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Carbapenemases-producing *Klebsiella pneumoniae* in hospitals of two regions of Southern Italy

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Carbapenem-resistant *Klebsiella pneumoniae* infections are reported with increasing frequency elsewhere in the world, representing a worrying phenomenon for global health. In Italy, there are hotspot data on the diffusion and type of carbapenemase-producing *Enterobacteriaceae* and *K. pneumoniae* in particular, with very few data coming from Apulia and Basilicata, two regions of Southern Italy. This study was aimed at characterizing by phenotypic and genotypic methods carbapenem-resistant *K. pneumoniae* isolated from several Hospitals of Apulia and Basilicata, Southern Italy. Antibiotic susceptibility was also evaluated. The relatedness of carbapenemase-producing *K. pneumoniae* strains was established by pulsed-field gel electrophoresis (PFGE). Among the 150 *K. pneumoniae* carbapenemase producers, KPC-3 genotype was the most predominant (95%), followed by VIM-1 (5%). No other genotypes were found and no co-presence of two carbapenemase genes was found. A full concordance between results obtained by both the phenotypic and the genotypic tests was observed. All strains were resistant to β-lactam antibiotics including carbapenems, and among antibiotics tested, only tetracycline and gentamycin showed low percentage of resistance (18% and 15%, respectively). Resistance to colistin was detected in 17.3% of strains studied. The analysis of PFGE profiles of the carbapenemases-positive strains shows that one group (B) of the five (A to E) main groups identified was the most prevalent and detected in almost all the hospitals considered, while the other groups were randomly distributed. Three different sequence types (ST 307, ST 258, and ST 512) were detected with the majority of isolates belonging to the ST 512. Our results demonstrated the wide diffusion of *K. pneumoniae* KPC-3 in the area considered, the good concordance between phenotypic and genotypic tests. Gentamicin and colistin had a good activity against these strains.

Key words: *Klebsiella pneumoniae*; carbapenemases; antimicrobial resistance; pulsed-field gel electrophoresis.

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Resistance to antibiotics is an important public health concern worldwide. Carbapenems have long been a last line of defense in the treatment of infections caused by extended-spectrum β-lactamases (ESBL) gram-negative bacteria. *Klebsiella pneumoniae, Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, the most important cause of hospital-acquired infections, have emerged as resistant to carbapenems elsewhere in the world (1, 2). The resistance to carbapenems is due to the ability to produce enzymes inactivating carbapenems, known as carbapenemases, and able to hydrolyze also other β-lactam antibiotics such as penicillins, cephalosporins, and monobactam. These enzymes have been described in *Enterobacteriaceae* and also in other gram-negative, non-fermenters bacteria such as *A. baumannii* and *P. aeruginosa*. Three main classes of carbapenemases have been identified: Ambler class A β-lactamases (KPC), class B (metallo-enzymes), and class D (OXA-48 type) (3). KPC genes are often flanked by transposons and are carried on transferable plasmids of Gram-negative bacteria facilitating their wide diffusion among many bacterial species (4, 5). *Klebsiella pneumoniae* is an opportunistic pathogen primarily attacking hospitalized patients. The emergence of multidrug-resistant

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(MDR) *K. pneumoniae* has been reported worldwide. In Italy, the spread of carbapenem-resistant *K. pneumoniae* has dramatically increased since 2009, the KPC-3 variant being the predominant clone circulating in Italy (6, 7). In this country, there are hotspot data on the diffusion and type of carbapenemases-producing *Enterobacteriaceae* and *K. pneumoniae* in particular, and few of these data come from Apulia and Basilicata (8–19).

The aims of this study were: (i) to compare the performance of phenotypic and genotypic tests for the detection of carbapenemases in *K. pneumoniae* isolated from several hospitals of the Apulia and Basilicata, Southern Italy; (ii) to determine the clones of the carbapenemases-producing *K. pneumoniae* circulating in different hospitals and wards of our area; and (iii) to evaluate the pattern of *K. pneumoniae* susceptibility to the antibiotics.

MATERIALS AND METHODS

Bacterial isolates

One hundred and fifty non-replicate *K. pneumoniae* strains isolated from patients admitted to 10 hospitals in the Apulia Region, Southern Italy and to 2 hospitals in the Basilicata Region, Southern Italy, were collected from January 2013 to July 2015. The manager of each clinical microbiology laboratory participated in the project on a voluntary basis.

