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2 **Title:**

3 LMB-1 producing *Citrobacter freundii* from Argentina, a novel player in the field of MBLs

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25

26 **Abstract**

27 Carbapenemase-producing Enterobacterales expressing OXA-48, KPC, NDM, VIM or IMP
28 enzymes are increasingly reported worldwide. We have characterized LMB-1, a novel metallo- β -
29 latamase (MBL) of Ambler class B3 from *Citrobacter freundii* 164 (*Cf164*) clinical isolate from Buenos
30 Aires, Argentina.

31 *Cf164* displayed reduced susceptibility to carbapenems but gave inconsistent results with
32 carbapenemase confirmatory tests, suggesting the presence of a weak carbapenemase. Analysis of WGS
33 of *Cf164* using Resfinder revealed four β -lactamase genes coding for CTX-M-8, PER-2, TEM-1 and
34 CMY-150, a novel chromosomally-encoded CMY variant. Kinetic parameters of purified CMY-150 did
35 not reveal any carbapenemase activity. However, CMY-150 conferred to *E. coli* higher MIC values for
36 ceftazidime and aztreonam as compared to CMY-2. The in-house developed β -lactamase search
37 software (ResMINER) in WGS data, revealed a novel subclass B3 MBL named LMB-1. LMB-1
38 conferred to *E. coli*, resistance to penicillins, to expanded-spectrum cephalosporins and reduced
39 susceptibility to carbapenems. The *bla*_{LMB-1} gene was located on a 176-kb IncA/C2 plasmid. LMB-1
40 shared 99% of amino acid sequence identity with the MBL encoded in the chromosome of
41 *Rheinheimera pacifica*, it's likely progenitor. Despite repeated attempts, LMB-1 could not be purified,
42 thus only specific activities could evidence hydrolysis of carbapenems.

43 Here we report CMY-150, a novel CMY-2 variant that confers increased ceftazidime and
44 aztreonam MICs to *E. coli* and the first description of LMB-1 in Argentina. This work underlines the
45 need for several CPE confirmatory tests, as this novel enzyme might have been missed using only one.

46

47 **1. Introduction**

48 Gram-negative bacteria (GNB), especially Enterobacterales, *Pseudomonas aeruginosa* and
49 *Acinetobacter baumannii*, have re-emerged as major players in antimicrobial resistance worldwide. In
50 these species, resistance may affect all major classes of anti-gram-negative agents, the multidrug
51 resistance (MDR) being relatively common. Currently, β -lactamase-mediated resistance does not spare
52 even the newest and most powerful β -lactams (carbapenems), whose activity is challenged by
53 carbapenemases [1].

54 Carbapenem-hydrolyzing β -lactamases encountered in Enterobacterales belong either to (i) the
55 Ambler class A including KPC, IMI and GES enzymes, (ii) Ambler class B metallo- β -lactamases (or
56 MBL) of NDM-, VIM- and IMP-type, and (iii) Ambler class D enzymes including OXA-48 and its
57 variants (mostly OXA-181, OXA-204 and OXA-232) [1]. Even though described for a few class C
58 enzymes, carbapenem-hydrolysis is very rare and weak [2,3].

59 MBLs are by far the most worrisome β -lactamases since their prevalence is increasing worldwide,
60 their broad substrate profile (including all β -lactams, except monobactams), and the lack of clinically-
61 useful inhibitors. The MBLs are divided into three subclasses (B1, B2, B3) based on primary amino
62 acid sequence homology [4]. There is relatively low sequence identity (< 20%) between the subclasses.
63 Within a subclass the sequence identity is higher and this, along with distinctive structural
64 characteristics within the active sites of B1, B2, and B3 enzymes, is the basis for the establishment of
65 the three subclasses. The B1 and B3 subclasses have a broad-spectrum substrate profile that includes
66 penicillins, cephalosporins, and carbapenems and contain two Zn^{2+} ions in their active site, while the B2
67 enzymes exhibit a narrow-spectrum profile that includes carbapenems and contain only one Zn^{2+} ion.

