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Molecular epidemiology and risk factors of bloodstream infections caused by extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* A case—control study☆

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Summary
Objectives: To study the prevalence, risk factors, outcome, and molecular epidemiology in patients with bacteremia caused by extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* (Kp) (cases), in comparison with patients with bacteremia caused by a susceptible Kp (controls).
Methods: This was a retrospective case—control study including all episodes of Kp bacteremia for the period 1993 to 2002 at a referral hospital for adults in Mexico. ESBL production was tested for by E-test. All isolates were typed by pulsed field gel electrophoresis (PFGE). A subset of isolates underwent plasmid analysis, conjugal transfer of cefotaxime resistance to *Escherichia coli* J53-2, isoelectric focusing bioassay, colony-blot hybridization, PCR, and sequencing.
Results: Of the 121 patients with bacteremia due to Kp included in the study, 17 (14.0%) had an ESBL-Kp isolate (cases). Multivariate analysis identified prior use of cephalosporins (OR 7.6, 95% CI 1.1—53.5; $p = 0.039$) and stay in the intensive care unit (ICU; OR 5.6, 95% CI 1.1—27.9; $p = 0.033$)

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as significant risk factors. No differences were observed in hospital stay or mortality after the event. Multi-drug resistance was more frequent in ESBL-Kp. There was no clonal predominance. A distinct β-lactamase profile was identified, which included a combination of TEM-1 (pI 5.4) and SHV-5 (pI 8.2) in 13/17 ESBL-Kp isolates. Cefotaxime resistance was transferred by conjugation in 14/17 isolates with a >120-kb plasmid encoding ESBL.

Conclusions: The prevalence of ESBL-Kp was found to be lower than that previously reported in Latin America. ESBL-Kp bacteremia was not associated with a worse clinical outcome. We were able to identify a plasmid-mediated horizontal dissemination over the 10-year period.

Introduction

Extended-spectrum β-lactamases (ESBL) are widely distributed among the *Enterobacteriaceae*, predominantly in *Klebsiella pneumoniae* and *Escherichia coli*. These enzymes are plasmid-mediated and constitute an increasingly important mechanism of antimicrobial resistance among nosocomial Gram-negative pathogens.[1,2] The highest prevalence of ESBL-producing *K. pneumoniae* (ESBL-Kp) worldwide has been observed in Latin America.[3] In Mexico, ESBL-Kp has mostly been reported causing outbreaks in neonatal intensive care units. In a multicenter study of urinary and bloodstream nosocomial infections, the rate of ESBL-Kp was found to be 65%.[4–6] Clonal and polyclonal plasmid-mediated horizontal dissemination of resistance have also been documented in the hospital setting.[7]

Resistance mediated by ESBL is of great concern for many reasons. First, ESBLs can hydrolyze broad-spectrum cephalosporins and monobactams.[2] Second, these enzymes are encoded in plasmids that confer resistance to multiple antibiotics.[1] Third, this type of resistance is not easily detected by routine antimicrobial susceptibility testing.[8] Fourth, severe infections caused by ESBL-Kp may be associated with a poor clinical outcome.[9,10] The aim of this study was to describe the prevalence, risk factors, outcome, and molecular epidemiology of the cases of bloodstream infections caused by ESBL-Kp in a tertiary-care hospital for adults in Mexico.

Patients and methods

Setting and patients

This study was conducted at the National Institute of Medical Sciences and Nutrition, a 250-bed referral hospital for adult patients in Mexico City. A retrospective case–control study was conducted to assess risk factors for bloodstream infections due to ESBL-Kp. All patients with positive blood cultures for *K. pneumoniae* during the period January 1993 to December 2002 were included. Cases were patients with bacteremia caused by ESBL-Kp and controls were patients with bacteremia due to susceptible *K. pneumoniae*. Only one clinical isolate per patient was included. Patients whose medical records were unavailable were excluded. The following data were obtained: age, gender, length of hospitalization, hospital stay prior to the bacteremia, use of catheters (central venous, arterial, or urinary), nasogastric tube, or mechanical ventilation, and co-morbid conditions (liver cirrhosis, malignancy, diabetes mellitus, renal failure (indicated by a creatinine level of ≥2.0 mg/dl or the need for dialysis), HIV/AIDS, immunosuppressive therapy, active leukemia or lymphoma, or abdominal sepsis). Antimicrobial therapy received during hospitalization was also recorded.

