Novel allelic variants of $bla_{OXA-48-like}$ carried on $IncN_2$ and $IncC_2$ plasmids isolated from clinical cases in Argentina. In vivo emergence of $bla_{OXA-567}$.

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PII: S2213-7165(24)00470-3
DOI: https://doi.org/10.1016/j.jgar.2024.12.008
Reference: JGAR 2450

To appear in: *Journal of Global Antimicrobial Resistance*

Received date: 31 October 2024
Revised date: 2 December 2024
Accepted date: 6 December 2024

Please cite this article as: Juan Manuel de Mendieta , Denise De Belder , Nathalie Tijet , Barbara Ghiglione , Roberto G. Melano , Melina Rapoport , Pablo Power , Adriana Di Bella , Estefanía Biondi , Fernando Pasterán , Alejandra Corso , Gomez A. Sonia , Novel allelic variants of $bla_{OXA-48-like}$ carried on $IncN_2$ and $IncC_2$ plasmids isolated from clinical cases in Argentina. In vivo emergence of $bla_{OXA-567}$., *Journal of Global Antimicrobial Resistance* (2024), doi: https://doi.org/10.1016/j.jgar.2024.12.008

HIGHLIGHTS

- Carbapenem resistant *E. coli* ST1196 and *K. pneumoniae* ST6838 were isolated from pediatric patients
- OXA-438 and OXA-567 were found as novel variants derived from OXA-48-like
- The new *oxa* enzymes were harbored in $IncN_2$ and $IncC_2$ conjugative plasmids
- Close genetic environment of $bla_{OXA-438}$ and $bla_{OXA-567}$ suggest a common origin
- $bla_{OXA-567}$ emerged *in vivo* during co-colonization with $bla_{OXA-163}$-*K. pneumoniae*

Novel allelic variants of *bla*$_{\text{OXA-48-like}}$ carried on IncN$_2$ and IncC$_2$ plasmids isolated from clinical cases in Argentina. *In vivo* emergence of *bla*$_{\text{OXA-567}}$.

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KEYWORDS: OXA-48-LIKE, OXA-163, CARBAPENEMASE, IncN, IncC, PLASMID, ENTEROBACTERALES

ABSTRACT

Background. The OXA-48-like enzymes are members of the class D β-lactamases, primarily detected in *Enterobacterales*, with the capacity to hydrolyze carbapenems. The allelic variant *bla*$_{OXA-163}$, which has low hydrolytic activity towards carbapenemes, was detected in Argentina in 2011 and spread successfully since then, giving sporadic origin to novel local variants.

Aim. To study the phenotypic profile and the dissemination strategies of two novel OXA enzymes, *bla*$_{OXA-438}$ and *bla*$_{OXA-567}$, harbored in *Escherichia coli* M17224 and *Klebsiella pneumoniae* M21014, isolated from two pediatric patients.

Methods. MICs were performed to determine the phenotypic profile of the clinical isolates, transcojugants and transformant cells. Biparental conjugation, PCR, Sanger and whole genome sequencing were performed to determine the complete genetic characteristics of the plasmids.

Results. Both isolates were found resistant to carbapenems and susceptible to ceftriaxone. *bla*$_{OXA-438}$ was located on an IncN$_2$ plasmid of 69 Kb while *bla*$_{OXA-567}$ on an IncC$_2$ plasmid of 175 Kb, both transferable by biparental conjugation. The close genetic environment of the *bla*$_{OXA}$ genes suggests a common origin, likely involving mobile genetic elements. Finally, the clinical case of M21014 revealed previous infections of the patient with two genetically related *K. pneumoniae* ST6838, that carried *bla*$_{OXA-163}$ on IncC$_2$ plasmid with equal size and genetic hallmarks than that of M21014, providing strong evidence for the intrapatient emergence of *bla*$_{OXA-567.}$ **Conclusions.** This research underscores the need for ongoing surveillance and integral studies to understand the emergence, biochemistry and dissemination capacity of OXA enzymes with the overarching aim to halt their spread.

INTRODUCTION

The OXA-48-like group of enzymes is composed of Class D beta-lactamases with the ability to hydrolyze carbapenems, described exclusively in *Enterobacterales* [1]. These enzymes were first reported in 2001 in Turkey, in a clinical isolate of *Klebsiella pneumoniae* producing OXA-48 [2]. Soon, OXA-48 was broadly disseminated in the north of Africa and in Mediterranean countries, as well as in Asia, Oceania and North America as responsible of casual intra hospital outbreaks [2]. Today, even though OXA-48 is the most reported variant of this group, a great variety of close related enzymes have emerged and disseminated. Examples of this are OXA-181, OXA-232, OXA-204 and OXA-244, detected along the years in different geographic regions with similar hydrolytic activities [3]. Different molecular studies suggest that the genes of these allelic variants may have originally been captured by mobile genetic elements (MGE) from the chromosome of *Shewanella*, an aquatic bacterium, and were later integrated in plasmids [4]. This is the case of Tn*1999*, commonly associated with plasmids from incompatibility group L (IncL) harboring $bla_{\text{OXA-48}}$, or Tn*2013*, associated with IncColE2 plasmids harboring $bla_{\text{OXA-181}}$ [4].

