Emergence of *Klebsiella pneumoniae* co-producing OXA-48, CTX-M-15, and ArmA in Greece.

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Abstract

Objectives: An extensively drug-resistant (XDR) Klebsiella pneumoniae isolate was sent to our Infectious Diseases Laboratory for further study. The strain was isolated from sequential blood cultures of a patient hospitalized in the ICU of a tertiary hospital in Athens.

Methods: Species identification and MIC determinations were performed with the BD Phoenix automated system. MICs of fosfomycin, tigecycline, colistin and meropenem were also determined using Etest (AB Biodisk), in accordance with the manufacturer's instructions. The *K.pneumoniae* isolate was submitted to EDTA-meropenem disk synergy test and boronic acid disk test as a screening for MBL and/or KPC production. The presence of carbapenemase, ESBL and 16S rRNA methylase was confirmed by polymerase chain reaction (PCR) with specific primers and conditions. Conjugation experiments were carried out by a filter method using a rifampin resistant *Escherichia coli* K12 laboratory strain as a recipient.

Results: The isolate was resistant to all antibiotics with the exception of fosfomycin (MIC = 8 mg/L). MICs to meropenem, colistin and tigecycline were 8, 3 and 6 mg/L as measured by E-test. High resistance was also observed in amikacin and gentamicin (>256 mg/L). The isolate harboured at least two different plasmids that were separately transferred to RC85 *E.coli* recipient. The first plasmid was carrying only the bla_{OXA-48} conferring resistance to ampicillin, temocillin, amoxicillin/clavulanate, ticarciline/clavulanate and reduced susceptibility to piperacilin/tazobactam and carbapenems. The second plasmid was carrying the armA conferring resistance to all aminoglycosides, the $bla_{CTX-M-15}$ conferring resistance or reduced susceptibility to all cephalosporins, and genes conferring resistance to tetracycline and chloramphenicol.

Conclusion: This is the first report of an *armA* - harboring *K. pneumoniae* in Greece, a country in which sporadic cases of *rmtB* bearing isolates have been already reported. Surveillance should be implemented to monitor the risk of emergence and spread of 16S rRNA methylases along with carbapenemases in this country.

Material and Methods

- Species identification and MIC determinations were performed with the BD Phoenix automated system.
- MICs of fosfomycin, tigecycline, colistin and meropenem were also determined using Etest (AB Biodisk), in accordance with the manufacturer's instructions.
- The *K. pneumoniae* isolate was submitted to EDTA-meropenem disk synergy test and boronic acid disk test as a screening for MBL and/or KPC production.
- Isoelectric focusing was performed in the *K.pneumoniae* clinical isolate and in the *E.coli* transconjugants.
- The presence of carbapenemase, ESBL and 16S rRNA methylase was confirmed by PCR with specific primers and conditions (Table 1).
- Conjugation experiments were carried out by a filter method using a rifampin resistant *Escherichia coli* K12 laboratory strain as a recipient.

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Results

- The isolate was resistant to all antibiotics with the exception of fosfomycin (MIC = 8 mg/L) (Table 2).
- MICs to meropenem, colistin and tigecycline were 8, 3 and 6 mg/L as measured by E-test (Table 2).
- High resistance was also observed in amikacin and gentamicin (>256 mg/L) (Table 2).
- EDTA-meropenem and boronic acid – meropenem disk synergy tests were negative for MBL and/or KPC production.
- Isoelectric focusing revealed the production of four β-lactamases with pI's 8.6, 7.6, 7.4 and 5.4 corresponding to CTX-M-15, SHV-11, OXA-48 and TEM-1.
- Polymerase chain reaction (PCR) with specific primers and conditions (as described in Table 1) and sequencing of PCR products, confirmed the presence of bla_{OXA-48}, $bla_{CTX-M-15}$, bla_{SHV-11} and bla_{TEM-1}.
- Multiplex PCR for 16S rRNA methylase genes revealed the carriage of *armA* gene in the *K.pneumoniae* isolate.
- Conjugation experiments with RC85 *E. coli* strain was successful at a frequency of $1.3x10^{-4}$ when selection was performed in media containing piperacillin (50mg/L) and $3.9x10^{-6}$ when selection was performed in media containing amikacin (40mg/L).
- The isolate harbored at least two different plasmids that were separately transferred to RC85 *E.coli* recipient.
- The first plasmid was carrying the bla_{OXA-48} conferring resistance to ampicillin, temocillin, amoxicillin/clavulanate, ticarcillin/clavulanate and reduced susceptibility to piperacillin/tazobactam and carbapenems.
- The second plasmid was carrying the *armA,* conferring resistance to all aminoglycosides, the $bla_{CTX-M-15}$ conferring resistance or reduced susceptibility to all cephalosporins, and genes conferring resistance to tetracycline and chloramphenicol.

