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Original Article

First report on the occurrence of SHV-12 extended-spectrum β-lactamase–producing *Enterobacteriaceae* in the Philippines

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Background and purpose: Soon after the introduction of extended-spectrum β-lactam antibiotics to treat infections caused by Gram-negative bacteria that were resistant to the existing β-lactams, resistant strains quickly emerged. This study investigated the antimicrobial susceptibility patterns, conjugative transferability, and molecular characterization of extended-spectrum β-lactamase (ESBL)–producing *Enterobacteriaceae* in the Philippines.
Methods: ESBL-producing *Enterobacteriaceae* were collected from patients at the Philippine General Hospital from June 2000 to August 2001. The antimicrobial susceptibility patterns were determined using the standard disk diffusion assay. ESBL production was confirmed using the double-disk synergy assay and the E-test, while the ESBLs were characterized for type using polymerase chain reaction, sequencing, and isoelectric focusing. Conjugation studies were conducted in vitro on solid medium.
Results: All of the ESBL-producing isolates were also resistant to most other classes of antimicrobials studied. The ESBL genes on conjugative and non-conjugative plasmids were bla_{SHV-12}. Fifteen isolates also carried bla_{TEM-1}. None of the isolates were positive for the CTX-M type of ESBL. Plasmids from cefoxitin-resistant isolates did not produce amplified products using CMY primers for the AmpC gene.
Conclusions: SHV-12 is the dominating ESBL among the *Enterobacteriaceae* studied. The gene bla_{SHV-12} is carried together with genes for multiple resistance to other classes of antimicrobials in conjugative and non-conjugative plasmids.

Key words: beta-Lactamase SHV-12; Conjugation, genetic; Drug resistance, microbial; *Enterobacteriaceae*; Isoelectric focusing

Introduction

Extended-spectrum β-lactam antibiotics, comprised of oxyimino-cephalosporins and oxyimino-monobactam, were introduced in the 1980s for treatment of serious infections caused by Gram-negative bacteria that were resistant to the existing β-lactams. However, a few years after their introduction, resistant strains producing extended-spectrum β-lactamases (ESBLs) emerged. Most ESBLs arise from mutations in the bla_{TEM} and bla_{SHV} genes carried in transmissible plasmids [1]. Recently, the emergence of CTX-M ESBLs has been reported worldwide [1-5].

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Currently, more than 200 different ESBLs have been characterized in different genera of *Enterobacteriaceae* and *Pseudomonas aeruginosa* [1]. Although some ESBLs confer high-level resistance, some result only in slight increases in the minimal inhibitory concentrations (MICs) of the antibiotics. However, organisms that produce ESBLs that confer low levels of resistance show resistant phenotypes in vivo when they are found in significant numbers, as in purulent secretions [6]. Moreover, the low levels of resistance of some ESBLs also create problems in their detection, since results with the routine disk-diffusion method and MIC determination may fail to reach the current Clinical and Laboratory Standards Institute (CLSI) breakpoints for resistance [7]. Inoculum concentration has also been shown to affect the results of in vitro ESBL determination [8]. Additional methods

are thus used to detect ESBL production. However, detection of ESBL is not routinely done in many hospital laboratories in the Philippines.

This study determined the antimicrobial susceptibility profiles of *Enterobacteriaceae* isolates, the conjugative transferability of the ESBL plasmids, and their molecular characteristics by polymerase chain reaction (PCR) and nucleotide sequencing, and by determining the isoelectric points (pI) of the enzymes. The bacterial isolates were from patients of the Philippine General Hospital (PGH), Manila, the Philippines. PGH is a 1500-bed university hospital that also serves as a referral center for the country. To the authors' knowledge, this is the first study to determine the molecular characteristics of ESBLs from isolates in the Philippines.