In 2011, a new variant of the OXA-48-like group, designated OXA-163, was reported in Argentina. This allelic variant showed a very low capacity to hydrolyze carbapenems, possibly due to a deletion of 4 amino acids in the β5-β6 loop involved in the hydrolysis of these antibiotics. However, it has high hydrolytic capacity against extended-spectrum cephalosporins, showing a particularly different hydrolytic profile than the other enzymes of this group [5, 6]. This phenotypic profile, together with the fact that OXA-163-like is not easily detected

by biochemical, colorimetric or phenotypic methods, permitted the silent dissemination of OXA-163 throughout the country [7]. This was confirmed in a multicenter, prospective study of carbapenemase-producing *Enterobacterales* (CPE) performed during the COVID-19 pandemic in Argentina where 7.5% of (61 out of 822) isolates, were Ambler Class D producers (*bla*$_{\text{OXA-48-like}}$) [8].

In subsequent years, new exclusively local variants have been identified in Argentina, all of which have been found located in a plasmid. At the moment, there are no genetic studies of the plasmids carrying the variants of the *bla*$_{\text{OXA-163-like}}$ subgroup [3]. Here, we aimed to perform a comparative study of the full plasmid sequences harboring novel variants of *bla*$_{\text{OXA-163-like}}$ subgroup that emerged in Argentina in order to understand the genetic content and dissemination strategies. Moreover, this study also describes the *in vivo* emergence of the novel *oxa* allelic variant *bla*$_{\text{OXA-567}}$.

2. Materials and Methods

2.1 Bacterial Isolates

The clinical isolates, M17224 and M21014, were referred to the National and Regional Reference Laboratory for Antimicrobial Resistance (NRRLAR) for further molecular characterization after following local derivation guidelines, due to unusual resistance profile towards carbapenems and cephalosporins. Bacterial identification was performed with the MALDI Biotyper kit (Bruker Daltonik, Bremen, Germany).

2.2 Susceptibility testing of the isolates

Routine phenotypic screening of the isolates was performed with Triton Hodge Test (THT), Blue Carba Test and double disk synergy test. Minimum inhibitory concentration (MIC) of all clinical isolates and strains used in this study were determined by microdilution using BD Phoenix automated microbiology system (NMIC/ID-406 panel). Results were interpreted according to Clinical & Laboratory Standards Institute (CLSI) [9].

The isolates were screened for *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{IMP}$ and *bla*$_{OXA-48-like,}$ using an in-house multiplex PCR protocol (Supplementary Table 1). Alleles were confirmed by Sanger sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit in an ABI PRISM 377 (Applied Biosystems, Foster City, California, USA).

2.3 PFGE AND MLST

The genetic relatedness among the isolates was evaluated by *Xba*I-digested pulsed-field gel electrophoresis (PFGE) using a CHEF-DR III System (Bio-Rad, Hercules, CA, USA) and interpreted as previously reported [10]. DNA fragments were resolved in 1% agarose gel by applying a switch time of 2.2 to 54.2 s for 20 h at 14 °C. To determine the international clones of the isolates, we performed multi-locus sequence typing (MLST) by PCR and sequencing according to the protocols and analysis established by EnteroBase (https://enterobase.warwick.ac.uk/) for *Escherichia coli* and PubMLST (https://pubmlst.org/) for *K. pneumoniae*.

2.4 Biparental conjugation and plasmid profile

Conjugation assays were performed using *E. coli* J53 resistant to sodium azide as acceptor strain in a 1:3 donor to acceptor ratio. Transconjugants were selected

with Trypticase Soy Agar supplemented with 50 µg/ml ampicillin and 200 µg/ml sodium azide, and incubated at 36 C° for 18 h. Gene transference and susceptibility profile of transconjugants were performed as explained in 2.2. The number and estimated size of the plasmids was evaluated in clinical and transconjugant isolates. Briefly, total DNA in agarose plugs was digested with nuclease S1 (Promega, Southampton, UK) and run in a PFGE equipment [11].

2.5 Cloning *bla*$_{OXA}$ variants, transformation and susceptibility testing of recombinant clones.

The *bla*$_{OXA}$ genes were amplified by PCR from whole DNA extracted from the respective clinical strains (Supplementary Table 2), using the cloning primers forward pk19-OXA-48-F-BamHI (5´-GGATCCCATGCGTGTATTAGCC-3´) and reverse pK19-OXA-48-R-EcoRI (5´-GAATTCTAGGGAATAATTTTTTCCTG-3´). PCR was performed using Phusion high-fidelity DNA polymerase (Thermo Scientific, Massachusetts, USA) and 1 µM of primers. The PCR products were first ligated in a pTZ57R/T vector and the inserts sequenced to verify the identity of *bla*$_{OXA-48-like}$ genes, the generated restriction sites and, as well as the absence of aberrant nucleotides. Resulting recombinant pTZ57R/T plasmids were then digested, and the released inserts were subsequently purified and cloned in a pK19 vector, previously digested with the appropriate restriction enzymes. Ligation mixtures were transformed into chemically competent *E. coli* DH5α cells. Transformed cells were selected in Lysogeny Agar plates supplemented with 30 µg/ml of kanamycin. Recombinant plasmids of the selected clones were extracted and sequenced to confirm the identity of *bla* genes and their correct insertion.

