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Characterization of Carbapenemase-Producing *Klebsiella pneumoniae* in Clinical Center University of Sarajevo, Bosnia and Herzegovina

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Klebsiella pneumoniae is the second most prevalent gram-negative rod that causes nosocomial infections in hospitalized or otherwise immunocompromised patients. It can develop multiple drug resistance that results in limited treatment options and increased use of carbapenems. Various mechanisms are related to the development of carbapenem resistance in *K. pneumoniae*. The aim of this study was to perform phenotypic and molecular characterization of clinical isolates of carbapenemase-producing *K. pneumoniae* from two outbreaks recorded in 2017 and 2018 in Clinical Center University of Sarajevo, Bosnia and Herzegovina. Identification of *K. pneumoniae* isolates was carried out on the basis of morphological, cultural, and biochemical characteristics. Interpretation of antimicrobial resistance was performed according to EUCAST breakpoints. There were four different resistotypes of carbapenemase-producing *K. pneumoniae* in this study and all were confirmed positive for $bla_{\text{OXA-48}}$ carbapenemase. Rep-PCR fingerprinting of these strains showed the presence of the two different genetic patterns with no similarity between them. The monitoring, surveillance, and molecular typing are essential to control the emergence of multidrug-resistant strains in nosocomial settings, and to reduce the frequency of outbreak occurrence.

Keywords: *Klebsiella pneumoniae*, carbapenemase, OXA-48, outbreak

Introduction

Klebsiella pneumoniae is the second most prevalent gram-negative rod that causes nosocomial infections in hospitalized or otherwise immunocompromised patients.[1,2]

In the past 40 years, treatment of infections caused by *K. pneumoniae* has been significantly impeded owing to the development of antimicrobial resistance. During the 2000s several global surveillance studies have shown that high percentage of *K. pneumoniae* isolates were resistant to first-line antibiotics, including cephalosporins, fluoroquinolones, and aminoglycosides. In addition, in the past 10 years, spreading of *K. pneumoniae* that produce extended-spectrum β-lactamase (ESBL) with limited treatment options has increased the use of carbapenems,[3] which has resulted in resistance to these antibiotics.

Various mechanisms are related to carbapenem-resistant *K. pneumoniae*.[4] Certain strains produce β-lactamases with very weak carbapenemase activity[5] but combined with permeability deformities can have greater influence in reduced carbapenem susceptibility (*e.g.*, Ambler class A or C ESBLs, CTX-M-15, CMY-2).[6]

Except from mentioned mechanisms, *K. pneumoniae* also produces enzymes that hydrolyze carbapenems and causes resistance without complementary permeability defects. These enzymes belong to Ambler molecular class A, B, or D.[7]

Data on carbapenem-resistant *K. pneumoniae* in Europe are different among regions. Low proportions of resistance were seen in most countries, whereas proportions between 25% and 50% were reported in Italy, Romania, Serbia, and Turkey, and proportions exceeding 50% were reported in Belarus and Greece.[8]

In several European countries, including France, Spain, Belgium, and Malta, OXA-48 is one of the most prevalent among the various acquired carbapenemases that can be found in carbapenem-resistant *K. pneumoniae* strains.[8,9] Its action results in efficient hydrolysis of β-lactams such as penicillins and poor hydrolysis of carbepenems and extended-spectrum cephalosporins.[10]

So far, among all members of Enterobacteriaceae, *K. pneumoniae* and *Escherichia coli* have been found to be the most common producers of OXA-48, particularly in nosocomial or community settings.[11] OXA-48-producing *K. pneumoniae* strains are indigenous in some Asian (Turkey),

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North African (Morocco, Tunisia), and European countries (Spain, Belgium) but differ in susceptibility profiles.[11] Indeed, the minimum inhibitory concentrations (MICs) of carbapenems may vary significantly among isolates, depending on the host susceptibility.[12]

The development of resistance to carbapenems is of greater concern, as these antibiotics are the last-line therapy in treatment of infections caused by multidrug-resistant (MDR) *K. pneumoniae*.[12] Carbapenamase genes are mostly carried on plasmids and can be shared among Enterobacteriaceae, including *K. pneumoniae*, and other gram-negative bacteria.[13]

