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Imported One-Day-Old Chicks as Trojan Horses for Multidrug-Resistant Priority Pathogens Harboring *mcr-9*, *rmtG*, and Extended-Spectrum β-Lactamase Genes

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ABSTRACT Antimicrobial resistance is a critical issue that is no longer restricted to hospital settings but also represents a growing problem involving intensive animal production systems. In this study, we performed a microbiological and molecular investigation of priority pathogens carrying transferable resistance genes to critical antimicrobials in 1-day-old chickens imported from Brazil to Uruguay. Bacterial identification was performed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry, and antibiotic susceptibility was determined by Sensititre. Antimicrobial resistance genes were sought by PCR, and clonality was assessed by pulsed-field gel electrophoresis (PFGE). Four multidrug-resistant (MDR) representative strains were sequenced by an Illumina and/or Oxford Nanopore Technologies device. Twenty-eight MDR isolates were identified as *Escherichia coli* (n = 14), *Enterobacter cloacae* (n = 11), or *Klebsiella pneumoniae* (n = 3). While resistance to oxyiminocephalosporins was due to $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-55}}$, and $bla_{\text{CMY-2}}$, plasmid-mediated quinolone resistance was associated with the *qnrB19*, *qnrE1*, and *qnrB2* genes. Finally, resistance to aminoglycosides and fosfomycin was due to the presence of 16S rRNA methyltransferase *rmtG* and *fosA*-type genes, respectively. Short- and long-read genome sequencing of *E. cloacae* strain ODC_Eclo3 revealed the presence of IncQ/*rmtG* (pUR-EC3.1; 7,400 bp), IncHI2A/*mcr-9.1*/$bla_{\text{CTX-M-2}}$ (pUR-EC3.2, ST16 [pMLST; 408,436 bp), and IncN2/*qnrB19*/*aacC3*/*aph(3″)-Ib* (pUR-EC3.3) resistance plasmids. Strikingly, the $bla_{\text{CTX-M-2}}$ gene was carried by a novel Tn*1696*-like composite transposon designated Tn*7337*. In summary, we report that imported 1-day-old chicks can act as Trojan horses for the hidden spread of WHO critical-priority MDR pathogens harboring *mcr-9*, *rmtG*, and extended-spectrum β-lactamase genes in poultry farms, which is a critical issue from a One Health perspective.

IMPORTANCE Antimicrobial resistance is considered a significant problem for global health, including within the concept of One Health; therefore, the food chain connects human health and animal health directly. In this work, we searched for microorganisms resistant to antibiotics considered critical for human health in intestinal microbiota of 1-day-old baby chicks imported to Uruguay from Brazil. We describe genes for resistance to antibiotics whose use the WHO has indicated to "watch" or "reserve" (AWaRe classification), such as *rmtG* and *mcr9.1*, which confer resistance to all the aminoglycosides and colistin, respectively, among other genes, and their presence in new mobile genetic

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elements that favor its dissemination. The sustained entry of these microorganisms evades the sanitary measures implemented by the countries and production establishments to reduce the selection of resistant microorganisms. These silently imported resistant microorganisms could explain a considerable part of the antimicrobial resistance problems found in the production stages of the system.

KEYWORDS extended-spectrum β-lactamase, multidrug-resistant pathogens, 1-day-old chicks, *mcr-9*, *rmtG*

Antimicrobial resistance is considered one of the 10 threats to global health in the WHO's 5-year strategic plan, within the 13th General Programme of Work (2019 to 2023) (https://www.who.int/news-room/spotlight/ten-threats-to-global-health-in-2019). In that sense, the United Nations has stated that antimicrobial resistance (AMR) is one of the biggest challenges we face as a global community (https://news.un.org/en/story/2018/11/1025511).

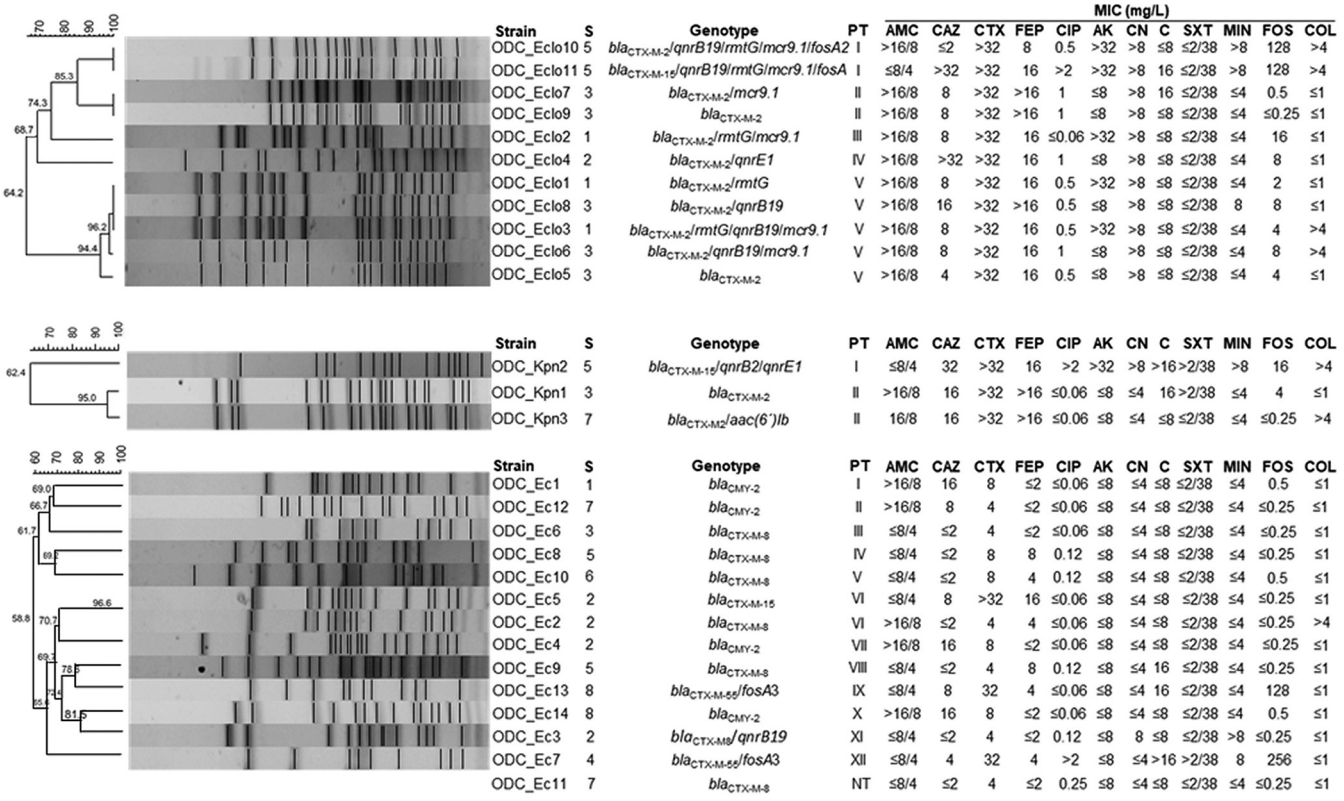
In 2017, the WHO established a list of microorganisms to be prioritized in regard to research and development of new antimicrobials, where third-generation cephalosporin-resistant *Enterobacterales* (TGCR-E) were included within the microorganisms of maximum interest (1); in addition, a list of important antimicrobials for human medicine categorized oxyiminocephalosporins, fluoroquinolones, colistin, and aminoglycosides as critically compromised drugs by the appearance and rapid spread of resistant bacteria (2).

Worryingly, most antibiotic use has been for livestock, and it is growing with the increase in global demand for animal protein, which is the main dietary trend in low- and middle-income countries (3). While in intensive animal production systems antimicrobials are used routinely to maintain health and productivity, a growing body of evidence has linked this practice with the occurrence of antimicrobial-resistant bacteria in food-producing animals (3, 4). In Latin America, several studies have shown the occurrence and transmission of critical-priority antibiotic-resistant *Enterobacterales* on poultry farms; however, the sources and routes of the acquisition have not been investigated (5–7). Here, we report the presence of transferable resistance genes for critically important antimicrobials in clinically relevant *Enterobacterales* isolated from the fecal matter of 1-day-old chicks imported from Brazil to Uruguay.

RESULTS

Identification of critical-priority MDR pathogens in the fecal matter of imported 1-day-old chicks. Seventy-two members of *Enterobacterales* were isolated and identified as *Escherichia coli* ($n = 37$), *Enterobacter cloacae* ($n = 30$), or *Klebsiella pneumoniae* ($n = 5$). Antibiotic susceptibility profiling and detection of antibiotic resistance genes were performed for the 72 isolates, and clonality was determined by pulsed-field gel electrophoresis (PFGE). To avoid duplicate results, from each shipment, only those isolates featuring phenotypic, and/or genotypic differences or different pulse types are presented. Accordingly, 28 isolates were selected, including 14 *E. coli*, 11 *E. cloacae*, and 3 *K. pneumoniae* isolates, which exhibited resistance to cefotaxime (100%), cefepime (75%), ceftazidime (67%), amoxicillin-clavulanic acid (64%), gentamicin (46%), and ciprofloxacin (39%). Lower resistance values (between 11% and 25%) were observed for trimethoprim-sulfamethoxazole, fosfomycin, minocycline, amikacin, and chloramphenicol. On the other hand, 7 isolates presented MIC values of >4 μg/ml for colistin. Conversely, all isolates were susceptible to carbapenems (Fig. 1).