68 MBLs of subclass B1, such as NDM, VIM, and IMP-like are the clinically-most relevant enzymes
69 in Enterobacterales, but sporadic isolations of other class B1 MBLs such as GIM, FIM, KHM, have also
70 be reported [4]. Subclass B2 contains only two enzymes that possess a narrow-spectrum of hydrolysis.
71 The Subclass B3 is a larger group of enzymes, of which the genes are mostly chromosomally-encoded,
72 and display a broad-spectrum of β -lactam hydrolysis. In this subclass only AIM, found in *P.*
73 *aeruginosa*, and SMB-1 in *Serratia marcescens* have been reported acquired [5,6].

74 While the increasing availability of commercial qPCR systems allows for the detection of the
75 most prevalent MBL families, molecular diagnostics clearly overlooks MBLs that belong to less
76 prevalent MBL-families. Furthermore, novel MBLs have recently been identified in clinical isolates:
77 TMB-1 in *Enterobacter hormaechei* and *Citrobacter freundii* from Tunisia [7] and LMB-1 in
78 *Enterobacter cloacae* from Austria [8]. In this work, we have characterized a novel chromosomally-
79 encoded CMY-variant: CMY-150 and the second clinical LMB-1-producing Enterobacterales, a *C.*
80 *freundii* clinical isolate recovered from a Hospital in Buenos Aires, Argentina.

81 **2. Materials and Methods**

82 **2.1. Bacterial strains.** *C. freundii* 164 (Cf164) was identified with matrix-assisted laser
83 desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MALDI Biotyper CA system,
84 Bruker Daltonics, Billerica, MA, USA). *Escherichia coli* TOP10 (Invitrogen, Saint-Aubin, France) was
85 used for cloning experiments and *E. coli* BL21 was used for over-expression of β -lactamases.

86 **2.2. Antimicrobial agents, susceptibility testing.** Antimicrobial susceptibilities were
87 determined by disk diffusion on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France) and
88 interpreted according to the EUCAST breakpoints, updated in 2018 (<http://www.eucast.org>). Minimal
89 inhibitory concentration (MIC) values were determined using the Etest technique (BioMérieux, Marcy-
90 l'Etoile, France).

91 **2.3. Confirmatory tests for carbapenemase-producing Enterobacteria.** Carbapenemase
92 activity was investigated using the in house Carba NP test, Blue Carba, Modified Hodge test, β Carba
93 Test (BioRad, Marnes-la-Coquette, France), rCIM, Maldi-TOF (MBT STAR-Carba™, Bruker), PCRs
94 and NG Carba5 (NG Biotech, Rennes, France) tests were performed following the manufacturer's
95 recommendations or as previously published [9-12].

96 **2.4. PCR, cloning experiments, and sequencing.** Whole-cell DNAs of Cf164 isolates were
97 extracted using the QIAamp DNA minikit (Qiagen, Les Ulis, France) and were used as templates for
98 PCR with the following primers: preCMY-150-A (5'-ATGATGAAAAAATCGAT-3') and preCMY-
99 150-B (5'-TCTGTCAGTTATTGCAGT-3') and preLMB-1-A (5'-ATGACGTTAGCTAAAAGTTTT -
100 3') and preLMB-1-B (5'-TACTGCGTTGTTTCCTTTATTTAAA-3') from amplifying *bla*_{CMY-150}, and
101 *bla*_{LMB-1}, respectively. The amplicon obtained was then cloned into the pCR®-Blunt II-TOPO® plasmid

130 (Qiagen) after quality trimming ($Q_s \geq 20$) according the manufacturer's recommendations (Qiagen,
131 Courtaboeuf, France). *C. freundii* Cf164 total DNA was also sequenced using PacBio long read
132 technology (Macrogen, Seoul, South Korea). PacBio subreads were assembled with both Canu [13] and
133 the RS_HGAP_Assembly.3 protocol from the SMRT analysis toolkit v2.3, while consensus accuracy
134 was further polished using Quiver [14] as was previously described [15]. The acquired antimicrobial
135 resistance genes were identified by uploading assembled genomes to the Resfinder server v2.1 that uses
136 BLAST for identification of acquired antimicrobial resistance genes and chromosomal mutations in
137 whole-genome data (<http://cge.cbs.dtu.dk/services/ResFinder-2.1>) [16]. Additionally, the whole genome
138 sequence of Cf164 was also uploaded to an in-house developed software, ResMiner (BI Iorga, pers.
139 comm.), that searches for flags specific of the four molecular classes of β -lactamases.

