Surveillance of antimicrobial susceptibility of Enterobacteriaceae pathogens isolated from intensive care units and surgical units in Russia

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A total of 473 strains of Enterobacteriaceae, including *Escherichia coli*, *Klebsiella* spp., *Proteus* spp., *Citrobacter* spp., *Enterobacter* spp., *Serratia* spp. and *Providencia* spp., were isolated from patients admitted to intensive care units and surgical units in Russia. About 90% of the isolates carried factors resistant to beta-lactams. The isolation rates of the extended-spectrum beta-lactamase (ESBL) producer defined in this study among *E. coli*, *Klebsiella* spp. and *Proteus* spp. were 45%, 48% and 17%, respectively. In the settings with high prevalence of the ESBL producer, flomoxef, which belongs to the oxacephem subgroup, and carbapenems retain their activity. The MIC_{50} of flomoxef, meropenem and imipenem against total isolates were 1 μg/mL, $\leq$0.063 μg/mL and 0.25 μg/mL, respectively. Fifty-five carbapenem-resistant strains were isolated in this study. The carbapenem resistant rates of *E. coli*, *Klebsiella* spp. and *Proteus* spp. were 3%, 16% and 29%, respectively.

Introduction

Global spreading of antimicrobial resistance is one of the most serious threats for the health-care system. The Russian Federation is one of the countries with an extremely high prevalence of multi-resistant bacteria in hospital settings. One surveillance study for selected European nations including Russia reported that beta-lactamase–mediated mechanisms have spread widely among Gram-negative bacilli, especially across the Eastern and Southern European nations[1)]. This is likely due to expanded use of beta-lactams without the activity against Gram-negative bacilli in

daily therapy.

One way of controlling the spread of antimicrobial resistance is by introducing known antibiotics, which are not widely used but have important properties, into clinical practice. One such antibiotic is flomoxef, a cephem antibiotic of the oxacephem subgroup. Flomoxef has not been marketed in European countries and Russia.

To investigate the latest *in vitro* susceptibility to beta-lactams widely used in clinical practice, we conducted an epidemiological study of clinically significant isolates of Enterobacteriaceae in Russia.

Materials and Methods

Bacterial isolates and identification

Isolates were collected in 2012 from local laboratories of 17 hospitals in the western part of Russia. Thirteen institutes were in Saint Petersburg and four were in Moscow, Republic of Karelia, Stavropol Krai and Kurgan. Isolates were obtained from patients with hospital infections (pneumonia, intraabdominal infections, skin and soft tissue infections, infections of urinary tract, and sepsis) admitted to intensive care units and surgical units specializing in various areas of surgery. Isolation and identification of bacteria were done in laboratories of healthcare institutions by means of widely accepted methods. The collected isolates were submitted to the laboratory of the Scientific Research Institute of Children's Infections. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has been used for the confirmation of identification of the submitted strains. Bacteria from isolated colonies were applied, using sterile loops, as thin films on polished stainless steel target plates (Bruker Daltonik GmbH, Leipzig, Germany) in three replicates. The bacteria were then left to dry at room temperature for 1 min. Subsequently 1.5 μL of the freshly prepared matrix solution, comprising a saturated α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid, was applied to the samples and cocrystallized with them at room temperature for 10 min. The samples were applied to a MicroFlex LT mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany), and the results were analyzed by MALDI Biotyper 2.0 software package (Bruker Daltonik GmbH, Leipzig, Germany) according to the manufacturer's instructions.

