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Original article

Comparison of genotypic and phenotypic antimicrobial profile in carbapenemases producing *Klebsiella pneumoniae*

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Abstract. *Background and aim:* Prompt administration of appropriate antibiotic therapy is crucial in improving outcomes, particularly for infections sustained by multi-drug resistant (MDR) strains. Although phenotypic antimicrobial susceptibility testing (AST) represents the gold standard to address antibiotics treatment, the long time required to obtained affordable results could negatively affect the prognosis. In contrast, rapid genotypic AST provides essential information for treatment choice and in surveillance programs. In order to evaluate the potential adoption of rapid AST in clinical routine, we compared the genotypic and phenotypic antimicrobial profiles of different carbapenemases-resistant *K.pneumoniae* (Cr-Kp) strains, characterized by different expression of carbapenemases-encoding genes: KPC, KPC+CTX-M, KPC+OXA-48. *Methods:* A set of 109 strains of Cr-Kp were tested for the antimicrobial drugs by the automatized Vitek II system and, in parallel, to the new combination of β-lactams/β-lactamases inhibitors (BL/BLI) by Etest. An antimicrobial resistance index (ARI) was calculated for each strain, assigning each 1 or 0 points based on observed resistance/susceptibility, and dividing the total by the number of antibiotics tested. Kruskal-Wallis test, followed by Dunn's post hoc test (Bonferroni correction), were used to compare quantitative variables among resistance gene subgroups. *Results:* We observed a higher ARI score in KPC/OXA-48 strains, similar profile in KPC alone and KPC/CTX-M groups and a significant lower resistance in no-carbapenemases-producing group. Same trend was observed considering BL/BLIs. *Conclusions*: These preliminary results showed a close link between genotypic and phenotypic AST, supporting the adoption of rapid AST in cases of severe infections, ensuring to saving time and providing, contextually, the surveillance of MDR strains and improving stewardship programs. (www.actabiomedica.it)

Key words: carbapenemases-encoding gene, rapid AST, *Klebsiella pneumoniae,* antimicrobial susceptibility testing

Introduction

The rapid emergence and spread of multi drug resistant bacteria pose a severe threat for global healthcare due to the extensive use of antibiotics (1). More than 60% of infections caused by MDR bacteria are associated with healthcare settings (2).

In Europe, it has been observed a gradient north-to-south and west-to-east, with higher rates of MDR bacteria recorded in the South and in the East (2), with one third of the infections caused by carbapenem-resistant *K. pneumoniae*, *Acinetobacter* spp. and *P. aeruginosa*, as highlighted by ECDC reports (3, 4). During last decades, a sixfold increase of the incidence of infection

and mortality caused by carbapenem resistant *Klebsiella pneumoniae* was recorded (2, 5). β-lactamases (bla) are the main cause of resistance for cr-Kp (6). The number of β-lactamases is constantly growing, and four classes of carbapenemases (A, B, C, and D) can be detected (7). Additionally, extended-Spectrum β-lactamases (ESBLs), encoded by *bla*CTX-M, could confer resistance to penicillins, third generation cephalosporins, and monobactams (8).

High rates of cr-Kp often show cross-resistance to other antibiotic classes, such as aminoglycosides, fluoroquinolones, and third-generation cephalosporins (9, 10), determining ineffective treatments, prolonged hospitalization, and decreasing survival rates (11).

Novel β-lactams/β-lactamases inhibitors (BL/BLIs), ceftazidime/avibactam (CZA), imipenem/relebactam (I/R), and meropenem/vaborbactam (M/V) have been developed for the treatment of severe infections sustained by carbapenem resistant bacteria. However, Despite the known effectiveness of BL/BLIs against carbapenemases, the emergence of resistance to novel combination have already been reported (12-14).

Phenotypic methods represent the gold standard for antimicrobial susceptibility test (AST), although it needs a long incubation time and potential difficulties for low-growth microorganisms. This delay contributes to AMR associated with the prescription of broad-spectrum antibiotics, as well as higher risk of empiric therapy failure and, worsening prognosis for patients. Currently, the most frequently used ASTs are based on the identification of specific MIC and the interpretation of pharmacokinetic profiles (15). In contrast, genotypic AST could be useful to predict phenotypic profile, by detection of AMR markers, can be performed directly on biological samples reducing time of analysis (from <2 hours to 1 day) and, concurrently, monitor local epidemiology and identify the emergence of new resistant clones (16, 17).

