Supplementary data

Bacterial strains and antimicrobial susceptibility testing

A total of 905 non-duplicated clinical isolates (569 *Escherichia coli*, 278 *Klebsiella pneumoniae*, 36 *Enterobacter cloacae* and *22 Citrobacter freundii)* collected from a tertiary hospital in Guangdong province in China between 2015 and March 2016 were randomly selected for molecular screening for *mcr-1*. The species identification and antimicrobial susceptibility testing were conducted using VITEK® 2 system (bioMerieux, France). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. The colistin susceptibility testing of *mcr-1* positive strains, their *E. coli* $J53^{AZ-R}$ transconjugants and pQF50 vector *E. coli DH5α* constructs were examined using broth microdilution method and interpreted according to the EUCAST breakpoints.

Analysis of *mcr-1* expression

The *mcr-1* genes with its natural promoter regions were amplified by PCR using the primers in **Table S2** and cloned into plasmid pQF50 using the Quick-Fusion Seamless Cloning Kit (Biotool, USA). Subsequently, the resultant recombinant plasmids were transferred into *E. coli* DH5α and selected on agar plates with ampicillin (100μg/ml), followed by PCR evaluation. Single colonies of the *E. coli* DH5α transformants were grown overnight in LB media with 100 μg/ml ampicillin at 37°C with shaking at 200 rpm. On the next day, overnight bacterial culture was 1:100 diluted in fresh LB media, and cultured at 37°C until mid-log phase (OD ~0.5). The RNA of *E. coli* DH5α

transformants was then extracted using the Trizol method, and the expression of *mcr-1* was evaluated by real-time reverse-transcription PCR using the PrimeScript™ RT reagent Kit and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) kit (TAKARA, China), using the primers listed in **Table S2**. The qRT-PCR was performed with three RNA sample preparations from three independent experiments. In addition, these *E. coli* DH5α transformants were subject to susceptibility testing using the broth microdilution method described above.

The *mcr-1* promoter activity analysis

In order to explore the relationship between sequence variation and promoter activity, the *mcr-1* promoter regions from different plasmids were amplified by PCR using the primers on **Table S2**, and then cloned into a promoterless lacZ reporter plasmid pQF50 using the Quick-Fusion Seamless Cloning Kit (Biotool, USA), followed by electroporation into *E. coli* BW25113 （another *E. coli* strain without endogenous β-galactosidase compared to DH5α. The β-gal activity of those strains driven by the *mcr-1* promoters was detected using the Multi-plate Reader (Biotek, USA), using *E. coli* BW25113 and *E. coli* BW25113- pQF50 as controls.

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41 **TABLE S1. Characteristics of patients and *mcr-1*-positive isolates**

