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Structure variability and phylogeny of *Klebsiella pneumoniae* OXA-48 Class D carbapenemases

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Summary

Carbapenem antibiotics are considered last resort options in the treatment of infections caused by multidrug-resistant *Enterobacteriaceae* producing Extended Spectrum β-Lactamases (ESBLs). The emergence of *Klebsiella pneumoniae* OXA-48 in particular is steadily expanding and represents a major public health concern. The aim of the present study is to analyze the variability and phylogeny of *K. pneumoniae* OXA-48 amino acids structures from different geographic areas of the world.

The data on *K. pneumoniae* OXA-48 amino acids structures were collected during the month of May 2019 from the Protein Data Bank (PDB). The alignment of protein sequences was carried using the Clustal Omega program available on the UniProt database. The phylogenetic analysis and dendrogram were realized using MEGA software version 6.
Among 58 structures, 8 representative OXA-48 variants were selected for the study. The alignment demonstrated that the conserved motifs were in general well conserved except for the two mutations S70G and S70A remarked respectively in the two chains 5HAQ and 5HAP from the United States. However, the OXA-181 and OXA-245 variants displayed mutations far away from the active sites. In comparison with OXA-48, OXA-181 variant showed four substitutions at Thr104Ala, Asn110Asp, Glu168Gln, and Ser171Ala; while OXA-245 had a single amino acid substitution Glu125Tyr. The phylogenetic analysis revealed three distinct clusters; the first one consists of four OXA-48 structures (Canada, Norway, United States and Italy) and one OXA-245 (Norway), the second includes two OXA-48 structures from the United States, while the third cluster is formed by an individual OXA-181 from Norway.
The results of this study confirm a similar evolutionary trend in the structure of *K. pneumoniae* OXA-48 variants worldwide. The current data on *K. pneumoniae* OXA-48 amino acids structures is limited to restricted geographic areas, and need broadening to provide the actual state of molecular changes and antimicrobial resistance evolution.

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Key words
Klebsiella pneumoniae, OXA-48, antibiotic resistance, phylogeny

Introduction

As antibiotic history entered its eighth decade, the selection of resistant bacterial strains increased by the overuse of antibiotics, and the lack of new antibiotic drug development progressively reduced the treatment options for bacterial infections. It is expected that the deaths caused by antibiotic resistant infections will exceed cancer deaths by 2050.[1-3] Based on the mechanism of hydrolysis and inactivation of β-lactam antibiotics and amino acid sequence homology, β-Lactamases enzymes are classified in four classes (A-D) known as metallo (Class B) with a Zn^{2+} ion(s) in the active site and serine β-lactamases (Classes A, C, and D).[4,5]

During the last decade, there is an increase of reports on Class D β-lactamase-mediated resistance to β-lactams, and an extended spread of oxacillinase enzymes or OXAs (Class D) among Gram-negative bacteria.[4,6,7] The emergence of resistance to carbapenems in Carbapenem-hydrolyzing class D β-lactamases (CHDLs), such as observed in *Acinetobacter* spp. and *Enterobacteriaceae*, represents a major public health concern worldwide, especially among immunocompromised individuals, is associated with a higher mortality rate and has been connected to outbreaks of nosocomial infections.[2,8-11]

The emergence of *K. pneumoniae* carbapenemases (KPCs) and carbapenemases of OXA-48-type in particular is increasing broadly, with varying geographical trends.[1,12] In addition to *K. pneumoniae*, this pattern of resistance was similarly observed in various *Enterobacteriaceae* species including *Enterobacter cloacae, Citrobacter freundii, Escherichia coli, Serratia marcescens, K. oxytoca*, and *Providencia rettgeri*.[11]

Acquired carbapenemases are enzymes able to break down carbapenems and can also hydrolyse almost all β-lactam antibiotics.[1,8,13] Currently, carbapenems antibiotics such as imipenem, meropenem, biapenem, ertapenem, and doripenem, are the last treatment options for the treatment of infections caused by multidrug-resistant *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs).[5,6,8,13] These pathogenic bacteria are frequently resistant to aminoglycosides, fluoroquinolones, trimethoprim-sulfamethoxazole and other antibiotics.[9]

