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FULL PAPER

Extended-spectrum β-lactamase & carbapenemase-producing fermentative Gram-negative bacilli in clinical isolates from a University Hospital in Southern Italy

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SUMMARY

The aim of this study was to determine the prevalence of extended-spectrum β-lactamases (ESBLs)- and carbapenemase-producing fermentative Gram-negative bacteria (FGNB) in a University Hospital in Southern Italy. These bacteria have the potential to disseminate bacterial resistance in healthcare settings and cause untreatable and prolonged infections associated with high rates of mortality. A retrospective observational study was carried out in a University Hospital in Sicily from January to December 2019.

A total of 1046 FGNB were recovered from different clinical samples among which 40%, 15% and 37% were, respectively, MDR, carbapenemase and ESBL producers. Antibiotic resistance profile of FGNB against the first-line drugs was remarkably high. *K. pneumoniae* (57%) followed by *E. coli* (27%) were found here as the major sources of ESBL producers. The highest proportion of ESBL producers was from ICU ward (72%), and were isolated from urine samples (63.6%) followed by blood samples (54%). Carbapenemase production among the FGNB in our study was about 0.9%, which is more than twice than the prevalence rate reported by the European Antimicrobial Resistance Surveillance Network (ECDC) (0.4%).

To our knowledge, this is the first report on the prevalence of ESBL and carbapenemase-producing FGNB in this region. Our data clearly indicate the importance of implementing antibiotic stewardship strategies in our region to reduce the unnecessary use of antibiotics.

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INTRODUCTION

The World Health Organization (WHO) declared that antimicrobial resistance (AMR) is a global health emergency and represents one of the major public health problems threatening humanity (Spera *et al.,* 2019). In 2017, to reduce the frequency of hospital and community infections caused by antibiotic-resistant microorganisms, Italy adopted its first National Action Plan on Antimicrobial Resistance (Nunez-Nunez *et al.,* 2018). In the last two decades, the problem of AMR has been rapidly worsening due to various factors such as: overuse of antibiotics (i.e., use without treatment indication), incorrect use of antibiotics in the veterinary sector, and excessive consumption of antibiotics in hospital (Sawa *et al.,* 2020). According to the ECDC/CDC definition, bacterial isolates are classified according to their degrees of antimicrobial resistance in multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) bacteria. MDR isolates are not susceptible to at least one agent in three or more antimicrobial categories, while XDR isolates are not susceptible to at least one agent in all but two or fewer antimicrobial categories (i.e., bacterial isolates remain susceptible to only one or two antimicrobial classes). Finally, PDR isolates are not susceptible to any agents in all antimicrobial categories (Magiorakos *et al.,* 2012). Antimicrobial resistance as well as of ESBL- and carbapenemase-producers in fermentative Gram-negative bacteria (FGNB) have been recognized by the World Health Organization among the greatest challenges to human health worldwide (Exner *et al.,* 2017). Mechanisms of antibiotic resistance in bacteria are different and include

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acquisition and transfer of antibiotic resistance genes that occurs through the transfer of bacterial mobile genetic elements such as conjugative plasmids, transposons and integrons (Malloy and Campos 2011; Reygaert 2018). β-Lactam antibiotics have been employed for several years for the treatment of bacterial infections because of their high specificity and safety compared to other classes (Livermore and Woodford 2006). However, the spread of extended-spectrum β-lactamase-producing FGNB is increasing worldwide, making these bacteria resistant to third-generation cephalosporins (cefotaxime, ceftriaxone, and ceftazidime), penicillins, but not carbapenems (Biehl *et al.,* 2016; Lin *et al.,* 2019). The widespread and indiscriminate use of broad-spectrum antibiotics not only in human medicine (i.e., prescription of antibiotics by physicians to treat an infection that may not be caused by bacteria) but also in agriculture and animal husbandry, may favour the selection of antibiotic-resistant bacteria that can easily be transferred from animals to humans (da Costa *et al.,* 2013; Economou and Gousia, 2015). The treatment of choice for ESBL-producing FGNB infections have been the carbapenems, a class of antibiotics belonging to the β-lactam family but structurally different from penicillins and cephalosporins (Walker *et al.,* 2018; Karaiskos and Giamarellou, 2020). However, in recent years, carbapenem-resistant FGNB have gradually increased, becoming an important public health problem, because these clinical isolates expressing multidrug resistance leave few treatment options (Tzouvelekis *et al.,* 2012; Beyene *et al.,* 2019).

