PREVALENCE OF MUTATIONS CONFERRING EXTENDED-SPECTRUM ACTIVITY ON SHV β-LACTAMASES PRODUCED BY NOSOCOMIAL ISOLATES OF *ENTEROBACTERIACEAE* FROM RUSSIAN NATIONWIDE SURVEY

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Revised Abstract

Background: This study was performed to assess the prevalence of ESBLs in Russian nosocomial isolates with special focus on the group of SHV β-lactamases. We also aimed to determine the relative frequencies of mutations conferring ESBL activity on SHV enzymes.

Methods: A total of 648 consecutive nonduplicate isolates of *Enterobacteriaceae* were collected in 26 ICUs in 19 Russian cities in 2003. Production of ESBLs in isolates of *Escherichia coli* and *Klebsiella* spp. was detected by the NCCLS MIC method and in isolates of other species by the double-disc synergy test. Detection of bla_{SHV} genes and specific mutations at positions 146, 149, 156, 179, 238, and 240 which are known to confer ESBL activity on SHV enzymes was performed using the real-time PCR with MGB Eclipse probes. Laboratory strains producing the SHV-1 (non-ESBL control), SHV-2, 3 (S238), SHV-4, 5 (S238, K240), SHV-18 (A238, K240), SHV-6 (A179), SHV-8 (N179) and strains carrying cloned bla_{SHV} fragments to which the naturally-occurring mutations G179, D156, S149 and V146 were introduced by site-directed mutagenesis were used as controls.

Results: On the basis of a synergy between clavulanate and oxyimino-cephalosporins, 384 (59.4%) isolates were found to produce ESBLs. *K. pneumoniae* and *E. coli* accounted, respectively, for 48.4% and 26% of ESBL-positive isolates. Among ESBL producers, 241 strains carried bla_{SHV} genes of which 153 (63.5%) contained a mutation corresponding to a G238S amino acid change, and one contained a mutation leading to a D157E substitution. The first mutation was associated with an additional E240K change in 100 strains. SHV ESBLs were detected in 99 (44.4%) *K. pneumoniae*, 31 (16.9%) *E. coli*, 18 (21.2%) *Enterobacter* spp., 2 (13.6%) *Proteus mirabilis*, 2 (13.3%) *K. oxytoca*, 1 (7.1%) *Citrobacter freundii*, and 1 (100%) *Pantoea* spp. Ninety three isolates, most of which were *K. pneumoniae*, carried both mutant and wild type bla_{SHV} genes.

Conclusions: The results of this study indicate an alarmingly high and ever growing incidence of ESBL-producers in Russian ICUs. Despite the previously reported spread of CTX-M β-lactamases, SHV ESBLs are still very common in our population of strains. A G238S substitution is predominant in SHV ESBLs.

Introduction and Objectives

Resistance of nosocomial *Enterobacteriaceae* to β-lactams mediated by production of extended-spectrum β-lactamases (ESBLs) has emerged as an important public health problem worldwide. Results of the previous country-wide surveillance in Russia in 1997-98 demonstrated high incidence of CTX-M-type ESBLs in nosocomial *E. coli* and *K. pneumoniae* strains (M. Edelstein, 2003). But until now, little was known about the prevalence of other ESBL types, in particular, TEM and SHV types in Russia. This study was performed with the following aims:

- to assess the incidence of ESBLs in nosocomial strains isolated in different regions of Russia in 2003;
- to determine the types and prevalence of mutations conferring extended-spectrum activity on SHV β-lactamases;
- to evaluate the usefulness of the recently developed multiplex real-time PCR with MGB Eclipse probes for detection of SHV ESBLs.

Materials and Methods

Bacterial strains. The reference strains used in this study are listed in Table 1. These strains carried either natural plasmids encoding previously characterised SHV and CTX-M variants or recombinant vectors to which mutations conferring ESBL activity were introduced by site-directed mutagenesis.

