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EVOLUTION OF BETA-LACTAMASES IN URINARY *KLEBSIELLA PNEUMONIAE* ISOLATES FROM CROATIA; FROM EXTENDED-SPECTRUM BETA-LACTAMASES TO CARBAPENEMASES AND COLISTIN RESISTANCE

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ABSTRACT

K. pneumoniae isolates often harbor various antibiotic resistance determinants including extended-spectrum β-lactamases (ESBLs), plasmid-mediated AmpC β-lactamases (p-Amp-C) and carbapenemases. In this study we analyzed 65 *K. pneumoniae* isolates obtained from urinary tract infections in the outpatients setting, with regard to antibiotic susceptibility, β-lactamase production, virulence traits and plasmid content.

Antibiotic susceptibility was determined by broth microdilution method. PCR was applied to detect genes encoding ESBLs, p-Amp-C and carbapenemases and plasmid incompatibility groups. Phenotypic methods were applied to characterize virulence determinants.

Increasing resistance trend was observed for amoxicillin/clavulanate, imipenem, meropenem and ciprofloxacin. The study showed that ESBLs belonging to the CTX-M family, conferring high level of resistance to expanded-spectrum cephalosporins (ESC) were the dominant resistance trait among early isolates (2013 to 2016) whereas OXA-48 carbapenemase, belonging to class D, emerged in significant numbers after 2017. OXA-48 producing organisms coharbored ESBLs. KPC-2 was dominant among isolates from Dubrovnik in the recent years. Colistin resistance was reported in three isolates. Inc L/M was the dominant plasmid in the later period, encoding OXA-48. Hyperviscosity was linked to KPC positivity and emerged in the later period.

This report describes evolution of antibiotic resistance in *K. pneumoniae* from ESBLs to carbapenemases and colistin resistance. The study demonstrated the ability of *K. pneumoniae* to acquire various resistance determinants, over time. The striking diversity of the UTI isolates could result from introduction of the isolates from the hospitals, transfer of plasmids and multidirectional evolution.

Key words: *Klebsiella pneumoniae*, extended-spectrum β-lactamase, OXA-48, KPC, resistance

INTRODUCTION

Klebsiella pneumoniae is an important hospital pathogen associated with a wide range of infections such as septicemia, meningitis, urinary tract infections (UTI), ventilator associated pneumonia and burn and wound infections [1]. In addition, it is an important causative agent of community acquired UTIs and UTIs in nursing home residents. *K. pneumoniae* isolates often harbor various antibiotic resistance determinants including extended-spectrum β-lactamases (ESBLs), plasmid-mediated AmpC β-lactamases (p-Amp-C) and carbapenemases [2-3]. Colistin is very often the last resort antibiotic, but emergence of colistin resistance in *K. pneumoniae* is limiting its therapeutic use. Colistin resistance determinants are usually found in ESBL or carbapenemase- producing *K. pneumoniae* [4].

The majority of ESBLs belong to three families: TEM, SHV, and CTX-M. TEM and SHV variants are derived by mutations of parental broad- spectrum TEM-1, TEM-2 and SHV-1 β-lactamases [2]. CTX-M β-lactamases are a growing family of plasmid-encoded ESBLs, that preferentially hydrolyze cefotaxime [2]. They are not closely related to TEM or SHV β-lactamases but are typical members of Ambler's class A which are derived from the genus *Kluyvera* [5]. In contrast to TEM and SHV β-lactamases, which rely on amino acid substitutions to extend their substrate profile, CTX-M enzymes have an intrinsic extended-spectrum profile. CTX-M β-lactamases are the dominant type of ESBLs in many countries such as UK, Italy, Spain, Greece, Poland, Bulgaria, Russia, Latvia, Brazil, China, Taiwan, and many other countries [5-6]. They are divided into five clusters based on their protein sequences: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 type. There are also rare types of ESBLs such as VEB, PER or IBC which are predominantly found in species

other than *K. pneumoniae* [2]. ESBL-producing *K. pneumoniae* strains can cause outbreaks of nosocomial infections, which are very difficult to control due to the multi-resistant phenotype of the causative bacteria and were reported in Mexico, Poland, and other countries [7-8]. Previous studies in Croatia revealed predominance of SHV-2, SHV-2a, and SHV-5 among *K. pneumoniae* isolates in 1994-1999 [9, 10] but they were replaced by CTX-M-15 post 2000 [11].

