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Genetic Features of MCR-1-Producing Colistin-Resistant *Escherichia coli* Isolates in South Africa

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A series of colistin-resistant *Escherichia coli* clinical isolates was recovered from hospitalized and community patients in South Africa. Seven clonally unrelated isolates harbored the *mcr-1* gene located on different plasmid backbones. Two distinct plasmids were fully sequenced, and identical 2,600-bp-long DNA sequences defining a *mcr-1* cassette were identified. Promoter sequences responsible for the expression of *mcr-1*, deduced from the precise identification of the +1 transcription start site for *mcr-1*, were characterized.

The recent identification of a plasmid-encoded polymyxin resistance mechanism (MCR-1) among human and animal enterobacterial isolates is a source of concern (1). Actually, polymyxins (colistin and polymyxin B) are the last-resort antibiotics for treating infections caused by carbapenemase producers. MCR-1 is a phosphoethanolamine transferase that modifies the lipopolysaccharide by adding phosphoethanolamine to lipid A, leading to resistance to polymyxins (1). This resistance trait is transferable and has been reported so far mostly in *Enterobacteriaceae* from animal isolates but also in those from human isolates and from food products (2–8). First identified in China as published in November 2015 (1), MCR-1-producing isolates are mostly *Escherichia coli* strains that have been reported in many different countries scattered throughout Europe, Asia, and North America. In Africa, PCR and *in silico* analysis identified a few MCR-1-positive *E. coli* isolates from chicken from Algeria and from a single human *E. coli* isolate from Nigeria (9).

Our study was initiated by the isolation of seven colistin-resistant enterobacterial *E. coli* isolates from patients hospitalized in different hospitals in Johannesburg and Pretoria, South Africa, and also from community patients in Johannesburg from March 2014 to June 2015. The clonal relationships of the isolates were first evaluated by pulsed-field gel electrophoresis analysis as described previously (10), and the results showed that the seven isolates belonged to six distinct clones (data not shown) (Table 1). Only isolates Af31 and Af48, both from Johannesburg, were indistinguishable. However, isolate Af31 was from a community patient whereas isolate Af48 was from a hospitalized patient. Multilocus sequence typing performed as described previously (10) confirmed that all isolates were distinct, with the exception of isolates Af31 and Af48 (Table 1). Among those isolates, Af31 and Af45 remained susceptible to all ß-lactams, Af23 and Af24 exhibited a penicillinase phenotype related to TEM-1 production, and isolates Af40 and Af49 exhibited an extended-spectrum-β-lactamase (ESBL) phenotype related to CTX-M-55 according to molecular analyses (11). Isolate Af48 exhibited an AmpC-type cephalosporinase phenotype related to CMY-2. Interestingly, isolate Af31 was resistant to florfenicol and possessed the *floR* gene that we previously identified in another MCR-1-positive *E. coli* isolate from Switzerland (12). It was noteworthy that all isolates were resistant to sulfonamides, tetracyclines, and fluoroquinolones (Table 1), which are antibiotics that are extensively prescribed in veterinary medicine (13).

Mating-out assays were performed with all *mcr-1*-positive isolates as donors and *E. coli* J53 (azide resistant) as the recipient as described previously (14). Selection was performed on Trypticase-soy agar plates supplemented with colistin (2 μg/ml) and sodium azide (100 μg/ml). Transconjugants were obtained for all donors except for Af49. They exhibited colistin MIC values of 4 to 8 μg/ml (resistance cutoff being at 2 μg/ml) compared to 0.25 μg/ml for the *E. coli* J53 recipient strain. No additional resistance marker was cotransferred along with the *mcr-1* gene in any of the *E. coli* transconjugants. PCR-based replicon typing (15) as well as primers specific for plasmid types IncX4 (X4-Fw [5′-AGCAAACAGGGAAAGGAGAAGACT-3′] and X4-Rv [5′-TACCCAAATCGTAACCTG-3′]) and IncI2 (I2-Fw [5′-TGCAGCTTGCTGTGATTAGC-3′] and I2-Rv [5′-TTCGCTGTTCATCATACGGC-3′]) identified different *mcr-1*-bearing plasmid backbones, including IncI2, IncHI2, and IncX4, differing in sizes and structures (Table 1). Surprisingly, the two clonally related Af31 and Af48 isolates harbored two different *mcr-1*-positive plasmid types corresponding to the IncHI2- and IncX4-type scaffolds, respectively.

