Short Communication

# Characterisation of *mcr-4.3* in a colistin-resistant *Acinetobacter nosocomialis* clinical isolate from Cape Town, South Africa

Yolandi Snyman [a,*], Sandra Reuter [b], Andrew Christopher Whitelaw [a,c], Lisa Stein [a], Motlatji Reratilwe Bonnie Maloba [d,e], Mae Newton-Foot [a,c]

[a] *Division of Medical Microbiology, Department of Pathology, Stellenbosch University, Cape Town, South Africa*
[b] *Institute for Infection Prevention and Hospital Epidemiology, Medical Center University of Freiburg, Freiburg, Germany*
[c] *National Health Laboratory Service, Tygerberg Hospital, Cape Town, South Africa*
[d] *Department of Medical Microbiology, University of the Free State, Bloemfontein, South Africa*
[e] *National Health Laboratory Service, Universitas Hospital, Bloemfontein, South Africa*

## ARTICLE INFO

## ABSTRACT

*Objectives:* Colistin resistance in *Acinetobacter* spp. is increasing, resulting in potentially untreatable nosocomial infections. Plasmid-mediated colistin resistance is of particular concern due to its low fitness cost and potential transferability to other bacterial strains and species. This study investigated the colistin resistance mechanism in a clinical *Acinetobacter nosocomialis* isolate from Cape Town, South Africa.

*Methods:* A colistin-resistant *A. nosocomialis* isolate was identified from a blood culture in 2017. PCR and Illumina whole-genome sequencing (WGS) were performed to identify genes and mutations conferring resistance to colistin. Plasmid sequencing was performed on an Oxford Nanopore platform. *mcr* functionality was assessed by broth microdilution after cloning the *mcr* gene into pET-48b(+) and expressing it in SHuffle® T7 *Escherichia coli* and after curing the plasmid using 62.5 mg/L acridine orange.

*Results:* The colistin minimum inhibitory concentration (MIC) of the *A. nosocomialis* isolate was 16 mg/L. The *mcr-4.3* gene was detected by PCR and WGS. No other previously described colistin resistance mechanism was found by WGS. The *mcr-4.3* gene was identified on a 24 024-bp RepB plasmid (pCAC13a). Functionality studies showed that recombinant *mcr-4.3* did not confer colistin resistance in *E. coli*. However, plasmid curing of pCAC13a restored colistin susceptibility in *A. nosocomialis*.

*Conclusion:* We describe the first detection of a plasmid-mediated *mcr-4.3* gene encoding colistin resistance in *A. nosocomialis* and the first detection of *mcr-4.3* in a clinical isolate in Africa. Recombinant expression of *mcr-4.3* did not confer colistin resistance in *E. coli*, suggesting that its functionality may be RepB plasmid-dependent or species-specific.

## 1. Introduction

*Acinetobacter* spp. has been increasingly reported as an opportunistic pathogen causing severe nosocomial infections, e.g. ventilator-associated pneumonia, catheter-related bacteraemia, urinary tract, wound and soft-tissue infections, postsurgical endocarditis and meningitis. *Acinetobacter* spp. infections are often difficult to treat owing to multidrug resistance. Colistin is considered a last-resort antimicrobial used to combat these infections; however, colistin resistance in *Acinetobacter* spp. has emerged worldwide, including in South Africa [1,2].

In *Acinetobacter* spp., chromosomally-encoded colistin resistance mechanisms include mutations in the *pmrB*, *pmrA* and *pmrC* operon genes and in genes encoding lipid A biosynthesis (*lpxA*, *lpxC* and *lpxD*). These mutations modify the lipid A of lipopolysaccharide (LPS) or lead to the complete loss of LPS [2–4], thereby conferring colistin resistance. Integration of the insertion sequence IS*Aba11* in *lpxC* or *lpxA* also causes inactivation of LPS production

[5]. Alternatively, overexpression of phosphoethanolamine transferase can result from the integration of IS*AbaI* upstream of a *pmrC* homologue (*eptA*), also leading to colistin resistance [6].

Colistin resistance due to plasmid-mediated mobile colistin resistance genes *mcr-1* to *mcr-10* has been described worldwide in numerous bacterial species from various sources [7–10]. In *Acinetobacter baumannii*, *mcr-1* and the *mcr-4.3* variant have been described [11]; however, there are conflicting data regarding the role of *mcr-4.3* in colistin resistance [12–14]. New *mcr* variants are being identified worldwide at an alarming rate, yet the functional role of some of these variants is not clear.

