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Fernando Cobo[1]
Juan Antonio Reguera-Márquez[1]
José Antonio Marín-Rodríguez[2]
Francisco José Martín-Pérez[2]
Patricia Pérez-Palacios[3,4,5]
Esther Recacha[3,4,5]
José María Navarro-Marí[1]

A 5-year study of bloodstream infections caused by carbapenemase-producing Gram-negative bacilli in southern Spain

[1]Department of Microbiology and Instituto de Investigación Biosanitaria Ibs.GRANADA, University Hospital Virgen de las Nieves. Granada, Spain.
[2]Department of Preventive Medicine. University Hospital Virgen de las Nieves. Granada, Spain.
[3]Unidad Clínica de Enfermedades Infecciosas y Microbiología, Hospital Universitario Virgen Macarena, Seville, Spain.
[4]Instituto de Biomedicina de Sevilla IBIS, Hospital Universitario Virgen Macarena/CSIC/Universidad de Sevilla, Sevilla, Spain.
[5]Centro de Investigación Biomédica en Red en Enfermedades Infecciosas (CIBERINFEC), Instituto de Salud Carlos III, Madrid, Spain

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ABSTRACT

Introduction. The aim of this study was to evaluate the microbiological epidemiology of carbapenemase-producing Gram-negative bacilli (CPGNB) isolated from blood during a 5-year period.

Methods. A total of 80 isolates from 78 patients were finally included; fifty-five (70.5%) were men and the mean age was 60 years. Detection of carbapenemase production was performed by immunocromatography (IC) and polymerase chain reaction (PCR). Genotyping was carried-out by pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST), and characterization of carbapenemase-producing isolates was performed by whole genome sequencing (WGS).

Results. The main microorganisms isolated were *K. pneumoniae* (29.4%), *E. cloacae* (28.2%), *A. baumannii* (17.9%) and *P. aeruginosa* (15.3%). Overall, the most common carbapenemase in Enterobacterales was OXA-48 group (57.7%). The most common carbapenemase in non-fermenting bacilli was OXA-23 (60.8%). The most common ST in *K. pneumoniae* producing OXA-48 types was ST45 and in *E. cloacae* ST114, while in *E. cloacae* producing VIM types was ST78. In OXA-23 types, the most common clone in *A. baumannii* was ST2, whereas in *P. aeruginosa* producing IMP types *was* ST253.

Conclusions. There was an increase in cases recorded in the years of highest incidence and severity of the SARS-CoV-2 pandemic, with a significant number of cases in patients admitted to the ICU. All bacteremias caused by A. baumannii were caused by the same clone, and 12 of the 14 cases caused by A. baumannii were part of outbreaks in the ICU.

Keywords: Carbapenemase; Enterobacterales; non-fermenting bacilli; OXA-48 type; OXA-23 type; ST-clones

Correspondece:
Dr. Fernando Cobo
Department of Microbiology and Instituto de Investigación Biosanitaria Ibs.GRANADA
University Hospital Virgen de las Nieves, Avda Fuerzas Armadas, 2. 18014 Granada, Spain
E-mail: fernando.cobo.sspa@juntadeandalucia.es

Estudio de 5 años de infecciones del torrente sanguíneo producidas por bacilos gramnegativos productores de carbapenemasas en el sur de España

RESUMEN

Introducción. El objetivo de este estudio fue evaluar la epidemiología microbiológica de los bacilos Gram negativos productores de carbapenemasas (CPGNB) aislados de sangre durante un período de 5 años.

Métodos. Se incluyeron finalmente 80 aislamientos de 78 pacientes; cincuenta y cinco (70,5%) eran hombres y la edad media fue de 60 años. La detección de la producción de carbapenemasas se realizó mediante inmunocromatografía (IC) y reacción en cadena de la polimerasa (PCR). El genotipado se llevó a cabo mediante electroforesis en gel de campo pulsado (PFGE) y tipificación de secuencia multilocus (MLST), y la caracterización de los aislados productores de carbapenemasas se realizó mediante secuenciación del genoma completo (WGS).

Resultados. Los principales microorganismos aislados fueron *K. pneumoniae* (29,4%), *E. cloacae* (28,2%), *A. baumannii* (17,9%) y *P. aeruginosa* (15,3%). En general, la carbapenemasa más común en Enterobacterales fue el grupo OXA-48 (57,7%). La carbapenemasa más común en los bacilos no fermentadores fue la OXA-23 (60,8%). El ST más común en *K. pneumoniae* que produce tipos OXA-48 fue ST45 y en *E. cloacae* ST114, mientras que en *E. cloacae* que produce tipos VIM fue ST78. En los tipos OXA-23, el clon más común en *A. baumannii* fue ST2, mientras que en *P. aeruginosa* tipo IMP fue ST253.