Nosocomial spread of carbapenemase-producing *K. pneumoniae* is very common and most prominent in intensive care units (ICUs) because of extended therapy with last-line antibiotics that suppress normal microbiota and lead to predominance of the resistant microbiota.[14] In case of outbreak, epidemiological typing is important for early detection and characterization of nosocomial epidemiology of this pathogen.[15]

So far, there are no published data on the prevalence of resistance to carbapenems and the types of carbapenemases among Enterobacteriaceae in Bosnia and Herzegovina. Therefore, the aim of this study was the phenotypic and molecular characterization of clinical isolates of carbapenemase-producing *K. pneumoniae* from two outbreaks recorded in 2017 and 2018 in Clinical Center University of Sarajevo, Bosnia and Herzegovina.

Materials and Methods

The first outbreak of carbapenem-resistant *K. pneumoniae*-producing carbapenemase OXA-48 in Clinical Center University of Sarajevo was registered in September 2017 (six isolates) and second in September 2018 (nine isolates).

Sample collection

Isolates were detected from different clinical specimens including wound swabs, sputum, bronchoalveolar lavage, and anal swabs from patients admitted to various clinics of Clinical Center, University of Sarajevo. A sample of inspiratory valve swab was taken from the operating room.

Culturing and species confirmation

All specimens were cultured onto standard culture media and incubated at 35°C (95°F). Further examination was carried out with standard microbiological methods and based on characteristic colony appearance. Identification of *K. pneumoniae* isolates was performed by morphological, cultural, and biochemical characterization.[16] Final identification was determined by VITEK 2 Compact System (bioMérieux, Marcy l'Étoile, France) using VITEK ID GN.

Antibiotic susceptibility testing

The antibiotic susceptibility of isolates was determined by the Kirby–Bauer disk diffusion method on Mueller–Hinton agar, using the EUCAST standard for ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefazolin, cefuroxime, ceftriaxone, ceftazidime, cefepime, amikacin, gentamicin, tobramycin, imipenem, meropenem, ciprofloxacin, levofloxacin, trimethoprim–sulfamethoxazole, and tigecycline.

In parallel, each isolate was tested with the VITEK 2 Compact System (bioMérieux), using a VITEK ID and AST card to determine the MICs. The MIC for colistin was determined by broth microdilution with MIC-Strip Colistin (Merlin, Diagnostika GmbH, Germany). We used *Pseudomonas aeruginosa* ATCC 27853 as quality control strain. Results were interpreted according EUCAST breakpoints.[16]

Phenotypic test for carbapenemase production

Isolates were tested for production of carbapenemases by combined-disk test containing meropenem ± various inhibitors (ROSCO Diagnostica A/S, Denmark). Class A carbapenemases were inhibited with boronic acid, class B carbapenemases with dipicolinic acid and ethylenediaminetetraacetic acid (EDTA). We identified putative OXA-48-like carbapenemase using temocillin with MIC >128 mg/L as a phenotypic marker. Because of its low specificity, the presence of OXA-48-like enzymes should be confirmed with other methods.[17]

Extraction of total bacterial DNA

Total bacterial DNA was extracted from colony-purified *K. pneumoniae* isolates and positive control strain (*K. pneumoniae* subsp. *pneumoniae*-producing OXA-48 carbapenemase; American Type Culture Collection, ATCC; No. BAA-2524™). A few colonies[5–7] were removed from fresh pure bacterial culture and suspended in 100 µL sterile distilled water, and then heated at 96°C for 15 minutes. After centrifugation at 12,000 × *g* and 5 minutes incubation at 4°C, the supernatant was used as a source of template DNA for PCR amplification. Prepared DNA extracts were used immediately or stored at −20°C for further analysis.

DNA quantification

Quantification was carried out after the total DNA extraction, using BioSpec-nano Small-volume UV Spectrophotometer (Shimadzu, Columbia, MD). The system measures the quantity of double-stranded DNA in ultra-small volume (1–2 µL) of sample supernatant preparations. For (rep-) PCR setup, 35 ng/µL DNA was used.

