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Short Communication

Prevalence and molecular characterisation of carbapenemase-producing Enterobacterales in an outbreak-free setting in a single hospital in Uruguay

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ABSTRACT

Objectives: This study aimed to characterise all carbapenemase-producing enterobacteria (CPE) isolates obtained from an outbreak-free setting in Uruguay.
Methods: We studied 12 CPE isolated from Hospital de Clínicas between 2012–2016. Bacterial identification and antibiotic susceptibility testing were performed using VITEK® 2 and Sensititre or agar dilution, respectively. Antimicrobial resistance genes and mobile genetic elements were identified by PCR and sequencing. Multilocus sequence typing was performed for *Klebsiella pneumoniae*. Plasmid conjugation was assessed, plasmid size was estimated by *S1*-PFGE and plasmid incompatibility groups were sought by PCR.
Results: Among 8364 enterobacteria, 12 CPE were isolated from urine, blood culture, wound, peritoneal fluid and punch samples. NDM-1 was the most prevalent carbapenemase, followed by VIM-2 and KPC-2. All isolates were resistant to gentamicin, cefotaxime, ceftazidime, trimethoprim/sulfamethoxazole, ciprofloxacin and imipenem and were susceptible to fosfomycin. We characterised six class 1 integrons: *dfrA12–orfF–aadA2*; *aacA4–bla*$_{OXA-2}$*–orfD*; *aadB–aadA2*; *dfrA1*; *aadB–bla*$_{OXA-10}$*–aadA1*; and *bla*$_{VIM-2}$*-dfrA7*. An association between various aminoglycoside, β-lactam and fluoroquinolone resistance genes were observed, some of them located in transferable plasmids belonging to incompatibility groups IncC, IncHI1 and IncM1. We described a new composite transposon (assigned Tn*6935*) including *bla*$_{NDM-1}$ flanked by two directly-oriented copies of a Tn*3*-like element IS*Kox2*-like family transposase. The sequence types of *K. pneumoniae* isolates were ST11, ST14 and ST661.
Conclusions: The presence of CPE is sporadic and could be due to measures taken by the Public Health Committee. Nevertheless, the coexistence of several resistance mechanisms and their presence in conjugative plasmids and high-risk clones is worrisome.

1. Introduction

Global dissemination of carbapenemase-producing enterobacteria (CPE) is a well-known problem. The World Health Organization (WHO) has classified CPE as a critical pathogen in its priority list of antibiotic-resistant bacteria for which there is an urgent need to develop new antimicrobial agents [1]. CPE are frequently associated with numerous resistance mechanisms such as extended-spectrum β-lactamases (ESBLs), methylases and plasmid-mediated quinolone resistance (PMQR) genes, and therefore are often multidrug-resistant, extensively drug-resistant or pandrug-resistant [2]. This association has become a great challenge from a therapeutic point of view since it restricts available antimicrobial options. Detection of CPE is also a burden for infection control teams since they are usually hard to remove from the hospital environment and humans owing to their persistence in the intestine, which may last up to a month [3,4].

The presence of carbapenemase-encoding genes either on conjugative plasmids or mobilisable platforms such as class 1 integrons and transposons, or in high-risk international clones favours their dissemination [3].

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In Latin America, the most frequent carbapenemases reported in enterobacteria are KPC-2, KPC-3 and NDM-1, each of them prevailing in different countries in the region [4].

The aim of this work was to describe the epidemiology and to characterise all of the CPE obtained from clinical isolates of patients treated in Hospital de Clínicas (Montevideo, Uruguay) from January 2012 to December 2016.

2. Materials and methods

2.1. Study setting

Isolates were obtained from Hospital de Clínicas 'Dr Manuel Quintela' clinical microbiology laboratory between January 2012 and December 2016. The hospital is a 250-bed teaching institution presenting approximately 8000 annual discharges.

2.2. Bacterial strains, antibiotic susceptibility testing and carbapenemase resistance screening

Bacterial identification was performed using a VITEK®2 Compact System (bioMérieux, Marcy-l'Étoile, France). Antibiotic susceptibility testing was performed using Sensititre™ (Thermo Fisher Scientific) for all available antibiotics, except for fosfomycin that was determined by agar dilution.

