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Exploring the complex interplay of polymyxin resistance mechanisms, lipid A variations, and virulence factors in *Escherichia coli* and *Klebsiella pneumoniae*

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ABSTRACT

The global emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Escherichia coli* and *Klebsiella pneumoniae* poses a serious public health concern due to limited therapeutic options. Polymyxins, often considered last-resort antibiotics, are increasingly compromised by resistance mechanisms such as lipid A modifications and plasmid-mediated genes like *mcr-1*. In this study, we investigated phenotypic and genotypic features of polymyxin-resistant clinical isolates from Brazilian hospitals. Seventeen Gram-negative bacilli (14 *E. coli*, 3 *K. pneumoniae*) were analyzed through antimicrobial susceptibility testing, lipid A profiling via mass spectrometry, whole-genome sequencing (WGS), and virulence assessment using the *Galleria mellonella* infection model. Resistance was associated with structural modifications of lipid A including phosphoethanolamine (PEtN), L-Ara4N, palmitate, and LpxO-mediated hydroxylation, and the presence of major resistance genes such as *mcr-1.1*, $bla_{\text{NDM-1}}$, and $bla_{\text{CTX-M-15}}$. Genetic context analysis revealed associations with mobile elements like IS*Ecp1* and IS*CR1*, as well as integron-associated gene cassettes (e.g., *aadA2, dfrA12*). MLST showed high clonal diversity among *E. coli* isolates, including ST10, ST131, ST354, and ST410, and detection of *K. pneumoniae* ST11 and ST4477, the former being a dominant high-risk clone in Brazil and worldwide. Virulence profiling revealed heterogeneous phenotypes, with some strains classified as hypervirulent. Overall, our findings underscore the complexity of resistance and virulence mechanisms in clinical *Enterobacterales* and highlight the importance of genomic surveillance to monitor the dissemination of MDR/XDR pathogens.

1. Introduction

Antimicrobial resistance (AMR) is widely recognized as a major global public health challenge. It arises as microorganisms adapt through Darwinian selection and the pressure of antimicrobial agents, acquiring resistance genes that can be transferred to other bacteria. Infections caused by these antibiotic-resistant pathogens are typically challenging to treat, prone to recurrence, and often result in considerable morbidity and mortality (Tacconelli et al., 2018).

Prior to the pandemic, rising cases of multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) pathogens drove the renewed clinical use of polymyxins. However,

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widespread overuse and misuse of colistin in both human healthcare and veterinary practices have now fueled the global spread of resistant microorganisms. Reports of resistance to this antimicrobial class have steadily increased, culminating in the alarming first report of *E. coli* harboring the *mcr-1* gene (mobilized colistin resistance) in 2016 (Liu et al., 2016).

The *mcr-1* gene encodes a phosphoethanolamine transferase enzyme that modifies the lipid A component of lipopolysaccharides (LPS) in the bacterial outer membrane, conferring resistance to polymyxins, a last-resort antimicrobial. The addition of a phosphoethanolamine (PEtN) group to lipid A reduces its negative charge, reducing the ability of colistin to bind and disrupt the bacterial membrane. Located primarily on plasmids, the *mcr-1* gene spreads between bacterial species such as *Escherichia coli, Klebsiella pneumoniae*, and *Salmonella* spp. through horizontal gene transfer mechanisms like conjugation, transformation, and transduction (El-Sayed Ahmed et al., 2020). Moreover, *mcr-1* is frequently co-located with other antibiotic resistance genes on multidrug-resistant plasmids, further complicating treatment (Kao et al., 2024; Nang et al., 2019). This widespread dissemination poses a critical public health threat, as it significantly limits therapeutic options for infections caused by Gram-negative bacteria (GNB).

In Brazil, the *mcr-1* gene has been increasingly identified across diverse sources, including human clinical isolates, food-producing animals, retail meat, and environmental samples. A recent review by Costa-Júnior et al. (2023) highlights its widespread distribution among *E. coli*, *K. pneumoniae*, and other Gram-negative bacilli, often carried by highly transferable plasmids such as IncI2 and IncX4 (Costa-Júnior et al., 2023). In a broader regional context, Lentz et al. (2021) documented the emergence and rapid dissemination of *mcr-1* across Latin America, reporting its presence in over ten countries, frequently associated with epidemic plasmid backbones and high-risk clones of Enterobacterales. Notably, Brazil appeared among the top contributors of mcr-1-positive isolates, further reinforcing the country's central role in the regional spread of colistin resistance (Lentz et al., 2021). These findings emphasize Brazil's critical role in the global dissemination of *mcr-1* and reinforce the importance of implementing robust local and regional surveillance strategies to monitor resistance dissemination.

This study aimed to elucidate the complex mechanisms underlying polymyxin resistance in hospital-acquired Gram-negative isolates in Brazil, including lipid A modifications and plasmid-mediated *mcr-1* dissemination, and to assess the virulence potential of these strains using *in vivo* and genomic tools. Our goal was to explore the interplay of resistance, virulence, and mobile genetic elements to inform surveillance and mitigation strategies.

2. Material and methods

2.1. Sample isolation and identification

Seventeen polymyxin B-resistant GNB strains were isolated from various culture sources of inpatients admitted to four different hospitals in São Paulo State, Brazil, between 2016 and 2018. Initial bacterial identification and antimicrobial susceptibility testing were conducted at the Adolfo Lutz Institute, a Public Health Laboratory located in Marília, São Paulo State, Brazil. The species were also identified by lipid A profiling using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF MS) in negative ion and reflectron mode as previously described (Yang et al., 2022). Lipid A spectra were acquired and processed using flexControl and flexAnalysis version 3.4 software (Bruker Daltonics Inc.). The strains that exhibited the addition of cationic groups (L-Ara4N and/or PEtN) and or mass difference (e.g. LpxO) related to polymyxin-resistant to lipid A were selected for further experiments. *Klebsiella pneumoniae* complex were confirmed by the MALDI TypeR platform from the Pasteur Institute (https://malditypeR.pasteur.fr/). This study was approved by the School of Pharmaceutical Sciences of Ribeirao Preto Ethics Committee under the number 20413419.2.0000.5403.