Genetic screening

For epidemiological typing purpose, the genetic similarities of carbapenemase-producing *K. pneumoniae* strains were further evaluated by rep-PCR, a semi-automated PCR technique for the amplification of the regions between the noncoding repetitive sequences in bacterial genomes. Rep-PCR was performed using the DiversiLab Klebsiella Kit (bioMérieux Inc., Durham, NC) according to the manufacturer's instructions.

Rep-PCR was performed on preheated thermal cycler (Eppendorf Mastercycler S) and DNA fingerprints were obtained according to the manufacturer's recommendations (bioMérieux). Rep-PCR fingerprinting products were compared with the manufacturer's preloaded library or a user-generated library to detect if an isolate clusters with a previously defined strain type by using the DiversiLab Microbial Genotyping System (bioMérieux). Rep-PCR product

fragments were separated using microfluidics electrophoresis in a small volume of sample. Genetic screening results were analyzed using the DiversiLab software (v3.4) to determine the distance matrices and then dendrograms were generated.

PCR amplification and detection of specific amplicons

PCR amplification was performed according to previously published procedure[18] with minor modification. In fact, reactions were modified to a single target detection instead of multiplex one, as it was originally developed[18] using a set of primers for bla_{OXA-48} genes (OXA-48-F sense primer 5′-GCGTGGTTAAGGATGAACAC-3′ and OXA-48-R antisense primer 5′-CATCAAGTTCAACCCAACCG-3′). In brief, a 50 μL reaction mixture contained 1 × PCR buffer II (10 mmol/L Tris–HCl [pH 8.3], 50 mmol/L KCl), 1.5 mmol/L $MgCl_2$, 0.125 mmol/L of each deoxynucleotide triphosphate, 10 μmol/L of each primer, 2 U AmpliTaq Gold Polymerase (Applied Biosystems by Life Technologies, Thermo Fisher Scientific, Waltham, MA). For each sample, 35 ng/μL DNA was added for PCR setup (10 μL of final reaction mixture). Amplification was carried out with the following thermal cycling conditions: 10 minutes at 94°C and 36 cycles of amplification consisting of 30 seconds at 94°C, 40 seconds at 52°C, and 50 seconds at 72°C, with 5 minutes at 72°C for the final extension. DNA fragments were analyzed by electrophoresis in a 2% agarose gel at 100 V for 45 minutes in 1 × TAE (40 mmol/L Tris–HCl [pH 8.3], 2 mmol/L acetate, 1 mmol/L EDTA) containing 1 × SYBR Safe gel stain (Invitrogen, Life Technologies, Carlsbad, CA; 10,000 × SYBR™ Safe stain concentrate). Ten microliters of PCR products were loaded into the gel and electrophoresed. In positive reactions, amplicons of 438 bp were generated.

Results

Carbapenemase-producing *K. pneumoniae* was detected in 15 patients during two outbreaks recorded in 2017 and 2018. Although all patients originated from different clinics, they were hospitalized at an ICU department at some point of time.

Each isolate was resistant to amoxicillin–clavulanic acid, cefepime, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, levofloxacin, and piperacillin/tazobactam antibiotics.

MIC for imipenem was 2–8 μg/mL and for meropenem >8 μg/mL. All isolates were susceptible to colistin and fosfomycin. During the first outbreak, strains showed susceptibility to trimethoprim/sulfamethoxazole (20–40 μg/mL), whereas in the second outbreak, isolates were intermediate or resistant to the same antimicrobials (MIC >80 μg/mL). In addition, they exhibited intermediate pattern to amikacin (MIC: 8 μg/mL) and tigecycline (MIC: 1 μg/mL). Furthermore, we have seen carbapenemase-producing *K. pneumoniae* isolates as resistotypes. These resistotypes were distinguished into four different groups. The first resistotype was present in 4 of 15 (26.67%) isolates indicating that it was intermediate to amikacin and tigecycline and sensitive to trimethoprim/sulfamethoxazole, colistin, and fosfomycin. The second resistotype (4/15; 26.67%) was intermediate to tigecycline and sensitive to amikacin, colistin, fosfomycin, and trimethoprim/sulfamethoxazole. The third resistotype (3/15; 20%) was intermediate to amikacin and tigecycline and sensitive to colistin and fosfomycin. The fourth resistotype (4/15; 26.67%) was intermediate to amikacin, trimethoprim/sulfamethoxazole, and tigecycline and sensitive to colistin and fosfomycin. First and second resistotypes were observed during the first outbreak and all four resistotypes were observed during the second outbreak. Results of antibiotic susceptibility testing and resistotypes of isolates are given in Table 1.