P-AmpC β-lactamases are derived from the chromosomal β-lactamases of the bacteria belonging to the genus *Enterobacter*, *Serratia*, *Citrobacter*, *Pseudomonas*, and *Acinetobacter* by the escape of the chromosomal gene to the plasmid [12]. They hydrolyze expanded-spectrum cephalosporins (ESC), monobactams, and cephamycins, but spare fourth generation cephalosporins and carbapenems. Unlike ESBLs, they are not susceptible to the inhibition with clavulanic acid, sulbactam or tazobactam [12]. The most frequent p-AmpC in *K. pneumoniae* is DHA-1 [12] which was also reported in Croatia [13].

Resistance to carbapenems is mediated mostly by two main mechanisms. The first involves the production of β-lactamase such as AmpC- cephalosporinases or ESBL with a very low level, combined with decreased permeability due to porin loss or alteration. The second mechanism is related to carbapenem hydrolyzing β-lactamases belonging to Ambler class A serin β-lactamases (KPC, GES), class B metallo-β-lactamases (MBL) of the IMP, VIM or NDM family, and OXA-48-like β-lactamases belonging to the class D or carbapenem-hydrolysing oxacillinases (CHDL) [14]. The Ambler class D OXA-48 β-lactamase initially identified from a carbapenem-resistant *K. pneumoniae* isolate from Turkey in 2004, hydrolyzes penicillins and imipenem, sparing ESC [15]. In the recent years, a remarkable increase of OXA- 48 producing organisms was reported in many countries all over the world with the highest rates observed in Turkey [16-19], France [20], and Germany [21]. The genes

encoding OXA-48 are located on Tn*1999*, a composite transposon made of two copies of the insertion sequence IS*1999* [22].

In 2008, the first carbapenemase identified in *K. pneumoniae* in Croatia was NDM-1 [23], while KPC-2 appeared in 2011 [24]. In the multicenter study conducted from 2012 to 2013, VIM-1 and NDM-1 were predominant carbapenemases in *K. pneumoniae* [25], followed by predominance of OXA-48 in 2015 and thereafter [26].

Colistin resistance in *K. pneumoniae* has emerged recently caused by the inactivation of the *mgr*B gene, encoding a negative feedback regulator of the *Pho*Q-*Pho*P signaling system which activates the *pmr* system that is responsible for the modification of the lipopolysaccharide polymyxin target and the acquisition of plasmid-mediated *mcr* genes, respectively [27]. The first colistin resistant *K. pneumoniae* isolates emerged in Croatia in 2013 and were associated with KPC or OXA-48 carbapenemases. The isolates were found to possess single nucleotide polymorphism (SNP) in the *pmr* and *pho*Q genes responsible for colistin resistance [28]. UTI isolates often possess various virulence determinants enabling the development of upper UTI, pyelonephritis and bloodstream infection [29].

During the routine laboratory work, we observed a change of the resistance phenotype among UTI *K. pneumoniae* isolates in two medical centers in Croatia in the last decade, which initiated a study to clarify the evolution and dynamic changes of antimicrobial resistance and virulence of this important urinary pathogen over an eight years period.

