ARTICLE ORIGINAL

Molecular profile of carbapenemase-producing *Enterobacterales* in burn patients

Profil moléculaire des *entérobactéries* productrices de carbapénèmases chez les brûlés

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RÉSUMÉ

Introduction: Les entérobactéries productrices de carbapénémases (EPC) représentent une menace pour la santé publique.
But: Déterminer la prévalence des EPC chez les patients hospitalisés au service de réanimation des brûlés du Centre de Traumatologie et des Grands Brûlés (CTGB), étudier leurs caractéristiques moléculaires et leurs résistances aux antibiotiques.
Méthodes: Il s'agit d'une étude rétrospective menée au laboratoire du CTGB entre juillet 2017 et décembre 2018. Tous les patients hospitalisés au service de réanimation des brûlés et infectés par des entérobactéries résistantes aux carbapénèmes étaient inclus. L'étude moléculaire était réalisée par PCR multiplex en temps réel de type GeneXpert® IV (Cepheid, Sunnyvale, USA) par le kit Xpert®Carba-R.
Résultats: Durant la période d'étude, parmi 574 entérobactéries, 64 souches (11,1%) étaient résistantes aux carbapénèmes dont 58 souches (90,6%) étaient productrices de carbapénémases. K. pneumoniae était la souche prédominante (n = 50) suivie par E. cloacae (n = 7), P. mirabilis (n = 3), E. aerogenes (n = 2), E. coli (n = 1) et P. rettgeri (n =1). Le gène blaNDM (58,6%) prédominait sur blaOXA48 (24,1%). Dix souches (17,3%) co-exprimaient ces deux gènes. Pour les 58 EPC, la résistance à l'ertapénème, à l'imipénème et au méropénem était de 100%, 18,4% et 36,2%, respectivement. Les taux de résistance les plus élevés étaient pour les céphalosporines de troisième génération (100%), la ciprofloxacine (95%) et la gentamicine (89,7%). La fosfomycine et la colistine avaient une activité conservée in vitro avec 5,2% et 4,8% de résistance, respectivement.
Conclusion: La prévalence élevée des EPC dans notre centre nécessite un dépistage continu et un renforcement d'hygiène.
Mots clés: Entérobactéries, carbapénémases, PCR, brûlés, Tunisie.

SUMMARY

Background: Carbapenemase-producing Enterobacterales (CPE) present a threat to public health worldwide.
Aim: To study their prevalence at the Trauma and Burn Center's Burn Unit and investigate their molecular characteristics and their associated antibiotics resistance patterns.
Methods: This is a retrospective study conducted at the Trauma and Burn Center's laboratory between july 2017 and december 2018. It included all patients hospitalized in the Trauma and Burn Center's Burn Unit infected with Enterobacterales resistant to carbapenems. The search of the carbapenemases genes was performed by PCR amplification GeneXpert® IV (Cepheid, Sunnyvale, CA, USA) by Xpert® Carba-R kit.
Results: During the study period, among 574 Enterobacterales, 64 strains (11.1%) were resistant to carbapenems, 58 strains (90.6%) of which were CPE. K. pneumoniae was the most predominant bacteria (n=50) fllowed by E. cloacae (n=7), P. mirabilis (n=3), E. aerogenes (n=2), E. coli (n=1) and P. rettgeri (n=1). The most common carbapenemase gene was blaNDM gene (58.6%) followed by blaOXA48 (24.1%). The co-existence of these two genes was identified in ten strains (17.3%). For the 58 CPE, resistance to ertapenem, imipenem and meropenem was 100%, 18.4% and 36.2%, respectively. The highest resistance rates were found to third-generation-cephalosporins (100%), ciprofloxacin (95%) and gentamicin (89.7%). Fosfomycin and colistin had the best susceptibility in vitro with only 5.2% and 4.8% of resistance, respectively.
Conclusion: The high prevalence of CPE in our center requires continued screening and reinforcement of hygiene measures.
Keywords: Enterobacterales, carbapenemase, PCR amplification, burns, Tunisia.