Combination disk test method showed no differences in the inhibition zone between meropenem disks with or without the inhibitors, whereas zone diameter of temocillin disk was <11 mm, indicating the phenotypic marker of OXA-48 (Fig. 1.).

Rep-PCR fingerprinting showed the occurrence of the two different genetic patterns with no similarity between them. Threshold of ≥92% was used.[19]

Figure 2 provides virtual gel image of dendrogram analysis. One cluster consisted of isolates 1, 2, and 3 isolated during the outbreak in 2017 and similarity among isolates was 95%. The second cluster included isolates 4 and 6 from the first outbreak and isolates 7–15 from the outbreak in 2018. Similarity among those isolates was ≥92%.

We have seen four different resistotypes of Carbapenemase-producing (CPE) *K. pneumoniae* in this study. The first resistotype of CPE *K. pneumoniae* that was intermediate to amikacin and tigecycline and sensitive to trimethoprim/sulfamethoxazole, colistin, and fosfomycin had included members from both genetic clusters (isolates 1, 2, 13, and 8).

The second resistotype that was intermediate to tigecycline and sensitive to amikacin, colistin, fosfomycin, and trimethoprim/sulfamethoxazole involved also the members from both genetic clusters (isolates 3, 14, 15, and 10). The third resistotype that was intermediate to amikacin and tigecycline and sensitive to colistin and fosfomycin had members from second genetic cluster (isolates 4, 9, and 11). The fourth resistotype that was intermediate to amikacin, trimethoprim/sulfamethoxazole, and tigecycline and sensitive to colistin and fosfomycin included members from second cluster (isolates 5, 6, 7, and 12).

By primer-specific PCR amplification all tested isolates were positive for bla_{OXA-48} and negative for other carbapenemases (Fig. 3).

Discussion

Carbapenemase-producing *K. pneumoniae* strains are an arising worldwide public health concern particularly among hospitalized patients from ICU.[20,21]

These infections are followed with limited therapeutic options and high mortality rate.[22,23]

Types of carbapenemases and frequency of carbapenem resistance vary between European countries. In most European countries there are sporadic cases or outbreaks of OXA-48-producing *K. pneumoniae*,[24] and in several of them OXA-48 is currently the most frequent carbapenemase.[9] OXA-48-type carbapenemases poorly hydrolyze carbapenems and broad-spectrum cephalosporins and aztreonam. Some strains with reduced permeability or the production of ESBLs can give resistance to extended-spectrum cephalosporins and lead to high-level resistance to carbapenems and cephalosporins.[7,24]

Table 1. Pattern of Resistance with MIC Values of *Klebsiella pneumoniae* Isolates