In South Africa, colistin resistance mechanisms have been investigated in *Acinetobacter* spp. [1], but *mcr* genes have not yet been detected. *mcr-1* has been identified in *Escherichia coli* and *Klebsiella* spp. from clinical isolates in multiple hospitals [7–9] and in *E. coli* isolated from broiler chickens, a pig and final effluents from wastewater treatment plants [15–18]. The *mcr-9.1* variant has been detected in *Enterobacter hormaechei* clinical isolates from South Africa but was not associated with colistin resistance [19].

As colistin is one of the few 'last-resort' therapeutic options, it is important to elucidate and monitor the mechanisms contributing to colistin resistance. Plasmid-mediated colistin resistance is of particular concern due to its low fitness cost and the potential for transfer to other strains and species of bacterial pathogens. This study investigated the colistin resistance mechanism in a clinical *Acinetobacter nosocomialis* isolate from Cape Town, South Africa.

## 2. Materials and methods

### 2.1. Isolate collection and clinical setting

A single colistin-resistant *A. nosocomialis* isolate was obtained from routinely collected clinical specimens processed at the National Health Laboratory Service (NHLS) laboratory at Tygerberg Hospital in 2017. The isolate formed part of a larger cohort of colistin-resistant *Acinetobacter* spp. isolates collected by convenience sampling over a 16-month period (2016–2017) [1]. Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) (Bruker Daltonik GmbH, Bremen, Germany) was used for species identification, and antibiotic susceptibility was initially determined using a VITEK®2 Advanced Expert System™.

### 2.2. Colistin susceptibility testing

Colistin broth microdilution (BMD) and SensiTest™ Colistin (Liofilchem, Roseto degli Abruzzi, Italy) were performed following European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines and breakpoints version 10 ($\leq$2 mg/L, susceptible; >2 mg/L, resistant) [20]. *Acinetobacter baumannii* EUCAST-20 [minimum inhibitory concentration (MIC), 0.5 mg/L] and *E. coli* NCTC® 13846 (MIC, 2–4 mg/L) were included as control strains.

### 2.3. PCR detection of mcr genes

After culturing isolate CAC13 on blood agar, genomic DNA was extracted using a crude boil–freeze extraction method [21]. PCR detection of *mcr-1, -2, -3, -4* and *-5* was performed as previously described [1]. The complete *mcr-4* gene was amplified and sequenced using previously published primers (Mcr-4 ext FW, 5′-ATC TGT TAA GTT TGT TGG TGA C-3′; and Mcr-4 ext R, 5′-TGA GAG CTA AAT GTA ACA ATA GA-3′) [22] using previously described PCR reagents and *mcr-1* amplification conditions [7]. Sanger sequencing was performed to confirm the *mcr-4* amplicons.

### 2.4. Whole-genome sequencing (WGS)

WGS was performed as previously described [1]. Briefly, DNA was extracted using a Roche Pure PCR Template Preparation Kit (Roche Diagnostics, Germany). The DNA concentration was determined using a Qubit dsDNA BR Assay Kit and Qubit 2.0 Fluorometer (Life Technologies, USA) and the DNA was diluted to 0.2 ng/$\mu$L. Library preparation was performed using a Nextera DNA Flex Library Preparation Kit (Illumina, Germany) and sequencing was performed using a MiSeq Reagent Kit v.2 on an Illumina MiSeq desktop sequencer for 26 h to produce paired-end sequences. Sequencing reads were assessed for sufficient coverage [>30 $\times$ based on mapping to reference genome A1 ( **CP010781**)] and assembly statistics ($N_{50} > 100\ 000$-bp; length ~4 M-bp; total number of contigs <100) (Supplementary Table S1a). Taxonomic identification was done using Kraken v.0.10.5-beta with the 4GB MiniKraken database [23]. Assemblies were generated using SPAdes v.3.13.1 [24] and were annotated using Prokka v.1.14.0 [25].