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INTRODUCTION

Burn patients are at high risk of infection [1]. This vulnerability is explained by the damage of the cutaneous barrier, a first line of defense against microbial invasion, the immunodepression caused by the burn injury, the length of the hospital stay, the invasive therapeutic procedures and the high pressure of antibiotics selection to which burn patients are subjected [2]. *Enterobacterales* constitute normal human intestinal flora. Once intestinal carriage is established, these opportunistic organisms can cause infections, especially in immunocompromised population, such as burn patients. Historically, these bacteria were susceptible to a wide range of antibiotics. During the past 25 years, multidrug-resistance has emerged and become widespread, especially carbapenem resistance in *Enterobacterales* (CRE) [3]. The spread of CRE presents devastating consequences for public health since there is no reliable treatment. Different mechanisms can explain this resistance, the most powerful ones are carbapemenases. The genes coding for these enzymes are carried by plasmids [4] that can carry resistance to other antimicrobial classes [5]. Since plasmids are readily transferred, these resistance genes can easily spread within species and even from species to species of *Enterobacterales.* In this context, it seems relevant for us to make a study, to determine the prevalence of CPE in the Trauma and Burn Center's Burn Unit and investigate their molecular characteristics and their associated antibiotics resistance patterns.

METHODS

This is a retrospective study conducted in the Trauma and Burn Center's Burn Unit from july 2017 to december 2018. All isolated strains of CRE in patients hospitalized in the Trauma and Burn Center's Burn Unit responsible for infection were collected. Strains isolated from rectal swabs were excluded. Samples were analyzed in the laboratory according to the "Référentiel En Microbiologie Médicale" (REMIC) [6]. Bacterial identification was based on morphologic, cultural and biochemical characteristics (Api systems, BioMérieux®).

Antimicrobial susceptibility testing was performed by the diffusion method on agar medium according to the CA-SFM standards, annually revised [7]. The minimal inhibitory concentrations (MIC) for ertapenem, meropenem, imipenem and tigecycline were determined by E-test method (BioMérieux®). The MIC for colistine were performed by using microdilution method (UMIC, Biocentric®). The results of MIC were interpreted according to the CA-SFM clinical breakpoints. The search for carbapenemases genes (bla_{VIM}, bla_{NDM}, bla_{IMP}, bla_{Oxa48} and bla_{KPC}) for our isolates was done by a multiplex real-time PCR GeneXpert® IV (Cepheid, Sunnyvale, CA, USA) by Xpert® Carba-R kit. The GeneXpert® system integrates sample preparation, DNA extraction, amplification and detection of target sequences, through ready-to-use, single-use cartridges. Xpert® Carba-R cartridges contain, in addition to the PCR buffer, DNA polymerase and two internal quality controls, the primers and probes which are able to detect the sequences hosting the genes encoding the five genes of carbapenemases. Absolute and relative frequencies were calculated for the qualitative variables. The evolution of CPE over time was studied by Spearman rank correlation coefficient (rs). For all statistical tests, the significance level (p) was set at 0.05.

RESULTS

During the study period, out of a total of 574 *Enterobacterales,* 64 CRE were recorded in 62 patients. The prevalence of CRE was 11.1%. Fifty-eight strains out of 64 CRE (90.6%) were producing of carbapenemases (Table 1). The other strains resistant to carbapenems (six out of 64) showed negative results in the multiplex PCR. The sex ratio was 1.9. The CPE were mainly isolated from intravascular catheters (34.5%), skin swab samples (31%), blood cultures (22.4%), urine samples (6.9%) and respiratory samples (sputum, protected tracheal sample) (5.2%). A statistically non-significant upward trend of the prevalence of CPE was observed in our center over time ($r_{s=}$0.35, p=0.1) (Figure 1). Many *Enterobacterales* species were identified among our isolates with dominance of *K. pneumoniae* (50/64, CRE= 78.1%, CPE=79.3%) flowed by *E. cloacae* (7/64, CRE=10.9%, CPE=8.7%), *P. mirabilis* (3/64, CRE=4.7%, CPE=5.2%), *E. aerogenes* (2/64, CRE=3.1%, CPE=3.4%), *E. coli* (1/64, CRE=1.6%, CPE=1.7%) and *P. rettgeri* (1/64, CRE=1.6%, CPE=1.7%) (Table 1).