Isolate no.	*Number*	*Clinic*	*Sample*	*MIC (μg/mL)*							
				AN	*TS*	*Col*[a]	*Imp*	*Mem*	*Tig*	*Fosf*	*Resistotypes*
First outbreak (2017)											
1	6653	Intensive care unit	Drain content	12 (I)	40 (S)	1 (S)	>8 (R)	>8 (R)	2 (I)	32 (S)	1
2	7272	Intensive care unit	Wound swab	16 (I)	20 (S)	0.5 (S)	>8 (R)	>8 (R)	2 (I)	16 (S)	1
3	7018	Intensive care unit	Wound swab	4 (S)	40 (S)	0.5 (S)	2 (R)	>8 (R)	2 (I)	32 (S)	2
13	6154	Intensive care unit	Wound swab	16 (I)	40 (S)	0.5 (S)	2 (R)	>8 (R)	2 (I)	32 (S)	1
14	6476	Thoracic surgery	Drain tip	4 (S)	40 (S)	0.25 (S)	2 (R)	>8 (R)	2 (I)	32 (S)	2
15	6480	Intensive care unit	Wound swab	4 (S)	40 (S)	0.5 (S)	>8 (R)	>8 (R)	2 (I)	16 (S)	2
Second outbreak (2018)											
4	6919	Abdominal surgery	Wound swab	8 (I)	160 (R)	0.125 (S)	>8 (R)	>8 (R)	2 (I)	32 (S)	3
5	6193	Intensive care unit	Abscess content	8 (I)	80 (I)	0.5 (S)	>8 (R)	>8 (R)	2 (I)	32 (S)	4
6	6204	Orthopedic surgery	Wound swab	8 (I)	80 (I)	0.25 (S)	2 (R)	>8 (R)	2 (I)	32 (S)	4
7	7384	Orthopedic surgery	Anal swab	8 (I)	80 (I)	0.5 (S)	2 (R)	>8 (R)	2 (I)	32 (S)	4
8	7393	Orthopedic surgery	Snal swab	8 (I)	20 (S)	0.5 (S)	2 (R)	>8 (R)	2 (I)	16 (S)	1
9	7190	Intensive care unit	BAL	8 (I)	160 (R)	0.25 (S)	2 (R)	>8 (R)	2 (I)	32 (S)	3
10	5936	Orthopedic surgery	Wound swab	4 (S)	40 (S)	0.5 (S)	2 (R)	12 (R)	2 (I)	16 (S)	2
11	794	Operating room	Inspiratory valve	8 (I)	80 (R)	0.5 (S)	2 (R)	>8 (R)	2 (I)	32 (S)	3
12	6393	Intensive care unit	Wound swab	8 (I)	80 (I)	0.25 (S)	2 (R)	>8 (R)	2 (I)	16 (S)	4

[a]Broth microdilution.

AN, amikacin; BAL, bronchoalveolar lavage; Col, colistin; Fosfo, fosfomycin; I, intermediate; Imp, imipenem; Mem, meropenem, MIC, minimum inhibitory concentration; R, resistant; S, susceptible; Tig, tigecycline; TS, trimethoprim/sulfamethoxazole.

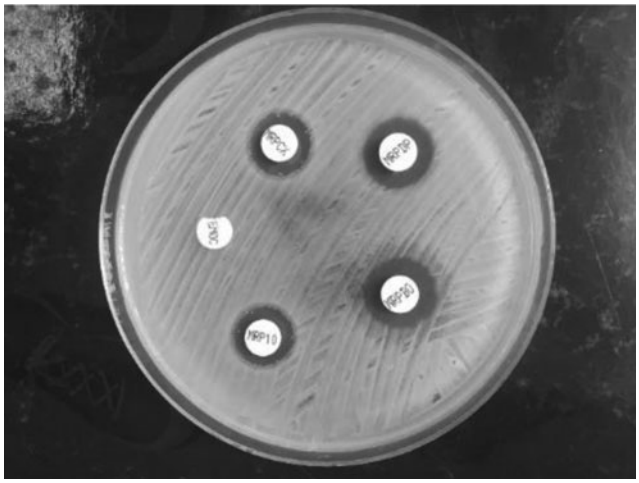

FIG. 1. Phenotypic detection of OXA-48 by combination disk test method.

In this study, we describe two outbreaks of *K. pneumoniae* OXA-48 recovered from patients who were at some point hospitalized in the ICU in Clinical Center University of Sarajevo. Patients in ICU are highly susceptible to infections because of multiple procedures, use of invasive devices, and the frequent use of antimicrobial agents.[25,26] In addition, these patients are commonly colonized with MDR microorganisms that remain for months in the gut and could be translocated through the gut epithelium, leading to infection or cross-transmission to other patients. This could result in outbreaks hard to control.[27]

As shown in our results, MIC of imipenem varied between 2 and 8 μg/mL, which is not unusual because of intermediate susceptibility and even susceptibility to carbapenems observed in producers of all types of carbapenemases,[28] especially of OXA-48/OXA-181 isolates not coproducing an ESBL.[7]

Although many carbapenem-resistant *K. pneumoniae* strains have been highly resistant, aminoglycosides can exhibit partial bactericidal activity against these isolates.[29] Isolates from our study were sensitive or intermediate to amikacin.