Microbiological methods

Isolates were identified using Vitek-1 (bioMerieux Vitek, Saint Louis, MO, USA). Antimicrobial susceptibility to aztrenonam, cefepime, ceftazidime, ceftriaxone, imipenem, piperacillin/tazobactam, and ticarcillin/clavulanate; amikacin and gentamicin; ciprofloxacin, gatifloxacin, and ofloxacin, was determined by microbroth dilution according to Clinical and Laboratory Standards Institute (CLSI) recommendations.[11] ESBL production was determined by microbroth dilution according to new guidelines from the CLSI. Briefly, *K. pneumoniae* isolates with a ceftazidime minimum inhibitory concentration (MIC) >1 μg/ml were suspected of harboring ESBLs. MICs for these isolates were confirmed with ceftazidime alone and in combination with clavulanic acid by E-test (AB Biodisk, Solna, Sweden). A decrease of >3-fold in the MIC value for ceftazidime in combination with clavulanic acid versus the MIC value for ceftazidime alone was considered as confirmation of ESBL production. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive controls for ESBL production, respectively.

Genome macro-restriction analysis by pulsed field gel electrophoresis

Macro-restriction of bacterial genomic DNA was done with *Xba*I (Bio-Rad, Richmond, CA, USA). Pulsed field gel electrophoresis (PFGE) separation was performed with CHEF-DR-III Variable Angle System (Bio-Rad). DNA was run at 6 V/cm for 24 h at 14 °C, and the pulse time was increased from 5 s to 40 s. A lambda ladder (New England BioLabs, Beverly, MA, USA) was used to determine molecular size. Genetic relatedness was estimated with the Dice coefficient of similarity. Isolates were clustered if the degree of relatedness was ≥0.70. PFGE patterns were analyzed by GelPrint AQ v. 4.0 (Ann Arbor, MI, USA).[12] A dendrogram was constructed by the unweighted pair group method with arithmetic averages.

Isoelectric focusing and bioassay

β-Lactamases were extracted by ultrasonic treatment. Isoelectric focusing (IEF) was conducted as previously described[13] using a Phast system minigel with a pH range of 3 to 10 (Pharmacia, Uppsala, Sweden). Extracts from *E.*

coli J53-2 encoding TEM-1 (pBR322), SHV-2 (pMG229), and SHV-5 (pAFF2) ESBLs were used as standards to determine the isoelectric points (pI) of 5.4, 7.6, and 8.2, respectively. To determine the ESBLs encoded in the isolates, a bioassay was performed using cefotaxime (1 μg/ml) in the gel after a mix of soft agar and a susceptible *E. coli* (J53-2) culture.

Plasmid isolation and conjugation experiments

Plasmid-DNA was extracted from all case isolates ($N = 17$) and a group of randomly selected controls ($N = 17$), using standard methods.[14] Matting was performed in filters placed on solid LB-Luria medium according to Miller,[15] using *E. coli* J53-2 (F^-, *pro*, *met*, Rif^r) as the receptor strain. In all cases, transconjugants were selected on Luria agar supplemented with rifampin (100 μg/ml) in combination with cefotaxime (1 μg/ml), or ampicillin (50 μg/ml). From each successful matting experiment, 25 independent transconjugants were obtained from each selection medium and tested on Luria plates supplemented with ampicillin (100 μg/ml), cefotaxime (1 μg/ml), kanamycin (25 μg/ml), tetracycline (25 μg/ml), chloramphenicol (10 μg/ml), or gentamicin (1 μg/ml). Transconjugants were denoted with an X before the donor strain designation.

Plasmid DNA fingerprinting

Plasmid DNA purification from transconjugants and control clinical isolates was performed with QIAGEN Plasmid Midi Kit (QIAGEN, Hilden, Germany) ion exchange columns following the manufacturer's procedure. Fingerprinting analysis was performed with *Pst*I restriction enzyme (Promega, Madison, WI, USA and Gibco BRL, Gaithersburg, MD, USA). The resulting DNA fragments were analyzed in 2% agarose gel.