Acquired antimicrobial resistance genes and mutations in the *lpxA, lpxC, lpxD, pmrA, pmrB* and *pmrC* genes were detected using ARIBA v.2.7.1 [26], CARD v.3.1.0 [27] and ResFinder 4.0 [28] databases as previously described [1]. ISFinder (https://www-is.biotoul.fr/blast.php) was used to screen for insertion sequence (IS) elements. Additionally, Artemis [29] was used to manually screen the annotated assemblies for known colistin resistance mutations and IS elements. The multilocus sequence typing (MLST) (https://github.com/tseemann/mlst) server was used to determine the sequence type (ST) based on WGS data using the Pasteur scheme (https://pubmlst.org).

### 2.5. Oxford Nanopore plasmid sequencing

Plasmid DNA was extracted using an alkaline lysis method (pH 8) followed by phenol:chloroform:isoamyl alcohol extraction (25:24:1) adapted from protocols from Addgene (https://www.addgene.org/protocols/purify-plasmid-dna) and IGEM (International Genetically Engineered Machine 2015) (Supplementary protocol 1). DNA library preparation was done using the 1D Native Barcoding genomic DNA protocol with two kits: EXP-NBD104 and SQK-LSK109 Kit R9 (Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's protocol. Sequencing was performed using a FLO-MIN106 flow cell on a MinION Mk1B sequencer with MinKNOW software v.1.15.1 (Oxford Nanopore Technologies).

Base calling and manual demultiplexing were performed using Guppy v.3.4.3 [30]. Unicycler v.0.4.8 (https://github.com/rrwick/unicycler) with dependencies (spades.py v.3.11.1, racon v.1.4.7, makeblastdb v.2.6.0+, tblastn v.2.6.0+, bowtie2-build v.2.3.4.1, bowtie2 v.2.3.4.1, samtools v.1.4.1, java v.11.0.8 and pilon v.1.23 for polishing) was used to hybridise the short Illumina and long Nanopore reads (Supplementary Table S1b).

Bandage v.0.8.1 (https://rrwick.github.io/Bandage/) [31] and CGView Server (http://cgview.ca/) with Prokka [25] annotation and Basic Local Alignment Search Tool (BLAST) capabilities were used to visualise the plasmids. Acquired antimicrobial resistance genes were detected using ARIBA [26], CARD [27] and ResFinder 4.0 [28] databases. The genomic raw reads and assemblies from the Illumina and Nanopore sequencing have been deposited in the National Center for Biotechnology Information (NCBI) database ( **PRJNA665160**) (Supplementary Table S1a).

### 2.6. Functional characterisation of the mcr-4.3 gene

The entire *mcr-4.3* coding sequence with *Xho*I and *Nde*I restriction sites was amplified using primers mcr-4YF (5′-CAT ATG ATT TCT AGA TTT AAG ACG TTA TC-3′) and mcr-4YR (5′-CTC GAG CTA ATA CCT GCA AG-3′) (IDT, USA) using a HIFI HotStart ReadyMix PCR

Kit (Kapa Biosystems, South Africa) with 0.2 $\mu$M of each primer and an annealing temperature of 56°C. The *mcr-1* gene was amplified from NCTC® 13846 as a control using primers mcr-1YF (5′-CAT ATG ATG ATG CAG CAT ACT TCT GTG TGG TAC CG-3′) and mcr-1YR (5′-CTC GAG TCA GCG GAT GAA TGC GGT-3′) using the same PCR conditions with an annealing temperature of 58°C.

The *mcr* genes were cloned into the *Xho*I and *Nde*I restriction sites of the pET-48b(+) expression vector using a CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Difco Laboratories, USA) [32] and were transformed into a colistin-susceptible SHuffle® T7 Competent *E. coli* strain [Inqaba Biotechnical Industries (Pty) Ltd., South Africa]. Colonies were selected on Luria–Bertani (LB) agar plates supplemented with 15 mg/L kanamycin and the insert was confirmed using PCR and Sanger sequencing. BMD was performed to assess the role of the *mcr* gene in colistin resistance.

### 2.7. Plasmid curing

Plasmid curing of CAC13 was performed in triplicate with 62.5 mg/L acridine orange (Glentham Life Sciences Ltd., UK) in 0.2 mL of culture at a 0.5 McFarland standard as previously described [33]. Serial dilutions ($10^{-1}$–$10^{-4}$) were performed and 0.1 mL was plated on LB agar plates with and without 4 mg/L colistin, followed by overnight incubation at 37°C. *mcr-4* PCR and plasmid extraction, as mentioned above, were performed on colonies from both agar plates to detect the *mcr-4.3* gene and pCAC13a plasmid, respectively. BMD was performed in triplicate to determine the colistin susceptibility of the plasmid-cured isolate.