Furthermore, all isolates were sensitive to colistin and fosfomycin. Fosfomycin is not an optimal treatment choice and could be highly appropriate only for urinary tract infections.[30] Because of nephrotoxicity and uncertain efficacy in pulmonary infections[15] colistin remains the last choice for the infections caused by CPE.[31] In our study, colistin resistance was not detected; however, in carbapenem-resistant Enterobacteriaceae this resistance was already detected.[31]

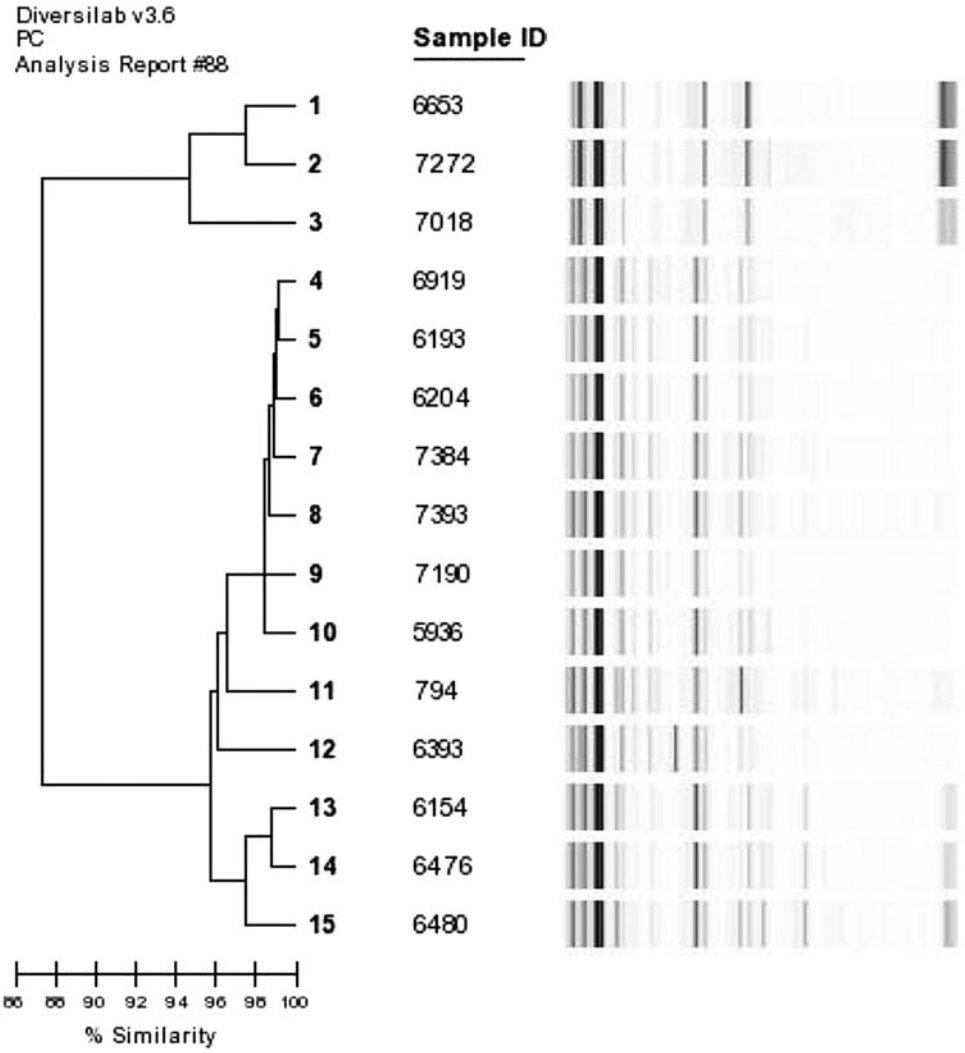

FIG. 2. Dendrograms and virtual gel images of *Klebsiella pneumoniae* isolates identified with web-based DiversiLab software.