2.5 Whole genome sequencing

Plasmidic DNA was extracted using QIAGEN Large-Construct Kit (Qiagen) according to the manufacturer's instructions. DNA concentration was determined by Qubit 2.0 Fluorometer (Thermo-Fisher Scientific), and DNA samples were stored at −20°C until further processing. The sequencing library was prepared with the Illumina Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA) as per manufacturer's instructions. Agilent 2100 Bioanalyzer was used to determine the quality of the DNA libraries. Sequencing was performed at Public Health Ontario Laboratory with an Illumina MiSeq platform with 600-cycle MiSeq Reagent Kit v3. Reads were assembled with SPAdes v.3.9.0. M21014 was additionally sequenced with Oxford Nanopore Technologies (ONT) in a MinION device. The chemistry used was number 8 and the flow cells FLO-MIN106 version R9.4. DNA extraction was made with the MasterPure Complete DNA & RNA Purification kit (Epicenter Illumina, Wisconsin, USA) and elution carried out to a final volume of 40 µL in TE buffer. Libraries were prepared with the Rapid Barcoding Kit SQK-RBK004 starting with 400 ng of high molecular weight DNA and according to Oxford Nanopore protocol (RBK_9054_V2_revE_23jan2018). Libraries were loaded and run for 48 hours. Basecalling was performed while sequencing or using Guppy. Nanoplot was used for quality control. Porechop (https://github.com/rrwick/Porechop) was used to split files by and to trim barcodes. Fastqc v.11.8 was used to determine the quality of the reads obtained (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and filtered (Phred>=30) with Trimmomatic v0.38 (http://www.usadellab.org/cms/?page=trimmomatic). Illumina-ONT hybrid

assemblies were performed with Unicycler v0.4.8 using default options (https://github.com/rrwick/Unicycler/). In the plasmid obtained from M17511, transcojugant of M17224, the gaps generated by short read sequencing and the relative transcription orientation of the fragmented sequences were solved performing PCR and Sanger sequencing using the primer combinations detailed in Supplementary table 1. In the case of M21014, the isolate was additionally sequenced by MinION as described above.

2.6 Bioinformatic analysis of the plasmid sequences

The sequences were annotated with Prokka v1.14.5 (1). Manual curation of the automated annotation was done with Artemis [12]. Resistance genes were searched with ResFinder v3.2 [13] and AMRFinderPlus v3.4.7. Incompatibility groups were searched with PlasmidFinder v2.1 [14]. Mobile elements and $bla_{\text{oxa-48-like}}$ immediate environment were analyzed manually and using online tools such as BLASTn and ISfinder [15]. OriTFinder was used to detect the transfer origin of the plasmids [16]. Multiple plasmids analysis and comparison between plasmids were generated by the BLAST Ring Image Generator (BRIG) v0.95 [17].

2.7 GenBank Accession numbers of plasmids

The sequences obtained here were submitted to GenBank as MN583554 (p17511_70) and ON925022 (p21014_170).

3. Results and Discussion

3.1 Epidemiological data and phenotypic/ genotypic features of the Isolates

The clinical isolates, M17224 and M21014, were recovered from two pediatric patients from two hospitals in the Buenos Aires City (CABA) and Buenos Aires Province, respectively. M17224 was identified as *E. coli* isolated in January 2014 from urine of a 12-year-old female patient with B-cell acute lymphoblastic leukemia. The patient was successfully treated with ciprofloxacin and discharged from the hospital with prophylactic trimethoprim-sulfamethoxazole. This patient had a history of frequent *E. coli* infections that lead to multiple hospitalizations during 2013 due to recurrent infections like bacteremia and abdominal inflammation [3]. Consequently, the patient was treated with several antimicrobials such as amikacin, imipenem, and fluconazole. The second isolate studied here, M21014, was recovered from a rectal swab of a baby that was born with a congenic malformation (bile duct atresia) and was hospitalized in the neonatology ICU. For simplicity, this clinical case will be described in section 3.6. Prior to the isolation of this strain, the patient had received vancomycin and amikacin for prophylaxis.

E. coli M17224 and *K. pneumoniae* M21014 were found resistant to imipenem, meropenem, ertapenem, cefoxitin, piperacillin/tazobactam, ampicillin/sulbactam, cefazolin, ciprofloxacin, and trimethoprim/sulfamethoxazole. Both isolates were susceptible to cefepime and ceftriaxone (Table 1). The susceptibility to aminoglycosides was variable. Both isolates showed carbapenemase activity using the THT with a meropenem disk, but negative results were observed for the double disk synergy test (between carbapenem disk and phenyl boronic acid and/or EDTA) and the colorimetric Blue Carba Test. PCR confirmed the presence of $bla_{\text{OXA-48-like}}$ in both isolates.

In Argentina, OXA-163-producing isolates usually present resistance to third-generation cephalosporins, and marginal resistance to carbapenemes. These isolates, M17224 and M21014, were susceptible to ceftriaxone and resistant to carbapenemes (Table 1). Therefore, a different OXA variant was suspected and as a result, the isolates were further studied.

Table 1. Antimicrobial susceptibility testing results of clinical isolates transcojugants and transformed strains.

Antimicrobial	ECO M17224	KPN M21014	TC-M17224	TC-M21014	ECO J53	ECO DH5α pk19-OXA-438	ECO DH5α pk19-OXA-567	ECO DH5α pk19
				mg/L				
Imipenem	> 8 R	> 8 R	2	1	0.5	2	0.5	0.5
Meropenem	16 R	32 R	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5
Ertapenem	> 1 R	> 1 R	≤ 0.25	0.5	≤ 0.25	≤ 0.25	0.5	≤ 0.25
Ceftriaxone	≤ 1 S	≤ 1 S	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Cefepime	4 S	4 S	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Cefoxitin	> 16 R	> 16 R	≤ 4	> 16	≤ 4	≤ 4	≤ 4	≤ 4
PTZ	> 64 R	> 64 R	> 64	64	≤ 4	64	32	≤ 4
AMS	> 16/8 R	> 16/8 R	> 16/8	> 16/8	≤ 4/2	> 16/8	> 16/8	≤ 4/2
Cefazolin	> 8 R	> 8 R	8	8	≤ 2	8	> 8	≤ 2
Ampicillin	> 16 R	NR	> 16	> 16	≤ 4	> 16	> 16	≤ 4
Ciprofloxacin	> 2 R	>2 R	> 2	2	≤ 0.125	≤ 0.125	≤ 0.125	≤ 0.125
Gentamicin	≤ 2 S	> 8 R	≤ 2	> 8	≤ 2	≤ 2	≤ 2	≤ 2
Amikacin	32 R	≤ 8 I/S	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8
TMS	> 2 R	> 2 R	> 2	> 2	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5