The hospitals are distinct and independent, located between 20 and 180 km apart and offer clinical support in all areas of medicine, surgery, and intensive cure (ICU) in particular. The strains were isolated from several clinical samples (Table 1).

The strains were identified as *K. pneumoniae* by Vitek 2 Automated System (bioMérieux, Florence, Italy), or MicroScan® Microbiology Systems (Siemens Healthcare Diagnostics, Milan, Italy) or BD Phoenix™ Automated Microbiology System (Becton, Dickinson, Milan, Italy) according to the methods used by the individual hospital. All strains included in this study were reported as carbapenemases producers by the automated systems used by the individual hospital.

Antimicrobial susceptibility testing

Susceptibility testing was performed using the disk-diffusion method on Mueller–Hinton agar (Biolife, Milan, Italy) and susceptibility breakpoints were determined and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (20). Minimum inhibitory concentration (MIC) of colistin sulfate (Sigma Chemicals, Milan, Italy) was determined by standard broth dilution method, because of possible overestimation of resistance by automated methods (21). MIC_{90} and MIC_{50} of meropenem and imipenem were determined by E-test (Liofilchem srl, Roseto degli Abruzzi, Teramo, Italy). *Escherichia coli* ATCC 25922 was used as the quality control strain.

Phenotypic investigations

The production of carbapenemases by *K. pneumoniae* isolates was performed by the Modified Hodge Test (MHT) (22). *Klebsiella pneumoniae* positive for carbapenemases were screened for the presence of the classes of carbapenemases or overexpression of AmpC-type β-lactamase or production of an ESBL in combination with porins loss by commercial synergy tests (Rosco Diagnostic, Biolife). All these investigations were performed according to the manufacturer's instructions.

Genotypic investigation

Primers to detect the genes bla_{KPC}, bla_{VIM}, bla_{NDM}, and bla_{OXA-48} encoding resistance for carbapenems were based on sequences available in GenBank and designed by using the Primer3 (version 4.0.0) software (http://primer3.wi.mit.edu) (Table 2). Genomic DNA was extracted as previously described (23). PCRs were performed in a total volume of 25 μL containing 50–100 ng of total DNA, 1X PCR buffer (10 mM Tris–HCl, 50 mM KCl, and 1.5 mM $MgCl_2$; pH 8.3), 200 μM of each deoxynucleoside triphosphate (dNTP), 20 μM of each primer, and 1 U Taq polymerase. All the PCR reagents were purchased from Takara (Takara Bio Inc., Otsu, Shiga, Japan). Positive controls for the four *bla* investigated genes were included.

Amplified products obtained for the only genes identified (bla_{KPC} and bla_{VIM} genes) were sequenced by the GATC Biotech AG (Sequencing Service, Köln, Germany). The resulting DNA sequences were analyzed for similarity by using the BLAST program available at the NCBI BLAST homepage (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Pulsed-field gel electrophoresis and multilocus sequence typing

All *K. pneumoniae* strains were typed by pulsed-field gel electrophoresis (PFGE). The employed protocol was

Table 1. Carbapenemase-producing *Klebsiella pneumoniae* isolated from different clinical samples

Materials	Apulia hospitals	Basilicata hospitals	Total (%)	Carba genes
Blood	70	5	75 (50)	68 bla_{KPC-3} 7 bla_{VIM-1}
Urine samples	29	5	34 (23)	34 bla_{KPC-3}
Pus	17	1	18 (12)	18 bla_{KPC-3}
Respiratory samples	10	3	13 (8.7)	13 bla_{KPC-3}
Central venous catheter	4	2	6 (4)	6 bla_{KPC-3}
Cerebrospinal fluid	1	1	2 (1.3)	2 bla_{KPC-3}
Feces	1	1	2 (1.3)	2 bla_{KPC-3}