Colony hybridization with bla_{TEM} and bla_{SHV} probes

All ESBL-Kp isolates and the transconjugants were tested for colony hybridization.[16] Two PCR products were used as probes, *tem* and *shv*. Amplification of bla_{TEM}-related genes was obtained with DNA of plasmid pBR322 and primers OT1 and OT2, as previously described.[17] Primers used for amplification of bla_{SHV}-related genes were SE5 and SB3 as previously described,[4] using DNA of plasmid pMG229 coding for SHV-2. Probes were labeled with [α-^{32}P] dCTP using the Rediprime II kit (Amersham Biosciences, Vienna, Austria) following the manufacturer's recommendations.

DNA sequencing of the extended-spectrum β-lactamases

To determine the enzyme encoded in clinical isolates, five isolates were selected representing each β-lactamase profile (2995, 2985, 2982, 2987, and 2989). PCR amplification was performed using the same primers used for DNA bla_{SHV} probe (SE5 and SB3). Amplification products were sequenced with fluorescent-based Taq FS Dye terminator cycle sequencing kit using the same primers. Sequence analysis was performed with Genetics Computer Group software and BLASTx searching (EMBL, SwissProt, and PIR databases).[18]

Statistical analysis

Continuous variables were compared using the *t*-test or Mann–Whitney U-test. Qualitative variables were compared using the Chi-square test or Fisher's exact test, as appropriate. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. Multivariate logistic regression analysis was performed to determine variables that were independently associated with the risk of bacteremia caused by ESBL-Kp. Data were analyzed using the SPSS software package v. 10.0.

Results

Demographic characteristics and risk factors

During the study period, 130 patients with *K. pneumoniae* bacteremia were identified. Of these, 121 patients had their medical records available for review and were included for further analysis; the median age was 48.1 years (range 15–85 years) and 64 (52.8%) were women. Of the 121 patients, 17 (14.0%) had bacteremia due to ESBL-Kp and were considered as cases and the other 104 (86.0%) patients were controls. Sixty-four (52.9%) bacteremia episodes were acquired within the hospital; all episodes caused by ESBL-Kp ($N = 17$) were hospital-acquired in contrast with 47 out of 104 (45.2%) episodes of bacteremia caused by non-ESBL-producing Kp ($p < 0.0001$).

Cases were significantly younger than controls. Additionally, cases were more likely to have had longer hospitalizations prior to bacteremia (mean 10.4 vs. 29.0 days, $p = 0.01$) and to have been admitted to the intensive care unit (ICU) than controls (Table 1). When co-morbid conditions of the two groups were compared, cases were significantly more likely to have leukemia/lymphoma and abdominal sepsis than controls. Cases were also more frequently intubated, had a central venous catheter, an arterial catheter, a nasogastric tube, or a urinary catheter. Cases had more frequently received cephalosporins, aminoglycosides, and quinolones than controls (Table 1). After multivariate analysis, only prior use of cephalosporins (OR 7.6, 95% CI 1.1–53.5; $p = 0.039$) and stay in the ICU (OR 5.6, 95% CI 1.1–27.9; $p = 0.033$) remained as independent risk factors for bloodstream infection with ESBL-Kp. A trend for association between ESBL-Kp bacteremia and previous use of quinolones (OR 6.8, 95% CI 0.9–50.4; $p = 0.058$) was observed.

Outcome

There was no significant difference between cases and controls in crude mortality rate (35.0% vs. 26.9%; $p = 0.47$) or in the mean length of hospitalization after the episode of bacteremia (mean 20.9 vs.15.9 days; $p = 0.49$).

Antimicrobial susceptibility

Resistance rates to aminoglycosides, quinolones, ticarcillin/clavulanate, and piperacillin/tazobactam were higher in ESBL-Kp. All the isolates were susceptible to imipenem. Results of antimicrobial susceptibility testing are shown in Table 2.