Antimicrobial susceptibility testing

The susceptibility of Enterobacteriaceae to antimicrobial agents was assessed by serial microdilution in Mueller-Hinton broth (Becton, Dickinson & Co., Maryland, USA) in accordance with Clinical and Laboratory Standards Institute (CLSI) recommendations and reading criteria in 96-well plates for immunological studies[2]. Substances of antibacterial products with known activity were used to prepare working antibiotic solutions. The following antibiotics were tested:

ampicillin, cefotaxime, ceftazidime, cefepime, cefoperazone, cefoperazone/sulbactam (fixed ratio of 2:1), flomoxef, imipenem, meropenem, amikacin, gentamicin, and ciprofloxacin. Flomoxef was gifted from Shionogi & Co., Ltd. (Osaka, Japan) and other antibiotics were obtained from Molekula GmbH (Munich, Germany). The following ranges of final antibiotic concentrations in the wells were used to determine the MIC: 0.063 to 64 μg/mL for ampicillin, cefotaxime, ceftazidime, cefepime, cefoperazone, flomoxef, amikacin, gentamicin and ciprofloxacin; 0.063/0.032 to 64/32 μg/mL for cefoperazone/sulbactam; 0.008 to 64 μg/mL for imipenem and meropenem. Each new series of plates underwent three types of testing: testing of the suitability of the antibacterial agents used with *Escherichia coli* strain ATCC 25922, monitoring of the growth of the strains under the test conditions, and monitoring of the sterility of the substrate applied (Mueller-Hinton broth).

Detection of ESBL-producing strain

ESBL production among *E. coli*, *Klebsiella* spp. and *Proteus mirabilis* was detected by the method recommended by the CLSI[3]. In screening for ESBL production, if the MIC of cefotaxime or ceftazidime was ≥2 μg/mL, the tested strain was classified as Screening (+). Otherwise it was classified as Screening (−). For confirmation of ESBL production, the ceftazidime MIC against Screening (+) strains was compared with the MIC of ceftazidime in combination with clavulanate (fixed clavulanate concentration of 4 μg/mL). A ≥8-fold (≥3 twofold dilution) decrease in the MIC of ceftazidime in combination with clavulanate versus the MIC of ceftazidime was defined as ESBL (+).

Results

Escherichia coli

The results of testing *E. coli* susceptibility to antibiotics are presented in Table 1. Most of the isolates were non-susceptible to ampicillin, over 70% of the isolates showed MIC >64 μg/mL. MIC_{50} values of the cephalosporins cefotaxime, ceftazidime, cefepime and cefoperazone were 64 μg/mL, 16 μg/mL, 8 μg/mL and 64 μg/mL, respectively. The MIC_{50} of cefoperazone/sulbactam was 8 μg/mL, which was 8 times lower than that of cefoperazone alone. A synergistic effect of cefoperazone with a beta-lactamase inhibitor (sulbactam) was observed. The MIC_{50} of flomoxef was 1 μg/mL and its mode of isolates in the susceptibility distribution was at 0.125 μg/mL.

The isolates were most susceptible to carbapenems with MIC_{50} of 0.25 μg/mL for imipenem and ≤0.063 μg/mL for meropenem.

***Klebsiella* spp.**

The susceptibility of *Klebsiella* spp. is presented in Table 2. MIC_{50} values of cephalosporins,

Table 1. Susceptibility distribution of *E. coli*

Antibiotic	MIC, μg/mL												MIC_{50}	MIC_{90}	Total
	≤ 0.063	0.125	0.25	0.5	1.	2.	4.	8.	16.	32.	64.	> 64			
Ampicillin					2	2	3	5	7	7	12	104	> 64	> 64	142
Cefotaxime	7	21	3	4	7	3	3	6	4	5	8	71	64	> 64	142
Ceftazidime	2	14	11	4	6	3	3	13	20	13	12	41	16	> 64	142
Cefepime	13	26	2	2	3	3	7	17	16	17	9	27	8	> 64	142
Cefoperazone	5	7	7	6	4	5	3	6	11	5	15	68	64	> 64	142
Cefoperazone/sulbactam	7	21	3	8	7	8	15	6	16	19	8	24	8	> 64	142
Flomoxef		49	5	5	14	10	12	12	3	7	10	15	1	> 64	142
Imipenem	32	34	37	19	14	4	2						0.25	1	142
Meropenem	104	5	14	9	6		1	1	2				≤ 0.063	0.5	142
Amikacin	2	3		8	11	15	21	26	8	1	4	43	8	> 64	142
Gentamicin	3	4	3	6	14	10	14	10	3	10	9	56	32	> 64	142
Ciprofloxacin	11	21	3	5	3	3	10	4	11	12	14	45	16	> 64	142

Table 2. Susceptibility distribution of *Klebsiella* spp.