2.2. Antimicrobial susceptibility testing, molecular characterization of ARGs and Inc plasmids, and molecular typing by Multilocus Sequencing Typing

Susceptibility testing was performed on 19 antimicrobials including 8 beta-lactams by the disk diffusion method: cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), cefpodoxime (CPD, 30 μg), cefepime (CPM, 30 μg), aztreonam (ATM, 30 μg), ertapenem (ETP, 10 μg), imipenem (IPM, 10 μg), and meropenem (MEM, 10 μg) and 11 non-beta-lactams: ciprofloxacin (CIP, 5 μg), nalidixic acid (NAL, 30 μg), amikacin (AMK, 10 μg), gentamicin (GEN, 10 μg), tobramycin (TOB, 10 μg), chloramphenicol (CHL, 30 μg), tetracycline (TET, 30 μg), sulbactam-ampicillin (SAM, 20/10 μg), sulfamethoxazole-trimethoprim (SXT, 1,25/23,75 μg), piperacillin-tazobactam (TZP, 100/10 μg), and ticarcillin-clavulanate (TIM), following the instructions in accordance with the recommendations of the Brazilian Committee on Antimicrobial Susceptibility Testing (BrCAST). Minimum inhibitory concentrations (MICs) were determined for polymyxin B and tigecycline by the reference broth microdilution method in a 96-well plate and using two-fold serial dilution (32–0.06 μg/mL), according to the BrCAST guidelines.

PCR was conducted to detect different ARGs, including acquired beta-lactamase genes ($bla_{\text{CTX-M-GROUP-1}}$, $bla_{\text{CTX-M-GROUP-2}}$, $bla_{\text{CTX-M-GROUP-8}}$, and $bla_{\text{CTX-M-GROUP-9}}$, bla_{TEM}, bla_{SHV}, bla_{PER}, bla_{GES}, bla_{BEL}, and bla_{VEB}), *tet* and *qnr* genes following primers used previously (Ballaben et al., 2022). PCR-based replicon typing (PBRT) was used to search for plasmids commonly found among *Enterobacterales* (Carattoli et al., 2005; García-Fernández et al., 2009). Multilocus Sequence Typing (MLST) was performed according to the *K. pneumoniae* (http://bigsdb.pasteur.fr/klebsiella/) and *E. coli* (https://pubmlst.org/organisms/escherichia-spp) databases.

2.3. In-vivo virulence evaluation

Galleria mellonella infection model was used to evaluate the virulence of the 17 polymyxin-resistant GNB strains according to available protocols (Paziani et al., 2019; Yang et al., 2018). Groups of 10 *G. mellonella* larvae at the sixth instar of development were selected at 225 ± 50 mg each, and the absence of cuticle pigmentation was selected to determine the virulence. A Hamilton syringe was used to inject a 10-μL inoculum containing PBS 1 × (control) and 1,5 × 108 CFU/mL of bacteria into the hemocoel of each larva through the last right proleg. Then, the larvae were maintained in glass containers in darkness at 37°C and monitored for survival daily. Survival curves and statistical analysis (Log-rank test, Mantel-Cox) were performed using GraphPad Prism, version 5.0 statistical software. P value < 0.05 was considered statistically significant.

2.4. Genome assembly and annotation, characterization of resistance and virulence factors

WGS was performed using Illumina MiSeq for short reads (2 ×250 bp, with an average depth coverage of approximately 100 ×) for two representative strains, *E. coli* 244/17 and *K. pneumoniae* 139/18. The strains were selected based on the evaluation of different Inc groups previously detected by PBRT, including the most prevalent Inc groups in the collection: *E. coli* 244/17, which possessed the IncN and IncFIIB groups, and *K. pneumoniae* 139/18, which carried the IncA/C group, as well as antimicrobial resistance profiles, representing key features of our collection. The genomes were assembled using SPAdes v3.15.5 (Bankevich et al., 2012) and annotated by Bakta v1.10.1 (Schwengers et al., 2021). The genome completeness was evaluated with CheckM v1.2.2 (Parks et al., 2015).

Resistance, virulence, and plasmid genes were identified using ResFinder, VirulenceFinder, and PlasmidFinder, all available at the Center for Genomic Epidemiology (CGE) (https://www.genomicepidemiology.

org). The Comprehensive Antibiotic Resistance Database was also used for ARGs search. Additional tools included Multi-Locus Sequence Typing (MLST), pMLST (plasmid typing), and MobileElementFinder. The Bio-Samples have been deposited at GenBank database under accession number PRJNA1248546.

3. Results and discussion

3.1. Bacterial strains, antimicrobial resistance profile, molecular characterization and typing

Fourteen *Escherichia coli* and 3 *Klebsiella pneumoniae* strains were previously identified and confirmed by MALDI-TOF-MS. The strains were recovered from urine, blood, surgical wound secretion, and rectal swab. All strains were resistant to polymyxin and/or produced MCR and had polymyxin B MICs between 2 and 8 μg/mL (Fig. 1). Most of the strains were considered MDR, defined by resistance to at least one antibiotic in three (or more) different antibiotic classes.

All strains except one (Ec 156/17) possessed ARGs, including *mcr-1*, $bla_{\text{CTX-M-GROUP-1}}$, $bla_{\text{CTX-M-GROUP-2}}$, $bla_{\text{CTX-M-GROUP-8}}$, $bla_{\text{TEM-1B}}$, bla_{NDM}, *tetA*, *tetB*, *aadA2b*, *catA1, inuF,* and *qnrB* (Fig. 1). Eight different plasmid Inc groups were found, being IncFIB (n = 15) the most frequently detected, followed by IncN (n = 10), IncA/C (n = 6), IncI2 (n = 5), IncP (n = 3), IncY and IncFIA (n = 2), and IncFIIs (n = 1) (Fig. 1). The co-expression of multiple ARGs is not a novel scientific finding; however, its significant epidemiological implications and consequent threats to public health remain a critical concern. The co-occurrence of *mcr-1* and $bla_{\text{CTX-M-like}}$ genes represents an escalating challenge in addressing antibiotic resistance. Several lines of evidence suggest that these resistance mechanisms are increasingly disseminating across diverse environments, including healthcare settings, agricultural environments, and community settings, underscoring the urgent need for coordinated strategies to mitigate this public health threat (Bastidas-Caldes et al., 2023; De La Cadena et al., 2023; Lentz et al., 2021; Mikhayel et al., 2024; Wang et al., 2021).