Table 1. Control strains

Enzyme (Mutations)	Host strain	Plasmid	Reference
SHV controls			
SHV-1 (WT)	*E.coli* J53	R1010[n]	G. A. Jacoby, 1991
SHV-2 (238S)	*E.coli* J53	pMG229[n]	G. A. Jacoby, 1991
SHV-5 (238S; 240K)	*E.coli* J53	pAFF2[n]	G. A. Jacoby, 1991
SHV-18 (238A)	*K.pneumoniae* K6	[n]	J. K. Rasheed, 2000
SHV-6 (179A)	*E.coli* J53	[n]	G. Arlet, 1997
SHV-8 (179N)	*E.coli* DH5a	pCTA-8[r]	C. C. Randegger, 2000
SHV (179G)	*E.coli* TOP10	pGEM-179G[r]	A. Ekimov, 2004
SHV (156D)	*E.coli* TOP10	pGEM-156D[r]	A. Ekimov, 2004
SHV (149S)	*E.coli* TOP10	pGEM-149S[r]	A. Ekimov, 2004
SHV (146V)	*E.coli* TOP10	pGEM-146V[r]	A. Ekimov, 2004
CTX-M controls			
CTX-M-3	*C.freundii* 2525	[n]	M. Gniadkowski, 1998
CTX-M-5	*E.coli* TOP10	[n]	M. Edelstein, 2004
CTX-M-9	*E.coli* MSP493	[n]	M. Sabaté, 2000

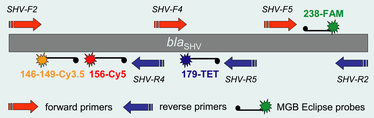
Consecutive nonduplicate nosocomial isolates of *Enterobacteriaceae* (n=648) were collected in 26 ICUs in 19 Russian cities: Cheliabinsk, Ekaterinburg, Irkutsk, Kazan, Krasnodar, Krasnoyarsk, Moscow, Novosibirsk, Omsk, Perm, Smolensk, St. Petersburg, Stavropol, Tolyatti, Tomsk, Tyumen, Ufa, Vladivostok and Voronezh in 2003. Species identification was performed using the API20E system (bioMerieux, France), and the strains were stored at -70°C until analysis.

Phenotypic ESBL detection. ESBL production was detected in *E.coli* and *K. pneumoniae* by the agar dilution procedure according to the NCCLS MIC methodology and by the double disk synergy test (DDST) with disks containing cefotaxime (30 μg), ceftazidime (30 μg), cefepime (30 μg) and amoxicillin-clavulanic acid (20+10 μg) in isolates of other species.

Preparation of template DNA for PCR. A 1 μl loopful of bacteria harvested from an agar plate was suspended in 100 μl of autoclaved MilliQ-water and heated at 99°C for 20 min. Heat-treated samples were centrifuged at 10000 g for 3 min. Two microliters of supernatant were used in PCR.

Detection of SHV-type ESBLs by real-time PCR and melting curve analysis. A single-tube PCR with four MGB Eclipse probes (Epoch Biosciences, USA) that perfectly match the wild-type (WT) bla_{SHV}-gene sequences at mutation sites corresponding to amino acids 146-149, 156, 179 and 238 was used to identify SHV ESBLs. The probes were labelled with different dyes (FAM, TET, Cy3.5 and Cy5) to allow multiplexing. Each reaction also included three pairs of primers complementary to the highly conserved regions flanking mutation sites (Fig. 1). Amplification was performed using the asymmetric (1:8) ratio of primers complementary to the same strand as the respective probes and opposite strand primers. A 50-cycle PCR was followed by melting curve analysis on a Rotor-Gene 2000 real-time PCR system (Corbett Research, Australia). Fluorescence detection was conducted on the four standard channels: FAM (410/510 nm), JOE (530/555 nm), ROX (585/610 nm) and Cy5 (625/660 nm).

Fig. 1. Primers and probes mapped to the bla_{SHV} gene internal sequence.



An additional PCR with primers SHV-F5 and SHV-R2 and the FAM-labeled probe complementary to the nucleotides 693-704 with respect to translation starting point of bla_{SHV}-1 was used to detect an E240K mutation.

The bla_{SHV} gene of a single *K.pneumoniae* isolate (1043) that produced an atypical melting profile with the probe 156-Cy5 was cloned in a pCC1 vector and sequenced on both strands using a BigDye Terminator Cycle Sequencing Kit v 3.1 on an ABI 310 automated sequencer (Applied Biosystems, USA).

Detection and subtyping of CTX-M-type ESBLs by PCR-RFLP. The bla_{CTX-M} genes were detected by PCR with primers CTX-M/F' and CTX-M/R' and assigned to either of the known genetic clusters on the basis of restriction analysis with endonucleases *Pst* I and *Pvu* II, as previously described (M. Edelstein, 2003).

Results and Discussion

Incidence of ESBL production. A total of 648 consecutive enterobacterial isolates were studied. Out of these isolates, 384 (59.4%) were found to produce ESBLs, as detected by phenotypic tests and confirmed by molecular methods. Another 16 isolates gave suspicious results in a DDST but were not confirmed as ESBL producers by either of molecular methods used. The incidence of ESBL producers in different ICUs varied from 20% to more than 90% (Fig. 2).

