were reported in different areas (14, 25). The concern of such resistant bacterial infection had extended to the nursing homes (43), geriatric (8) and pediatic (10, 12, 13) populations, transplant recipients (12) and oncology patients (16). Those infected were often fragile and intolerable to infectious diseases. Failure to identify ESBL producers by routine susceptibility testing leading to inappropriate antimicrobial treatment may result in increased mortality.

Gram-negative aerobic bacteria accounted for the majority of cases of nosocomial infection in the National Taiwan University Hospital (NTUH) (5). The frequency of extended spectrum β-lactam resistant *Klebsiella pneumoniae* at NTUH

increased from 3.4% in 1993 to 10.3% in 1997 according to the disk diffusion method (18). Increasing prevalence of extended spectrum β-lactam resistant *Escherichia coli* from 2.5% in 1993 to 6.7% in 1997 was also observed (24). Compared with the average data from the hospital-wide surveillance, the frequencies of extended spectrum β-lactam resistant *K. pneumoniae* and *E. coli* isolates in a pediatric ward were found three to five times higher (see below). In the current study, a retrospective survey with detailed molecular analysis was conducted to investigate the clinical significance of ESBL-producing *K. pneumoniae* and *E. coli* bacteremia in children with malignancy.

Materials and Methods

Patients and bacterial strains

We searched the computerized database at the National Taiwan University Hospital, a 1800-bed acute care medical center, for the *E. coli* and *K. pneumoniae* blood isolates resistant or intermediately susceptible to aztreonam or the third generation cephalosporins in a pediatric oncology ward between 1993 and 1997. The pediatric oncology ward has 35 beds and most patients were admitted for evaluation and management of malignant diseases. The medical records of patients harboring the studied microorganisms were reviewed. Neutropenia was defined as a polymorphonuclear cell count of $\leq$ 500 /mm^3. Bacterial strains were stored at -70°C before retrieval for testing.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by both the agar dilution and disk diffusion tests according to the National Committee for Clinical Laboratory Standards (NCCLS) (30, 31). For susceptibility testing by the agar dilution method, the following antimicrobial agents were obtained as standard reference powders of known potency for laboratory use: amoxicillin, ampicillin and cephalothin (Sigma Chemical Co., St. Louis, Mo.); clavulanic acid (SmithKline Beecham, Brockhans Park, Surry, UK); piperacillin and tazobactam (Lederle Laboratories, Pearl River, N.Y.); cefmetazole (Sankyo Co., Hiratsuka, Japan); imipenem (Merck, Sharp & Dohme, West Point, Pa.); cefotaxime, gentamicin and cefpirome (Hoechst Marion Roussel, Frankfurt, Germany); ceftazidime (Glaxo Group Research Limited, Greenford, UK); cefepime, amikacin and aztreonam (Bristol-Myers-Squibb Laboratories, Princeton, NY, USA); meropenem (Sumitomo Pharmaceuticals Co., Osaka, Japan); ciprofloxacin (Bayer Co., Leverkusen, Germany). All drugs were incorporated into Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) in serial two-fold concentrations from 0.03 to 128 μg/ml. Two control strains, *E. coli* ATCC 35218 and ATCC 25922, were included in each test run. Inoculated plates were incubated in ambient air at 35°C for 16 to 18 h. The MIC of each antimicrobial agent was defined as the lowest concentration that inhibited visible growth of the organism.

Screening tests for ESBLs

Double-disk synergy test and Etest for ESBLs were used as screening tests to detect ESBLs producing strains. In the double-disk synergy test, the following antimicrobial disks were placed on Mueller-Hinton agar (BBL Microbiology Systems)

adjacent to an amoxicillin/clavulanic acid disc (20 μg of amoxicillin plus 10 μg of clavulanate): cefotaxime (30 μg), ceftazidime (30 μg), aztreonam (30 μg), and cefepime (30 μg). All disks were purchased from Becton Dickinson Microbiology System (Sparks, Md.). The procedures and interpretation of the double-disk synergy test were as previously described (19).

The Etest ESBL screen (PDM Epsilometer; AB Biodisk, Solna, Sweden) based on the recognition of a reduction of ceftazidime MIC in the presence of clavulanic acid was performed according to the manufacturer's instructions.

Genomic fingerprinting by pulsed field gel electrophoresis (PFGE)

Total DNA was prepared and PFGE was performed as described previously (1, 41). The restriction enzyme *XbaI* (New England Biolabs, Beverly, Mass.) was used at the manufacturer's suggested temperature. Restriction fragments were separated by PFGE in 1% agarose gel (Bio-Rad, Hercules, Calif.) 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 apparatus (Bio-Rad Laboratories, Richmond, Calif.). The initial pulse time of 1 s was increased linearly to 35 s in 20 h at 200 V at 4°C. Gels were then stained with ethidium bromide and photographed under ultraviolet light.

Band patterns were visually compared and classified as indistinguishable (clonal), closely related (clonal variants, three or less band differences), possibly related (four to six band differences), and unrelated according to previously described criteria (42).

Plasmid isolation and resistance transferal

Plasmid profile analysis was performed using the alkaline extraction method (20). Resistance transferal was carried out by conjugation. A rifampicin-resistant strain of *E. coli* (JP-995) was used as the recipient. Recipients and donors were separately inoculated into brain heart infusion broth (Oxoid, Basingstoke, Hampshire, England) and incubated at 37°C for four hours. They were then mixed at a volume ratio of 1:1 for overnight incubation at 37°C. A 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).

Isoelectric focusing

Procedure of isoelectric focusing was generally according to the previously described (28). Bacteria were harvested from 20-h brain heart infusion broth culture by centrifugation and the pellet was resuspended in 1 ml of phosphate buffer (0.05 M, pH 7). Enzymes were released by two cycles of freezing (-70^{0}C) and thawing (room temperature), and sonication for 5 min in a sonicator in ice cold water. Isoelectric focusing was performed in ampholine gel (pH 3.0 to 10.0, Pharmacia, Uppsala, Sweden). 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.

Restriction enzyme digested profile of plasmids

Plasmid DNA from the transconjugant was prepared as described previously (1). Restriction enzyme analysis of the plasmids from the transconjugants was performed according to manufacturer's instructions. The restriction enzymes *Pvu*II and *Pst*I (Gibco BRL, NY, USA) were used. *Hin*dIII + *Eco*RI digested λ – phage DNA were used as molecular weight markers.

PCR amplification for bla_{TEM} and bla_{SHV} and direct DNA sequencing

Oligonucleotide primers (Gibco BRL) used for PCR assay were as follows: 5'-ATAAAATTCTTGAAGACGAAA (primer A), 5'-GACAGTTACCAATGCTTAATCA (primer B), 5'-GGGTAATTCTTATTTGTCGC (primer C) and 5'-TTAGCGTTGCCAGTGCTC (primer D). Primers A and B were known to be specific for bla_{TEM} (26). Primers C and D were known to be specific for bla_{SHV} (37). Reactions were performed in a DNA thermal cycler (Bio-Rad) 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 of each deoxynucleoside triphosphate, and 2 μM each oligonucleotide primer. Thirty-five cycles were performed for each reaction, with the following temperature profiles: 94°C, 1 min; 58°C, 1 min; and 72°C, 1 min.