Commonly, OXA-48 enzymes show high-level of hydrolytic activity against penicillins and carbapenems at a low level, sparing expanded-spectrum activity against cephalosporins, and are not susceptible to β-lactamase inhibitors.[6,12,13] The kinetics analysis of these enzymes demonstrated low level of hydrolytic activity against carbapenems, with higher activity against imipenem than meropenem.[10]

Currently, the OXA-48-like subgroup has seen an important evolution in new enzyme variants; they are grouped by amino acid identity and include a heterogeneous group of enzymes.[7] In addition to OXA-48, 10 variants with similar enzymatic profiles were identified and named OXA-48b, OXA-162, OXA-163, OXA-181, OXA-199, OXA-204, OXA-232, OXA-244, OXA-245, OXA-247.[5,10,11] The $bla_{\text{OXA-48}}$ gene is carried by the Tn*1999* composite transposon or its variants Tn*1999.2* and Tn*1999.3*, is made of two copies of the insertion sequence IS*1999* and is located on a transferrable plasmid.[13,15]

In 2001, the first isolate of *K. pneumoniae* OXA-type β-lactamase was identified and named OXA-48 in Istanbul, Turkey. It subsewuently spread in several Turkish cities[16] and has since been described in other enterobacterial species throughout the Mediterranean area.[6] In the following years, outbreaks of OXA-48-producing *K. pneumoniae* have been reported in countries such as Algeria,[17] India,[18] Spain,[13] Japan,[19] Argentina,[20] Saudi Arabia,[21] Italy,[22] USA,[23] Canada[24] and Norway.[2]

Although numerous studies described the emergence of *K. pneumoniae* OXA-48 producers worldwide, their amino acid structure analysis and phylogeny is still lacking in the literature, limiting the observation of the evolutionary relationship inside this class of enzymes. The present study explores the enzyme structure variability and phylogeny of class D *K. pneumoniae* OXA-48 from different geographic areas of the world to provide additional information and understanding of their evolutionary features.

Materials and Methods

The data on OXA-48 *K. pneumoniae* structures was gathered from Protein Data Bank (PDB) (https://www.rcsb.org) during the month of May 2019. The corresponding Ipdb of each enzyme, FASTA sequence of chain A, variant name, country of origin and references were registered. The sequences were analyzed for close homology by the Clustal Omega program available on the database of protein sequence and functional information UniProt (http://www.uniprot.org/align/). The Phylogenetic analysis and dendrograms were carried using MEGA software version 6.[25]

Results

A total of 58 *K. pneumoniae* OXA-48 variants were observed in PDB during the month of May 2019, among them, OXA-48 was the most dominant variant with 56 structures, while only one OXA-181 and one OXA-245 structures were found. Among these variants, 8 representatives OXA-48 isolates were selected for the study, formed by six OXA-48 structures, one OXA-181 and one OXA-245. To ensure the geographical diversity, the variants were taken from different regions in the world. The corresponding Ipdb of each enzyme, variant name, country of origin and references are summarized in Table 1.

Alignment results displayed 88.679 % of identity with 5 similar positions and 235 identical positions. The longest chain belongs to three structures 3HBR, 5OE0 and 5OE2 with a length of 265 amino acids residues, followed by 5QA4 with 243 residues, 4WMC with 242 residues, while the three structures 5HAQ, 5HAP and 5FAQ contain 241 residues (Figure 1).

The alignment of bla OXA-48 variants showed the four conserved motifs $_{70}$STFK$_{73}$, $_{118}$SVV$_{120}$, $_{144}$YGN$_{146}$, $_{208}$KTG$_{210}$ in all enzyme structures, in addition to the two conserved loops: Ω loop 157W-X-X-X-X-X-X-I-X164 and β5-β6 loop across the residues 212-220.