PCR mapping was performed by referring to the sequence of the reference plasmid identified in China (pHNSHP45; GenBank accession no. KP347127) (1) in order to characterize the genetic environment of the *mcr-1* gene among the different *E. coli* isolates. Insertion sequence IS*Apl1* was identified upstream of *mcr-1* in all but one isolate (Af48). Downstream of *mcr-1*, the closely related

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genetic environment previously identified for plasmid pHNSHP45 was found in all isolates.

Considering that several plasmid types were identified, two of them were entirely sequenced. Sequencing was performed by using Illumina technology (Fasteris, Plan-les-Ouates, Switzerland). The *mcr-1*-positive pAf23 plasmid from isolate Af23 was retained since it was an IncI2-type plasmid, as was plasmid pHNSHP45 from the pioneer study (1). The two plasmids were almost completely identical (99% identical at the nucleotide level), with pAf23 being 61.1 kb in size and pHNSHP45 being 64.1 kb, with an additional IS*683* element (Fig. 1). No other resistance gene was identified on pAf23 (Table 1). Therefore, the occurrence of the *mcr-1* gene in Af23 was related to the acquisition of a plasmid that may be considered structurally related to the Chinese index plasmid.

The second sequenced *mcr-1* plasmid, plasmid pAf48 from isolate Af48, was 31.8 kb in size and exhibited an IncX4-type scaffold (Fig. 1). Again, no resistance gene other than the *mcr-1* gene was identified on that plasmid (Table 1). A similar (92.3% overall identity at the nucleotide level) but *mcr-1*-negative plasmid backbone was identified in the United Kingdom, which was plasmid pSAM7 from *E. coli* recovered from cattle and harboring the $bla_{\text{CTX-M-14}}$ ESBL gene (16).

In order to understand the process of acquisition of the *mcr-1* gene in different plasmid backbones, a detailed comparison of the different *mcr-1*-positive plasmid sequences was established. Identical 2,600-bp sequences that might be defined as representative of a *mcr-1* cassette were identified in plasmids pAf23 and pAf48 but also in the pHNSHP45 IncI2-type plasmid from China (1) and the pKH457-3 IncP-type plasmid from Belgium (17) through our *in silico* analysis. The 5′ extremity of that cassette started with CA AAT, and the 3′ extremity ended with AAGTT (Fig. 2). At those extremities, no putative inverted repeat sequence was identified that might have corresponded to features usually identified at insertion sequence extremities or at the extremities of mobile insertion cassette (*mic*) elements such as that containing the *qnrS2* quinolone resistance gene (18). In addition, no putative target site duplication was identified on both extremities of that cassette, likely ruling out an in *trans* transposition process.

A detailed analysis of the *mcr-1* cassette showed that the *mcr-1* start codon was identified at position 79 and that 791 bp separated the stop codon of *mcr-1* gene from the right end of the cassette (Fig. 2). A putative promoter region (called P_1-*mcr*-1) was identified within the first 79 bp of the cassette, corresponding to sequences −35 (TGGATT) and −10 (TATAAT) being separated by a 16-bp sequence. Therefore, expression of *mcr-1* may be driven by a promoter which is part of the mobile cassette, thus making this element autonomous in term of transcription. In addition, by analysis of the sequences located upstream of the *mcr-1* cassette in plasmids pAf23 and pHNSHP45, the IS*Apl1* insertion sequence element was identified, being located 9 bp upstream of the *mcr-1* cassette. In order to confirm that P_1-*mcr*-1 was indeed the correct promoter leading to *mcr-1* expression, and also to verify whether the occurrence of IS*Apl1* could modify the +1 transcription start of *mcr-1*, mapping of this transcription start site was performed by 5′ rapid amplification of cDNA ends (5′-RACE), as described previously (19). Total RNA was isolated from the different strains studied using an RNeasy Midi kit (Qiagen, Courtaboeuf, France) and the manufacturer's recommendations. 5′-RACE reactions were performed using 5 μg of total RNA of each strain (Af23 and Af48) and a 5′/3′ RACE kit (2nd generation; Roche Diagnostics,

TABLE 1 Features of the MCR-1-producing colistin-resistant *E. coli* isolates and patient characteristics[a]