Material and methods

Bacterial isolates

The *K. pneumoniae* isolates from outpatient's urinary specimens were collected in two centers located in different geographic regions: Public Health Institute of Dubrovnik-Neretva County in southern- coastal region and University Hospital Centre Zagreb (UHC Zagreb) in northern-continental Croatia. The isolates were identified to the species level by conventional biochemical testing and MALDI-TOF. The patients had symptoms of UTI, significant bacteriuria, *K. pneumoniae* in pure culture (>10^5 CFU/ml) and pyuria (>5 white blood cells/high power field). Only in the case of urine obtained from catheters, multiple organisms were analyzed as well. The isolates were collected during two periods: 2013 to 2016 and 2018 to 2021. In total, 65 non duplicate (one per patient) isolates were collected: 8 from Dubrovnik and 57 from UHC Zagreb. There were 20 isolates in the first and 45 isolates in the second period.

The age range of the patients was 1 month to 93 years. Eleven patients were residents of the nursing home in Zagreb, which sends specimens for microbiology analysis to UHC Zagreb, whereas one urine sample was from an orphanage near Zagreb. All other specimens were from non-institutionalized patients. Five urine samples were from urinary catheters, while the rest were middle stream urine samples. Five samples from catheters had mixed cultures. There were 27 males and 38 females, included in the study.

Antimicrobial susceptibility testing and phenotypic tests for detection of ESBLs, plasmid-mediated AmpC β-lactamases, and carbapenemases

The susceptibility testing to a wide range of antibiotics (Table 1) was determined by the broth microdilution method in Mueller-Hinton broth and 96 wells microtiter plates, according to CLSI standards from 2019 [30] and for colistin according to the EUCAST standard (Http://www.eucast.org). Minimum inhibitory concentrations (MICs) were read as the lowest concentration of an antibiotic that inhibited visible growth after 18h at 37 ºC. The susceptibility to ceftazidime/avibactam, sulphamethoxazole/trimethoprim, tetracycline, and chloramphenicol was determined by disk-diffusion test. The isolates were classified as multidrug-resistant (MDR), extensively drug-resistant (XDR) or pandrug-resistant (PDR) as described previously by Magiorakos et al [31].

The double disk synergy test (DDST) [32] was carried out in the frames of routine laboratory analysis of the isolates. ESBL production was confirmed by CLSI combined disk test using disks with expanded-spectrum cephalosporins (ceftazidime, cefotaxime, ceftriaxone) or ESC alone and with addition of clavulanic acid [30] whereas p-AmpC β-lactamases were detected by combined disk test using cephalosporin disks with 3-aminophenylboronic acid (PBA) [33]. A modified Hodge test (MHT) and the carbapenem-inactivation method (CIM) were used to screen for the presence of carbapenemases [34, 35]. Additionally, the isolates were tested by combined disk tests with imipenem and meropenem alone and combined with PBA, 0.1 M EDTA or both to screen for KPC, MBLs, or simultaneous production of KPC and MBL, respectively [36, 37].

Conjugation

Conjugal transfer of ertapenem and cefotaxime resistance was carried out in mixed broth cultures at 35 °C. *E. coli* J65 resistant to sodium- azide was used as recipient [38]. The ESBL and carbapenemase- producing transconjugants were selected on MacConkey agar containing either ertapenem (0.5 mg/L) or cefotaxime (2 mg/L) and sodium azide (100 mg/L). The

frequency of conjugation was determined relatively to the number of donor cells. Cotransfer of resistance to gentamicin, tetracycline, sulfamethoxazole/trimethoprim, chloramphenicol, and ciprofloxacin was determined as well.

