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Emergence of *Klebsiella pneumoniae* Carrying OXA-48 Carbapenemase in Bulgaria

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Abstract
In the beginning of June 2013 we identified a carbapenem-nonsusceptible *Klebsiella pneumoniae* isolate (OXA48BG), according to the Clinical and Laboratory Standards Institute 2013 recommendations. The strain was isolated from the urine sample of female patient in a University hospital in Sofia, Bulgaria. Antimicrobial susceptibilities were determined by the Etest (LIOFILCHEM). Modified Hodge test, Carba NP test and phenotypic tests for carbapenemase detection by using the KPC, MBL and OXA-48 Confirm Kit (Rosco Diagnostica) were performed. PCR amplification and DNA sequencing of carbapenemase genes ($bla_{\text{OXA-48}}$) were carried out. OXA48BG was resistant to ampicillin, amoxicillin/clavulanic acid, piperacillin, piperacillin/tazobactam, cephalothin, cefuroxime, cefotaxime, trimethoprim/sulfamethoxazole; susceptible to ceftazidime, cefepime, aminoglycosides, fluoroquinolones and colistin; and had borderline susceptibility to imipenem and meropenem. Using phenotypic tests and molecular-genetic techniques OXA48BG was identified as OXA-48 producer. So far there are only single reports for detection of *K. pneumoniae* strains carrying genes for OXA-48 carbapenemase in Bulgaria.

1. Introduction

Nowadays, an emergence of carbapenem resistance in Enterobacteriaceae is reported, mostly related to the spread of carbapenemases [8]. Clinically significant carbapenemases belong to the Ambler class A (e.g. KPC), class B (e.g. IMP, VIM and NDM), and class D (e.g. OXA-48 and its variants possessing weaker but significant carbapenemase activity) [9]. OXA-48 had first been identified from a clinical Klebsiella pneumoniae isolate recovered in Istanbul, Turkey, in 2001 [10]. In recent years, there have been a constantly growing number of reports of isolation of OXA-48 carbapenemase-producing *Klebsiella pneumoniae*, mainly in Turkey, the Middle East and North Africa, but also in many European countries – [11, 14].

Here, we report an isolation and identification of *K. pneumoniae* strain carrying OXA-48 carbapenemase from a patient underwent renal transplantation.

2. Materials and Methods

The strain (OXA48BG) was isolated in Aleksandrovska University Hospital in Sofia, Bulgaria, in the beginning of June 2013 from urine sample of a 37 years old female outpatient with clinical symptoms of urinary tract infection. The patient underwent renal transplantation in the beginning of March 2013 in Turkey.

Species identification was done by ID 32 GN system (bioMérieux, France).

The initial antimicrobial susceptibility testing was performed by disk-diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI) 2013 recommendations [1]. Additionally, the minimum inhibitory concentrations (MICs) of several antibiotics were determined by the Etest (LIOFILCHEM). *Esherichia coli* ATCC 25922 was used as a control strain.

Phenotypic detection of carbapenemase production was done by using the Modified Hodge test [1], Carba NP test [2] and KPC, MBL and OXA-48 Confirm Kit (ROSCO Diagnostica A/S).

The molecular basis of the resistance phenotype for a putative OXA-48-like carbapenemase gene was determined by polymerase chain reaction (PCR) analysis. The total DNA from OXA48BG isolate was extracted by boiling. The amplification was performed with OXA-48 specific primers (5'–TTGGTGGCATCGATTATCGG–3' and 5'–GAGCACTTCTTTTGTGATGGC–3') [10]. PCR was carried out with 3 µl of the template DNA, 0.25 µM of each primer (Alpha DNA, Canada), 0.2 mM deoxyribonucleoside triphosphates, 1x Reaction Buffer, 2 mM $MgCl_2$ and 1.0 U of Taq DNA polymerase (Prime TaqTM DNA Polymerase, GENET BIO) in a total volume of 25 µl. The DNA was amplified using the following protocol: an initial denaturation (94°C, 5 min) followed by 35 cycles of denaturation (94°C, 45 s), annealing (60°C, 45 s) and extension (72°C, 45 s), and a single final extension of 7 min at 72°C. The obtained PCR product was prepared for sequencing by purification through columns S-400 (GE Healthcare, UK) and was sent to Macrogen Inc. (Republic of Korea).

