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Occurrence of the mobile colistin resistance gene *mcr-3* in *Escherichia coli* from household pigs in rural areas

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Sir,

Polymyxins are regarded as one of the few last-resort antibiotics for the treatment of serious infections caused by MDR Gram-negative bacteria, such as carbapenem-resistant Enterobacteriaceae. However, the discovery of the plasmid-mediated colistin resistance gene *mcr-1* further reduced the treatment options.[1] The gene *mcr-1* has been identified in Enterobacteriaceae from different sources in more than 45 countries on five continents.[2,3] The gene *mcr-2* was identified in *Escherichia coli* from pigs and cattle in Belgium.[4] This gene is rarely detected and seems to be limited to some European countries.[4,5] Recently, the third plasmid-mediated colistin gene, *mcr-3*, has been reported in China.[6] The *mcr-4* and *mcr-5* genes have been newly identified in Enterobacteriaceae from animals and humans in European countries.[7,8] The gene *mcr-3* shares 45% and 47% nucleotide sequence identity with *mcr-1* and *mcr-2*, respectively, and the corresponding protein shares 33% and 32% amino acid identity with MCR-1 and MCR-2, respectively. To date, the knowledge about the occurrence of *mcr-3* genes in Enterobacteriaceae from humans or animals is still limited. Here, for the first time (to the best of our knowledge), we report the occurrence of *mcr-3* in backyard pigs from households in rural areas of China.

We screened 417 pig faecal samples from 254 household backyard farms in 12 villages in the Shandong province in 2015, using ESwabs (Copan, Brescia, Italy). The faecal sample was first added to 1 mL of Nutrient broth (Hangzhou Binhe Microorganism Reagent Co., Ltd, Hangzhou, China) and was incubated overnight at 37°C. Total DNA from 500 μL of the enrichment culture of each sample was extracted using the TIANamp Bacteria DNA Kit (TIANGEN Biotech Co., Beijing, China). The total DNA was screened for *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* genes by PCR as previously described.[1,4,6–8] A total of 71 (71/417, 17%) samples from 56 households were positive for *mcr-1*. Five (5/417, 1.2%) samples from five households were positive for *mcr-3*. All samples were negative for *mcr-2*, *mcr-4* and *mcr-5*. A 1 μL sample of each *mcr-3*-positive culture was plated on *Salmonella Shigella* (SS) agar (Hangzhou Binhe Microorganism Reagent Co., Ltd) containing 2 mg/L colistin. Colonies with different morphology on the SS agar were picked and checked for the presence of the *mcr-3* gene by PCR and sequencing. Five *mcr-3*-positive isolates, AZ20, BZ5, CZ11, LZ11 and IZ43, were detected from five different villages and all of them were identified as *E. coli* through 16S rDNA sequencing and MALDI-TOF MS analysis.[2] Testing for susceptibility to eight antimicrobial agents by agar dilution and confirmation by broth dilution[9,10] revealed that all isolates were resistant to colistin and gentamicin, and isolates AZ20 and BZ5 were also resistant to ciprofloxacin. All isolates were susceptible to meropenem and intermediate to ceftazidime (Table 1).

DNA from the five *mcr-3*-positive strains was extracted using the TIANamp Bacteria DNA Kit. Paired-end sequencing was conducted using an Illumina HiSeq 2500 (Berry Genomic Company, Beijing, China). Reads were assembled *de novo* using SPAdes 3.9.[11] The MLST profiles, putative plasmids and antibiotic resistance genes (Table 1) were predicted by uploading the assembly to the bacterial analysis pipeline at the Center for Genomic Epidemiology website (http://www.genomicepidemiology.org/). The *mcr-3* gene was identical in the three isolates CZ11, LZ11 and IZ43, whereas the amino acid sequence encoded by the *mcr-3* variant in isolates BZ5 and AZ20 differed from MCR-3 at three amino acid positions (M23V, A457E and T488I). This variant gene showed 100% nucleotide sequence identity to *mcr-3.5* (GenBank accession no. NG_055782).[12]

S1-PFGE and Southern blot analysis revealed that *mcr-3* and *mcr-3.5* were located on different plasmids in the five isolates with their sizes ranging from ~83 to ~246 kb (Table 1). To test the transferability of the *mcr-3* and *mcr-3.5* genes, filter mating was performed with *E. coli* J53Az^R as recipient.[13] Transconjugants were selected on Brain Heart Infusion agar (Beijing Land Bridge Technology Ltd, Beijing, China) supplemented with 2 mg/L colistin and 300 mg/L sodium azide. Only isolates AZ20 and BZ5 carried conjugative plasmids, the IncP1-type plasmid pBZ5 and plasmid pAZ20 of an unknown Inc type. The two transconjugants exhibited 4- and 8-fold increased colistin and polymyxin B MICs compared with recipient *E. coli* J53, but were susceptible to the other six antimicrobial agents (Table 1). Electrotransformation was used for the remaining three *mcr-3*-carrying isolates. Two IncHI2 plasmids, pCZ11 and pLZ11, derived from isolates CZ11 and LZ11, respectively, were successfully transferred into *E. coli* DH5α (Takara Bio Inc., Beijing, China). The two transconjugants exhibited 16-fold

Table 1. Antimicrobial susceptibility and resistome profiles of the five *mcr-3*-/*mcr-3.5*-carrying strains and their transconjugants