In the conserved motifs regions, the whole of OXA-48 variants were stable excepted for the two mutations S70G and S70A observed respectively in the two chains 5HAQ and 5HAP from the United States; both mutations were located in the conserved motif STFK. However, the OXA-181 and OXA-245 variants di-

Table 1

Klebsiella pneumoniae OXA-48 variants gathered from PDB during the month of May 2019: variant name, Ipdb, country of origin and references.

Variant name	Ipdb	Country of origin	References
OXA-48	3HBR	Italy	8
OXA-48	4WMC	United States	23
OXA-48	5HAQ*	United States	29
OXA-48	5HAP**	United States	29
OXA-48	5FAQ	Canada	19
OXA-48	5QA4	Norway	3
OXA-181	5OE0	Norway	2
OXA-245	5OE2	Norway	2
*: mutant - S70G, **: mutant - S70A			

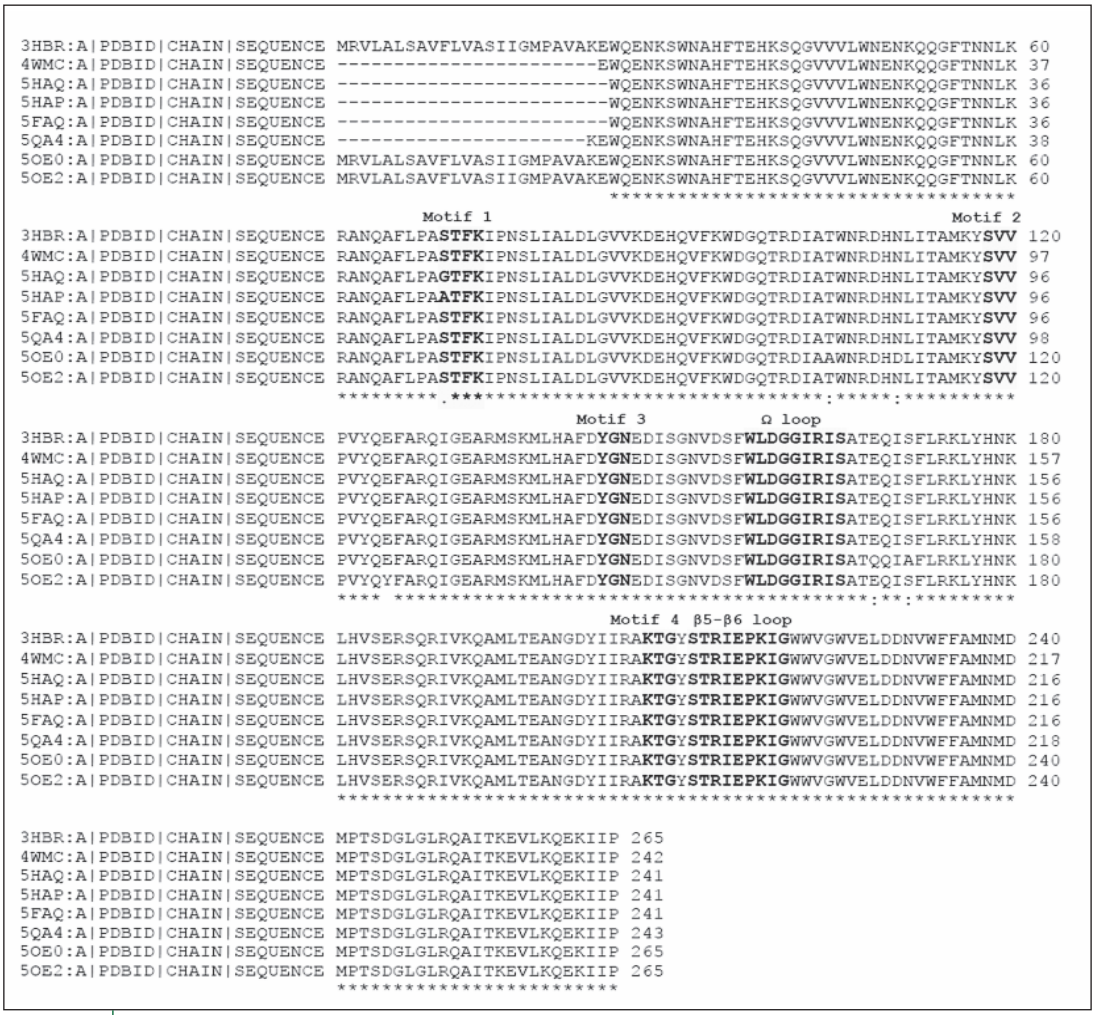

Figure 1 Amino acid alignment of 8 representative *Klebsiella pneumoniae* OXA-48 variants from different areas in the world: OXA-48 (3HBR/Italy, 4WMC, 5HAQ and 5HAP/United States, 5FAQ/Canada, 5QA4/Norway); OXA-181(5OE0) and OXA-245(5OE2)/Norway. Stars indicate residues identical among all the amino acid sequences. Amino acid motifs which are well conserved (even if possibly variable) are indicated by gray shading. Numbering is according to DBL.[35]

splayed mutations far from the active sites and none of the conserved motifs seemed to be affected. In comparison with OXA-48, OXA-181 variant (5OE0 from Norway) showed four substitutions at Thr104Ala, Asn110Asp, Glu168Gln, and Ser171Ala; while OXA-245 has a single amino acid substitution at Glu125Tyr.

The phylogenetic tree revealed three distinct clusters (Figure 2); the first cluster regroups four OXA-48 structures in addition to OXA-245. The second cluster regroups two OXA-48 structures and the third cluster is formed by an individual OXA-181 variant. In general the evolutionary