We previously reported a shift from the expression of solely KPC to the co-expression of KPC and CTX-M, followed by the emergence of *K. pneumoniae* strains carrying both KPC and OXA-48 (18, 19).

An observational study was carried out to evaluate phenotypic profiles of *K. pneumoniae* strains based on the expression of different resistance genes, including KPC, KPC/CTX-M and KPC/OXA-48 co-producing isolates. A cumulative Antimicrobial Resistance Index (ARI) was calculated to assess AMR trends among different genotypic profiles.

Materials and methods

A monocentric observational study was carried at the university hospital of Sassari (Italy), between 2018 and 2022. A set of 109 *K. pneumoniae* strains, detected from clinical specimens was selected. Samples were isolated from rectal swabs, blood, and respiratory tract specimens. Only one sample was used per patient.

Identification and characterization of carbapenem resistant Enterobacteriaceae

Identification of colonies and antimicrobial susceptibility tests were performed using the Vitek II system. The following antibiotics were tested using the VITEK®2 AST-N397 card: amikacin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefepime, cefotaxime, ceftazidime, ceftolozane/tazobactam, meropenem, imipenem, gentamicin, tobramycin, ciprofloxacin, trimethoprim/sulfamethoxazole (20). Simultaneously the Etest assays were carried out to verify the susceptibility for the new β-lactams/β-lactamases inhibitors combination, (bioMérieux, Inc., Durham, NC) for ceftazidime/avibactam, imipenem/relebactam, and meropenem/vaborbactam, not included in the available Vitek AST cards (21). Results were interpreted according to the clinical breakpoints of the European Union Committee on Antimicrobial Susceptibility testing, EUCAST v.13.0 (22).

All *K. pneumoniae* strains resistant to at least one carbapenem were defined as carbapenem resistant; subsequently ESBL (*bla*CTX-M) and carbapenemase encoding genes (*bla*KPC, *bla*NDM, *bla*VIM, *bla*IMP, *bla*OXA-48) were detected via RT-PCR, using the commercial kit Allplex Entero DR assay (23).

Statistical analysis

Medians and 25^{th}-75^{th} percentiles (interquartile range, IQR) were used to describe quantitative variables, and absolute and relative (percentages)

frequencies for qualitative ones. The Kruskal-Wallis test, followed by Dunn's post hoc test, was used to compare quantitative variables among different resistance gene subgroups. According to De Socio et al. an Antimicrobial Resistance Index (ARI) was calculated per strain (24). For each antibiotic tested in the study, a point of 0 (for susceptibility), 0.5 (for intermediate) and 1 (for resistance) was assigned; subsequently, the ARI score was calculated dividing the sum of each score by the total number of tested antibiotics, resulting in a final score ranging from 0 to 1. A two tailed P value less than 0.05 was considered statistically significant. Statistical analyses were performed with STATA version 17 (StatsCorp, TX, USA).

Results

A total of 109 *K. pneumoniae* strains were selected, mainly isolated from rectal swabs (48/109; 44.0%), blood cultures (38/109; 34.9%), and broncholavage (23/109; 25.1%).

Following the detection of carbapenemases encoding genes, 31.2% (34/109) of strains were classified in the KPC/OXA-48 resistance group, followed by KPC (32/109; 29.4%), KPC/CTX-M (31/109; 28.4%), and No Carbapenemase (No CP) group (12/109; 11.0%) (Table 1).

Overall, we observed a marked resistance profile among carbapenemases-producing groups, for almost all antibiotics tested. In detail, a percentage of resistance >90% was observed for amoxicillin/clavulanic acid (93.6%), piperacillin/tazobactam and cefotaxime (90.8%); the majority of strains showed high resistance for cefepime (89.9%), ceftazidime and ceftolozane/tazobactam (89%), ciprofloxacin (84.4%), trimethoprim/sulfamethoxazole (78%); >85% were resistant to carbapenems, with 87.2% and 85.3% of resistance to meropenem and imipenem, respectively. A slightly lower rate of resistance was found for aminoglycosides (amikacin and gentamicin with 60.6% and 62.4%, respectively), with the only exception of tobramycin (~82%). Considering novel BL/BLIs, the percentage of resistance to CZA, I/R, and M/V was 16.5%, 25.7%, and 22.9%, respectively (Table 2).