Antibiotic	MIC, μg/mL												MIC_{50}	MIC_{90}	Total
	≤ 0.063	0.125	0.25	0.5	1.	2.	4.	8.	16.	32.	64.	> 64			
Ampicillin			1					2	5	5	8	167	> 64	> 64	188
Cefotaxime	2	12		2	3	1		5	5	7	3	148	> 64	> 64	188
Ceftazidime	2	6	4	3	1	1	1	15	17	27	23	88	64	> 64	188
Cefepime		15	2	1	4	1	6	21	30	36	24	48	32	> 64	188
Cefoperazone	8	4	3	4	4	2	2	1	2	13	10	135	> 64	> 64	188
Cefoperazone/sulbactam	8	11	1	3	3	2	7	13	25	29	29	55	32	> 64	186
Flomoxef	1	58	6	12	9	11	11	13	16	9	6	36	2	> 64	188
Imipenem	22	29	38	37	20	14	9	4	9	5			0.5	4	187
Meropenem	88	16	16	16	15	7	6	4	7	4	8	1	0.125	16	188
Amikacin	1	3		4	18	13	8	16	7	7	9	102	> 64	> 64	188
Gentamicin		2	6	2	15	2	8	8	8	5	6	126	> 64	> 64	188
Ciprofloxacin	5	16	1	2	4	3	3	7	16	23	16	92	64	> 64	188

cefotaxime, ceftazidime, cefepime and cefoperazone were ＞64μg/mL, 64μg/mL, 32μg/mL and ＞64μg/mL, respectively. The MIC_{50} of cefoperazone/sulbactam was 32μg/mL, which was lower than that of cefoperazone alone. The MIC_{50} of flomoxef was 2μg/mL and its mode of isolates in the susceptibility distribution was at 0.125μg/mL.

The isolates were most susceptible to carbapenem antibiotics with MIC_{50} of 0.5μg/mL to imipenem and 0.125μg/mL to meropenem. The susceptibility distribution of *Klebsiella* spp. showed some carbapenem-resistant isolates (MIC ≥4μg/mL). The resistance rate was 14% (27/187) to imipenem and 16% (30/188) to meropenem.

***Proteus* spp.**

The results of *Proteus* spp. were separately described by indole production in Table 3. One was an indole-negative strain, *P. mirabilis*, and the other was an indole-positive group. Of the 52 isolates, 43 were *P. mirabilis*.

Most of the *P. mirabilis* isolates were non-susceptible to ampicillin, over 70% were showing MIC ≥64μg/mL. MIC_{50} values of cephalosporins, cefotaxime, ceftazidime, cefepime and cefoperazone were 32μg/mL, 2μg/mL, 4μg/mL and 32μg/mL, respectively. Addition of sulbactam to cefoperazone resulted in a MIC_{50} shift to a lower value. The MIC_{50} of cefoperazone alone, 32μg/mL, was shifted to 1μg/mL by addition of sulbactam. The MIC_{50} of flomoxef was 0.5μg/mL and its susceptibility distribution pattern was similar to cefepime; both modes of isolates in the susceptibility distribution were at 0.125μg/mL.

The indole-positive group was less susceptible to antibiotics than *P. mirabilis*. MIC_{50} of cefotaxime, ceftazidime, cefepime, cefoperazone and flomoxef were ＞64μg/mL, 8μg/mL, 8μg/mL, 64μg/mL and 32μg/mL, respectively.