Methods

Bacterial isolates

Enterobacteriaceae isolates with the classical ESBL phenotype, defined as clavulanate-susceptible β-lactamases conferring resistance to oxyimino-cephalosporins and monobactam, and susceptibility to cefoxitin [9,10], as well as those with the unusual phenotype of ESBL-positivity but cefoxitin resistance (for possible carriage of the plasmid-captured AmpC gene) were studied. Of the 185 *Enterobacteriaceae* isolates collected from patients from June 2000 to August 2001 that were available in the hospital culture collection, 75 non-repetitive isolates (1 from each patient) were phenotypically confirmed to be ESBL-positive and were further characterized.

The following strains were provided as reference strains for PCR and isoelectric focusing (IEF): strains with the classical β-lactam plasmids TEM-1 and SHV-1, and ESBL plasmids SHV-3, SHV-5, and SHV-7 from the Instituto Nacional de Salud Publica (INSP), Mexico; *Escherichia coli* 00EEC-1147 with CMY-2 (AmpC plasmid) from the National Cheng Kung University Hospital, Taiwan; *E. coli* MISC336, *Proteus mirabilis* MISC182, *Enterobacter aerogenes* MISC253, and *E. coli* MISC419 with ESBL CTX-M groups 1, 2, 3, and 4, respectively, from the University of Texas Health Center, San Antonio, Texas, United States. *E. coli* J532 (Na^r, Rif^r) from the INSP was used as the recipient strain for conjugation and as a negative control for PCR and IEF clavulanate inhibition studies.

Antimicrobial susceptibility testing

The standard disk diffusion method was used to determine the susceptibility of the *Enterobacteriaceae* isolates to 14 antimicrobial agents following the recommendations of the CLSI [7]. The means of 3 replicates per organism were taken and interpreted using the CLSI interpretative standards.

The antibiotics tested were β-lactams: ceftazidime 30 μg, cefotaxime 30 μg, ceftriaxone 30 μg, aztreonam 30 μg, cefoxitin 30 μg, cefuroxime 30 μg, cefepime 30 μg, and imipenem 10 μg; fluoroquinolones: ciprofloxacin 5 μg; aminoglycosides: gentamicin 10 μg, tobramycin 10 μg, and amikacin 30 μg; tetracycline 30 μg; and sulfamethoxazole-trimethoprim 25 μg (Mast Diagnostics, Bootle, UK).

Extended-spectrum β-lactamase detection

All bacterial isolates with zones of inhibition produced by any one of the following β-lactams — aztreonam, ceftazidime, cefotaxime, and ceftriaxone — that met the CLSI screening criteria [7] for potential ESBL producers were further tested using the phenotypic confirmatory double-disk synergy method and the E-test.

For the double-disk synergy method, the bacterial inocula were prepared following the CLSI recommendations [7]. The amoxicillin–clavulanic acid disk (with 10 μg clavulanic acid, Mast Diagnostics) was placed in the center of the inoculated Mueller Hinton plate. Ceftazidime, cefotaxime, aztreonam, and ceftriaxone disks were placed 14 mm edge-to-edge (or 20 mm center-to-center) from the disk. After 18 to 24 h of incubation at 37°C, the diameters of the zones of inhibition of oxyimino–β-lactam disks on the side facing away from the amoxicillin–clavulanic acid disk were taken and compared with those on the side facing the disk. An enhancement of at least 5 mm in the diameter of inhibition by clavulanic acid in any of the oxyimino–β-lactam disks was interpreted as positive for ESBL production in the phenotypic confirmatory test. The test was repeated with the oxyimino–β-lactam disks placed at 20 mm edge-to-edge (or 26 mm center-to-center) from the amoxicillin–clavulanic acid disk for results with overlapping zones of inhibition. Each test was done in triplicate.