The aim of the present study was to investigate the prevalence of extended-spectrum β-lactamase and carbapenemase production among FGNB recovered from patients admitted to University Hospital, Southern Italy, from January to December 2019. The reliable identification of these microorganisms plays a crucial role in the management and control of healthcare-associated infections and in the implementation of antimicrobial stewardship programs.

MATERIALS AND METHODS

Study design

From January to December 2019, non-replicate, FGNB clinical isolates recovered from hospitalized patients were collected by the Clinical Microbiology Laboratory of Messina, Southern Italy. Clinical samples were submitted to the laboratory and processed following standard procedures. Specimens collected were inoculated onto appropriate isolation culture media (Blood culture broth, Blood agar, Chocolate agar, and MacConkey agar plate) and incubated at 35/37°C according to standard protocols for each sample. In cases where a delay in culturing was unavoidable, appropriate transport media were used. All commonly isolated fermentative gram-negative bacilli recovered from the various clinical specimens during the study period were included. According to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2014) and the European Antimicrobial Resistance Surveillance Network (ECDC, 2013), only the first isolate from each patient was included in this study.

Identification and antimicrobial susceptibility tests

Bacterial identification and antimicrobial susceptibility testing were carried out by the Vitek-2 System using GN^R cards, as per the manufacturer's instructions (BioMérieux, Marcy l'Etoile, France) (Bonura *et al.,* 2015; Ferranti *et al.,* 2018). The antimicrobial susceptibility profile of isolates was confirmed by Kirby-Bauer disc diffusion method and the data were interpreted using clinical breakpoints according to EUCAST criteria 2019 [EUCAST breakpoint tables v 9.0 (2019)]. The following antibiotics were used: cefotaxime (30μg), ceftazidime (30μg), amoxicillin/clavulanic acid (20/10μg), amikacin (30μg), cefepime (10μg), ciprofloxacin (5μg), gentamycin (10μg), sulfa/trimeth (1.25/23.75μg), piperacillin-tazobactem (100μg), imipenem (10μg), meropenem (10μg), ertapenem (10μg), fosfomycin (10μg). In agreement with previous reports, the term MDR "multidrug resistance" among gram-negative bacteria was used to indicate resistance to three or more of the following antimicrobials: ceftazidime, ciprofloxacin, meropenem, gentamicin, ampicillin/sulbactam, or piperacillin/tazobactam (Pop-Vicas *et al.,* 2008).

Screening for ESBL producers

ESBL-producing organisms are defined as ones that confer resistance to most beta-lactam antibiotics, including penicillin, cephalosporins, and aztreonam (Bush 2018). They generally remain susceptible to carbapenems and may be inhibited in vitro by beta-lactam/beta-lactamase inhibitor combinations (Ho *et al.,* 2019). ESBL testing was performed with VITEK2, which uses the analysis of MIC patterns evaluated by the Advanced Expert System to detect ESBL expression (Sanders *et al.,* 2000). FGNB suspected to be producers of ESBL enzymes were subjected to a confirmation test according to EUCAST guidelines for the detection of resistance mechanisms (Bavelaar *et al.,* 2021). The confirmatory test was performed using disks containing cephalosporin (cefotaxime AND ceftazidime, or cefepime) alone and in combination with clavulanic acid (CA) (10μg) to distinguish isolates harbouring ESBLs from those resistant for other reasons. Briefly, the antibiotic discs were placed on Mueller-Hinton agar plates seeded with a suspension of each isolate adjusted to match the turbidity standard of 0.5 McFarland. The test is considered positive for an ESBL producer if the inhibition zone diameter is ≥5 mm larger with

clavulanic acid than without (Rawat and Nair, 2010; Malloy and Campos, 2011).