ECO, *E. coli*; KPN, *K. pneumoniae*; PTZ, Piperacillin/Tazobactam; TMS, Trimethoprim/Sulfametoxazole; AMS, Ampicillin/Sulbactam; TC, transconjugant; R, resistant; S, susceptible; I, intermediate; NR, natural Resistance.

3.2 Sequence types and molecular characterization of the isolates

Molecular epidemiology identified *E. coli* M17224 as part of the international clone ST1196, which has been previously reported in various hosts and environments, associated with carbapenemases such as NDM-1 and KPC-2 in humans [18, 19] and OXA-48 in animals [20]. *K. pneumoniae* M21014 belonged to ST6838, a single locus variant of ST26, a virulent clone associated with respiratory infections and pyogenic liver abscess disease in humans [21]. Both *E. coli* and *K. pneumoniae* clones are unusual in our region.

In terms of the molecular analysis of the bla_{OXA} allelic variants, *E. coli* M17224 harbored $bla_{OXA-438}$, a new variant of $bla_{OXA-163}$, which was previously biochemically

characterized [3]. *K. pneumoniae* M21014 carried another new variant called *bla*$_{OXA-567}$. Both new alleles harbored mutations that affected the β5 strand or the loop connecting the β5 and β6 strand (Figure 1), which are responsible for substrate specificity in class D β-lactamases [22]. Compared to OXA-163, the new variant OXA-567 has an amino acid insertion (KYG) in the loop between the β5 and β6 strands, while OXA-438 has an insertion (GY) in the β5 strand. As seen in the previously detected variant OXA-247 from Argentina, these mutations occur near the conserved KTG motif, which likely enhances carbapenem hydrolysis and reduces oxyimino-cephalosporin inactivation when compared to OXA-163 [3, 23]. To the best of our knowledge, the OXA variants detected in Argentina appear to be unique, with no similar mutations reported elsewhere.

Figure 1. Amino acid alignment of OXA-48, OXA-163, OXA-567 and OXA-438 variants.The red arrow indicates the amino acids that conform the β5 strand and the green arrow indicates those of β6 strand.

3.3 Horizontal transfer of *bla*$_{OXA}$ genes

Biparental conjugation of both *bla*$_{OXA}$ plasmids was successful. In relation to susceptibility to carbapenems, the MIC of transconjugant TC-M17224 and transformant *E. coli* DH5α pk19-OXA-438 to imipenem were 2 dilutions above that of the acceptor strain *E. coli* J53 and *E. coli* DH5α pk19 MIC (Table 1). For TC-M21014 and its transformant *E. coli* DH5α pk19-OXA-567, only an increase of one dilution in ertapenem was observed. Transconjugants and transformants remained susceptible to cefepime and ceftriaxone, and presented MIC values to piperacillin/tazobactam, ampicillin/sulbactam, cefazolin and ampicillin similar to the clinical isolates. In the transformants, where *bla*$_{OXA-438}$ and *bla*$_{OXA-567}$ were cloned, there are no additional resistance mechanisms to β-lactams (enzymatic and/or non-enzymatic) and the increase in MIC values compared to *E. coli* DH5α pk19 (only vector) is inferred only by the production of these enzymes.

In addition, both transconjugants strains showed high MICs to ciprofloxacin, trimethoprim/sulfamethoxazole, suggesting the co-selection of additional resistance mechanism.

3.4 Analysis of the plasmids harboring *bla*$_{OXA-438}$ and *bla*$_{OXA-567}$

To estimate the number and size of plasmids in the clinical isolates and transconjugants, S1-PFGE was performed. As shown in Figures 2B and 3B, the clinical isolates exhibited between 1 and 3 bands ranging from approximately 20 kb to 260 kb. Specifically, isolate M17224 harbored three plasmids of ~70, 90, and 105 kb, while isolate M21014 presented two bands, one ~170 kb and the other

~260 kb. The transconjugant strains showed at least one band similar in size to the respective clinical strain, indicating that the conjugative plasmid retained its original size, suggesting no rearrangements occurred.

The plasmids carrying *bla*$_{OXA-438}$ (p17511_70) and *bla*$_{OXA-567}$ (p21014_170) were further analyzed and compared. Sequencing results (Supplementary Table 3) confirmed the plasmid sizes matched those seen on the S1-PFGE gels. p17511_70 and p21014_170 belonged to the IncN$_2$ and IncC incompatibility groups, respectively. The biparental conjugation findings were corroborated by S1-PFGE and the identification of *tra* genes, the replication protein *repA and ori*T *in silico* (Figures 2A and 3A). Both plasmids also contained replication and maintenance genes, along with multiple resistance genes conferring resistance to β-lactams, fluoroquinolones, aminoglycosides, sulfonamides, tetracycline, and macrolides (Supplementary Table 3). Many of these resistance genes were associated with mobile genetic elements, such as compound transposons and insertion sequences, illustrated in detail in Figures 2A and 3A for both plasmids.