Conclusiones. Hubo un incremento de casos registrados en los años de mayor incidencia y gravedad de la pandemia por SARS-CoV-2, con un número importante de casos en pacientes ingresados en UCI. Todas las bacteriemias producidas por *A. baumannii* lo fueron por el mismo clon, y 12 de los 14 casos producidos por *A. baumannii* formaron parte de brotes en la UCI.

Palabras clave: Carbapenemasa; Enterobacterales; bacilos no fermentadores; OXA-48 type; OXA-23 type; clones ST

INTRODUCTION

The increasing prevalence of carbapenemase-producing gram-negative bacilli (CPGNB) is a major public health threat worldwide, causing significant morbidity and mortality [1]. This trend is particularly, but not limited, to Enterobacterales, especially *Klebsiella pneumoniae* and extensively drug-resistant strains of *Pseudomonas aeruginosa*. In addition, the increasing prevalence of nosocomial infections caused by multidrug-resistant bacteria complicates the choice of appropriate treatment [2].

The importance of monitoring carbapenem-resistant gram-negative bacteria is due to their potential for widespread transmission of resistance through mobile genetic elements. In fact, WHO considers these microorganisms as a priority 1 (critical) in the list of antibiotic-resistant "priority pathogens", that responds to urgent public health needs (https://who.int). Particularly serious are the bloodstream infections caused by these bacteria due to their potential higher risk of morbidity and mortality. Mortality and clinical outcomes associated with carbapenem-resistant gram-negative bacteria can be severe. It is important to understand factors that may help guide earlier detection and therefore earlier treatment of infections caused by these pathogens [3,4].

Moreover, the economic impact could be higher than the annual cost of many chronic diseases and many acute diseases. Costs rise proportionally with the incidence of these infections, so prevention and control strategies should be considered.

Currently, carbapenemases are classified into three groups and the most frequently isolated enzymes are as follows: class A enzymes that includes KPC, class B metallo-β-lactamases (MBLs) represented by the groups VIM, IMP and NDM, and class D enzymes such as OXA-48-like [3]. In European countries, current data on these enzymes are diverse and the distribution of different types of carbapenemases varies in different countries of this world region [4]. In a recent report of carbapenem-resistant *K. pneumoniae* in Europe, KPC was predominant in some countries such as Italy, Greece, Portugal and Israel, while OXA-48 was the main carbapenemase enzyme in Romania, Turkey and Spain [5]. OXA-48 is by far the most common carbapenemase in *K. pneumoniae* circulating in Spain, followed by MBLs and KPCs [6]. Regarding carbapenemase-producing *P. aeruginosa* in Spain, the prevalence was low, accounting for approximately 0.4% of carbapenem-resistant isolates [7]. Due to the great diversity, it is important to know the patterns of CPGNBs especially in bloodstream infections, because early detection may allow timely approaches to adopt preventive measures and controlling possible outbreaks. This manuscript addresses the assessment of the burden of CPGNBs isolated from blood in our hospital over a 5-year period.

METHODS

Setting and demographic data. This study was conducted in the Department of Microbiology of the University Hospital Virgen de las Nieves located in Granada, southeastern Spain. This center is a tertiary care facility with approximately 1,100 beds and serves as a primary care facility for nearly 550,000 inhabitants. Only samples obtained from blood of hospitalized patients were included and analyzed. Data about age, sex, ward of admission, infections in other location, colonization before or after the infection, risk factors for carbapenemase acquisition, presence of septic shock, antimicrobial treatment applied, and outcome were recorded.