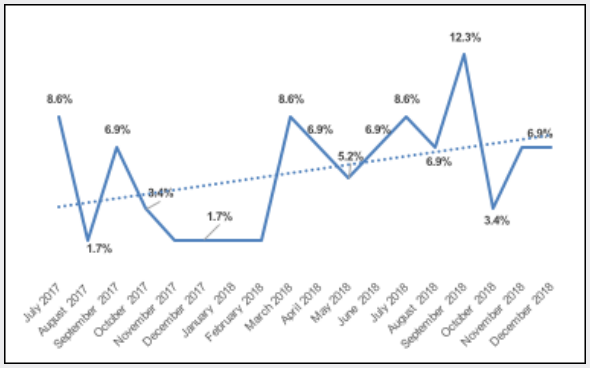

Figure 1. Monthly prevalence evolution of carbapenemase-producing- Enterobacterales

Table 1. Distribution of carbapenemase-producing-*Enterobacterales* among Carbapenems-resistant-strains

Strains	Carbapenems-resistant- *Enterobacterales*	Carbapenemase-producing- *Enterobacterales* (%)
K. pneumoniae	50	46 (79.3%)
E. cloacae	7	5 (8.7%)
P. mirabilis	3	3 (5.2%)
E. aerogenes	2	2 (3.4%)
E. coli	1	1 (1.7%)
P. rettgeri	1	1 (1.7%)
Total	**64**	**58**

Molecular study had shown that the most common carbapenemase gene was bla_{NDM} gene (58.6%) followed by bla_{OXA48} (24.1%). The co-existence of these two genes was identified in ten strains (17.3%). Among the 46 carbapenemase-producing-*K. pneumoniae,* 30 (65.2%) harboured bla_{NDM} gene, eight (17.4%) bla_{OXA48} and eight (17.4%) both genes. The *NDM*-producing-*K. pneumoniae* were not more resistant to carbapenems than those producing OXA_{48}. Indeed, the comparison of MIC values of imipenem and meropenem tested according to the type of carbapenemases (*NDM* or OXA_{48}) did not show a statistically significant difference (p = 0.34 for imipenem and p = 0.08 for meropenem). For *E. cloacae,* four strains carried the bla_{OXA48} gene only and one strain harboured bla_{OXA48} gene associated with bla_{NDM} gene. All strains of *P. mirabilis* had bla_{NDM} gene. For the two strains of *E. aerogenes,* one carried the bla_{NDM} gene and the other bla_{OXA48} gene. The strain of *E. coli* and that of *P. rettgeri* had bla_{OXA-48} gene and bla_{OXA48} gene associated with bla_{NDM} gene, respectively (Table 2).

Overall resistance to ertapenem, imipenem and meropenem for CRE was 100%, 18.5% and 34.4%, respectively. For CPE, 58 (100%) strains were resistant to ertapenem ((MIC ranged from 0.75 to 32 mg/L), nine (18.4%) to imipenem (MIC ranged from 2 to 32 mg/L) and 21 (36.2%) to meropenem (MIC ranged from 2 to 32 mg/L).

All CPE were resistant to penicillins, third-generation-cephalosporins and amoxicillin-clavulanic acid. Resistance rates of 100% to ticarcillin-clavulanic acid, 97.9% to piperacillin-tazobactam, 88.9% to aztreonam, and 86.1% to cefepime were recorded in CPE. Regarding aminoglycosides, resistance was high for tobramycin (95.7%), gentamicin (89.7%) and netilmicin (84.8%). Resistance to amikacin was observed in 100% of cases. For fluoroquinolones, resistance rates were 95% for ciprofloxacin, 96.5% for norfloxacin. The lowest resistance rates were recorded for tigecycline (44.8%), fosfomycin (5.2%). In addition, two strains were susceptible to colistin according to MICs (4.8%). Indeed, the two strains resistant to colistin were *K. pneumoniae.* One of them harboured bla_{NDM} gene and the other bla_{OXA48} gene.