### 3. Results

A single colistin-resistant *A. nosocomialis* isolate (CAC13) was identified from a larger study of colistin-resistant clinical *Acinetobacter* spp. in Cape Town, South Africa [1]. This isolate had a colistin MIC of 16 mg/L and was obtained from a blood culture from a patient at a district hospital in Cape Town in 2017. In addition to colistin resistance, this isolate was phenotypically resistant to cefotaxime and trimethoprim/sulfamethoxazole but was susceptible to amikacin, cefepime, ceftazidime, ciprofloxacin, gentamicin, piperacillin/tazobactam and carbapenems based on VITEK®2 Advanced Expert System™ antimicrobial susceptibility testing.

The *mcr-4* gene was detected in CAC13 using PCR. Sanger sequencing of the entire *mcr-4* gene showed 100% amino acid identity and coverage (430/430) to the *mcr-4.3* allele and the sequence was deposited in GenBank ( **MG948623**). Based on WGS, CAC13 was identified as ST279 and harboured no known colistin resistance mutations in the *pmrBAC* operon or *lpx* genes.

Nanopore plasmid sequencing showed that *mcr-4.3* was encoded on a 24 024-bp plasmid (pCAC13a; GenBank **JACYVX010000081.1**) with >50% coverage and <98.85% identity to *mcr-4.3*-harbouring plasmids from other *A. baumannii* isolates [12–14] (Fig. 1). pCAC13a encodes the replication initiation protein RepM from the RepB family, which is part of the Rep-3 superfamily (Pfam:01051), and is identical to a GR22 pAb-MCR4.3 plasmid [13]. The plasmid also contains an IS*3* family transposase (IS*Aba19*) and copies of two different type II toxin–antitoxin systems (PhD/YefM and RelE/ParE) that are involved in plasmid stabilisation and maintenance. *mcr-4.3* was found downstream of a putative recombinase family protein and a Tn*3* family transposase (IS*Psy42*). No mobile elements were found downstream of the *mcr-4.3* gene.

No additional antibiotic resistance gene were detected on pCAC13a. However, an additional sulfonamide resistance gene (*sul2*) was detected on a separate 17 330-bp RepB plasmid (pCAC13b; GenBank **JACYVX010000080.1**).

In *E. coli*, recombinant *mcr-4.3* (in pET-48b) did not result in an increase in the colistin MIC, in contrast to the 8-fold increase in colistin MIC conferred by *mcr-1* (Table 1). The pCAC13a plasmid was cured from the *A. nosocomialis* isolate at an acridine orange concentration of 62.5 mg/L. All colonies screened from the colistin-containing LB retained the pCAC13a plasmid containing the *mcr-4.3* gene, while colonies that were cultured on the LB-only agar plates were *mcr-4.3*-negative and had lost the pCAC13a plasmid. The plasmid-cured *A. nosocomialis* isolate showed a 16-fold reduction in MIC (Table 1) in comparison with the parental strain.

### 4. Discussion

This study investigated the colistin resistance mechanism in an *A. nosocomialis* clinical isolate from Cape Town, South Africa. The *mcr-4.3* gene was detected on a 24 024-bp RepB plasmid (pCAC13a). No other previously described colistin resistance mechanisms and/or possible colistin resistance determinants, such as mutations, insertions, deletions or stop codons in the *pmrCAB* or *lpx* genes, were identified.