Characteristics	GZ49260	GZ49263	GZ49266	GZ49269	GZ49271	GZ49273
Source of isolate	Urine	Blood	Urine	Urine	Cervical fluid	Ascites
Age (year)/gender	81/F	48/M	66/M	59/F	43/F	56/F
Ward	Nephrology	Radiotherapy	Urinary Surgery	Oncology	Gynaecology	Hepatology
Infection	Bacteriuria	Sepsis	Urinary tract infection	Urinary tract infection	Cervical infection	Intra-abdominal infection
Species	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*K. pneumoniae*	*E. coli*
ST	744	7572	156	4204	976	1196
Acquired Resistance genes	*aac(3)-IId, strAB, aadA1, bla*$_{\text{TEM-1}}$, *bla*$_{\text{CTX-M-130}}$, *mcr-1*, *floR*, *sul1, sul2, tet(A), tet(B), dfrA1*	*aadA1, aph(3')-Ia, strAB*,*bla*$_{\text{OXA-10}}$, *mcr-1, qnrS1, cmlA1,arr-2, sul2, sul3, tet(A), dfrA14*	*aadA2, aph(3')-IIa, aph(3')-IId, strAB, rmtB*, *bla*$_{\text{CTX-M-65}}$, *bla*$_{\text{TEM-1}}$, *mcr-1, floR, sul1, sul2, tet(A), dfrA12*	*aadA16, aph(3')-IIa, aph(3')-IId, strAB, aac(6')Ib-cr*,*bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{CTX-M-55}}$, *mcr-1, mphA, floR, arr-3, sul1, sul2, dfrA27*	*strAB*, *bla*$_{\text{SHV-27}}$, *mcr-1, qnrS1, fosA, floR, sul2, tet(A)*	*mcr-1*
Plasmid replicons	FIB, X4, I1, FIC, Q1, FII	HI2, FIC, FIB, HI2A	I1, I2, X1, FIB, p011	N, X4, FII, FIB	FII, FIB, X4	I1, I2, FII
mcr-1 plasmids replicon	X4	Unknown	I2	X4	X4	I2
Antimicrobial susceptibility testing (μg/ml) [a]						
Amikacin	≤2	≤2	≥64	≤2	≤2	≤2
Ampicillin	≥32	≥32	≥32	≥32	≥32	≥32
Ampicillin-sulbactam	≥32	16	≥32	≥32	4	4
Piperacillin-tazobactam	≤4	≤4	≤4	≤4	≤4	≤4
Cefazolin	≥64	≤4	≥64	≥64	≤4	≤4
Cefatriaxone	≥64	≤1	≥64	≥64	≤1	≤1
Ceftazidime	4	≤1	4	16	≤1	≤1
Cefepime	4	≤1	2	4	≤1	≤1
Cefotetan	≤4	≤4	≤4	≤4	≤4	≤4
Imipenem	≤1	≤1	≤1	≤1	≤1	≤1
Ertapenem	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Levofloxacin	≥8	2	≥8	≥8	1	≥8
Ciprofloxacin	≥4	2	≥4	≥4	≤0.25	≥4
Trimethoprim-sulfamethoxazole	≥320	≥320	≥320	≥320	≤20	≤20
Gentamicin	≥16	≤1	≥16	≥16	≤1	≤1

Aztreonam	16	≤1	16	16	≤1	≥32
Tobramycin	≥8	≤1	≥16	8	≤1	≤1
Nitrofutantoin	≤16	≤16	≥32	128	≤16	64
Colistin	8	16	4	4	16	8
Polymyxin B	8	8	4	8	32	8
E. coli J53 transconjugants						
Colistin	4	-	4	4	8	4

[a] The species identification and antimicrobial susceptibility testing were conducted using VITEK® 2 system (bioMerieux, France), and interpreted by the standards of Clinical and Laboratory Standards Institute (CLSI, 2016) . *E. coli* ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 were used as quality control strains. The colistin susceptibility testing was examined using broth microdilution method and interpreted according to the EUCAST breakpoints.

TABLE S2. Primers used in this study

Primers	Primer sequence* (5'→3')
mcr-1 promoter_m-F (*Bam* HI)	GACGTCGACTCTAGA*GGATCC*CTTAAAAAATTGCAGTA
pGZ49260/49271*mcr-1*promoter-F (*Bam* HI)	GACGTCGACTCTAGA*GGATCC*ATACTCTCAAGTGTATATTC
pGZ49263*mcr-1*promoter-F (*Bam* HI)	GACGTCGACTCTAGA*GGATCC*GCTGAATTTACAATCCAAG
pGZ49266*mcr-1*promoter-F (*Bam* HI)	GACGTCGACTCTAGA*GGATCC*CCGCTGAATTTACAATCCAA
pGZ49269*mcr-1*promoter-F (*Bam* HI)	GACGTCGACTCTAGA*GGATCC* TTAAAAATAAGCCCACATA
pGZ49273*mcr-1*promoter-F (*Bam* HI)	GACGTCGACTCTAGA*GGATCC*GATGTCCCTCTGGGATGCGC
*mcr-1*promoter-R (*Hind*III)	ATACCCTCTAGCTAG*AAGCTT*GAGAAACTACTCAAAAA
mcr-1-R (*Hind*III)	ATACCCTCTAGCTAG*AAGCTT*AATACGAATGGAGTGTGCGG
ropD-F (for qRT-PCR)	GGGATCAACCAGGTTCAATG
*rop*D-R (for qRT-PCR)	GGTGCCAGATCTTCTTCTGC
mcr-1-F (for qRT-PCR)	CAGTTTCTTTCGCGTGCATA
mcr-1-R (for qRT-PCR)	GCGTCTTTGGCGTGATAAAT

*The underlined sequences denote restriction enzyme sites