The *bla*$_{OXA-438}$-bearing plasmid, p17511_70, belonged to incompatibility group N$_2$ (Figure 2A). Its final nucleotide sequence was 69,304 bp with a GC content of 50%, identifying 77 ORFs. This plasmid carried seven resistance genes, including dfrA16 and sul1, within the class 1 integron In194 (http://integrall.bio.ua.pt/?acc=MN583554), and *bla*$_{OXA-9}$, *bla*$_{TEM-1}$, *aadA1*, and *aac(6´)-Ib-cr7*, all within the Tn*3*-related transposon Tn*6238*, which was interrupted by a Tn*Ec1*-like element (Figure 2A). *bla*$_{OXA-438}$ was located near Tn*6238*. The closest match to p17511_70 in the NCBI database was the 71 kb IncN$_2$ plasmid pEC448_OXA163 (CP015078.1), isolated in an *E. coli* ST131 isolate in 2011 from Buenos Aires, Argentina, which harbored *bla*$_{OXA-163}$ [25]. Both plasmids shared

high sequence identity (100%), with differences only in acquired mobile genetic elements and resistance determinants (Figure 2). While p17511_70 contained the class 1 integron In194, pEC448_OXA163 housed a complex class 1 integron with *qnrB10* and a duplicated 3' conserved segment. Additionally, the Tn*6238* transposon in pEC448_OXA163 was intact, unlike its interrupted counterpart in p17511_70. $IncN_2$ plasmids are commonly associated with bla_{NDM-1} carbapenemase genes in *K. pneumoniae* and *E. coli* [26, 27]. To date, only $bla_{OXA-181}$ has been reported in $IncN_1$ plasmids from an *E. coli* isolate in China [4].

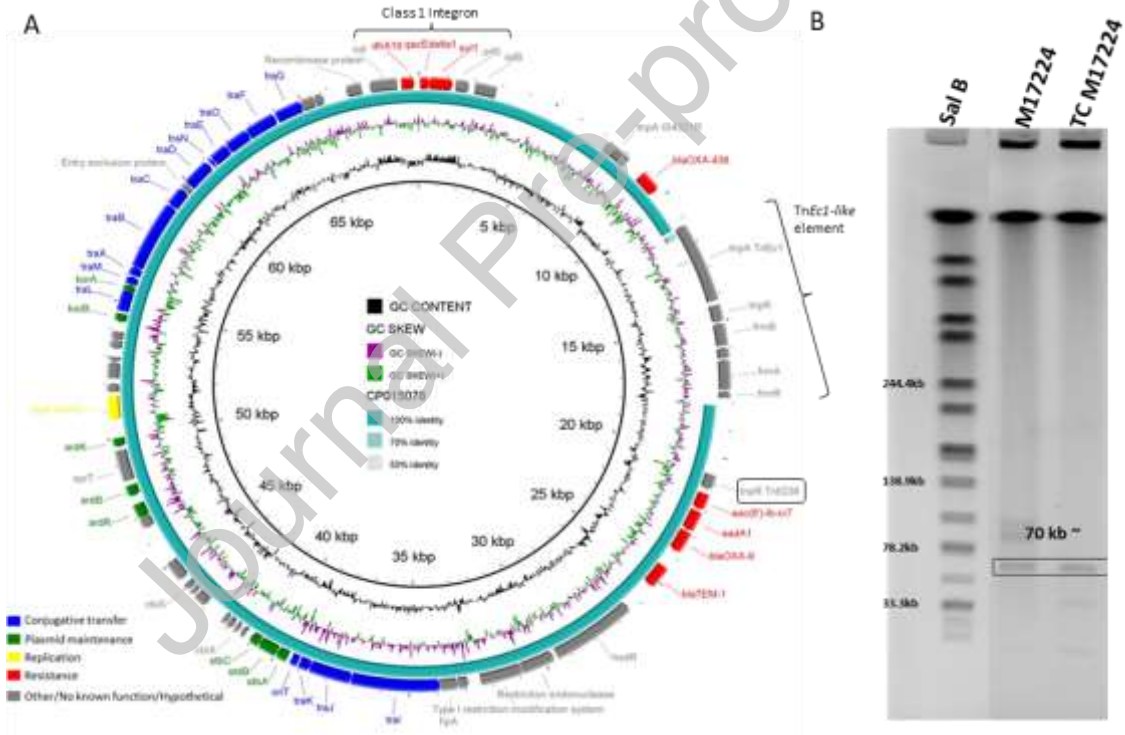

Figure 2. A: BRIG alignment of p17511_70 $IncN_2$ plasmid harboring $bla_{OXA-438}$ vs CP015078. The comparison was relative to p17511_70 (inner ring in black). At the center of the ring, color levels indicate BLAST result with a matched degree of shared regions. The outermost ring depicts genes as arrows in the corresponding transcription orientation present in p17511_70 as follows: blue, conjugative transfer; green, plasmid maintenance; yellow, replication; red, resistance; grey, other, no known function or hypothetical. Of note, the BRIG alignment only shows the annotation of those genes and/or elements present in the inner circle plasmid

(p17511_70). Class 1 integron and Tn*6238* interrupted by Tn*Ec1-like* element are indicated (more details in the text). Disrupted or partial genes are omitted from the BRIG figure. **B:** PFGE of S1-nuclease digested genomic DNA of clinical isolate M17224 and transconjugant of M17224 (TC-M17224). Sal B: *Salmonella* Branderup. The rectangle indicates the inferred size of the plasmid by PFGE-S1

The plasmid harboring *bla*$_{\text{OXA-567}}$, p21014_170, had a final nucleotide size of 175,492 bp and belonged to the IncC type 2 incompatibility group. p21014_170 showed the typical IncC hallmarks such as the antimicrobial resistant island (ARI-B), upstream *parA* and *parB* genes (Figure 3A) [24]. Interestingly, *bla*$_{\text{OXA-567}}$ was located outside the ARI-B. Additionally, some antimicrobial resistance determinants were found associated with genetic structures and rearrangements associated with IS*26*. Virulence genes were not found in the plasmids.