Table 1. Primers used in PCR experiments

Gene	Primer (5'→3')	PCR product
armA	5'-ATTCTGCCTATCCTAATTGG-3' 5'-ACCTATACTTTATCGTCGTC-3'	315bp
rmtB	5'-GCTTTCTGCGGGCGATGTAA-3' 5'-ATGCAATGCCGCGCTCGTAT-3'	173bp
rmtC	5'-CGAAGAAGTAACAGCCAA AG-3' 5'-ATCCCA ACATCTCTCCCACT-3'	711bp
rmtA	5'-CTAGCGTCCATCCTTTCCTC-3' 5'-TTGCTTCCATGCCCTTGCC-3'	635bp
rmtD	5'-CGGCACGCGATTGGGAAGC-3' 5'-CGGAAACGATGCGACGAT-3'	401bp
npmA	5'-GGGCTATCTAATGTGGTG-3' 5'-TTTTTATTTCCGCTTCTTCGT-3'	229bp
bla_{OXA-48}	5'-TTGGTGGCATCGATTATCGG-3' 5'-GAGCACTTCTTTTGTGATGGC-3'	743bp
bla_{KPC}	5'-ATGTCACTGTATCGCCGTCT-3' 5'-TTTTCAGAGCCTTACTGCCC-3'	893bp
bla_{VIM}	5'-ATGGTGTTTGGTCGCATATC-3' 5'-TGGGCCATTCAGCCAGATC-3'	509bp
bla_{VEB}	5'-CGACTTCCATTTCCCGATGC-3' 5'-GGACTCTGCAACAAATACGC-3'	642bp
bla_{OXA-10}	5'-GTCTTTCGAGTACGGCATTA-3' 5'-ATTTTCTTAGCGGCAACTTAC-3'	720bp
bla_{SHV}	5'-ATGCGTTATATTCGCCTGTG-3' 5'-GTTAGCGTTGCCAGTGCTCG-3'	862bp
bla_{TEM}	5'-TAGAATTCCATTCAAATATGTATCCGCTCATG-3' 5'-TAGAATTCCATCCATAGTTGCCTGACTCCCC-3'	880bp

***bla*$_{OXA-48}$**

- was located on a conjugative plasmid (frequency of conjugation $1.3x10^{-4)}$

armA

- was located on a conjugative plasmid (frequency of conjugation $3.9x10^{-6)}$
- on the same plasmid was located the $bla_{CTX-M-15}$ and genes responsible for chloramphenicol and tetracycline resistance

*The size of the two plasmids has to be determined

Table 2. Susceptibilities of *K.pneumoniae* clinical isolate and the two transconjugant *E.coli* isolates.

Antimicrobial agent	MIC (mg/L)			
	E.coli RC85	*K.pneumoniae* b 1352	*E.coli* transconjugant 1	*E.coli* transconjugant 2
Amikacin	<=8	>32 (>256*)	>32 (>256*)	<=8
Amoxicillin/Clavulanate	<=4/2	>16/8	8/4	>16/8
Ampicillin	<=4	>16	>16	>16
Aztreonam	<=2	>16	8	<=2
Cefepime	<=2	>16	>16	2
Cefoxitin	<=4	>16	<=4	<=4
Ceftazidime	<=1	>16	4	<=1
Ceftriaxone	<=1	>32	>32	32
Cefuroxime	<=4	>16	>16	>16
Ciprofloxacin	<=0.5	>2	<=0.5	<=0.5
Colistin	<=0.5	>4 (3)	<=1	<=1
Gentamicin	<=2	>8 (>256*)	>8 (>256*)	<=2
Ertapenem	<=0.25 (0.015*)	>4	<=0.25	1
Imipenem	<=1(0.25*)	8	<=1	<=1(0.5*)
Meropenem	<=1 (0.06*)	>8 (8*)	<=1	<=1 (0.125*)
Piperacillin/Tazobactam	<=4/4	>64/4	<=4/4	64/4
Tigecycline	<=1 (0.25*)	>4 (6*)	2 (0.75*)	<=1 (0.25*)
Trimethoprim/Sulfamethoxazole	<=1/19	>4/76	<=1/19	<=1/19
	Zone Diameter (mm)			
Temocillin 30μg	23	6	23	6
Chloramphenicol 30μg	27	6	6	26
Tetracycline 30μg	27	6	6	25
Minocycline 30μg	22	10	10	21
Acquired genes detected		$bla_{CTX-M-15}$, bla_{OXA-48}, $bla_{TEM-like}$, *armA*	$bla_{CTX-M-15}$, *armA*	bla_{OXA-48}

* MICs in parenthesis have been identified by E-test

Conclusions

- This is the first report of an *armA* - harboring *K. pneumoniae* in Greece, a country in which sporadic cases of *rmtB* - bearing isolates have been already reported.
- The *K.pneumoniae* isolate was also a OXA-48 carbapenemase producer.
- bla_{OXA-48} and *armA* were located on different conjugative plasmids.
- 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.
- Surveillance should be implemented to monitor the risk of emergence and spread of 16S rRNA methylases along with carbapenemases in this country.