Antibiotic resistance genes. Resistance to beta-lactams was due to the presence of $bla_{\text{CTX-M-2}}$ ($n = 12$), $bla_{\text{CTX-M-8}}$ ($n = 7$), $bla_{\text{CTX-M-15}}$ ($n = 4$), $bla_{\text{CMY-2}}$ ($n = 4$), and $bla_{\text{CTX-M-55}}$ ($n = 2$). Plasmid-mediated quinolone resistance genes were detected in eight isolates (Fig. 1), of which five carried *qnrB19*, whereas *qnrE1*, *qnrB2*, and *qnrE1* plus *qnrB19* were detected in one isolate each. Concerning aminoglycoside resistance, the 16S rRNA methyltransferase (16S-RMTase) *rmtG* was detected in 5 out of 6 amikacin- and gentamicin-resistant isolates (Fig. 1). On the other hand, the aminoglycoside acetylase gene



Strain	S	Genotype	PT	MIC (mg/L) AMC	CAZ	CTX	FEP	CIP	AK	CN	C	SXT	MIN	FOS	COL
ODC_Eclo10	5	$bla_{CTX-M-2}$/*qnrB19*/*rmtG*/*mcr9.1*/*fosA2*	I	>16/8	≤2	>32	8	0.5	>32	>8	≤8	≤2/38	>8	128	>4
ODC_Eclo11	5	$bla_{CTX-M-15}$/*qnrB19*/*rmtG*/*mcr9.1*/*fosA*	I	≤8/4	>32	>32	16	>2	>32	>8	16	≤2/38	>8	128	>4
ODC_Eclo7	3	$bla_{CTX-M-2}$/*mcr9.1*	II	>16/8	8	>32	>16	1	≤8	>8	16	≤2/38	≤4	0.5	≤1
ODC_Eclo9	3	$bla_{CTX-M-2}$	II	>16/8	8	>32	>16	1	≤8	>8	≤8	≤2/38	≤4	≤0.25	≤1
ODC_Eclo2	1	$bla_{CTX-M-2}$/*rmtG*/*mcr9.1*	III	>16/8	8	>32	16	≤0.06	>32	>8	≤8	≤2/38	≤4	16	≤1
ODC_Eclo4	2	$bla_{CTX-M-2}$/*qnrE1*	IV	>16/8	>32	>32	16	1	≤8	>8	≤8	≤2/38	≤4	8	≤1
ODC_Eclo1	1	$bla_{CTX-M-2}$/*rmtG*	V	>16/8	8	>32	16	0.5	>32	>8	≤8	≤2/38	≤4	2	≤1
ODC_Eclo8	3	$bla_{CTX-M-2}$/*qnrB19*	V	>16/8	16	>32	>16	0.5	≤8	>8	≤8	≤2/38	8	8	≤1
ODC_Eclo3	1	$bla_{CTX-M-2}$/*rmtG*/*qnrB19*/*mcr9.1*	V	>16/8	8	>32	16	0.5	>32	>8	≤8	≤2/38	≤4	4	>4
ODC_Eclo6	3	$bla_{CTX-M-2}$/*qnrB19*/*mcr9.1*	V	>16/8	8	>32	16	1	≤8	>8	≤8	≤2/38	≤4	8	>4
ODC_Eclo5	3	$bla_{CTX-M-2}$	V	>16/8	4	>32	16	0.5	≤8	>8	≤8	≤2/38	≤4	4	≤1

Strain	S	Genotype	PT	AMC	CAZ	CTX	FEP	CIP	AK	CN	C	SXT	MIN	FOS	COL
ODC_Kpn2	5	$bla_{CTX-M-15}$/*qnrB2*/*qnrE1*	I	≤8/4	32	>32	16	>2	>32	>8	>16	>2/38	>8	16	>4
ODC_Kpn1	3	$bla_{CTX-M-2}$	II	>16/8	16	>32	>16	≤0.06	≤8	≤4	16	>2/38	≤4	4	≤1
ODC_Kpn3	7	bla_{CTX-M2}/*aac(6')Ib*	II	16/8	16	>32	>16	≤0.06	≤8	≤4	≤8	≤2/38	≤4	≤0.25	>4

Strain	S	Genotype	PT	AMC	CAZ	CTX	FEP	CIP	AK	CN	C	SXT	MIN	FOS	COL
ODC_Ec1	1	bla_{CMY-2}	I	>16/8	16	8	≤2	≤0.06	≤8	≤4	≤8	≤2/38	≤4	0.5	≤1
ODC_Ec12	7	bla_{CMY-2}	II	>16/8	8	4	≤2	≤0.06	≤8	≤4	≤8	≤2/38	≤4	≤0.25	≤1
ODC_Ec6	3	$bla_{CTX-M-8}$	III	≤8/4	≤2	4	≤2	≤0.06	≤8	≤4	≤8	≤2/38	≤4	≤0.25	≤1
ODC_Ec8	5	$bla_{CTX-M-8}$	IV	≤8/4	≤2	8	8	0.12	≤8	≤4	≤8	≤2/38	≤4	≤0.25	≤1
ODC_Ec10	6	$bla_{CTX-M-8}$	V	≤8/4	≤2	8	4	0.12	≤8	≤4	≤8	≤2/38	≤4	0.5	≤1
ODC_Ec5	2	$bla_{CTX-M-15}$	VI	≤8/4	8	>32	16	≤0.06	≤8	≤4	≤8	≤2/38	≤4	≤0.25	≤1
ODC_Ec2	2	$bla_{CTX-M-8}$	VI	>16/8	≤2	4	4	≤0.06	≤8	≤4	≤8	≤2/38	≤4	≤0.25	>4
ODC_Ec4	2	bla_{CMY-2}	VII	>16/8	16	8	≤2	≤0.06	≤8	≤4	≤8	≤2/38	≤4	≤0.25	≤1
ODC_Ec9	5	$bla_{CTX-M-8}$	VIII	≤8/4	≤2	4	8	0.12	≤8	≤4	16	≤2/38	≤4	≤0.25	≤1
ODC_Ec13	8	$bla_{CTX-M-55}$/*fosA3*	IX	≤8/4	8	32	4	≤0.06	≤8	≤4	16	≤2/38	≤4	128	≤1
ODC_Ec14	8	bla_{CMY-2}	X	>16/8	16	8	≤2	≤0.06	≤8	≤4	≤8	≤2/38	≤4	0.5	≤1
ODC_Ec3	2	bla_{CTX-M8}/*qnrB19*	XI	≤8/4	≤2	4	≤2	0.12	≤8	8	≤8	≤2/38	>8	≤0.25	≤1
ODC_Ec7	4	$bla_{CTX-M-55}$/*fosA3*	XII	≤8/4	4	32	4	>2	≤8	≤4	>16	>2/38	8	256	≤1
ODC_Ec11	7	$bla_{CTX-M-8}$	NT	≤8/4	≤2	4	≤2	0.25	≤8	≤4	≤8	≤2/38	≤4	≤0.25	≤1

FIG 1 Pulsed-field gel electrophoresis (PFGE) profile of 28 1-day-old chicks (ODC). "Genotype" refers to resistance genes detected by PCR. Eclo, *E. cloacae*; Kpn, *K. pneumoniae*; Ec, *E. coli*; S, shipment; PT, pulsotype; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; CIP, ciprofloxacin; AK, amikacin; CN, gentamicin; C, chloramphenicol; SXT, trimethoprim-sulfamethoxazole; MIN, minocycline; FOS, fosfomycin; COL, colistin.

aac(6')Ib was detected in one additional isolate (Fig. 1). Two *E. coli* isolates harbored $bla_{CTX-M-55}$ plus *fosA3*, one *E. cloacae* isolate carried $bla_{CTX-M-15}$ plus *fosA*, and $bla_{CTX-M-2}$ and *fosA2* were identified in one *E. cloacae* strain (Fig. 1).

It is noteworthy that the colistin-resistance gene *mcr-9.1* was identified in four of the seven colistin-resistant isolates (MIC $>$ 4 μg/mL) and in two isolates displaying colistin MICs of $\leq$1 μg/mL (Fig. 1).

PFGE results. PFGE analysis of 11 *E. cloacae* isolates revealed 5 different pulsotypes (PTs). Two of the five pulsotypes (PT-I and PT-V) presented resistance genes to the three high-priority critical antibiotics studied. Interestingly, PT-V was detected in isolates recovered from two different shipments and had a wide variety of resistance genes, including the *mcr-9.1* and *rmtG* genes.

Of the 14 *E. coli* isolates, 13 were typed by PFGE and one was nontypeable. Twelve pulsotypes were identified; two isolates belonged to PT-VI, and although both were recovered from the same shipment, they carried different extended-spectrum β-lactamase (ESBL) genes ($bla_{CTX-M-8}$ versus $bla_{CTX-M-2}$) (Fig. 1). On the other hand, two *E. coli* isolates carrying $bla_{CTX-M-55}$ and *fosA3* belonged to different pulsotypes (i.e., PT-IX and PT-XII) (Fig. 1).

Regarding the three *K. pneumoniae* isolates, 2 pulsotypes were detected. Pulse type II comprised two strains, recovered from different shipments (3 and 7), which carried $bla_{CTX-M-2}$ ESBL genes (Fig. 1).

Whole-genome sequencing (WGS) analysis. *E. cloacae* isolates ODC_Eclo3 and ODC_Eclo10, showing resistance to colistin, oxyiminocephalosporins, and aminoglycosides, *K. pneumoniae* isolate ODC_Kpn3, showing resistance to colistin and oxyiminocephalosporins, and *E. coli* isolate ODC_Ec7, displaying resistance to oxyiminocephalosporins and fosfomycin, were sequenced. Isolates ODC_Eclo3 and ODC_Eclo10 belonged to sequence type 145 (ST145) and ST32, respectively, and shared the occurrence of four

FIG 2 Distribution of plasmid replicons and resistance genes among the four sequenced strains. Black cells indicate presence and white cells indicate absence.

plasmids of incompatibility groups IncQ1, IncHI2/2A, IncN2, and ColE1-like (Fig. 2). These isolates harbored 12 antibiotic resistance genes conferring resistance to beta-lactams, fluoroquinolones, colistin, aminoglycosides, fosfomycin, and folate pathway inhibitors (Fig. 2). Among such genes, *mcr-9.1*, *rmtG*, and $bla_{\text{CTX-M-2}}$ stand out on account of their relevance. Additionally, both isolates carried *fosA2* and $bla_{\text{ACT-90}}$ and $bla_{\text{ACT-52}}$-type AmpC genes.

On the other hand, *K. pneumoniae* ODC_Kpn3 belonged to ST37 and carried three plasmids of incompatibility groups IncFIB, IncN, and ColE1-like. This isolate harbored genes conferring resistance to four different antibiotic families: aminoglycosides [*aac(6′) Ib*, *aph(3″)Ib*, and *aph(6)Id*], beta-lactams ($bla_{\text{CTX-M-2}}$, $bla_{\text{OXA-2}}$, and $bla_{\text{SHV-11}}$), sulfonamides (*sul1*), and fosfomycin (*fosA2*) (Fig. 2). ODC_Kpn3 showed a MIC for colistin of greater than 4 μg/mL. Although we did not find resistance genes transferable to colistin, ODC-Kpn3 had three mutations in regulatory genes: two in *pmrB* (R256G and T246A) and one in *ccrB* (C68S). No mutations were observed in *pmrA*, *mgrB*, *phoP*, or *phoQ*.