140 For the reconstruction of pCf164_LMB-1 and pCf164_CTX-M-8 gaps were filled by PCR
141 amplification and Sanger sequencing. Open reading frames (ORFs) were annotated using the RAST
142 server (rast.nmpdr.org) followed by manual comparative curation and determination of sequence
143 similarity using the BLAST web server. Alignments were performed by using the BRIG tool [17]

144 **2.6. Plasmid characterization and conjugation assay.** Plasmid DNA of the clinical isolates
145 Cf164 was extracted using the Kieser method [18]. The Kieser extract was used to transform *E. coli*
146 TOP10 strain by electroporation. The electroporants were plated on a TSA plate containing 100 μ g/ml
147 ampicillin. Transformants were analyzed by PCR using the primers pre-LMB-1A and pre-LMB-1B.
148 From the transformants harboring the *bla*_{LMB-1} gene, plasmid DNA extraction was done using the
149 Kieser's method and subsequently analyzed on 0.7% agarose gel stained with ethidium bromide.
150 Plasmids of ca. 154, 66, 48, and 7 kb of *E. coli* NCTC 50192 were used as plasmid size markers [19].

151 To perform the quantitative filter mating-out assay, the clinical isolates Cf164 was used as
152 donor and *E. coli* J53 as recipient. Both isolates were each grown overnight in brain heart infusion
153 (BHI) (BioMérieux). A 0.25 ml of each donor and recipient donor culture were diluted in 4.75 ml BHI
154 broth and incubated at 37°C for 5 h without shaking. Subsequently, 0.25 ml of the donor culture was
155 gently mixed with 2.5 ml of the recipient culture, and 200 μ l of this mating mix was spotted on a 0.45-
156 μ m filter (Millipore, Paris, France) placed on a prewarmed MH plates and incubated at 37°C for 2 h.
157 The mating assays were terminated by placing the filters in 4 ml of an ice-cold 0.9% NaCl solution,

158 followed by vigorous agitation for 30 s. The number of transconjugants per donor cell was determined
159 by plating dilutions of the mating mixture onto plates containing antibiotics. Strain donor cells were
160 selected with 100µg /ml ampicillin. Transconjugants were selected with 100µg/ml ampicillin and
161 100µg/ml azide. Transfer frequencies were calculated by dividing the number of transconjugants by the
162 number of donor cells.

163 **2.7. β -Lactamase purification.** An overnight culture of *E. coli* strain BL21 (DE3) harboring
164 pET41b-CMY-150, pET41b-LMB-1 and pET28a-LMB-1 were used to inoculate 2 L of LB broth
165 containing 50 µg/ml kanamycin. The bacteria were cultured at 37°C until reaching an OD of 0.6 at 600
166 nm. Expression of both enzymes was induced overnight at 25°C with 0.2 mM IPTG, as previously
167 described [20]. For pET41b-LMB-1 additional induction conditions were tested (temperature: 16°, 25°,
168 37°; IPTG: 0.2mM, 0.5mM, 1.0mM; cells: *E. coli* BL21 (DE3), *E. coli* BL21 (DE3) pLysS,
169 *E. coli* Rosetta™ (DE3)). Cultures were centrifuged at 6000 g for 15 min and the pellets were
170 resuspended in 10 mL of Buffer A (20 mM phosphate buffer, 175 mM potassium sulfate (K₂SO₄),
171 40mM Imidazol, pH 7.40). The cells were disrupted by sonication and bacterial debris was removed by
172 two consecutive centrifugation steps: at 10.000 g for 1 h at 4°C, and at 48.000 g for 1 h at 4°C. CMY-
173 150 and LMB-1 were purified in one step pseudo-affinity chromatography using a NTA-Ni column (GE
174 Healthcare, Les Ulis, France) [20]. Protein purity was estimated by SDS-PAGE, pure fractions were
175 pooled and dialyzed against 20mM Hepes, K₂SO₄ 50mM buffer (pH 7) and concentrated using
176 Vivaspin® columns (GE Healthcare). Protein concentration was determined by Bradford Protein assay
177 (Bio-Rad) [20].

