Detection of the SHV genotype polymorphism of the extended-spectrum β-lactamase-producing Gram-negative bacterium

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Abstract. The prevalence of extended-spectrum β-lactamases (ESBLs) is due to the extensive usage of the extended-spectrum cephalosporins and leads to huge financial loss worldwide, whilst presenting a challenge to the clinical treatment. The aim of the present study was to delineate the frequency of ESBL occurrence in Enterobacteriaceae and confirm the SHV genotype. A random collection of 153 *Escherichia coli* isolates (*E. coli*) and 70 *Klebsiella pneumoniae* isolates were tested. The amplification products obtained by polymerase chain reaction were sequenced. Isolates with novel mutations were transformed to *E. coli* DH5α. The minimum inhibitory concentration (MIC) was obtained by a microdilution method. The relevance ratio of ESBL was 67.7% and the proportion of the SHV β-lactamase gene (bla_{SHV}) was 18.5%. A new genotype of β-lactamase was demonstrated and submitted to GenBank. A total of 12 mutational sites were found in 28 ESBL-producing isolates, including four nonsense mutations. Sensitive-rates of 28 ESBL-producing isolates to imipenem were 100%, and resistant-rates to penicillin, amoxicillin and oxacillin were 100%. The MIC of DH5α-F8 to penicillin, oxacillin, cefoxitin, cefotaxime, cefepime, cefoperazone/sulbactam, imipenem and netilmicin was 512, 512, 2, 0.03, 0.06, 4, 0.015 and 32 respectively. In conclusion, ESBL and SHV-28 is the most prevalent *bla*. Imipenem is the most effective antibiotic to ESBL, and the 4th-generation cephalosporins and β-lactamase inhibitor compound are also effective. ESBL is mediated by plasmids and able to spread among different Enterobacteriaceae. In conclusion, new mutations of the bla_{SHV} gene exist from at least 2010.

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Introduction

Extended-spectrum β-lactamases (ESBLs) are the plasmid-mediated bacterial enzymes that have been found in enteric Gram-negative bacteria and are important pathogens in nosocomial infections (1). The importance of ESBLs in the spread of multiple resistances among Gram-negative bacteria was thoroughly recognized (2). The majority of ESBLs are derived from broad-spectrum β-lactamases, TEM-1 and SHV-1. CTX-M and OXA-type enzymes are novel families of ESBLs (3). There are nine distinct structural families, TEM, SHV, CTX-M, PER, VEB, GES, TLA, BES and OXA, which are classified based on comparisons of deduced amino acid sequences (4). TEM and SHV enzymes were included in the first ESBL variant and they remain in the most prevalent types of ESBL (5-7). The first SHV type of an ESBL isolate was reported in Germany in 1983 (8). SHV is one of the most common genotypes of ESBL (9,10).

Recently, ESBLs have increased in prevalence due to the use of extended-spectrum cephalosporins (ESC). The resistance to ESC among Enterobacteriaceae has occurred worldwide (11). β-lactams were the most frequently prescribed antibiotics accounting for ~50% of global antibiotic administration. Therefore, ESBLs presented a big challenge to treatment (12). ESBL-producing isolates caused outbreaks of infections that lead to serious antibiotic management concerns with significant economic causatives (13). The main predictor of mortality caused by ESBL-producing isolates was initially inadequate antimicrobial treatment (14). *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) were the most popular ESBL-producing isolates (15). In addition, ESBLs have been found in other Enterobacteriaceae and *Pseudomonas aeruginosa* (3).

The resistance to the 3rd-generation cephalosporins was mainly due to the production of ESBLs enzymes, which are hydrolyzing oxyimino-cephalosporins inhibited by clavulanic acid (16). ESBL enzymes, such as SHV, TEM and CTX-M, were

all mediated by the specific plasmids. Conjugation experiments and plasmid identification were conducted to examine the transferability of resistance to ESCs (11). Traditionally, the scope of the problem caused by ESBLs was assessed by considering the microbiological width (the type of microorganisms involved), antibiotic depth (the number of antibiotics hydrolyzed by ESBL) and geographical breadth (the global burden of resistance). Otherwise, the clinical impact of antibiotic resistance, such as the effect on mortality, was extremely difficult to define (1).