Regarding the *E. coli* ODC_Ec7 strain, multilocus sequence typing (MLST) analysis showed that it belonged to the international ST224. Its resistance profile (beta-lactams,

including oxyiminocephalosporins, fosfomycin, trimethoprim-sulfamethoxazole, chloramphenicol, and ciprofloxacin) was associated with the presence of $bla_{CTX-M-55}$, bla_{TEM-1b}, *fosA3*, the tandem *dfr12/sul3*, *cmlA1*, and mutations in *gyrA* (D87N and S83L), *parC* (S80I), and *parE* (S458A). Additionally, strain ODC_Ec7 carried the *tetA* and *aadA1/aadA2* genes, responsible for resistance to tetracycline and streptomycin, respectively (Fig. 2). Furthermore, this strain belonged to serotype O114:H23, and based on its virulence profile, it corresponded to the extraintestinal pathogenic *E. coli* pathotype. *In silico* analysis indicated the presence of the following virulence genes: *papC*, *afaD*, *hra*, *iha*, and *lpfA* (coding for adhesins); *iucC*, *iutA*, and *sitA* (coding for siderophores and iron transporters); *iss* and *traT* (related to the survival to the bactericidal power of serum); the gene coding for EAST-1 toxin; and the genes *celB*, *cia*, *cma*, and *cvaC* (coding for colicins and microcins).

Taking into account the peculiar coexistence of resistance genes present in some isolates (i.e., *rmtG*, *mcr-9* and $bla_{CTX-M-2}$), we selected isolate ODC_Eclo3 for hybrid sequencing and analysis. In this regard, hybrid sequencing yielded the complete nucleotide sequences of the chromosome and the plasmids present in the aforementioned isolate.

Overall, the genome of isolate ODC_Eclo3 consisted of an ~4.8-Mb chromosome (GC content, 54.9%) and four plasmids, namely, pIncHI2/2A (408.4 kb; GC content, 50.28%), pIncN2 (59.3 kb; GC content, 52.82%), pIncQ (7.4 kb; GC content, 63.82%), and pColE1-like (11.5 kb; GC content, 63.82%).

Ten of the 12 resistance genes present in ODC_Eclo3 were distributed among three plasmids belonging to incompatibility groups IncQ, IncHI2/2A, and IncN2.

IncQ plasmid. The plasmid belonging to incompatibility group Q (pUR-EC3.1) featured a region spanning 6,137 bp with 99% identity to plasmid pRSF1010 (GenBank accession no. HE654726); this region was composed of 7 open reading frames (ORFs) coding for replication-related proteins RepA and RepC, the mobility proteins MobABC, two hypothetical proteins, and 394 bp belonging to the gene *sulII* (Fig. 3). Furthermore, genes *repC*, *mobA*, and *mobC* feature three chi sequences (5′-GCTGGTGG-3′) which act as targets for the *recBCD* products that mediate single-strand recombination events (8).

The *sulII* gene was interrupted by a 1,283-bp region comprising the genes *rmtG* and *rsmI*-like (Fig. 3). Both genes code for *S*-adenosylmethionine-dependent methyltransferases (*S*-AdoMet MTases); however, the former stands out on account of conferring resistance to a wide range of aminoglycosides. Interestingly, this 1,283-bp region was identical to that found in plasmid pEc13 (GenBank accession no. MH325469), detected in a Brazilian *Enterobacter hormaechei* isolate obtained from a urinary tract infection (UTI) (9).

IncHI2/2A plasmid. As previously mentioned, the plasmid belonging to incompatibility group IncHI2/2A, designated pUR-EC3.2, had a size of 408,436 bp, and the GC content was 50.28%. BLASTn analysis of pUR-EC3.2 showed that the closest matches corresponded to a series of "superplasmids" conferring carbapenem resistance, recently reported in Australia (10) (Fig. 4); interestingly, while the former were all typed as ST1, plasmid pUR-EC3.2 was assigned to the newly designated ST16 (11).

In order to characterize this new plasmid, we followed the scheme proposed by García-Fernández and Carattoli, based on the detection of 11 backbone amplicons and on the nucleotide sequence of *repA* (58). In this regard, pUR-EC3.2 replicon was 100% identical to HIA-R478 (its counterpart in plasmid pR478), consisting of the two iteron-based replication determinants RepHIA and RepHI2; however, when applying the aforementioned *in silico* scheme, we detected several interesting differences. While we found the five amplicons common to IncHI2 plasmids, pR478-related amplicons *tnsD* and *smrD* were missing; conversely, O1R_160, related to HI2-APEC-O1-R-type plasmids, was present in pUR-EC3.2. Based on these findings, we hypothesize that pUR-EC3.2 ST16 could be a new subtype of the IncHI2/2A plasmid pR478. In this context, $bla_{CTX-M-2}$ would constitute part of such a "hybrid" structure, since this ESBL gene is mainly associated with HI2-APEC-O1-R-type plasmids (10, 12, 13). So, to the best of our knowledge, this is the first description of a CTX-M-2-R478-derived plasmid.

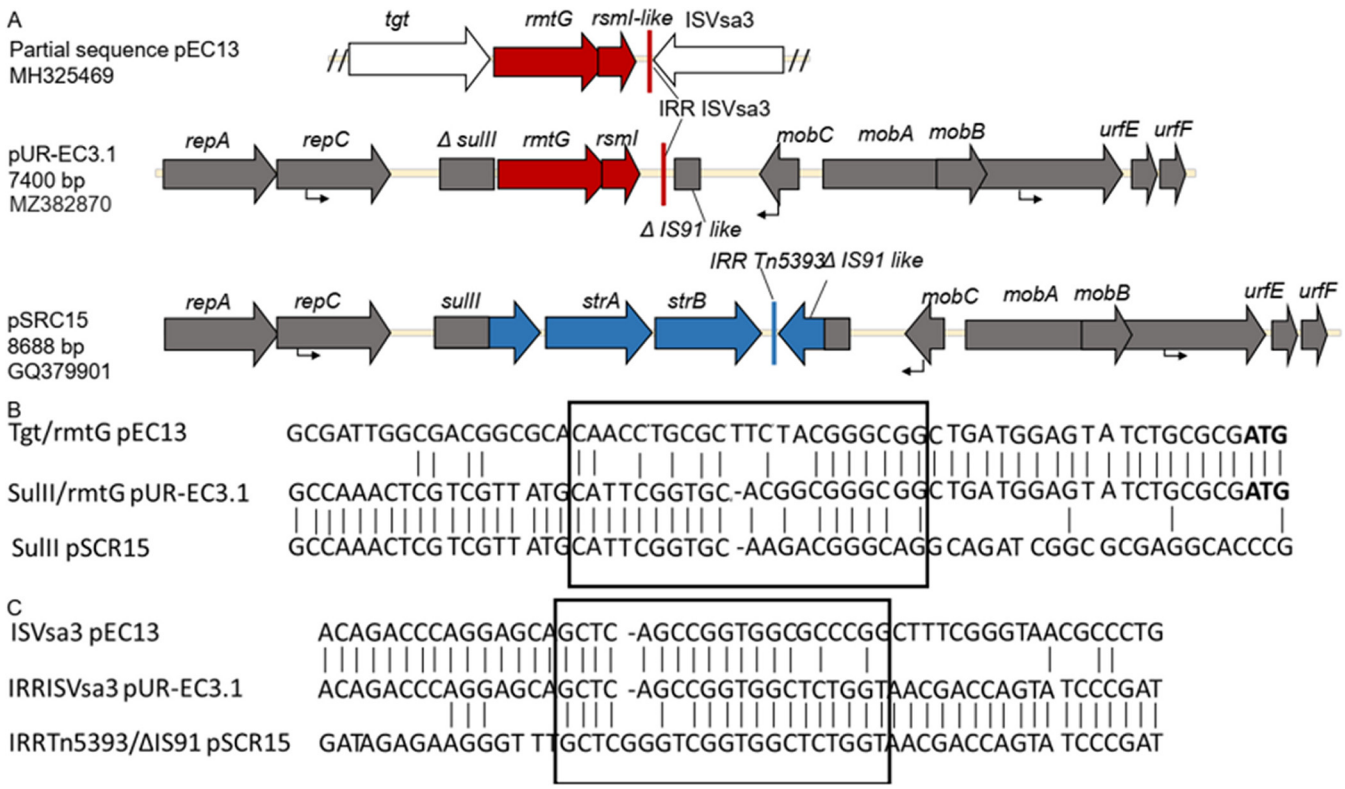


FIG 3 Schematic map of pUR-EC3.1 and comparison with pSRC15 and the *rmtG*-bearing region present in plasmid pEC13 (partial sequence). (A) Regions of nucleotide identity between pUR-EC3.1 and pSRC15 are shown in gray, whereas regions of nucleotide identity between pUR-EC3.1 and pEC13 are depicted in crimson. In blue are the genes putatively lost during recombination events. Black arrows indicate the location of Chi sequences, located in *repC*, *mobA*, and *mobC* genes and oriented in their respective direction of transcription. (B) Alignment of the 5′ terminus of the *rmtG*-coding region present in pUR-EC3.1 (Δ*sulII-rmtG*) with the corresponding *sulII* region in pSRC15 and the *tgt-rmtG* region present in pEC13. (C) Alignment of the 3′ terminus of the *rmtG*-coding region found in pUR-EC3.1 (IRR IS*Vsa3*::ΔIS*91*) with the region corresponding to IRR Tn*5393*::ΔIS*91-sulII* present in pSRC15 and with IS*Vsa3* in pEC13. The boxed sequences correspond to zones of high nucleotide identity between the three analyzed plasmids.

Regarding resistance genes, pUR-EC3.2 harbored several operons conferring resistance to heavy metals (mercury) and metalloids (arsenic and tellurium), as well as genes conferring resistance to oxyiminocephalosporins, aminoglycosides, sulfonamides, and polymyxins. In this context, resistance genes to the first 3 antibiotic families were encoded in a complex class 1 integron carrying *aadA1*, *aac(3′)-VIa*, as part of its variable region *qacEΔ1*, *sul1* as part of 3′CR, $bla_{CTX\text{-}M\text{-}2}$, linked to an IS*CR1*, and a second copy of the 3′CR formed by a *orf3*/*qacEΔ1* fusion and *sul1*. This integron has already been described fir plasmid pESBL3227-IncI (13), flanked by Tn*As3* and IS*91*; nevertheless, in pUR-EC3.2 it was embedded in a novel Tn*1696*-like composite transposon designated Tn*7337* (Fig. 5).