For direct DNA sequencing, PCR products were purified with microspin S-300 HR PCR purification columns (Pharmacia). Sequencing reactions were performed with corresponding primers specific for the bla_{TEM} and bla_{SHV} gene (26, 37) according to the method of Sanger et al (38). An automated sequencer (377, ABI Prism, Perkin-Elmer, Connecticut, USA) was used.

Results

Bacterial strains and clinical features

Forty-nine (56.3%) of 87 isolates of *K. pneumoniae* and 21 (18.6%) of 113 *E. coli* isolates obtained from all unselected clinical specimens from the pediatric oncology ward submitted to the clinical microbiology laboratory between 1993 and 1997 were resistant or intermediately susceptible to aztreonam or the third generation cephalosporins by the disk diffusion method. Of the blood isolates, 46.2% (12/26) of *K. pneumoniae* and 23.1% (6/26) of *E. coli* were not susceptible to the extended spectrum β-lactams. Of the 18 resistant blood isolates recovered from storage, one *E. coli* isolate was missing and another *E. coli* isolate was excluded because its MIC against aztreonam and the third generation cephalosporins was in the susceptible range (≤ 2) when tested by the agar dilution method. Twelve clinical isolates of *K. pneumoniae* (kp1 - 12) and four isolates of *E. coli* (ec13 - 16) were included for this study. Two isolates of *E. coli* (ec13 and ec14) were obtained from the same patient on different occasions (2 days apart). Similarly, 2 pairs of *K. pneumoniae* isolates (kp1 and kp2, kp10 and kp11) were from 2 individual patients and were recovered 2 and 4 days apart respectively. A total of three episodes of *E. coli* blood stream infection and 10 episodes of *K. pneumoniae* blood stream infection in the pediatric ward from 1993 to 1997 were reviewed. Clinical data on 13 patients are summarized in Table 1. All patients had malignancies and received chemotherapy before their bacteremia occurred. Only one (patient 6) was not neutropenic when the multiply resistant strains were isolated. All these infection episodes were nosocomial. The infection focus

could not be determined in most cases except for two episodes of catheter-related infection and two episodes of urinary tract infection (patients 1,3, 4 and 12).

Patients had previously received piperacillin and/or ceftazidime with amikacin and/or cefazolin. Nine of the 10 patients were receiving an extended spectrum β-lactam when they developed ESBL-producing *Enterobacteriaceae* bacteremia. Thereafter, nine patients received imipenem containing regimens. Out of these nine, four patients recovered, three died of causes not attributable to ESBL producer infection and two died of mixed bacteremia or *K. pneumoniae* infection within one day of blood culturing. The other four patients (out of a total of 13) were treated with an extended spectrum β-lactam containing regimen, only one of whom died of ESBL producing *K. pneumoniae* infection. One patient survived and one died of fungemia and one died of *Stenotrophomonas maltophilia* infection.

Three patients (patients 1, 3, and 4) who did nbt die of ESBL producing *K. pneumoniae* bacteremia and recieved extended spectrum β-lactams had identified sources of infection, i.e., urinary tract infection or catheter-related infection. Their infected catheters were removed and their antimicrobial regimens contained effective antibiotics. In the case of two patients (patients 1 and 4) with catheter-related ESBL producing *K. pneumoniae* infection, cefmetazole (MIC = 1 μg/ml for kp1) or amikacin (MIC = 0.5 μg/ml for kp5) were used. For patient 3, who had a urinary tract infection and bacteremia due to ESBL producing *K. pneumoniae*, the isolate was susceptible to amikacin (MIC = 16 μg/ml) and he was successfully treated with amikacin with cefazolin and ceftazidime.

Three patients (patients 1, 9, and 11) had subsequent bacteremia 2 to 4 days after a prior bacteremic episode. When the second episode of bacteremia occurred, one patient (patient 1) was not receiving effective therapy for ESBL producer and two patients (patients 9 and 11) had just begun to receive imipenem within 1 day.

Plasmid profile and transferal of resistant determinants

Comparison of plasmids isolated from clinical strains revealed that each contained one or two plasmids with molecular weights >90 kb. The gene encoding the ESBL could be transferred with either ceftazidime or aztreonam selection in most donors except for four *K. pneumoniae* isolates (kp1-4). Only one plasmid was transferred for each strain and the resistant gene in each transconjugant was found to be located in a plasmid of > 90kb (data not shown).

PFGE analysis of donors and plasmid profile of transconjugants

Two *E. coli* isolates (ec13, ec14) from the same patient had exactly the same total DNA profile with *Xba*I digestion in PFGE (lanes 13 and 14, Fig. 1A). Among the three *E. coli* bacteremia patients, three different DNA profiles were identified in PFGE (Lanes 15, 13 and 14, and 16, Fig. 1A). Regarding the *K. pneumoniae* isolates, 4 isolates (kp1, 2, 3, 4) had indistinguishable PFGE patterns. The other 8 *K. pneumoniae* isolates (Lanes 5 - 12), including the kp10 and kp11 isolates from the same patient, had unrelated PFGE patterns (Fig. 1B). Restriction enzyme digestion of plasmids showed that all the 4 *E. coli* (Fig. 2A) and 8 *K. pneumoniae* (Fig. 2B)

transconjugants (kp5-12) had different *Pst*I or *Puv*II-digested profiles, indicating that their plasmids were all different.

Antimicrobial susceptibility of clinical isolates and transconjugants

Results of the in vitro antimicrobial susceptibility tests are shown in Table 2. All the 4 *E. coli* and 12 *K. pneumoniae* isolates were resistant to ampicillin and cefazolin and susceptible to cefepime, cefmetazole, imipenem, meropenem and ciprofloxacin. The percentages of isolates susceptible to amoxicillin/clavulanic acid and piperacillin/tazobactam were 66.6% and 93.8%, respectively. Not all the clinical isolates had MIC values against extended spectrum β-lactams above the resistance breakpoint of NCCLS criteria (31). A total of 20%, 26.6%, 46.6% and 100% clinical isolates were susceptible to aztreonam, ceftazidime, cefotaxime and cefepime, respectively. However, when clavulanic acid at a fixed concentration of 4 μg/ml was combined with ceftazidime, cefotaxime, aztreonam or cefepime individually for susceptibility testing, the MIC values decreased by more than 16 times in all isolates and all became susceptible. If the susceptibility criteria of cefepime were applied to cefpirome, only one of the 16 isolates would be resistant to cefpirome. The MICs of the 12 transconjugants to various antimicrobial agents is similar to the clinical isolates from which they were derived.

All 16 isolates from 13 patients yielded positive results with the double disk synergy test and the Etest ESBL screening test.

Isoelectric focusing

Isoelectric focusing of sonic extracts of the strains followed by nitrocefin screening revealed two major bands, pI 5.4 and pI 8.2, in both the clinical isolates and transconjugants of two *K. pneumoniae* isolates (kp9, kp12) and all four *E. coli* isolates. Five isolates (kp1-4, 10) had only a band of pI 7.6 and five isolates (kp5-8, 11) had a band of pI 8.2 (Table 3).

