Prevalence of 16S rRNA methylase genes in Enterobacterial isolates in two Greek hospitals in a three year period

Galani I[1], Moraitou H[2], Orlandou K[1], Nafplioti K[1], Vogiatzakis E[2], Petrikkos G[1], Souli M[1]

[1]4th Department of Internal Medicine, Athens University School of Medicine

[2]Department of Clinical Microbiology, 'Sotiria' General and Chest Diseases Hospital

National and Kapodistrian University of Athens

ABSTRACT

Objectives: The aim of this study was to investigate the prevalence of 16S rRNA methylase genes in consecutively collected Enterobacteriaceae in Athens, Greece. RmtB has been reported previously in 0.2% of *Klebsiella pneumoniae* and 0.4% of *Proteus mirabilis* strains isolated from November 2007 to October 2009 in "Attikon" University Hospital.

Methods: Enterobacteriaceae isolates resistant to both amikacin and gentamicin (n=105) consecutively collected during a three year period (March 2010 - Feb 2013) in our Infectious Diseases Laboratory, as well as 70 MDR (including aminoglycosides) *K. pneumoniae* strains isolated in another tertiary hospital in Athens during 2011, 2012 and 2013, were tested further for MIC determination to amikacin, gentamicin, tobramycin, netilmicin, apramycin and neomycin with the broth dilution technique. Isolates with MICs >=512 mg/L to at least the first four aminoglycosides were examined for the presence of 16S rRNA methylase genes (*armA*, *rmtB, rmtC, rmtA, rmtD* and *npmA*) by PCR. Molecular typing was performed by REP-PCR. Carbapenemase production was confirmed by PCR.

Results: Thirty one (4.7%) *K. pneumoniae*, fifteen (40.5%) *Providencia stuartii* and three (1.1%) *Proteus mirabilis* isolates among Enterobacteriaceae consecutively collected were positive for *rmtB* and highly resistant to all clinical used aminoglycosides tested (MICs >=512 mg/L). All *rmtB* - bearing *K.pneumoniae* strains were KPC producers with 87.5% of them being also resistant to colistin. All but one *P. stuartii* and one of the three *P.mirabilis* isolates were VIM producers. Among the MDR *K. pneumoniae* isolates of the second hospital, twenty eight (40%) were positive for *rmtB*. Of those isolates twenty were KPC producers, five were VIM producers and three were no carbapenemase producers. The 48.3% of the *rmtB*-positive *K. pneumoniae* isolates belonged to the same clone while two other clones included 22.4% and 13,8% of the isolates, each. All VIM producing *rmtB*-*K.pneumoniae* isolates were clonally unrelated. Three strains belonged to a fourth clone (5.2%) similar to one isolated in our previous study in 2009. All but one *P.stuartii* isolates were clonally related while the three *P.mirabilis* strains although isolated at distant intervals were possibly related.

Conclusions: Our data demonstrate an increased prevalence of *rmtB*-positive KPC-producing *K. pneumoniae* and *rmtB*-positive *P.mirabilis* since 2009 as well as a high prevalence of *rmtB* in *P.stuartii*, which have been increasingly isolated in Greek hospitals as a result of the extensive use of antibiotics of last resort such as colistin and tigecycline. The spread of XDR isolates producing both carbapenemases and 16S rRNA methylases raises clinical concern and may become a major therapeutic threat in the future.

CONTACT

Irene Galani
National and Kapodistrian University of Athens, Faculty of Medicine
4th Department of Internal Medicine, Infectious Diseases Laboratory
Email: egalani@med.uoa.gr

INTRODUCTION

- Methylation of 16S ribosomal RNA (rRNA) has emerged as a novel aminoglycoside resistance mechanism in pathogenic gram-negative bacteria since 2003 and is increasingly reported worldwide.
- 16S rRNA methyltransferases (RMTs) are responsible for very high level resistance to all parenterally administered aminoglycosides that are currently in clinical use. The responsible genes are mostly located on transposons within transferable plasmids, which provides them with the potential to spread horizontally and may in part explain the rapid worldwide distribution among Enterobacteriaceae and non-glucose-fermenting bacilli of human and animal origin.
- One great concern is the multidrug resistance development in RMT-producing pathogenic bacteria through further accumulation of various antimicrobial resistance genes such as carbapenem-hydrolyzing β-lactamases. More worrisome is the emergence of members of Enterobacteriaceae family co-producing NDM-1 metallo-β-lactamase and RMTs (RmtB, RmtC and ArmA) often located on the same broad-range conjugative plasmid.
- Thus far, RMTs have been reported from at least 30 countries or regions. The worldwide dissemination is becoming a serious global concern and this implies the necessity to continue investigations on the trend of RMTs to restrict their further distribution.
- The aim of this study was to investigate the prevalence of 16S rRNA methylase genes in consecutively collected Enterobacteriaceae in Athens, Greece. RmtB has been reported previously in 0.2% of *Klebsiella pneumoniae* and 0.4% of *Proteus mirabilis* strains isolated from November 2007 to October 2009 in "Attikon" University Hospital.