MLST analysis identified 13 distinct sequence types (STs) among the *Escherichia coli* isolates: ST10, ST48, ST131, ST224, ST312, ST354, ST393, ST410, ST624, ST1140, ST1640, and ST3024. These were distributed across eight clonal complexes (CCs): CC10, CC131, CC354, CC31, CC23, CC648, CC350, and CC101. ST224 and ST1140 were not assigned to any known CC.

The most represented lineage was CC10, encompassing ST10 and ST48. This complex is globally distributed and genetically diverse, with strains commonly detected in humans, food-producing animals, and aquatic environments (Nüesch-Inderbinen et al., 2017; Pérez-Etayo et al., 2022; Reid et al., 2019; Sanz et al., 2022a). It is often associated with multidrug resistance, particularly due to the presence of ESBL genes such as $bla_{\text{CTX-M}}$ variants and, in some cases, *mcr-like* genes. CC10 has also been recognized as a reservoir for extraintestinal pathogenic *E. coli* (ExPEC), with isolates capable of causing human infections and

Isolate ID/Year (source)[a]	Resistances[b]	MIC POL B (μg/mL)[c]	Resistance genes[d]	Virulence Profile	Inc group (Plasmid)	ST (CC)	Lipid A spectra (MALDI-TOF-MS)	Pattern
E. coli 379/17 (urine)	CTX, GEN, NA, CIP, NOR	8	*mcr-1*, $bla_{\text{CTX-M group 2}}$	Avirulent	I2, FIB, Y	410 (23)		(A)
E. coli 381/17 (urine)	SAM, NA, CIP, NOR, GEN, TOB, TET, SXT	8	*mcr-1*, $bla_{\text{CTX-M group 1}}$, *tetA*		N, FIB, P, A/C	624 (648)		
E. coli 56/18 (urine)	SAM, ATM, CTX, CPD, CAZ, CPM, CIP, NA, NOR, GEN, TOB, SXT, TIM	8	*mcr-1*, $bla_{\text{CTX-M group 2}}$		FIB	1640 (350)		
E. coli 155/17 (urine)	SAM, ATM, CTX, CPD, CPM, CIP, NOR, NA, GEN, TOB, TET, SXT, TIM	4	*mcr-1*, $bla_{\text{CTX-M group 2}}$, *tetA*	Moderately virulent	FIB, P	354 (354)		
E. coli 464/18 (urine)	TIM	4	*mcr-1*		I2, FIB	131 (131)		
E. coli 231/17 (urine)	SAM, ATM, CTX, CPD, CPM, NA, TIM	2	*mcr-1*		I2, FIA, FIB	48 (10)		
E. coli 152/17 (urine)	NA, CIP, NOR, TET, TIM	8	*mcr-1*, *tetA*, *tetB*	Moderately virulent	N, FIB, Y	3024 (101)		(B)
E. coli 157/17 (urine)	NA, CIP, NOR, TIM	8	*mcr-1*		N, FIB	393 (31)		
E. coli 244/17 (blood)#	SAM, ATM, CTX, CPD, CPM, CAZ, ETP, IPM, MER, AMK, TOB, CHL, TET, TZP, SXT, TIM	4	*mcr-1*, $bla_{\text{TEM-1B}}$, *aadA2b*, *catA1*, *inuF*	Avirulent	N, FIB	3076 (-)		
E. coli 973/16 (urine)	SAM, ATM, CTX, CPD, CAZ, CPM, ETP, CIP, TZP, SXT, TIM	4	*mcr-1*, $bla_{\text{CTX-M group 1}}$		N, FIB, A/C	1140 (-)		
E. coli 156/17 (urine)	NA, CIP, NOR	4	-	Avirulent	FIB	393 (31)		(C)
E. coli 816/16 (surgical wound secretion)	SAM, ATM, CTX, CPD, CAZ, CPM, CIP, CHL, TET, SXT, TIM	4	$bla_{\text{CTX-M group 8}}$, *tetA*	Virulent	I2, N, FIA, FIB	10 (10)		
E. coli 153/17 (urine)	SAM, ATM, CTX, CPD, CPM, CIP, TET, TZP, SXT, TIM	8	*mcr-1*, $bla_{\text{CTX-M group 8}}$, *tetA*	Avirulent	I2, FIB, A/C	224 (-)		(D)
E. coli 622/17 (urine)	NA, CIP, NOR, TET, SXT, TIM	4	*mcr-1*, *tetA*	ND	N, FIB	312 (354)		ND†
K. pneumoniae 139/18 (unknown)#	SAM, ATM, CTX, CPD, CAZ, CPM, EPT, IPM, MER, GEN, TOB, AMK, NA, CIP, LEV, CHL, TET, SXT, TIM, TZP	8	*aac(6')-Ib-cr*, *aadA2*, *aac(3)-IIa*, $bla_{\text{OXA-1}}$, $bla_{\text{SHV-11}}$, $bla_{\text{SHV-67}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{NDM-1}}$, *fosA*, *erm(C)*, *catA1*, *catB3*, *oqxA*, *oqxB*, *qnrB1*, *sul1*, *sul2*, *tetA*, *dfrA12*, *dfrA14*	Moderately virulent	A/C	147 (-)		(E)
K. pneumoniae 73/17 (rectal swab)	SXT, TET	4	*tetA*	Hypervirulent	N, FIIs	11 (11)		(F)
K. pneumoniae 136/17 (urine)	CTX, AMC, CAZ, ATM, NA, CIP, NOR, SXT, TET, TIM	4	$bla_{\text{CTX-M group 1}}$	Avirulent	N, A/C	4477 (-)		(G)