178 **2.8. Steady-state kinetic parameters.** Kinetic parameters of purified CMY-150 were
179 determined at 30°C in 100mM sodium phosphate buffer (pH 7). The k_{cat} and K_m values were determined
180 by analyzing hydrolysis of β -lactams under initial-rate conditions with an ULTROSPEC 2000 model
181 UV spectrophotometer (GE Healthcare) using the Eadie–Hoffstee linearization of the Michaelis–
182 Menten equation, as previously described [20]. The different β -lactams were purchased from Sigma–
183 Aldrich (Saint-Quentin-Fallavier, France).

184 The specific activities of the β -lactamase LMB-1 for β -lactams were determined on the
185 supernatant of a whole cell crude extract obtained from an overnight culture of pTOPO-LMB-1 in *E.*
186 *coli* TOP 10 as previously described [19].

187 **2.9. Nucleotide sequence accession number.** The whole genome sequence of *Cf164* generated
188 in the study have been submitted to the Genbank nucleotide sequence database, under the accession
189 number of NZ_QCWX01000001. The nucleotide sequence of *bla*_{CMY-150} genes along with plasmid
190 pCf164_LMB-1 and pCf164_CTX-M-8 have been submitted to the EMBL/Genbank nucleotide
191 sequence database under the accession numbers NG_060513, MH475146 and MN187903, respectively.

192

193

194 **3. Results and discussion**

195 **3.1. Clinical case.** A 68-year-old patient with Non-Hodgkin lymphoma, type I diabetes and
196 history of previous myocardial infarction, was admitted in a tertiary care hospital in Buenos Aires. After
197 12 days of the hospitalization, he presented febrile neutropenia and a catheter-related bacteraemia due
198 to a methicillin resistant *Staphylococcus haemolyticus*. At the same time, the urine culture revealed a
199 bacterial count of 10⁵ CFU/ml of a *C. freundii* isolate. The patient was treated with vancomycin and
200 amikacin that led to the resolution of the urinary tract infection and the bacteraemia.

201 **3.2. Characteristics of *C. freundii* 164.** Routine antibiogram and MIC values of *Cf164*
202 revealed penicillins, aztreonam, expanded-spectrum cephalosporin resistance and reduced susceptibility
203 to imipenem (Table 1). As the MIC for imipenem was 0.5 μ g/ml and even though the MIC for
204 meropenem was below 0.125 μ g/ml, which is the EUCAST screening cut off for carbapenemases, the
205 blue carba carbapenemase confirmatory test was performed. The latter gave a weak positive result
206 suggesting the presence of a carbapenem-hydrolyzing β -lactamase. These results were confirmed using
207 different biochemical confirmatory tests based on imipenem hydrolysis (β Carba, rCIM and MBT
208 STAR-CarbaTM tests) which were consistently positive except for the Carba NP that remained
209 repeatedly negative. PCRs detecting the five most prevalent carbapenemases (*bla*_{OXA-48}, *bla*_{KPC}, *bla*_{NDM},
210 *bla*_{VIM}, *bla*_{IMP}) and the immunochromatographic NG Carba5 test were negative, suggesting the presence
211 of a minor carbapenemase. WGS data of *Cf164* was analyzed using CLC genomic workbench (Qiagen)

212 and revealed a genome of 5,229,602 bp, with an average 50 X coverage (NZ_QCWX01000001).
213 Resistome was analyzed by searching acquired genes using Resfinder and point mutations involved in
214 chromosomal resistance. Four β -lactamase genes were identified, a novel chromosomal and natural
215 *bla*_{CMY-2-like} gene, *bla*_{CTX-M-8} and *bla*_{PER-2} coding for two ESBLs, and *bla*_{TEM-1}, coding for a broad-
216 spectrum penicillinase. The isolate harbored also two *qnr* resistance genes (*qnrE* and *qnrB38*), a
217 rifampicin resistance gene (*aar-3*), a sulfonamide resistant gene (*sul1*) and a trimethoprim resistance
218 gene (*dfrA27*). The finding of these genes matched well with the resistance phenotype of *Cf164* to
219 trimethoprim and to nalidixic acid. Mutations in the QRDR, known to confer fluoroquinolone resistance
220 were not found explaining the susceptibility to fluoroquinolones.