PCR amplification and sequencing of PCR products for bla_{TEM} and bla_{SHV}

All four *E. coli* transconjugants were positive for both bla_{TEM} and bla_{SHV} amplification. The entire bla_{TEM} sequence including the promoter region from the four *E. coli* isolates was found to be identical to the bla_{TEM-1} encoding *Tn*2 (11). Comparison of sequences at the bla_{SHV} region of the four *E. coli* strains to the published SHV-1 gene sequence (29) revealed three nucleotide substitutions causing two amino acid changes (238, GlyGGC→SerAGC; 240, GluGAG→LysAAG), with the remaining one being silent (268, ThrACG→ThrACC). The amino acid sequences for the bla_{SHV} sequence in all four *E. coli* isolates were found to be identical to the published SHV-5 sequence (2). Two isolates of *K. pneumoniae* (kp9, kp12) also harbored both TEM-1 and SHV-5 genes as these *E. coli* isolates. The other 10 *K. pneumoniae* isolates had only one SHV type gene (See Table3). Five isolates had the SHV-5 gene. Five isolates had an SHV-2 gene with the characteristic of one nucleotide substitution causing one amino acid changes (238, GlyGGC→SerAGC). Four SHV-2 producing *K. pneumoniae* isolates (Kp1 – 4) did not have silent mutation (268, ThrACG→ThrACC) whereas another SHV-2 producer (kp10) and all the SHV-5 containing isolates had the same silent mutation as the *E. coli* isolates had.

Discussion

In the hospital studied, routine susceptibility testing was performed by the Kirby-Bauer disk diffusion test and cefotaxime was the representative antibiotic for third generation cephalosporins as recommended by the NCCLS (30) unless a special request was made to test for another antimicrobial or the isolate was resistant to cefotaxime. The double disk synergy test was not routinely used in our clinical microbiology laboratory. Since some ESBL producers may not be detectable by the routine use of only one extended spectrum β-lactam, the prevalence of ESBL-producing *Enterobacteriaceae* might have been even higher than what was reported.

In this study, clonal spread was detected by PFGE in 4 isolates of *K. pneumoniae* of 3 patients (patients1 - 3) that produced SHV-2 in 1994. The four isolates also shared the characteristic of not transferring their resistance by conjugation and lack of silent mutation (268, ThrACG→ThrACC). The other *K. pneumoniae* isolates from different patients proved to be unrelated by PFGE. The difference of their plasmid digestion profiles also ruled out the possibility of plasmid dissemination in the studied ward.

Two plasmid-mediated β-lactamases, TEM-1 and SHV-5, were simultaneously found in two *K. pneumoniae* (kp9, kp12) and in all the four *E. coli* isolates. These resistant determinants were conjugatively transferable and a plasmid of size >90 kb was identified in all six transconjugants. However, plasmid digestion profiles of these isolates revealed six different patterns, even for the two isolates from the same patient (ec13, ec14). Circulation of one resistant plasmid harboring two β-lactamase genes was thus excluded.

It is estimated that worldwide about 50% of clinical *E. coli* isolates produce TEM-1 β-lactamase (23, 36) and TEM-1 accounts for about 80% of all plasmid-encoded β-lactamases in clinical *Enterobacteriaceae* (9). Although the prevalence of TEM-1 in Taiwan was not studied, considering the worldwide distribution of TEM-1 and the finding of 82% *E. coli* isolates in 1998 in our hospital being ampicillin-resistant as determined by the routine disk diffusion method, the existence of TEM-1 in our *E. coli* isolates seems reasonable. SHV-5 has been reported as the most common ESBL gene in *K. pneumoniae* isolates in another Taiwan hospital (22) and was the predominant ESBL gene in our *K. pneumoniae* isolates as well. That the spread of ESBL was mainly by patient-to-patient transfer rather than by direct selection of point mutation derivatives has been postulated based on clinical interventional study (34). The coexistence of TEM-1 and SHV-5 instead of the occurrence of TEM-1 mutant ESBLs in our *E. coli* isolates suggests a greater ease of acquisition of another ESBL gene over the development of a TEM-1 mutant ESBL gene.

There were three pairs of sequential isolates from three patients (Table 3). Two *E. coli* isolates from patient 11 recovered 2 days apart were of the same clone but had different plasmids. Although the existence of completely different plasmids in the two isolates was not impossible, this finding suggests the possible existence of a unit smaller than a plasmid, such as integrons or transposons (15) or the existence of a unstable plasmid that may change easily. The two *K. pneumoniae* isolates (kp1, kp2) from patient 1 and another two strains (kp3, kp4) had the same macrorestriction pattern by PFGE, suggesting a clonal origin. As for kp10 and kp11 isolated 4 days

apart from patient 9, the totally different macrorestriction patterns, ESBL genes, and plasmid digestion patterns suggested that these two strains were acquired independently of each other. There were thus three different bacteriologic patterns in patients having bacteremia with consecutive ESBL producers.

In the current study, most of the ESBL-producers had initially been reported as intermediately susceptible or resistant to cefotaxime. With even further determination of MIC values by the agar dilution method, some isolates were susceptible to cefotaxime, ceftazidime or aztreonam. According to the new recommendations by the NCCLS in 1999 (32) on susceptibility testing for *E. coli* and *K. pneumoniae*, strains that are inhibited to a lesser degree than the normal susceptible population, even though the MIC is lower than the standard resistance breakpoint, should be screened for potential ESBL. Resistance should be reported if the strain showed a positive inhibition test (32). Since the SHV-5 β-lactamase is generally classified as a ceftazidimase and is less efficient in hydrolyzing cefotaxime (4), if organisms harboring this enzyme are not tested according to the above guidelines but with a cefotaxime disk only, inability to detect resistance and treatment failure might ensue (21).

Schiappa et al (39) found that patients with ESBL-producing *E. coli* bacteremia were more likely to survive if they received appropriate treatment within 3 days of onset of the infection. A pediatric patient with leukemia who developed bacteremia with a cefotaxime-susceptible but ceftazidime-resistant *E. coli* had been documented to die after receiving cefotaxime for less than one day (39). In addition, another study suggested that mortality was significantly lower when a carbapenem

was used in the first 5 days of bacteremia compared to a non-carbapenem regimen (35). Delay in using antibiotics effective against ESBL producers may result in delayed clearance of bacteremia was demonstrated by the three patients (patients 1, 9, and 11) with two episodes of bacteremia. Patient 9 even had a fatal outcome in the second bacteremia episode due to mixed ESBL producing *K. pneumoniae* and *Citrobacter freundii* infection. Thus, timely identification of ESBL producer is important and policies for laboratory performance and antimicrobial therapy may need to be re-evaluated to take this into account, especially when the prevalence of ESBL producer is increasing in a specific setting.