For the E-test, E-test strips TZ/TZL and CT/CTL (AB Biodisk, Solna, Sweden) were used. The tests were done according to the manufacturer's instructions. The procedure for inoculum preparation followed that of the CLSI guidelines [7]. Mueller Hinton agar plates were used. The MICs of ceftazidime and cefotaxime with and without clavulanic acid were taken after 18 to 24 h of incubation at 37°C. A ≥3 $\log_2$ dilution (≥8x) decrease in the MIC of ceftazidime or

cefotaxime in the presence of clavulanic acid compared with the MIC in the absence of the β-lactamase inhibitor was interpreted as confirmed positive for ESBL production [7]. Test results of MICs for ceftazidime >32 μg/mL, or for cefotaxime >16 μg/mL were read as undetermined. The double-disk synergy results were taken as the results for these 12 of the 75 isolates studied. Each test was run in triplicate.

Conjugation studies

Fifteen clinical isolates of *Enterobacteriaceae* that were phenotypically confirmed to be ESBL-positive, and were susceptible to nalidixic acid were tested for the conjugative transferability of their antimicrobial resistance to *E. coli* J532. The remaining 60 of the 75 ESBL-positive isolates were resistant to nalidixic acid, and were thus not included in the assay.

The donor isolates and *E. coli* J532 were grown in tryptic soy broth to the log phase at 37°C for 4 to 6 h. Approximately 3×10^8 cells/mL donor and recipient cells, at a ratio of 1:10 (20:200 μL), were spread plated on tryptic soy agar (TSA) plates, and incubated at 37°C for 6 to 8 h. The transconjugant mixture was harvested after adding 1.5 mL sterile normal saline solution, and 100 μL of each mixture were inoculated into selective plates containing 30 μg/mL nalidixic acid and 10 μg/mL of ceftazidime, cefotaxime, or aztreonam. Suspected transconjugants growing in the plates were further tested for their antimicrobial susceptibility patterns and for ESBL production using the double-disk synergy method and the E-test. Donors that failed to transfer their resistance were tested at least 3 times for conjugative transfer.

Curing of antimicrobial resistance

Enterobacteriaceae isolates that failed to transfer their antimicrobial resistance through conjugation were tested for the stability of their resistance to an elevated temperature of 44.5°C. Organisms grown to the log phase at 37°C were further incubated at 44.5°C for 16 to 18 h. Ten-fold serial dilutions of the culture were plated in TSA and incubated at 44.5°C. Well-isolated colonies were inoculated with sterile toothpicks into TSA with 30 μg/mL of ceftazidime, cefotaxime, or aztreonam, and incubated at 37°C to screen for colonies that have been cured of their resistance. Cured colonies were recovered from TSA plates without antibiotics. The antibiograms of the cured suspects were determined using the disk-diffusion method.

Plasmid extraction

Plasmids were extracted using the alkaline lysis method [11]. The extracts were run on 0.6% agarose gel, stained with ethidium bromide, and viewed using an ultraviolet transilluminator.

Polymerase chain reaction

Plasmids extracted from the clinical isolates and their transconjugants were used as templates for PCR reactions. Oligonucleotide primers that are specific for bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and $bla_{CMY\text{-}1}$ were used. The sequences were: TEM forward 5' ATAAAATTCTT GAAGACGAAA 3'; TEM reverse 5' GACAGTTAC CAATGCTTAATC 3' [10,12,13]; SHV forward 5' GGG TTATTCTTATTTGTCGC 3'; SHV reverse 5' TTAG CGTTGCCAGTGCTC 3' [10,13,14]; CTX-M forward 5' TTTGCGATGTGCAGTACCAGTAA 3'; CTX-M reverse 5' CGATATCGTTGGTGGTGCCATA 3' [2,4]; CMY forward 5' ATGCAACAACGACAATCCATC3'; and CMY reverse 5' GTTGGGGTAGTTGCGATTGG 3' [14]. Reactions were performed in a thermal cycler (MJ Research, Waltham, MA, USA) in 25-μL reaction mixtures containing 1X PCR buffer, 0.5 mM of each dNTP, 3 mM magnesium chloride, 0.5 μM each of forward and reverse primers, 0.05 U/μL Taq polymerase, and 5 ng of plasmid sample. For SHV, TEM, and CMY, the cycling parameters included a hot-start initial denaturation at 95°C for 5 min; 30 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and final extension at 72°C for 5 min. For CTX-M, the annealing was carried out at 52°C for 1 min. The PCR products were viewed after electrophoresis using 1.2% agarose gel and staining with ethidium bromide, using an ultraviolet transilluminator. The molecular weight of the PCR products was determined by comparing with the 100-bp ladder molecular weight standard.