This study showed that recombinant *mcr-4.3* does not confer colistin resistance in *E. coli*, however plasmid curing of the *mcr-4.3*-encoding pCAC13a plasmid restored susceptibility to colistin in *A. nosocomialis*. There are conflicting data regarding the contribution of *mcr-4.3* to colistin resistance (Supplementary Table S2); two other studies have also shown that recombinant expression of *mcr-4.3* in *E. coli* does not impact the colistin MIC [34,35], while one has found the gene to result in colistin resistance [13]. Similar to this study, none of these studies confirmed the expression of *mcr-4.3* in *E. coli*. Therefore, the absence of a change in colistin MIC might be attributed to the phosphoethanolamine transferase not being expressed from the pET-48 plasmid in *E. coli*, rather than *mcr4.3* not playing a role in colistin resistance. Alternatively, the native genomic environment of the plasmid may contribute to colistin resistance, and/or *mcr-4.3*-mediated colistin resistance may be species-dependent. For example, the native promoter and regulatory elements involved in *mcr-4.3* expression may be specific to *Acinetobacter*. This is supported by reports that in *Acinetobacter* spp., the *mcr-4.3* gene has only been detected in colistin-resistant isolates, while in *Shewanella frigidimarina* and *Leclercia adecarboxylata, mcr-4.3* has been detected in colistin-susceptible isolates [12,36]. In *Acinetobacter* spp., *mcr-4.3* has always been detected on a RepB plasmid, which is in contrast to the *mcr-4.3* genes in other hosts, which were all harboured on ColE10-type plasmids [34–36] or were chromosomally-encoded [12]. Therefore, the activity of the *mcr-4.3* gene may be dependent on other factors encoded on the RepB plasmid, which may alter its expression or modify the expressed protein to cause colistin resistance. There could also be an alternative colistin resistance mechanism encoded on pCAC13a causing colistin resistance, although no potential candidates were identified by plasmid sequencing and annotation.

Plasmid-mediated colistin resistance is a major threat due to its low fitness cost and ability to be transferred between different strains and species. No conjugation-related open-reading frames were detected on the pCAC13a plasmid, which is consistent with other *mcr-4.3* plasmids (pEH_mcr4.3, pEC_mcr4.3 and pAB18PR065) that were found to be non-conjugative and non-transformable [12,14]. However, pCAC13a, pAB18PR065 [14], pAb-MCR4.3 [13] and pEH_mcr4.3 [14] contain an IS*3* family transposase (IS*Aba19*) and a Tn*3* family transposase (IS*Psy42*), which could possibly assist in translocation of the gene to the chromosome or other plasmids.

Previously, only *mcr-1* and *mcr-9.1* genes have been reported in Enterobacterales isolates in southern Africa. Detection of *mcr-4.3* in other Gram-negative bacterial species suggests that *mcr-4.3* is not limited to China, Singapore, the Czech Republic and Brazil
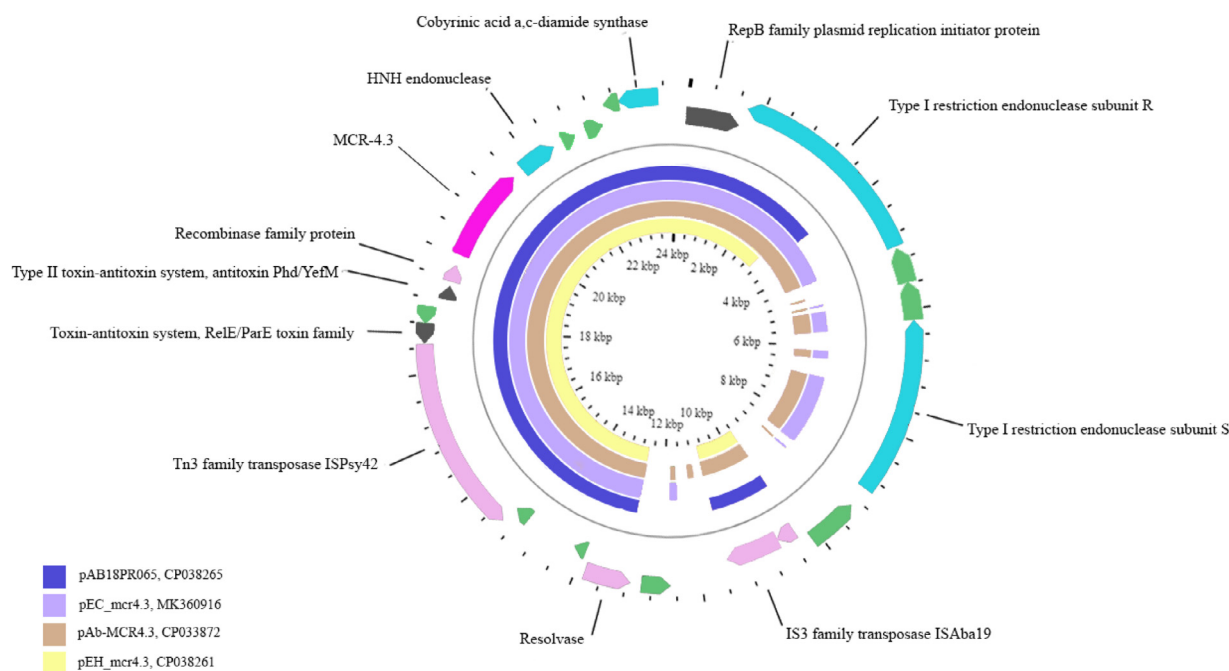
**Fig. 1.** Plasmid characterisation of the *mcr-4.3*-encoding plasmid (pCAC13a) from *Acinetobacter baumannii* CAC13. The inner rings represent four other known *A. baumannii mcr-4.3*-encoding plasmids: pAB18PR065 (25 602-bp; **CP038265**); pEC_mcr4.3 (43 093-bp; **MK360916**); pAb-MCR4.3 (35 502-bp; **CP033872**); and pEH_mcr4.3 (18 786-bp; **CP038261**).