Two of nine patients receiving imipenem containing regimens died within 1 day of septicemia and three of four patients having non-carbapenem therapy did not die of ESBL producer bacteremia. No increased mortality in patients treated with ESBL susceptible antibiotics was found. However, we could not give any definite suggestion about the treatment regimen for ESBL producer bacteremia from this study due to small number of cases and lack of case control design. Among the four patients receiving ESBL susceptible antibiotics, two had catheter-related infection and one had urinary tract infection. For the patients with catheter-related ESBL producer bacteremia, immediate removal of the infected catheter and use of an active antimicrobial agent other than imipenem may still produce a good outcome. Naumovski et al. reported that seven children with non-bacteremic urinary tract infections were successfully treated with ceftazidime therapy and they proposed that high urine level of ceftazidime was the reason for successful therapy (33).

Ceftazidime and amikacin therapy for bacteremic urinary tract infection was shown effective in one patient in the study.

Prior administration of any antibiotic and prior ceftazidime or aztreonam administration were reported to be risk factors for acquiring ESBL-producing organisms (39). Every patient included in current study had received the standardized regimen containing an anti-pseudomonal β-lactam, amikacin and cefazolin three weeks prior to bacteremia. However, the retrospective nature of this study could not prove definitely that prior antimicrobial administration was the risk factor. Nevertheless, the finding that 9 of 13 patients developed ESBL-producing bacteremia while under extended spectrum β-lactam therapy argues for the occurrence of selection pressure.

In conclusion, we report the clonal spread of ESBL-producing *K. pneumoniae* and the identification of *E. coli* and *K. pneumoniae* blood isolates with transferable resistant plasmids carrying both extended (SHV-5) and restricted spectrum β-lactamase (TEM-1) genes in a pediatric oncology ward. SHV-5 was the predominant ESBL detected in this study. The possibility that the ESBL gene may disseminate to other members of *Enterobacteriaceae* necessitates close monitoring of the ESBL producer to prevent such occurrence. In a ward with high percentages of ESBL producing *E. coli* and *K. pneumoniae* isolates, if symptomatic improvement is not seen during the empirical combination of an anti-pseudomonal β-lactam and an aminoglycoside, changing antibiotics to effectively treat for ESBL producers such as imipenem should be promptly considered. The new recommendation by NCCLS for screening for ESBL in *E. coli* and *K. pneumoniae* isolates should also be strictly

enforced to avoid false designation of susceptibility to third generation cephalosporins and translation to clinical failure.

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Table 1. Clinical features and review of antimicrobial agents use of patients with respect to detection of ESBL-producing bacteremia.

Patient	Isolate	Age	Sex	Infection focus	Malignancy	Antibiotics when bacteremia occurred	Antibiotics after detection (timing after bacteremia)[a]	Outcome
P1	kp1, kp2 (2 days after kp1 isolated)	14	F	Catheter related infection	Ewing's sarcoma	Cefazolin, Amikacin, Piperacillin, Amphotericin B,	No change in antibiotics Cefmetazole Aztreonam Amphotericin B (within 1 day)	Died of *C. tropicalis* fungemia on the 34th Day after *K. pneumoniae* (Kp2) bacteremia
P2	kp3	2	M	FUO	AML	Piperacillin, Cefazolin, amikacin	**Imipenem** Gentamicin Amphotericin B (within 1 day)	Recovered
P3	kp4	1	F	Urinary tract infection	Neuro-blastoma	Nil	Cefazolin Amikacin Ceftazidime (2 days)	Recovered
P4	kp5	9	M	Catheter related infection	ALL	Cefazolin, Amikacin, Piperacillin, Ceftazidime	Vancomycin Amikacin Ceftazidime Amphotericin B (within 1 day)	Died of *Stenotrophomonas maltophilia* infection on the 36th Day after *K. pneumoniae* (Kp5) bacteremia

P5	kp6	6	M	FUO	AML	Cefazolin, Amikacin, Ceftazidime, Amphotericin B Vancomycin	**Imipenem** Cefmetazole, Amphotericin B (1 days)	Died of *S. maltophilia* infection on the 74th Day after *K. pneumoniae* (Kp6) bacteremia
P6	kp7	13	M	FUO	Ewing's sarcoma	Amikin, Piperacillin, Amphotericin B Cefazolin	**Imipenem** Gentamicin (4 days)	Died of seizure and apnea on the 34th Day after *K. pneumoniae* (Kp7) bacteremia
P7	kp8	16	M	FUO	ALL	Cefazolin, ceftazidime, Amikacin	Vancomycin **Imipenem** Ciprofloxacin (within 1 day)	Died on the 2nd day after *K. pneumoniae* bacteremia
P8	kp9	13	M	FUO	ALL	Cefazolin, Netilmicin, Piperacillin, Amphotericin B	**Imipenem** Cefmetazole Amphotericin B (2 day)	Recovered
P9	kp10, kp11 (4 days after kp10 isolated)	2	F	FUO	AML	Cefazolin, Ceftazidime, Amikacin **Imipenem**	**Imipenem** (3 days) **Imipenem** Amphotericin B (within 1 day)	Died on the 2nd Day after *K. pneumoniae* (Kp11) and *Citrobacter freundii* bacteremia. (*K. pneumoniae* and *C. freundii* isolated on two separate days.)

P10	kp12	4	M	FUO	AML	Cefazolin, Amikacin, Ceftazidime, Amphotericin B	No change in antibiotics	Died on the day of *K. pneumoniae* bacteremia
P11	ec13	5	F	FUO	ALL	Ceftazidime	**Imipenem** (1 day)	Recovered
	ec14 (2 days after kp13 isolated)					**Imipenem**	**Imipenem,** Gentamicin (1 day)	
P12	ec15	3	M	Urinary tract infection	ALL	Nil	**Imipenem,** Amikacin (2 days)	Recovered
P13	ec16	2	M	FUO	AML	Nil	Cefazolin, Piperacillin, Tobramycin (within 1 day); then Imipenem, Vancomycin (3 days)	Died of sepsis of unidentified pathogen on the 116th Day after *E. coli* (Ec16) bacteremia

a. The antibiotics after bacteremia including empirical antibiotics use and adjusted antibiotics after the culture report available. The timing of changing antibiotics when adjusting antibiotics on the day of bacteremia is presented as "within 1 day".

FUO: fever of unknown origin, AML: acute myeloid leukemia, ALL: acute lymphoid leukemia.

Table 2. In vitro susceptibilities of the four *E. coli*, 12 *K. pneumoniae* isolates and their transconjgants determined by the agar dilution method.