Fig. 2. PIncidence of ESBL production among nosocomial Enterobacteriaceae from various Russian cities.Only the cities that provided 10 consecutive enterobacterial isolates are shown. Data from the hospitals located in the same cities are pooled together.

The majority of ESBL-positive strains belonged to *K. pneumoniae* (48.4%) and *E. coli* (26%) - the most commonly isolated enterobacteria. At the same time, ESBLs were also found in all the other species of *Enterobacteriaceae*, including those with intrinsic production of molecular class C β-lactamases: *Enterobacter* spp., *Serratia marcescens*, *Citrobacter freundii*, *Morganella morganii* and *Providencia* spp. which jointly comprised 19% of ESBL producers (Fig. 4).

Fig. 3. Production of ESBLs in different species

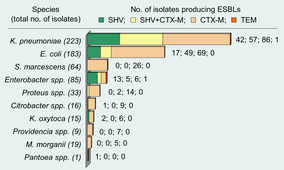
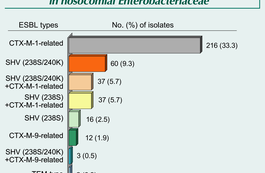


Fig. 4. Frequencies of various ESBL types in nosocomial Enterobacteriaceae

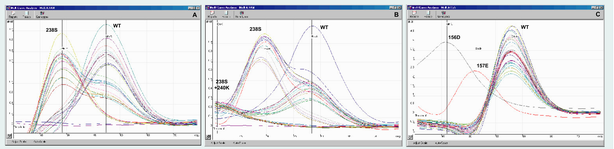


In 2003 the rates of ESBL production increased up to 84.3% in *K. pneumoniae*, 54.7% in *E. coli*, and 60.9% in *P. mirabilis*, as compared to 60.2%, 15.8% and 18.9%, respectively, in the late 1990-s.

Distribution of ESBL types. The relative frequencies of different ESBL types in nosocomial *Enterobacteriaceae* are shown in figure 4. The most common ESBL types were those belonging to the CTX-M-1 genetic cluster, namely the CTX-M-3 and CTX-M-15. Their proportion increased more than twice since 1998. β-Lactamases of the CTX-M-9 cluster were detected for the first time in Russia. Fourteen *E. coli* and a one *K. pneumoniae* isolate producing the CTX-M-9-related enzymes were isolated in 5 geographically distant cities: Moscow, St. Petersburg, Tyumen, Novosibirsk and Krasnodar.

Using the real-time PCR, 241 strains ESBL-producing strains were found to carry bla_{SHV} genes. A mutation corresponding to a G238S amino acid change in SHV β-lactamase was detected either alone or in combination with an E240K substitution in 53 and 100 strains, respectively (Fig 4, 5A, 5B). An atypical melting profile of the probe 156-Cy5 obtained with a single *K.pneumoniae* isolate from Moscow indicated the presence of previously unknown mutation (Fig. 5C).

Fig. 5. Detection of mutations conferring ESBL activity on SHVs by melting curve analysis with probes 238-FAM (A), 238-240-FAM (B) and 156-Cy5 (C).



Sequencing of the whole gene from these isolate revealed a new SHV variant differing from SHV-1 by two mutations: D157E and S196N. Their effect on the activity of enzyme is currently under investigation. No other mutations at amino acid positions known to be associated with ESBL activity on SHV enzymes were detected.

Ninety three isolates, including 89 *K. pneumoniae* and 4 enterobacters, carried both mutant and wild type bla_{SHV} genes. A co-production of SHV- and CTX-M-type ESBLs was detected in 78 isolates of five species.

ESBLs of TEM-type were found only in one *K. pneumoniae* and one *Enterobacter aerogenes* isolate.

Conclusions

- The results of this study indicate a very high incidence (59.4%) of ESBL production among nosocomial *Enterobacteriaceae* isolated in Russian ICUs in 2003. To our knowledge, this is the highest incidence reported worldwide.
- The CTX-M-type β-lactamases identified in 47.2% of *Enterobacteriaceae* have become the most common ESBLs in Russian ICUs, while the SHV-type ESBLs were detected in 23.6% of strains.
- A G238S substitution was found in almost all SHV ESBLs and was commonly associated with an additional E240K substitution.
- A real-time PCR with MGB Eclipse probes proved to be highly effective for detection of SHV ESBLs. The advantages of this method over the other molecular tests include high speed and processivity, broad range of mutations that can be accuracy detected and discriminated in a single reaction, a possibility to identify multiple bla_{SHV} alleles and new mutations at given sites and reduced risk of false positive results due to PCR contamination.

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