Fig. 1. MALDI-TOF MS lipid A profiles, associated virulence phenotypes, and sequence types (STs) of polymyxin-resistant *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates. Representative lipid A spectra (A–G) illustrate distinct structural modifications, including phosphoethanolamine (PEtN), 4-amino-4-deoxy-L-arabinose (L-Ara4N), phosphate, palmitate, and LpxO-mediated hydroxylation. These molecular alterations correlate with variable virulence levels observed in the Galleria mellonella infection model, ranging from avirulent to hypervirulent profiles. STs determined by multilocus sequence typing (MLST) are indicated for each isolate. a: # Isolate sequenced by Illumina. ARGs were predicted by WGS data. b: SAM: sulbactam-ampicillin, TZP: piperacillin-tazobactam, TIM: ticarcillin-clavulanate, CTX: cefotaxime, CAZ: ceftazidime, CPD: cefpodoxime, CPM: cefepime, ATM: aztreonam, ETP: ertapenem, IPM: imipenem, MER: meropenem, GEN: gentamicin, TOB: tobramycin, AMK: amikacin, TET: tetracycline, NA: nalidixic acid, CIP: ciprofloxacin, NOR: norfloxacin, SXT: sulfamethoxazole-trimethoprim, CHL: chloramphenicol. c: MIC for POL B: polymyxin B; according to BrCAST, the recommended cut-off points are ≤ 2 (Susceptible) and > 2 (Resistant). POL B MIC was determined by broth microdilution as recommended. d: -, no target genes were detected, † ND: not determined (This isolate was lost during the experiment).

carrying virulence markers typical of clinical lineages (Nüesch-Inderbinen et al., 2017; Pérez-Etayo et al., 2022).

Among the detected lineages, ST131 (CC131) stands out as a globally dominant and clinically significant clone (Gladstone et al., 2021). It is a pandemic ExPEC lineage frequently associated with high rates of urinary tract and bloodstream infections in both community and hospital settings (de Castro et al., 2025). Its global success is driven by the expansion of fluoroquinolone-resistant sub lineages, particularly those carrying $bla_{CTX\text{-}M\text{-}15}$ and other ESBL genes (García-Meniño et al., 2024). Despite extensive antimicrobial resistance, ST131 retains a rich repertoire of virulence factors, enhancing its transmissibility and pathogenic potential (Balbuena-Alonso et al., 2023; Hassuna et al., 2024). Its detection in this study reinforces its critical role in resistance surveillance and treatment considerations.

Two isolates in our dataset, ST312 and ST354, belong to CC354, a group increasingly recognized in Brazil for its association with multidrug resistance and circulation in both clinical and environmental contexts (Fuga et al., 2022). ST354 has been frequently reported carrying $bla_{CTX\text{-}M\text{-}24}$ and resistance to fluoroquinolones, with detections in human infections, poultry meat, livestock, and water (Pérez-Etayo et al., 2022; Soncini et al., 2022). The detection of these two STs reflects the growing relevance of CC354 in local dissemination of resistance traits.

ST393 (CC31) and ST410 (CC23), both recognized as pandemic *E. coli* lineages with high-risk potential were also detected (Pérez-Etayo et al., 2022). ST410 has been reported in a wide range of human and non-human sources and is often associated with $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, and carbapenemase genes such as bla_{NDM} and $bla_{OXA\text{-}48}$ (Fuga et al., 2022). Its detection in human isolates underscores its expanding relevance in both environmental and clinical settings. ST393, part of CC31, is a globally disseminated multidrug-resistant ExPEC clone, identified across humans, animals, and environmental samples, including remote regions such as the Brazilian Amazon (Vianello et al., 2021). ST393 isolates typically carry $bla_{TEM\text{-}1}$, aminoglycoside-modifying enzymes, *tetB*, and mutations associated with fluoroquinolone resistance, often linked to IncQ1 and IncF-type plasmids (Vianello et al., 2021). These features support its persistence and global dissemination.

ST624, assigned to CC648, represents another lineage of growing concern. Although less studied than ST13, CC648 has been implicated in both community and hospital infections and is known for harboring a broad resistance repertoire, including ESBLs, carbapenemases (bla_{CTX}, bla_{KPC}) (Sanz et al., 2022b; Seenama et al., 2019). Its detection in a human clinical isolate further supports its status as an emerging MDR clone.

ST3024, affiliated with CC101, adds to this pattern of emerging high-risk lineages. ST101, the most well-characterized member of CC101, has been found in human and animal infections and is often associated with ESBL and carbapenemases such as bla_{OXA}, bla_{KPC}, and bla_{NDM} (Peng et al., 2022). It is known for rapid dissemination in healthcare environments and for its ability to colonize multiple hosts (Ku et al., 2023). Although ST3024 is less studied, its inclusion in CC101 suggests similar genetic features and public health relevance.

Finally, ST1640 (CC350) was also detected, and while it is less frequently reported in literature, it has been identified in ESBL-producing E. coli isolated from environmental, food, and clinical sources (Ojer-Usoz et al., 2017). The presence of this clonal complex among human clinical isolates underscores the need to include lesser-known lineages in surveillance efforts, as they may serve as silent reservoirs for antimicrobial resistance.

Taking all findings together, the clonal diversity observed in this study reflects the wide-ranging evolutionary trajectories of *E. coli* involved in human infections. Although all isolates originated from clinical human sources, several sequence types such as ST10, ST354, ST410, and ST393 have also been reported in animals, food, and environmental settings. These findings emphasize the interconnected nature of reservoirs in the dissemination of resistant *E. coli* and reinforce the importance of integrating a One Health perspective into antimicrobial resistance surveillance efforts.

According to MLST, two STs were identified among the *K. pneumoniae* isolates: ST11 and ST4477.

ST11 is one of the most widespread and clinically significant *Klebsiella pneumoniae* lineages worldwide. It belongs to clonal complex 11 (CC11), a group strongly associated with the dissemination of carbapenem resistance, particularly via KPC-type enzymes (Xie et al., 2021). Globally, ST11 has been frequently linked to hospital outbreaks, especially in Asia, where it plays a dominant role in the spread of $bla_{KPC\text{-}2}$ and bla_{NDM} genes (Yuan et al., 2024). Notably, this lineage has demonstrated the ability to acquire virulence plasmids encoding siderophores (such as aerobactin and yersiniabactin), *rmpA/rmpA2*, and other hypervirulence determinants, giving rise to multidrug-resistant hypervirulent variants, including the well-characterized ST11-KL64 sub lineage (Chen et al., 2025).