To delineate the trends of ESBL-producing isolates, the frequency of SHV in Chengdu (China) were detected by assessing the clinical isolates obtained from hospitals in Chengdu between 2010 and 2011. Confirmation testing of ESBLs-producing isolates was performed by the double-disk synergy test. The minimum inhibitory concentration (MIC) of the ESBLs-producing isolates to antibiotics was obtained by a microdilution method. Polymerase chain reaction (PCR) and DNA sequencing measurement were also performed to determine the genotypes and mutations of the SHV β-lactamase gene (bla_{SHV}). Additionally, we hypothesized that the ESBL gene may be transferred among Enterobacteriaceae.

Materials and methods

Bacterial isolates. A total of 223 isolates, including *E. coli* (n=153) and *K. pneumoniae* (n=70), were collected from hospitals in Chengdu between 2010 and 2011 (all the patients provided written consent). The ethics committee of Sichuan University approved the study. *E. coli ATCC25922*, *K. pneumoniae ATCC700603* and a SHV *E. coli* isolate were used as quality control strains.

Screening and confirmation for ESBL. The ESBL phenotype screening test was performed according to the disk diffusion method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) on Mueller-Hinton agar (Sigma-Aldrich, St. Louis, MO, USA) using commercial antibiotic disks. The antibiotics used were ceftazidime, aztreonam, cefotaxime and ceftriaxone (all 30 μg/disk; all Binhe Microorganism Reagent Co., Ltd., Hangzhou, Zhejiang, China). The size of the inhibition holes was measured after the Mueller-Hinton plates (Aoboxing Bio-Tech Co., Ltd., Beijing, China) were incubated at 35˚C for 16 h. When the size of the inhibition hole was 22 mm for ceftazidime, ≤27 mm for aztreonam, ≤27 mm for cefotaxime or ≤25 mm for ceftriaxone, the occurrance of any of these circumstances showed the possibility of ESBLs. The double-disk synergy testing method was performed to confirm ESBL. The antibiotics that were used for confirmation testing in ESBLs were cefotaxime (30 μg/disk), cefotaxime-clavulanate (30 μg/disk) (Binhe), ceftazidime (30/10 μg/disk) and ceftazidime-clavulanate (30/10 μg/disk) (Binhe Microorganism Reagent Co., Ltd.). When the difference of diameter in any pair of disks was ≥5 mm, the isolate was confirmed to produce ESBLs.

Extracting the plasmid DNA and amplification of ESBL genes. The plasmid DNA of ESBL-producing isolates was extracted with the Plasmid Mini Kit I (Omega Bio-Tek, Norcross, GA, USA) and stored at -20˚C. The presence of β-lactamase genes was investigated by PCR (Bio-Rad, Hercules, CA, USA). The primers aiming to amplify the SHV genes were designed by the Primer Premier 5.0 (PREMIER Biosoft, Palo Alto CA, USA) according to the original SHV gene sequence from GenBank; accession no. AF124984. The primers were as follows: bla_{SHV1}, 5'CTTATTTGTCGCTTC TTT3'; and bla_{SHV2} 5'TTATGGCGTTACCTTTG3'. In the 25 μl PCR system was 1 μl (10 pmol/μl) primer bla_{SHV1}, 1 μl (10 pmol/μl) primer bla_{SHV2}, 1 μl (10 ng/μl) plasmid, 12.5 μl 2X Taq Master mix (Tiangen Biotech, Co., Ltd., Beijing, China) and 9.5 μl sterile water. PCR amplification was performed under the following conditions: Initial denaturation at 94˚C for 3 min, denaturation at 94˚C for 45 sec, annealed at 58˚C for 45 sec and extended at 72˚C for 1 min. After 35 cycles, the amplification was finally extended at 72˚C for 7 min. The PCR products were detected by 2% agarose gel electrophoresis (Sigma-Aldrich).

Transconjugation experiments. The amplification products of *K. pneumoniae* F8, including novel mutations, and *K. pneumoniae* F25, including the bla_{SHV-18} gene to the carrier pUCm-T (Sangon Biotech, Shanghai, China) with ampicillin-resistance, were conjugated. The carrier, including the target segments, were transformed to competent cell: *E. coli* DH5α (Sangon Biotech). The bacterial strains transformed were selected on Mueller-Hinton agar with ampicillin-resistance with a concentration of 50 μg/ml ampicillin (Binhe Microorganism Reagent Co., Ltd.). The combinations are known as DH5α-F8 and DH5α-F25 in the following section. The plasmid DNA of DH5α-F8 and DH5α-F25 were extracted after incubation at 35˚C for 16-18 h. The plasmids were amplified and primer bla_{SHV1} and bla_{SHV2} were analyzed with 2% agarose gel electrophoresis (Sigma-Aldrich).