Tn*7337* maintains the genes *tnpA*, *tnpR*, the IRR (inverted repeat right) and IRL (inverted repeat left) inverted repeats (the former interrupted by IS*4321R*), the first 30 nucleotides of IS*6100* IRL, and a truncated copy of the mercury resistance operon *merRTPCADE* (Tn*7337* is missing the 3′ region of *merE*). Unlike Tn*1696*, reported by Partridge et al. (14), Tn*7337* features a copy of IS*26* instead of the 1,733-bp region including IS*6100*, *urf2*, and 101 bp corresponding to the 3′ region of *merE* (Fig. 5).

Furthermore, the insertion of IS*26* resulted in the deletion of the 3′ terminus of the *merE* gene; further studies would be required in order to determine whether the insertion of IS*26* has hampered the function of the *mer* operon.

On the other hand, putative plasmid resistance to polymyxins was accounted for by the occurrence of phosphoethanolamine transferase gene *mcr-9.1*. This gene was flanked upstream by an IS*903*-like transposase and downstream by the cupin fold metalloprotease gene, *wbuC*, followed by IS*26*; a similar context has been described for various IncHI2/2 plasmids in *E. cloacae* (GenBank accession no. CP020529.1), *E. hormaechei*

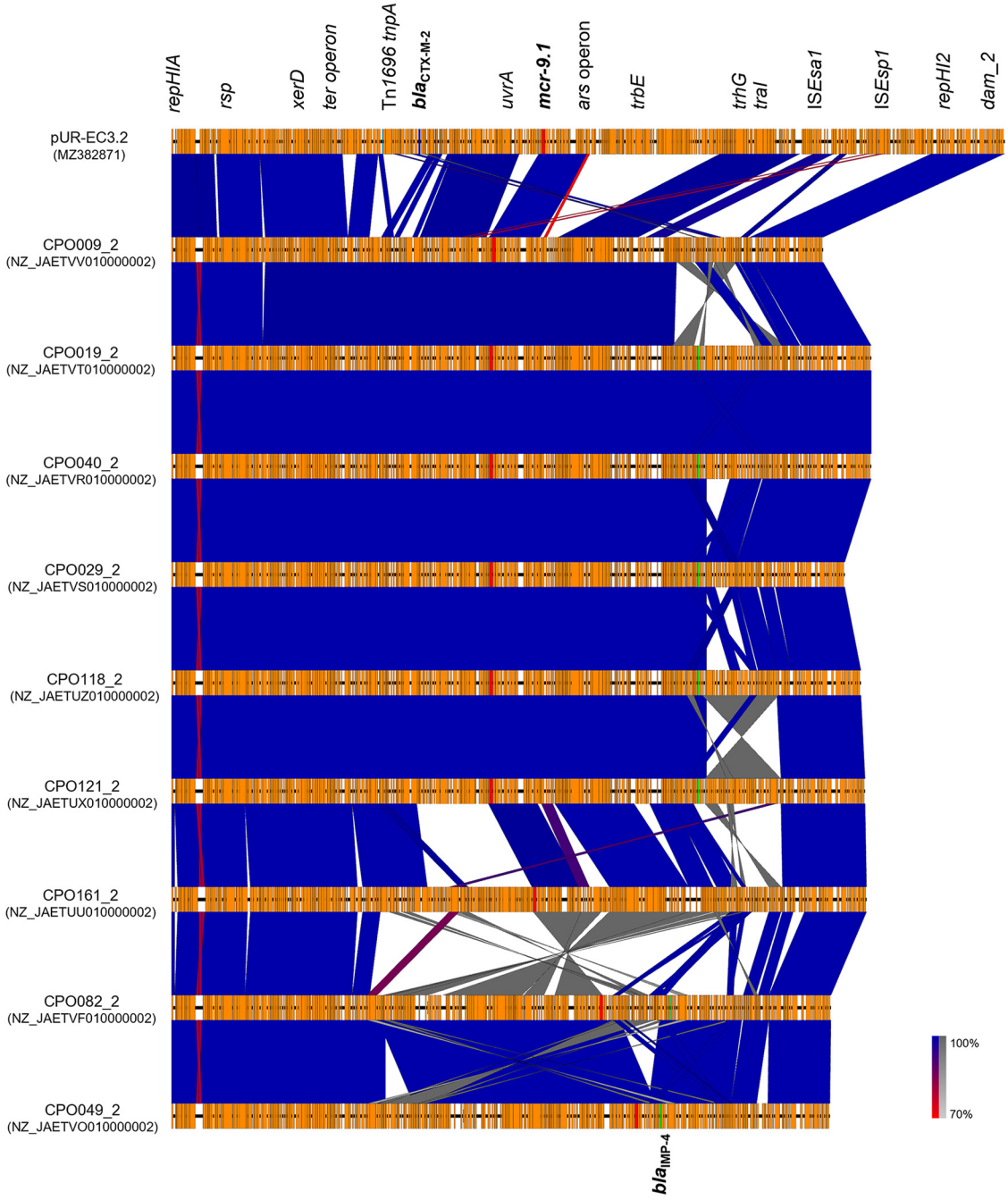


FIG 4 Linear alignment of plasmid pUR-EC3.2 with a group of recently described *mcr-9.1*-bearing plasmids (accession numbers are in parentheses). Regions of maximum nucleotide identity are indicated in deep blue, and regions of minimum identity are depicted in red. Inverted regions between different plasmids are shown in gray. Genes conferring resistance to critically important antibiotics in pUR-EC3.2, $bla_{CTX\text{-}M\text{-}2}$ and *mcr-9.1*, are shown in cyan and red, respectively; likewise, *mcr-9* alleles in the other plasmids are depicted in the same color. Carbapenem resistance gene $bla_{IMP\text{-}4}$ is shown in bright green.

(GenBank accession no. CP031575.1), and *Cronobacter sakazakii* (GenBank accession no. CP028975.1), among others (10, 15). Nevertheless, compared to such plasmids, the orientation of IS*26* in pUR-EC3.2 was reversed (i.e., located in the same strand as *wbuC*, *mcr-9.1*, and IS*903*-like). Although the genetic context of *mcr-9.1* in pUR-EC3.2 also included an ~30-kb conserved upstream region (containing the *rcnR/rcnS* nickel-cobalt efflux transporters, sensory proteins, and the arsenic resistance operon), the *qseB/qseC* two-component system that takes part in the expression of *mcr-9.1* was missing. *In silico*

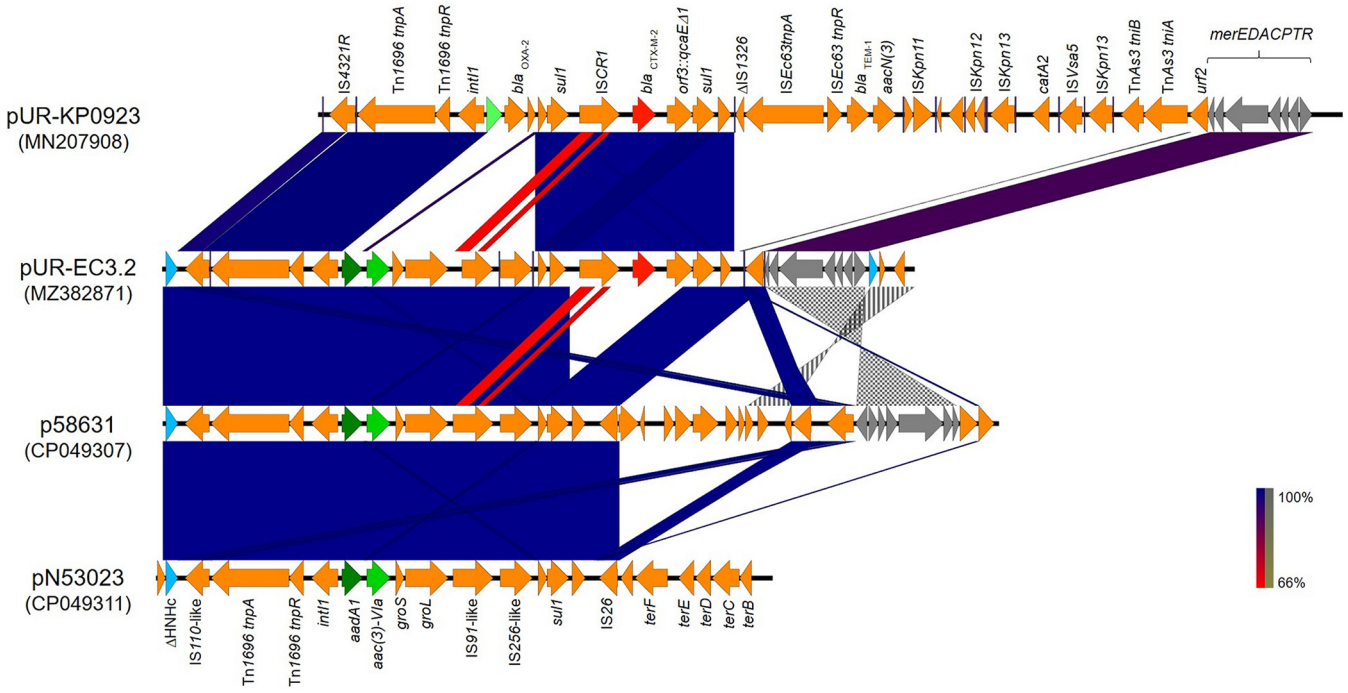


FIG 5 Comparison between the Tn*7337* containing the complex class 1 integron carrying $bla_{CTX\text{-}M\text{-}2}$ in pUR-EC3-2, and similar genetic contexts described for other plasmids (accession numbers are in parentheses). The alignment is drawn according to the best BLAST; homology blocks in deep blue indicate maximum nucleotide identity, whereas red blocks indicate regions of minimum nucleotide identity. Gray checkered or striped blocks show inverted regions. CDS color code: red, $bla_{CTX\text{-}M\text{-}2}$; hues of green, aminoglycoside-modifying genes; slate gray, mercury resistance operon; cyan, 5′ and 3′ regions of a truncated HNHc endonuclease. Vertical lines depict inverted repeats associated with the various insertion sequences.

promoter prediction analyses indicated the occurrence of putative promoter regions close to a gene; further studies are required in order to assess the expression of *mcr-9.1*. In this regard, plasmid transfer assays have proven unsuccessful so far. The occurrence of $bla_{CTX\text{-}M\text{-}2}$ and *mcr-9.1* has already been documented, albeit separately, in IncHI2/2A plasmids; to the best of our knowledge, this constitutes the first report of cocarriage of these two resistance genes in such a plasmid.