MIC median values were significantly different (P< 0.0001) among different carbapenemases-producing groups. The lowest MIC median values for CZA, I/R, and M/V were found in the No CP group (0.10, 0.13 and 0.02, respectively), similar MIC values were found in KPC and KPC/CTX-M groups, whereas the KPC/OXA-48 group showed the highest MIC values (6, 3, 8, for CZA, I/R, and M/V, respectively).

Table 1. Group characteristics.

Variables		Sample (n=109)
Resistance gene subgroups, n (%)	No carbapenemase	12 (11.0)
	KPC	32 (29.4)
	KPC/CTX-M	31 (28.4)
	KPC/OXA	34 (31.2)

Antimicrobial resistance index (ARI) for antimicrobial resistance trend

Median (IQR) ARI score for all samples included in the study was 0.8 (0.7-0.9) points, with significant differences (P=0.0001) among groups. The ARI score revealed a statistically significant increasing

Table 2. Percentage of antibiotic resistance observed in *K. pneumoniae* strains.

Antibiotic	n (%) of resistant strains
Amoxicillin/clavulanic acid	102 (93.6)
Piperacillin/tazobactam	99 (90.8)
Cefepime	98 (89.9)
Cefotaxime	99 (90.8)
Ceftazidime	97 (89.0)
Ceftolozane/tazobactam	97 (89.0)
Meropenem	95 (87.2)
Imipenem	93 (85.3)
Amikacin	66 (60.6)
Gentamicin	68 (62.4)
Tobramycin	90 (82.6)
Ciprofloxacin	92 (84.4)
Trimethoprim/sulfamethoxazole	85 (78.0)
Ceftazidime/avibactam	18 (16.5)
Imipenem/relebactam	28 (25.7)
Meropenem/vaborbactam	25 (22.9)

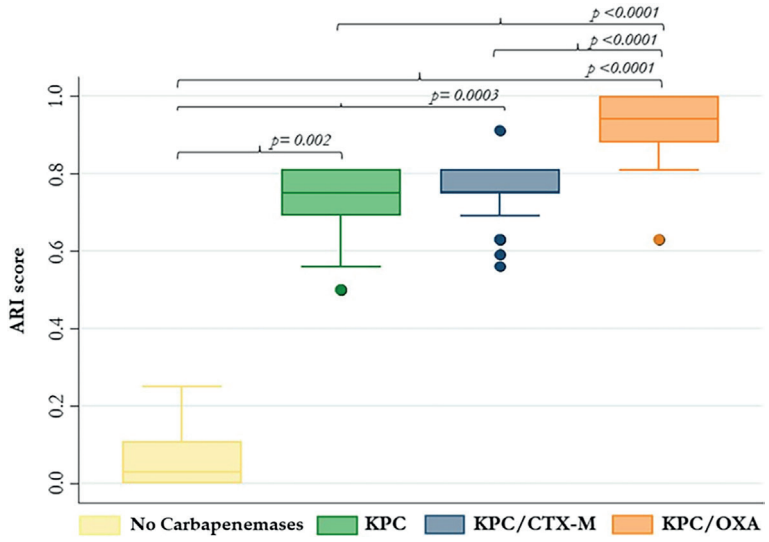

Figure 1. ARI score among resistance gene subgroups – post hoc analysis.

trend among the different groups, with the lowest median recorded in the non-carbapenemase-producing group (0.03), intermediate values in the KPC and KPC/CTX-M isolates (0.75 for each group), and the highest value in the KPC/OXA-48 cluster (0.94) (Figure 1).

Discussion

The global spread of antimicrobial resistant bacteria, mainly due to the inappropriate use of antimicrobial drugs, enforces the optimization of antibiotic use and highlights the importance of antimicrobial stewardship programs. Moreover, delays of proper therapy administration and consequent use of broad-spectrum antibiotics, could both increase the emergence of resistant clones and the risk of treatment failure (15, 16).

Growing evidence highlights the advantages of genotypic detection of AMR determinants particularly in cases of infection sustained by MDR bacteria (25). Moreover, the potential adoption of rapid AST provides valid information for addressing the most suitable therapy, monitoring changes in AMR over time through the detection of carbapenemases and new emerging MDR clones (26).

We found a relevant difference in the phenotypic antibiotic susceptibility profile when different groups of carbapenemase-producing *K. pneumoniae* were compared. A marked resistance profile was observed in KPC/OXA-48 co-producing strains, whereas similar profile among KPC/CTX-M and KPC groups, and a higher antimicrobial susceptibility for non carbapenemase-producing isolates. The same resistance profile was well described by the ARI score, suggesting the potential adoption of this tool to compare different resistance profiles.