In Brazil, ST11 has also emerged as a dominant high-risk clone associated with healthcare-associated infections. Molecular epidemiology studies have documented a shift in clone prevalence over time, with ST11 increasingly replacing other clones in clinical settings and beyond this environment (Esposito et al., 2023). Brazilian ST11 isolates frequently harbor multiple carbapenemase genes (bla_{KPC}, bla_{NDM}), including emergent KPC variants (e.g., KPC-33, KPC-103 to KPC-143), and present extensive resistance profiles along with virulence genes related to adherence, capsule formation, and siderophore production (de Oliveira et al., 2023; Lobato et al., 2023).

No information is currently available in the literature regarding ST4477, suggesting this may represent a novel or underreported lineage. The isolate assigned to this sequence type (Kp136/17), recovered from a human urine sample, harbored $bla_{CTX\text{-}M\text{-}group1}$. The presence of this ESBL in this uncommon lineage highlights its potential clinical relevance, particularly in urinary tract infections. Although the evolutionary background and epidemiological distribution of ST4477 remain unknown, its detection in a clinical context reinforces the need for continuous genomic surveillance to identify emerging *K. pneumoniae* clones with resistance potential.

Together, these findings illustrate the coexistence of a globally dominant high-risk clone (ST11) and a poorly characterized lineage (ST4477) among the *K. pneumoniae* isolates. This highlights both the persistence of well-established epidemic clones and the emergence of potentially novel sequence types in clinical settings.

3.2. Lipid A analysis and polymyxin resistance

Lipid A analysis revealed that all MCR-1-producing *E. coli* strains presented an addition of PEtN ($\Delta m/z$ 123) and/or L-Ara4N ($\Delta m/z$ 131) and/or phosphate ($\Delta m/z$ 80) (Fig. 1 A, B, D). This modification reduces the membrane's net negative charge, diminishing the binding affinity of polymyxins, which target negatively charged lipids (Sun et al., 2018). Strains with lipid A pattern D exhibited a L-Ara4N addition. This cationic group is incorporated into lipid A via a biosynthetic pathway that includes ArnA for its synthesis and the *arnBCADTEF* operon for its transfer. This modification markedly changes the charge properties of lipid A, enhancing bacterial survival in adverse environments (Rogga and Kosalec, 2023). The phosphate addition was observed within lipid A patterns B, D, and E (Fig. 1). LpxT, an enzyme associated with lipid A modification, catalyses the phosphorylation of lipid A, resulting in the formation of lipid A 1-diphosphate (Valvano, 2022). A study reported that in *E. coli*, the interplay between EptA- and LpxT-dependent modifications of lipid A is crucial, as LpxT enhances the net negative charge, while its inhibition by PmrR under specific conditions allows polymyxin B resistance and increases resistance to deoxycholate, a secondary bile acid produced in the intestines, impacting intestinal colonization and survival (Tian et al., 2021).

Although the addition of PEtN and L-Ara4N to lipid A is commonly observed in polymyxin-resistant bacteria, other molecules can also be associated with this phenotype. For example, *K. pneumoniae* strains

presented the addition of palmitate (Δ*m/z* 238) and/or phosphate and/or LpxO (Δ*m/z* 16) (Fig. 1 E–G). This highlights the complex pathways involved in the development of polymyxin resistance. The enzyme PagP (phospholipid lipid A palmitoyltransferase) is essential in the adaptation of GNB to environmental stress, plays a significant role in immune evasion and bacterial pathogenesis, and contributes to antibiotic resistance, particularly to polymyxins (Huang et al., 2020). PagP transfers a palmitate chain from phospholipids to lipid A, a component of lipopolysaccharides (LPS) in the outer membrane. This palmitoylation increases membrane hydrophobicity and reduces the negative charge, limiting the electrostatic interaction and binding of polymyxins to lipid A (Bishop, 2005). This modification, regulated by systems like PhoP/-PhoQ, enhances bacterial survival in the presence of polymyxin, making it a key resistance mechanism (Huang et al., 2020; Sun et al., 2022). LpxO is a lipid A-modifying enzyme essential for the pathogenicity of *Klebsiella pneumoniae* (Kidd et al., 2017). This enzyme hydroxylates secondary acyl chains, such as myristate, which are incorporated into lipid A by LpxL2, a late-stage acyltransferase (Mills et al., 2017). Moreover, LpxO-mediated hydroxylation reduces recognition by the host immune system. *lpxO* mutants elicit stronger inflammatory responses and exhibit increased susceptibility to phagocytosis. Additionally, LpxO-dependent lipid A hydroxylation enhances resistance to polymyxins and other cationic antimicrobial peptides by decreasing membrane permeability. The expression of *lpxO* is regulated by the PhoPQ two-component system, which is activated in response to environmental stressors, including antimicrobial peptides (Mills et al., 2017).

Despite the distinct lipid A profile patterns observed between the *E. coli* and *K. pneumoniae* strains, no correlation was found between the addition of specific molecules to lipid A and the MICs for these organisms. For instance, as shown in Fig. 1, strains exhibiting the same lipid A pattern (pattern A), displayed polymyxin B MICs ranging from 2 to 8 μg/mL. Furthermore, strains lacking any modifications to lipid A (pattern C) exhibited an MIC of 4 μg/mL, which is considered resistant to polymyxin B. These findings highlight the complexity of the resistance mechanisms to polymyxins and the challenge in correlating the phenotype and genotype. The mechanisms underlying polymyxin B resistance in GNB remain complex and only partially understood. GNB can develop resistance through intrinsic mechanisms, mutations, adaptive responses, or horizontally acquired resistance mediated by the *mcr-1* gene and its variants (El-Sayed Ahmed et al., 2020). Previous studies have examined the correlation between the presence of the *mcr-1* gene and the minimum inhibitory concentration (MIC) of polymyxin B/colistin, reporting that strains harboring the gene typically exhibit MIC values between > 2 and 4 μg/mL (Pillonetto et al., 2019). These findings align with the observations from this study (Fig. 1). Furthermore, strain Ec231/17 was confirmed as an MCR-1 producer but exhibited an MIC of 2 μg/mL, classifying it as susceptible to polymyxin B. This phenotype-genotype discordance, previously reported in other studies, underscores the importance of robust antimicrobial resistance surveillance programs that integrate phenotype and genotype analysis (Nang et al., 2019; Pillonetto et al., 2019). Another important point to highlight is the case of strains Ec816/16 and Ec156/17 that did not present any cationic group addition to lipid A but showed a polymyxin B MIC of 4 μg/mL. Polymyxin resistance in GNB also involves various mechanisms beyond enzymatic modifications to lipid A (Olaitan et al., 2014). The intrinsic regulator RamA in *K. pneumoniae* is recognized for its crucial role in the bacterial response to antimicrobial agents. It controls genes associated with permeability barriers, which may contribute to diminished antibiotic susceptibility. Recent studies have demonstrated that elevated levels of this regulator lead to modifications in LPS, resulting in a decreased susceptibility to polymyxin antibiotics (Doi and van Duin, 2020). Together, these mechanisms, alongside enzymatic modifications such as lipid A alterations, contribute to the complex strategies that bacteria employ to evade polymyxin antibiotics, presenting significant challenges for treatment and control.

