

**Introduction**

Among Enterobacteiraeae, *Klebsiella pneumoniae* has been identified as the most important pathogens causing drug-resistant infections in hospital setting, especially in intensive care unit (ICU). Being able to colonize a wide range of body sites, *K. pneumoniae* is traditionally associated with healthcare-related illnesses, including pneumonia, bacteremia, respiratory and urinary tract infections, but additional syndromes (i.e., pyrogenic liver abscess, haemorrhagic colitis, Lemierre’s syndromes) occur, which are more often community-acquired, linked to well defined virulence characteristics of select clonal or pathotypes, and require specific screening and/or identification tests [4].

Resistance to carbapenems represents a global and emerging threat for public health [5], particularly among hospitalized patients from intensive care unit [6-9]. In addition to the resistance to beta-lactams, *K. pneumoniae* are frequently resistant to other classes of antibiotics, reducing or eliminating the effectiveness of available therapeutic options [10-12]. Risk factors related to carbapenems resistance include: the previous use of carbapenems; the elderly; length of hospital...
stays and surgery before admission in the intensive care unit; urinary and central venous catheterization; mechanic ventilation; tracheotomy; underlying diseases and immunosuppression; and transfer from settings where carbapenem resistance is endemic [13].

Despite global attempts to control the spread of carbapenem-resistance, population-weighted mean throughout the EU/EEA has shown an increasing trend of carbapenemase producing K. pneumoniae from 6% in 2011 to 7.3% in 2014 [14]. Countries reporting the highest rates of carbapenem resistance also showed combined resistance to fluoroquinolones, third-generation cephalosporins and aminoglycosides [15]. In Italy, carbapenem resistant K. pneumoniae are highly endemic, and healthcare-associated outbreaks have been described [16], with a mean prevalence of 34.3% in 2013 [10].

Multiple mechanisms are involved in the carbapenems resistance, including the production of various types of carbapenemases (KPC, NDM, VIM, OXA-48-like), which are the most mechanism in Europe [9,17]. However there are alternative resistance mechanisms including reduced permeability of outer membrane mediated by the loss of porins, and the up-regulation of efflux systems, and these often occur with the over-expression of genes coding for AmpC beta-lactamases [13] or extended-spectrum beta-lactamases [18].

Based on the experiences from national and international surveillance networks, in April 2014, the World Health Organization (WHO) published the first global report on surveillance of antimicrobial resistance. This report concluded that surveillance data, if available, are essential for treatment choices, understanding trends, identifying priority areas for interventions, and monitoring the impact of interventions to limit resistance [19]. Hence, the implementation of the epidemiological and laboratory surveillance are essential to reduce the spread of multiresistance. In this context, molecular characterization of K. pneumoniae is crucial both for the identification of strains responsible for outbreaks, to inform therapeutic options and to implement epidemiological surveillance [20].

Since no previous investigations were conducted in the Molise Region, Central Italy, the aim of the present study was to characterize K. pneumoniae cultures isolated from hospitalized patients with respiratory tract infections in an intensive care unit.

Materials and Methods

Selection of K. pneumoniae cultures

Molise is the second smallest region of Italy (4,438 sq km), located in a central-east position with the city of Campobasso as the regional capital. The regional healthcare system is managed by the regional health authority, known as the Azienda Sanitaria Regionale del Molise (A.S.Re.M), and the study was conducted in the “Antonio Cardarelli” Hospital in Campobasso, which contains 336 beds, of which 287 for acute care and 49 for day-hospital/surgery. During 2010, in monitoring K. pneumoniae infections, the hospital reported isolates which were either sensitive or resistant to carbapenems.

Between May and November 2010 a total of twenty-two cultures of K. pneumoniae were selected (Table 1): all cultures were previously isolated and identified from the same hospital intensive care unit (ICU), consisting of 6 beds. The bacterial cultures were recovered from patients with respiratory tract infections, and the mean age was 64±16.38 years (median 71 years, interquartile range 21-87 years). Seventeen patients (77%) were male. The clinical specimens from respiratory secretions were subcultured on purified on McConkey (Biolife, Milan, Italy) agar, and plates were incubated at 37°C overnight. Pure cultures were stored at 4°C until the use for further DNA isolation and amplification.