Carbapenemase production was suspected according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) algorithm. Phenotypic methods such as Rosco KPC/MBL confirmation kit and/or double disk synergy test with ethylene diamine tetra-acetic acid (EDTA) and boronic acid were used to confirm carbapenemase production. Susceptibility results were interpreted according to EUCAST breakpoints (http://www.eucast.org) [5].

2.3. Resistance mechanisms detection

Molecular confirmation of carbapenemases (KPC, VIM, IMP, NDM, GES and OXA-48) was performed by multiplex real-time PCR according to Monteiro et al. [6]. Carbapenemase genes were sequenced with the following primers: KPC-2F (5'-AACAAGGAATATCGTTGATG-3') and KPC-2R (5'-AGATGATTTTCAGAGCCTTA-3'); VIM-F (5′-TAGGAATTCACCATGTTCAAACTTTTGAGTAAGT-3′) and VIM-R (5′-ATAAAGCTTAGCTACTCAACGACTGAGCGA-3′); NDM-1F (5′-GGTTTGGCGATCTGGTTTTC-3′); and NDM-1R (5'-CGGAATGGCTCATCACGATC-3') [7]. Additionally, PMQR genes [*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrE*, *qnrS*, *qnrVC* and *aac(6′)-Ib*-cr], ESBL genes ($bla_{\text{CTX-M}}$ group, $bla_{\text{PER-1, -2}}$, bla_{TEM} and bla_{SHV}), 16S rRNA methyltransferase genes (*armA*, *npmA* and *rmtA–D*), class 1 and 2 integrons, variable regions and insertion sequence IS*CR1* were searched by PCR and sequencing [8,9]. Primers used for amplification and sequencing of *qnrE* were QnrE1-F (5′-GGCATTGATTTTTGAAGGCGA-3′) and QnrE1-R (5′-GTGGGTAAAATTGGCCGCTC-3′).

2.4. Multilocus sequence typing (MLST)

MLST of *Klebsiella pneumoniae* isolates was performed following the guidelines in the MLST database (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html).

2.5. Conjugation assays and plasmid characterisation

Conjugation assays were carried out using *Escherichia coli* J53-2 (rifampicin-resistant) as recipient strain. Transconjugants were selected on Luria–Bertani agar plates supplemented with 2 mg/L ceftazidime and 150 mg/L rifampicin. Plasmid size was estimated by treatment with *S1* nuclease followed by pulse-field gel electrophoresis (PFGE). Additionally, plasmid incompatibility (Inc) groups were determined for all transconjugant strains. To differentiate the incompatibility groups IncA/C and IncL/M, we performed PCR using the primers RepAF/R and M-L/M employed by Hancock et al. and Carattoli et al., respectively [9,10]. We then sequenced and compared the obtained sequences with the database http://www.genomicepidemiology.org [9–12].

2.6. Plasmid sequencing and annotation

Plasmid DNA of *Citrobacter freundii* HC2 was extracted from its transconjugant using a QIAGEN® Large-Construct Kit following the manufacturer's instructions. Whole-genome sequencing of plasmid DNA was performed using an Ion Torrent PGM Sequencer (Life Technologies, Carlsbad, CA, USA) with an Ion 314 Chip (Life Technologies). The generated data were analysed using the Torrent Server (Torrent Suite 3.2.1) and reads were assembled using SPAdes 3.1.

Contigs were compared with the GenBank database (BLASTn) to inspect those containing $bla_{\text{NDM-1}}$ and were manually reassembled using Vector NTI Advance 11.0 (Invitrogen). To confirm the assembly and gap filling, a PCR and Sanger sequencing (Institut Pasteur, Montevideo, Uruguay) approach was performed, using primers designed in this work and NDM-1F/R [7] (Supplementary Table).

3. Results and discussion

3.1. Bacterial strains and antimicrobial susceptibility testing

We studied 8364 non-duplicate enterobacteria (Enterobacteriaceae and Morganellaceae) clinical strains isolated between 2012–2016. Of the 8364 strains isolated during this period, 46% were *E. coli*, 30% *K. pneumoniae*, 7% *Proteus mirabilis*, 6.5% *Enterobacter cloacae*, 3% *Morganella morganii*, 2% *Serratia marcescens*, 1% *Proteus* spp. and *Providencia* spp. and 4.5% other species non-colistin resistant.