3.3. In vivo virulence assay

All polymyxin-resistant GNB strains (except strain Ec 622/17) were evaluated for *in vivo* virulence using the *Galleria mellonella* model (Supplementary Figure 1). Among them, only *K. pneumoniae* strains 816/16 and 73/17 were able to kill all larvae within 48 h and were thus classified as highly virulent (Group C). *E. coli* strains 231/17, 381/17, 155/17, 464/18, and 152/17, as well as *K. pneumoniae* strain 139/18, caused progressive larval death and were considered moderately virulent (Group B). In contrast, *E. coli* strains 244/17, 379/17, 973/17, 153/17, 156/17, 157/17, 56/18, and *K. pneumoniae* strain 136/17 were categorized as avirulent (Group A), as they killed fewer than 70 % of the larvae or failed to cause any lethality during the 10-day observation period. Notably, strain Ec 244/17, despite carrying several virulence determinants according to WGS, did not cause any larval mortality. In this context, although the strain exhibited multiple virulence determinants, no clear correlation between genotype and phenotype could be established.

The *G. mellonella* model was selected due to its scalability, low cost, ethical feasibility, and the presence of a sequenced genome with a conserved innate immune system, which allows for comparative assessments of bacterial virulence (Wright and Kavanagh, 2022). Although this model lacks adaptive immunity and cannot fully recapitulate human infection, it has been widely used to evaluate virulence in a range of Gram-negative bacteria, including *Enterobacterales* (Ballaben et al., 2022; Elizalde-Bielsa et al., 2023; Ménard et al., 2021; Pereira et al., 2020).

3.4. WGS analysis

Sequencing was performed using short reads. All the metadata are summarized in Supplementary Table 1.

E. coli 244/17 was isolated from the bloodstream of a hospitalized patient in São Paulo in 2017. The strain was only susceptible to GEN and classified as multidrug-resistant (MDR) (Fig. 1). According to *in silico* analysis, ResFinder revealed five ARGs: *aadA2b*, $bla_{\text{TEM-1B}}$, *mcr-1.1*, *Inu (F)*, and *catA1* while CARD detected 56 ARGs, including resistance-nodulation-cell division (RND), major facilitator superfamily (MFS), small multidrug resistance (SMR), and ATP-binding cassette (ABC) efflux-pumps related genes.

VirulenceFinder identified virulence determinants related to adhesion (*fdeC, nlpI, tia*), fimbriae (*fimH, lpfA, papC, yehA, yehB, yehC, yehD*), haemolysis (*hlyE*), tellurium tolerance (*terC*), and immune evasion (*traT, traJ*). The VFDB database identified 70 virulence determinants, including genes for invasion (*ibeB, ibeC*) and secretion systems. PlasmidFinder detected IncX4, IncR, and IncN plasmid groups. According to MLST, *E. coli* 244/17 belongs to ST3076. In 2019, emergence of IncX3 plasmid-harboring $bla_{\text{NDM-5}}$ in *E. coli* from different STs, including ST 3076, was reported from China (Liu et al., 2019). To the best of our knowledge, there is no available information regarding this ST to date. Although Ec 244/17 is not an NDM-producing strain, these results demonstrate that this ST may be associated with various ARGs, including $bla_{\text{NDM-5}}$ and *mcr-1*, carried on different Inc group plasmids.

MobileElementFinder identified various mobile genetic elements (MGEs), such as IS*630*, IS*10*, IS*3*, IS*605*, and others, some of which were associated with ARGs or virulence genes. The *mcr-1.1* gene and IncX4 replication site were located on the same contig (k_141_87), suggesting that *mcr-1.1* is carried by the IncX4 plasmid. The relationship between MGEs and ARGs has been widely explored, underscoring the pivotal role of MGEs in the spread of diverse ARGs, including *mcr-1* (Goodman et al., 2023; Koonin et al., 2020). Since its initial detection in 2016, multiple *mcr* variants have been identified across all continents except Antarctica in many bacterial species such as *E. coli, K. pneumoniae, Salmonella enterica*, and *Shigella sonnei*. These variants have been located on plasmids of various Inc groups, particularly IncX4 and IncHI2, as well as integrated into the chromosome (Girardello et al., 2021; Nang, Li, &

Velkov, 2019).

The genetic environment of *mcr-1.1* in *E. coli* isolate Ec244/17 is shown in Fig. 2A. Unlike the canonical Tn*6330* structure, which typically features *mcr-1* flanked by two IS*Apl1* insertion sequences and a downstream *pap2* gene (Snesrud et al., 2018), no IS*Apl1* elements were detected in this isolate. This suggests that *mcr-1.1* is embedded in a distinct, possibly stabilized, genetic context. Notably, the *pap2* gene was identified downstream of *mcr-1.1*, maintaining the typical adjacency observed in many IncI2- and IncX4-type plasmids. As previously demonstrated by Choi et al. (2020), the presence of an intact *pap2* gene appears to be functionally important for the expression of colistin resistance, reinforcing the potential phenotypic relevance of this arrangement (Choi et al., 2020).

This study identified eight Inc groups via PBRT among the strains carrying the *mcr-1* gene. However, additional experiments are necessary to determine the precise genetic location of the gene and, if plasmid-associated, which specific Inc plasmid harbours it.