**Table 1**
Effect of recombinant *mcr-4.3* (in pET-48b) and pCAC13a plasmid curing on the colistin minimum inhibitory concentration (MIC) determined by broth microdilution

| Isolate | Colistin MIC (mg/L) [category [a]] |
|---|---|
| *Escherichia coli* ATCC 25922 (susceptible control) | 0.5 [S] |
| *E. coli* NCTC® 13846 (*mcr-1* control) | 4 [R] |
| *Acinetobacter nosocomialis* CAC13 | 16 [R] |
| *E. coli* SHuffle® T7 | 0.5 [S] |
| pET-48b(+)-SHuffle® T7 | 0.5 [S] |
| pET-48b(+)-*mcr-1*-SHuffle® T7 | 4 [R] |
| pET-48b(+)-*mcr-4.3*-SHuffle® T7 | 0.5 [S] |
| Plasmid-cured CAC13 (*mcr-4.3*-negative) | 1 [S] |
| Uncured CAC13 (*mcr-4.3*-positive) | 16 [R] |

[a] S, susceptible (MIC ≤ 2 mg/L); R, resistant (MIC > 2 mg/L) [20].

and may be disseminated worldwide [13,34–36]. Other *mcr* variants may also be present in southern Africa and intensive clinical surveillance is necessary to unearth these and pre-empt further escalation.

## 5. Conclusion

This study describes the first report of a plasmid-mediated *mcr-4.3* gene in a colistin resistant clinical isolate from Africa. The *mcr-4.3* gene is encoded on a RepB plasmid in a clinical ST279 *A. nosocomialis* isolate. The pCAC13a plasmid containing *mcr-4.3* conferred colistin resistance in the *A. nosocomialis* isolate, although recombinant *mcr-4.3* did not confer resistance in *E. coli*, suggesting that colistin resistance is dependent on the native genomic environment of the pCAC13a plasmid or on the bacterial species.

## Acknowledgments

## Competing interests

None declared.

## Ethical approval

This study was approved by the Health Research Ethics Committee (HREC) at Stellenbosch University [S18/10/259]. Informed

consent was waivered by the HREC since the study was observational and patient care was not influenced.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2021.03.002.

## References

[1] Snyman Y, Whitelaw AC, Reuter S, Dramowski A, Maloba MRB, Newton-Foot M. Clonal expansion of colistin-resistant *Acinetobacter baumannii* isolates in Cape Town, South Africa. Int J Infect Dis 2020;91:94–100. doi:10.1016/j.ijid.2019.11.021.

[2] Adams MD, Nickel GC, Bajaksouzian S, Lavender H, Murthy AR, Jacobs MR, et al. Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. Antimicrob Agents Chemother 2009;53:3628–34. doi:10.1128/AAC.00284-09.

[3] Moffatt JH, Harper M, Harrison P, Hale JDF, Vinogradov E, Seemann T, et al. Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. Antimicrob Agents Chemother 2010;54:4971–7. doi:10.1128/AAC.00834-10.