Among the *Proteus* spp., only one isolate (*P. mirabilis*) was resistant to meropenem (MIC ≥ 4μg/mL) but 15 isolates including 3 indole-positive isolates were resistant to imipenem (MIC ≥ 4μg/mL). Lower susceptibility to imipenem, which is a natural feature of *Proteus* species, was observed.

Enterobacteriaceae encoding chromosomal AmpC beta-lactamases

Table 4 presents results for *Citrobacter* spp., *Enterobacter* spp., *Serratia* spp. and *Providencia* spp. Bacteria of these species were classified into one group based on the mechanism of resistance to beta-lactam antibiotics. Bacteria in this group have inducible gene expression of chromosomal AmpC beta-lactamases[4]. MIC_{50} of cephalosporins, cefotaxime, ceftazidime, cefepime and cefoperazone were ＞64μg/mL, 8μg/mL, 8μg/mL and ＞64μg/mL, respectively. The MIC_{50} of cefoperazone with sulbactam was 8μg/mL, which was 16 times lower than that of cefoperazone alone. The MIC_{50} of flomoxef against these species was 4μg/mL and the susceptibility distribution was as broad as that of the cepharosporins.

Table 3-A. Susceptibility distribution of *Proteus mirabilis*

Antibiotic	MIC, μg/mL												MIC_{50}	MIC_{90}	Total
	≤ 0.063	0.125	0.25	0.5	1.	2.	4.	8.	16.	32.	64.	> 64			
Ampicillin			1					3	4	4	3	28	> 64	> 64	43
Cefotaxime	3	9		3	3	1		1	1	1	2	19	32	> 64	43
Ceftazidime	1	8	4	2	3	5	4	5	1		1	9	2	> 64	43
Cefepime	3	14		1	1	2	5	4	4	2	2	5	4.	> 64	43
Cefoperazone	4	4		2	3	2	1	1	1	3	2	19	32.	> 64	42
Cefoperazone/sulbactam	3	9	6	1	5	3	1	4		3	3	4	1.	64	42
Flomoxef		19	2	4	5	3	3	3	3			1	0.5	8	43
Imipenem	4	3	5	6	6	7	10	2					1.	4	43
Meropenem	25	7	5	4	1		1						≤ 0.063	0.5	43
Amikacin		1			1	6	8	6	1	2	4	14	8	> 64	43
Gentamicin	1		1	3	6	5	3	2	1	1	2	18	16	> 64	43
Ciprofloxacin	2	8		2		3	1	4	8	5	4	6	16	> 64	43

Table 3-B. Susceptibility distribution of *Proteus* spp. (indole-positive group)

Antibiotic	MIC, μg/mL												Total
	≤ 0.063	0.125	0.25	0.5	1.	2.	4.	8.	16.	32.	64.	> 64	
Ampicillin					1							8	9
Cefotaxime		2										7	9
Ceftazidime		2				2		1		1	1	2	9
Cefepime		2						3		1	2	1	9
Cefoperazone				1	1				1	1	1	4	9
Cefoperazone/sulbactam		1			1	1	1	1		3		1	9
Flomoxef		2					1			2	1	3	9
Imipenem		1	1	1	1	2	2	1					9
Meropenem	4	1		2	2								9
Amikacin					1			1	1		1	5	9
Gentamicin		1							1	1	2	4	9
Ciprofloxacin				1			1	3			1	3	9

Table 4. Susceptibility distribution of Enterobacteriaceae encoding chromosomal AmpC beta-lactamases*