IncN2 plasmid. The third resistance plasmid was pUR-EC3.3, a plasmid belonging to the IncN2 incompatibility group; this plasmid spanned 59.3 kb and had a GC content of 52.82%. IncN plasmids are genetic platforms which play an important part in disseminating resistance genes among *Enterobacterales*; these plasmids can be subdivided into three groups (i.e., IncN1, IncN2, and IncN3), and although they share similar backbones, the nucleotide sequence homology is limited (16). Only a few IncN2 plasmid sequences are available in the GenBank database; alignment of pUR-EC3.3 with such plasmids shows several conserved regions corresponding to backbone genes, interspersed by the different insertion sequences and resistance genes (Fig. 6). In this regard, pUR-EC3.3 featured two antibiotic resistance "blocks," conferring resistance to quinolones, aminoglycosides, sulfonamides, and quaternary ammonium compounds. The first block spans approximately 10 kb and consists of IS*Ec11*, *qnrB19*, *aacC3*, a tunicamycin resistance protein, two uncharacterized ORFs, IS*Kpn12*, an IS*1133*-like transposase, *aph(3″)-Ib*, and *aph(6)-Id* truncated by the insertion of an incomplete IS*Kox11*. Nucleotide similarity searches yielded only partial matches, one consisting of the region comprehended between *aacC3* and ΔIS*Ko11*, another corresponding only to *qnrB19*, and a third region consisting of IS*Ec11*. This suggests at least two insertion events, the first consisting of the recruitment of the *aacC3*-ΔIS*Ko11* segment from either *Salmonella enterica* strain AR_0127 plasmid unnamed1 (GenBank accession no. CP021952.1) or the chromosome of *E. coli* (the closest BLASTn matches). The other insertion event would correspond to the recruitment of *qnrB19* by IS*Ec11*. The frequent occurrence of *qnrB19* in ColE-like plasmids makes them a likely source for this event.

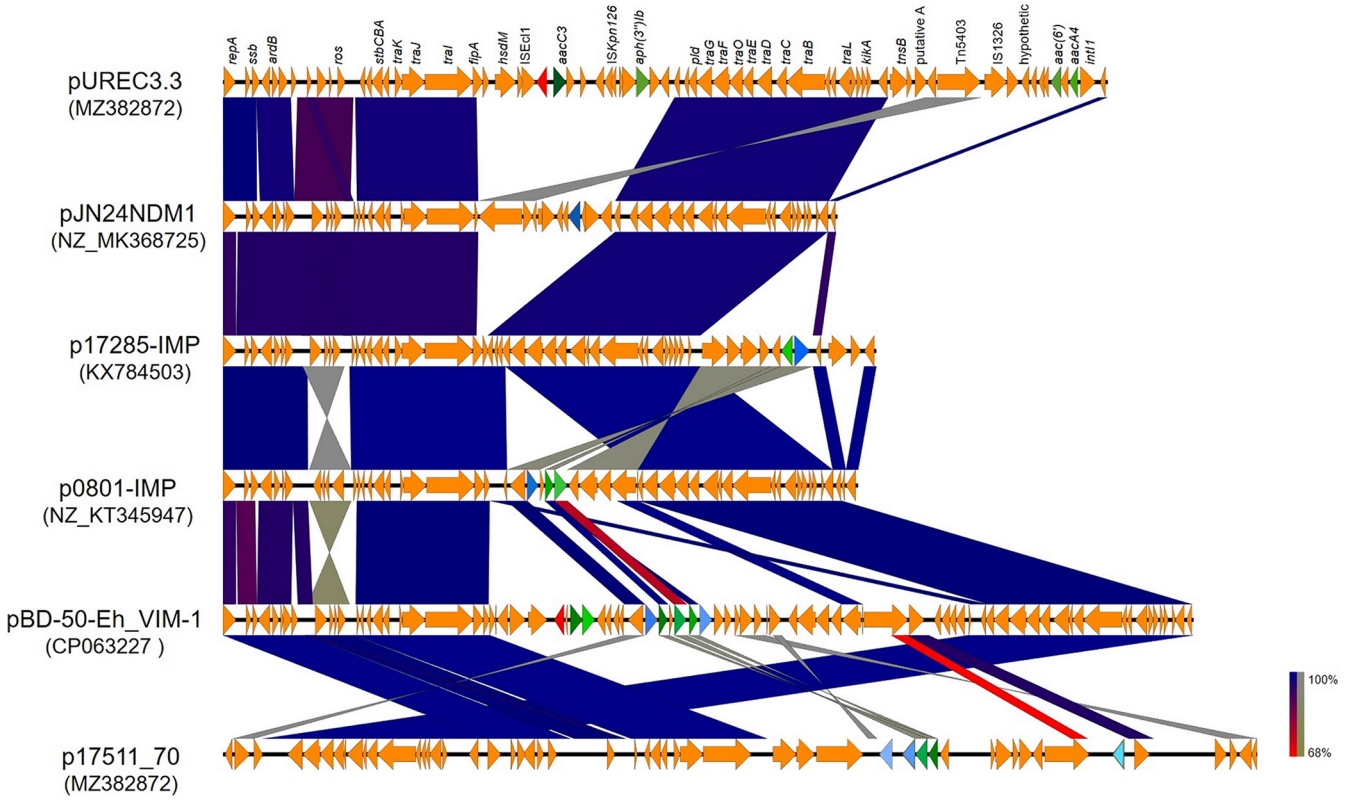


FIG 6 Linear alignment of plasmid pUR-EC3.3 against other IncN2 plasmids available in public databases (accession numbers are in parentheses). Regions of maximum nucleotide identity are indicated in deep blue; conversely, regions of minimum identity are indicated in red. Inverted regions between different plasmids are indicated in gray. CDS color code: red, *qnrB19*; hues of green, aminoglycoside-modifying genes; hues of blue, beta-lactamase genes.

On the other hand, the second resistance block consisted of a class 1 integron carrying the following gene cassettes: *aacA4*, an uncharacterized *orf*, an *aac(6′)-30/aac(6′)-Ib* fusion, *qacEΔ1*, and *sul1*.

The available IncN2 plasmids in GenBank are mostly associated with carbapenemase genes, mainly bla_{IMP} variants, bla_{NDM} and bla_{VIM}; other beta-lactamases detected in these plasmids include bla_{OXA} and bla_{SHV} derivatives. The presence of *qnrB19* genes has been mostly associated with small ColE-like plasmids; so far, only two IncN2 plasmids have been reported carrying this gene, namely, pBD-50-Eh_VIM-1 (GenBank accession no. CP063227) and pUR-EC3.3, detected in our study. Although both plasmids were detected in *E. cloacae* isolates, pBD-50-Eh_VIM-1 was recovered from a clinical isolate in Germany (16), whereas pUR-EC3.3 was recovered from 1-day-chicken feces in Uruguay.

DISCUSSION

In the year 2010, approximately 63,200 tons of antibiotics was consumed by livestock industry animals globally (3). Predictions indicate that by 2030, antibiotic consumption will reach 105,600 tons, due to the increase in animal production needed to meet the demands of population growth (17). China, the United States, and Brazil are currently the largest consumers of antibiotics in livestock (https://cddep.org/tool/country-level-trends-in-antibiotic-use-2000-2015/).

In Uruguay, several measures have been implemented to reduce the use of antibiotics in animal production. In this regard, in 2011, presidential decree 98/011 (https://www.impo.com.uy/bases/decretos/98-2011) banned the employment of antibiotics as a growth factor for cattle and sheep. Later in 2019, decree 141/019 (https://www.impo.com.uy/bases/decretos/141-2019) prohibited the import, export, manufacture, sale, use, possession, and marketing of veterinary products containing colistin in its formula.

However, countries that import live production animals risk the entry of multidrug-resistant (MDR) microorganisms included in their microbiota, thus circumventing health policies aimed at reducing the selection of such MDR microorganisms (18).

In this setting, Brazil is one of the main producers and exporters of chicken meat worldwide (https://www.wattagnet.com/articles/38644-brazilian-breeders-brand-launched-during-siavs-2019), and the occurrence of resistant microorganisms has been reported for retail chicken meat on several occasions (17–19). In parallel, on account of being considered free from avian influenza and Newcastle disease, Brazil is also one of the main suppliers of 1-day-old chicks worldwide and Uruguay's main supplier (https://www.wattagnet.com/articles/38644-brazilian-breeders-brand-launched-during-siavs-2019).

Among the main transferable mechanisms of resistance to critically important antimicrobials are extended-spectrum β-lactamases (ESBLs) and plasmid-mediated class C β-lactamases (pAmpC) for oxyiminocephalosporins (20), Qnr variants not capable of acetylating fluoroquinolones (i.e., *A*, *B*, *C*, *D*, *E*, *S*, and *VC*) and AAC(6′)-Ib-cr capable of acetylating ciprofloxacin and norfloxacin among fluoroquinolones (21), and MCR variants (1 to 10) for colistin resistance and 16S rRNA methylases for aminoglycoside resistance (21, 22).

The occurrence of enterobacteria carrying $bla_{\text{CTX-M-2}}$ or $bla_{\text{CTX-M-8}}$ and $bla_{\text{CMY-2}}$ has been previously described for the United Kingdom and Denmark, for chicken meat imported from Brazil (23). Likewise, the presence of $bla_{\text{CTX-M-2}}$ or $bla_{\text{CMY-2}}$ has been documented for nontyphoidal *Salmonella* serovars isolated from poultry, whereas $bla_{\text{CTX-M-2}}$, $bla_{\text{CMY-2}}$, and $bla_{\text{CTX-M-15}}$ have been detected in *E. coli* isolates recovered from chicken carcasses produced by four Brazilian poultry producers (24, 25). However, this is the first report in Uruguay of antibiotic resistance genes present in 1-day-old chickens imported from Brazil.

Recently, we reported in our country the presence of antibiotic-resistant *E. coli* isolates obtained from livestock, including broiler and layer flocks. Our results showed that 90% of the *E. coli* isolates obtained from poultry displayed resistance to fluoroquinolones, whereas 15% of such isolates displayed resistance to oxyiminocephalosporins (5). The resistance mechanisms detected in those isolates were *qnrB19* and *qnrE1* for fluoroquinolones and $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$, $bla_{\text{CMY-2}}$, $bla_{\text{CTX-M-55}}$, and $bla_{\text{SHV-2a}}$ for oxyiminocephalosporins. Interestingly, the same resistance genes (except $bla_{\text{SHV-2a}}$) were also detected in this study. Considering that 1-day-old chickens are transported to broiler farms and subsequently to production flocks housing thousands of birds, the hypothesis that the resistant microorganisms detected in adult birds were introduced as part of the microbiota of 1-day-old chickens is perfectly plausible. However, new prospective studies capable of demonstrating this are needed.