METHODS AND MATERIALS

- One hundred and five (n=105) Enterobacteriaceae isolates resistant to both amikacin and gentamicin consecutively collected during a three year period (March 2010 - Feb 2013) in our Infectious Diseases Laboratory, as well as
- 70 MDR (including aminoglycosides) *K. pneumoniae* strains isolated in 'Sotiria' General and Chest Diseases Hospital in Athens during 2011, 2012 and 2013, were tested.
- Only one isolate per patient was included in the study.
- Species identification of isolated bacteria and primary MIC determinations were performed using an automated system (BD Phoenix automated microbiology system; BD Diagnostic Systems).
- MIC determinations to amikacin, gentamicin, tobramycin, netilmicin, apramycin and neomycin were performed with the broth microdilution technique.
- Isolates with MICs ≥512 mg/L to the first four of the above aminoglycosides were examined for the presence of 16S rRNA methylase genes (*armA, rmtB, rmtC, rmtA, rmtD* and *npmA*) by multiplex PCR.
- Genomic fingerprinting was carried out by repetitive-element PCR (rep-PCR) analysis using primers 5' III GCG CCG ICA TCA GGC 3', and 5' ACG TCT TAT CAG GCC TAC 3'.
- Beta-lactamases were determined by isoelectric focusing (IEF) and the presence of the responsible genes were identified by PCR.
- The PCR primers used in this study for amplification of genes are reported in Table 1.
- Sequencing of the PCR products were performed by MWG-THE Genomic Company.

Gene	Primer (5'→3')	PCR product
armA	5'-ATTCTGCCTATCCTAATTGG-3'	315bp
	5'-ACCTATACTTTATCGTCGTC-3'	
rmtB	5'-GCTTTCTGCGGGCGATGTAA-3'	173bp
	5'-ATGCAATGCCGCGCTCGTAT-3'	
rmtC	5'-CGAAGAAGTAACAGCCAAAG-3'	711bp
	5'-ATCCCAACATCTCTCCCACT-3'	
rmtA	5'-CTAGCGTCCATCCTTTCCTC-3'	635bp
	5'-TTGCTTCCATGCCCTTGCC-3'	
rmtD	5'-CGGCACGCGATTGGGAAGC-3'	401bp
	5'-CGGAAACGATGCGACGAT-3'	
npmA	5'-GGGCTATCTAATGTGGTTG-3'	229bp
	5'-TTTTTATTTCCGCTTCTTCGT-3'	
bla_{KPC}	5'-ATGTCACTGTATCGCCGTCT-3'	893bp
	5'-TTTTCAGAGCCTTACTGCCC-3'	
bla_{VIM}	5'-ATGGTGTTTGGTCGCATATC-3'	509bp
	5'-TGGGCCATTCAGCCAGATC-3'	
bla_{VEB}	5'-CGACTTCCATTTCCGATGC-3'	642bp
	5'-GGACTCTGCAACAAATACGC-3'	
bla_{OXA-10}	5'-GTCTTTCGAGTACGGCATTA-3'	720bp
	5'-ATTTTCTTAGCGGCAACTTAC-3'	
bla_{SHV}	5'-ATGCGTTATATTCGCCTGTG-3'	862bp
	5'-GTTAGCGTTGCCAGTGCTCG-3'	
bla_{TEM}	5'-TAGAATTCCATTCAAATATGTATCCGCTCATG-3'	880bp
	5'-TAGAATTCCATCCATAGTTGCCTGACTCCCC-3'	

Table 1. Primers used in PCR experiments.

RESULTS

Infectious diseases laboratory collection

Isolates	*Total*	*Aminoglycoside resistant*	*rmtB-harboring*
Klebsiella pneumoniae	660	67	31
Escherichia coli	612	2	0
Proteus mirabilis	275	8	3
Enterobacter spp	167	2	0
Morganella morganii	84	1	0
Citrobacter spp	63	1	0
Providencia stuartii	37	19	15
Serratia spp	31	1	0

K. pneumoniae

- ❖Thirty one *K. pneumoniae isolates (4.7%)*, were positive for *rmtB*
- ❖All *rmtB* - bearing isolates were highly resistant to all clinical used aminoglycosides tested (MICs ≥512 mg/L)
- ❖Apramycin MICs ranged from 4 to 64μg/ml, with an MIC_{50} of 16 and an $MIC_{90}$of 64μg/ml
- ❖Neomycin MICs ranged from 2->1024, with both MIC_{50} and MIC_{90} ≥512
- ❖The vast majority of *K.pneumoniae* isolates (87.5%) were also resistant to colistin
- ❖All *rmtB* - bearing *K. pneumoniae* strains were KPC producers and belonged to 4 distinct clones.
- ❖All harbored the bla_{KPC}, the bla_{OXA-10} and bla_{TEM} β-lactamase genes, while most of them (29/31) and the bla_{VEB} gene.