Molecular detection of resistance genes

DNA templates were prepared by thermal lysis. Specific primers were used for the detection of broad spectrum and extended-spectrum β-lactamases (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{OXA-9}$, *bla*$_{OXA-1}$ and *bla*$_{PER-1}$) [39-42], p-AmpC β-lactamases [43], carbapenemases of class A (*bla*$_{KPC}$), B (*bla*$_{VIM}$, *bla*$_{IMP}$ and *bla*$_{NDM}$) and D (*bla*$_{OXA-48-like}$), [44] and fluoroquinolone resistance genes (*qnr*A, *qnr*B, *qnr*S) [45], as described previously. More specific primers for each cluster of CTX-M family were then used for isolates which tested positive for *bla*$_{CTX-M}$ genes with general MA-1 and MA-2 primers [46]. Representative CTX-M amplicons (number 3, 18, 19 and 20) were sent to Eurofin for sequencing. Plasmid-encoded colistin resistance genes *mcr-1* and *mcr-2* were analyzed in three isolates resistant to colistin [47]. PCRs were carried out in an AC196 - Alpha Cycler (PCR max, UK). PCR mapping was performed with primers for IS*1999* combined with forward and reverse primers for *bla*$_{OXA-48}$ [22]. The size of the product was determined by the gel electrophoresis, after staining with ethidium bromide. The flanking regions of *bla*$_{CTX-M}$ genes were analyzed by PCR mapping with forward primer for IS*Ecp1* and IS26 combined MA-3 primer (universal reverse for *bla*$_{CTX-M}$ genes) [48].

Characterization of plasmids

First, plasmid DNA was extracted from donor strains, and their respective transconjugants with the Machery Nagel nucleospin kit according to the manufacturer's instructions. The plasmid DNA was subjected to electrophoresis in 1% agarose gel. After staining with ethidium bromide, the DNA was visualized by ultraviolet light. PCR-based replicon typing (PBRT) [49] was applied to determine the plasmid content of the tested strains. Plasmid

extractions obtained from transconjugant strains were subjected to PCR for the detection of OXA-48 and ESBLs in order to determine the resistance gene content of the transconjugants. PBRT was also applied on transconjugants to identify incompatibility groups, such as in their respective donors.

Whole genome sequencing (WGS)

WGS was done on two representative isolates (4423 and 3822). The bacterial genomes were sequenced using the IonTorrent PGM platform (Life Technologies, Carlsbad, USA) according to the manufacturer's instructions. The Ion Xpress Plus Fragment Library Kit was used to enzymatically shear 100 ng of the genomic DNA. The target fragment size was 400 bp. Subsequently, the fragmented DNA was processed using the Ion DNA Barcoding kit (Life Technologies) and its size selected using the E-Gel SizeSelect 2% Agarose kit (Life Technologies). The size distribution of the DNA fragments was analyzed using the High Sensitivity Kit (Agilent, Santa Clara, USA). Further sample processing was performed using the Ion OneTouch Kit (Life Technologies). Finally, the amplified DNA was sequenced using the 318 chip (Life Technologies) [50]. The single reads obtained were *de novo* assembled using MIRA 3.9.9. The contigs were analyzed using online services of the Center of Genomic Epidemiology (http://www.genomicepidemiology.org/services/). ResFinder 4.0 was used to identify antibiotic resistance mechanisms and MLST 2.0 to determine the sequence type.

Determination of virulence factors

String test was attempted by stretching a mucoviscous string from the colony using a standard bacteriologic loop, as described previously. If a viscous string >5 mm was formed, the isolate was defined as hypermucoviscous.

The production of hemolysin was tested on human blood agar plate and was considered positive when bacteria were stabbed with a sterile straight wire into 5% human blood agar, and after 18 to 24 h of incubation at 37 ºC, a clearing zone was observed.

Bacterial susceptibility to serum killing was measured by assessing regrowth after incubation in normal human serum according to the Schiller and Hatch method.