Bacterial isolates: identification and antimicrobial susceptibility testing. Eighty CPGNBs isolates, from January 2017 to February 2022, were included from 78 patients with bacteremia due to CPGNB. All isolates were initially identified by MALDI-TOF MS (Bruker Biotyper, Bellerica, MA, USA) according to the manufacturer's recommendations. Antimicrobial susceptibility was determined using the panel N96 of MicroScan WalkAway system (Beckman Coulter, USA). The isolates with a MIC to meropenem > 0,12 mg/L were initially selected for the study. Phenotypic detection of carbapenemase production was performed using the βCarba assay (BioRad, Spain) and commercial combined disk test (Rosco Diagnostica A/S, Denmark), containing meropenem and inhibitors (phenyl-boronic acid, dipicolinic acid and cloxacillin) as well as a disk of temocillin for Enterobacterales, and imipenem and inhibitors (phenyl-boronic acid, dipicolinic acid, EDTA and cloxacillin) for *P. aeruginosa*. The NG test Carba5 (NG Biotech, Guipry, France) was used to detect the carbapenemase group (KPC, VIM, IMP, NDM and OXA-48). All isolates were sent to the Reference Laboratory of Andalusia (located in Hospital Universitario Virgen Macarena, Seville, Spain) for phenotypic and molecular characterization, as part of PIRASOA programme, an antimicrobial stewardship programme. The PIRASOA programme was developed to prevent and control healthcare-associated infections and promote the appropriate use of antimicrobials throughout the Andalusian public health

Pulsed-field electrophoresis (PFGE). Genotyping was assessed by pulsed field gel electrophoresis (PFGE) after digestion of total chromosomal DNA with *XbaI* (Enterobacterales), *SpeI* (*Pseudomonas* spp) and *ApaI* (*Acinetobacter* spp.). DNA fragments were separated using a CHIEF DR-II system (Biorad, Madrid, Spain). Normalization strain used were *E. coli* ATCC25922 for *K. pneumoniae*, *Salmonella* braenderup for the other Enterobacterales species and *P.aeruginosa* ATCC27853 for *Pseudomonas* spp. Banding patterns of the gel were analyzed using Bionumerics 8.1 software (AppliedMaths, Austin, TX, USA) using Dice's coefficient (1%) to measure the genetic similarity between the isolates.

Molecular detection of carbapenemase and MLST. The presence of carbapenemases (NDM, VIM, KPC, IMP and OXA-48 types) was assessed by PCR with gene-specific primers and direct sequencing by Sanger until 2017. From October 2017, molecular detection and identification of carbapenemase genes was performed using whole genome sequencing (WGS) with MiSeq system (Illumina®, San Diego, CA, USA). DNA library was prepared

Table 1 Demographic data of cases of bacteremia with carbapenemase-producing Gram-negative bacilli (2017-2022).

Demographic data	n	%
Average age (years)	60	
Sex		
Men	55	70.5
Women	23	29.5
Ward of admission		
Medical ward	43	55.1
Surgical ward	3	3.8
Intensive care unit	32	41
Microorganisms		
K. pneumoniae	23	29.5
E. cloacae	22	28.2
A. baumannii	14	17.9
P. aeruginosa	12	15.3
E. coli	3	3.8
P. putida	2	2.5
K. oxytoca	1	1.2
C. freundii	1	1.2
Colonized patients		
Before infection	14	17.9
After infection	10	12.8
Without colonization	54	69.2
Septic shock		
No	55	70.5
Yes	23	29.5
Risk factors for infection		
Use of invasive procedures	47	60.2
ICU admission	39	50
Immunosuppression	35	44.8
Other (surgery, long antibiotic treatment)	7	8.9
Infection in other location	35	44.8
Empirical therapy*		
Correct	52	66.6
Incorrect	26	33.3
Outcome		
Successful	42	53.8
Death	36	46.1

*** Empirical therapy was considered correct when the antibiotic applied was susceptible to this pathogen.**

with the Nextera DNA flex (Illumina®). Raw reads were assembled *de novo* using the CLC Genomic Workbench v9 (Qiagen). Antimicrobial resistance genes were determined using ResFinder4.1 database [8-10]. MLST was determined by PCR and sequencing or by WGS data (from 2017) using MLSTfinder 2.0 [11, 12].

Outbreaks' study. During the period of study, the outbreaks declared were analyzed. An outbreak was considered when two or more cases of an infection were caused by the same microorganism with a time-space association and suspicion of an epidemiologic link [13]. Initially, isolates of the same species with similar resistance pattern were studied with different molecular techniques to consider them belonging to an outbreak.

RESULTS

Demographic data. Seventy-eight patients with bacteremia due to CPGNB were finally included in the study; 55 (70.5%) isolates belonged to male patients with an average age of 60 years (range 19-94). Most of samples came from medical units (n=43; 55.1%) and intensive care unit (ICU) (n=32; 41%). Moreover, 35 (44.8%) patients had an infection for the same microorganism in other location. Twenty-three (29.5%) patients had septic shock and 36 (46.1%) patients died as a consequence of this infection. The main risk factor for infection due to microorganisms carbapenemase-producing was the use of invasive procedures (n=47; 60.2%) followed by ICU admission (n=39; 50%). An incorrect empirical therapy was applied in 26 (33.3%) patients and in 17 (65.3%) of them the outcome was unsuccessful (Table 1).