Table 1 Clinical characteristics of cases and controls before the episode of *Klebsiella pneumoniae* bacteremia

Characteristic	Cases ($N = 17$)	Controls ($N = 104$)	p
Age, years ± SD	35.29 ± 18.32	50.21 ± 17.41	0.001 [a]
Male, *n* (%)	7 (41.2)	50 (48.1)	0.597 [b]
Stay in ICU, *n* (%)	15 (88.2)	17 (16.3)	<0.001 [b]
Co-morbid conditions			
Leukemia/lymphoma	7 (41.1)	15 (14.4)	0.008 [b]
Immunosuppressive treatment	5 (29.4)	15 (14.4)	0.123 [b]
Abdominal sepsis	4 (23.5)	6 (5.8)	0.014 [b]
Diabetes mellitus	3 (17.6)	0 (0)	0.239 [b]
Pancreatitis	2 (11.8)	6 (5.8)	0.356 [b]
End-stage renal disease	1 (5.9)	12 (11.5)	0.485 [b]
Other malignancies	1 (5.9)	19 (18.3)	0.202 [b]
HIV infection	1 (5.9)	4 (3.8)	0.696 [b]
Liver cirrhosis	0 (0)	12 (11.5)	0.140 [b]
Interventions during hospitalization			
Central venous catheter	13 (76.5)	49 (47.1)	0.025 [b]
Endotracheal tube	12 (70.6)	30 (28.8)	0.001 [b]
Urinary catheter	12 (70.6)	21 (20.2)	<0.001 [b]
Nasogastric tube	6 (35.3)	8 (7.7)	0.001 [b]
Arterial catheter	5 (29.4)	10 (9.6)	0.022 [b]
Surgery	4 (23.5)	35 (33.7)	0.408 [b]
Antibiotic use			
Cephalosporins	14 (82.4)	16 (15.4)	<0.001 [b]
Aminoglycosides	12 (70.6)	12 (11.5)	<0.001 [b]
Quinolones	5 (29.4)	9 (8.7)	0.013 [b]
Ureidopenicillins	2 (11.8)	9 (8.7)	0.679 [b]
Carbapenems	1 (5.9)	1 (1.0)	0.140 [b]

ICU, intensive care unit. Cases: patients with bacteremia caused by extended-spectrum β-lactamase producing *K. pneumoniae*; controls: patients with bacteremia due to susceptible *K. pneumoniae*.
[a] Student's *t*-test or Mann–Whitney U-test.
[b] Chi-square or Fisher's exact test.

Molecular typing

All the 121 isolates were typed by PFGE. Fifty-four different genotypes were identified; 21 isolates were unique and the remaining 100 were clustered in 33 different genotypes containing two to four isolates found throughout the entire study period. The 17 ESBL-Kp isolates were clustered in 11 genotypes, three of them with two or three isolates but without epidemiological relatedness after a critical review (Table 3).

Table 2 Antimicrobial drug resistance of *Klebsiella pneumoniae* among cases and controls

Antibiotic	Cases ($N = 17$)			Controls ($N = 104$)			p
	Range μg/ml	MIC 50/90	% Resistant	Range μg/ml	MIC 50/90	% Resistant	
Amikacin	1–64	16/64	29	0.25–16		0	<0.001
Gentamicin	0.125–16	8/16	70	0.062–16	0.25/8	17.3	<0.001
Ofloxacin	0.031–16	1/8	29	0.062–32	0.062/1	7.6	0.007
Gatifloxacin	0.031–16	0.062/16	29	0.015–8	0.031/0.5	5.7	0.002
Ticarcillin/clavulanate	64–128	128/128	100	1–128	16/128	44.2	<0.001
Piperacillin/tazobactam	2–128	128/128	94	0.125–128	4/64	20	<0.001
Imipenem	0.031–1	0.031/0.125	0	0.031–0.25	0.031/0.125	0	-

MIC, minimum inhibitory concentration. Cases: patients with bacteremia caused by extended-spectrum β-lactamase producing *K. pneumoniae*; controls: patients with bacteremia due to susceptible *K. pneumoniae*.

Table 3 Molecular characteristics of the 17 extended-spectrum β-lactamase producing *Klebsiella pneumoniae* clinical isolates