Detection of Carbapenemase production

Carbapenemase-producing FGNB were screened using VITEK AST-N202 cards. An isolate was considered a carbapenemase producer if after screening with a VITEK AST-N202 card it showed intermediate or high resistance to more than one of the carbapenems (ertapenem, imipenem, or meropenem) (Ferranti *et al.,* 2018). Detection of CPE was confirmed following the guidelines of the EUCAST disc diffusion method (2017),which indicate screening cut-offs using meropenem MIC >0.12 mg/L and/or a < 25 mm meropenem (10 µg) disc diffusion zone diameter, with an increased meropenem zone diameter cut-off of <27 mm recommended for areas where OXA-48 producers are endemic (Haldorsen *et al.,* 2018).

FilmArray blood culture identification panel

Fifty random positive blood culture bottles (BACTEC Plus Aerobic/F bottles) were also analysed using the FilmArray BCID panel to identify pathogens and their antibiotic susceptibility status. The FilmArray BCID panels were processed according to the manufacturer's instructions. Briefly, 200 µL of the positive blood culture was aspirated (within eight hours of positivity), mixed with sample buffer, and loaded into the FilmArray pouch. The FilmArray BCID panel was loaded onto the FilmArray instrument for nucleic acid extraction, amplification, and DNA melting curve analysis to identify the possible presence of both bacterial targets and related resistance genes in the tested culture.

Data analysis

The prevalence of MDR, carbapenemase- or ESBL-producing FGNB was calculated by dividing the number of MDR, carbapenemase- or ESBL-positive samples by the total number of FGNB samples. The data were expressed as absolute frequencies and percentages. For each parameter (MDR, ESBL and carbapenemase), the comparison between urine and other clinical specimens (including respiratory samples, blood, etc.) was performed using Z-test for difference of proportions; the same test was applied to compare the prevalence of MDR, ESBL and carbapenemase in antibiotic-treated and non-treated patients. A p-value less than 0.05 was considered statistically significant. All statistical analyses were performed by using SPSS for Window package, version 22.

RESULTS

Of the 6928 clinical samples received during the indicated period, 3452 were found to be positive for bacterial pathogens, among which 1046 were fermentative gram-negative bacteria. Among the latter, the majority of the isolates (81.2%) were from the inpatient department, while the rest (17.8%) were from the outpatient department. Moreover, out of 1046 isolates, 58.6% were isolated from male patients, while 41.4% were recovered from female patients. As shown in Table 1, although different types of clinical specimens were collected and analysed, the majority of the isolates were isolated from urine (58.6%) followed by blood (20.9%) and respiratory samples (15.8%). The predominant species were *E. coli* (53.3%) and *K. pneumoniae* (38%) (*Table 1*).

Antimicrobial susceptibility testing of bacterial isolates is summarized in Table 2. Resistance to amoxicillin/clavulanic acid was the highest (62%), followed by ciprofloxacin (52.3%), whereas lower resistance rates were recorded for amikacin and piperacillin-tazobactam combination (9.6% and 32.3%, respectively). Of the total of 1046 FGNB identified during the study period, 40.2%, 19%, and 37% were, respectively, MDR, carbapenemase and ESBL producers. Moreover, out of 1046 FGNB isolates, 43.6%, 53.1% and 32.6% displayed resistance to cephalosporins, quinolones and aminoglycosides, respectively.