Characteristics of carbapenemase-producing isolates. During the period of study, a total of 80 strains were included in the study (two patients had one isolate producing two types of carbapenemases each one). From these isolates, Enterobacterales represented 64% (n=52) of them while non-fermenting Gram-negative bacilli represented 36% (n=28). Among Enterobacterales, *K. pneumoniae* was the microorganism most common isolated (n=23; 29.4%), followed by *E. cloacae* (n=22; 28.2%). Among non-fermenting Gram-negative bacilli, *A. baumannii* was the bacterium most common isolated (n=14; 17.9%) followed by *P. aeruginosa* (n=12; 15.3%) (Table 1). Overall, the most common carbapenemases detected were OXA-48 (n=26; 32.5%) and VIM (n=25; 31.2%), followed by OXA-23 (n=14; 17.5%). In Enterobacterales, regarding to the prevalence through the years, VIM-like had the highest prevalence in 2017 (8 cases), meanwhile OXA-48-like had the highest prevalence in 2020 (12 cases) and OXA-23 had the highest prevalence in 2021 (11 cases). The carbapenemase less frequent through all period of study was NDM-like (only 2 cases). In non-fermenting Gram-negative bacilli, the most common carbapenemase was OXA-23 (n=14; 17.5%) followed by IMP-like (n=6; 26.1%). Only in 9 isolates, co-production of an additional associated resistance mechanism was detected, especially penicillinases in 8 strains of *K. pneumoniae* (TEM-1). One isolate of *K. pneumoniae* harbored two carbapenemases: OXA-48 and NDM-5, and other isolate of *C. freundii* produced also two carbapenemases: OXA-48 and VIM-63 (Table 3).

Regarding to clonal lineages of carbapenemase-producing Enterobacterales, *K. pneumoniae* ST45/OXA-48 and *E. cloacae* ST114/OXA-48 were the most prevalent (n= 6, each one),

Table 2 Types of carbapenemases in Gram-negative bacilli causing bacteremia (2017-2022).

Type of carbapenemase	2017	2018	2019	2020	2021	2022	TOTAL
VIM	8	4	4	3	5	1	25
OXA-48	-	-	1	12	10	3	26
OXA-23	-	-	1	2	11	-	14
IMP	-	5	1	1	2	-	9
KPC	3	-	-	-	1	-	4
NDM	-	-	1	1	-	-	2
TOTAL	11	9	8	19	29	4	80

followed by *E. cloacae* ST78/VIM-1 (n=5). Moreover, two isolates of *K. pneumoniae* ST258/KPC-1 and KPC-3 were detected. In non-fermenting Gram-negative bacilli, the most frequent variant was *A. baumannii* ST2/OXA-23 (n=14) followed by *P. aeruginosa* ST253/IMP-16 (n=5) (Table 4).

Outbreaks detected. The data obtained through PFGE allowed us to establish which strains were part of the different outbreaks. During the period of study, four outbreaks were declared; the first of them was caused by *P. aeruginosa* ST253/IMP-16 in March 2018 in the ICU (three cases); the other three outbreaks were caused by *A. baumannii* ST2/OXA-23 in the ICU in December/January 2020, at the beginning of February 2021 and at the end of March and beginning of April 2021 (four cases each one).

DISCUSSION

This is a report of the bacteremias due to CPGNB isolated in a tertiary-care hospital in southeast Spain in a 5-year period. It was performed from routine testing of 80 Gram-negative bacilli isolates belonging to Enterobacterales and non-fermenting Gram-negative bacilli species isolated from blood cultures during this period.

CPGNB represent a global threat to healthcare systems, especially when they are causing severe infections such as bloodstream infection. Regarding to the prevalence of CPGNB, there is currently considerable heterogeneity across different countries and regions. Several surveillance studies focused on epidemiological situation of CPGNB are available in the scientific literature and, recently, a report about the epidemiological status in carbapenemase-producing Enterobacterales in 37 European countries has been recently published [1]. Overall, four countries such as Greece, Italy, Malta and Turkey were in "endemic" situation and other countries, including Spain, were in an "inter-regional spread" stage. The result of this report indicates that 11 countries reported a worsened epidemiological situation compared with the previous report [5]. These trends are highlighted in Enterobacterales, especially in *K. pneumoniae*; in these bacteria, carbapenemases are the main contributing factor to extensive drug resistance.

