MULTILOCUS SEQUENCE TYPING OF *ACINETOBACTER* STRAINS FROM RUSSIA AND BELARUS PRODUCING ACQUIRED OXA CARBAPENEMASES

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INTRODUCTION

Nosocomial infections due to *Acinetobacter* strains represent a major therapeutic challenge. Nowadays, such infections are renowned for being difficult to treat because of emergence of multiple drug resistant Acinetobacters. Particularly, resistance to carbapenems that is often mediated by production of acquired carbapenem-hydrolysing class D β-Lactamases (CHDLs) of three groups: OXA-23, OXA-40 and OXA-58, is becoming an increasing problem in Acinetobacters.

The aim of this study was to assess by multilocus sequence typing (MLST) the genetic diversity of clinical CHDL-producing *Acinetobacter* strains isolated in Russia and Belarus.

METHODS

Bacterial isolates. The 15 isolates studied represented 1 sporadic case and 10 nosocomial outbreaks of carbapenem-resistant Acinetobacter infections that occurred in 9 hospitals of 6 cities of Russia and in 2 cities of Belarus in 1998-2008. The sources, dates of isolation and types of infections caused by these isolates are shown in Table 2. The geographic origins of these isolates are also shown in Fig. 1.

Detection of CHDL genes. The genes for acquired OXA-type carbapenemases were detected by PCR with primers listed in Table 1 [Martinovich et al., ECCMID 2008, P1505].

MLST. Isolates were typed according to MLST scheme of Bartual et al. [JCM 2005, 43:4382-90]. The typing protocol, however, was modified by the introduction of new primers for amplification and sequencing of all 7 loci: *gltA, gyrB, gdhB, recA, cpn60, gpi*, and *rpoD* of *A. baumannii* (Table 1).

The new primers were designed to anneal at highly conserved positions flanking each locus to be sequenced and thus to contain no degeneracy. Using these primers, all loci were amplified under the same conditions. The PCR mixtures (25 µl) contained: 0.5 µM each primer, 200µM dNTPs, 1.5 mM $MgCl_2$, 1.5U of Taq-F DNA-polymerase (Interlabservice, Russia), 67 mM Tris-HCl (pH=8.3), 17 mM $(NH_4)_2SO_4$, 0.1% Tween-20, 0.12 mg/ml BSA, 8% glycerol, 2% DMSO and 3 µl of template DNA prepared by rapid boiling of bacteria (1-3 colonies) in TE buffer. Thermal cycling protocol in DNA Engine PTC-200 (Bio-Rad, Hercules, CA) included initial 15-min denaturation at 95°C, then 30 cycles of 20-sec denaturation at 95°C, 30-sec annealing at 54°C, and 45-sec elongation at 72°C (with final elongation step extended to 3 min). PCR products were purified using ExoSAP-IT reagent (USB Corp., Cleveland, OH) and sequenced on both strands using the same amplification primers, BigDye Terminator v3.1 Cycle Sequencing Kits and ABI310 model DNA sequencer (Applied Biosystems, Foster, CA).

Alleles of each locus were identified using A. baumannii MLST profiles database (http://pubmlst.org/abaumannii/). BURST clustering of allelic profiles and UPGMA clustering of concatenated sequences were used to identify related sequence types.

RESULTS

Among the 15 isolates studied, 3, 2, and 10 were found by PCR to carry the genes for OXA-23-, OXA-40-, and OXA-58-like β-lactamases, respectively (Table 2)

Using the new MLST primers, PCR products of expected sizes were obtained for the 7 housekeeping genes from all but one (MW2-3577) isolate.

Figure 1. Geographic origins of the isolates studied.

This latter isolate was biochemically identified as *A. baumannii* but produced only the cpn60 and recA PCR products. By BLAST analysis, the cpn60 and recA sequences of MW2-3577 shared only 83% to 85% identity with the corresponding sequences of *A. baumannii* strains, while the recA gene sequence of MW2-3577 was completely identical to that of *Acinetobacter* Genomospecies 10 (GenBank Acc. No. AF191139).