The production of extended spectrum beta-lactamases was detected in 57%, 27%, 10.7%, and 11.5%, respectively, of *K. pneumoniae, E. coli, E. cloacae, and C. freundii* strains. The distribution of ESBL and carbapenemase isolates was different among different species and depended on whether the isolates came from the outpatients or inpatients department. In outpatients, the most frequent ESBL-producing species were detected in 41 *E. coli* (26.3%), 40 *K. pneumoniae (*17.8%), 2 *E. cloacae (*27.3%), and 1 *C. freundii* (33.3%), respectively. In inpatients, the frequency of ESBL production per species was the following: 111 *E. coli* (73%), 186 *K. pneumoniae (*82.3%), 5 *E. cloacae* (71.4%), and 2 *C. freundii* (66%). None of the *C. freundii* strains produced carbapenemase, but car-

Table 1 - *Distribution of commonly isolated fermentative Gram-negative bacteria among different specimen types.*

	Urine	*Blood*	*Lower respiratory tract samples*	*Pus*	*Sputum*	*Intraoperative material*	*Other specimen*	*Total*
E. coli	411	82	25	4	15	10	11	558
K. pneumoniae	186	102	74	1	22	4	8	397
E. cloacae	11	22	12	1	14	2	3	65
C. freundii	5	13	3	1	1	2	1	26
Total	613	219	114	7	52	18	23	1046

Table 2 - *Antimicrobial resistance profiles of commonly isolated FGNB.*

	E. coli N (%)	*K. pneumoniae* N (%)	*E. cloacae complex* (N %)	*C. freundii* N (%)	*Total* N (%)
Amikacin	12 (2.1)	81 (19.8)	0 (0)	1 (3.8)	94 (8.9)
Amoxicillin/Clavulanic acid	286 (51.2)	287 (72.2)	65 (100)	26 (100)	664 (63.4)
Ciprofloxacin	241 (43.1)	261 (65.7)	12 (18.4)	9 (34.6)	523 (50)
Ertapenem	13 (2.3)	129 (32.4)	14 (21.5)	0 (0)	156 (14.9)
Fosfomicin	15 (2.6)	125 (31.4)	27 (41.5)	0 (0)	167 (15.9)
Gentamycin	86 (15.4)	164 (41.3)	5 (8.1)	1 (3.8)	256 (24.4)
Imipenem	2 (0.3)	84 (21.1)	6 (9.1)	0 (0)	92 (8.7)
Meropenem	4 (0.8)	157 (39.5)	8 (12.3)	0 (0)	169 (16.1)
Piperacillin-Tazobactem	47 (8.4)	236 (59.4)	26 (40)	3 (11.5)	312 (29.8)
Cefepime	109 (19.5)	190 (47.8)	10 (15.3)	0 (0)	309 (29.5)
Cefotaxime	189 (33.8)	261 (65.7)	29 (44.6)	11 (42.3)	490 (46.8)
Ceftazidime	177 (31.7)	267 (67.2)	28 (43)	10 (38.4)	482 (46)
Sulfamethoxazole-trimethoprim	194 (34.7)	153 (38.5)	11 (17)	3 (11.5)	361 (34.5)
Total	558	397	65	26	1046

bapenemases were produced by 0.9%, 40.8% and 18.1%, respectively, of *E. coli, K. pneumoniae* and *E. cloacae* strains.

As shown in *Figure 1*, the prevalence of MDR, ESBL and carbapenemase-producing FGNB was different among different wards. Most of the multidrug-resistant bacteria were isolated from patients hospitalised in the Intensive Care Unit (ICU) (31.3%) followed by the Internal Medicine Ward (25.7%), Surgical Ward (22.2%), and Neonatal Unit (NU) (7%). Similarly, ESBL and carbapenemase-producing isolates were more frequently recovered in samples from patients