Strain no.	Date of isolation	Site of isolation	Gender	Age range of the patient (yrs)	Origin (city)	MIC of colistin (μg/ml)	β-lactam resistance phenotype	PFGE profile	Sequence type	*mcr*-1-positive plasmid type	*mcr*-1-positive plasmid size (ca. kb)	Coresistance markers
Af23	March 2014	Blood	M	50–59	Pretoria	4	Penicillinase (TEM-1)	B	ST10	IncI2	70	CIP, TET, SXT
Af24	March 2014	Pus	F	40–49	Johannesburg	4	Penicillinase (TEM-1)	C	ST1007	IncI2	65	CIP, TET, SXT
Af31	October 2014	Urine	F	60–69	Johannesburg	8	Susceptible	A	ST624	IncHI2	150	CIP, TET, SXT, CHL, FLO, KAN
Af40	March 2015	Wound	F	60–69	Johannesburg	4	ESBL (CTX-M-55)	D	ST57	IncI2	ND	CIP, TET, CHL, FOS, KAN, TOB, SXT
Af45	May 2015	Urine	F	60–69	Johannesburg	8	Susceptible	E	ST101	IncI2	70	TET, SXT
Af48	May 2015	Urine	F	60–69	Johannesburg	4	AmpC (CMY-2)	A	ST624	IncX4	30	CIP, TET, SXT, CHL
Af49	May 2015	Urine	F	30–39	Pretoria	4	ESBL (CTX-M-55)	F	ST226	ND	ND	CIP, TET, SXT, CHL, FOS, KAN, TOB

[a]PFGE, pulsed-field gel electrophoresis; M, male; F, female; ND, not determined; CIP, ciprofloxacin; TET, tetracycline; SXT, trimethoprim plus sulfamethoxazole; CHL, chloramphenicol; FLO, florfenicol; KAN, kanamycin; TOB, tobramycin; FOS, fosfomycin; CHL, chloramphenicol.