221 **3.3. Characterization of β -lactamase CMY-150.** CMY-150 differs from CMY-2 by 15 amino-
222 acid substitutions (L6I, V52I, Q55E, Q122R, R125S, R146T, A150E, H153R, A182V, P185H, N214S,
223 D218N, H262L, A273E, V368A) and for CMY-65 by two amino-acid substitutions (P185H, H262L).
224 CMY-150 and CMY-2 were cloned into the pCR®-Blunt II-TOPO® plasmid (pTOPO-CMY-150, and
225 pTOPO-CMY-2) and transformed into *E. coli* TOP10. CMY-150 conferred a higher level of resistance
226 to cefotaxime (>256 μ g/ml vs 8 μ g/ml for CMY-2), ceftazidime (>256 μ g/ml vs 24 μ g/ml for CMY-2)
227 and aztreonam (32 μ g/ml vs 3 μ g/ml for CMY-2) (Table 1). MICs for carbapenems were
228 heterogeneous, with imipenem being the highest (MIC 0.38 μ g/ml), meropenem (MIC 0.047 μ g/ml) and
229 ertapenem (MIC 0.047 μ g/ml) remaining in the susceptibility range. The MICs for carbapenems
230 conferred by pTOPO-CMY-150, even though in the susceptibility range, were slightly higher than those
231 conferred by CMY-2 (Table 1). In front of these results, MICs for imipenem for *Cf164* was performed
232 on cloxacillin-containing (250 μ g/ml) MH plates and revealed the exact same value, suggesting that
233 another enzyme capable of hydrolyzing carbapenems might be present in these bacteria.

234 To compare the kinetic properties of CMY-2 and the new CMY-150, the steady state
235 parameters of the pure proteins were determined (Table2). Overall, the catalytic efficiencies were very
236 similar between the two enzymes, except for ampicillin (40-fold higher for CMY-2). The kinetic
237 parameters for aztreonam could not be obtained since no hydrolysis was observed. For this reason, we
238 determined the IC₅₀ values for CMY-2 and CMY-150, revealing similar results for both enzymes (0.2
239 μ M for CMY-2 and 0.31 μ M for CMY-150). These results cannot explain the difference observed in

240 the MIC values obtained and although further analysis are required to understand this phenotype, we
241 suggest that the CMY-150 could present a higher affinity for aztreonam than CMY-2. Regarding the
242 kinetic parameters for imipenem, in both enzymes the K_m values were highly similar as well as the
243 activity, further suggesting that another enzyme capable of hydrolyzing carbapenems might be present
244 in *Cf164*.

245 **3.4. Identification of bla_{LMB-1} gene in the WGS of *Cf164*.** Using an in-house developed
246 software, ResMiner (BI Iorga, pers. comm.), that searches for flags specific of the four molecular
247 classes of β -lactamases, found in addition to $bla_{CMY-150}$, $bla_{CTX-M-8}$, bla_{PER-2} and bla_{TEM-1} genes, an
248 additional gene coding for a putative MBL of sub class B3. Blast searches revealed that this MBL had
249 100 % sequence identity with LMB-1, a MBL found only once if an *E. cloacae* isolate from Linz in
250 Austria (Accession number KU646836) [8].

251 **3.5. Susceptibility of LMB-1-producing isolates and kinetic properties of LMB-1.** The
252 expression of LMB-1 in *E. coli* Top10 resulted in increased MICs of penicillins, cephalosporins and
253 carbapenems, but not of aztreonam (Table 1). A slight inhibition of β -lactamase inhibitors on the MICs
254 could be detected. Of note, the MIC of cefepime was lower than that of ceftazidime and MICs of
255 aztreonam were comparable to that of *E. coli* TOP 10, confirming the MBL behavior of LMB-1.