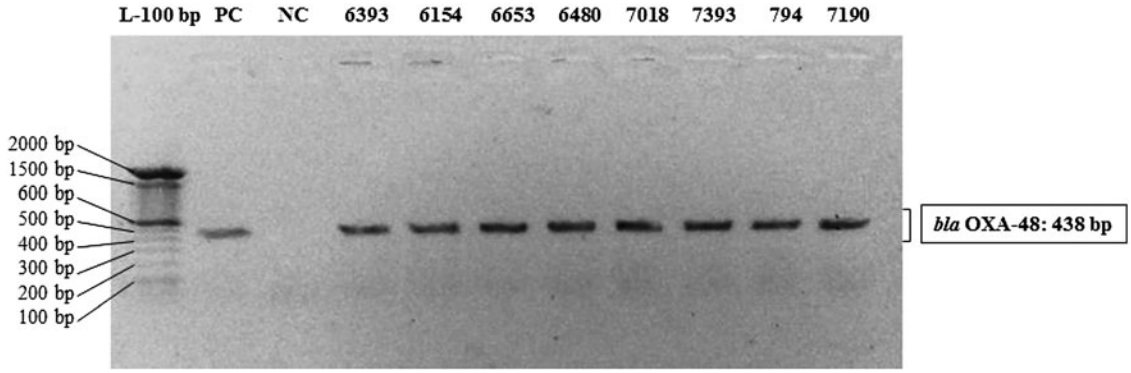

FIG. 3. Confirmation of *Klebsiella pneumoniae* subsp. *pneumoniae*-producing OXA-48 carbapenemase by primer-specific PCR amplification. Agarose gel electrophoresis (2% agarose in 1×TAE, 100 V, 45 minutes) of PCR amplified products using specific primer set for *K. pneumoniae* subsp. *pneumoniae*-producing OXA-48 carbapenemase. From left to right: L-100 bp size ladder (Invitrogen, Life Technologies, Carlsbad, CA); PC, positive control: *K. pneumoniae* subsp. *pneumoniae* strain producing OXA-48 carbapenemase (American Type Culture Collection, ATCC; No. BAA-2524™); NC, negative control: Rnase/Dnase free water; clinical human/environment samples IDs: 6393, 6154, 6653, 6480, 7018, 7393, 794, and 7190. Size of specific PCR product was 438 bp.

EuSCAPE survey in 2013–2014 showed that in Italy 43% of KPC-producing *K. pneumoniae* isolates were resistant to colistin, and 13% of isolates from blood cultures were reported to EARS-Net.[31,32]

Corbellini *et al.*[15] reported that all isolates were sensitive to colistin and tigecycline, whereas in our study strains were intermediate to tigecycline. Tigecycline possesses significant therapeutic limitations because it is not useful in urinary infections owing to low urinary levels.[15,33]

Rep-PCR-based analyses provided information to evaluate the epidemiological status of nosocomial infection. For this purpose, we used DiversiLab System for tracking the spread of carbapenem-resistant *K. pneumoniae* strains in the clinical setting. DiversiLab System has the ability to distinguish isolates from different phylogenetic clonal groups,[34] which is valuable in detecting relationships among clones during a bacterial outbreak.[35]

Results revealed the presence of two different genetic patterns with no similarity between them. Second cluster included isolates from both outbreaks. These strains were detected from different body sites of patients hospitalized at different hospital wards, although all of them were at some point of hospitalization in the ICU. This finding indicates the possible role of clonal dissemination from colonized patients and intrahospital transmission of the bacteria. To establish effective control measures for *K. pneumoniae* dissemination, it is important to understand how transmission occurs.[15]

All isolates included in this study were confirmed to produce $bla_{\text{OXA-48}}$ carbapenemase. The high rate of *K. pneumoniae* spreading in the hospital settings and the capacity of $bla_{\text{OXA-48}}$-carrying plasmid for the horizontal gene transfer can make outbreaks difficult to control.[36]

Conclusions

Emergency of *K. pneumoniae*-producing carbapenemases in various hospital departments emphasizes an urgent need for infection prevention control and management of antibiotic use. After these outbreaks, new measures were implemented involving the rectal screening of the patients attending ICU, and strict patient isolation, to improve epidemiological and hygienic situation in the clinics.

The monitoring, surveillance, and molecular typing are essential to control the appearance of MDR strains in nosocomial settings, and to reduce the frequency of outbreak occurrence.[21]

Because there is no similar research about carbapenemase-producing strains in our country, this study has an important role in characterization of *K. pneumoniae* OXA-48 in Bosnia and Herzegovina.

Disclosure Statement

No competing financial interests exist.

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