Of the 8364 isolates, 12 (0.14%) displayed a positive carbapenemase production confirmation test. Characteristics of carbapenemase-positive strains are shown in Table 1. There was no statistical difference in annual CPE prevalence (data not shown).

All CPE were obtained from different samples and comprised various species of Enterobacterales (i.e. Enterobacteriaceae and Morganellaceae). None of them was observed during an outbreak or presented epidemiological relatedness.

As expected, most CPE were of nosocomial origin, although four isolates were obtained from patients from the emergency or dermatology departments, hence circulating in the community. Thus, community dissemination of CPE is a possibility in our country as has already been postulated in other regions of the world [13].

The only antibiotic active against all 12 isolates was fosfomycin, being the only therapeutic option in two strains. On the other hand, colistin was active against eight isolates, whereas the remaining four isolates were naturally resistant to colistin (*Proteus* spp., *Providencia* spp. and *M. morganii*). Beyond fosfomycin and colistin, amikacin and minocycline only covered three isolates each; additionally, all isolates were resistant to oxyimino-cephalosporins, piperacillin/tazobactam, amoxicillin/clavulanic acid, imipenem, meropenem, ertapenem, aztreonam, ciprofloxacin, gentamicin and trimethoprim/sulfamethoxazole (Table 1).

3.2. Antimicrobial resistance mechanisms

The most prevalent carbapenemase was NDM-1 (n = 10), followed by VIM-2 (n = 1) and KPC-2 (n = 1).

Table 1
Characteristics of carbapenemase-producing Enterobacterales isolates, samples, carbapenemase (CARB) type, antimicrobial susceptibility profile, associated resistance genes, and plasmid size and incompatibility group.