This plasmid presented a GC content of 51.98% and 204 ORFs were identified, 10 of which were resistance genes: *tet(A), aph(6)-Id, aph(3")-Ib, sul2, msr*(E) and *mph*(E) were located in a structure designated in the IncC plasmids as antibiotic resistance island B (ARI-B) (Figure 3A). [25]. The genes *tmrB, aac (3)-IIe, bla*$_{\text{OXA-1,}}$ *aac (6')-Ib-cr5, tet(A), qnrB1 and dfrA14* were found immersed in a fragment of approximately 30Kb associated with multiple copies of IS*26* (Figure 3A). This fragment was identical (100% nucleotide identity and 100% coverage) with that of other IncFIB plasmids isolated in *K. pneumoniae* (CP067949.1, OX030699.1, both unpublished), suggesting that this module can be mobilized autonomously as a transposition unit. Finally, *bla*$_{\text{OXA-567}}$ was found in the vicinity of the *rhs2* gene which is considered a hot spot region of the IncC plasmids associated with the acquisition of different resistance determinants [25].

p21014_170 presented a nucleotide identity ≥ 99.9% and a coverage of 84% compared to a plasmid from a *K. pneumoniae* (MZ465528.1) isolated in 2004 in the City of Buenos Aires from a broncho-alveolar lavage clinical sample

(unpublished). The comparative genetic analysis between the plasmids showed a high degree of conservation between the backbones but significant differences in the composition of mobile genetic elements and resistance determinants. IncC plasmids, sometimes mentioned as IncA/C, have been reported harboring OXA-48 and OXA-204 variants in *P. rettgeri* and *K. pneumoniae* isolates from Turkey and Tunisia, respectively [4].

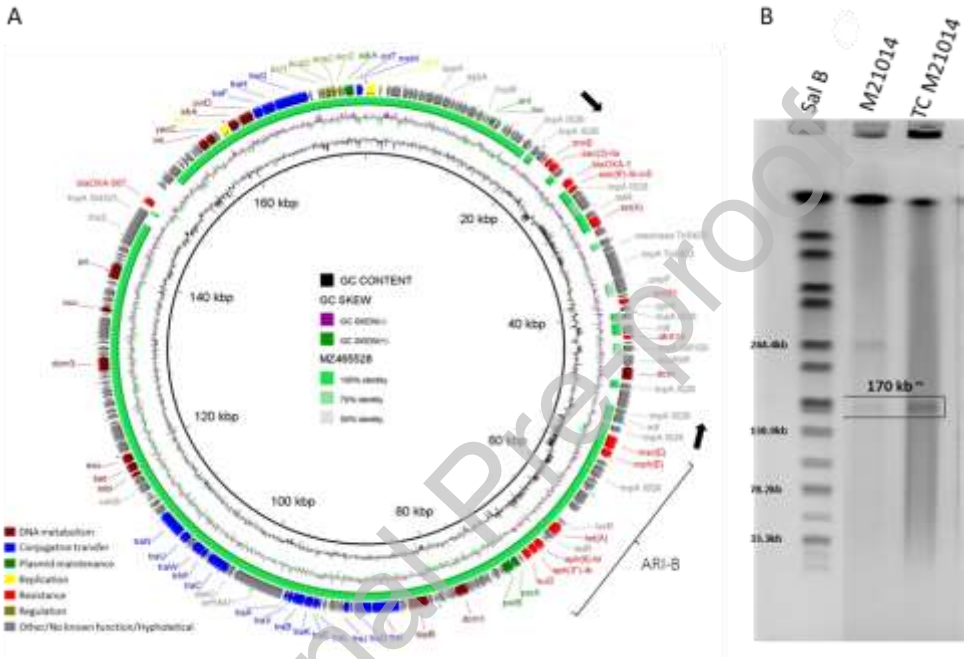

Figure 3. A: BRIG alignment of p21014_170 IncC type 2 plasmid harboring *bla*$_{OXA-567}$ vs MZ465528. The comparison was relative to p21014_170 (inner ring in black). At the center of the ring, color levels indicate BLAST result with a matched degree of shared regions. The outermost ring depicts genes as arrows in the corresponding transcription orientation as follows: brown, DNA metabolism; blue, conjugative transfer; green, plasmid maintenance; yellow, replication; red, resistance; green olive, regulation; grey, other, no known function or hypothetical. ARI-B is indicated with a black key. Solid black arrows outside the ring demark the IS26 rich region mentioned in the text. Disrupted or partial genes are omitted from

the BRIG figure. **B:** PFGE of S1-nuclease digested genomic DNA of clinical isolate M21014 and TC M21014 (transconjugant of M21014). Sal B: *Salmonella* Branderup. The rectangle indicates the inferred size of the plasmid by PFGE-S1.