The PCR products were extracted from the gel and purified using the NucleoTrap® Nucleic Acid Purification Kit (BD Biosciences, Palo Alto, CA, USA) for sequencing.

Sequencing of polymerase chain reaction products

The sequences used were: TEM forward 5' GGGG AGCTCATAAAATTCTTGAAGAC 3'; TEM reverse 5' GGGGGATCCTTACCAATGCTTAATCA 3'; SHV forward: 5' GTTCATATGCGTTATATTCGCCTGTG 3'; and SHV reverse: 5' ATAGGATCCTTAGCGTTGC CAGTGCT 3' [15]. The CMY and CTX-M primers used for the detection of the ESBL genes were also used in the sequencing reactions.

The nucleotide sequences of bla_{SHV-12} of *Enterobacter cloacae* 04 and of bla_{SHV-7} of the reference strain *E. coli* were deposited in Genbank under the accession numbers EU 155390 and EU 155391, respectively.

Isoelectric focusing and enzyme inhibition assay

Crude protein extracts were prepared as follows. Bacterial isolates were grown in 4 mL of brain heart infusion broth (BHIB, BBL™; Becton Dickinson, Sparks, MD, USA) with 30 µg/mL ceftazidime at 37°C with shaking at 120 rpm for 18 to 24 h. The bacterial pellet was washed once with 0.1 M phosphate buffer (pH, 7.4) after centrifugation at 4000 rpm at 4°C for 15 min. Sample rehydration buffer 1 mL (125 µL ampholyte; pH, 3-10 [Invitrogen, Carlsbad, CA, USA]; 100 µL of 0.1% bromphenol blue; and deionized distilled water to make up to 25 mL) was added to suspend the pellet. The culture was frozen and thawed 3 times and sonicated for 15 min. The protein extract was diluted 10^{-2} with the sample rehydration buffer. IEF was performed using the ZOOM IPG Runner System (Invitrogen) with ampholyte of pH 3.0 to 10.0. Enzyme activity of the β-lactamases was detected by overlaying the gel with 500 µg/mL nitrocefin in 0.1 M phosphate buffer (pH, 7.0) and noting the color change to red. For broad bands, measurement of the distance traveled was taken from the middle of the band to the edge of the gel. Extracts from strains producing TEM-1, SHV-7, CMY-1, and SHV-5 were used as standards for pIs of 5.4, 7.6, 8.0, and 8.2, respectively. In addition, Invitrogen IEF markers were run along with the samples. Inhibition assay was carried out by overlaying the gels with 0.3 mM clavulanic acid in 0.1 M phosphate buffer (pH, 7.0) for 15 min, followed with 500 µg/mL nitrocefin.

Results

Antimicrobial susceptibility patterns

Aside from resistance to the oxyimino-cephalosporins and monobactam, all of the ESBL-producing isolates were also resistant to most other classes of antimicrobials tested (Table 1). Resistance to netilmicin, sulfamethoxazole-trimethoprim, tetracycline, cefoxitin, amikacin, ciprofloxacin, and gentamicin were shown by 99%, 96%, 81%, 80%, 67%, 65%, and 64% of the isolates, respectively. On the other hand, 8% were resistant to cefoperazone, while 3% were resistant to imipenem.

Table 1. Characteristics of *Enterobacteriaceae* isolates from the Philippine General Hospital.