Strain no.	Isolation date (M/Y)	Sample	Species	CARB	MIC (mg/L)														5′CS-3′CS VR	Integron	Other genes	Genes in Tc	Plasmid size (kb)	Inc	bla_{NDM-1} environment[a]
					CTX	CAZ	FEP	TZP	ETP	MEM	IPM	CIP	GEN	AMK	SXT	COL	TIG	FOS							
HC1	03/2012	Urine	*Providencia rettgeri*	NDM-1	>32	>32	>16	>64/4	>2	≥16	≥16	>2	>8	>32	>2/38	NR	NR	32	*aadB–bla*$_{OXA-10}$*–aadA1*	In*907*	*qnrVC*, *aac(6′)-Ib*, *bla*$_{TEM-1}$	*bla*$_{NDM-1}$, *aadB–bla*$_{OXA-10}$*–aadA1*, *qnrVC*, *aac(6′)-Ib*, *bla*$_{TEM-1}$	170	IncC	A
HC2	03/2013	Urine	*C. freundii*	NDM-1	>32	>32	>16	>64/4	>2	≥16	≥16	2	>8	32	>2/38	≤1	1	0.25	*aadB–bla*$_{OXA-10}$*–aadA1*	In*907*	*qnrVC*, *aac(6′)-Ib*, *bla*$_{TEM-1}$	*bla*$_{NDM-1}$, *aadB–bla*$_{OXA-10}$*–aadA1*, *qnrVC*, *aac(6′)-Ib*, *bla*$_{TEM-1}$	200	IncC	A
HC3	08/2013	Urine	*Moraxella morganii*	NDM-1	>32	>32	4	>64/4	≤1	4	≥16	>2	>8	32	>2/38	NR	NR	4	–	–	*qnrD1*	No Tc	No Tc	No Tc	A
HC4	12/2013	Blood	*Enterobacter cloacae*	VIM-2	>32	>32	>16	>64/4	>2	≥16	≥16	>2	>8	>32	>2/38	≤1	>2	2	*bla*$_{VIM-2}$*–dfrA7*/*aadB–aadA2*	In*1687*/In*293*	*bla*$_{TEM-1}$, *bla*$_{CTX-M-9}$, *aac(6′)-Ib-*cr, *qnrA1*	*bla*$_{VIM-2}$*–dfrA7*, *bla*$_{TEM-1}$	200	IncC	
HC5	02/2014	Surgical wound	*Proteus mirabilis*	NDM-1	>32	>32	>16	>64/4	>2	≥16	≥16	2	>8	≤8	>2/38	NR	NR	0.25	*aadB–bla*$_{OXA-10}$*–aadA1*	In*907*	*qnrVC*, *aac(6′)-Ib*, *bla*$_{TEM-1}$	*bla*$_{NDM-1}$, *aadB–bla*$_{OXA-10}$*–aadA1*, *qnrVC*, *aac(6′)-Ib*, *bla*$_{TEM-1}$	200	IncC	A
HC6	02/2015	Peritoneal fluid	*Klebsiella aerogenes*	NDM-1	>32	>32	>16	>64/4	>2	≥16	8	>2	>8	16	>2/38	≤1	>2	1	–	–	*bla*$_{CTX-M-15}$	*bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$	500	IncHI1	A
HC7	06/2016	Blood	*E. cloacae*	NDM-1	>16	>32	>16	>64/4	>2	≥16	≥16	1	>8	32	>2/38	≤1	2	2	*aadB–bla*$_{OXA-10}$*–aadA1*	In*907*	*qnrVC*, *aac(6′)-Ib*, *bla*$_{CTX-M-15}$, *aac(6′)-Ib-*cr, *qnrB1*	*bla*$_{NDM-1}$, *aadB–bla*$_{OXA-10}$*–aadA1*, *qnrVC*, *aac(6′)-Ib*	104/55	IncC-IncM1	A
HC8	07/2016	Urine	*Klebsiella pneumoniae* ST11	KPC-2	4	8	8	>64/4	2	≥16	≥16	>2	>8	≤8	>2/38	≤1	≤0.5	8	*dfrA1*	*In183*	*bla*$_{TEM-1}$, *aac(6′)-Ib*	No Tc	No Tc	No Tc	
HC9	08/2016	Urine	*P. mirabilis*	NDM-1	>32	>32	>16	>64/4	>2	≥16	≥16	>2	>8	16	>2/38	NR	NR	0.25	*aac(6′)-Ib-bla*$_{OXA-2}$*–orfD (gcuD)*	In*35*	*bla*$_{CTX-M-2}$, *aac(6′)-Ib*	*bla*$_{NDM-1}$	33	NT	A
HC10	11/2016	Skin (punch)	*E. cloacae*	NDM-1	>32	>32	>16	64/4	≤1	≥16	≥16	>2	>8	16	>2/38	≤1	>2	4	*dfrA12–orfF (gcuF)–aadA2*	In*27*	*bla*$_{CTX-M-15}$, *aac(6′)-Ib-*cr, *qnrA1*, *qnrB1*	No Tc	No Tc	No Tc	B
HC11	12/2016	Blood	*K. pneumoniae* ST14	NDM-1	>32	>32	16	>64/4	>2	≥16	≥16	2	>8	16	>2/38	≤1	≤0.5	2	*aadB–bla*$_{OXA-10}$*–aadA1*	In*907*	*bla*$_{CTX-M-15}$, *aac(6′)-Ib*, *aac(6′)-Ib-*cr, *qnrB1*	*bla*$_{NDM-1}$, *aadB–bla*$_{OXA-10}$*–aadA1*	200	IncC	A
HC12	12/2016	Blood	*K. pneumoniae* ST661	NDM-1	>32	>32	>16	>64/4	>2	≥16	≥16	>2	>8	≤8	>2/38	≤1	>2	4	*aadB–bla*$_{OXA-10}$*–aadA1*	In*907*	*bla*$_{CTX-M-15}$, *aac(6′)-Ib-*cr, *qnrA1*, *qnrB1*	*bla*$_{NDM-1}$, *aadB–bla*$_{OXA-10}$*–aadA1*, *qnrA1*	170	IncC	B

MIC, minimum inhibitory concentration; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; TZP, piperacillin/tazobactam; ETP, ertapenem; MEM, meropenem; IPM, imipenem; CIP, ciprofloxacin; GEN, gentamicin; AMK, amikacin; SXT, trimethoprim/sulfamethoxazole; COL, colistin; TIG, tigecycline; FOS, fosfomycin; VR, variable region; Tc, transconjugant; NT, not determined; Inc, plasmid incompatibility group.
[a] See Fig. 1.