Drug-sensitivity testing of ESBL-producing isolates, DH5α-F8 and DH5α-F25. The MIC was obtained by the microdilution method according to the standards for antimicrobial susceptibility testing (NCCLS document M100-S9, 2008). The antibiotic concentrations were 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03 and 0.015 mg/l, respectively. The antibiotics used were penicillin, amoxicillin, oxacillin, cefoxitin, ceftazidime, ceftriaxone, cefotaxime, cefoperazone, cefepime, ampicillin-sulbactam, piperacillin-tazobactam, cefoperazone-sulbactam, imipenem, aztreonam, netilmicin, levofloxacin and gentamicin (all Binhe Microorganism Reagent Co., Ltd.).

Results

Detection of ESBLs. A total of 151 ESBLs were detected in 112 (74.67%) *E. coli* and 39 (55.7%) of *K. pneumoniae* isolates. The total relevance ratio was 67.7%. PCR products were ~1,021 base pairs and there were 28 isolates, including bla_{SHV} gene in 16 *E. coli* and 12 *K. pneumoniae* isolates. The proportion of gene bla_{SHV} was 18.5%.

Blast search of SHV. The results of Blast searches revealed that there were nine isolates of SHV-28, seven of SHV-11, four of SHV-1, three of SHV-12, one of SHV-31 and one of SHV-18. There were another three isolates that included novel mutations and their nucleotide sequence had no difference between each other and cannot be found in the NCBI reference genome. The

Table I. Mutations of the known SHV genotypes of *bla*.

Mutation sites	Quondam bases	Bases after mutation	Quondam amino acid	Amino acid after mutation	Mutational rates (%)
7 (Y7F)	A	T	Tyr	Phe	32.1
8 (I8F)	A	T	IIe	Phe	3.6
35 (L35Q)	T	A	Leu	Gln	39.2
43 (R43S)	C	A	Arg	Ser	3.6
238 (G238S or G238A)	G	A or C	Gly	Ser or Ala	10.7, 3.6
240 (E240K)	G	A	Glu	Lys	17.9

Table II. Results of 28 extended-spectrum β-lactamases (ESBL)-producing isolates to 17 antibiotics.

	28 ESBL-producing strains		
Antibiotics	Resistance (%)	Intermediary (%)	Sensitive (%)
Oxacillin	100.0	0.0	0.0
Penicillin	100.0	0.0	0.0
Amoxicillin	100.0	0.0	0.0
Cefoxitin	95.9	4.1	0.0
Cefotaxime	92.7	0.0	8.3
Ceftazidime	45.8	16.7	37.5
Cefoperazone	87.5	4.1	8.3
Ceftriaxone	92.7	0.0	8.3
Cefepime	11.0	12.5	76.5
Amoxicillin/clavulanate	83.4	8.3	8.3
Piperacillin/tazobactam	8.3	16.6	75.0
Cefoperazone/sulbactam	4.2	12.5	83.3
Aztreonam	59.9	0.0	40.1
Imipenem	0.0	0.0	100.0
Levofloxacin	66.7	8.3	33.0
Netilmicin	37.5	20.8	41.7
Gentamicin	40.3	8.3	33.0

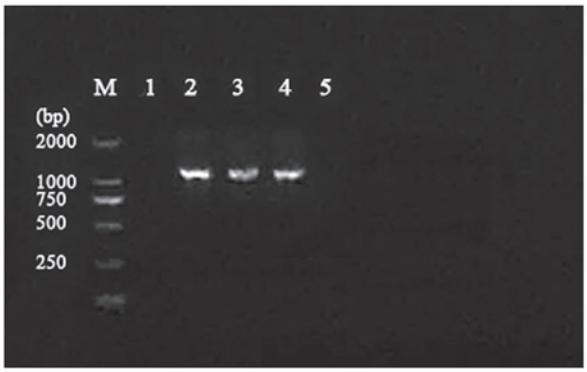

Figure 1. Plasmid DNA of DH5α-F8 and DH5α-F28 was observed by agarose gel electrophoresis. The plasmid was successully transformed. Lane M, marker; 1, positive control; 2, SHV positive strains; 3, DH5α-F8; 4, DH5α-F25; 5, SHV negative strains.