3.5 Immediate genetic environment of *bla*$_{\text{OXA-438}}$ and *bla*$_{\text{OXA-567}}$

In both plasmids, the immediate genetic environment of *bla*$_{\text{OXA-438}}$ and *bla*$_{\text{OXA-567}}$ was highly similar; IS*4321R* was upstream *bla*$_{\text{OXA-438}}$ and IS*4321* upstream *bla*$_{\text{OXA-567}}$, two highly related insertion sequences (Figure 4) known to belong to the IS*1111* family. It has been documented that they perform a site-specific transposition by entering the inverted repeat sequences of other transposons of the Tn*21* family [26]. In our analysis, IS*4321* and IS*4321R* were found flanked by a vestige of a Tn*21-like* element, which would have acted as a target for the acquisition of these MGEs. In this sense, we tested *in silico* that the repeated inverted sequence of the Tn*21-like* element can be restored by "eliminating" both ISs. *bla*$_{\text{OXA-567}}$ was followed by a truncated IS*4-like* while *bla*$_{\text{OXA-438}}$ by a ΔIS*4-like*-ΔIS*6100.* These partial sequences of IS*4-like* element presented a high nucleotide identity (≥ 97%) with other ISs found in chromosomes of *Shewanella* spp. (CP041151.1, CP022089.2 and CP043902.1) and *Shewanella decolorationis* (CP037898.1 and CP031775.3).

As shown in Figure 4, immediately upstream and downstream of *bla*$_{\text{OXA}}$ gene, both environments share an identical intergenic region of 61 and 201 pb, respectively. This fragment belongs to the chromosome of *Shewanella* spp. Interestingly, two plasmid sequences, CP015078.1 (mentioned above, harboring *bla*$_{\text{OXA-163}}$) and KJ488943.1, which is a partial sequence of an IncF plasmid carrying *bla*$_{\text{OXA-370}}$

isolated in Brazil in May 2013 from an *Enterobacter hormaechei*, share the same fragments of the *Shewanella* chromosome upstream and downstream of the gene. This could suggest that these *bla*$_{oxa}$-gene variants come from a common capture and mobilization event, independently of others already reported in *bla*$_{OXA-48}$, *bla*$_{OXA-181\ or\ 232}$ and *bla*$_{OXA-204}$ associated with the mobile genetic elements Tn*1999*, Tn*2013* and Tn*2016*, respectively [4].

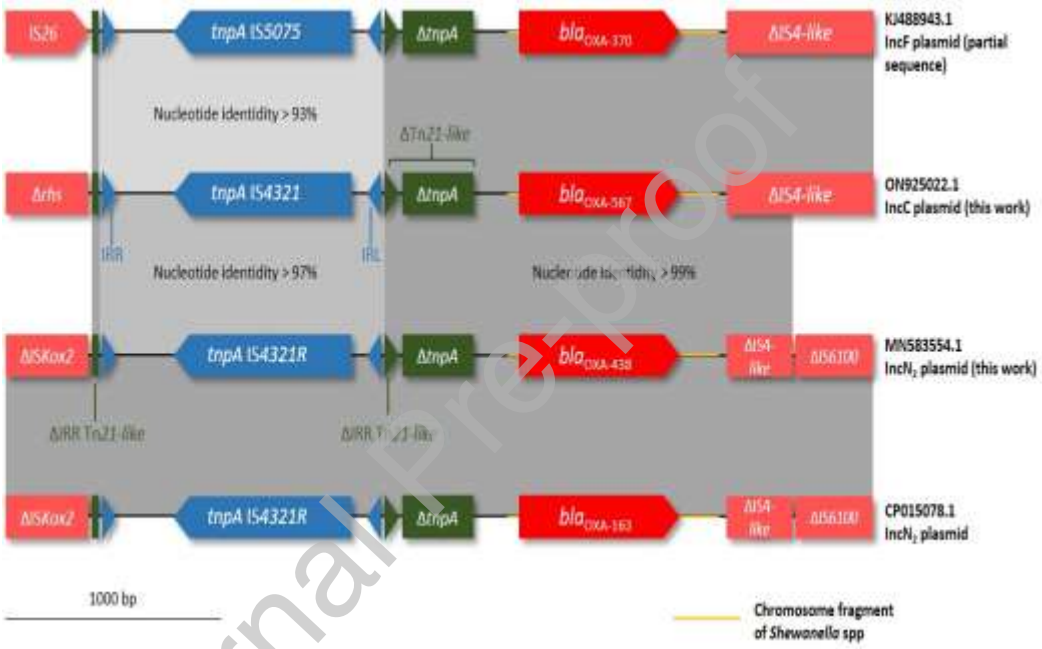

Figure 4. Genetic environment of local variants in the OXA-48-like group. Top: Immediate genetic environment of *bla*$_{OXA-567/370}$ on IncC and IncF plasmids. Bottom: Immediate genetic environment of *bla*$_{OXA-163/438}$ on IncN$_2$ plasmids. IRR: Inverted repeat sequence right; IRL: Inverted repeat sequence left. Upstream and downstream of the *bla*$_{OXA}$ variants, the same DNA fragments from the *Shewanella* chromosome are indicated with a light blue line.