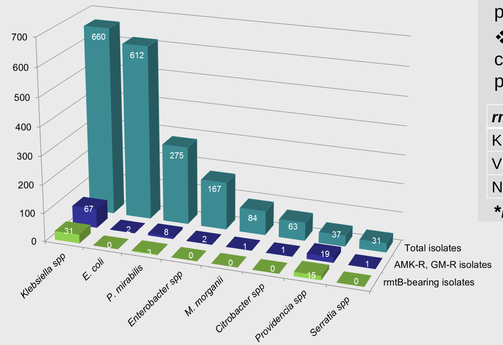

Chart 1. Enterobacterial isolates of Infectious Diseases Laboratory.

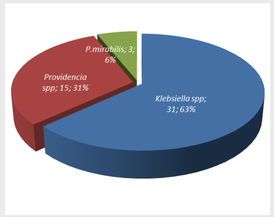

Figure 1. *rmtB* - bearing isolates of Infectious diseases laboratory.

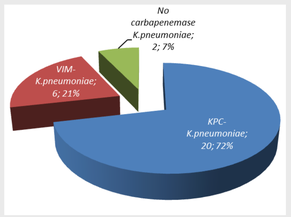

Figure 2. *rmtB* – bearing *K. pneumoniae* isolates of 'Sotiria' General and Chest Diseases Hospital.

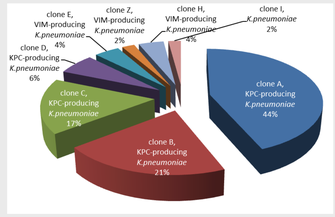

Figure 3. Clonality of *rmtB* – bearing *K. pneumoniae* isolates.

RESULTS

P. miirabilis

- ❖Three *P. mirabilis isolates* (1.1%) were positive for *rmtB* and highly resistant to all clinical used aminoglycosides tested
- ❖Apramycin MICs ranged from 16 to 128μg/ml, while Neomycin MICs ranged from 256-≥512
- ❖One of the three *P. mirabilis* isolates produced the VIM carbapenemase, while all three isolates harbored the bla_{VEB}, bla_{OXA-10} and bla_{TEM} genes .

P. stuartii

- ❖Fifteen *P. stuartii* isolates(40.5%) were highly resistant to all clinical used aminoglycosides tested and found positive for *rmtB*
- ❖Apramycin MICs ranged from 4 to 64μg/ml, with MIC_{50}/MIC_{90} 16/64μg/ml respectively
- ❖Neomycin MICs ranged from 16-≥512μg/ml, with MIC_{50}/MIC_{90} 128/ ≥512 respectively
- ❖All *P. stuartii* isolates harbored the bla_{VIM}, bla_{SHV}, bla_{VEB}, bla_{OXA-10} and bla_{TEM} β-lactamase genes and all but one were clonally related

'Sotiria' General and Chest Diseases Hospital collection

- ❖Seventy MDR (including aminoglycosides) *K. pneumoniae* isolates
- ❖Twenty eight isolates (40%) were positive for *rmtB*
- ❖Twenty of them (71.4%) were KPC producers belonging to the same 4 distinct clones as in the previous collection
- ❖Six isolates (21.4%) were VIM producers belonging to 4 different clones
- ❖Two *rmtB*-harboring *K. pneumoniae* isolates did not produce a carbapenemase
- ❖All isolates harbored the bla_{OXA-10} and bla_{TEM} genes, while only the VIM-producing isolates harbored the bla_{VEB}
- ❖bla_{SHV} of ESBL-type was present in the two isolates that did not produce a carbapenemase, in all VIM-producing isolates and in 6 (30%) of the KPC-producing isolates

***rmtB- K.pneumoniae* isolates**	**Number**	**Clones**	**β-lactamase genes**
KPC-producing	20	4	bla_{KPC}, bla_{SHV}*, bla_{OXA-10}, bla_{TEM}
VIM-producing	6	4	bla_{VIM}, bla_{SHV}*, bla_{VEB}, bla_{OXA-10}, bla_{TEM}
No carbapenemase producing	2	2	bla_{SHV}*, bla_{OXA-10}, bla_{TEM}

*bla_{SHV} : ESBL-type

CONCLUSIONS

- ❑ Increased prevalence of *rmtB*-positive KPC-producing *K. pneumoniae* and *rmtB*-positive *P. mirabilis* since 2009
- ❑ High prevalence of *rmtB* in *P. stuartii*, which have been increasingly isolated in Greek hospitals as a result of the extensive use of antibiotics of last resort such as colistin and tigecycline.
- ❑ The spread of XDR isolates producing both carbapenemases and 16S rRNA methylases raises clinical concern and may become a major therapeutic threat in the future.