We found high rates of XDR (Extensively Drug Resistant) strains among KPC and KPC/CTX-M producing groups, whereas ~50% of *K. pneumoniae* co-producing KPC/OXA-48 strains were classified as PDR (Pan Drug Resistant), including resistance to CZA, M/V, and I/R (27).

We found a higher rate of resistance among carbapenemase-producing isolates in comparison with estimates reported in Eastern, South-Western Europe, and Mediterranean countries, where the percentage of resistance for third generation cephalosporins, fluoroquinolones, and aminoglycosides were approximately 50–60% (28).

The expression of *bla*KPC is confirmed as main mechanism of carbapenem resistance in *K. pneumoniae*,

whose transmission inter- and intra-species is facilitated by mobile genetic elements (29).

In our study, no-carbapenemases-producing group was susceptible to carbapenems, unlike KPC, KPC/CTX-M and KPC/OXA-48 strains; this finding excludes other resistance mechanisms and confirms the ability of *bla*KPC to inhibit the activity of β-lactams.

The resistance to usual BL/BLIs combination (i.e., amoxicillin/clavulanic acid and piperacillin/tazobactam) was found in all carbapenemase-producing groups, confirming as traditional β-lactamases inhibitors (i.e., clavulanic acid, tazobactam) are not active against KPC, VIM, IMP, NDM and OXA-48 enzymes (30).

The recent introduction of new BL/BLIs represents a promising alternative for difficult to treat infections. In our study, the co-expression of multiple carbapenemases (KPC/OXA-48) highlighted an alarmingly lower susceptibility to novel BL/BLIs, and the emergence of cross resistance between M/V, I/R and CZA. In these cases, genotypic AST could be a useful tool for the identification of CZA resistance determinants (31). Moreover, several reports describe the restoration of carbapenem activity in CZA resistant strains (32, 33). However, the co-existence of carbapenemase variants (i.e., KPC-31, OXA-181) and porin mutations may explain the persistence of carbapenems-resistance in our data (13). Further studies should be addressed to identify the best therapeutic option in case of marked resistant profile, evaluating the effectiveness of mono- vs combined-therapy, also considering the potential adverse effects (34).

Overall, we showed a close link between specific resistance genes and the antimicrobial phenotypic profiles, considering novel BL/BLIs combinations as well. These results, although preliminary, support the adoption of genotypic AST for the identification of the mechanisms of resistance and drive the prompt administration of the most effective therapy, improving patient outcomes (35).

We introduce, for the first time in our setting, the ARI score, an easy and quantitative measure of AMR, potentially useful to observe trends in antimicrobial resistance among different species and hospital wards, over time, especially when correlated with other clinical variables or to assess the effectiveness of stewardship intervention (24). The gold-standard for AST is the phenotypic analysis of isolates (i.e., cultural-based methods and MIC definition), which also supports possible combination therapies based on the pharmacokinetic/pharmacodynamic profile. Moreover, the adoption of genotypic ASTs in clinical practice has some limitations: firstly, the limited number of targets that are searched for and, consequently, the risk of false-negative results; secondly, the presence of a resistance determinant can not necessarily translate into an increase in the MIC value, as it may not be expressed and, consequently, the antibiotic could be wrongly classified as "resistant". On this basis, the integration of genotypic and phenotypic analysis could accelerate and facilitate the diagnosis and treatment of the most severe infections.

Our study shows several limitations: firstly, the monocentric design did not allow us to compare our results with different settings; the use of automated Vitek 2 system for AST did not provide the exact MIC value for single antibiotic and, as a result, did not allow to do further consideration besides the classification of strains in the categories susceptible/resistant; an important limitation was the lack of clinical data did not allow to correlate AST profile and ARI score with patient outcomes, due to the retrospective design of the study.

We highlighted the potential value of genotypic AST as a predictor of phenotypic susceptibility profile in *K. pneumoniae* strains.

Rapid AST can optimize time of antibiotic testing, reducing the risk of inappropriate treatment administration and poor outcome.

Conflict of Interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

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M.P., N.M., I.S., and L.S.; writing—review and editing, N.M., G.S., A.P.; visualization, A.D.R., M.P., N.M., I.S., L.S., L.C., G.S., and A.P.; supervision, L.C., G.S. and A.P. All authors have read and agreed to the published version of the manuscript.

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