Antibiotics	Range (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	Percentages of susceptible strains (%)
ATM	2 - 64	16	64	25
CAZ	4 - ≥256	128	≥256	25
CAZ+CLA	0.06 - 4	0.5	4	-
CTX	2 - 32	16	32	43.8
CTX+CLA	≤0.03 - 2	0.5	2	-
FEP	0.12 - 2	1	2	100
AMP	16 - ≥256	≥256	≥256	0
AMX+CLA	2 - 32	8	32	62.5
CFZ	16 - ≥256	64	128	0
CMZ	0.5 - 16	2	16	100
AMK	0.5 - 16	4	16	100
IPM	0.06 - 0.5	0.12	0.25	100
CIP	≤0.03 - 1	≤0.03	0.5	100
CPR	0.12 - 16	4	8	93.8
PIP +TZB	1 - 128	2	16	93.8

Footnote: ATM: aztreonam, CAZ: ceftazidime, CTX: cefotaxime, FEP: Cefepime, AMP: ampicillin, AMX: amoxicillin, CFZ: cefazolin, CMZ: cefmetazole, AMK: amikacin, IPM: imipenem, CIP: ciprofloxacin, CPR: cefpirone, 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 ESBL-producing *E.coli* and *K. pneumoniae* isolates

Patient	Isolate	SHV	TEM	Macrorestriction genotype[a]	Plasmid digestion profiles[b]	β-lactamase pI[c]
P1	kp1	SHV-2		B	ND	7.6
	kp2	SHV-2		B	ND	7.6
P2	kp3	SHV-2		B	ND	7.6
P3	kp4	SHV-2		B	ND	7.6
P4	kp5	SHV-5		E	e	8.2
P5	kp6	SHV-5		F	f	8.2
P6	kp7	SHV-5		G	g	8.2
P7	kp8	SHV-5		H	h	8.2
P8	kp9	SHV-5	TEM-1	I	i	5.4, 8.2
P9	kp10	SHV-2		J	j	7.6
	kp11	SHV-5		K	k	8.2
P10	kp12	SHV-5	TEM-1	L	l	5.4, 8.2
P11	ec13	SHV-5	TEM-1	A	a	5.4, 8.2
	ec14	SHV-5	TEM-1	A	c	5.4, 8.2
P12	ec15	SHV-5	TEM-1	C	b	5.4, 8.2
P13	ec16	SHV-5	TEM-1	D	d	5.4, 8.2

a. Macrorestriction genotypes were determined by PFGE after digestion with *Xba*I.

b. Plasmid digestion profile for tranconjugcants with *Pvu*II and *Pst*I. ND: not determine.

c. Isoelectric point of β-lactamases determined by isoelectric focusing.

Legends of Figures

Fig.1: Profiles produced by PFGE of *Xba*I macrorestriction fragments of ESBL-producing *E. coli* (Fig. 1A) and *K. pneumoniae* (Fig. 1B) isolates. Lane M: *Sma*I digested *Staphylococcus aureus* NCTC 8325 DNA ladder served as molecular size marker. See Table 1 for origins of the isolates. Lane 15 refers to patient 12, lanes 13 and 14 refer to patient 11 and lane 16 refers to patient 13.

Fig. 2: *Pst*I and *Pvu*II digestion profiles of plasmids from transconjugants of *E. coli* (Fig. 2A) and *K. pneumoniae* (Fig. 2B). Lane M: *Eco*RI + *Hin*dII digested λ DNA ladder. Lanes 5 – 12 and 13 - 16 refer to the transconjugants of clinical isolates kp5 - 12 and ec13 – 16. See Table 1 for origins of the clinical isolates.

Part Two:

Cefotaxime-Resistant *Escherichia coli* Bacteremia：Correlation of High Level Expression AmpC Beta-lactamase, SHV- and TEM-Types Extended Spectrum Beta-lactamase and Their Clinical Features

Introduction

With the increasing usage of the oxyimino-cephalosporins (extended spectrum cephalosporins), more and more reports concerned about the emergence of resistance to oxyimino-cephalosporins among *Klebsiella pneumoniae* and *Escherichia coli* clinical isolates world widely (Medeiros AA 1997 CID 24 Suppl1,S19-45). Plasmid-mediated extended spectrum beta-lactamase (ESBL) was the most frequently reported mechanism for the emergence of resistance to oxyimino-cephalosporins for *K. pneumoniae* and *E. coli*, two common community- and hospital-acquired pathogens, in many areas (Jacoby JA 1997 Infect Dis Clin N Am 11:875-887, Livermore DM, 1996 Antibiotics in Lab Medicine 4th ed. Jarlier V 1988 Rev. Infect Dis. P867-878).

The prevalence of ESBL producing *K. pneumoniae* in Taiwan ranged from 8.5% to 30.0% in different hospitals from 1997 to 1999 [Jan IS, JFMA 1998, Liu PYF JCM 1998, Yan JJ, AAC 2000]. Besides the SHV-5 and SHV-12 ESBLs, a plamid-mediated AmpC enzyme (CMY-8) was identified as the mechanism of resistance for *K. pneumoniae* isolates in Taiwan (Yan JJ, AAC 2000). The prevalence of oxyimino-cephalosporin resistant *E. coli* strains and the proportion of ESBL producing strains to cause such resistance in Taiwan is rarely reported. As *E. coli* contains chromocomal AmpC which is often constitutive and not inducible (Coudron PE, JCM 2000, 38:p1791-6), AmpC hyperproducer strain caused by strong AmpC promoter is noted to be a possible mechanism to cause resistance to the oxyimimo-cephalosporins (Nelson EC 1999 AAC 43:957-9). The recent increasing documentation of plasmid-mediated AmpC enzymes conferring resistance to the extended spectrum beta-lactam in many countries [Coudron PE, JCM 2000, 38:p1791-6] warned that ESBLs producing Enterobacteriacea was not

the only problem we will face. It is of interest to understand the role of other resistance mechanisms such as AmpC hyperproducing and changes of outer membrance protein in the development of resistance to oxyimino-cephalosporins. We retrospectively study the prevalence of resistance to oxyimino-cephalosporins of *E. coli* at NTUH during 1993 and June 1998. The collected *E. coli* blood isolates were further analyzed for the mechanisms of resistance, dissemination with clonal or plasmid-mediated transmission, and the related patients' clinical characteristics.

Materials and Methods

Bacterial strains

We searched the computerized database at NTUH, which has 1800-bed capacity and is a primary and tertiary care medical center, for *E. coli* blood isolates resistant or intermediately resistant to aztreonam or the third generation cephalosporins determined by disc diffusion method (NCCLS) from Jan 1, 1993 to June 31, 1998. The medical records of patients having aztreonam or the third generation cephalosporins resistant *E. coli* bacteremia were reviewed.

Definitions

Nosocomial infection was defined according to the 1988 Centers for Disease Control (CDC) definition for nosocomial infection [Garner JS, Jarvis WR]. Appropriate antimicrobial therapy was defined as the patients receiving antimicrobial agents to which the isolates were susceptible *in vitro*. Treatment failure is defined when no clinical improvement after antimicrobial therapy for more than two days.