[4] Mu X, Wang N, Li X, Shi K, Zhou Z, Yu Y, et al. The effect of colistin resistance-associated mutations on the fitness of *Acinetobacter baumannii*. Front Microbiol 2016;7:1715. doi:10.3389/fmicb.2016.01715.

[5] Moffatt JH, Harper M, Adler B, Nation RL, Li J, Boyce JD. Insertion sequence IS*Aba11* is involved in colistin resistance and loss of lipopolysaccharide in *Acinetobacter baumannii*. Antimicrob Agents Chemother 2011;55:3022–4. doi:10.1128/AAC.01732-10.

[6] Trebosc V, Gartenmann S, Tötzl M, Lucchini V, Schellhorn B, Pieren M, et al. Dissecting colistin resistance mechanisms in extensively drug-resistant *Acinetobacter baumannii* clinical isolates. mBio 2019;10:e01080 19. doi:10.1128/mBio.01083-19.

[7] Newton-Foot M, Snyman Y, Maloba MRB, Whitelaw AC. Plasmid-mediated *mcr-1* colistin resistance in *Escherichia coli* and *Klebsiella* spp. clinical isolates from the Western Cape region of South Africa. Antimicrob Resist Infect Control 2017;6:78. doi:10.1186/s13756-017-0234-8.

[8] Coetzee J, Corcoran C, Prentice E, Moodley M, Mendelson M, Poirel L, et al. Emergence of plasmid-mediated colistin resistance (MCR-1) among *Escherichia coli* isolated from South African patients. S Afr Med J 2016;106:35–6. doi:10.7196/SAMJ.2016.v106i5.10710.

[9] Poirel L, Kieffer N, Brink A, Coetze J, Jayol A, Nordmann P. Genetic features of MCR-1-producing colistin-resistant *Escherichia coli* isolates in South Africa. Antimicrob Agents Chemother 2016;60:4394–7. doi:10.1128/AAC.00444-16.

[10] Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 2016;16:161–8. doi:10.1016/S1473-3099(15)00424-7.

[11] Hameed F, Khan MA, Muhammad H, Sarwar T, Bilal H, Rehman TU. Plasmid-mediated *mcr-1* gene in *Acinetobacter baumannii* and *Pseudomonas aeruginosa*: first report from Pakistan. Rev Soc Bras Med Trop 2019;52:e20190237. doi:10.1590/0037-8682-0237-2019.

[12] Ma F, Shen C, Zheng X, Liu Y, Chen H, Zhong L, et al. Identification of a novel plasmid carrying *mcr-4.3* in an *Acinetobacter baumannii* strain in China. Antimicrob Agents Chemother 2019;63:e00133 19. doi:10.1128/AAC.00133-19.

[13] Martins-Sorenson N, Snesrud E, Xavier DE, Cacci LC, Iavarone AT, McGann P, et al. A novel plasmid-encoded *mcr-4.3* gene in a colistin-resistant *Acinetobacter baumannii* clinical strain. J Antimicrob Chemother 2020;75:60–4. doi:10.1093/jac/dkz413.

[14] Bitar I, Medvecky M, Gelbicova T, Jakubu V, Hrabak J, Zemlickova H, et al. Complete nucleotide sequences of *mcr-4.3*-carrying plasmids in *Acinetobacter baumannii* sequence type 345 of human and food origin from the Czech Republic, the first case in Europe. Antimicrob Agents Chemother 2019;63:e01166 18. doi:10.1128/AAC.01166-19.

[15] Mendelson M, Brink A, Gouws J, Mbelle N, Naidoo V, Pople T, et al. The One Health stewardship of colistin as an antibiotic of last resort for human health in South Africa. Lancet Infect Dis 2018;18:e288–94. doi:10.1016/S1473-3099(18)30119-1.

[16] Igwaran A, Iweriebor BC, Okoh AI. Molecular characterization and antimicrobial resistance pattern of *Escherichia coli* recovered from wastewater treatment plants in Eastern Cape South Africa. Int J Environ Res Public Health 2018;15:1237. doi:10.3390/ijerph15061237.

[17] Perreten V, Strauss C, Collaud A, Gerber D. Colistin resistance gene *mcr-1* in avian-pathogenic *Escherichia coli* in South Africa. Antimicrob Agents Chemother 2016;60:4414–15. doi:10.1128/AAC.00548-16.