Results

Antimicrobial susceptibility testing and phenotypic tests for detection of ESBLs, plasmid-mediated AmpC β-lactamases and carbapenemases

An increasing resistance trend between two periods was observed for amoxicillin/clavulanic acid, cefuroxime, cefepime, imipenem, meropenem, and ciprofloxacin with 55%, 95%, 80%, 10%, 15%, and 70% of resistant isolates, respectively, in the first period, and 69%, 100%, 87%, 24%, 31% and 95% in the second period, respectively (Table 1). A decreasing trend was reported for piperacillin/tazobactam and gentamicin, with 60% and 85% of resistant isolates in the first period, respectively, and 58% and 80%, respectively, in the second period as shown in Table 1. The isolates from both periods showed uniform resistance to ceftazidime, cefotaxime, and ceftriaxone. Colistin resistance did not exhibit temporal variations. There was a higher rate of ESBL positivity detected by DDST and combined disk test in the first compared to the second period (95% vs 88%). On the contrary, the rate of carbapenemase positivity detected by modified Hodge and CIM tests was higher in the second period compared to the first (47% vs 15%) (Table 1).

Conjugation

Cefotaxime resistance was transferable from 30 isolates (2 from the first and 28 from the second period) (10% vs 62%). The frequency of conjugation ranged from 1.4×10^{-5} to 9.3×10^{-3}. Resistance to tetracycline was co-transferred alongside with cefotaxime resistance from 20 strains, to sulphamethoxazole/trimethoprim from 16, and to gentamicin from nine strains. Resistance to chloramphenicol was not transferable. Transfer of ertapenem resistance to *E. coli* recipient strain was successful from 13 strains with OXA-48 carbapenemase (2 from the first period and 11 from the second period, 100% vs 69%) with frequencies ranging from 1.5×10^{-5} to 4.3×10^{-2}. The transfer of ertapenem resistance was not successful from KPC-producing organisms. Resistance determinants to non-β-lactam antibiotics among OXA-48-producing organisms were not transferable.

Molecular detection of resistance genes

The rate of *bla*$_{\text{CTX-M}}$ positivity remained very high during both periods (95% in the first and 88% in the second period) as shown in Table 1. On the other hand, *bla*$_{\text{OXA-48}}$ genes were found in only 10% of the early isolates, but increased to 35% among the late isolates. *bla*$_{\text{TEM}}$ genes were detected only in the first period as shown in Table 1. *bla*$_{\text{KPC}}$ genes were only sporadic in the first period and emerged in significant numbers in the second period (5% vs 20%). There was only one strain harboring *bla*$_{\text{VIM}}$ gene identified in 2021.

IS*1999* preceded *bla*$_{\text{OXA-48}}$ genes in eight isolates demonstrating reduced susceptibility to meropenem. *Mcr* genes encoding transferable colistin resistance and *qnr* genes were not found. Insertion sequences IS26 and IS*Ecp1* were not identified. Transconjugant strains harbored the same β-lactamases as their respective donors.

Characterization of plasmids

IncFIA, IncN, and IncFII were dominant plasmid types detected in 10% of the isolates in the earlier period as shown in Table 1. On the other hand, IncL/M dominated in the second period with 35% of the isolates being positive, all positive for OXA-48. IncFII was the second most prevalent plasmid type in the later period with 9% positive isolates (Table 1). The IncA/C plasmid was identified in 2021 and encoded VIM- MBL. There was only sporadic occurrence of IncW, IncFIB and IncI1 plasmids in both periods.

Whole genome sequencing

WGS of two isolates 4423 (NCBI Gen Bank accession number: JAMKEO000000000) and 3822 (NCBI Gen Bank accession number: JAMZQT000000000) revealed a wide variety of aminoglycoside and fluoroquinolone resistance genes (Table 2). Both isolates possessed *aac(*6')Ib-cr, *aac*(3)-IId, and *str*B genes encoding aminoglycoside acetylases and *oqx*A and *oqx*B encoding efflux pumps, responsible for fluoroquinolone resistance as shown in Table 2. *Sul*2 encoding dihydropteroate synthetase and *tet*B coding for tetracycline resistance protein were identified as well. One isolate had the *cat*B3 gene encoding chloramphenicol acetyltransferase. Among the β-lactam resistance determinants, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1b}}$, and, $bla_{\text{OXA-1}}$ were detected in both isolates. The two sequenced ESBL- positive organisms 4423 and 3822 were found to belong to ST15.