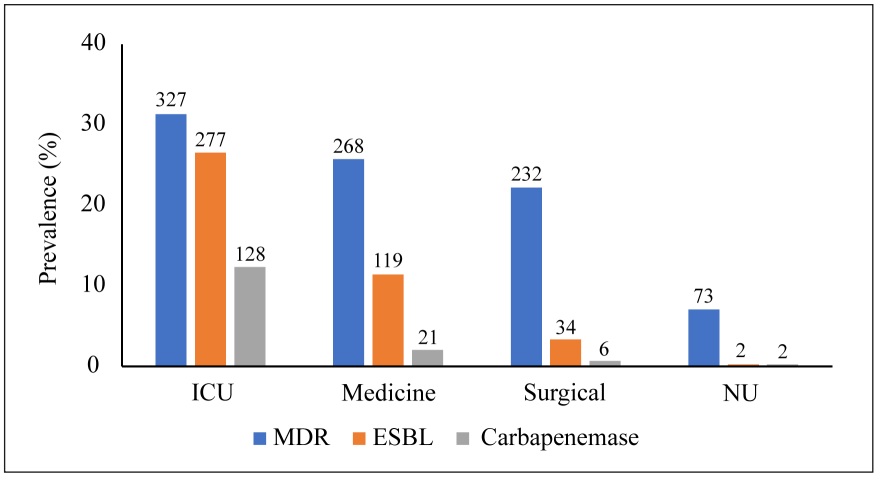

Figure 1 - *Prevalence of MDR, ESBL and carbapenemase-producing FGNB among different wards. The numbers above each column indicate the number of positive samples.*

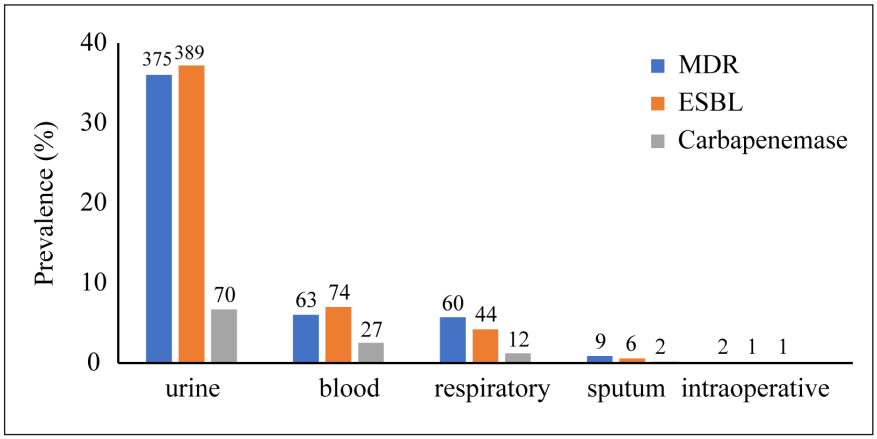

Figure 2 - *Prevalence of MDR, ESBL and carbapenemase-producing FGNB among different clinical specimens. The numbers above each column indicate the number of samples tested.*

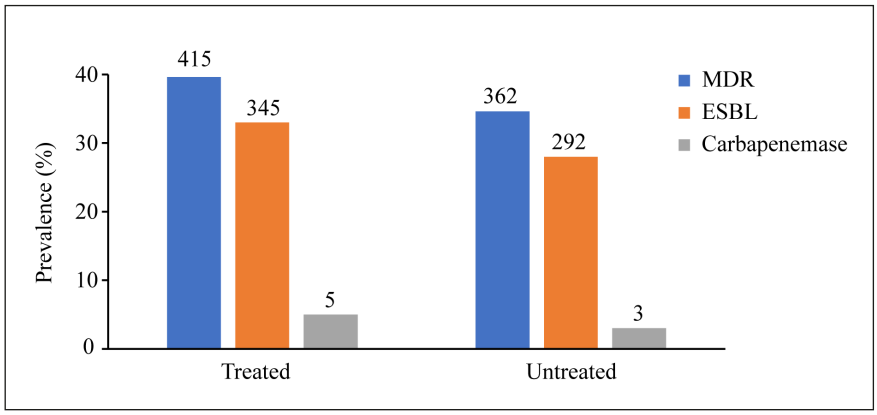

Figure 3 - *Prevalence of MDR, ESBL and carbapenemase-producing FGNB in antibiotic-exposed and non-exposed patients. The numbers above each column indicate the number of samples tested.*