novel sequence was submitted to GenBank with an accession no. JX192924. The gene sequence used, was: 1 ctttgggctc ctcatgatgt attgtg gtta tgcgtcatat tcgcctgtgt attatctccc 61 tgttagccac cctgccgctg gcggtacaag ccagcccgca gccgcttgag caaattaaac 121 aaagcgaaag ccagctgtcg ggccgcgtag gcatgataga aatggatctg gccagcggcc 181 gcacgctgac cgcctggcgc gccgatgaac gctttcccat gatgagcacc tttaaagtag 241 tgctctgcgg cgcagtgctg gcgcgggtgg atgccggtga cgaacagctg gagcgaaaga 301 tccactatcg ccagcaggat ctggtggact actcgccggt cagcgaaaaa caccttgccg 361 acggcatgac ggtcggcgaa ctctgcgccg ccgccattac catgagcgat aacagcgccg 421 ccaatctgct gctggccacc gtcggcggcc ccgcaggatt gactgccttt ttgcgccaga 481 tcggcgacaa cgttacccgc cttgaccgct gggaaacgga actgaatgag gcgcttcccg 541 gcgacgcccg cgacaccact accccggcca gcatggccgc gaccctgcgc aagctgctga 601 ccagccagcg tctgagcgcc cgttcgcaac ggcagctgct gcagtggatg gtggacgatc 661 gggtcgccgg accgttgatc cgctccgtgc tgccggcggg ctggtttatc gccgataaga 721 ccggagctgg cgaacggggt gcgcgcggga ttgtcgccct gcttggcccg aataacaaag 781 cagagcgcat tgtggtgatt tatctgcggg atacgccggc gagcatggcc gagcgaaatc 841 agcaaatcgc cgggatcggc gcggcgctga tcgagcactg gcaacgctaa cccggcggtg 901 gcgcgcgcgt tatccggctc gtagcactcg cagctgccgg gcgatatgac tggcggcggc 961 atcggagaga tgccgtcggt aatgatggtg gtgaaccggg tcaaagtatc acccccataa 1021 c; the coding region was from 33 to 893.

These five known genotypes of bla_{SHV} included 10 mutational sites. However, these changes do not lead to amino acid changes at position of 112, 260, 268 and 274, which were so-called nonsense mutations. Other mutations are represented in Table I. Three isolates, including novel mutations, contain five mutational sites: Base 121 at amino acid position 35 changes from 'T' to 'A' and the amino acid Leu is substituted by Gln; recorded as L35Q in GenBank. Base 36 in the coding region changes from 'T' to 'C', with a Tyr-to-His substitution; base 89 from 'C' to 'A', with a His-to-Gln substitution; base 353 from 'T' to 'C' and base 494 from 'C' to 'T', but the amino acid of these two positions does not change.

Extraction of DH5α plasmids. The plasmid DNA of DH5α-F8 and DH5α-F28 were extracted, as shown in Fig. 1. The results showed that the gene sequence was identical to that of JX192924. This accounted for the gene segments with novel mutations successfully transconjugating to *E. coli* DH5α. There are three mutational amino acids that differ from the original (accession no. EU441172); H3F, Q20H and Q31L. Additionally, this mutation has not emerged since the first SHV-28 strain was detected.

Table III. Minimum inhibitory concentration (MIC) of the transconjugation isolates, DH5α-F8 and DH5α-F25, to 17 antibiotics.

	MIC, mg/l	
Antibiotics	DH5α-F8 L35Q 36th base 89th base	DH5α-F25 G238A E240K
Oxacillin	512	512
Penicillin	512	512
Amoxicillin	256	256
Cefoxitin	2	4
Cefotaxime	0.03	4
Ceftazidime	0.25	8
Cefoperazone	4	32
Ceftriaxone	0.125	0.25
Cefepime	0.06	0.06
Amoxicillin/clavulanate	32	16
Piperacillin/tazobactam	64	64
Cefoperazone/sulbactam	4	4
Aztreonam	16	8
Imipenem	0.015	0.015
Levofloxacin	64	0.03
Netilmicin	32	0.5
Gentamicin	128	0.06

Antimicrobial susceptibility testing. The results of the antimicrobial susceptibility testing are shown in Table II. The MIC of DH5α-F8 and DH5α-F25 to 17 antibiotics are shown in Table III. A total of 28 ESBL-producing isolates were all sensitive to imipenem, and all resistant to penicillin, amoxicillin and oxacillin. The sensitive-rates to cefoperazone/sulbactam, piperacillin/tazobactam and cefepime were all >75%. The resistant-rates to the 2nd and 3rd-generation cephalosporins and levofloxacin were high. DH5α-F8 and DH5α-F25 were resistant to penicillin, amoxicillin and oxacillin, and sensitive to imipenem, the 3rd and 4th-generation cephalosporins and the β-lactamase inhibitor compound, except for piperacillin/tazobactam. Compared to DH5α-F25, DH5α-F8 was sensitive to levofloxacin, netilmicin and gentamicin.