distances between *K. pneumoniae* enzymes structures were considerably low and did not exceed 0.0021, expressing a near phylogenetic linkage despite the geographic origins. Particularly, there was a perfect identity between the four OXA-48 structures: 5FAQ (Canada), 5QA4 (Norway), 4WMC (United States) and 3HBR (Italy). The two OXA-48 structures: 5HAQ and 5HAP from United States were clustered together manifesting a similar evolutionary trend. However, the individual OXA-181 from Norway had a unique evolutionary feature compared to the other OXA-48 variants. The evolutionary state of OXA-245 was intermediate to the whole of variants.

Discussion

Klebsiella pneumoniae OXA-48 variants structures

The low number of *K. pneumoniae* OXA-48 protein structures available on PDB could be explained by the typical association of this class of enzymes with *Klebsiella* spp.[26] Indeed, Antunes and Fisher[7] reported that OXA-48 phenotype have not yet been described in other bacteria such as *Acinetobacter* and *Pseudomonas*. On the another hand, among the 12 subgroups of class D Carbapenemases (OXA-23, OXA-24/40, OXA-48, OXA-51, OXA-58, OXA-134a, OXA-143, OXA-211, OXA-213, OXA-214, OXA-229, and OXA-235), only OXA-23, OXA-48, OXA-51, and OXA-58 subgroups were reported in *K. pneumoniae*.[27] Among OXA-48-like β-lactamases, more than 10 OXA-48 variants have been described and named OXA-48b, OXA-162, OXA-163, OXA-181, OXA-199, OXA-204, OXA-232, OXA-244, OXA-245, OXA-247, with OXA-48 being the most prevalent.[10,14] Currently, among the members of OXA-48-like enzymes, all the variants OXA-181 and OXA-245 have been established in *K. pneumoniae*.[2,5] Moreover, the low number of *K. pneumoniae* OXA-48 protein structures available suggests that the costly sequencing limits their study and exploration.