256 As purification of LMB-1 failed despite repeated attempts in different conditions, crude lysates
257 of *E. coli* TOP10 pTOPO-LMB-1 were used to determine β -lactam specific activities. It revealed strong
258 specific activity against carbapenems, oxyimino cephalosporins and aminopenicillins (Table 3). No
259 specific activity against aztreonam nor cefepime, as previously reported (8), could be detected. When
260 compared to NDM-1 expressed in the same conditions, LMB-1 had slightly higher (2-fold) activity
261 against ceftazidime, whereas the activity against carbapenems was c.a. 8-fold lower (Table3). It is
262 important to highlight that the MIC values for carbapenems in *Cf164* were lower than those obtained
263 with *E. coli* Top10 carrying the natural plasmid pCf164_LMB-1 and with *E. coli* Top10 carrying
264 pTOPO-LMB-1. The latter results, together with the low specific activities against carbapenems as
265 compared to NDM-1, may explain the negative results obtained with NP Carba test, a test based on
266 imipenem hydrolysis.

267 Most of these results correlate well with the results of the comparative MIC studies confirming
268 activity of LMB-1 against aminopenicillins, ceftazidime, cefotaxime and carbapenems. However, in the
269 comparative MIC studies, expression of LMB-1 accounted for an 8-fold increase in the MIC of
270 cefepime, which indicates that LMB-1 does indeed present activity against cefepime. The fact that no
271 specific activity could be detected against cefepime within 10 min may indicate that the hydrolysis rates
272 of LMB-1 for this substrate may be very low yet the hydrolysis rate appears to have been sufficiently
273 high to account for a substantial increase in the MIC of cefepime, or the affinity of LMB-1 for cefepime
274 may be high enough to trap the antibiotic, which is reflected in the raise of the MIC value.

275 **3.6. Characterization of plasmids and genetic environments.** PacBio assembly analysis
276 revealed 12 contigs, two of them belonging to plasmidic DNA. The presence of two plasmids was
277 confirmed by Kieser extraction (Figure 1). *bla*_{LMB-1} gene was inserted onto a conjugative IncA/C type
278 plasmid of 175,712bp, pCf164_LMB-1 (accession number MH475146.1) (Figure 1), that also carried
279 *bla*_{PER-2} gene. pCf164_LMB-1 was different from the IncFIB(K)-type pNRZ-10170-LMB-1 plasmid
280 found in *E. cloacae* NRZ-10170 from Austria (accession number MH056209.1) [8] except for the
281 immediate genetic environment of *bla*_{LMB-1} gene.

282 The comparison of the immediate genetic environment of *bla*_{LMB-1} in pNRZ-10170-LMB-1 and
283 in pCf164_LMB-1 revealed an identical area between a *qacG* gene upstream of *bla*_{LMB-1} gene and *sulI*
284 gene downstream of *bla*_{LMB-1} gene (Figure 2). This genetic organization is similar to a complex class 1
285 integron with the presence of *ISCR1* element. Toleman *et al.* reported the mobilization of class 1
286 integrons by *ISCR1* through the transposition of *ISCR1* next to the 3' end of the class 1 integron
287 (*qac/sul* genes) and the transposase misreading of the *terIS* sequence [21]. The segment of DNA
288 involving the 3' end of the class 1 integron and the 5' end of the *ISCR1* is deleted erasing the normal
289 termination site, *terIS* and thereby creating an integron-*ISCR1* fusion. From this point on, *ISCR1* is
290 able to mobilize the integron [21]. It is likely that the similarities observed in the genetic environment
291 of *bla*_{LMB-1}, in pNRZ-10170-LMB-1 and in pCf164_LMB-1, might be the result of *bla*_{LMB-1} gene
292 mobilization mediated by an *ISCR1*, among other events. LMB-1 shares 99% sequence identity with a
293 predicted MBL (SEI10464) from the marine bacterium *R. pacifica*, as previously shown [8].