Regarding to the type of carbapenemase, different reports showed different distribution in Europe [3]; while KPC-like is predominant in Italy, Greece, Portugal and Israel, VIM-like is more frequent in Hungary and OXA-48-like in the main carbapenemase in Romania, Turkey and Spain [3].

From the point of view of CPGNB that cause bacteremia, there are some studies reporting this item. A multicenter study conducted in the USA demonstrated that the majority of cases were due to *K. pneumoniae* KPC-2 and KPC-3, and the ST258 predominated among KPC-producing *K. pneumoniae* [14]. Other study showed that in cases of bacteremia caused by carbapenemase-producing *K. pneumoniae* (CPKP), 61.1% of these bacteria were KPC [15]. In Korea, in 131 cases of bacteremia due to carbapenemase-producing Enterobacterales, 69% were caused by *K. pneumoniae*, followed by *Enterobacter* spp (10%) and *E coli* (8%); regarding to the type of carbapenemase, KPC was the most commonly observed (66%) followed by NDM (20%) [16]. Moreover, other study stated that from 146 carbapenem-resistant *K. pneumoniae* strains, KPC-2 was the main mechanism of carbapenem resistance (n=127) [17]. Finally, a report recently published of bacteremia with carbapenemase-producing Enterobacterales in immunocompromised patients previously colonized, 12.7% (28 from 221) developed bloodstream infections of carbapenemase-producing Enterobacterales [18]. Most of these infections were caused by *K. pneumoniae*, and KPC was the most frequent type of carbapenemase, followed by NDM.

A multicenter study of CPKP and carbapenemase-producing *E. coli* conducted in Spain in 2019, revealed that CPKP was the most frequent carbapenemase-producing bacteria found in blood (50 out of 52 isolates), although in this work only 52 isolates were collected from bacteremia [19]. The main limitation of this study to know the true prevalence of CPGNB in blood is that only isolates of *K. pneumoniae* and *E. coli* were included. Regarding to the type of carbapenemase, almost 70% of isolates expressed OXA-48, although no data of carbapenemases from type of samples were reported. However, in this study, the distribution of carbapenemase-producing

Table 3 **Main variants and clonal lineages of carbapenemase-producing Enterobacterales (n=45).**

Carbapenemase (n/%)	Variants (n)	Species and clones (n)
KPC (2/4.4)	KPC-1 (1)	*K. pneumoniae*, ST258/KPC-1 (1)
	KPC-3 (1)	*K. pneumoniae*, ST258/KPC-3 (1)
VIM (13/28.8)	VIM-1 (12)	*K. pneumoniae*, ST25/VIM-1 (1)
		K. pneumoniae, ST469/VIM-1 (1)
		E. cloacae, ST78/VIM-1 (5)
		E. cloacae, ST111/VIM-1 (1)
		E. cloacae, ST90/VIM-1 (2)
		E. cloacae, ST311/VIM-1 (1)
		K. oxytoca, ST36/VIM-1 (1)
	VIM-63 (1)	*E. cloacae*, ST22/VIM-63 (1)
OXA (24/53.3)	OXA-48 (24)	*K. pneumoniae*, ST307/OXA-48 (4)
		K. pneumoniae, ST45/OXA-48 (6)
		K. pneumoniae, ST405/OXA-48 (2)
		K. pneumoniae, ST6446/OXA-48 (1)
		E. cloacae, ST114/OXA-48 (6)
		E. cloacae, ST307/OXA-48 (1)
		E. cloacae, ST110/OXA-48 (1)
		E. coli, ST405/OXA-48 (2)
		E. coli, ST307/OXA-48 (1)
NDM (2/4.4)	NDM-5 (2)	*K. pneumoniae*, ST15/NDM-5 (2)
NDM + OXA (2/4.4)	NDM-5 + OXA-48	*K. pneumoniae*
		ST15/NDM
		ST307/OXA-48
VIM + OXA (2/4.4)	VIM-63 + OXA-48	*C. freundii*
		ST22/VIM-63
		ST405/OXA-48