Isolate/ isolate no.	ESBL type	Isoelectric points	Conjugative transferability	Resistance phenotype
Klebsiella pneumoniae				
67	SHV-12	8.2	+	Aztreonam, ceftazidime,cefotaxime, ceftriaxone, cefuroxime, tetracycline, tobramycin, sulfamethoxazole-trimethoprim
245	SHV-12	8.2	+	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, amikacin, tobramycin, sulfamethoxazole-trimethoprim
259	SHV-12	8.2	+	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, tobramycin, sulfamethoxazole-trimethoprim
154	SHV 12 TEM-1	8.2 5.4	Not tested[b]	Aztreonam, ceftazidime,cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, sulfamethoxazole-trimethoprim, naladixic acid
97	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
213	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, naladixic acid
226	SHV-12 TEM-1	8.2 Not detected	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, cefoperazone, amikacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
227	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, cefoperazone, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid

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235	SHV-12	8.2	+	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, tetracycline, amikacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin
117	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoperazone, cefoxitin, tetracycline, ciprofloxacin, imipenem, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
179	SHV-12 TEM-1	8.2 5.4	+	Aztreonam, ceftazidime,cefotaxime, ceftriaxone, cefuroxime, tetracycline, amikacin, tobramycin, gentamicin
222	SHV-12	8.2	+	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoperazone, tobramycin, sulfamethoxazole-trimethoprim, gentamicin
188	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
223	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
225	SHV-12 TEM-1	8.2 5.4	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
228	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
241	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
229	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
231	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
232	SHV-12 TEM-1	8.2 5.4	–	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, amikacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin
234	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
236	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
237	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
233	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
238	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, amikacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
239	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, amikacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid

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244	SHV-12 TEM-1	8.2 5.4	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
246	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
247	SHV-12	8.2	–	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, tetracycline, amikacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin,
248	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, amikacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
249	SHV-12	Not done	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
250	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
251	SHV-12	Not done	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
252	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
253	SHV-12	Not done	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim gentamicin, nalidixic acid
256	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
257	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
258	SHV-12	8.2	–	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, tetracycline, amikacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin
260	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
261	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
262	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
263	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
264	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid

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Klebsiella ozaenae				
255	SHV-12	8.2	–	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoperazone, tetracycline, amikacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin
102	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
221	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
224	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
43	SHV-12 TEM-1	8.2 5.4	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoperazone, cefoxitin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
137	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime,ceftriaxone, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
177	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
55	SHV-12	8.2	+	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, tobramycin, sulfamethoxazole-trimethoprim
150	SHV-12	8.2	+	Aztreonam, ceftazidime,ceftriaxone, cefuroxime, cefoxitin, amikacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin
10	SHV-12 TEM-1	8.2 5.4	–	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, tobramycin, sulfamethoxazole-trimethoprim, gentamicin,
64	SHV-12	Not done	Not tested[b]	Aztreonam, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, gentamicin, nalidixic acid
164	SHV-12	Not done	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
166	SHV-12	Not done	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
180	SHV-12	Not done	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
Enterobacter cloacae				
4	SHV-12 TEM-1	8.2 Not detected	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
28	SHV-12 TEM-1	8.2 Not detected	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
35	SHV-12 TEM-1	8.2 Not detected	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid

(Table continued on page 81)

(Table continued from page 80)

52	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
134	SHV-12 TEM-1	8.2 Not detected	–	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, imipenem, tobramycin, sulfamethoxazole-trimethoprim, gentamicin
158	SHV-12 TEM-1	8.2 Not detected	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
170	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
181	SHV-12	8.2	+	Aztreonam, ceftazidime,cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, tobramycin, gentamicin
135	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime,ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, naladixic acid
136	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime,cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, naladixic acid
Escherichia coli				
168	SHV-12 TEM-1	8.2 5.4	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
174	SHV-12	Not done	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
112	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
71	SHV-12 TEM-1	8.2 5.4	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
88	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
92	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
106	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, gentamicin, nalidixic acid
153	SHV-12	8.2	Not tested[b]	Aztreonam, ceftazidime, ceftriaxone, cefuroxime, cefoxitin, tetracycline, amikacin, ciprofloxacin, tobramycin, sulfamethoxazole-trimethoprim, nalidixic acid
SHV-7[a]	SHV-7	7.6		Aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefuroxime, ampicillin, kanamycin
TEM-1[a]	TEM-1	5.4		Ampicillin

[a]Reference isolates.