294 IncA/C plasmids were among the earliest antimicrobial resistance-encoding plasmids identified
295 in Gram-negative bacteria and have received attention due to their broad host range and their
296 association with the emergence of multidrug resistance in enteric pathogens of humans and animals
297 [22]. IncA/C plasmids are of high-molecular-weight low-copy-number and are considered responsible
298 for the dissemination of the clinically-relevant β -lactamase genes e.g. *bla*_{CTX-M}, *bla*_{CMY}, *bla*_{NDM}, *bla*_{IMP},
299 *bla*_{VIM}, and *bla*_{KPC} genes, among others [23,24]. In a nucleotide BLAST search for closely related
300 plasmids to pCf164_LMB-1, two IncA/C plasmids were found, namely in, *Salmonella enterica* strain
301 CFSAN064034 plasmid pGMI17-002_1 (CP028170) and in *Vibrio cholerae* genome assembly *V.*
302 *cholerae* 116-17a plasmid pNDM-116-17 both isolated in India (LN831185). In addition, plasmid
303 pCf587 harboring PER-2 (MG053108) recovered in Argentina from *C. freundii* 33587, also belongs to
304 IncA/C type plasmids and is related to pCf164_LMB-1 [25]. PER-2 has been the second most prevalent
305 ESBL, after the pandemic CTX-Ms, in Argentina, accounting for nearly 10% and 5% of the oxyimino-
306 cephalosporin resistance in *K. pneumoniae* and *E. coli*, respectively [25]. Comparative sequence
307 analysis revealed a conserved backbone length and similar overall genetic arrangements (Figure 3).
308 pNRZ-10170-LMB-1 was also included in the analysis, showing similarities only in the immediate
309 environment of *bla*_{LMB-1} gene (Figure 3).

310 The second plasmid carried by Cf164, pCf164_CTX-M-8 (accession number MN187903), was
311 fully sequence by PacBio as a single contig. This 68,453 bp plasmid belonged to the IncM
312 incompatibility group and encoded plasmid replication, maintenance, and transfer genes and two
313 resistance gene regions (Figure 4). The first one contained the *bla*_{TEM-1A} penicillinase and the
314 second one of 3,560 bp, contained the *bla*_{CTX-M-8} gene bracketed by two copies of IS26 likely forming a
315 composite transposon [2]. Overall, the nucleotide sequence of pCf164_CTX-M-8 was extremely similar
316 to that of pKp145-11b recovered from a *K. pneumoniae* clinical isolate from Brazil (GenBank accession
317 number KX118608) [26]. The differences between these two plasmids are based on the resistance genes
318 present next to the *bla*_{TEM-1A} gene. Indeed, pKp145-11b harbored a Tn1331-like element, that carried in
319 addition to *bla*_{TEM-1A} gene, three other resistance genes *bla*_{OXA-9} gene, *ant*(3')-Ia gene and *aac*(6')-Ib
320 gene [26], as previously described [27]. Finally, as reported pKp145-11b had high nucleotide sequence

321 identity with plasmid pKp41M (accession number KY781949), another IncM plasmid carried by a *K.*
322 *pneumoniae* isolate recovered from a parrot in Brazil [26]. To the best of our knowledge, this is the first
323 report of *bla*_{CTX-M-8} gene carried by an IncM type plasmid reported in Argentina, suggesting that this
324 epidemic plasmid is not only circulating amongst human and animal in Brazil, but is now also spreading
325 to the south of the continent.

326 **4. Conclusion**

327 In conclusion, this is the first report of LMB-1, a novel MBL of sub class B3, carried by a
328 IncA/C type plasmid. Our data suggests that *R. pacifica* is likely the progenitor of LMB-1, and that two
329 independent mobilization events have occurred on two different plasmids, in two different species, and
330 on two distantly located continents. The dissemination of the conjugative pCf164_LMB-1 represents a
331 public health threat as it combines a prevalent ESBL gene (*bla*_{PER-2}) with a difficult to detect
332 carbapenemase gene and that both genes are harbored by a broad-spectrum and conjugative IncA/C
333 plasmid that may spread to many human pathogens.

334 LMB-1 displays hydrolytic activities against most β -lactams, especially carbapenems and
335 cephalosporins, that are important antimicrobial agents for the treatment of severe nosocomial
336 infections caused by Enterobacterales. Even tough in the susceptibility range, animal models of
337 infections are required to evaluate whether carbapenems may be a suitable therapeutic option. The fact
338 that molecular assays, nor ResFinder, nor immunochromatography tests failed to identify this novel
339 MBLs, and that biochemical tests gave inconsistent results may lead to silent spread of this type of
340 enzyme. Thus, epidemiological studies to evaluate the extend of the dispersion of LMB-1 in Europe and
341 the Americas should be undertaken.