Antimicrobial susceptibility testing

K. pneumoniae isolates were tested for antibiotic susceptibility using the Phoenix Automated Microbiology System (Becton Dickinson Diagnostic Systems, Sparks, United States) in collaboration with the hospital microbiology laboratory. The Minimal inhibitory concentrations (MICs) determinations were performed for different classes of antibiotics, testing imipenem and meropenem (carbapenems); ampicillin, amoxicillin-clavulanate, piperacillin and piperacillin-tazobactam (penicillins); cefazidime, cefepime and cefotaxime (cephalosporins); amikacin and gentamicin (aminoglycosides); ciprofloxacin and levofloxacin (fluoroquinolones), and aztreonam (monobactams).

Results were interpreted according to the breakpoints values recommended by the European Committee on Antimicrobial Susceptibility Testing [21], classifying the strains in three susceptibility categories as “sensitive”, when the strain is inhibited by a concentration of an antibacterial agent associated with a high likelihood of therapeutic success; “intermediate” inhibited by a concentration of an antibacterial agent associated with an uncertain therapeutic effect, and “resistant” inhibited by a concentration of an antibacterial agent associated with a high likelihood of therapeutic failure.

Molecular Epidemiology Study

Pulsed-field gel electrophoresis (PFGE) with XbaI enzyme
macrorestriction of genomic DNA was performed to establish clonal relationships between *K. pneumoniae* isolates, according to the PulsNet protocol. PFGE conditions were as follows: pulse times ranged from 5 to 40s over 24h at 6.0V/cm and at 14°C. The PFGE profiles obtained were converted to TIFF files and subjected to cluster analysis using BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium). Patterns interpretation was carried out according to Tenover criteria [22], and following the analysis approach recently described for food borne disease investigations and surveillance [20,23]. Clustering was based on the un weighted pair-group method with arithmetic averages (UPGMA). The Dice correlation coefficient was used to analyze the similarity of banding patterns with 1.0% tolerance and optimization. A 95% cut-off similarity was considered for the interpretation of chromosomal DNA restriction patterns, thus, isolates with <95% similarity profiles were characterized as different PFGE subtypes. In the dendrogram analysis, the assignment of clusters was based on ≥85% similarity level.

DNA extraction
A bacterial suspension was obtained with pure *K. pneumoniae* growth into 400 μl Trypton Soy (Biolife) broth medium. Genomic DNA purification was carried out following the protocol of Maxwell® 16 Cell DNA Purification Kit (Promega Corporation, Milan) using the Maxwell® 16 Instrument (Promega).

Molecular analysis of resistance-associated genes
PCR assays were performed to detect class A carbapenemases (bla<sub>KPC</sub> and bla<sub>GES</sub> genes), class B metallo-beta-lactamases (bla<sub>IMP</sub>, bla<sub>VIM</sub>, and bla<sub>NDM-1</sub> genes), class D (bla<sub>OXA-48</sub>, bla<sub>MBL-EXT</sub>, and bla<sub>AmpC</sub> plasmid-mediated <br>β-lactamases encoding genes. The presence of target genes was investigated in single PCRs using specific oligonucleotides listed in Table 2, as previously described [24]. Amplifications were carried out using 2 μl of DNA template in 50 μl of final reaction volume containing 25 μl of PCR Master Mix 1× (Promega, Milan, Italy), and 1.0 μmol l<sup>-1</sup> of each primer (Eurogentec, Biosense srl, Milan, Italy). Target genes were amplified using the same conditions of initial denaturation of 95°C for 2 min, followed by 35 cycles at 95°C for 1 min, extension at 72°C for 1 min, and final extension step at 72°C for 5 min. The annealing phase was different depending on the amplified product: bla<sub>KPC</sub> at 52 °C for 1 min; bla<sub>GES</sub> and bla<sub>ACT-MIR</sub> at 54 °C for 1 min; bla<sub>VIM</sub> and bla<sub>OXA-48</sub> at 56°C for 1 min; bla<sub>IMP</sub> and bla<sub>DHA</sub> at 45°C for 1 min; bla<sub>NDM-1</sub>, bla<sub>ACC</sub>, bla<sub>LAT-BIL-CMY</sub>, bla<sub>MOX-CMY</sub>, bla<sub>FOX</sub>, and bla<sub>DHA</sub> at 47°C for 1 min; bla<sub>VIM</sub> at 53°C for 1 min. The correct size amplified product was detected by agarose gel electrophoresis (1.0-1.5% m/v concentration, 1X TAE buffer at 100 V for 1 hour). Positive and negative controls and a 100 bp DNA ladder (Promega) were included in each batch of reactions.