[b]Not tested in conjugation studies. Isolate is resistant to nalidixic acid, the marker used for selection of transconjugants.

Abbreviation: ESBL = extended-spectrum β-lactamase.

Conjugation studies

Nine of the 15 ESBL isolates tested either transferred all or most of their resistance genes, converting the *E. coli* transconjugants into ESBL strains with concurrent resistance to different classes of antimicrobials (Table 2). None of the 4 isolates that were resistant to cefoxitin transferred the resistance. This was expected since resistance to cefoxitin is known to be usually due to chromosomal mutation that leads to porin changes. The remaining 6 isolates failed to transfer their resistance in all 3 trials conducted. However, these isolates were cured of their resistance when exposed to the elevated temperature of 44.5°C, suggesting plasmid carriage.

Detection and identification of extended-spectrum β-lactamase genes

PCR of the plasmids from all 75 clinical isolates with the SHV primers produced the expected product of approximately 800 bp, while 15 also produced the expected PCR product of approximately 1200 bp with the TEM primers. None of the isolates were positive for the CTX-M type of ESBL. All of the SHV-7, TEM-1, and CTX-M reference strains produced the expected results.

National Center for Biotechnology Information nucleotide BLAST analysis identified all of the strains to belong to SHV-12. When translated, all the sequences showed the leucine (L) to glutamine (Q) substitution at amino acid 35 (using Ambler et al's classification scheme [16]), glycine (G) to serine (S), and glutamate (E) to lysine (K) substitutions at positions 238 and 240 when compared with the classical β-lactamase SHV-1. These are the typical mutations of SHV-12 ESBL [17]. IEF of the crude protein extracts and reaction with nitrocefin also showed the expected pI of 8.2 for SHV-12. Treatment with clavulanate inhibited the expression of the pI 8.2 protein, confirming it to be an ESBL.

Studies with the SHV-7 reference identified it as SHV-7 with 99% alignment in the nucleotide blast. Translation of the sequences showed a silent mutation

Table 2. Resistance transferred from extended-spectrum β-lactamase–positive *Enterobacteriaceae* through conjugation.[a]

Organism/isolate no.	Resistance phenotype	Resistance transferred to *Escherichia coli* J532
Enterobacter cloacae		
181	Ceftazidime, aztreonam, cefotaxime, tetracycline, gentamicin, ceftriaxone, cefuroxime, tobramycin, cefoxitin	All resistance except cefoxitin
Klebsiella ozaenae		
55	Ceftazidime, aztreonam, cefotaxime, tetracycline, amikacin, ceftriaxone, cefuroxime, tobramycin, sulfamethoxazole-trimethoprim, cefoxitin	All resistance except cefoxitin
150	Ceftazidime, aztreonam, amikacin, gentamicin, ceftriaxone, cefuroxime, tobramycin, sulfamethoxazole-trimethoprim, cefoxitin	All resistance except cefoxitin
Klebsiella pneumoniae		
67	Ceftazidime, aztreonam, cefotaxime, tetracycline, ceftriaxone, cefuroxime, tobramycin, sulfamethoxazole-trimethoprim	All resistance
179	Ceftazidime, aztreonam, cefotaxime, tetracycline, amikacin, gentamicin, ceftriaxone, cefuroxime, tobramycin	All resistance except tetracycline
222	Ceftazidime, aztreonam, cefotaxime, ceftriaxone, tobramycin, gentamicin, cefepime, sulfamethoxazole-trimethoprim, cefuroxime	All resistance except cefepime and sulfamethoxazole-trimethoprim
235	Ceftazidime, aztreonam, cefotaxime, ceftriaxone, tobramycin, gentamicin, tetracycline, amikacin, sulfamethoxazole-trimethoprim, cefuroxime	All resistance except gentamicin, tetracycline, sulfamethoxazole-trimethoprim
245	Ceftazidime, aztreonam, cefotaxime, ceftriaxone, tobramycin, amikacin, sulfamethoxazole-trimethoprim, cefuroxime	All resistance except amikacin
259	Ceftazidime, aztreonam, cefotaxime, amikacin, ceftriaxone, cefuroxime, tobramycin, tetracycline, sulfamethoxazole-trimethoprim, cefoxitin	All resistance except cefoxitin