Virulence factors

The rate of hemolysin production was higher in the earlier period (40% vs 31%) whereas string tests, indicating hyperviscousity tested positive in two KPC-producing organisms from the later period (Table 1). All isolates exhibited resistance to serum bactericidal activity as shown regardless of the period as shown in Table 1.

Discussion

ESBLs were identified as a dominant resistance determinant among our isolates throughout the study period, whereas carbapenemases and colistin resistance emerged in later years.

The production of ESBLs was associated with a high level of resistance to ESC and to non-β-lactam antibiotics which could be explained by the fact that these resistance genes are usually encoded on the same plasmid carrying *bla*$_{\text{CTX-M}}$ genes. CTX-M β-lactamases are typical cefotaximases, but the CTX-M-15 variant identified in some of the isolates very efficiently hydrolyzed also ceftazidime, and thus, the majority of isolates exhibited high level resistance to all ESC. IS*Ecp* increases the expression of *bla*$_{\text{CTX-M}}$ genes and mediates the mobilization of the genes. In spite of the lack of an insertion sequence, the transfer of cefotaxime resistance was successful in half of the isolates. IncFIA and IncFIB plasmids carried *bla*$_{\text{ESBL}}$ genes similarly as in the previous studies on ESBL *E. coli*.

The majority of ertapenem-resistant isolates were positive for OXA-48 which emerged as carbapenem-resistant determinant among UTI isolates already in 2013, but increased significantly in 2020. The isolates were resistant to penicillins and ESC, but exhibited different resistance patterns to carbapenems, with ertapenem resistance, whereas imipenem and meropenem tested susceptible, intermediate susceptible or resistant depending on the strain. All isolates coharbored additional ESBLs belonging to the CTX-M family, responsible for resistance to ESC. The L/M plasmid was found in all OXA-48-positive organisms which is in concordance with earlier publications [26]. OXA-48 was detected only in isolates from Zagreb. IS*1999* enables mobilization events and was found in eight isolates resistant to meropenem leading to the conclusion that it increases the expression of *bla*$_{\text{OXA-48}}$ genes. KPC-2-producing strains originated mostly from Dubrovnik. KPC was very frequent in northwest Croatia at the beginning of carbapenemase spread ten years ago but in the UHC Zagreb

occurred sporadically [25]. It evolved to a dominant carbapenem resistance trait in the southern region of Croatia. The presence of carbapenemases in the specimens from both centers could be in part explained by the fact that the majority of outpatients whose specimens are processed in the participating institutions were previously hospitalized in different clinical wards where they were probably colonized with MDR isolates. A VIM-producing isolate was obtained from a child with vesicoureteral reflux which had multiple previous hospitalizations. The emergence of community-acquired UTI due to carbapenem-resistant isolates can be explained by the prolonged intestinal carriage of such isolates upon release from the hospital. VIM-1 was the dominant carbapenemase genes among Croatian *K. pneumoniae* isolates until 2011, but in the last decade it got outnumbered by OXA-48. Ertapenem resistance was transferable only from OXA-48-producing organisms, but not from KPC or VIM producers. This could be attributed to the high mobility of the L/M plasmid. Colistin resistance was associated with the production of the KPC-2 carbapenemase. Plasmid-mediated resistance was excluded, and thus, we can assume that resistance was due to the inactivation of *mgr*B genes as previously reported, but the clarification of this resistance trait was beyond this study. Phenotypic tests such as Hodge, CIM, and inhibitor-based tests with clavulanic acid showed a high sensitivity in recognizing carbapenemases and ESBLs. The majority of the patients with OXA-48-producing *K. pneumoniae* were residents of nursing homes which are important reservoirs of MDR bacteria. Colistin and ceftazidime/avibactam are in the most cases the only therapeutic options left for the treatment of UTIs caused by carbapenemase-producing pathogens, although some of the isolates with OXA-48 exhibited very low MICs of imipenem and meropenem, and thus, UTIs associated with them, are supposed to respond to carbapenem therapy. An XDR phenotype was identified in KPC-producing organisms. Moreover, hyperviscosity was associated with KPC carbapenemases as shown in previous

investigations [29]. Resistance to serum bactericidal activity enables the isolates to survive in the bloodstream and cause septicemia as complication of UTI.