342

343

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350

351 **Conflict of interest**

352 LD is co-inventor of the Carba NP Test, which patent has been licensed to bioMérieux (La Balmes les
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354

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358

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433

434 **Table 1** MIC values of β -lactams for *C. freundii* 164, *E. coli* TOP10 pTOPO-CMY-150, *E. coli*
 435 TOP10 pTOPO-CMY-2, *E. coli* TOP10 pN-LMB-1, *E. coli* TOP10 pTOPO-LMB-1 and *E. coli*
 436 TOP10.

Antibiotic	MIC ($\mu\text{g/mL}$)					
	<i>C. freundii</i> 164	<i>E. coli</i> TOP10				<i>E. coli</i> TOP10
		(pTOPO- CMY-150)	(pTOPO- CMY-2)	(pCf164_ LMB-1)	(pTOPO- LMB-1)	
Amoxicillin	>256	>256	>256	>256	>256	2
Amoxicillin + CLA ^a	64	64	32	>256	24	2
Cefotaxime	>32	>32	8	>32	4	0.06
Ceftazidime	128	>256	24	>256	48	0.12
Cefepime	2	0.5	0.125	8	0.38	0.023
Imipenem	0.5	0.38	0.24	1.5	0.75	0.25
Meropenem	0.094	0.047	0.023	0.8	0.25	0.016
Ertapenem	0.064	0.047	0.012	0.5	0.19	0.003
Temocillin	64	24	16	>1024	512	4
Aztreonam	32	32	3	>256	0.064	0.047

437 ^aCLA, clavulanic acid (2 $\mu\text{g/ml}$)

438

439

440 **Table 2 .** Kinetic parameters of CMY-150 and CMY-2.

441

Substrate	<i>K_m</i> (μ M)		<i>k_{cat}</i> (s^{-1})		<i>k_{cat}/K_m</i> ($mM^{-1} s^{-1}$)	
	CMY-150	CMY-2	CMY-150	CMY-2	CMY-150	CMY-2
Ampicillin	55	16	0.30	3.2	5.4	204
Ceftazidime	13	15	0.04	0.05	3.1	3.3
Imipenem	3.7	2.5	0.02	0.02	5.8	9.6
Aztreonam	NH ^a	NH	NH	NH	-	-

442 ^a No hydrolysis

443

444 **Table 3.** Specific activities for β -lactamases NDM-1 and LMB-1.

Antibiotic	Specific Activity (mU/mg)	
	NDM-1	LMB-1
Cefalotine	0.41	0.44
Cefoxitine	0.44	0.81
Ceftazidime	0.38	0.24
Imipenem	7.42	0.88
Meropenem	5.95	0.88
Ertapenem	6.55	0.66

445 Experiments were done in triplicate. Error rates were within 10%

446

447

448 **Figure legends**

449

450 **Figure 1.** Plasmid extraction. Migration of the extracted pCf164_LMB-1 from the *Cf164* (WT) and
451 the transconjugant *E. coli* J53 (Tc). M, marker.

452

453 **Figure 2.** Genetic environment of *bla*_{LMB-1}. Comparison of the genetic environment of *bla*_{LMB-1} gene
454 presents in *Cf164_LMB-1* with the environment of *bla*_{LMB-1}-like sequence in *Rheinheimera pacifica*
455 and *bla*_{LMB-1} gene in pNRZ-10170-LMB-1. Truncated ORFs are indicated by Δ.

456

457 **Figure 3.** Comparative genome analyses of several IncA/C group plasmids. Structural comparison of
458 pCf164_LMB-1 with those of the most similar IncA/C group plasmids and pNRZ-10170-LMB-1.

459

460 **Figure 4.** Plasmid sequence schematic representation. Major structural features of the plasmids
461 pKp145-11b (KX118608) and pCf164_CTX-M-8 (MN1879903). Common structures are
462 highlighted in gray. Resistance genes and boxed in black. The *bla*_{CTX-M-8} composite transposon is
463 also indicated.

464