Discussion

Currently, antibiotic resistance is a severe problem. As opposed to methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci*, ESBLs-producing strains are another class of primary pathogenic bacteria (17,18). ESBLs mediated by plasmids are common in Enterobacteriaceae, particularly in *E. coli* and *K. pneumoniae*, which have been reported to cause serious infections in hospitals, as well as in the community in different parts of the world (19,20). A previous study indicated that the present disk diffusion criteria underestimate the prevalence of ESBL-producing strains (21). All the phenomena suggest that the trends of ESBLs-producing isolates strains may be underestimated.

SHV-2 is the first reported genotype of *bla*, which has only one mutation site at codon 238. The change from glycine to serine induces the hydrolysis of the 3rd-generation cephalosporin (15). Subsequent to the identification of SHV-2, numerous types of SHV genotypes have been found successively. One study identified SHV-8 with an Asp-to-Asn substitution at amino acid position 179 in a bacteremia patient that had received multiple courses of antimicrobial agents, including ceftazidime. The collection of blood isolates from the same patient suggested the *in vivo* evolution of resistance under selective pressure of treatment with cephalosporins (1).

With regards to the high presence ratio in Chengdu of the ESBL SHV genotypes, SHV-1, SHV-11 and SHV-28 were the most popular types. A total of 28 ESBL-producing isolates showed a high resistance-rate to oxacillin, amoxicillin, penicillin, cefoxitin, levofloxacin and gentamicin. Similarly, Jain *et al* (22) also reported that ESBL-producing organisms were resistant to ampicillin, cotrimoxazole, tetracycline and gentamicin. According to our study, the results indicated that broad-spectrum penicillins, such as amoxicillin and penicillin, were ineffective to the infection of ESBL-producing isolates. In addition, β-lactamase inhibitor compounds, such as clavulanic acid and sulbactam, are able to enhance the sensitivity of ESBL-producing isolates to β-lactamase (23). Tazobactam enhanced the antibacterial function of piperacillin as a new type of β-lactamase inhibitor. In the present study, the drug-sensitivity testing showed that piperacillin/tazobactam was superior to amoxicillin/clavulanate. For the prior use of clavulanate, is is possible that the bacterium was less sensitive compared to tazobactam and sulbactam. The 4th-generation cephalosporin and β-lactamase inhibitor compound are effective to ESBL, while the carbapenems, such as imipenem, are most available to ESBL. DH5α-F8 and DH5α-F25 were resistant to penicillin, oxacillin, amoxicillin, amoxicillin/clavulanate, aztreonam and piperacillin/tazobactam, and sensitive to 3rd and 4th-generation cephalosporins and cefoperazone/sulbactam. The MIC of DH5α-F25 to the 3rd-generation cephalosporin was higher than that of DH5α-F8. DH5α-F25 includes two mutations at codons 238 and 240. These codons did not mutate in DH5α-F8. A previous study reported that mutations at codons 238 and 240 are relevant to the resistance to cefotaxime and ceftazidime (24). The MIC of the original isolates of F8 and F25 showed a high resistant-rate compared to DH5α-F8 and DH5α-F25, which suggested that the original isolates performed other resistant mechanisms leading to the resistance to the 3rd-generation cephalosporin. Further study is required to confirm their resistant mechanism. Multi-resistance isolates are now common in the clinic, so the choosing of antibiotics should be based on the drug-sensitivity testing. The 3rd-generation cephalosporin should be replaced by cephamycin, a β-lactamase inhibitor compound and carbapenem.

In conclusion, the present study demonstrated that ESBL was mediated by the plasmids. The ESBL-producing members, such as *E. coli* and *K. pneumoniae*, showed a high resistance-rate to numerous types of antibiotics. Novel

mutations have existed in Chengdu from at least 2010. Further study is required to determine its biochemical character.

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