Table 2. Primers designed to detect the *bla* genes

Primers	5′–3′ sequence	Amplicon size (pb)
$bla_{\text{KPC-F}}$	TCGCCGTCTAGTTCTGCTGTCTTGTC	871 pb
$bla_{\text{KPC-R}}$	TTACTGCCCGTTGACGCCCAATC	871 pb
$bla_{\text{VIM-F}}$	TGCTTTTGATTGATACAGCGTGGGGT	402 pb
$bla_{\text{VIM-R}}$	CATCGGCCACGTTCCCCGCAGA	402 pb
$bla_{\text{VIM A-F}}$	TGCCGATGGTGTTTGGTCGCATATC	600 pb
$bla_{\text{VIM A-R}}$	AGCAAGTCTAGACCGCCCGG	600 pb
$bla_{\text{VIM B-F}}$	TGACCGCGTCTATCATGGCTATTGCG	760 pb
$bla_{\text{VIM B-R}}$	ACGACTGAGCGATTTGTGTGCGCTTT	760 pb
$bla_{\text{NDM-F}}$	CGCATTAGCCGCTGCATTGATGCTGA	699 pb
$bla_{\text{NDM-R}}$	ATCACGATCATGCTGGCCTTGGGGAA	699 pb
$bla_{\text{OXA48-F}}$	ACGGGCGAACCAAGCATTTTTACCCG	567 pb
$bla_{\text{OXA48-R}}$	AGCCCTAAACCATCCGATGTGGGCAT	567 pb

performed by combining the standard protocol designed by the Centre for Disease Control and Prevention Atlanta for *Salmonella* (http://www.cdc.gov/pulsenet/pathogens/index.html) with that reported by Durmaz et al. (24).

The lysis of cells in agarose was performed as following: plugs were incubated in 4 mL of cell lysis solution 1 (CLS-1: 50 mM Tris–HCl, 50 mM EDTA, lysozyme 2.5 mg/mL, proteinase K 0.1 mg/mL) at 37 °C in a shaking water bath for 1 h. The plugs were then transferred into a fresh tube containing 4 mL of cell lysis solution 2 (CLS-2: 0.5 M EDTA, 1% sarcosyl, and proteinase K 400 μg/mL) at 55 °C in a shaking water bath for 2 h. Digital images of the PFGE profiles were analyzed using algorithms available in the BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium) and compared by cluster analysis using the Dice coefficient and the unweighted pair group method with arithmetic means (UPGMA), with a position tolerance limit and optimization of 1%.

Representative *K. pneumoniae* isolates for each different PFGE profile were subjected to multilocus sequence typing (MLST). MLST typing was performed as previously described (25) at the Department of Medical Biotechnologies, University of Siena, Siena, Italy. Sequence types (STs) were assigned using the MLST website (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

RESULTS

One hundred and fifty *K. pneumoniae* strains were isolated from 150 patients admitted to 10 hospitals in Apulia and two hospitals in Basilicata. The distribution of the hospitals is reported in Fig. 1. *Klebsiella pneumoniae* carbapenemases producers were isolated in particular from blood (50%), urinary samples (23%), respiratory samples (8.7%),

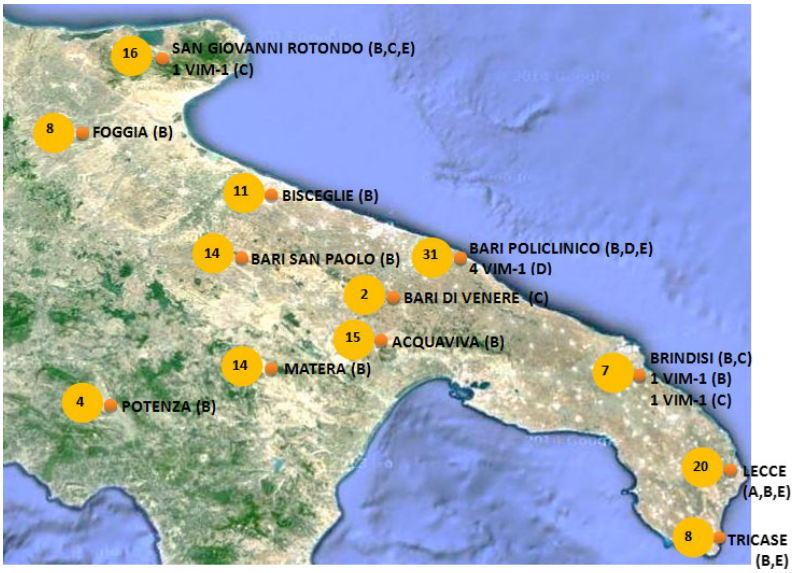

Fig. 1. Distribution of Hospitals of Apulia and Basilicata (Southern Italy) participating in the study and the number of strains collected. In brackets are showed the PFGE groups identified.

pus (12%), central venous catheter (CVC) (4%), cerebrospinal fluid (1.3%), and feces (1.3%). *Klebsiella pneumoniae* carbapenemases producers were mainly isolated from patients admitted to the Intensive Care Unit (ICU) (49%), followed by the Department of Medicine (38%) and Surgery (13%) (Table 1).