Antimicrobial susceptibility testing

Antimicrobial susceptibilities of these isolates and their transconjugated isolates were determined concomitantly by the disk diffusion and agar dilution methods described in the National Committee for Clinical Laboratory Standards (NCCLS) documents (NCCLS, NCCLS). For susceptibility testing by the agar dilution method, the following antimicrobial agents were obtained as standard reference powders of known potency for laboratory use: amoxicillin, ampicillin, cephalothin from Sigma Chemical Co. (St. Louis, MO, USA); clavulanic acid

from SmithKline Beecham (Brockhans Park, Surry, UK); piperacillin and tazobactam from Lederle Laboratories (Pearl River, NY, USA); cefmetazole from Upjohn Co. (Kalamazoo, MI, USA); imipenem from Merck Sharp & Dohme (West Point, PA, USA); cefotaxime, gentamicin and cefpirome from Hoechst Marion Roussel (Frankfurt, Germany); ceftazidime from Glaxo Group Research Limited (Greenford); cefepime, amikacin and aztreonam from Bristol-Muers-Squibb Laboratories (Princeton, NY, USA); meropenem from ICI pharmaceutical, Inc. (Cheshire, UK); ciprofloxacin from Bayer Co. (Leverkusen, Germany). All drugs were incorporated into Mueller-Hinton agar (Becton Diuckson Microbiology Systems, Sparks, MD, USA) in serial two-fold concentrations from 0.03 to 128 μg/ml, Two control strains, *Escherichia coli* ATCC 35218 and 25922, were included in each set of tests. The plates were incubated in ambient air at 35^0C for 16 to 18 h. Minimum inhibitory concentration (MIC) of each antimicrobial agent was defined as the lowest concentration which inhibited visible growth of the organism. A fine, barely visible haze or a single colony was disregarded.

Screen tests of ESBLs

For double-disk synergy test, Mueller-Hinton agar (BBL Microbiology Systems) and the following antimicrobial disks were included: cefotaxime (30μg), ceftazidime (30μg), aztreonam (30μg), cefepime (30μg), and amoxicillin/clavulanic acid (20μg of amoxicillin plus 10μg of clavulanate). All disks were purchased from Becton Dickson Microbiology System (Cockeysville, Md). The procedure and interpretation of the double-disk synergy test were

performed as previously described [Piddock LJ, Walters RN, 1997, JAC: 39 p177].

The Etest ESBLs screen (AB Biodisk, Solna, Sweden) based on the recognition of a reduction in the ceftazidime MIC in the presence of clavulanic acid was performed following the manufacture's instruction.

Isoelectric focusing

Cells were harvested from 20-h brain heart infusion broth cultures by centrifugation and the pellet was resuspended in 1ml 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, and sonication for 5 min in a sonicator in ice cold water. Isoelectric focusing was performed in ampholine gel (pH 3.0 to 10.0, Pharmacia). Preparations from standard strains known to harbor TEM-1, SHV-1 and SHV-5 were used as standard. After isoeclectric focusing, β-lactamases were detected by spreading nitrocefin (50μg/ml) on the gel surface (Matthew 1976, J Gen Microbiol).

PCR amplification for *bla*$_{TEM}$ and *bla*$_{SHV}$, PCR for the regulatory regions of ampC genes and direct DNA sequencing

Oligonucleotide primers used for PCR assay were as follows: 5'-ATAAAAT TCTTGAAGACGAAA(primer A), 5'-GACAGTTACCAATGCTTAATCA (primer B); 5'-GGTAATTCTTATTTGTCGC (primer C). Oligonucleotides were synthesized by GIBCO BRL, New York. Primer A and B were known to be specific for *bla*$_{TEM}$ (Mabilat, 1993). Primer C and D were known to be specific for *bla*$_{SHV}$ (Rasheed et 1997).

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 deoxynucleoside triphosphate, and 2 µM each oligonucleotide primer. Thirty-five cycles were performed for each reaction, with the following temperature profiles: 94°C, 1 min; 58°C, 1 min; and 72°C, 1 min.

For direct DNA sequencing, PCR products were purified with mircospin S-300 HR PCR purification columns (Pharmacia, Uppsala, Sweden). Sequencing reactions were performed with consecutive primers specific for the bla_{TEM} and bla_{SHV} gene (Mabilat et al; Rasheed et al) according to the method of Sanger et al ( ). An automatic sequencer (377, ABI Prism, PerkinElmer, Connecticut) was used.

Transferring resistance and restriction enzyme digested profile of plasmids

Resistance transferring was carried out by Conjugation. A rifampicin-resistant strain of *E. coli* (JP-995) was used as the recipient. Recipients and donors were separately inoculated into brain heart infusion broth (Oxoid) and incubated at 37°C for four hours. They were then mixed at a volume ratio of 1:1 for overnight incubation at 37°C. A 0.01 ml-volume of the overnight broth mixture was then spread on a MacConkey agar plate containing rifampicin (100µg/ml) and either Ceftazdime (1µg/ml) or Aztreonam (2µg/ml).

Restriction enzyme analysis of the plasmids from the transconjugants was performed according to manufacturer's instructions. The restriction enzymes *PstI* from Gilco were used. λ-*Hin*dIII + *Eco*RI cleavage products were used as

molecular weight marker. plasmid DNA from the transconjugant was prepared as described previously (Ausubel, 1995).

Genomic fingerprinting by Pulse field gel electropheresis (PFGE)

PFGE was performed as described previously [Ausubel 1995, Schoonmaker D, 1992, JCM]. Two restriction enzymes, XbaI and SfiI (Biolabs,), were used under the manufacture's suggested temperature. Restriction fragments were separated by PFGE in 1% agarose gel (Bio-Rad, Mercules, CA, USA) in 0.5X TBE buffer (45mM Tris, 45mM boric acid, 1.0 mM EDTA, pH 8.0) with use of the Bio-Rad CHEF-DRII (Bio-Rad, Mercules, CA, USA). The initial pulse time of 1 s was increased linearly to 35 s over 20 h at 200 V at 4^0C. Gels were then stained with ethidium bromide and photographed under ultraviolet light.

To reveal PFGE polymorphism, each sample was analyzed by the Molecular Analyst Fingerprinting, Fingerprinting Plus, and Fingerprinting DST Software (Bio-Rad Laboratories, Richmond, Calif., USA). The grouping method was done to deduce a dendrogram from the matrix via the Unweighted Pair Group Method using Arithmetic average (UPGMA) clustering technique after calculation of similarities using Person correlation coefficient between every pair of organisms.

RNA expression study

Total RNA was extracted according to Elisha et al. (?) and 15 μg was electrophoresed in 1.2% agarose-0.66 M formaldehyde with 40 mM morpholinepropanesulfonic acid-10mM sodium acetate-1mM EDTA (pH 7.7) and tansferred to Hybond N+ (Amersham International) in 20x SSC (Nelson). Primers 5'-TACTGGCGTGCTTGGTG-3' and 5'-GACTCTCGCTGGATTGG-3',

corresponding to nucleotides +314 to +330 and +1141 to +1157, respectively, were used to amplify and 884bp internal portion of *ampC* probe (Nelson). Similarly, an internal fragment of the 16S rRNA probe of E. coli was amplified by using the universal primers (Edwards). The prehybridization and hybridization procedures were recommended by the manufacturer (Amersham International).

Results

Bacterial strains

The percentage of *E. coli* isolates resistant to aztreonam or the third generation cephalosporins by disc diffusion method increased year by year, from 2.47% in 1993 to 6.67% in 1997. Forty-four *E. coli* blood isolates were found insensitive to cefotaxime or aztreonam by disc diffusion method [NCCLS disc method] from Jan. 1993 to June 1998. Among them, 38 isolates were recovered from stocks under -70^0C. Seven isolates were excluded for not having elevated MIC $\geq$2 μg/ml to aztreonam, cefotaxime and ceftazidime after further determination of MIC [NCCLS, agar dilution]. For the medical records of four infection episodes were inadequate to analyze or missing, a total of 27 bacteremic episodes due to ESBLs producing *E. coli* were reviewed.