Additionally, these resistance genes were among the most frequently detected in our country in *Salmonella* isolates of human origin (26). Bearing in mind that *Salmonella* is considered a primary pathogen to humans (albeit with a life cycle mainly associated with birds), and the amount of resistance genes detected in the microorganisms reported in this study, the findings of Cordeiro et al. could be regarded as the tip of the iceberg in terms of transfer of resistant microorganisms from poultry to humans.

On the other hand, we recently described the genetic platforms associated with $bla_{\text{CTX-M-2}}$ in microorganisms of human origin (27). This ESBL is mainly encoded in IncC plasmids, associated with a complex class 1 integron (In35) featuring in its variable region the genes *aac(6′)Ib-*$bla_{\text{OXA-2}}$*-orfD* (28). These integrons are inserted in Tn*1696*-derived complex transposons and form hybrid transposons (Tn*7057* and Tn*7058*) featuring three Tn*3* family transposases (27).

Nevertheless, in this work we describe a different platform (i.e., Tn*7337*) encoded in a R478-related IncHI2/2A plasmid belonging to ST16, which, to the best of our knowledge, has never been reported before. Although Tn*7337* derives from Tn*1696*, the associated class 1 integron and the occurrence of IS*26* between the mercury resistance operon and such integron constitute a new genetic arrangement.

The concept of One Health regards human and animal health and the environment as a whole. The occurrence of known resistance genes in novel genetic platforms highlights the importance of keeping in mind not only the presence of antibiotic resistance genes but also the entire genetic platform mobilizing such genes, in order to establish possible links between resistance genes present in humans, in animals, and in the environment. In view of these findings, more effort should be dedicated to studying plasmids and genetic platforms associated with $bla_{\text{CTX-M-2}}$, especially in those cases where this ESBL gene is not present in IncC plasmids (29–31).

On the other hand, we also detected the occurrence of resistance genes so far unknown in our country, such as the 16S rRNA methylase gene *rmtG* (which confers high resistance to almost all aminoglycosides) and the phosphoethanolamine transferase gene *mcr-9.1* (responsible for polymyxin resistance).

The various features of pUR-EC3.2, which harbored $bla_{\text{CTX-M-2}}$ and *mcr-9.1*, have been presented in previous sections within this report. So far, the occurrence of *mcr-9* alleles had been reported in our neighboring countries, Argentina (in human isolates) (32) and Brazil (isolates of human and animal origin) (33). In relation with polymyxin resistance, only *mcr-1* had been reported so far in Uruguay, in isolates of human and animal origin (swine and poultry) (5, 34); nevertheless, the arrival of other plasmid-mediated genes conferring resistance to these antibiotics should raise the alarm in both human and veterinary medicine.

Among the different and alarming findings, we should also point out the presence of *rmtG*, carried on a small IncQ1 plasmid. So far, in Uruguay only *rmtC* had been reported in *Acinetobacter baumannii* (35); nevertheless, *rmtG* had already been reported in Brazil and Chile (36). Although this IncQ1 plasmid lacks the necessary genes for self-transfer, their coexistence (in a bacterial cell) with conjugative plasmids, such as IncHI2A and/or IncN2, may facilitate the horizontal transfer of resistance genes in the former. Considering that this plasmid presented chi sequences, and according to what was previously reported by Zaman and Boles, this homologous recombination event might have been mediated by RecBCD enzymes and oriented by the Chi sequences present either in *repC* or *mobC* following a model similar to the one proposed by Yau et al. (Fig. 3) (37, 38).

Finally, we would also like to highlight the detection of *E. coli* ST224 in one of the studied 1-day-old chicken shipments. According to the virulence genes present in this isolate, *E. coli* ODC_Ec7 should be considered extraintestinal pathogenic *Escherichia coli* (ExPEC) and highly capable of zoonotic expansion, keeping in mind the definition proposed by Johnson et al. (39).

Oxyiminocephalosporin-resistant *E. coli* ST224 has already been detected in birds and pets and even in up to 10% of the ESBL-producing *E. coli* isolates obtained from community-acquired urinary tract infections (CA-UTIs) (40–42).

On the one hand, strain ODC_Ec7 harbors virulence genes that can promote the colonization of the human urinary tract and genes conferring resistance to first-line antibiotics such as beta-lactams ($bla_{\text{CTX-M-55}}$), fosfomycin (*fosA3*), trimethoprim-sulfamethoxazole (*dfr12/sul3*), and quinolones (*gyrA* [D87N and S83L], *parC* [S80I], and *parE* [S458A]). On the other hand, strain ODC_Ec7 also carries several genes coding for colicins and microcins, thus allowing this strain to compete against gut-colonizing intestinal microorganisms.

Additionally, as an animal pathogen, strain ODC_Ec7 carries genes conferring resistance to antibiotics common in veterinary medicine, namely, tetracycline (*tetA*) and streptomycin (*aadA1/aadA2*).

With such an impressive array of genes, there is a high probability of *E. coli* ST224 becoming an important pathogen in the context of One Health.

Summing up, out of a small sample obtained from a shipment of 1-day-old chickens, we detected the arrival in our country of microorganisms carrying multiple mechanisms of resistance to critically important antibiotics in human medicine. Some of these resistance genes were encoded in relatively uncommon plasmids and/or carried by primary human and animal pathogens. The surveillance of MDR microorganisms in

animals destined for human consumption should be implemented at the beginning of the food production chain and in later steps, in order to assess the penetration of such microorganisms in the production chain.

A permanent and sustained entry of MDR microorganisms into our country could counteract the measures implemented by our government and producers, aimed at reducing the amount of antibiotic consumption and, in turn, lowering the incidence of antimicrobial resistance; in this setting, imported animals carrying such microorganisms in their digestive tracts act as modern equivalents of the Trojan horse.

MATERIALS AND METHODS

Sampling, identification, and antibiotic susceptibility testing. From January 2018 and May 2019, eight shipments containing 1-day-old chicks imported to Uruguay from Brazil were randomly sampled by swabbing fecal matter from the bottoms of packing boxes.

Samples were cultured in Luria-Bertani broth (Oxoid Ltd., Basingstoke, UK) for 24 h at 37°C. Next, 10 μL of broth was subcultured on MacConkey agar plates (Oxoid Ltd.) supplemented with 3 μg/mL colistin (Sigma-Aldrich, St. Louis, MO), 1 μg/mL ceftriaxone (Libra, Montevideo, Uruguay), or 0.125 μg/mL ciprofloxacin (ION, Montevideo, Uruguay). Up to 5 colonies of different morphologies were studied from each plate.

Bacterial identification was performed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Bruker, MA). Antibiotic susceptibility was determined by the Sensititre system using ARGNF plates (Thermo Fisher Scientific, MA) as per the manufacturer's recommendations. Susceptibility to fosfomycin and colistin was determined by agar dilution methods and colistin agar test, respectively. The colistin agar test was performed according to the Clinical and Laboratory Standards Institute (CLSI). A 0.5 McFarland suspension of the strain to be studied was prepared. Subsequently, 10 μL of a 1/10 dilution of this suspension was plated in Mueller-Hinton agar (MHA) medium supplemented with colistin (0, 1, 2, and 4 μg/mL) and incubated for 20 h at 35°C (43).

All results were interpreted according to the CLSI (43), except for susceptibility to fosfomycin, which was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org). Results of intermediate susceptibility were considered resistant.

ARG detection. Antimicrobial resistance genes (ARG) were screened by PCR using primers to amplify ESBL (bla_{CTX-M}, bla_{TEM}, bla_{SHV}, and bla_{PER-2}-type), pAmpC β-lactamase (bla_{CMY}, bla_{DHA}, $bla_{MOX,}$ bla_{ACC}, bla_{EBC}, and bla_{FOX}), and aminoglycoside [*aac(6)Ib*, *armA*, *rmtA-H*, and *npmA*], quinolone [*aac(6*$^{-}$*)Ib-cr*, *qnrA*, *-B*, *-C*, *-D*, *-E*, *-S*, and *-VC*, and *qepA*], and fosfomycin (*fosA*, *fosA3*) resistance genes (5, 34). Plasmid-mediated colistin resistance genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5*) were detected by real-time PCR or conventional PCR (44–46).

PFGE. Clonality was assessed by pulsed-field gel electrophoresis (PFGE) following digestion with the restriction enzyme XbaI (Thermo Scientific, Waltham, MA). Band patterns were analyzed with BioNumerics v6.6 (Applied Maths, Sint-Martens-Latem, Belgium) with 2% tolerance and 0% optimization. Strains were considered related with 85% similarity (29).

Short- and long-read genome sequencing. Genomic DNA was obtained with the NZY microbial genomic DNA (gDNA) isolation kit, following the manufacturer's instructions (NZYTech Genes & Enzymes, Lisbon, Portugal). DNA quality and quantity were assessed with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Four isolates were selected for WGS based on their resistance profiles and their pulsed-field gel electrophoresis profiles. Next-generation sequencing was performed by using Illumina MiSeq-I with Nextera XT libraries. Reads were assembled with SPADES v.3.11 using k-mers 21, 33, 55, 77, 99, and 127 with the "careful" option turned on and the following cutoffs for final assemblies: minimum contig/scaffold size = 500 bp and minimum contig/scaffold average nucleotide coverage = 10-fold. In parallel, selected isolates were also sequenced using an Oxford Nanopore Technologies device. In brief, DNA libraries were prepared with a rapid sequencing kit (SQK-RAD004) following the manufacturer's instructions. Libraries were loaded onto R9.4.1 flow cells (FLO-MIN106) and sequenced for 8 h on a MinION device (Oxford Nanopore Technologies, Oxford, UK). Basecalling was performed using Guppy with a high-accuracy model, integrated into the MinKNOW software v4.1.22. The quality of generated data was assessed with NanoPlot v1.33.1 (47), and Filtlong v0.2.0 (https://github.com/rrwick/Filtlong) was applied to remove reads shorter than 2,000 bp and reads with a mean quality score of <93. *De novo* genome assembly was achieved using Flye assembler v2.8.2 (48) with four polishing iterations and using the plasmid option to recover any short unassembled plasmid. On the other hand, genomic sequences of isolates harboring both *rmtG* and *mcr-9* genes were assembled *de novo*, using a hybrid approach, with Unicycler v0.4.8 (49).