One hundred and fifty *K. pneumoniae* strains, reported as probable carbapenemases producers by automated systems, were confirmed as carbapenemase producers by phenotypic tests.

One hundred and forty-three K. pneumoniae strains were KPC strains on the basis by synergy with the association meropenem–boronic acid (125 strains from hospitals in Apulia and 18 strains from hospitals in Basilicata). Seven *K. pneumoniae* strains were Metallo-β-lactamases (MBL)-producer strains on the basis by synergy with the association meropenem–dipicolinic acid. All these seven strains were isolated from Apulia (four strains from the ICU of Policlinico Bari, two from Brindisi Hospital, and one from San Giovanni Rotondo Hospital) (Fig. 1). The molecular analysis of the carbapenemases gene fully confirmed the results obtained by the phenotypic tests. No OXA-48 or NDM-1 were detected and no co-presence of two or more carbapenemase genes was found.

Sequencing of PCR amplicons was used to identify the carbapenemases subtype of *K. pneumoniae*. All the 143 *K. pneumoniae* strains identified as KPC by phenotypic methods carried the bla_{KPC-3} gene and all the seven isolates identified as MBL by phenotypic methods carried the bla_{VIM-1} gene.

Seven *K. pneumoniae* strains harboring the $bla_{\text{VIM-1}}$ gene were detected only among strains isolated from blood, whereas bla_{KPC-3} gene was found in all the clinical samples considered (Table 1).

Susceptibility testing revealed that all *K. pneumoniae* isolates were resistant to β-lactam antibiotics including carbapenems (meropenem and imipenem). *Klebsiella pneumoniae* isolates were resistant to: aminoglycosides (amikacin 73%, kanamycin 93%, and gentamicin 15%), chloramphenicol 93%, fluoroquinolones (nalidixic acid 99%, ciprofloxacin 81%, and levofloxacin 98%), trimetoprim-sulfamethoxazole 81%, and tetracycline 18% (Fig. 2). Resistance to colistin was 17.3% and MIC_{50} and MIC_{90} were 1 mg/L and 32 mg/L, respectively (range 0.5–256 mg/L). MIC_{90} and MIC_{50} were >32 mg/L for both meropenem and imipenem.

The PFGE pulsotypes of the 150 *K. pneumoniae* strains were clustered, assuming a threshold of similarity ≥80%, in five main groups (A to E) (Fig. 3). The group B was that prevalent and accounted for