Table 2. Distribution of carbapenemases encoding genes in carbapenemase-producing-*Enterobacterales*

Carbapemenases	%	*K. pneumoniae*	*E. cloacae*	*P. mirabilis*	*E. aerogenes*	*E. coli*	*P. rettgeri*	Total
bla_{NDM}	**58,6**	30	0	3	1	0	0	34
$bla_{OXA\ 48}$	**24,1**	8	4	0	1	1	0	14
bla_{NDM}+ $bla_{OXA\ 48}$	**17,3**	8	1	0	0	0	1	10
Total = 58	**100**	**46**	**5**	**3**	**2**	**1**	**1**	**58**

Globally, CPE were more resistant to antibiotics than carbapenemase free *Enterobacterales*. This difference was statistically significant for piperacillin-tazobactam, norfloxacin and tobramycin ($p < 0.05$).

DISCUSSION

Antibiotic resistance has become a major concern worldwide. In recent years, widespread outbreaks of CRE have been increasingly reported [8]. It differs from most other multidrug-resistant bacteria in that there is no reliable treatment. Two types of acquired resistance mechanisms to carbapenems have been identified in CRE: either a defect accumulation of antibiotic associated with the production of cephalosporinase and/or extended spectrum β-lactamases or production of a carbapenemase [9]. The genes coding for these enzyme are carried by plasmids that often carry other resistance.

In our study, we noticed a high prevalence of CPE among CRE (90.6%). This prevalence was similar to that reported by a study made in the Military Hospital of Tunis (81.6%) [10]. In our center the study of the monthly prevalence in CPE has shown a statistically non-significant upward trend over time ($r_s = 0.35$, $p=0.1$). This evolution was accordant with results posted by the European survey on CRE which insisted in the upward trend in four European countries (Greece, Italy, Malta, Turkey), which are facing a situation where CPE are endemic [11].

In Tunisia, few studies were interested in the prevalence of CPE, even less in intensive care units. Our study describes the CPE in the Trauma and Burn Center's Burn Unit and investigates their molecular characteristics and their associated antibiotics resistance patterns. However, a prospective study including a large sample size with a multicentric character is needed to better identify the CPE with its phenotypic and molecular aspects and to draw more relevant conclusions.

Even though CPE have been reported as a serious public health, it is in developing countries that the situation is more critical due to the lack of both national policies rationalizing antibiotic therapy and surveillance of antibiotic resistance [12,13]. In Tunisia, there are a limited number of studies, without any real mapping of the territorial dissemination of CPE. However, the absence of a national study could lead to an underestimation of the risk, a quick dissemination of CPE, a lack of adequate preventive measures and unexplained therapeutic failures.

The molecular method that we have adopted in our study for the detection of carbapenemase-producing strains is the most recommended one [14]. It has excellent sensitivity and specificity. The multiplex real-time PCR GeneXpert® IV (Cepheid, Sunnyvale, CA, USA) by Xpert® Carba-R kit can detect the main genes coding for carbapemenases: bla_{VIM}, bla_{NDM}, bla_{IMP}, bla_{Oxa48} and bla_{KPC}. It is an easy test that does not require qualified personnel.

In our work, *Klebsiella pneumoniae* was the most common CPE (79.3%) flowed by *E. cloacae* (8.7%), *P. mirabilis* (5.2%), *E. aerogenes* (3.4%), *E. coli* (1.7%) and *P. rettgeri* (1.7%). Our results were similar to those reported by studies made in the Military Hospital of Tunis [10] and in Sfax [15], showing that carabapenemase-producing *Klebsiella pneumoniae* was the most frequent species, isolated in 85.2% and 60.2%, respectively. At present, there are no data on the prevalence of EPC in intensive care units (ICUs) in Tunisia. However, the prevalence of *K. pneumoniae* resistant to ertapenem is 38.9% in Tunisian ICUs [16]. The fragility of patients, the length of the hospital stay, the presence of invasive devices and the overuse of antibiotic therapy explain this high prevalence of CRE especially in the ICUs [17].