[a]Only 15 of the 75 extended-spectrum β-lactamase–positive isolates were susceptible to nalidixic acid and were tested for conjugation; 6 were negative for conjugative transfer of the antimicrobial resistance.

from ACC to ACG, resulting in the same threonine amino acid. The SHV-7 protein showed the expected pI of 7.6 after IEF.

Fifteen isolates were shown to also have TEM plasmids using PCR (Table 1). Nucleotide BLAST of the sequences identified these to be TEM-1, a classical β-lactamase. This was supported by the expected pI of 5.4 shown by the extracted proteins, the activities of which were inhibited by clavulanate. It was noted that 6 of the 15 isolates did not produce the TEM-1 protein when assayed using IEF, suggesting that these were not expressed. None of the isolates had TEM-derived ESBL.

Cefoxitin-resistant isolates were also studied for the presence of *cmy* genes coding for resistance to the cephamycins in the plasmids extracted. None of the isolates were positive for these using PCR and IEF. The reference CMY-2 isolate gave the expected results.

Discussion

The CLSI 2005 recommendation is for all ESBL-producing strains to be reported as resistant for all penicillins, cephalosporins, and aztreonam [7]. Antimicrobials belonging to other groups are used for treatment of infections caused by these isolates. However, all of the isolates in the study were also found to be resistant to most of the antimicrobials in the aminoglycoside, fluoroquino-lone, tetracycline, and sulfamethoxazole-trimethoprim groups. The occurrence of most multiclass resistance among the ESBL-positive isolates was higher than in other studies [18,19]. Therefore, use of combination therapy may also result in treatment failure. Imipenem was the only antimicrobial to which 97% of the isolates were susceptible.

Moreover, the antimicrobial resistance genes were shown to be carried in plasmids, some of which were transmissible through conjugation. This poses an additional problem in controlling the dissemination of ESBL-producing microorganisms, since horizontal transfer of resistance genes through conjugation may occur in a clinical setting at a high frequency [20]. This converts susceptible organisms to ESBL producers, and also confers resistance to antimicrobials belonging to different classes. The heavy use of antimicrobials in a hospital setting further positively selects for these resistant strains to survive and be disseminated.

SHV-12 was shown to be the only ESBL type among the *Enterobacteriaceae* isolates studied. Although CTX-M ESBL has been reported to be emerging worldwide [1-5], it was not found among the isolates in this study. SHV-12 was first identified by Nüesch-Inderbinen et al in 1997 in 1 strain of *Klebsiella pneumoniae* and 1 strain of *E. coli* among 30 ESBL-producing *Enterobacteriaceae* isolates collected over a 2-year period in Switzerland [21]. The results of the present study are similar to those of studies in other countries where SHV-12 has been found to be the prevalent type [22-25].

SHV-12 ESBL has the G238S and E240K mutations in addition to the L35Q mutation. Positions 238 and 240 are the preferred sites of mutation to evolve an ESBL phenotype in SHV [26]. Mutation in G238S in SHV-2 is said to preserve the efficient catalytic activity against both penicillins and cephalosporins. This mutation, together with that of E240K, is also associated with increased steady-state β-lactamase expression, compared with the sole presence of G238S mutation. The demonstration of these mutations in all ESBLs in this study suggests their efficiency in rendering the isolates to be resistant to penicillins and cephalosporins.

These results underscore the need for vigilance in infection control at the PGH, improved and sustained surveillance of antimicrobial resistance among hospital isolates, and the regular review and proper handling of antimicrobials by medical practitioners and compliance by patients.

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