Antibiotic	MIC, μg/mL														
	≤ 0.063	0.125	0.25	0.5	1.	2.	4.	8.	16.	32.	64.	> 64	MIC_{50}	MIC_{90}	Total
Ampicillin								2	3	5	6	75	> 64	> 64	91
Cefotaxime	2	9	1	2	3	4	3	5	1	2	6	52	> 64	> 64	90
Ceftazidime	1	5	8	6	11	6	5	4	5	10	7	23	8	> 64	91
Cefepime	5	15	2	1	4	1	10	13	15	9	10	6	8	> 64	91
Cefoperazone	4	3	1	1	6	2		3	8	5	8	50	> 64	> 64	91
Cefoperazone/sulbactam	3	8	3	2	4	10	9	7	7	13	11	14	8	> 64	91
Flomoxef	1	10	2	7	8	11	9	4	7	8	8	16	4	> 64	91
Imipenem	14	10	11	20	18	8	4	1	1	4			0.5	4	91
Meropenem	61	9	7	2	3		3		1	5			≤ 0.063	1	91
Amikacin	1	2		4	8	13	13	11	6	5	1	26	8	> 64	90
Gentamicin	1		6	3	4	4	9	4	3	4	8	45	64	> 64	91
Ciprofloxacin	8	7	1	4	5	6	3	6	12	10	7	22	16	> 64	91

* *Citrobacter* spp., *Enterobacter* spp., *Serratia* spp., *Providencia* spp.

Table 5-A. Detection of ESBL-producing strain

Bacteria	Total number	Screening (+)			Carbapenem resistant number (%)
		Sub-total number (%)	ESBL (+) number (%)	ESBL (-) number (%)	
E. coli	142	111 (78)	64 (45)	47 (33)	4 (3)
Klebsiella spp.	188	176 (94)	91 (48)	85 (45)	30 (16)
Proteus spp.	52	38 (73)	9 (17)	29 (56)	15 (29)

Screening (+): MIC of ceftazidime ≥2μg/mL or cefotaxime ≥2μg/mL.
ESBL (+): ≥8-fold decrease in MIC of ceftazidime in combination with clavulanate versus their MIC of ceftazidime alone among Screening (+) strains.
Carbapenem resistant contains strains of MIC of meropenem ≥4μg/mL or imipenem ≥4μg/mL.

Table 5-B. Activity of flomoxef and reference antibiotics for Screening (+) and Screening (−)

Antibiotic	Screening (+) n = 325		Screening (-) n = 57	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
	μg/mL	μg/mL	μg/mL	μg/mL
Ampicillin	> 64	> 64	32.	> 64
Cefotaxime	> 64	> 64	0.125	0.5
Ceftazidime	64.	> 64	0.125	0.5
Cefepime	32.	> 64	0.125	1.
Cefoperazone	> 64	> 64	0.5	32.
Cefoperazone/sulbactam	32.	> 64	0.125	8.
Flomoxef	2.	> 64	0.125	8.
Imipenem	0.25	4.	0.25	0.5
Meropenem	≤ 0.063	4.	≤ 0.063	0.25
Amikacin	> 64	> 64	4.	64.
Gentamicin	> 64	> 64	1.	16.
Ciprofloxacin	> 64	> 64	0.125	32.

Screening (+): MIC of ceftazidime ≥ 2 μg/mL or cefotaxime ≥ 2 μg/mL.
Screening (-): MIC of ceftazidime < 2 μg/mL or cefotaxime < 2 μg/mL.

Table 5-C. Susceptibility distribution of ESBL (+) strains

Antibiotic	MIC, μg/mL												MIC_{50}	MIC_{90}	Total
	≤ 0.063	0.125	0.25	0.5	1.	2.	4.	8.	16.	32.	64.	> 64			
Cefotaxime	2	3	1		5	3	3	4	4	11	4	124	> 64	> 64	164
Ceftazidime					4	1	1	16	23	32	28	59	64	> 64	164
Cefepime	7	4		1	5	3	8	25	29	31	17	34	16	> 64	164
Flomoxef		61	8	10	13	12	11	10	10	9	7	13	1	64	164
Meropenem	97	17	22	11	10	1	2	1	2		1		≤ 0.063	1	164

ESBL (+): ≥ 8-fold decrease in MIC of ceftazidime in combination with clavulanate versus their MIC of ceftazidime alone among Screening (+) strains.
Screening (+) contains strains of MIC of ceftazidime ≥ 2 μg/mL or cefotaxime ≥ 2 μg/mL.