In our study, molecular study had shown that the most common carbapenemase gene was bla_{NDM} gene (58.6%) followed by bla_{OXA48} gene (24.1%). The co-existence of these two genes was identified in ten strains (17.3%). The clear predominance of bla_{NDM} gene is not common under our skies. In Tunisia, the bla_{NDM} gene was first reported in 2013 [18]: an isolate of *K. pneumonia* that coharboured bla_{OXA48} and bla_{NDM} genes was isolated in a patient from Lybia. Recent tunisian studies showed the epidemic spread of bla_{NDM} gene at Sahloul Hospital in Sousse, Tahar Sfar Hospital in Mahdia (Center East of Tunisia) [19] and in Djerba (south-east of Tunisia) [20], that was associated with bla_{OXA48} gene in some cases. Bla_{OXA48} *gene* was first identified in Turkey, in 2003, in *K. pneumoniae* [21]. Indeed, bla_{OXA48} gene was considered to be the predominant carbapenemase gene in Tunisia [22]. The first OXA_{48} mediated carbapenem resistance in Tunisia was described in 2008 [23]. Because of the preferential location of bla_{OXA48} gene in north africa, Tunisia

is exposed to a high risk of their spread [24]. This contrast in results can be explained by a north-south gradient with a predominance of the *bla*$_{OXA48}$ gene in the north of Tunisia and the *bla*$_{NDM}$ gene in the south of Tunisia. Our center received patients from all over the country and even neighboring countries, which clarified our results. In our study*, K. pneumoniae* was the most frequent CPE (79.3%). The prevalence of carbapenemase production by strains of *K. pneumoniae* was 92%. Although not naturally resistant to antibiotics, since it produces only moderate amounts of chromosomal penicillinase, *K. pneumoniae* is a "collector" of plasmids carrying multiple resistances. The successive addition of genetic elements coding for resistance to aminoglycosides and β-lactams, combined with the rapid accumulation of chromosomal mutations conferring resistance to fluoroquinolones, has left carbapenems as the drugs of choice for the treatment of severe infections caused by *K. pneumoniae* [25]. *K. pneumoniae* remains the *Enterobacterales* in which most of carbapemenase genes have been identified [4].

In our study, the three strains of *P. mirabilis* had *bla*$_{NDM}$ gene. A study carried out in France found only one strain of *P. mirabilis* out of a total of 1075 CPE [26]. Our only isolated strain of *P. rettgeri* had *bla*$_{OXA48}$ gene associated with *bla*$_{NDM}$ gene. The production of carbapenemase by *P. mirabilis* and *P. rettgeri* is alarming as they have natural resistance to colistin and tigecyclin, which make therapeutic options limited [27]. One of our three strains of *P. mirabilis* was sensitive to a single antibiotic, aztreonam. This antibiotic has already been reported to be the only antibiotic active against *NDM*-producing-*P. mirabilis* [27].

CPE are generally resistant to most, if not all available antibiotics [28]. Our study confirmed 100% resistance to ertapenem, which is the most reliable indicator for the detection of this resistance. Antibiotic resistance rates were as follows: 18.4% to imipenem, 36.2% to meropenem, 100% third-generation-cephalosporins 97.9% to piperacillin-tazobactam, 89.7% to gentamicin and 95% to ciprofloxacin. These results were consistent with what was reported in a multicenter study carried out in 83 hospitals in Spain [29]. The lowest resistance rates of our CPE were recorded for tigecycline (44.8%), fosfomycin (5.2%) and colistin (4.8%). Indeed, *NDM*-producing-*Enterobacterales* are often only sensitive to two bactericidal antibiotics, colistin and fosfomycin, and to tigecyclin, a bacteriostatic antibiotic [30].

In our study, the two strains resistant to colistin were *K. pneumoniae.* One of them harboured *bla*$_{NDM}$ gene and the other *bla*$_{OXA48}$ *gene*. Indeed, colistin resistance in *K. pneumoniae* has been reported mostly together with carbapenem resistance [31]. The transposition of genes encoding carbapenemases, leading to disruption of the chromosomal *mgrB* gene, was reported as a source of resistance to colistin in *K. pneumonia* [31].

CONCLUSION

Resistance to carpabenems is rapidly spreading in many *Enterobacterales* species in our country. A high prevalence of CPE in the Trauma and Burn Center's Burn Unit with the predominance of *bla*$_{NDM}$ gene in more than half of the strains was observed. Therefore an early identification of CPE carriers and global national strategies are imperative, to establish adequate infection control precautions and stop outbreaks of these multidrug-resistant bacteria.

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