Alignment of Klebsiella pneumoniae OXA-48 variants

Despite the variability observed in the whole of Class D β-lactamases, several conserved amino acid residues and motifs are exhibited.[7] Each monomer harbors an independent active site made of four conserved motifs including $_{70}$STFK$_{73}$, $_{118}$SVV$_{120}$, $_{144}$YGN$_{146}$, $_{208}$KTG$_{210}$.[2] The highly conserved motif STFK (Ser-Thr-Phe-Lys) located at positions 70-73 (DBL numbering system) is

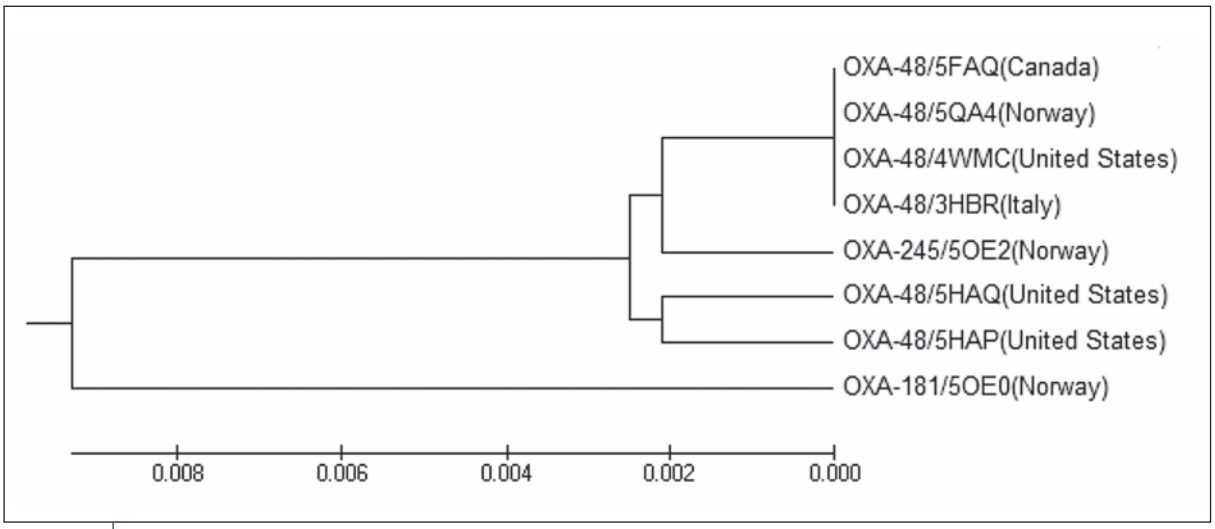

Figure 2 Dendrogram obtained from 8 representative OXA-48 *Klebsiella pneumoniae* variants isolated from different geographical regions worldwide. The evolutionary history was inferred using the UPGMA method. The evolutionary distances were computed using the Poisson correction method. Evolutionary analyses were conducted in MEGA6.[25]

present in the majority of Class D β-lactamases and possesses the active-site serine situated at position 70.[4,7] Serine has been established as the nucleophile in charge for the creation of the acyl-complex with β-lactam antibiotics, while the deacetylation is ensured by lysine (Lys73) which has a conserved post-translational carboxylation function.[2,5] The YGN motif is located at the positions 144 to 146; however it can be replaced by the FGN motif in several OXAs. The highly conserved KTG motif is present at the positions 216 to 218 in class D β-lactamases.[4]

The members of OXA-48-like variants differ by only a few amino acid substitutions or deletions (one to five amino acids).[5,11,12,14] Potron *et al*.[28] demonstrated four substitutions at Thr104Ala, Asn110Asp, Glu168Gln, and Ser171Ala) in OXA-181 variant, identified from a *K. pneumoniae* isolate in India. Compared to OXA-48, OXA-245 showed a single amino acid substitution Glu125Tyr from *K. pneumoniae* isolates collected in Spain.[13] The variant OXA-245 emerges from random mutations in the *K. pneumoniae* OXA-48 gene. On another hand, the substitutions in OXA-181 and OXA-245 protein sequences were observed far from the active-site residues, and none of the conserved motifs seems to be affected;[2] despite the amino acid sequence variability, the active-site residues involved in hydrolysis are almost completely conserved.[5]