Interestingly, AmpC β-lactamases which are very prevalent among outpatient urinary *P. mirabilis* isolates were not found in *K. pneumoniae*. ST15 was previously identified in a CTX-M-15-producing high risk clone in Brazil and in KPC-positive colistin-resistant isolates from Spain, showing the capability of this ST to acquire various resistance determinants. The strength of the study is detailed molecular analysis of the isolates, but the limitation is relatively small number of isolates and the fact that WGS was done only on two isolates.

Conclusion

The study found that carbapenemases increased in the last four years in addition to ESBLs which were detected in the early period as the sole β-lactam resistance determinant and persisted among the isolates throughout the whole study period. Moreover, the shift from incFIA and IncFIB plasmid types to a predominance of L/M plasmid type was noticed. Our study demonstrated the ability of *K. pneumoniae* to acquire different resistance traits over time. The striking diversity of the UTI isolates could result from the introduction of the isolates from the hospitals, transfer of plasmids, and multidirectional evolution. Some of the virulence traits such as serum resistance persisted over a prolonged time with the additional acquisition of new determinants like hyperviscosity linked with carbapenemase positivity in the recent period.

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ETHICAL STATEMENT

The study was approved by the Ethical Committee of the University Hospital Centre Zagreb. The Ethical permission was obtained from the University Hospital Centre Zagreb (number: 02/21 AG, class 8-1-15/122-2). It is a retrospective, *in vitro* study, not involving human or animal subjects. Urine samples were collected for purpose of routine diagnostic and thus informed consent was not necessary.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included here and are available from the corresponding author on reasonable request. Public access to the University repository is open: **http://medlib.mef.hr/**

Gene sequences of two strains (4423 and 3822) are deposited in the NCBI Gene bank with the numbers JAMKEO000000000 and JAMZQT000000000)

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CONFLICT OF INTEREST

None to declare.

AUTHOR'S CONTRIBUTION

B.B. I.B.: experimental work and manuscript preparation

L.P; M.K; S.S; N.B; Z.V; D.B.P and S.F.Z: experimental work and data collection

All authors read and agreed to the final version of the manuscript.

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Table 1. Antibiotic susceptibility, β-lactamase production and plasmid incompatibility groups of *K. pneumoniae* isolates.

Abbreviations: AMC-amoxycilin/clavulanic acid; TZP-piperacillin/tazobactam; CXM-cefuroxime; CAZ-ceftazidime; CTX-cefotaxime; CRO-ceftriaxone; FEP-cefepime; IMI-imipenem; MEM-meropenem; GM-gentamicin; CIP-ciprofloxacin; COL-colistin; resist-resistance. 1st period: 2013-2016, 2nd period: 2018-2021.