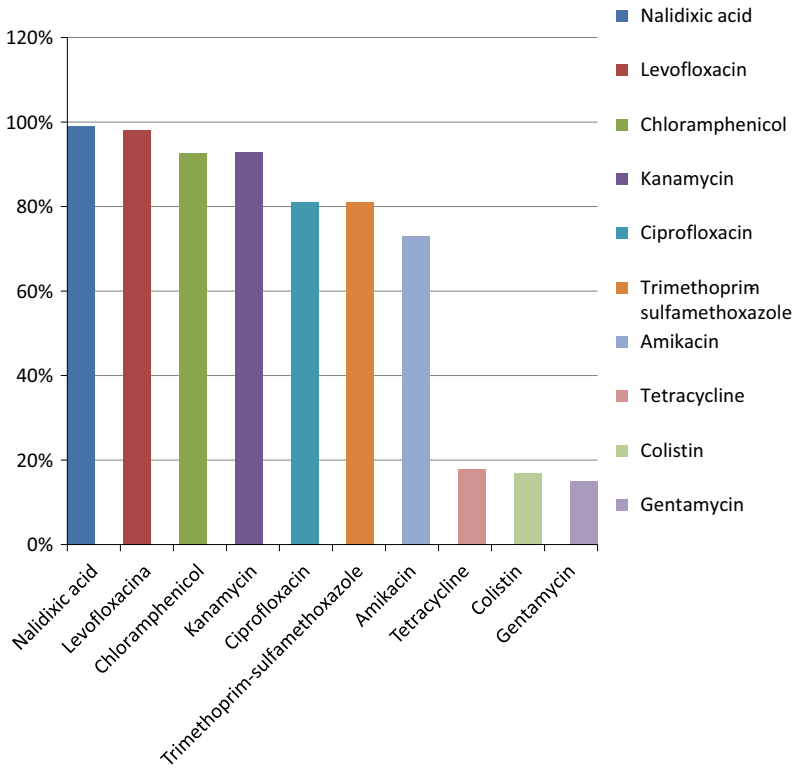

Fig. 2. Percentage of resistance in *Klebsiella pneumoniae*. The 100% of resistance to β-lactam antibiotics is not reported.