The other 14 isolates were confirmed as *A. baumannii* and were distributed by analysis of MLST profiles into 9 novel sequence types (STs). Seven STs encompassing 12 isolates were found to be double-locus variants of several known STs (Table 2). Analysis of concatenated sequences of seven loci also confirmed the relatedness of six newly identified STs and the known STs: ST20, ST22, ST33, ST37, ST41 and ST44 that include epidemic clones from Europe, Asia and South America. Interestingly, two STs included 3 and 4 isolates from geographically distant sites (Fig. 2) and one of these STs included isolates with different CHDLs. On the other hand, two local outbreaks in Moscow and Novosibirsk were shown to involve 2 and 3 OXA-58-producing clones each

CONCLUSIONS

- As part of this study the existing *A. baumannii* MLST scheme was optimized by the introduction of new amplification and sequencing primers. The optimized typing protocol offers the advantage of more reliable amplification and sequencing of all 7 housekeeping genes using the same non-degenerate primers and uniform PCR conditions.

- The results of our study illustrate that the epidemiology of carbapenem-resistant Acinetobacter infections is highly complex and involves both the dissemination of epidemic clones and inter-clonal transmission of OXA carbapenemases.

Table 1. Primers used in this study.

Primer	Sequence, 5'-3'	Target	Amplicon size, bp
OXA-23-F	TTTCTTTCTGGTTGTACGGTTCA	bla_{OXA-23}	723
OXA-23-R	CATTTCTGACCGCATTTCCA		
OXA-40-F	GATGAAGCTCAAACACAGGGTG	bla_{OXA-40}	587
OXA-40-R	TTTCCATTAGCTTGCTCCACC		
OXA-58-F	GGGCTTGTGCTGAGCATAGT	bla_{OXA-58}	739
OXA-58-R	CCGTAGAGCAATATCATCACCAGC		
ABA-gltA-f	ACAGTGGCACATTAGGTCCC	*gltA*	718
ABA-gltA-r	GCAGAGATACCAGCAGAGATACA		
ABA-gyrB-f	AACCATCTCAACGAAATCTTCC	*gyrB*	548
ABA-gyrB-r	GCTGGGTCTTTTTTCCTGACA		
ABA-recA-f	GGTCCTGAATCTTCTGGTAAAAC	*recA*	456
ABA-recA-r	GAATTTAAGAGCATTACCACCAGT		
ABA-cpn60-f	CAACTGTACTTGCTCAAGC	*cpn60*	500
ABA-cpn60-r	CGCTTCACCTTCAACATCTTC		
ABA-gpi-f	AAAATCCATGCTGGCAATA	*gpi*	451
ABA-gpi-r2	CAATACAAGACCAAAGAGAATAACG		
ABA-rpoD-f	GTGAAGGTGAAATCAGCATTGC	*rpoD*	652
ABA-rpoD-r	GCAATTTGTTCATCTAACCAAGC		
ABA-gdhB-f1	CCACATGCTTTGTTATGGGG	*gdhB*	526
ABA-gdhB-r1	GATTTAACGCGTAATACTTTACCCAT		

Table 2. Epidemiological data and genotyping results for the *Acinetobacter* isolates.