According to Evans and Amyes[10] even for the same enzyme, the important variation between studies in the kinetic properties obtained, complicates the feasibility of valid comparisons. Stojanoski *et al*.[29] evaluated the effect of side chain removal at the active-site serine by a glycine substitution on stability. They confirmed that all glycine mutants enzymes showed enhanced thermostability compared to the wild type, and their catalytic efficiency decreased considerably. Compared with OXA-48, it seems that the modifications in OXA-181 and OXA-245 protein sequences did not significantly change the enzymatic kinetics characteristics, exhibiting a broadly similar activity.[7,10,12] Nevertheless, it is well known that changes in distal sites can affect the enzymatic catalytic activity.[2]

Phylogeny of Klebsiella pneumoniae OXA-48 variants

Since the first reports in the Mediterranean countries of Europe and North Africa, the number of OXA-48 and OXA-48-like enzymes have increased worldwide and have recently disseminated to United States, Canada and Japan, with OXA-48 being the most geographically prevalent member of the class D β-lactamases.[10,11]

While *K. pneumoniae* isolates responsible for OXA-245 were identified in restricted areas such as Spain [13,30] and South Africa,[31] OXA-181 variant has been described worldwide. In all cases the concerned regions are known for the high prevalence of *K. pneumoniae* OXA-48 producers.[1]

Evolution of antibiotic resistance in time is due to the development of new mutations under antibiotic selection in clinical environment or usually by acquisition of mobile genetic elements such as plasmids or transposons increasingly spreading in community and environment.[12,26] Furthermore, the pOXA-48a plasmid containing the unique antibiotic resistance gene $bla_{\text{OXA-48}}$, is self-conjugative with high conjugation rate, which may explain its global dissemination in *K. pneumoniae*.[1]

Traditionally, *K. pneumoniae* OXA-48 producers are commonly identified from patients with a history of travel to countries known for increased prevalence of OXA-48 producers, even without contact with the local healthcare system.[6,9,12] However, cases of patients who had not travelled abroad have been reported, suggesting potential nosocomial transmission.[15] The major risk factors associated with OXA-48 carbapenemase-producing *K. pneumoniae* outbreaks are previous hospitalization, previous carbapenem treatment, bloodstream infections, fecal carriage, ventilator use, intensive care unit and personnel movement between infectious diseases and internal medicine wards.[12]

K. pneumoniae frequent cause of human infections. It is largely present in the environment, found in water, soil and sewage, and can survive for prolonged periods of time in extreme environments.[1,2] Indeed, the increase of antibiotic residues in the environment due to the worldwide rise of antibiotic use both in humans and animals, creates a selective pressure responsible for the emergence and spread of bacterial resistance.[12] Consequently, humans can contact antibiotic-resistant bacteria, antibiotics or antibiotic resistance genes through various environmental pathways. Food chain, agricultural and veterinary systems are anthropogenic potential sources of antibiotic presence in the environment.[12,26] Interestingly, Prado *et al*.[32] detected the presence of ESBL-producing *K. pneumoniae* in effluents and sludge of hospital sewage treatment plant.

On the another hand, wildlife could play an essential role in the proliferation of resistant bacteria , particularly through wild birds which may in fact contribute to the intercontinental transmission of antibiotics resistance.[33] Moreover, insects such as houseflies are possible vectors for the dissemination of Multi Drug Resistant bacteria (MDR) in different environments.[34]

In conclusion, the alignment of *K. pneumoniae* OXA-48 enzymes exhibited a low level of amino acid variability. The main mutations were located far from the active sites in all variants, except for the two mutant

S70G and S70A from United States where the substitutions occurred in the active site STFK. The phylogenetic analysis showed a near linkage of protein structures despite the geographic origins of *K. pneumoniae* isolates except for the individual variant OXA-181. These observations confirm a similar evolutionary trend of *K. pneumoniae* OXA-48 variants worldwide.

However, the present data on *K. pneumoniae* OXA-48 amino acids structures is limited to restricted geographic areas, and need expansion to demonstratethe actual state of molecular changes and antimicrobial resistance evolution. In addition, future studies must focus on the effect of molecular modifications on enzyme stability and kinetics to evaluate carbapenems antibiotics efficiency.