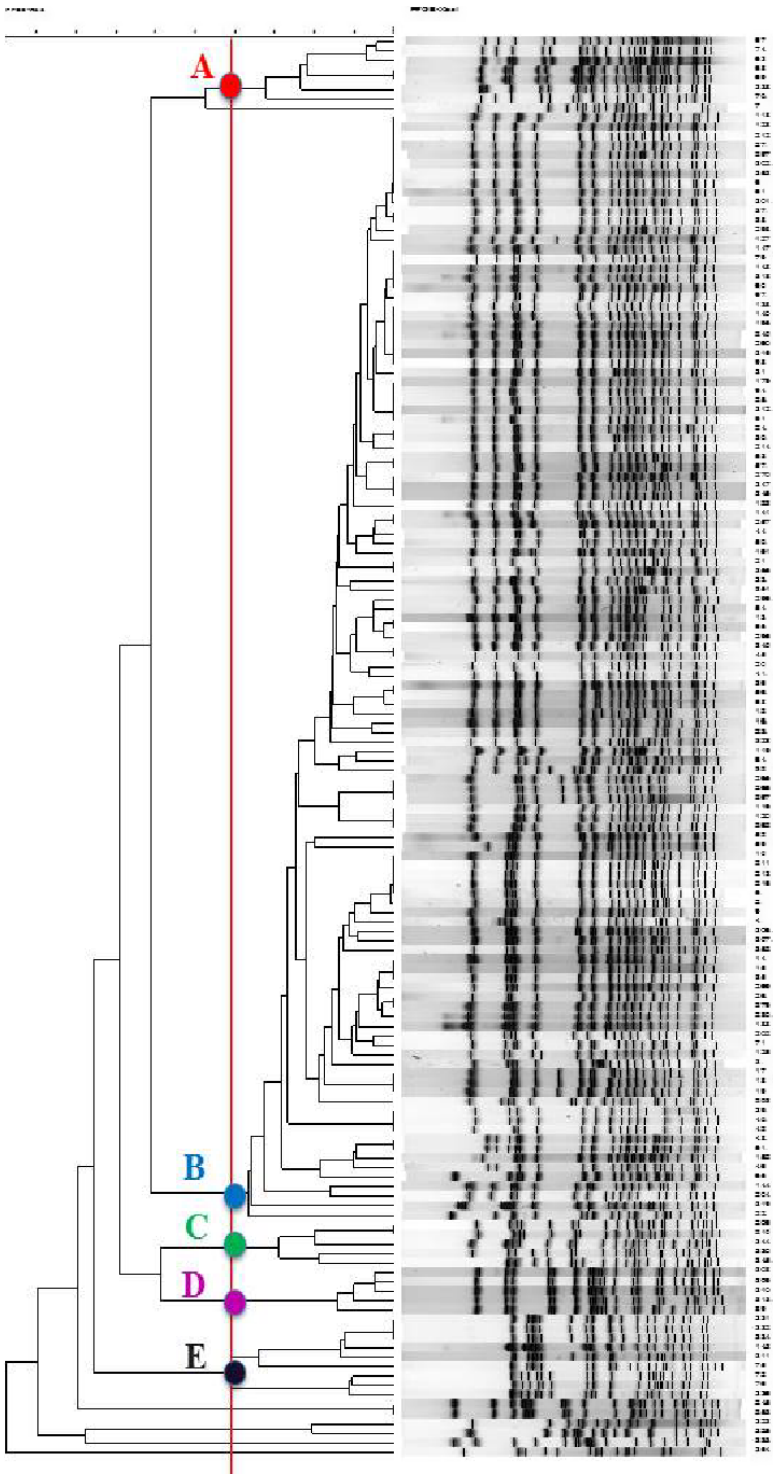

Fig. 3. Dendrogram based on PFGE profiles of 150 carbapenemase-producing *Klebsiella pneumoniae* isolates. PFGE profiles were clustered, based on a threshold of similarity ≥80%, into five groups (A to E). The group A included 7 strains containing bla_{KPC-3} gene; the group B (117 strains) included 1 isolate carrying bla_{VIM-1} and 116 isolates carrying bla_{KPC-3}; the group C (5 strains) consisted of 2 isolates positive for bla_{VIM-1} and 3 isolates positive for bla_{KPC-3}; the group D (5 strains) included 4 isolates possessing bla_{VIM-1} gene and 1 isolate possessing bla_{KPC-3} gene; and the group E consisted of 9 isolates harboring bla_{KPC-3} gene.

Table 3. Distribution of the five groups identified by PFGE among the three main wards considered for each hospital located in Apulia (10) and Basilicata (2)

Hospitals	Wards		
	Intensive care unit	Medicine	Surgery
Francesco Miulli (Acquaviva delle Fonti, Bari)	B	B	B
Di Venere (Bari)	C	/	/
Policlinico (Bari)	B, D	B, D, E	B
San Paolo (Bari)	B	/	/
Vittorio Emanuele II (Bisceglie, Bari)	B	B	/
Antonio Perrino (Brindisi)	B, C	B	/
Ospedali Riuniti (Foggia)	B	B	B
Vito Fazzi (Lecce)	A, B, E	A, B, E	A, B, E
Casa Sollievo della Sofferenza (S. Giovanni Rotondo, Foggia)	B, C, E	B	/
Cardinale G. Panico (Tricase, Lecce)	B	B	E
Madonna delle Grazie (Matera, Basilicata)	B	B	B
San Carlo (Potenza, Basilicata)	B	B	B

78% of the analyzed strains. It was virtually detected in all hospitals with the exception of one (Di Venere Hospital, Bari), while the presence of the other four identified groups was randomly distributed between the hospitals. Seven *K. pneumoniae* strains carrying the VIM gene were identified; four of these were detected in the group D and only present in the ICU of Policlinico Hospital, Bari. Two strains were detected in the group C and one in the group B (Fig. 1).

Distribution of the five identified groups was clustered within single wards investigated in each hospital suggesting a wide inter-ward spread of *K. pneumoniae* strains (Table 3).

MLST analysis attributed the isolates of PFGE profile A to ST258, of PFGE profiles B and E to ST512, of PFGE profile C to ST 307, and of PFGE profile D to ST 512.

DISCUSSION

Klebsiella spp. is an opportunistic pathogen and is ubiquitous in nature. In humans, the nasopharynx and the intestinal tract are the sites most commonly colonized. In the hospital environment, colonization rates increase in direct proportion to the length of stay both in hospitalized patients and hospital personnel. The emergence of carbapenem-resistant *K. pneumoniae* as a cause of nosocomial infections has been reported with increasing frequency worldwide (7). These bacteria are almost always resistant also to many other classes of antibiotics. Several papers have dealt with the emergence and the spread of carbapenem-resistant *K. pneumoniae* and the presence of several genes in the strains isolated in several countries. In Italy, carbapenem-resistant *K. pneumoniae* has dramatically increased after the first isolation of a *K. pneumoniae*-producing bla_{KPC-3} reported in 2008 by Giani et al. (18). The ECDC report an increase of percentage of invasive isolates of *K. pneumoniae* carbapenemases producers from 6.7% in 2011 to 32.9% in 2014 (26) and an increase of spread of other Gram-negative carbapenemase producers has been observed. Several surveys conducted in Italy have shown that the KPC-type enzymes most commonly encountered are KPC-3 (9, 13, 17, 27) followed by KPC-2 (28). The bla_{VIM} gene has been reported with a minor prevalence (9, 11–15, 17, 29). A survey conducted in 2011 involving 25 Italian cities, distributed throughout the nation and covering most Italian regions including two hospitals in Apulia, Southern Italy, reported that among 13 749 clinical isolates of *Enterobacteriaceae*, carbapenemases were found in 2.0% of the isolates and *K. pneumoniae* contributed to the majority of carbapenem-resistant *Enterobacteriaceae* (CRE) (86.7%) representing the 11.9% of the isolates. KPC producers were the most frequent (86%) followed by VIM (7%) and OXA-48 (2%) (29). Three years of surveillance (2011–2014) conducted in central Italy, identified 73 *K. pneumoniae* strains harboring the bla_{KPC} gene, 44 the bla_{VIM} gene, 3 the bla_{NDM}, and 2 strains the bla_{OXA-48} gene. These genes were found also in other strains of carbapenem-resistant *Enterobacteriaceae*. (14).