Characteristics	2013	2014	2015	2016	2018	2019	2020	2021	Total number	1st period	2nd period	Trend
Total number of isolates	13	1	4	2	6	6	7	26	65	20 (31%)	45 (69%)	
Total number of ESBL positive isolates	13	1	3	2	6	2	7	25	59	19 (95%)	40 (88%)	↓
Total number of carbapenemase positive isolates	1	0	2	0	1	6	3	11	24	3 (15%)	21 (47%)	↑
AMC resist	6	0	4	2	3	6	5	17	43	12 (55%)	31 (69%)	↑
TZP-resist	5	1	4	2	3	6	3	14	38	12 (60%)	26 (58%)	↓
CXM-resist	13	1	4	1	6	6	7	26	64	19 (95%)	45 (100%)	↑
CAZ-resist	13	1	4	2	6	6	7	26	65	20 (100%)	45 (100%)	
CTX-resist	13	1	4	2	6	6	7	26	65	20 (100%)	45 (100%)	
CRO-resist	13	1	4	2	6	6	7	26	65	20 (100%)	45 (100%)	
FEP-resist	9	1	4	2	5	5	5	23	54	16 (80%)	39 (87%)	↑
IMI-resist	1	0	1	0	1	5	0	5	13	2 (10%)	11 (24%)	↑
MEM-resist	1	0	2	0	1	5	1	7	17	3 (15%)	14 (31%)	↑
GM-resist	11	1	3	2	4	4	6	22	53	17 (85%)	36 (80%)	↓
CIP-resist	7	1	4	2	6	5	7	25	57	14 (70%)	54 (95%)	↑
COL-resist	0	0	1	0	0	2	0	0	3	1 (5%)	2 (4%)	
$bla_{\text{CTX-M}}$	13	1	3	2	6	2	7	25	59	19 (95%)	40 (88%)	↓
bla_{TEM}	0	0	0	2	0	0	0	2	4	2 (10%)	2 (4%)	↓
$bla_{\text{oxa-48}}$	1	0	1	0	1	2	4	9	18	2 (10%)	16 (35%)	↑
bla_{KPC}	0	0	1	0	0	4	0	0	5	1 (5%)	9 (20%)	↑
bla_{VIM}	0	0	0	0	0	0	0	1	1	0 (0%)	1 (2%)	↑
hemolysin	6	0	2	0	3	0	0	11	22	8 (40%)	14 (31%)	↓
String test	0	0	0	0	0	2	0	0	2	0 (0%)	2 (4%)	↑
Resistance to serum bactericidal activity	13	1	4	2	6	6	7	26	65	20 (100%)	45 (100%)	
IncI1 plasmid	0	0	0	1	0	0	0	1	1	0 (0%)	1 (2%)	↑
IncL/M plasmid	1	0	1	0	1	2	4	9	18	2 (10%)	16 (35%)	↑
IncN plasmid	2	0	0	0	0	0	0	0	2	2 (10%)	0 (0%)	↓
IncFIA plasmid	2	0	0	0	0	1	0	4	7	2 (10%)	5 (11 %)	↑
IncFIB plasmid	1	0	0	0	0	1	0	0	2	1 (5%)	1 (2%)	↓
IncW plasmid	1	0	0	0	0	0	0	1	2	1 (5%)	0 (0%)	↓
IncA/C plasmid	0	0	0	0	0	0	0	1	1	0 (0%)	1 (2%)	↑
IncFII plasmid	2	0	0	0	1	0	1	2	6	2 (10%)	4 (9%)	↓

Table 2. Whole genome sequencing of *K. pneumoniae* isolates. Abreviatons of resistance genes: AG-aminoglycosides; FQ-fluoroquinolones; CHL-chloramphenicol; SUL-sulphonamides; TET-tetracyclines.

Isolate	**AG**	**β-lactam**	**FQ**	**FOS**	**CHL**	**SUL**	**TET**
K. pneumoniae 4423	*aac(6')Ib-cr* *aac(3)-IId* *strB*	*bla*$_{\text{CTX-M-15}}$ *bla*$_{\text{SHV-28}}$ *bla*$_{\text{TEM-1-B}}$ *bla*$_{\text{OXA-1}}$	*aac(6')Ib-cr* *oqxB* *oqxA*	*fosA*		*Sul2*	*tetA*
K. pneumoniae 3882	*aac(6')Ib-cr* *aac(3)-IId* *strB*	*bla*$_{\text{TEM-1B}}$ *bla*$_{\text{CTX-M-15}}$ *bla*$_{\text{SHV-28}}$ *bla*$_{\text{OXA-1}}$	*aac(6')Ib-cr* *oqxB* *oqxA*	*fosA*	*catB3*	*Sul2*	*tetA*