K. pneumoniae 139/18 was isolated from a hospitalized patient in 2018. The strain was resistant to all tested antimicrobials thus classified as XDR according to the literature (Magiorakos et al., 2012) (Fig. 1). Preliminary analysis identified the presence of $bla_{\text{CTX-M-group 1}}$, bla_{NDM}, *mcr-1*, and an IncA/C plasmid. Surprisingly, the *mcr-1* gene was not detected in WGS data despite repeated PCR confirmation prior to sequencing. ResFinder identified 21 ARGs, including *aac(6′)-Ib-cr, aadA2, aac(3)-IIa*, $bla_{\text{OXA-1}}$, $bla_{\text{SHV-11}}$, $bla_{\text{SHV-67}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{NDM-1}}$, *fosA*, *erm(C), catA1, catB3, oqxA, oqxB, qnrB1, sul1, sul2, tetA, dfrA12*, and *dfrA14*. Moreover, amino acid mutations were found in *ompK36* (N49S, L59V, G189T, F198Y, F207Y, A217S [associated with carbapenem resistance], T222L, D223G, Q227S, L228K, E232R, and N304E) and in *ompK37* (I70M and I128M [both associated with carbapenem resistance]). CARD detected 47 ARGs, including genes encoding resistance-nodulation-cell division (RND), major facilitator superfamily (MFS), small multidrug resistance (SMR), and ATP-binding cassette (ABC) efflux pumps.

VirulenceFinder detected three virulence determinants: *fimH, mrkA*, and *iutA* (using the *Escherichia* database). The VFDB database (specific to *Klebsiella*) revealed numerous virulence factors, including those related to adhesion (*mrkA–mrkJ, fimA–fimK, pilW*), iron acquisition (*iutA, entA–entF, fepA–fepG, iroE, iroN*), regulation (*rcsA, rcsB*), and secretion systems (*clpV/tssH, dotU/tssL, hcp/tssD, impA, ompA*, and others). PlasmidFinder identified IncC (ST03), IncFIB, IncFII, Col4401, and RepB plasmid groups.

According to MLST, *K. pneumoniae* 139/18 belongs to ST147. MobileElementFinder detected MGEs associated with ARGs or virulence genes, including IS*Kpn1*, IS*Kpn13*, IS*Kpn21*, IS*Kpn26*, IS*Kpn28*, IS*5075*.

Klebsiella pneumoniae ST147 is a globally recognized, high-risk clone associated with multidrug resistance (MDR) and severe hospital-acquired infections (Lapp et al., 2021; Park et al., 2023). This lineage is particularly concerning due to its capacity to harbor a wide array of resistance genes, including carbapenemase genes such as bla_{NDM}, bla_{KPC}, and $bla_{\text{OXA-48}}$, as well as extended-spectrum beta-lactamase (ESBL) genes like $bla_{\text{CTX-M}}$. Additionally, some ST147 strains exhibit resistance to colistin, attributed to the acquisition of the *mcr-1* gene or mutations in the *mgrB* gene. The dissemination of these resistance determinants is facilitated by large plasmids and MGEs, enabling horizontal gene transfer and rapid adaptation. Beyond its resistance profile, certain ST147 strains possess virulence factors such as siderophores (yersiniabactin and aerobactin), which enhance pathogenicity and survival in host environments (Falcone et al., 2022). Clinically, ST147 has been implicated in life-threatening infections, including pneumonia, bloodstream infections, and urinary tract infections, particularly in immunocompromised patients. Outbreaks caused by ST147 have been reported globally, posing significant challenges to healthcare systems due to limited treatment options, which often rely on combination therapies involving last-resort antibiotics such as colistin, tigecycline, or fosfomycin (Elgayar et al., 2024; Martin et al., 2021; Peirano et al., 2020). The remarkable genetic plasticity of ST147, combined with its resistance and virulence potential, highlights the urgent need for enhanced global surveillance, stringent infection control measures, and accelerated development of innovative antimicrobial strategies.

The genetic environment of $bla_{\text{CTX-M-15}}$ in Kp139/18 is depicted in Fig. 2B. The $bla_{\text{CTX-M-15}}$ gene was preceded by the insertion sequence IS*Ecp1*, a well-characterized mobile element known to mediate the mobilization and transposition of $bla_{\text{CTX-M-type}}$ gene (Zhao et al., 2020). This arrangement, with IS*Ecp1* immediately upstream of $bla_{\text{CTX-M-15}}$, has been frequently reported in a range of pathogenic bacterial species and plays a key role in the horizontal dissemination of this resistance determinant (Awosile and Agbaje, 2021; Cerezales et al., 2020; Ebmeyer