The sequences were analysed for close homology by the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI) (http//:www.nbi.nlm.nih.gov/BLAST). The sequencing data were compared and verified by the OXA gene sequences deposited in NCBI GenBank for *K. pneumoniae* strains: OXA48 – KJ481797.1, KJ481798.1, KC335143.1, JN626286.1, KC757417.2N2, KC757416.2N1, HE617182.1 (*Escherichia coli*), JX423832.1 (*Enterobacter cloacae*); OXA162 – HM015773.1, GU197550.1; OXA244 – KJ187475.1, JX438000.1; OXA247 – JX893517.1, OXAp204B – JQ809466.1, OXA181 – JQ996150.1 (*Citrobacter freundii*), HM992946.1 (*Enterobacter cloacae*).

The model for construction of nucleotide phylogenetic tree and phylogenetic analyses were performed в последовательност as described previously [12] with the following programs: MUSCLE [3], J Model Test 0.1.1 [13], PhyML 3.0 [5] by Phylemon 2 [16] and 1000 bootstrap replications. The phylogenetic tree (Figure 1) was drawn by FigTree1.4.0 software (http://tree.bio.ed.ac.uk/).

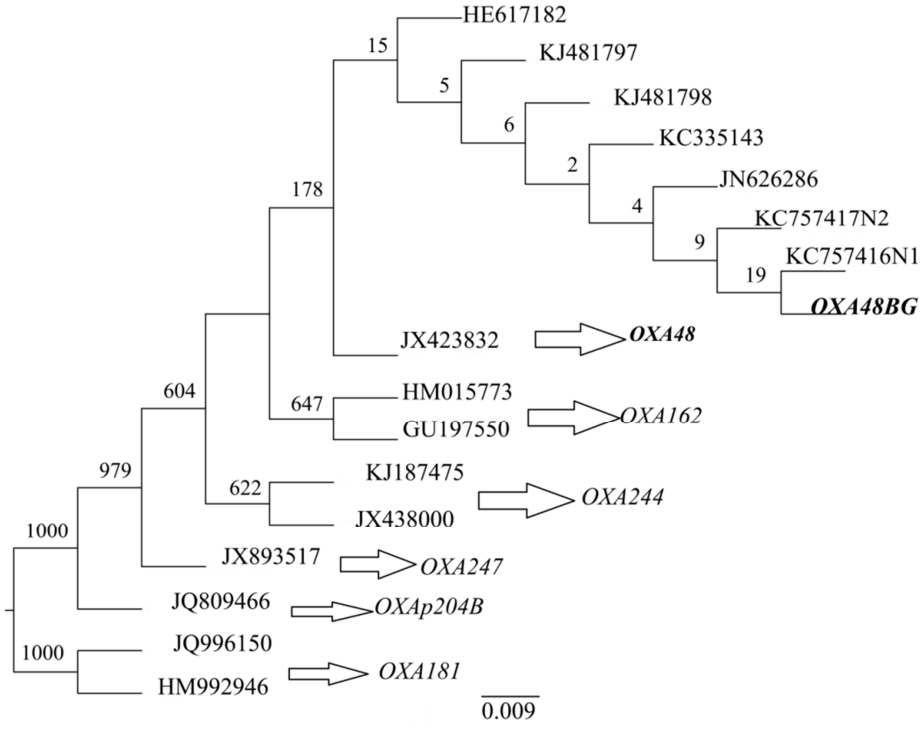

Figure 1. *Phylogenetic tree based on nucleotide (nt) sequences of the blaOXA-48-like genes in K. pneumoniae isolates. The tree was calculated from 699 nt aligned positions in the final data set. For building of phylogenetic tree were used TIM1 model and one thousand bootstrap replications.*

3. Results

OXA48BG *K. pneumoniae* was resistant to ampicillin, amoxicillin/clavulanic acid, piperacilln, piperacillin/tazobactam, cephalothin, cefuroxime, cefotaxime, trimethoprime/sulfamethoxazole and susceptible to ceftazidime, cefepime, aztreonam, ciprofloxacin, gentamicin, amikacin, tobramycin, colistine, tetracycline and tigecycline, and had borderline susceptibility to imipenem and meropenem (Table 1). The Modified Hodge test and Carba NP test were positive. The tests for synergy with phenylboronic acid, dipicolininc acid and cloxacilln were negative and the strain was resistant to temocillin.

The amplification with OXA-48 specific primers yielded a 744 bp product. After processing sequence data for further analysis a product with size 699 nt was used. Our fragment covers the section of the gene between 52 and 751 nt, according to the reference sequence KC757416.2. BLAST analysis of the obtained nucleic acid sequence provided an identity value of 100% with beta-lactamase OXA-48 of *K. pneumoniae*.