**Results**
Antibiotic susceptibility patterns among *K. pneumoniae* isolates
The *K. pneumoniae* isolates showed a multidrug-resistant antibiotype (Table 3). All the strains were resistant to ampicillin,
amoxicillin-clavulinate, piperacillin, piperacillin-tazobactam, ceftazime, cefotaxime, and aztreonam. Concerning the carbapenems, 73\% (n=16) were classified as intermediate for imipenem, and 18\% (n=4) as resistant, while the majority (n=20, 91\%) of isolates resulted sensitive to meropenem. In addition to VIM, no isolate harbored the bla\(\text{KPC}\) gene encoding AmpC plasmid-mediated beta-lactamases. The class B carbapenemase was detected in 90.9\% of isolates. The bla\(\text{NDM-1}\) encoding class B carbapenemases, or the genes GES genes, coding for class A carbapenemases were not detected among the isolate, however, bla\(\text{KPC}\) and bla\(\text{VIM}\) genes were identified among the isolates (Figure 1). The MICs results (S=Sensitive; R=Resistant; I=Intermediate) were interpreted according to EUCAST as follows: S ≤2, R > 8, I when S < x > R for IMP and MER; S ≤8, R > 16, I when S < x > R for PIP TZP and AN; S ≤1, R > 4, I when S < x > R for CAZ, PEP and ATM; S ≤1, R > 2, I when S < x > R for CTX and LVX; S ≤8, R > 16, I when S < x > R for AMP and AMC; S ≤1, R > 4, I when S < x > R for GM; S ≤0.5 and R > 1 for CIP.

### Table 3: Results of MICs (ug/mL) evaluation of 22 K. pneumoniae strains.

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**Abbreviations:** IMP: Imipenem; MER: Meropenem; AMP: Ampicillin; AMC: Amoxicillin-clavulanate; PIP: Piperacillin; TZP: Piperacillin-tazobactam; CAZ: Ceftazidime; PEP: Cefepime; CTX: Cefotaxime; AN: Amikacin; GM: Gentamicin; CIP: Ciprofloxacin; LVX: Levofloxacin; ATM: Aztreonam.

The MICs results (S=Sensitive; R=Resistant; I=Intermediate) were interpreted according to EUCAST as follows: S ≤2, R > 8, I when S < x > R for IMP and MER; S ≤8, R > 16, I when S < x > R for PIP TZP and AN; S ≤1, R > 4, I when S < x > R for CAZ, PEP and ATM; S ≤1, R > 2, I when S < x > R for CTX and LVX; S ≤0.5 and R > 1 for CIP.

**Discussion**

The emergence and spread of multi-resistant *K. pneumoniae* underlines that there is an urgent need to implement rapid and sensitive strategies for the identification and control of resistant isolates, and the application of molecular typing methods is required to define the structure and dynamics of the bacteriums population,

The detection of VIM characterized by susceptible and/or intermediate profile to carbapenems was similarly reported in a recent study conducted in Spain [29], where 43 K. pneumoniae cultures harbored the bla VIM gene, although the 63.9% and 49.5% were sensitive to meropenem and imipenem, respectively. In a previous study [16], 234 K. pneumoniae isolates were characterized as resistant to ertapenem, and the other strains as intermediate or susceptible to imipenem and meropenem; the isolates with a susceptible or intermediate phenotype to imipenem and/or meropenem were VIM and OXA-48 producers and the other carbapenemases were not detected, while the strains harboring KPC were resistant to both imipenem and meropenem. Hence, these data suggest that VIM producing K. pneumoniae are still confined to sporadic cases or small outbreaks in Italy, and are not widely disseminated [27,30].

The blaKPC gene was not detected on K. pneumoniae isolates, and this finding is in agreement with previous reports indicating among class B carbapenemase a more frequent detection of VIM instead of IMP, which was not identified in any resistant strains [31]. Indeed, IMP and VIM production is still relatively rare amongst members of the Enterobacteriaceae [8] except for K. pneumoniae circulating in the European Mediterranean basin, with VIM carbapenemase mostly detected in Greece, Italy, and Spain, and E. coli IMP-producers in Taiwan and Japan [31].

The NDM-1 class B and OXA-48 class D carbapenemases were also not detected in the 22 K. pneumoniae tested here, and previous reports have also described that isolates resistant to carbapenems, particularly to ertapenem, do not contain these genes coding for beta-lactamase. In a recent study [32], K. pneumoniae strains were resistant to beta-lactam antibiotics, including carbapenems and penicillins/inhibitors, colistin and ciprofloxacin; however, among genes encoding carbapenemases and AmpC plasmid-mediated beta-lactamase, only the presence of blaKPC-3, blaTEM-1 and blaSHV-1 genes was identified. Moreover, the isolation of 15 K. pneumoniae cultures resistant to carbapenems and colistin in an Italian hospital was reported [33], and all isolates were negative for AmpC, metallo-beta-lactamase and ESBL, but contained blaKPC and were positive by the modified Hodge test [34,35] and were therefore considered as KPC producers.