admitted to the ICU (26.5% and 12.3%, respectively) than those in other medical wards (*Figure 1*). *Figure 2* shows the distribution of MDR, ESBL and carbapenemase production in the various samples. The MDR isolation rate was significantly higher in urine (36%) than in other clinical specimens, including respiratory samples (5.7%) and blood (6%) ($p<0.001$ for each comparison). Similarly, the highest prevalence of ESBL-producing isolates was in urine samples (37.2%) followed by blood (7%) and respiratory samples (4.2%); this result is statistically supported by a highly significant p-value ($p<0.001$). The prevalence of carbapenemase-producing isolates was significantly greater in urine (6.7%) than in blood (2.5%) or respiratory specimens (1.2%) ($p<0.001$ for both comparisons). In selected experiments, we compared the results of conventional methods with those of BioFire FilmArray Blood Culture Identification (BCID) panel for identifying the pathogens and their antibiotic susceptibility status. Among the 50 positive random blood cultures, 97% of FilmArray BCID panel results were consistent with the conventional methods, while the sensitivity and specificity for bla_{KPC} (the only available carbapenem-resistance gene on the FilmArray BCID panel) was 100%.

The prevalence of MDR, ESBL and carbapenemase-producing FGNB among antibiotic-treated and non-treated patients is shown in Figure 3. The prevalence of MDR, ESBL and carbapenemase production was significantly higher among previously-treated compared to non-treated patients ($p=0.018$, $p=0.013$ and $p=0.020$, respectively) (*Figure 3*).

DISCUSSION

Nosocomial bacterial infection is one of the most common complications in hospitalized patients and are a major public health concern because these infections are often due to MDR bacteria that are difficult to treat (Reygaert 2018). FGNB are members of the family of Enterobacteriaceae, which due to their ability to spread quickly in healthcare settings are among the most common hospital-acquired pathogens (Wang *et al.,* 2015). In addition, these bacteria, exhibiting multidrug resistance, cause infections associated with high rates of morbidity and mortality (Tzouvelekis *et al.,* 2012; Falagas *et al.,* 2014). In the present study, a total of 1046 clinical FGNB isolates were identified from patients admitted to Messina University Hospital and these bacteria were tested against 13 antibacterial drugs. The antibiotic resistance profile of FGNB against first-line drugs was remarkably high. The resistance profile of FGNB against drugs of β-lactam/β-lactamase inhibitor combinations ranges from 29.8% for piperacillin/tazobactam to 63.4% for Amoxicillin/Clavulanic acid, indicating that the first β-lactam with β-lactamase inhibitor demonstrates better activity. The antibiotic resistance profile of FGNB against other first-line drugs such as cephalosporins and quinolones was also high (both above 60% for *K. pneumoniae*). On the other hand, the percentage antibiotic resistance rates of FGNB against aminoglycosides were below 40% (above 30% only for *K. pneumoniae*). The surveillance of antimicrobial resistance in Italy for 2019 reported by the European Centre for Disease Prevention and Control (ECDC) showed that the resistance rates of *K. pneumoniae* were high: 54.7% of isolates were resistant to fluoroquinolones, 57.6% to cephalosporins, 32.6% to aminoglycosides, and 28.5% to carbapenemase. In our study, we found slightly higher resistance rates to all four classes of antibiotics: 67% to fluoroquinolones, 62% to cephalosporins, 37.3% to aminoglycosides, and 40.8% to carbapenemase. The data reported by the ECDC for *E. coli* in Italy in 2019 are also high: 44.5% to fluoroquinolones, 30.9% to cephalosporins, 15.9% to aminoglycosides, and 0.4% to carbapenemase. In our study, for *E. coli* we observed more frequent resistance to fluoroquinolones (44.5%) and carbapenemase (0.9%), whereas resistance to cephalosporins and aminoglycosides was less frequent (29% and 8.6%, respectively).