After multiple alignment of isolates of OXA-48 group differences in the nucleotide sequences of the research region was not found among them. Differences were identified among OXA-48 group and other groups included in the study. Specific mutations of different groups are in positions:

For OXA181: A→G (10, 277, 297, 366, 492); C → T (249, 259, 270, 280, 285, 342, 357, 393, 417, 447, 507, 618); T→ C (273, 279, 300, 369, 372, 384, 396, 486, 490, 510, 516, 519); A → T (498); G→ A (360); G→T (501); A→C (438, 469, 471); C→G (522); G→C (450, 451); AGC→GCT (460-462);

For OXA 204: GAC → TCG (243-245), G → T (450); T→ G (633);

For OXA 247: GAPS on position 581-592; T→ C (593), G→ A (595), AC→TA (597-598);

For OXA 162: A→G (586);

For OXA 244: A→G (589);

General mutations: for OXA 181 and 204: C → T (426); T→G (576); for OXA 181, 204 and 247: T→C (552);

Analysis of nucleotide sequencing by JModelTest showed that TIM1 model is the most suitable for phylogenetic analysis. Phylogenetic tree based on nucleotide (nt) sequences of the blaOXA-48-like genes in *K. pneumoniae* isolates was constructed (Figure 1).

The sequence of OXA48BG has been submitted to the GenBank, NCBI with accession number KJ959619.

Table 1. *MICs[a] of selected antibiotics against the OXA48BG strain of K. pneumoniae.*

Antimicrobial agents	MIC (µg/mL) against OXA48BG (interpretation[b])
Amoxicillin/clavulanic acid (2/1)	>256 (R)
Piperacillin	>256 (R)
Piperacillin/tazobactam[c]	>256 (R)
Cefotaxime	16 (R)
Ceftazidime	1.5 (S)
Cefepime	4 (S)
Imipenem	1.5 (I)
Meropenem	1.5 (I)
Aztreonam	1 (S)
Amikacin	1.5 (S)
Gentamicin	0.38 (S)
Tobramycin	0.75 (S)
Ciprofloxacin	0.47 (S)
Trimethoprim/sulfamethoxazole (1/19)	>32 (R)
Colistin	0.25 (S)

[a]MICs – minimum inhibitory concentrations, [b]interpretive criteria according to the CLSI-2013 recommendations, [c]tazobactam at a fixed concentration of 4 µg/mL; S – susceptible, I – intermediate resistant, R – resistant.

4. Discussion and Conclusion

In the performed nucleotide phylogenetic analyses was found that OXA48BG belongs to the group of *K. pneumoniae* OXA-48.

During the last years, a growing number of Bulgarian patients with terminal renal insufficiency are transplanted abroad, mainly in Afghanistan and Turkey. This is associated with increased risk of importation of multidrug-resistant, in particular carbapenem-resistant, bacteria [4]. In this case the patient was successfully treated with 10 days course of ciprofloxacin.

So far there are only single reports for detection of *K. pneumoniae* strains carrying genes for OXA-48 carbapenemase in Bulgaria. In 2014, during the 24th ECCMID congress in Barcelona, Markovska et al. reported the detection of two isolates *K. pneumoniae* producing class D carbapenemase in the University Hospital of Varna [6]. These isolates produced OXA-48 and a CTX-M-type ESBL of the CTX-M-9 subgroup. For both isolates was shown the presence of a plasmid of the L/M replicon-type, but transconjugation experiments were unsuccessful.

Recently, Sabtcheva et al. reported the identification of OXA-48-producing *Klebsiella pneumoniae*, causing peritonitis in a cancer patient admitted to the Oncology Hospital in Sofia [14]. The presence of $bla_{\text{OXA-48}}$ gene was confirmed with PCR and sequencing. The $bla_{\text{OXA-48}}$ gene was flanked by two intact copies of IS1999 on truncated

ΔTn1999.1. This transposon was located on unusual non-typeable 29-kb plasmid that could be transferred only by transformation. Multilocus sequence typing (MLST) indicated the presence of the sequence type ST530.

The absence of mutations between the representatives of OXA-48 and the presence of those between the groups shows that the included region in the survey is suitable for the determination of group membership but not to study the isolates in group OXA-48. This will be most likely possible after sequencing the whole gene. The established nucleotide differences between groups can be used as a basis for multiplex Real Time or conventional PCR group determination.

These studies, as well as other recent reports [7, 15, and 17] provide accumulating evidence of rapid spread of carbapenem-resistant Enterobacteriaceae, producing different types of carbapenemases in Bulgaria, and prompts for widespread epidemiologic surveillance.

Protection of human and animal subjects. The authors declare that no experiments were performed on human or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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