bacteria was different depending on the geographical location; founding in southern Spain more KPC-producing isolates. Moreover, in this report 8 high-risk CPKP clones were detected, especially ST307/OXA-48 (16.4%) and ST11/OXA-48 (16.4%), although in bacteremias ST512-258/KPC and ST15/OXA-48-like were the most frequent bacteremia-producing clones (24 and 16%, respectively). In our study, *K. pneumoniae* was also the most frequent carbapenemase-producing isolated bacteria (29.5%) followed by *E. cloacae* (28.2%) and *A. baumannii* (17.9%). However, the most frequent type of carbapenemase was OXA-48, followed by VIM-like, and, in *A. baumannii*, the main type of carbapenemase was OXA-23. These data contrast with those previously mentioned, and show the differences between countries or regions respect to the prevalence of carbapenemases in bloodstream infections and the different measures to be taken to prevent its spread, reinforcing the need for epidemiological control at regional level.

Respect to the high-risk bacteremia-producing clones, in our study the most frequent association between high-risk clone and carbapenemase-producing *K. pneumoniae* was ST307/OXA-48 (4 cases), followed by *K. pneumoniae* ST258/KPC-1 (1 case).

Regarding to non-fermenting Gram-negative bacilli (NF-GNB), data about carbapenemase-producing NFGNB in bloodstream infections are scarce. An Italian nationwide survey on *P. aeruginosa* from invasive infections included 935 clinical isolates from bloodstream infections and lower respiratory tract infections collected 20 centres between 2014 and 2023; they showed that 12 and 32 isolates were positive for carbapenemase types VIM and IMP, respectively [20]. However, the authors do not specify the type of samples for each group of carbapenemases. In *A. baumannii*, a study found that the acquired carbapenemase detected most frequently was OXA-23,

Table 4 Main variants and clonal lineages of carbapenemase-producing non Enterobacterales (n=23).

Carbapenemase (n/%)	Variants (n)	Species and clones (n)
IMP (6/26.1)	IMP-16 (5)	*P. aeruginosa*, ST253/IMP-16 (5)
	IMP-23 (1)	*P. aeruginosa*, ST175/IMP-23 (1)
VIM (3/13.1)	VIM-1 (2)	*P. aeruginosa*, ST115/VIM-1 (1)
		P. putida, ST78/VIM-1 (1)
	VIM-2 (1)	*P. aeruginosa*, ST277/VIM-2 (1)
OXA (14/60.8)	OXA-23 (14)	*A. baumannii*, ST2/OXA-23 (14)

which was present in 69% of the isolates, but the type of sample was not specified either [21].

A Spanish nationwide survey on *P. aeruginosa* showed that 2.1% of isolates produced carbapenemases, especially GES type; bloodstream samples only represented 5.9% of all isolated tested, but no more details were shown [7]. However, in our bloodstream isolates, the main type of carbapenemase was IMP-like, particularly IMP-16, and all strains belonged to ST253 clone (Table 4). Respect to this, three of five cases belonged to the same outbreak. These data demonstrated that epidemiological studies should be performed in all health areas, in order to know the differences in the prevalence of carbapenemases in each region.

The rapid detection of carbapenemases is an important factor in order to establish a rapid and adequate empirical therapy, especially in bloodstream infections and other severe infections. In our patients, an incorrect empirical therapy was applied in 26 (33.3%) patients and in 17 (65.3%) of them the outcome was unsuccessful. Thus, taking into account the epidemiology of carbapenemase-producing Gram-negative bacilli in our area, an improvement in the initiation of empirical treatment should occur in patients with risk factors for acquiring carbapenemases, although this improvement may be due to a rapid microbiological diagnosis.

In our study, the great increase in cases registered during the years 2020 and 2021 is striking, which were the years with the highest incidence and severity of the pandemic produced by SARS-CoV-2. It is also striking that a very significant number of cases occurred in patients admitted to the ICU, a service that was especially pressured during this period. All bacteremias caused by *A. baumannii* were caused by the same clone and occurred in these three years (2019, 1 case and 2020-21, thirteen cases). Twelve of the fourteen cases caused by *A. baumannii* were from outbreaks (two or three) that occurred in the ICU in 2020 and 2021.

The main limitations of the present study is that the data are limited to one health area; further studies including a great number of hospitals will be necessary to corroborate these data.

In summary, respect to the prevalence of CPGNB, our results add information to several studies published last years in Spain and in other countries, emphasizing the need for epidemiological surveillance both at regional and local level. A rapid detection of carbapenemases in bloodstream infections can help physicians to establish the best therapeutic strategy as well as to implement control measures to prevent the spread of these strains in order to reduce the mortality of these patients.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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