No. strain	Year of isolation	PFGE pattern	Plasmid (kb)	β-Lactamase pattern pI[a]	Transfer of CTX by conjugation	β-Lactamase produced by transconjugant[a]	Plasmid profile	Type of ESBL
2984	1993	19	200,170,115	5.4,7.6,(8.2)	+	5.4,(8.2)	A	
2985	1993	15	170,70	5.4,7.6,(8.2)	+	5.4,(8.2)	A	SHV-5
2981	1993	27	120,<40	5.4,(8.2)	+	5.4,(8.2)	A1	
2982	1994	14	120,65	5.4,(8.2)	+	5.4,(8.2)	A1	SHV-5
2990	1997	14	170,120,86	5.4,(8.2)	+	5.4,(8.2)	A2	
2991	2000	16	170	5.4,(8.2)	+	5.4,(8.2)	A3	
2993	2000	27	170	5.4,(8.2)	+	5.4,(8.2)	A3	
2986	1995	3	170,90	5.4,7.7,(8.2)	+	5.4,(8.2)	A3	
2989	1997	14	170	5.4,7.7,(8.2)	+	5.4,(8.2)	A3	SHV-5
2995	1993	23	170	7.6	+	7.6	A3	SHV-2
2997	1997	18	170,<40	5.4,(8.2)	+	5.4,(8.2)	A4	
2999B	2002	12	170,<40	5.4,7.8,8.0,(8.2)	+	5.4,(8.2)	B	
2999I	2002	19	170,120,54	7.6, (8.2)	+	(8.2)	C	
2987	1997	4	120,70	7.6, (8.2)	+	7.6, (8.2)	D	SHV-5
2992	2000	30	120	5.4,7.6,(8.2)	–	-	E[b]	
2983	1993	18	120	5.4,(8.2)	–	-	E[b]	
2994	2000	??	200,120,86	7.6	–	-	F[b]	

PFGE, pulsed field gel electrophoresis; CTX, cefotaxime resistance; ESBL, extended-spectrum β-lactamase.
[a] () Indicate the enzyme with cefotaximase activity (ESBL).
[b] The plasmid restriction pattern was obtained from the clinical isolate as these strains were not able to transfer resistance by conjugation.

Plasmid profiles and transfer of resistance

Each of the 17 ESBL-Kp contained one to three plasmids with sizes between 40 and 200 kb. We were able to transfer resistance to ampicillin and cefotaxime in 14 of the 17 isolates studied. Resistance genes were encoded within the largest plasmid identified. All transconjugants also expressed resistance to tetracycline, kanamycin, gentamicin, and chloramphenicol. We performed an enzyme restriction analysis to seek relationships between these plasmids. A major pattern A, with four subtypes, was identified in 11 plasmids of 14 transconjugants. This pattern was related in 10 of 11 plasmids expressing the same β-lactamase profile, which contained two enzymes with pIs of 5.4 and 8.2, with the exception of transconjugant 2995, which expressed a β-lactamase with a pI of 7.6. The other three plasmid profiles (B to D) obtained from the transconjugants were different. Restriction analysis of the remaining three plasmids that did not transfer resistance by conjugation showed the same pattern in two of them (isolates 2992 and 2983) and a completely unrelated pattern in the last one (isolate 2994) (Table 3, Figure 1).

Seventeen isolates randomly obtained from the controls contained from one to three plasmids with sizes between 40 and 200 kb. We could not obtain transconjugants from these isolates, therefore we prepared a plasmid from each clinical isolate that was digested with the same restriction enzyme (*Pst*I). We did not find a pattern among the 17 plasmids from controls or to those corresponding to the ESBL-Kp plasmids.

IEF analysis and enzyme inhibition test

Results of the IEF analysis are shown in Table 3. Two isolates exclusively showed an enzyme with a pI of 7.6; another two isolates showed a profile with pIs of 7.6 and 8.2. The remaining 13 isolates had a predominant profile that contained at least two enzymes with pIs of 5.4 and 8.2; six of these 13 isolates showed an additional enzyme. In all cases, enzymes with pIs of 8.2 or 7.6 hydrolyzed cefotaxime in the bioassay.