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Conflict of Interest

The authors declare no conflict of interest.

Περίληψη

Φυλογενετική ανάλυση και ποικιλομορφία των OXA-48 class D καρβαπενεμασών παραγόμενων από στελέχη *Klebsiella pneumoniae*

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Οι καρβαπενέμες θεωρούνται η τελευταία επιλογή στη θεραπεία των λοιμώξεων που προκαλούνται από εντεροβακτηριακά που παράγουν ευρέως φάσματος β-λακταμάσες (ESBLs). Η εμφάνιση στελεχών *Klebsiella pneumoniae* που παράγουν OXA-48 καρβαπενεμάσες εξαπλώνεται σταθερά, με αποτέλεσμα να αποτελεί μεγάλο κίνδυνο για τη δημόσια υγεία. Σκοπός της παρούσας μελέτης ήταν η φυλογενετική ανάλυση OXA-48 καρβαπενεμασών προερχόμενων από στελέχη *K. pneumoniae* διαφόρων γεωγραφικών περιοχών. Τα δεδομένα για τη δομή των αμινοξέων των συγκεκριμένων καρβαπενεμασών συλλέχθηκαν από την Τράπεζα Πρωτεϊνών (Protein Data Bank, PDB), όπως αυτά βρέθηκαν κατατεθειμένα τον Μάιο 2019. Η κατάταξη των αλληλουχιών έγινε με το πρόγραμμα Clustal Omega, διαθέσιμο στη βάση δεδομένων UniProt. Η φυλογενετική ανάλυση και το δενδρόγραμμα πραγματοποιήθηκαν με τη χρήση του λογισμικού MEGA (version 6). Μεταξύ 58 κατατεθειμένων αλληλουχιών, για τη μελέτη επελέγησαν 8 αντιπροσωπευτικές παραλλαγές (variant) των OXA-48. Η μελέτη έδειξε ότι γενικά οι σταθεροί τύποι ήταν καλά διατηρημένοι, με εξαίρεση δύο μεταλλάξεις S70G και S70A, που παρατηρήθηκαν στις δύο αλύσους 5HAQ και 5HAP στελεχών από τις ΗΠΑ. Εντούτοις, τα OXA-181 και OXA-245 variants εμφάνισαν μεταλλάξεις πολύ μακριά από τις ενεργείς θέσεις. Σε σύγκριση με το OXA-48, το OXA-181 variant έδειξε τέσσερεις αντικαταστάσεις και συγκεκριμένα: Thr104Ala, Asm110Asp, Gly168Glu και Ser171Ala. Αντίθετα το OXA-245 variant είχε μόνο μια αντικατάσταση στη θέση 125 και συγκεκριμένα Gly125Tyr. Η φυλογενετική ανάλυση έδειξε τρεις ξεχωριστές ομάδες. Η πρώτη αποτελούταν από τέσσερα OXA-48 variants από Καναδά, Νορβηγία, ΗΠΑ και Ιταλία, και ένα OXA-245 από Νορβηγία. Η δεύτερη περιελάμβανε δύο OXA-48 variants από τις ΗΠΑ και η τρίτη ομάδα ένα OXA-181 variant από τη Νορβηγία. Τα αποτελέσματα της παρούσας μελέτης επιβεβαίωσαν την παγκόσμια εξελικτική τάση στη δομή των OXA-48 variants. Τα δεδομένα σήμερα για τη δομή των αμινοξέων των OXA-48 γενικά είναι περιορισμένα σε συγκεκριμένες περιοχές και υπάρχει ανάγκη περαιτέρω μελετών, έτσι ώστε να γίνει γνωστή η κατάσταση σχετικά με τις μοριακές μεταβολές και την εξέλιξη της αντιμικροβιακής αντοχής.

Λέξεις κλειδιά
Klebsiella pneumoniae, OXA-48 variants, αντιμικροβιακή αντοχή

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