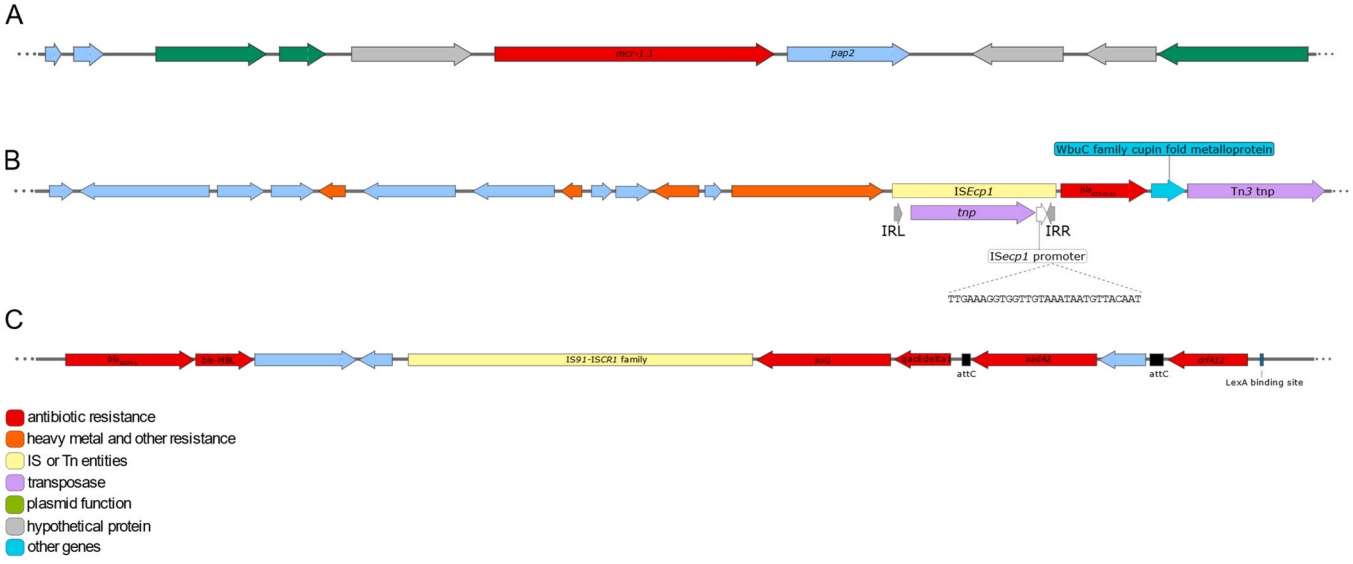

Fig. 2. Representation of genetic arrangements carrying antimicrobial resistance genes in clinical isolates. (A) Genetic context of the *mcr-1.1* gene in *E. coli* isolate Ec244/17, showing the downstream presence of *pap2*, a gene previously implicated in colistin resistance expression. (B) Genetic environment of $bla_{\text{CTX-M-15}}$ in *K. pneumoniae* isolate Kp139/18, associated with an IS*Ecp1* element suggestive of mobilization via transposition. (C) Multidrug resistance region from the same *K. pneumoniae* isolate, carrying bla_{NDM}, *sul1, ΔqacE, aadA2*, and *dfrA12*, the latter two exhibit their respective attC sites. IS*91*/I*SCR1* element was detected upstream. Arrows represent predicted coding sequences (ORFs), with colours indicating functional categories.

et al., 2021). Notably, chromosomal integration of IS*Ecp1*–$bla_{CTX\text{-}M\text{-}15}$ units has also been described in *Enterobacteriaceae* such as *E. cloacae* and *E. coli*, contributing to stable inheritance of multidrug resistance traits and potentially complicating clinical outcomes (Shawa et al., 2021).

The multidrug resistance region identified in Kp139/18 (Fig. 2C) harbors bla_{NDM}, *sul1, ΔqacE, aadA2*, and *dfrA12* with attC sites adjacent to *aadA2* and *dfrA12*, characteristic of gene cassettes. These cassettes are frequently embedded in class 1 integrons and have been widely reported in clinical *Enterobacterales* isolates (Cerezales et al., 2020). An upstream IS*CR1* element, belonging to the IS*91* family, was also detected. IS*CR1* plays a central role in the mobilization and expression of adjacent resistance genes via rolling-circle transposition and internal promoter activity (TnPedia Team, 2025). Its association with cassette arrays encoding aminoglycoside and trimethoprim resistance, such as *aadA* and *dfrA*, has been extensively documented (Domínguez et al., 2019). This configuration illustrates a well-established genetic arrangement involved in the dissemination of multidrug resistance in Gram-negative bacteria.

3.5. Limitations

This study is limited by the relatively small number of isolates analyzed and the use of short-read sequencing, which may constrain full plasmid reconstruction and the precise localization of resistance genes. Nonetheless, the integration of multiple analytical approaches provides strong evidence for the resistance mechanisms and virulence traits characterized here.

4. Conclusions

This study highlights the multifaceted nature of polymyxin resistance and virulence in clinical *Escherichia coli* and *Klebsiella pneumoniae* isolates from Brazilian hospitals. Through the integration of phenotypic assays, lipid A structural profiling, and whole-genome sequencing, we identified key resistance determinants such as *mcr-1.1,* $bla_{NDM\text{-}1}$, and $bla_{CTX\text{-}M\text{-}15}$ as well as diverse lipid A modifications including phosphoethanolamine (PEtN), palmitate, and LpxO-mediated hydroxylation, which contribute to reduced susceptibility to polymyxins. Virulence profiling revealed the presence of siderophores, adhesins, and secretion system genes, with notable strain-to-strain variability in pathogenic potential, as demonstrated in the *Galleria mellonella* model. The detection of high-risk clones such *as K. pneumoniae* ST147 co-harboring resistance and virulence genes exemplifies the convergence of traits that pose major challenges to clinical management. MLST analysis revealed high clonal diversity among *E. coli* isolates, including globally relevant lineages such as ST10, ST131, ST354, and ST410, many of which have been reported across human, animal, and environmental sources. Among *K. pneumoniae*, we identified ST11 a dominant carbapenemase-producing clone in Brazil and ST4477, a poorly characterized lineage carrying $bla_{CTX\text{-}M}$, suggesting the emergence of underreported resistance reservoirs. In the present study, IS*Ecp1* and IS*CR1* were observed in close genetic proximity to key resistance genes and gene cassettes, indicating co-localization. Nevertheless, our data do not demonstrate mobilization by these elements. To our knowledge, this is among the few studies to combine phenotypic, genotypic, and functional characterization of polymyxin-resistant strains in Brazil, offering valuable insights into the epidemiology of critical-priority pathogens. These findings reinforce the urgency of comprehensive antimicrobial surveillance, strengthened infection control policies, and continued research into novel therapeutic strategies.

CRediT authorship contribution statement

Anelise Stella Ballaben: Writing – review & editing, Writing – original draft, Visualization, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Joseane Cristina Ferreira**: Writing – review & editing, Methodology. **Ludmilla Tonani**: Writing – review & editing, Methodology. **Fabiana Caroline Zempulski Volpato**: Writing – review & editing, Methodology. **Afonso Luís Barth**: Writing – review & editing, Resources, Methodology. **Doroti de Oliveira Garcia**: Writing – review & editing, Resources. **Marcia R. von Zeska Kress**: Writing – review & editing. **Yohei Doi**: Writing – review & editing, Data curation. **Robert K. Ernst**: Writing – review & editing, Software, Resources, Methodology, Data curation. **Ana Lúcia da Costa Darini:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.microb.2025.100555.

Data availability

The BioSamples have been deposited at GenBank database under accession number PRJNA1248546 (SAMN47861066 and SAMN47861065).

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