In the present study, out of 1046 FGNB, 39.5% were MDR and, among these, the highest MDR strains were detected in *K. pneumoniae* (48.9%) followed by *E. cloaceae* (41%), *Citrobacter freundii* (38.4%) and *E*

coli (33.1%). In our study, higher MDR *K. pneumonia* and *E. coli* were recovered compared to the data obtained from the ECDC, where lower MDR *K. pneumoniae* (30.3%), and *E. coli* (11.6%) were reported.

Our study reported collectively a high prevalence of ESBL-producing FGNB (37%). *K. pneumoniae* (57%) followed by *E. coli* (27%) were found as the major sources of ESBL producers. This is almost in line with the Euro surveillance study, where the prevalence of these two microorganisms resistant to 3rd generation cephalosporins was reported to be 57.6% and 30.9%, respectively. The highest proportion of ESBL producers was from the ICU ward (72%), and were isolated from urine samples (63.6%) followed by blood samples (54%). There is a considerable variation in ESBL prevalence in different parts of the globe, and the detection rate of ESBL producers varies considerably, ranging from 3.8% in Australia to as high as 80.6% in India (Sharif *et al.,* 2016; Djim-Adjim-Ngana *et al.,* 2020).

Carbapenemase production among the FGNB in our study was about 0.9%, which is more than twice the prevalence rate reported by the ECDC (0.4%). However, the difference in the prevalence of CPE might be due to different types of samples analysed (i.e., ECDC data are based exclusively on invasive isolates obtained from blood or cerebrospinal fluid) ((ECDC). 2019). On the other hand, CPE production among FGNB reported in the present study was much lower than that reported in other studies from various parts of the globe (Bassetti and Peghin, 2020; Gorgulho *et al.,* 2020). This difference in the prevalence of CPE in different studies might be due to various factors such as: improper use of carbapenem antibiotics in different geographic area, inadequate infection prevention and control programs, and inappropriate carbapenemase detection methods (Meletis, 2016; Gurung *et al.,* 2020; Hammoudi Halat and Ayoub Moubareck, 2020).

In our study, the ICU was the hospital unit with the highest isolation rate of MDR strains, ESBL, and carbapenemase-producing strains of FGNB, followed by the Medicine ward. The results of our study are consistent with those of previous studies in which length of ICU stay, excessive use of invasive medical devices, unsuccessful implementation of infection prevention and control programs, and overuse of antibiotics are considered the main risk factors for development of MDR, ESBL, and carbapenemase-producing strains (Hsueh *et al.,* 2002; Paskovaty *et al.,* 2005). Finally, our data indicate a prevalence of MDR, ESBL and carbapenemase-producing FGNB in antibiotic-treated compared with untreated subjects as a result of long-term exposure of bacteria to antimicrobial agents.

The findings of our study emphasize the importance of continuous monitoring and surveillance programmes, including at the local level, to prevent, detect and contain the spread of ESBL and carbapenemase among FGNB (Schrijver *et al.,* 2018). To our knowledge, there is little to no information regarding the prevalence of ESBL and carbapenemase-producing FGNB in a University Hospital in Southern Italy. These data also have potential clinical impact, since they highlight the importance of detecting the distribution of ESBL and carbapenemase-producers in different wards of hospitals. Therefore, strategies must be implemented to improve the rational use of antibiotics in the hospital and contain the increasing burden of drug resistance in bacterial strains.

Conflicts of interest

The authors declare no conflicts of interest.

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