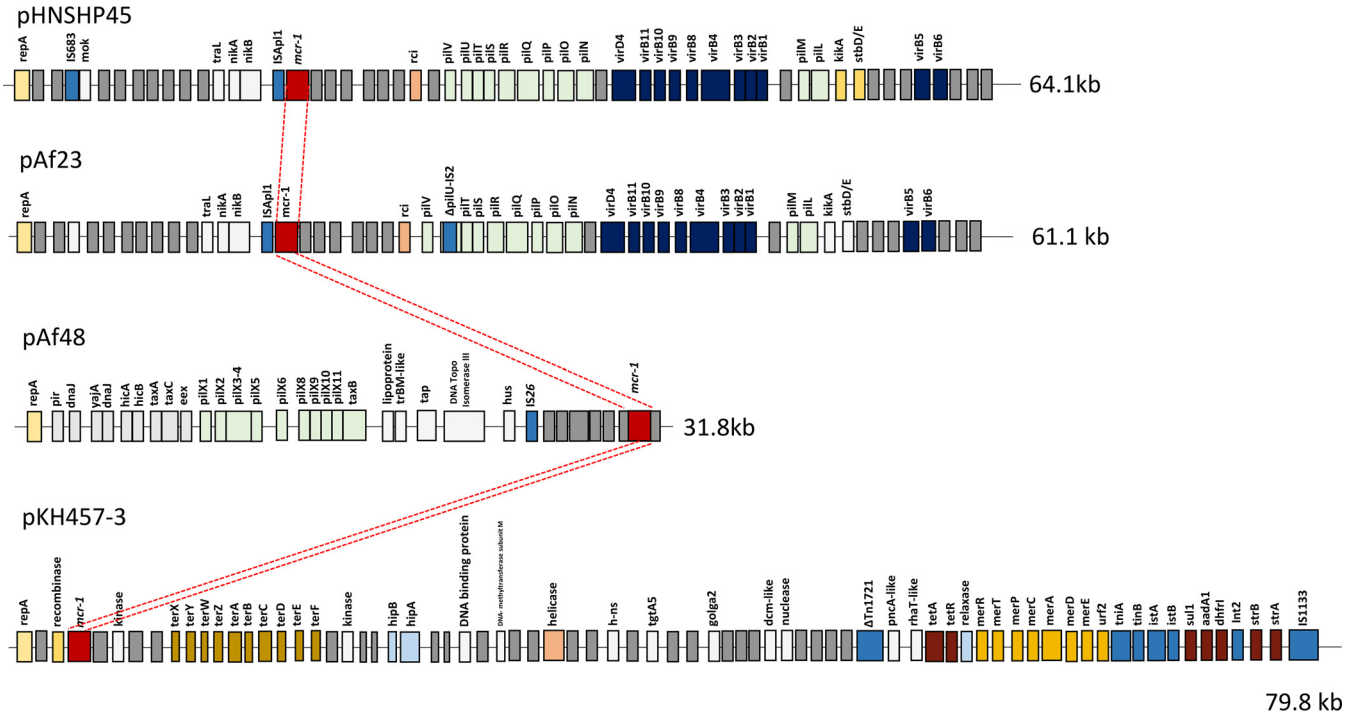

FIG 1 Schematic map of the *mcr-1*-bearing plasmids. pHNSHP45 is the reference plasmid from China (GenBank accession no. KP0347127) (1), the IncP-type pKH457-3 is from Belgium (17), pAf23 is the IncI2-type plasmid recovered from isolate Af23 (GenBank accession no. KX032519) (pAf23), and pAf48 is the IncX4-type plasmid from isolate Af48 (GenBank accession no. KX032520) (pAf48). Open reading frames are indicated by small and vertical rectangles. Colors correspond to loci combining genes acting for the same function (e.g., *pil*-type genes for the pilus apparatus). The *mcr-1* cassette is represented by a red box, and the conserved *mcr-1* cassette identified on the different plasmids is highlighted by red dashed lines. The sizes of the respective plasmids are indicated.

Rotkreuz, Switzerland) following the manufacturer's recommendations and using primers SP1 (5′-AAAATAACTGGTCACCGC GC-3′), SP2 (5′-ACAGGCTTTAGCACATAGCG-3′), and SP3 (5′-AAAGAGCACGACAGCGATCG-3′). Considering the +1 transcription site identified, results confirmed that P_1-*mcr*-1 was indeed the promoter of *mcr-1* expression (Fig. 2). Also, the same site was identified in both isolate Af23 and isolate Af48, thus indicating that the occurrence of IS*Apl1* in Af23 did not modify the promoter sequence of *mcr-1* (data not shown).

Our report describes the first MCR-1-producing *E. coli* isolates from South Africa, recovered from community and distantly located hospitalized patients. We identified the same clone (ST624) in both hospital- and community-acquired isolates, that clone being identified as an avian pathogenic *E. coli* isolate causing colibacillosis in poultry in Spain and China (20, 21), but also in isolates from patients in France and Japan (22). This feature, together with the identification of the *floR* gene associated with resistance to florfenicol (that antibiotic being given only in veterinary medicine), further suggests the animal origin of that resistance trait (23).

Overall, this study further showed the wide spread of the *mcr-1* gene on different plasmid backbones and in different *E. coli* clonal

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CAAATTATAAATACTCTCAAGTGTATATTCAGTATGGGATTGCGCAATGATTGCCTAATAAAATTTCTGAAATATTTCTG

                                        -35       P1-mcr-1      -10         +1
TATCGCATAATTTTTTATATCAGATAAATTGTACTGGATTTCTTAAAAAATTGCAGTATAATTGCCGCAATTATCCCACC

GTTTATTTTTTGAGTAGTTTCTCATGATGCAGCATACTTCTGTGTGGTACCGACGC-----------//-----------
                         M  M  Q  H  T  S  V  W  Y  R  R
                   --> mcr-1

TGATGTCACCGCGGACAAAGTCAAAGACCGCACCGCATTCATCCGCTGATTTCTCCCTGTATTTTTTCCAAACCCACCGC
  D  V  T  A  D  K  V  K  D  R  T  A  F  I  R  *

-------------------------//640 bp //-----------------------------------------------

GGCTGCTATAAGATGATTATTGGCGATCATTTTTTGAGCCATACGGTGGTGTCGATGATGCTTGCGTGGGCGATGTCGGC

AGGGCTTGCGTGGGTGTTTTTTAAGAAGGGTGAACAAGTT
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FIG 2 Sequence of the *mcr-1* cassette. The 2,600-bp-long sequence is shown, with the start and stop codons of *mcr-1* being in bold. The sense of transcription of *mcr-1* is indicated by an arrow. Amino acids of the MCR-1 sequence are indicated below the nucleotide sequence. The P_1-*mcr-1* promoter sequences are indicated, with the corresponding −35 and −10 boxes being underlined, as well as the +1 transcription start.

backgrounds. A detailed genetic analysis identified an *mcr-1*-containing cassette that could have been mobilized from its original (and still unknown) reservoir to its host and that could be a source of transferable polymyxin resistance. The mechanism of mobilization of this *mcr-1* cassette remains to be determined, since it might correspond to an unknown genetic process.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this work have been deposited in the GenBank nucleotide database under accession no. KX032519 (pAf23) and KX032520 (pAf48).

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