[18] Founou LL, Founou RC, Allam M, Ismail A, Essack SY. Extended-spectrum $\beta$-lactamase-producing *Escherichia coli* harbouring *mcr-1* gene isolated from pigs in South Africa. S Afr Med J 2018;108:796–7. doi:10.7196/SAMJ.2018.v108i10.13508.

[19] Osei Sekyere J, Maningi NE, Modipane L, Mbelle NM. Emergence of *mcr-9.1* in extended-spectrum-$\beta$-lactamase-producing clinical Enterobacteriaceae in Pretoria, South Africa: global evolutionary phylogenomics, resistome, and mobilome. mSystems 2020;5:e00148 20. doi:10.1128/mSystems.00148-20.

[20] European Committee on Antimicrobial Susceptibility Testing (EUCAST) Recommendations for MIC determination of colistin (polymyxin E) as recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group. EUCAST; 2016. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf [accessed 22 March 2021].

[21] Ndlovu T, Le Roux M, Khan W, Khan S. Co-detection of virulent *Escherichia coli* genes in surface water sources. PLoS One 2015;10:e0116808. doi:10.1371/journal.pone.0116808.

[22] Carattoli A, Villa L, Feudi C, Curcio L, Orsini S, Luppi A, et al. Novel plasmid-mediated colistin resistance *mcr-4* gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. Euro Surveill 2017;22:30589. doi:10.2807/1560-7917.ES.2017.22.31.30589.

[23] Wood DE, Salzberg SL. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol 2014;15:R46. doi:10.1186/gb-2014-15-3-r46.

[24] Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 2012;19:455–77. doi:10.1089/cmb.2012.0021.

[25] Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics 2014;30:2068–9. doi:10.1093/bioinformatics/btu153.

[26] Hunt M, Mather AE, Sánchez-Busó L, Page AJ, Parkhill J, Keane JA, et al. ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads. Microb Genom 2017;3:e000131. doi:10.1099/mgen.0.000131.

[27] McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, et al. The Comprehensive Antibiotic Resistance Database. Antimicrob Agents Chemother 2013;57:3348–57. doi:10.1128/AAC.00419-13.

[28] Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 2012;67:2640–4. doi:10.1093/jac/dks261.

[29] Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, et al. Artemis: sequence visualization and annotation. Bioinformatics 2000;16:944–5. doi:10.1093/bioinformatics/16.10.944.

[30] Wick RR, Judd LM, Holt KE. Deepbinner: demultiplexing barcoded Oxford Nanopore reads with deep convolutional neural networks. PLoS Comput Biol 2018;14:e1006583. doi:10.1371/journal.pcbi.1006583.

[31] Wick RR, Schultz MB, Zobel J, Holt KE. Bandage: interactive visualization of de novo genome assemblies. Bioinformatics 2015;31:3350–2. doi:10.1093/bioinformatics/btv383.

[32] Michelsen BK. Transformation of *Escherichia coli* increases 260-fold upon inactivation of T4 DNA ligase. Anal Biochem 1995;225:172–4. doi:10.1006/abio.1995.1130.

[33] Dasmeh H, Baserisale M, Emami A. Plasmid curing assay in clinical isolates of antibiotic resistant *Acinetobacter baumannii*. Microbiol J 2015;5:43–8. doi:10.3923/mj.2015.43.48.

[34] Teo JWP, Kalisvar M, Venkatachalam I, Ng OT, Lin RTP, Octavia S. *mcr-3* and *mcr-4* variants in carbapenemase-producing clinical Enterobacteriaceae do not confer phenotypic polymyxin resistance. J Clin Microbiol 2018;56:e01562 17. doi:10.1128/JCM.01562-17.

[35] Chavda B, Lv J, Hou M, Chavda KD, Kreiswirth BN, Feng Y, et al. Coidentification of *mcr-4.3* and bla$_{NDM-1}$ in a clinical *Enterobacter cloacae* isolate from China. Antimicrob Agents Chemother 2018;62:e00649 18. doi:10.1128/AAC.00649-18.

[36] Sun Q, Wang H, Shu L, Dong N, Yang F, Zhou H, et al. *Leclercia adecarboxylata* from human gut flora carries *mcr-4.3* and bla$_{IMP-4}$-bearing plasmids. Front Microbiol 2019;10:2805. doi:10.3389/fmicb.2019.02805.