The absence of NDM-1 in our strains is in agreement with the low detection rate of this determinant in Italy, where the first NDM-1 identification was described in E. coli isolated from a patient who had an epidemiological link with areas endemic for this carbapenemase. Moreover, during 2008-2010, a total of 77 cases infected by K. pneumoniae containing NDM-1 from 13 European countries were reported [36]. Similarly, the OXA-48 enzyme was reported in Italy among strains from cross-border origin but only in the last years with a limited distribution [27]. The genes coding for AmpC enzymes were also not detected. In Italy, previous studies only confirmed the presence of blaFOX gene [37], while other AmpC beta-lactamases were frequently reported in other countries [27].

At state of the art, carbapenemases are mainly found in K. pneumoniae and to a lesser extent in E. coli and other members of the Enterobacteriaceae, with higher prevalence in southern Europe and Asia. The high prevalence of carbapenemase producing enterobacteria in a hospital environment will globally contribute to persistent spread of K. pneumoniae producing all types of carbapenemases, mainly KPC, VIM, NDM-1 and OXA-48. Since these enzymes are able to hydrolyze almost all beta-lactam antibiotics and isolates are often especially in hospital settings [26].

The present study describes the results of characterization of 22 K. pneumoniae isolated from patients with respiratory tract infection in an intensive care unit. The antibiotic profile or resis
type was determined for each isolate by testing against a panel of fourteen antibiotics, including two carbapenems. The identified antibiotic resistance profiles were supported by PCR detection of resistance-associated genes, while PFGE typing allowed describing the intraspecific structure of the tested isolates.

The PFGE analysis revealed a high genomic diversity among isolates characterized by similar or identical antibiotic-resistance types, suggesting a wide heterogeneity in strains circulation within the hospital ward during the study period. Although at the time of the study, KPC carbapenemase is considered as the most epidemiologically important and widespread determinant conferring resistance to carbapenems in Italy [27], none of the tested strains harbored the blaKPC gene. However, the lack of this gene was partially supported by the intermediate and sensitive antibiotic phenotypes for both imipenem and meropenem detected for the majority of the isolates tested. In Italy, compared to the continued dissemination and transmission of KPC class A carbapenemase, the other carbapenemase resistance mechanisms are still considered minor or emerging concerns, being associated with sporadic reports [18] and linked to mobility and travel from areas where such resistance is endemic.

In this study, the presence of GES carbapenemase was not detected in any isolate examined, and this observation is in agreement with the available recent epidemiological data, showing the circulation of these enzymes mostly in specific European countries (i.e., France, Greece, Portugal), as well as in South Africa and Southeast Asia [28]. Conversely, our analysis indicated that about 91% of isolates harbored bla VIM, suggesting the production of VIM carbapenemase as the only mechanism for the intermediate and resistant phenotypes. Only two isolates were bla VIM negative, and both were classified as intermediate to imipenem and sensitive to meropenem, respectively.
resistant to other classes of antibiotics, the selective pressure and the likelihood of persistence of such strains may increase. In addition, some "successful" clones and hypervirulent strains may increase the likelihood of dissemination and change the endemicity characteristics at global level [38].

In conclusion, this study reports, for the first time, the results of biomolecular analysis conducted on multidrug-resistant K. pneumoniae isolated from patients with respiratory tract infections and hospitalized in an intensive care unit in the Molise Region of Central Italy. The results provide hitherto unavailable information for the implementation of surveillance and control of infections and for limiting the dissemination of resistance genes, not only at a local level. The monitoring, surveillance and molecular typing are, therefore, essential to control the emergence of multidrug-resistant strains in nosocomial settings, and to reduce the frequency of outbreaks. Exploring the antibiotic resistance phenotype, the resistance-mediated gene profiles and investigating the dynamics of K. pneumoniae population in a hospital setting can allow an understanding of the evolution and the complex interchange between genes, plasmids and clones. Moreover, the implementation of rigorous control measures, the appropriate management of antibiotic therapy or "antimicrobial stewardship", and the promotion of awareness of the hazard represented by K. pneumoniae resistant strains are crucial to preserve the latest therapeutic options available for treating these infections.

References