Antimicrobial susceptibility of clinical isolates

Results of the in vitro antimicrobial susceptibility tests are shown in Table 1. All *E. coli* isolates were resistant to ampicillin and cefazolin except one which was susceptible. All isolates were susceptible to cefepime, and imipenem. The percentages of isolates susceptible to amoxicillin/clavulanic acid and

piperacillin/tazobactam were 33.3 % and 92.6% respectively. Not all the clinical isolates had MIC values against extended spectrum β-lactams above the resistance breakpoint of NCCLS criteria (31). A total of 74.1%, 25.9%, 81.3% and 100% clinical isolates were susceptible to aztreonam, ceftazidime, cefotaxime and cefepime, respectively. However, when clavulanic acid at a fixed concentration of 4 μg/ml was combined with ceftazidime, cefotaxime, aztreonam or cefepime individually for susceptibility testing, the MIC values decreased by more than 16 times in 14 isolates. The MIC values of the other isolates decreased by less than 4 times.

Molecular typing with PFGE method

Among 27 isolates, PFGE revealed that no identical pattern was observed. Except two isolates from the same patient 5 months apart had the same PFGE pattern, all the other isolates had more than three band difference and their similarity was less than 88% correlation (Figure 1).

Screen test for ESBL

Ten isolates and their related transconjuganrs yielded positive results with the Etest ESBL screening test (Table 3).

PCR amplification and sequencing of PCR products for *bla*$_{TEM}$ and *bla*$_{SHV}$

Among 27 isolates, 21 and 8 were positive to *bla*$_{TEM}$ and *bla*$_{SHV}$ PCR amplification respectively. The entire *bla*$_{TEM}$ amino sequence from all *E. coli* except one isolate was found to be identical to the *bla*$_{TEM-1}$ encoding *Tn*2 (11). Substitutions at amino acid 164 (~~Arg~~His) and 240 (~~Glu~~Lys) where have an

identical amino acid sequence to TEM-28 were found in one isolate. For 8 isolates positive to bla_{SHV} PCR amplification, all of them have an identical amino acid sequence to SHV-5 and all except one coexisted with TEM-1 β-lactamase. Transconjugants of isolates with TEM28 and SHV5 gene were also positive for these ESBL genes.

Transferring resistance and restriction enzyme digested profile of plasmids

Plasmids of TEM-28 and SHV-5 ESBL-gene carriers were found to be transferred with either ceftazidime or aztreonam selection. In addition, six isolates with transferable resistance were found not positive to bla_{TEM} and bla_{SHV} PCR amplification. Only one plasmid was transferred for each strain and the resistant gene in each transconjugant was found to be located in a plasmid of > 90kb (data not shown).

Restriction enzyme digestion of plasmids showed that all 15 transconjugants had different *PstI* digested profiles. The result obtained from digested plasmids indicating that 7 transconjugants which coexisted with TEM-1 and SHV-5 β-lactamase were also all different (data not shown).

Isoelectric focusing

Isoelectric focusing revealed that transconjugants possessing TEM-1 and SHV-5 β-lactamase have a pI point at 5.4 and 8.2, respectively. For the isolate with TEM-28, a pI at 6.1 was observed. Besides that nine isolates were TEM or SHV type ESBL producers, there were six transconjugants having plasmids each

carrying more than one beta-lactamases identified by having different pI points (table3).

RNA expression and DNA sequence analysis of the regulatory regions of *ampC* genes

Strains other than TEM- or SHV-ESBL producer were subsequently selected for *ampC* expression study. In order to compare transcription of the *ampC* genes, we performed Northern hybridizations of equal amounts of RNA extracted. Nine strains were confirmed to have a high expression of *ampC* RNA after the hybridization with 16sRNA probe and *ampC* probe.

Correlation of characteristics of susceptibility and the related resistance mechanism

With adding fixed concentration (4 μg/ml) of clavulanic acid, More than 4 fold dilution (including 4 folds) Decrease of MIC of cefotaxime, ceftazidime, aztreonam and cefepime were observed over 13, 10, 12, 16, respectively, of the 27 isolates. A total of 20 (74.1%) isolates had synergy effect (>= 4 fold decrease of MIC) when clavulanic acid were added with either one of the above four extended spectrum beta-lactams. Ceftazidime is the only one of the four extended spectrum beta-lactams to have clavulanic acid synergy effect among the 9 isolates producing SHV5 or TEM28.

Patient characteristics

The age distribution of the 27 patients was between 2 months old and 83 years old. 10 (37.0%) patients were younger than 5 years old and 8 (29.6%)

patients were older than 65 years old. The most common underlying disease of these patients was malignancy (19/27, 70.4%) and seven patients were under neutropenic state. Six patients were receiving corticosteroid treatment; three patients had DM and three patients had liver cirrhosis. Twenty-six infection episodes were regarded as nosocomial infection. About their infection foci, nine patients had bacteremia without identified infection source and seven patients had urinary tract infection. There were three pneumonia, three biliary tract infection, three intraabdominal infection and three soft tissue infection. These blood isolates were recovered on the 3rd to the 99th hospital day (mean, 35 days). Twenty-five (92.6%) patients received multiple antibiotics before *E. coli* bacteremia occurred.

The relationship of antibiotic usage and the resistance mechanism

When categorizing these patients to the TEM or SHV derived ESBLs group, AmpC hyperproducers group, and a group not related to the above two mechanisms, we found the prvious usage (2 months before bacteremia occurred) of aminoglycoside, ciprofloxacin, any beta-lactam, or specifically the use of extened spectrum beta-lactam, or any antibiotics were not significantly related to the mechanisms with Chi-Square test.

The relationship of outcome and the antibiotics those patients received

The used antibiotics includes those prescribed empirically when patients' bacteremic symptoms occurred and those adjusted according to the disc diffusion susceptibility testing results [NCCLS, disc, old]. Among the fourteen patients received imipenem containing regimens, thirteen were successfully treated except that one patient died of fungal infection. Only one patient was treated with

ciprofloxacin and that patient had a favorable outcome. Eight patients were ever treated with regimens containing amoxicillin/clavulanate or ampicillin/sulbactam and the prescription of amoxicillin/clavulanate, ampicillin/sulbactam for the aztreonam or cefotaxime resistant *E. coli* bacteremia was according to the susceptible results shown by disc susceptible testing. There was only one patient with soft tissue infection were successfully recovered form the bacteremic episode with Beta-lactam/inhibitor combination. Seven patients were treated with cefmetazole and three patients were successfully recovered from primary bacteremic episodes. Ten (37.0%) patients had fatal outcome and 18 patients survived. Seven (25.9%) patients' death was attributable to the resistant *E. coli* infection.

Discussion

In this study, we identified the resistance to the extended spectrum beta-lactams in these *E. coli* blood isolates were equally due to either ESBLs, AmpC hyperproducing, or mechanisms unrelated with these beta-lactams. Almost all of these patients received multiple antibiotic treatment course and no difference in the received antibiotics among the 3 group was found.