In a study conducted in the Turin area, Piedmont, northwestern Italy, the KPC *K. pneumoniae* was 17.5% of strains, and in line with those reported in 2012 in Italy (11.9–19.4%) (6, 11, 29). A progressive increase in the detection of carbapenemases-producing *K. pneumoniae* strains from 2012 to 2014 in the screening rectal swab samples in one hospital of Padua, north-east Italy, was reported. KPC strains were the most frequently isolated (99.1%), followed by OXA-48 and NDM-1 (16). In the Valle D' Aosta region (northwestern, Italy), KPC-2- followed by KPC-3-producing

K. pneumoniae were the most prevalent, whereas the VIM-1 gene was detected only in *E. coli*. (30). A study from Catania, Sicily, Southern Italy detects only KPC carrying the $bla_{\text{KPC-3}}$ gene (10), whereas in Padua area, KPC-3 followed by KPC-2 were the prevalent genotypes found (15).

Carbapenemases producers *K. pneumoniae* were 1.3% of a survey conducted in 2011–2012, in the Bolzano area (Northern Italy), VIM-1 gene and KPC-3 were the most prevalent genotypes detected (31). Additionally, 17.5% of *K. pneumoniae* isolates from different samples collected in a survey in the north-west of Italy were KPC (11). The KPC *K. pneumoniae* sequence type (ST) 258 is the most frequently encountered in the world. In Italy, other different STs such as ST307 and ST323 have also been detected (13). Our results are similar to that reported by a recent countrywide survey performed in Italy. The majority (97%) of the *K. pneumoniae* isolated from blood were positive for the bla_{KPC} gene (87% carrying the bla_{KPC-3} variant, while the remaining the bla_{KPC-2} variant). Only 1.2% were positive for the bla_{VIM-1} gene. OXA-48 and NDM variants were not detected (32).

In the area considered in this work, we found *K. pneumoniae* isolates producing KPC-3 carbapenemase followed by VIM-1 producers that were identified only in three hospitals located in three different areas of Apulia. Recently, the emergence of *K. pneumoniae* co-producing several classes of carbapenemases (NDM-1 and OXA-48), ESBL, and other resistance genes has been described but in our strains no co-presence of different classes of carbapenemases was observed.

In our work no discrepancies were found between the results of the phenotypic assays and those of the molecular tests suggesting that the former may be suitable to confirm the type of carbapenemases produced by *K. pneumoniae* in a laboratory of limited capacity, it being simple and not expensive to perform, considering the cost of the molecular systems and the expert personnel required for them.

All *K. pneumoniae* KPC were classified into five main PFGE groups. The main group B was detected in the two regions investigated so highlighting the wide spread of this group of *K. pneumoniae* clonally related among different hospitals. The *K. pneumoniae* strains carrying the bla_{VIM} gene were restricted to only three geographically distinct hospitals. Three different sequence type (ST 307, ST 258, and ST 512) were detected with the majority of isolates belonging to the ST 512, according to a recent countrywide Italian survey reporting a diffusion of ST 512 lineage (32).

In conclusion, our study, the first conducted in several hospitals of Apulia and Basilicata, Southern Italy, has demonstrated the diffusion of *K. pneumoniae* KPC-3 and VIM-1 in our area. A good performance of phenotypic methods in detecting the mechanism of resistance was also seen. The PFGE results suggest a clonal dissemination of *K. pneumoniae* harboring the carbapenemases gene across the hospitals of Apulia and Basilicata.

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