Species [a]	Strain	City (Hospital)	Date of isolation	Type of infection	CHDL group	MLST allelic profile [b]							Related STs	
						gltA	*gyrB*	*gdhB*	*recA*	*cpn60*	*gpi*	*rpoD*	Min. nt difference [c]	DLVs [d]
Aba	SL-248	Stavropol (1)	17-Aug-1998	Pneumonia	OXA-58	1	15	23x	72	4	12x	2	20	20
Aba	IR-547	Irkutsk (1)	02-Dec-2002	Pneumonia	OXA-23	1x	15	16x	11	15x	3x	2	-	-
Aba	MW1-816	Moscow (1)	24-Dec-2002	Pneumonia	OXA-58	1	15	23x	10	14	12x	18	37	34, 35, 37, 48, 49
Aba	MW1-2282	Moscow (1)	02-Nov-2006	Pneumonia	OXA-58	1	15	2x	15	1	14x	18	-	45
Aba	MW1-797	Moscow (1)	18-Dec-2002	Pyelonephritis	OXA-58	1	15	2x	15	1	14x	18	-	45
Aba	NS2-1777	Novosibirsk (2)	02-May-2006	Pneumonia	OXA-58	1	15	2x	15	1	14x	18	-	45
Aba	NS1-2280	Novosibirsk (1)	28-May-2003	Pneumonia	OXA-58	10	12	4x	11	4	9x	5	16, 25, 43, 44	44
Aba	EK1-386	Ekaterinburg (1)	13-Jun-2006	Sepsis	OXA-58	10	12	4x	11	4	9x	5	16, 25, 43, 44	44
Aba	EK2-1283	Ekaterinburg (2)	19-Jun-2006	Pneumonia	OXA-58	10	12	4x	11	4	9x	5	16, 25, 43, 44	44
Aba	MG-25674	Mogilev (1)	26-Nov-2007	Pneumonia	OXA-40	10	12	4x	11	4	9x	5	16, 25, 43, 44	44
Aba	NS2-1671	Novosibirsk (2)	28-Apr-2006	Pneumonia	OXA-58	10	12	4x	11	14	9x	5	16, 25, 43, 44	-
Aba	NS2-1744	Novosibirsk (2)	08-Sep-2006	Pneumonia	OXA-58	1	12	3x	2	2	19x	3	33, 6	4, 21, 33
Aba	MI-25818	Minsk (1)	13-Feb-2008	Pneumonia	OXA-40	1	3	3x	2	2	7x	3	22, 41	22, 41, 53
Aba	KR-26517	Krasnodar (1)	19-Jul-2008	Peritonitis	OXA-23	10	25x	4x	11	4	23x	5	-	-
AG10	MW2-3577	Moscow (2)	09-Mar-2004	Pneumonia	OXA-23	-	-	-	-	-	-	-	-	-

a: Aba, *A. baumannii* ; AG10, *Acinetobacter* Genomospecies 10.
b: Mutant alleles are indicated by "x" after a number.
c: <=8-nt difference for concatenated sequences.
d: DLVs, double-locus variants

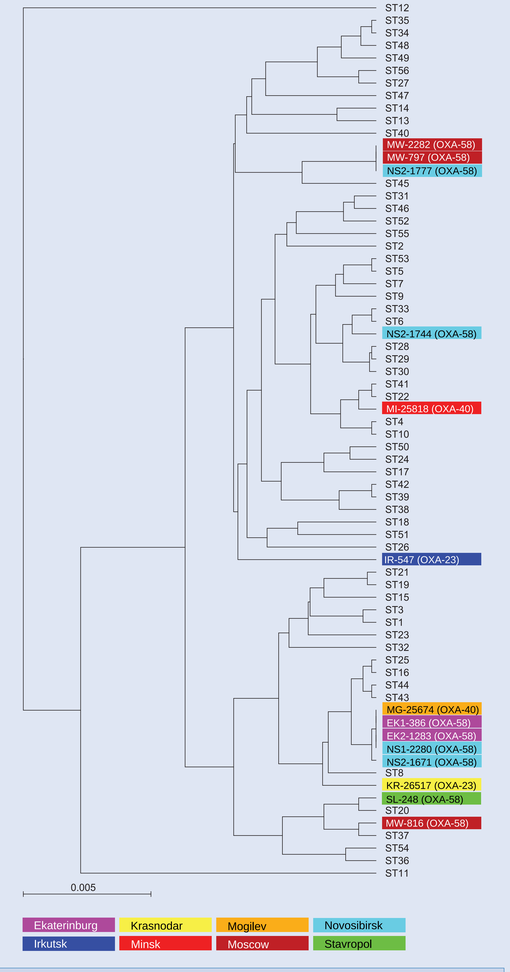

Figure 2. UPGMA clustering of concatenated sequences of 7 housekeeping genes of the known sequence types (STs) and clinical *A. baumannii* isolates studied. Color coding corresponds to the geographic origins of the isolates.