This low proportion of CPE is similar to that previously described in America and Europe, with a relatively low prevalence of NDM [13].

Ten strains with class 1 integrons were detected with variable regions already described in the INTEGRALL database as In*27* (*dfrA12–orfF–aadA2*), In*183* (*dfrA1*), In*35* (*aacA4–bla*$_{OXA-2}$*–orfD*), In*293* (*aadB–aadA2*) and In*907* (*aadB–bla*$_{OXA-10}$*–aadA1*). Additionally, a new rearrangement was described, containing *bla*$_{VIM-2}$*–dfrA7*, assigned as In*1687*. The six isolates with integrons belonging to In*907* were associated with NDM-1 and four of them with *qnrVC* and *aac(6′)-Ib*.

Coexistence of carbapenemases and different resistance genes to aminoglycosides [*aac(6′)-Ib*, *aac(6′)-Ib*-cr], β-lactams (*bla*$_{TEM-1}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-15}$) and fluoroquinolones [*qnrA1*, *qnrB1*, *qnrD1*, *qnrVC*, aac*(6′)-Ib*-cr] was found. No 16S rRNA methyltransferases were detected (Table 1).

Among the PMQR genes, *qnrVC* has been described mainly in the Vibrionaceae family as a gene cassette. It has the particularity of presenting its own promoter, unlike other *qnr* variants. Recently, we reported the first isolate, previously named CF638 (HC2 in this work), carrying a new genetic background of *qnrVC6* within a complex class 1 integron associated with In*907* and the insertion sequence IS*CR1* [14]. In this work, we describe three non-related additional isolates presenting *qnrVC* within the same background.

3.3. MLST

Sequence types were determined only for *K. pneumoniae* isolates, which belonged to ST11, ST14 and ST661 (Table 1).

The sequence types of the three *K. pneumoniae* strains showed clonal variety. Worldwide, *K. pneumoniae* ST11 (CC258, single-locus variant of ST258) has been associated with NDM-1, VIM-2, OXA-48, KPC-2 and KPC-3, among others, and is considered an epidemic clone [8,13,15]. This differs from a previous report in Uruguay where dissemination of *K. pneumoniae* carrying KPC-2 was due to ST258 [16].

Regionally, ST11 is replacing ST258, together with ST25 and ST307, which are sequence types considered more virulent according to Cejas et al. [17].

Klebsiella pneumoniae ST14 (CC292) is one of the sequence types most frequently associated with NDM-1 metallo-β-lactamase and is also considered as a high-risk clone [13,18]. We have previously reported the presence of ST14 associated with CTX-M-15, CTX-M-8 and SHV-2 [12].

Finally, the third sequence type observed in *K. pneumoniae* was ST661, which has already been reported with VIM-1 and KPC-2 in Europe, the latter responsible for an outbreak [19]. In our case it was associated with NDM-1.

3.4. Plasmid characterisation and replicon typing

Nine transconjugants were obtained from the conjugation assays with the twelve CPE isolates, harbouring carbapenemases in plasmid with sizes ranging from 33 kb up to 500 kb and belonging to incompatibility groups IncC, IncHI1 and IncM1. Conjugation assay results, susceptibility and genes detected in transconjugants are shown in Table 1.

Related to *K. pneumoniae* ST14 and ST661, each of them presented the carbapenemase gene in a conjugative IncC plasmid. IncC was the most prevalent incompatibility group, observed in seven of nine transconjugants. Among these isolates, four carried *bla*$_{NDM-1}$, *qnrVC* and a class one integron In*907* (Table 1).

Other plasmids were characterised as IncHI1 and IncM1, also already associated with NDM-1 [13]. Since its emergence in 2015, the IncL/M group has spread globally and harboured various types of carbapenemases. Lately, the presence of *bla*$_{OXA-48}$ in IncL/M plasmids showed a higher transfer efficiency compared with those carrying *bla*$_{NDM-1}$ owing to Tn*1999*, increasing the potential level of dissemination of these carbapenemases [3]. One of the transconjugants obtained carried *bla*$_{NDM-1}$ and two plasmids belonging to incompatibility groups IncC and IncM1. We could not determine which of the two plasmids harboured the carbapenemase gene.