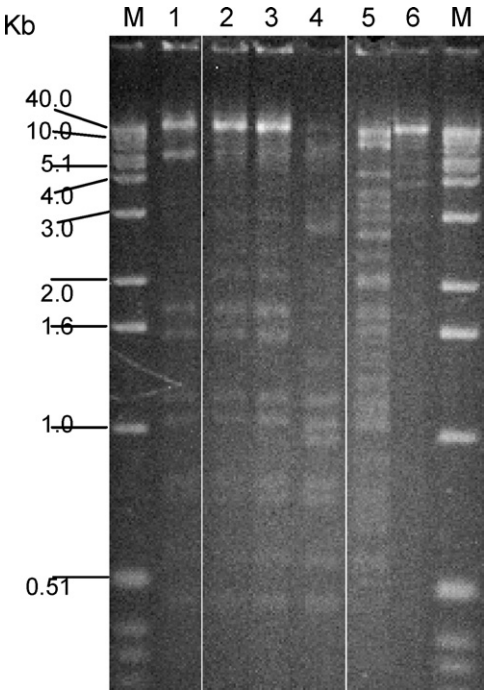

Figure 1 Representative gel from *Pst*1 restriction patterns of plasmids obtained from transconjugant SHV-derived producing *Klebsiella pneumoniae*. Lane M, DNA molecular size marker 1-kb ladder; lane 1, X2986; lane 2, X2984; lane 3, X2985; lane 4, X2986; lane 5, X2999A; lane 6, X2999B.

In the 14 transconjugants, we identified 13 with a pI 8.2 enzyme suggestive of ESBL activity; in 11 of them, an additional enzyme with a PI of 5.4 but without ESBL activity was also identified. Only one transconjugant showed the 7.6 pI enzyme with ESBL activity.

Molecular hybridization with bla_{TEM} and bla_{SHV} genes

After molecular hybridization, 11 ESBL-Kp isolates expressing enzymes with a pI of 5.4 were identified as TEM β-lactamase producers. All the ESBL-Kp isolates hybridized with the bla_{SHV} probe, as expected.

Sequencing of ESBL-encoding genes

To identify the type of ESBL encoded in ESBL-Kp, we selected five of the isolates according to the β-lactamase pattern obtained by IEF: 2982, 2985, 2987, 2989, and 2995 (Table 3). Isolates 2982, 2985, 2987, and 2989 expressed the 8.2 ESBL and contained the Gly238Ser and Glu240Lys substitutions that corresponded to the SHV-5 β-lactamase. DNA sequence of the PCR product of isolate 2995 producing the ESBL with a pI of 7.6 had the amino acid substitution of Gly238Ser, which corresponded to an SHV-2 enzyme.

Discussion

In this retrospective case—control study, we found that previous use of cephalosporins ($p = 0.039$) and stay in the ICU ($p = 0.033$) were independent risk factors for bloodstream infection with ESBL-Kp. Also, we observed a greater rate of crude mortality in those cases with an ESBL-producing organism (35.0%) compared with controls (26.9%), although this difference was not statistically significant. These factors have often been reported in other settings, however, they have been strongly associated with hospital-acquired infections within outbreaks and with higher crude mortality rates.[19—21] In our hospital, there does not appear to have been an outbreak situation, as we have demonstrated with molecular epidemiological tools, but horizontal dissemination of plasmids that contain ESBLs.[22]

The outcome in patients infected with ESBL-Kp was similar, in general terms, to the outcome of controls. There are several reasons that may explain this finding: (1) Our results clearly indicate that these isolates are diverse since they do not seem to be a part of an outbreak or a specific clone; (2) usually we initially prescribe at least two antibiotics to treat any episode of bacteremia, almost always including a β-lactam and an aminoglycoside; (3) once the presence of an ESBL-producing organism is confirmed, the initial therapy is modified accordingly; (4) the number of ESBL-Kp bacteremias may not be large enough to ensure statistical power.

These findings support the idea that bacteremia caused by ESBL-producing *K. pneumoniae* is not necessarily associated with a worse outcome. However, in our study and in other similar studies, other factors may influence the outcome, mainly patient characteristics (co-morbidities), the hospital site where the bacteremia was acquired (ICU vs. medical ward), length of hospitalization, the use of adequate vs. inadequate initial therapy, and an efficient infection control program.[23,24]

In contrast to other reports from Latin America, we identified the most common risk factors for infections due to ESBL-producing bacteria, but with a much lower prevalence.[25] The spread and intensity of cephalosporin resistance among enteric bacteria depend on several factors, and in our hospital, the horizontal and prolonged carriage of plasmid-encoded SHV-2 and -5 type β-lactamases seem to be the source of cephalosporin and multi-drug resistance.[26]

In summary, our findings highlight the need for a continuous microbiological and molecular screening in ESBL-producing organisms. This knowledge contributes to the understanding of the evolution and dissemination of SHV genes in the hospital setting.

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Ethical approval: The work describe here was evaluated and approved by the institutional review board.

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