In this regard, since *mcr-9* has been previously described for colistin-susceptible isolates (32), we specifically designed primers aimed at detecting this gene in all the studied isolates (mcr9-FW, 5′-TTTGAT TGCAGGTGTTGCCG-3′, and mcr9-RV, 5′-AGATATAGCCCGCTTTCGCC-3′).

***In silico* analysis.** Prediction of antibiotic resistance genes was performed using both the Comprehensive Antibiotic Resistance Database (select criteria, perfect and strict; sequence quality, high quality/coverage) (https://card.mcmaster.ca/) and the ResFinder 4.1 suite (50). Detection of plasmid incompatibility groups was achieved using the PlasmidFinder 2.1 suite (threshold for minimum identity, 95%; minimum coverage, 60%) (51), whereas sequence types were predicted by MLST 2.0 (52). Plasmids were annotated using the RAST 2.0 suite (Rapid Annotation using Subsystem Technology) (53) and

manually curated with Artemis software (54). Plasmid comparison was performed using the Python application Easyfig 2.2.2; in this regard, BLASTn files were generated with a maximum E value of 10−3 (55). Prediction of promoter regions was carried out using the online analysis tool BacPP (56).

Data availability. The nucleotide sequences of plasmids pUR-EC3.1, pUR-EC3.2 and pUR-EC3.3, and their corresponding annotations, were deposited in GenBank under accession numbers MZ382870, MZ382871, and MZ382872, respectively. The composite transposon present in pUR-EC3.2 was designated Tn*7337* by The Transposon Registry (https://transposon.lstmed.ac.uk/) (GenBank accession number MZ396394) (57).

REFERENCES

1. World Health Organization. 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. https://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf.
2. WHO. 2017. Critically important antimicrobials for human medicine: 5th revision. World Health Organization, Geneva, Switzerland.
3. Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A, Laxminarayan R. 2015. Global trends in antimicrobial use in food animals. Proc Natl Acad Sci U S A 112:5649–5654. https://doi.org/10.1073/pnas.1503141112.
4. Landers TF, Cohen B, Wittum TE, Larson EL. 2012. A review of antibiotic use in food animals: perspective, policy, and potential. Public Health Rep 127:4–22. https://doi.org/10.1177/003335491212700103.
5. Coppola N, Freire B, Umpiérrez A, Cordeiro NF, Ávila P, Trenchi G, Castro G, Casaux ML, Fraga M, Zunino P, Bado I, Vignoli R. 2020. Transferable resistance to highest priority critically important antibiotics for human health in Escherichia coli strains obtained from livestock feces in Uruguay. Front Vet Sci 7:588919. https://doi.org/10.3389/fvets.2020.588919.
6. Cunha MV, Lincopan N, Cerdeira L, Esposito F, Dropa M, Franco LS, Moreno AM, Knöbl T. 2017. Coexistence of CTX-M-2, CTX-M-55, CMY-2, FosA3, and QnrB19 in extraintestinal pathogenic Escherichia coli from poultry in Brazil. Antimicrob Agents Chemother 61:e02474-16. https://doi.org/10.1128/AAC.02474-16.
7. Dominguez JE, Redondo LM, Figueroa Espinosa RA, Cejas D, Gutkind GO, Chacana PA, Di Conza JA, Fernández Miyakawa ME. 2018. Simultaneous carriage of mcr-1 and other antimicrobial resistance determinants in Escherichia coli from poultry. Front Microbiol 9:1679. https://doi.org/10.3389/fmicb.2018.01679.
8. Cheng K, Wilkinson M, Chaban Y, Wigley DB. 2020. A conformational switch in response to Chi converts RecBCD from phage destruction to DNA repair. Nat Struct Mol Biol 27:71–77. https://doi.org/10.1038/s41594-019-0355-2.
9. Martins ER, Bueno MFC, Francisco GR, Casella T, de Oliveira Garcia D, Cerdeira LT, Gerber AL, de Almeida LGP, Lincopan N, de Vasconcelos ATR, Nogueira MCL, Estofolete CF. 2020. Genome and plasmid context of two rmtG-carrying Enterobacter hormaechei isolated from urinary tract infections in Brazil. J Glob Antimicrob Resist 20:36–40. https://doi.org/10.1016/j.jgar.2019.06.020.
10. Macesic N, Blakeway LV, Stewart JD, Hawkey J, Wyres KL, Judd LM, Wick RR, Jenney AW, Holt KE, Peleg AY. 2021. Silent spread of mobile colistin resistance gene mcr-9.1 on IncHI2 'superplasmids' in clinical carbapenem-resistant Enterobacterales. Clin Microbiol Infect https://doi.org/10.1016/j.cmi.2021.04.020.
11. García-Fernández A, Carattoli A. 2010. Plasmid double locus sequence typing for IncHI2 plasmids, a subtyping scheme for the characterization of IncHI2 plasmids carrying extended-spectrum β-lactamase and quinolone resistance genes. J Antimicrob Chemother 65:1155–1161. https://doi.org/10.1093/jac/dkq101.
12. Guo Q, Ding B, Jové T, Stoesser N, Cooper VS, Wang M, Doi Y. 2016. Characterization of a novel IncHI2 plasmid carrying tandem copies of blaCTX-M-2 in a fosA6-harboring Escherichia coli sequence type 410 strain. Antimicrob Agents Chemother 60:6742–6747. https://doi.org/10.1128/AAC.01173-16.
13. Darphorn TS, Bel K, Koenders-van Sint Anneland BB, Brul S, Ter Kuile BH. 2021. Antibiotic resistance plasmid composition and architecture in Escherichia coli isolates from meat. Sci Rep 11:2136. https://doi.org/10.1038/s41598-021-81683-w.
14. Partridge SR, Brown HJ, Stokes HW, Hall RM. 2001. Transposons Tn1696 and Tn21 and their integrons In4 and In2 have independent origins. Antimicrob Agents Chemother 45:1263–1270. https://doi.org/10.1128/AAC.45.4.1263-1270.2001.
15. Kieffer N, Royer G, Decousser JW, Bourrel AS, Palmieri M, De La Rosa JMO, Jacquier H, Denamur E, Nordmann P, Poirel L. 2019. *mcr-9*, an inducible gene encoding an acquired phosphoethanolamine transferase in *Escherichia coli*, and its origin. Antimicrob Agents Chemother 63:e00965-19. https://doi.org/10.1128/AAC.00965-19.
16. Jiang X, Yin Z, Yin X, Fang H, Sun Q, Tong Y, Xu Y, Zhang D, Feng J, Chen W, Song Y, Wang J, Chen S, Zhou D. 2017. Sequencing of blaIMP carrying IncN2 plasmids, and comparative genomics of IncN2 plasmids harboring class 1 integrons. Front Cell Infect Microbiol 7:102–109. https://doi.org/10.3389/fcimb.2017.00102.
17. WHO. 2014. Antimicrobial resistance. Global report on surveillance. Bull World Health Organ 61:383–394.
18. Lammie SL, Hughes JM. 2016. Antimicrobial resistance, food safety, and One Health: the need for convergence. Annu Rev Food Sci Technol 7:287–312. https://doi.org/10.1146/annurev-food-041715-033251.
19. Casella T, Cerdeira LT, Fernandes MR, Souza TA, Haenni M, Madec JY, Lincopan N, Nogueira MCL. 2017. Draft genome sequence of a CTX-M-15-producing Escherichia coli ST345 from commercial chicken meat in Brazil. J Glob Antimicrob Resist Elsevier Ltd 9:124–125. https://doi.org/10.1016/j.jgar.2017.04.002.
20. Bush K. 2018. Past and present perspectives on β-lactamase. Antimicrob Agents Chemother 62:e01076-18. https://doi.org/10.1128/AAC01076-18.
21. Ruiz J. 2019. Transferable mechanisms of quinolone resistance from 1998 onward. Clin Microbiol Rev 32:1–59. https://doi.org/10.1128/CMR.00007-19.
22. Mendes Oliveira VR, Paiva MC, Lima WG. 2019. Plasmid-mediated colistin resistance in Latin America and Caribbean: a systematic review. Travel Med Infect Dis 31:101459. https://doi.org/10.1016/j.tmaid.2019.07.015.
23. Rabello RF, Bonelli RR, Penna BA, Albuquerque JP, Souza RM, Cerqueira AMF. 2020. Antimicrobial resistance in farm animals in Brazil: an update overview. Animals (Basel) 10:552. https://doi.org/10.3390/ani10040552.
24. Penha Filho RAC, Ferreira JC, Kanashiro AMI, Berchieri Junior A, da Costa Darini AL. 2019. Emergent multidrug-resistant nontyphoidal Salmonella serovars isolated from poultry in Brazil coharboring blaCTX-M-2 and qnrB or blaCMY-2 in large plasmids. Diagn Microbiol Infect Dis 95:93–98. https://doi.org/10.1016/j.diagmicrobio.2019.04.003.
25. Botelho LAB, Kraychete GB, e Silva JLC, Regis DVV, Picão RC, Moreira BM, Bonelli RR. 2015. Widespread distribution of CTX-M and plasmid-mediated AmpC β-lactamases in Escherichia coli from Brazilian chicken meat. Mem Inst Oswaldo Cruz 110:249–254. https://doi.org/10.1590/0074-02760140389.
26. Cordeiro NF, Nabón A, García-Fulgueiras V, Álvez M, Sirok A, Camou T, Vignoli R. 2016. Analysis of plasmid-mediated quinolone and oxyiminocephalosporin resistance mechanisms in Uruguayan Salmonella enterica isolates from 2011–2013. J Glob Antimicrob Resist 6:165–171. https://doi.org/10.1016/j.jgar.2016.06.002.
27. Papa-Ezdra R, Cordeiro NF, Di Pilato V, Chiarelli A, Pallecchi L, Garcia-Fulgueiras V, Vignoli R. 2021. Description of novel resistance islands harbouring blaCTX-M-2 in IncC type 2 plasmids. J Glob Antimicrob Resist 26:37–41. https://doi.org/10.1016/j.jgar.2021.03.031.
28. Bado I, Cordeiro NF, Robino L, García-Fulgueiras V, Seija V, Bazet C, Gutkind G, Ayala JA, Vignoli R. 2010. Detection of class 1 and 2 integrons, extended-spectrum β-lactamases and qnr alleles in enterobacterial isolates from the digestive tract of intensive care unit inpatients. Int J Antimicrob Agents 36:453–458. https://doi.org/10.1016/j.ijantimicag.2010.06.042.
29. Vignoli R, García-Fulgueiras V, Cordeiro NF, Bado I, Seija V, Aguerrebere P, Laguna G, Araújo L, Bazet C, Gutkind G, Chabalgoity A. 2016. Extended-spectrum β-lactamases, transferable quinolone resistance, and virulotyping in extra-intestinal E. coli in Uruguay. J Infect Dev Ctries 10:43–52. https://doi.org/10.3855/jidc.6918.