3.5. Genetic environment of *bla*$_{NDM-1}$

A DNA fragment of 16444 bp harbouring *bla*$_{NDM-1}$ was assembled from HC2 plasmid sequence analysis, which included a new composite transposon of 15802 bp assigned as Tn*6935* [20]. Tn*6935* is composed of a 9393-bp conserved region that includes the *bla*$_{NDM-1}$ gene, identical to that reported by Hu et al. [21], flanked by two directly-oriented copies of an IS*Kox2*-like (Tn*3* family). The direct repeats 5′-TGATT-3′ were found adjacent to the transposon boundaries, suggesting its mobilisation through a conventional transposition event (Fig. 1). The presence of Tn*6935* was assessed by PCR mapping in each NDM-positive isolate, being confirmed in 8/10 isolates (including HC2) (Fig. 1A) (GenBank accession no. MT897966).

The most frequent transposon-carrying plasmid belonged to the IncC incompatibility group and was 170–200 kb in size. It was accompanied by In*907* associated with IS*CR1–qnrVC* in *Providencia rettgeri*, *C. freundii* and *P. mirabilis* (isolates HC1, HC2 and HC5, respectively). A similar plasmid but without the IS*CR1–qnrVC* element was detected in *K. pneumoniae* ST14 (HC11). The fact that the first isolate reported was obtained in March 2012 and the last one in December 2016, and its finding in several bacterial species, suggests that it might be a successful plasmid regarding the dissemination and maintenance of NDM-1. Further studies are needed to establish the similarity of these plasmids in detail.

On the other hand, Tn*6935* was detected in an IncHI plasmid, a small non-typeable 33-kb plasmid, and in a strain in which conjugation was not possible. These findings suggest the mobilisation capacity of the transposon per se.

Finally, a shorter environment of *bla*$_{NDM-1}$ was amplified in the remaining two NDM-positive strains (HC10 and HC12) with a size of 2352 bp (Fig. 1B).

The arrangement described in this work comprising IS*Aba125*, *bla*$_{NDM-1}$, *ble* and *trpF* is similar to other *bla*$_{NDM-1}$ genetic

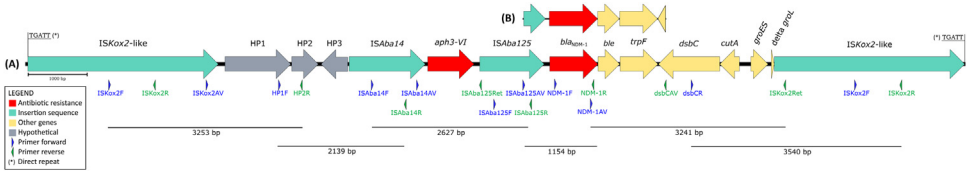

Fig. 1. Composite transposon Tn*6935* of *Citrobacter freundii* HC2 obtained by plasmid sequencing. Segments amplified for PCR-based mapping and primers used both for PCR and sequencing are shown below. (A) Transposon Tn*6935* found in isolates HC1, HC2, HC3, HC5, HC6, HC7, HC9 and HC11. (B) Segment mapped in HC10 and HC12. Abbreviations: HP1, 2 and 3 (grey) are genes encoding hypothetical proteins. GenBank accession no. MT897966.

environments previously described in Latin America [22] and is conserved as mentioned above [21]. The presence of IS*Kox2* is novel within this platform.

4. Conclusions

Among the micro-organisms reported in this work, there are some particularly worrisome aspects: (i) the presence of high-risk clones of *K. pneumoniae* producing carbapenemases; (ii) the combination of multiresistance plasmids in micro-organisms naturally resistant to colistin; and (iii) the wide dissemination capacity of NDM, not only by mobilisation of a successful plasmid but also by mobilisation of the composite transposon first described in this paper.

In our country, the diversity and sporadic presence of CPE could be due to the fast measures taken by the infection control committee, including contact isolation precautions and surveillance cultures, among others [16].

Given the similarities in the plasmids carrying $bla_{\text{NDM-1}}$, and since it is not an outbreak scenario, we might be in the presence of a successful plasmid responsible for the dissemination and maintenance of the carbapenemase through different species. Its acquisition by a high-risk clone could bring a new challenge for our hospital.

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Conflict of interest

None declared.

Ethical approval

Not required.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2020.11.006.

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