3.6 Intrapatient emergence of *bla*$_{OXA-567}$

As mentioned in section 3.1, the patient was colonized with isolate M21014, a mucous *K. pneumoniae* harboring the new OXA-567 variant. Briefly, on February 14th, 2016, a *K. pneumoniae* (M19969), identified as a flat colony, was isolated from a blood culture of the baby. This infection originated in a surgical wound and was successfully treated with meropenem and amikacin. On March 2nd 2016, *K. pneumoniae* M21013 was obtained as a flat colony together with *K. pneumoniae* M21014 (mucous colony) obtained from the rectal swab as mentioned in section 3.1. As a result, M19969 and M21013 were further studied to determine the possible inpatient emergence of OXA-567. M19969 and M21013 were positive by THT and negative for the Blue Carba Test and the double disk synergy test. As shown in Table 2, disc diffusion results were typical of OXA-163 producers, with low resistant to carbapenems and resistance to cephalosporins. In fact, $bla_{\text{OXA-163}}$ was confirmed by PCR and sequencing.

Table 2. Intra patient emergence of $bla_{\text{OXA-567}}$

Antimicrobial	M21014		M21013		M19969	
	mg/L	mm	mg/L	mm	mg/L	mm
Imipenem	> 8 R	17 R	ND	23 S	ND	26 S
Meropenem	32 R	10 R	ND	15 R	ND	28 S
Ertapenem	> 1 R	6 R	ND	6 R	ND	21 I
Ceftazidime	ND	24 S	ND	6 R	ND	13 R
Cefotaxime	ND	24 I	ND	7 R	ND	16 R
Ceftriaxone	≤ 1 S	ND	ND	ND	ND	ND
Cefepime	4 S	23 S	ND	8 R	ND	17 R
Cefoxitin	> 16 R	8 R	ND	6 R	ND	23 S
Piperacillin/tazobactam	> 64 R	9 R	ND	8 R	ND	10 R
Ciprofloxacin	> 2 R	6 R	ND	6 R	ND	16 R
Gentamicin	> 8 R	6 R	ND	6 R	ND	6 R
Amikacin	≤ 8 I/S	17 I	ND	18 S	ND	18 S
Trimethoprim/Sulfametoxazole	> 2/38 R	6 R	ND	6 S	ND	6 R
Date of isolation	March 2rd 2016		March 2nd 2016		Feb. 14th 2016	
Sample	Screening		Screening		Blood	
Kpn colony type	Mucous		Flat		ND	
Blue Carba Test	Negative		Negative		Negative	

THT-Meropenem	Positive	Positive	Positive
EDTA or Boronic Acid-Meropenem/Imipenem synergy disk test	Negative	Negative	Negative
K-Set or PCR for OXA-48-like	Positive	Positive	Positive
Sequenced variant	*bla*$_{OXA-567}$	*bla*$_{OXA-163}$	*bla*$_{OXA-163}$

ND, not determined; R, resistant; S, susceptible; I, intermediate.

Furthermore, the three isolates, M19969, M21013 and M21014 were genetically related by PFGE (Supplementary figure 1). This was further confirmed by MLST, given that all isolates belonged to ST6838. Then, the plasmid profile was inspected by S1-PFGE, and we found that the three isolates harbored two plasmids of equal size, one of 250 kb band the other of ca. 170 kb, coherent with the size of the sequenced plasmid detailed in section 3.7 (Figure 3). Furthermore, M19969 and M21013 harbored conjugative IncC$_2$ plasmids. Finally, *bla*$_{OXA163}$ gene was embedded in the same immediate genetic environment as *bla*$_{OXA-567}$ platform flanked by TnpA-IS*4321*–ΔIS4-like. Altogether, these results strongly suggest the intrapatient emergence of *bla*$_{OXA-567}$, an event that has already been observed in the past for OXA enzymes in Argentina [23].

4. Conclusions

We fully characterized two novel plasmids from the incompatibility group N$_2$ and C$_2$, harboring *bla*$_{OXA-438}$ and *bla*$_{OXA-567}$, the last one an *in vivo* mutant of *bla*$_{OXA-163}$. Experimental and *in silico* studies confirmed that these plasmids are transferable and harbored additional clinically relevant antimicrobial resistance determinants. Analysis of the immediate genetic environment suggests that *bla*$_{OXA-438}$ and *bla*$_{OXA-567}$ could have arisen from a founding capture event considering that they share identical genetic hallmarks. It is difficult to establish how these variants have been mobilized to reach IncN$_2$ (*bla*$_{OXA-438}$ and *bla*$_{OXA-163}$), IncC (*bla*$_{OXA-567}$) and incF

(*bla*$_{OXA-370}$) plasmids, considering that there are clearly no mobile genetic structures, only genetic scars from genetic events. Considering the wide dissemination of *bla*$_{OXA-163}$ in Argentina as well as the increasing number of allelic variants emerging as a consequence of active bacterial activity at the nosocomial level, more studies are required to prevent further emergence of these enzymes.

ACKNOWLEDGEMENTS

This study was supported by the National Agency for the Promotion of Research, Technological Development and Innovation (ANPCyT), PICT-2017-0321 to S.A.G. and the Ministry of Health, annual budget assigned to Antimicrobial Division (NRRLAR). S.A.G., B.G. and P.P. are members of the National Scientific and Technical Research Council (CONICET), Argentina. We would like to thank Stella Cristaldo for the technical assistance.

FUNDING

National Agency for the Promotion of Research, Technological Development and Innovation (ANPCyT), PICT-2017-0321 and the annual budget provided by the Ministry of Health, to Antimicrobial Division (NRRLAR).

ETHICS STATEMENT

All authors have no conflicts of interest and no ethics statement to declare

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Graphical Abstract