30. García-Fulgueiras V, Bado I, Mota MI, Robino L, Cordeiro NF, Varela A, Algorta G, Gutkind G, Ayala JA, Vignoli R. 2011. Extended-spectrum beta-lactamases and plasmid-mediated quinolone resistance in enterobacterial clinical isolates in the paediatric hospital of Uruguay. J Antimicrob Chemother 66:1725–1729. https://doi.org/10.1093/jac/dkr222.
31. Garcia-Fulgueiras V, Araujo L, Bado I, Cordeiro NF, Mota MI, Laguna G, Algorta G, Vignoli R. 2017. Allodemic distribution of plasmids co-harbouring bla CTX-M-15/aac(6′)-Ib -cr/qnrB in Klebsiella pneumoniae is the main source of extended-spectrum β-lactamases in Uruguay's paediatric hospital. J Glob Antimicrob Resist 9:68–73. https://doi.org/10.1016/j.jgar.2017.01.008.
32. Faccone D, Martino F, Albornoz E, Gomez S, Corso A, Petroni A. 2020. Plasmid carrying mcr-9 from an extensively drug-resistant NDM-1-producing Klebsiella quasipneumoniae subsp. quasipneumoniae clinical isolate. Infect Genet Evol 81:104273. https://doi.org/10.1016/j.meegid.2020.104273.
33. Fuentes-Castillo D, Sellera FP, Goldberg DW, Fontana H, Esposito F, Cardoso B, Ikeda J, Kyllar A, Catão-Dias JL, Lincopan N. 2021. Colistin-resistant Enterobacter kobei carrying mcr-9.1 and bla CTX-M-15 infecting a critically endangered franciscana dolphin (Pontoporia blainvillei), Brazil. Transbound Emerg Dis 2021:1–7. https://doi.org/10.1111/tbed.13980.
34. Papa-Ezdra R, Grill Diaz F, Vieytes M, García-Fulgueiras V, Caiata L, Ávila P, Brasesco M, Christophersen I, Cordeiro NF, Algorta G, Galiana A, Vignoli R. 2020. First three Escherichia coli isolates harbouring mcr-1 in Uruguay. J Glob Antimicrob Resist 20:187–190. https://doi.org/10.1016/j.jgar.2019.07.016.
35. Bado I, Seija V, Gonzalez-Zorn B, Gutiérrez C, Araújo Pirez L, Delgado-Blas JF, Gaudio M, Papa-Ezdra R, García-Fulgueiras V, Vignoli R, Medina JC, Cordeiro NF, Rieppi G. 2018. Molecular characterization of carbapenem-resistant Acinetobacter baumannii in the intensive care unit of Uruguay's university hospital identifies the first rmtC gene in the species. Microb Drug Resist 24:1012–1019. https://doi.org/10.1089/mdr.2017.0300.
36. Poirel L, Labarca J, Bello H, Rioseco ML, Bernabeu S, Nordmann P. 2014. Emergence of the 16S rRNA methylase RmtG in an extended-spectrum-β-lactamase-producing and colistin-resistant Klebsiella pneumoniae isolate in Chile. Antimicrob Agents Chemother 58:618–619. https://doi.org/10.1128/AAC.02059-13.
37. Zaman MM, Boles TC. 1996. Plasmid recombination by the RecBCD pathway of Escherichia coli. J Bacteriol 178:3840–3845. https://doi.org/10.1128/jb.178.13.3840-3845.1996.
38. Yau S, Liu X, Djordjevic SP, Hall RM. 2010. RSF1010-like plasmids in Australian Salmonella enterica serovar typhimurium and origin of their sul2-strA-strB antibiotic resistance gene cluster. Microb Drug Resist 16:249–252. https://doi.org/10.1089/mdr.2010.0033.
39. Johnson JR, Kuskowski MA, Owens K, Gajewski A, Winokur PL. 2003. Phylogenetic origin and virulence genotype in relation to resistance to fluoroquinolones and/or extended-spectrum cephalosporins and cephamycins among *Escherichia coli* isolates from animals and humans. J Infect Dis 188:759–768. https://doi.org/10.1086/377455.
40. Cao X, Zhang Z, Shen H, Ning M, Chen J, Wei H, Zhang K. 2014. Genotypic characteristics of multidrug-resistant Escherichia coli isolates associated with urinary tract infections. APMIS 122:1088–1095. https://doi.org/10.1111/apm.12260.
41. He DD, Cui MM, Zhang TL, Hu GZ, Liu JH, Pan YS. 2021. Characterization of blaCMY-2-carrying IncC and rmtB-carrying IncI1/ST136 plasmids in an avian Escherichia coli ST224 strain. Plasmid 114:102555. https://doi.org/10.1016/j.plasmid.2021.102555.
42. Kidsley AK, White RT, Beatson SA, Saputra S, Schembri MA, Gordon D, Johnson JR, O'Dea M, Mollinger JL, Abraham S, Trott DJ. 2020. Companion animals are spillover hosts of the multidrug-resistant human extraintestinal Escherichia coli pandemic clones ST131 and ST1193. Front Microbiol 11:1968. https://doi.org/10.3389/fmicb.2020.01968.
43. CLSI. 2020. Performance standards for antimicrobial susceptibility testing, 30th ed. CLSI supplement M100. CLSI, Wayne, PA.
44. Li J, Shi X, Yin W, Wang Y, Shen Z, Ding S, Wang S. 2017. A multiplex SYBR green real-time PCR assay for the detection of three colistin resistance genes from cultured bacteria, feces, and environment samples. Front Microbiol 8:2078. https://doi.org/10.3389/fmicb.2017.02078.
45. Carattoli A, Villa L, Feudi C, Curcio L, Orsini S, Luppi A, Pezzotti G, Magistrali CF. 2017. Novel plasmid-mediated colistin resistance *mcr-4 g* ene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. Eurosurveillance 22:30589. https://doi.org/10.2807/1560-7917.ES.2017.22.31.30589.
46. Borowiak M, Fischer J, Hammerl JA, Hendriksen RS, Szabo I, Malorny B. 2017. Identification of a novel transposon-associated phosphoethanolamine transferase gene, mcr-5, conferring colistin resistance in d-tartrate fermenting Salmonella enterica subsp. enterica serovar Paratyphi B. J Antimicrob Chemother 72:3317–3324. https://doi.org/10.1093/jac/dkx327.
47. De Coster W, D'Hert S, Schultz DT, Cruts M, Van Broeckhoven C. 2018. NanoPack: visualizing and processing long-read sequencing data. Bioinformatics 34:2666–2669. https://doi.org/10.1093/bioinformatics/bty149.
48. Kolmogorov M, Yuan J, Lin Y, Pevzner PA. 2019. Assembly of long, error-prone reads using repeat graphs. Nat Biotechnol 37:540–546. https://doi.org/10.1038/s41587-019-0072-8.
49. Wick RR, Judd LM, Gorrie CL, Holt KE. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol 13:e1005595. https://doi.org/10.1371/journal.pcbi.1005595.
50. Bortolaia V, Kaas RS, Ruppe E, Roberts MC, Schwarz S, Cattoir V, Philippon A, Allesoe RL, Rebelo AR, Florensa AF, Fagelhauer L, Chakraborty T, Neumann B, Werner G, Bender JK, Stingl K, Nguyen M, Coppens J, Xavier BB, Malhotra-Kumar S, Westh H, Pinholt M, Anjum MF, Duggett NA, Kempf I, Nykäsenoja S, Olkkola S, Wieczorek K, Amaro A, Clemente L, Mossong J, Losch S, Ragimbeau C, Lund O, Aarestrup FM. 2020. ResFinder 4.0 for predictions of phenotypes from genotypes. J Antimicrob Chemother 75:3491–3500. https://doi.org/10.1093/jac/dkaa345.
51. Carattoli A, Hasman H. 2020. PlasmidFinder and in silico pMLST: identification and typing of plasmid replicons in whole-genome sequencing (WGS). Methods Mol Biol 2075:285–294. https://doi.org/10.1007/978-1-4939-9877-7_20.
52. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, Jelsbak L, Sicheritz-Pontén T, Ussery DW, Aarestrup FM, Lund O. 2012. Multilocus sequence typing of total-genome-sequenced bacteria. J Clin Microbiol 50:1355–1361. https://doi.org/10.1128/JCM.06094-11.
53. Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, Stevens R. 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res 42:D206–D214. https://doi.org/10.1093/nar/gkt1226.
54. Carver T, Berriman M, Tivey A, Patel C, Böhme U, Barrell BG, Parkhill J, Rajandream MA. 2008. Artemis and ACT: viewing, annotating and comparing sequences stored in a relational database. Bioinformatics 24:2672–2676. https://doi.org/10.1093/bioinformatics/btn529.
55. Sullivan MJ, Petty NK, Beatson SA. 2011. Easyfig: a genome comparison visualizer. Bioinformatics 27:1009–1010. https://doi.org/10.1093/bioinformatics/btr039.
56. Avila S, Echeverrigaray E, Gerhardt G. 2011. BacPP: bacterial promoter prediction—a tool for accurate sigma-factor specific assignment in enterobacteria. J Theor Biol 287:92–99. https://doi.org/10.1016/j.jtbi.2011.07.017.
57. Tansirichaiya S, Rahman MA, Roberts AP. 2019. The Transposon Registry. Mobile DNA 10:1–6. https://doi.org/10.1186/s13100-019-0182-3.
58. García Fernández A, Cloeckaert A, Bertini A, Praud K, Doublet B, Weill FX, Carattoli A. 2007. Comparative analysis of IncHI2 plasmids carrying $bla_{CTX-M-2}$ or $bla_{CTX-M-9}$ from *Escherichia coli* and *Salmonella enterica* strains isolated from poultry and humans. Antimicrob Agents Chemother 51:4177–4180. https://doi.org/10.1128/AAC.00603-07.