The MIC_{50} of carbapenems was 0.5 μg/mL for imipenem and ≤0.063 μg/mL for meropenem.

Detection and susceptibility of ESBL-producing strain

Comparison of the susceptibility distribution between cefoperazone and that of cefoperazone with sulbactam indicated the presence of ESBL-producing strains in *E. coli*, *Klebsiella* spp. and *Proteus* spp. To further understand the results, we classified possible ESBL producers and carbapenem-resistant strain producers (Table 5-A).

For ESBL screening, cephalosporin non-susceptible isolates were classified as Screening (+). For ESBL confirmation, isolates showing synergy between ceftazidime and clavulanate in Screening (+) were defined as ESBL (+). The ESBL (+) detection rate was 45% in *E. coli*, 48% in *Klebsiella* spp. and 17% in *Proteus* species.

The carbapenem-resistant rate was 3% for *E. coli*, 16% for *Klebsiella* spp. and 29% for *Proteus* spp. Most of them (39/49) were included in the ESBL (−) subgroup, which did not show synergy between ceftazidime and clavulanate (data not shown).

To clarify the unique activity of flomoxef among cephems, we compared activities against Screening (+) and Screening (−) subgroups (Table 5-B). The MIC_{50} of cephems, except for flomoxef, against the Screening (+) subgroup was 32 μg/mL or higher. Intriguingly, the MIC_{50} of flomoxef was 2 μg/mL. The activities of flomoxef and major beta-lactams against ESBL (+) are presented in Table 5-C. Meropenem was the most potent with an MIC_{50} ≤0.063 μg/mL and the mode of the isolates in the susceptibility distribution was at ≤0.063 μg/mL. The MIC_{50} of flomoxef was 1 μg/mL and the mode of the MIC distribution was 0.125 μg/mL.

Discussion

This study showed that beta-lactam non-susceptible Enterobacteriaceae had spread widely in intensive care units and surgical units in Russia. The obtained results generally correspond to those published earlier concerning the spread of ESBL in Russia. In 1999, it was reported that the prevalence of ESBL among *Klebsiella* spp. was more than 40%[5], and in 2009, this parameter reached 67%[6].

In the settings with high prevalence of ESBL-producer in Russia, flomoxef retained activity against cephalosporin-resistant isolates of Enterobacteriaceae. The advantages of flomoxef over other cephalosporins can be explained by its resistance to ESBL hydrolysis[7]. In Eastern Asia, flomoxef is recently being re-evaluated in the face of globally spreading ESBL-producing bacteria[8~11]. Of course, it should be noted that flomoxef is not a universal antibiotic. The range of flomoxef MIC_{50} against Enterobacteriaceae, except for the indole-positive *Proteus* group, is 0.5–4 μg/mL, which is lower than those of cepharosporins. However, the MIC_{90} values of flomoxef in this study against *E. coli*, *Klebsiella* spp. and Enterobacteriaceae encoding chromo-

somal AmpC beta-lactamases were $>64\mu$g/mL. It has been reported from Taiwan that high rates of resistance were observed for flomoxef among Enterobacteriaceae carrying AmpC beta-lactamases and/or carbapenemase such as metallo-beta-lactamase or KPC (*Klebsiella pneumoniae* carbapenemase)[12]. The non-susceptible strains in our study could carry those beta lactamases.

Our findings show that flomoxef may be considered as a treatment option of community- and hospital-acquired localized infections caused by Enterobacteriaceae in Russia. This should help prevent the overuse of carbapenems.

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Ethical approval: Not required.

Conflicts of interest

This study was conducted at Scientific Research Institute of Children's Infections under the contract research for Nycomed Russia/CIS.

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