In 1997, 10.3% *K. pneumoniae* isolates were of ESBL phenotypes in National Taiwan University Hospital (NTUH)[Jan IS]. In this study, we report the epidemiology of ESBL producing E. coli in a nonepidemic hospital setting.

The study is limited by that only isolates from blood specimen were collected during 5 and half a year study period, therefore the isolates number is small for epidemiologic molecular typing and the significance of the patient characteristics were limited to blood stream infection due to ESBL producing *E. coli*. Besides ESBL producers often have low minimum inhibitory concentrations to beta-lactam antibiotics, they could appear sensitive on routine susceptibility testing by disc diffusion method [*J Infect 1998;36:255-258*]. The selection of isolates from the cefotaxime or aztreonam resistant strain determined by disc method might miss the ESBL producers which showed susceptible by the disc method.

TEM-28 was first reported in United States in 1996 (Bradford PA 1996 AAC) and later found in a United States hospital surveillance of ceftazidime-resistant strains in 1998 (Yang Y, 1998, AAC). The surveillance identified the first TEM-derived ESBL in Taiwan and it was a rare isolate containing TEM-28 outside United States. It was generally thought unlikely that two mutations appear with spontaneous mutations on a beta-lactamase gene [Maria-Cristina Negri AAC

2000]. The finding of only one TEM28 producing isolates may be due to the limited small collected isolate number from blood isolates and suggested there might be prevalence of other TEM1-derived ESBL genes with single mutation in this hospital with more extensive surveillance and investigation.

The identified SHV-5 and TEM28 were all classified as ceftazidimase []. The preference of ceftazidimase in this hospital may be related to the larger amount of clinical usage of ceftazidime than other third generation cephalosporins [data from ??], especially in oncology ward [LK Siu JCM 1999].

In agreement with the previous reports [Livermore DM JAC 1996 p409], the ceftazidime/ceftazidime+clavulante MIC ratio >= 16 may stratify the ESBL producers. However, the synergy effect of ceftazidime and clavulanate is more specific for the identified ESBLs than that of other oxyiminocephalosporin and clavulanate. It may be related to that isolates with two or three different beta-lactamses are more effective to hydrolyze cefotaxime and aztreonam than ceftazidime, thus made the ratio of drug/ drug+clavulanate higher when cefotaxime or aztreonam were used.

As hyperproduction of TEM-1 enzyme may be responsible for the resistance to amoxicillin clavulanate combinations [Stapleton P AAC 1995] and both ceftazidime and inhibitor drug combination resistance attributable to ESBL plus the classic beta lactamase [JID1996 173:151-8 Rice LB], the high prevalence of TEM-1 and SHV-5 in transferable plasmids among *E. coli* and *K. pneumoniae* in the hospital suggested inhibitor drug combination is not a favorable choice for empirical usage for extended spectrum beta-lactam resistant situation.

Comparing with other Taiwan reports about ESBLs in *K. Pneumoniae* isolates [Yan JJ, Liu PYF, LK Siu], SHV5 was prevalent in two hospitals,

whereas the plamid size is quite different [36 kb and >90kb, respectively]. Besides, plasmid is referred as the main mechanism of transmission in one hospital but not in the other hospital. SHV12 is prevalent in another hospital whereas diversity of the transferable plasmid were seen. Fingerprinting of the plasmids carrying ESBL gene for these *E.coli* isolates is similarly diversified like the previous study for K. pneumoniae isolates in the same hospital. The above investigation of ESBL in Taiwan revealed that the prevalent ESBL gene and the mechanism of transmission differed in different hospitals in this small island. It also suggested that ESBL is mainly related with nosocomial infection but not related with island-wide community transmission, although the emergence of resistance to the extended spectrum beta-lactamase raised concern in many hospitals at the same period.

A novel AmpC type enzyme (CMY-8) with pI point of 8.25 was identified to have endemic spread in *K. pneumoniae* isolates in a Taiwan hospital [Yan JJ AAC 2000]. However, the AmpC hyperproducing *E.coli* isolates in this study have variety of pI points > 8.0 suggested Various AmpC type beta-lactamase, including chromosomal and plasmid-mediated, existed in the studied hospital.

In animal model, imipenem and combination of cefperazone and sulbactam were more effective than oxyiminocephalosporin monotherapy [Rice LB, AAC 1991, 35:p1243-44]. It was suggested to avoid extended-spectrum cephalosporins as single agents when treating serious infections due to these organisms [Rice LB, AAC 1991, 35:p1243-44]. The clinical response of combination of oxyiminocephalosporin and beta-lactamase inhibitor need further investigation. We focused on the role of ESBL blood stream infection, an evidence of invasive infection, and also found imipenem showed the most promising clinical response for patients having extended spectrum beta-lactam resistant *E. coli* bacteremia.

More than half of patients using beta-lactamase inhibitor combination or cefmetazole according the susceptibility test had clinical failure. Although larger scale clinical investigation is needed to understand the role of beta-lactamase inhibitor combination and cefamycin group antibiotics in treating oxyiminocephalosporin-resistant *Enterobacteriacea* infection. Some TEM type ESBLs like TEM-28 possessed resistance to both ceftazidime and beta-lactamase inhibitor combination [Bradford PA. Jacobus NV] make the chance of beta-lactamase inhibitor combination as a treatment option lower. Our observation may alert clinicians to notice the importance to identify resistance and treatment difficulty for such resistant pathogen.

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Table 2. In vitro susceptibilities of 28 *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	64	74.1
ATM+CLA	≤0.03 - 16	0.28	8	-
CAZ	0.5- ≥256	8	≥256	25.9
CAZ+CLA	≤0.03 - 16	0.5	8	-
CTX	0.125 – 16	4	16	81.4
CTX+CLA	≤0.03 - 16	0.25	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	128	≥256	7.4
CMZ	0.25 - 128	8	128	70.4
AMK	0.25 - ≥256	1	128	92.6
IPM	0.03 - 0.5	0.12	0.25	100
CIP	≤0.03 - 128	≤0.03	64	66.7
PIP +TZB	0.5 - 32	4	32	92.6

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	pI	ESBL Etest
P1	-	SHV-5	8.2	+
P8	TEM-1	SHV-5	5.4, >8.2	+
P9	TEM1		5.4, 6.0	-
P10	TEM-1	-	5.3, 5.4, >8.2	+
P11	TEM-1	-	5.4, 8.2, >9.0	-
P13	TEM-1	SHV-5	5.4, 8.2	+
P15	TEM-1		5.4	-
P18	TEM1		5.4, 5.6, 8.2, 8.7	-
P21	TEM-28	-	6.1	+
P23	TEM-1	SHV-5	5.4, 8.2	+
P25			6-7, 7.8, 8.2, 8.7	-
P28	TEM-1	SHV-5	5.4, 8.2	+
P32	TEM-1	SHV-5	5.4, 8.2	+
P34	TEM-1	SHV-5	5.4, 8.2	+
P40	TEM-1	SHV-5	5.4, 8.2	+