Strain	MLST	Variant and size of *mcr-3*-carrying contig (kb)	Plasmid size (kb)	Plasmid type	MIC (mg/L)[a]								Resistome profiles[c]
					CST	PMB	MEM	RIF	GEN	CIP	FFC	CAZ	
AZ20	ST155	*mcr-3.5*, 2.7	~83	unknown	**8**[b]	4	0.008	4	**256**	**32**	128	4	*aac(3)-IId*, *aadA1*, *aadA2*, *aph(3′)-Ia*, bla_{TEM-1B}, *cmlA1*, *floR*, *fosA*, *oqxAB*, *qnrS2*, *strA*, *strB*, *sul2*, *sul3*, *tet*(A)
BZ5	ST165	*mcr-3.5*, 49.9	~91	IncP1	**8**	4	0.016	>64	**32**	**4**	8	8	*aac(6′)Ib-cr*, *aadA1*, *aadA2*, *arr-3*, bla_{TEM-1B}, *cmlA1*, *dfrA12*, *oqxAB*, *qnrS2*, *sul3*, *tet*(A)
CZ11	ST3933	*mcr-3*, 2.6	~246	IncHI2	**8**	8	0.016	<2	**128**	0.5	4	2	*aph(3′)-IIa*, bla_{TEM-1B}, *oqxAB*, *strA*, *strB*, *sul1*, *sul2*, *tet*(A)
LZ11	ST3933	*mcr-3*, 2.6	~246	IncHI2	**8**	8	0.016	<2	**256**	1	4	8	*aph(3′)-IIa*, bla_{TEM-1B}, *oqxAB*, *strA*, *strB*, *sul1*, *sul2*, *tet*(A)
IZ43	ST10	*mcr-3*, 7.1	~180	unknown	**4**	4	0.016	16	>**256**	0.25	8	8	*aac(6′)Ib-cr*, *aacA4*, *aph(3′)-Ic*, *arr-3*, bla_{OXA-1}, bla_{TEM-1B}, *catB3*, *dfrA14*, *qnrS1*, *strA*, *strB*, *sul1*, *sul3*, *tet*(A)
J53-AZ20	–	–	~83	unknown	**8**	4	0.008	4	1	0.008	4	0.5	ND
J53-BZ5	–	–	~91	IncP1	**4**	4	0.016	4	1	0.032	4	1	ND
J53	–	–	–	–	1	0.125	0.016	4	1	0.004	8	1	–
DH5α-CZ11	–	–	–	IncHI2	**4**	4	0.008	2	**16**	0.032	4	2	ND
DH5α-LZ11	–	–	–	IncHI2	**4**	4	0.008	2	**16**	0.064	4	4	ND
DH5α	–	–	–	–	0.25	0.25	0.008	2	0.25	0.032	4	0.25	–

[a]Abbreviations and resistance breakpoints: CST, colistin (R >2 mg/L); PMB, polymyxin B; MEM, meropenem (R ≥4 mg/L); RIF, rifampicin; GEN, gentamicin (R ≥16 mg/L); CIP, ciprofloxacin (R ≥4 mg/L); FFC, florfenicol; CAZ, ceftazidime (R ≥16 mg/L). CST: EUCAST interpretation criteria. MEM, GEN, CIP and CAZ: CLSI interpretation criteria. PMB, RIF and FFC: no interpretation criteria.

[b]Bold formatting indicates resistance to the respective antimicrobial agents.

[c]*aadA1*, *aadA2*, *aacA4*, *strA*, *strB*, *aac(3)-IId*, *aph(3′)-Ia*, *aph(3′)-IIa* and *aph(3′)-Ic*: aminoglycoside resistance genes. *sul1*, *sul2* and *sul3:* sulphonamide resistance genes. *dfrA12* and *dfrA14*: trimethoprim resistance genes. *tet*(A): tetracycline resistance gene. *cmlA1*, *floR* and *catB3*: phenicol resistance genes. bla_{TEM-1B} and bla_{OXA-1}: β-lactam resistance genes. *fosA*: fosfomycin resistance gene. *arr-3*: rifampicin resistance gene. *oqxAB*, *qnrS1*, *qnrS2* and *aac(6′)-Ib-cr*: quinolone resistance genes. ND, not determined.

increased colistin and polymyxin B MICs and exhibited 64-fold increased gentamicin MICs compared with *E. coli* DH5α (Table 1). WGS analysis of the five *mcr-3*- and *mcr-3.5*-carrying isolates revealed contigs ranging in size from 2.6 to 49.9 kb (Table 1). The core structure, *mcr-3-dgkA*, was identified in all five contigs, four of which exhibited >99% nucleotide sequence identity to the corresponding region of the original *mcr-3*-carrying plasmid pWJ1. The largest contig (49.9 kb) of plasmid pBZ5 showed 99% nucleotide sequence identity to that in plasmid pMCR3_WCHEC-LL123 (GenBank accession no. MF489760) of human *E. coli* from China.[12] Plasmid pBZ5 contained toxin–antitoxin systems (*higB* and *higA*) as well as conjugative elements, which can maintain *mcr-3.5* in its host and facilitate the horizontal transfer of this *mcr-3* variant to other bacteria.[14]

Two of the *E. coli* isolates (CZ11 and LZ11) had identical resistome profiles, the same MLST type (ST3933) and similar MIC values (Table 1). The Parsnp tool in the Harvest suite was applied to compare the homology of the *mcr-3*-positive isolates.[15] It revealed that the two isolates CZ11 and LZ11 were almost identical in their core-genome SNPs. The two *E. coli* isolates were from two different villages separated by a distance of ∼10 km, suggesting a clonal spread of *mcr-3*-carrying isolates among pigs from different villages, possibly by trade of their respective animals.

In summary, this is—to the best of our knowledge—the first report of *mcr-3* and *mcr-3.5* in *E. coli* in backyard pig husbandry. This study revealed that *mcr* genes are not restricted to pigs from large-scale commercial farms, but also occur in pigs from small-scale backyard holdings. Therefore, adequate measures, such as raised awareness of rational usage of antimicrobial agents in both animals and humans, prudent usage of colistin in pigs for disease treatment and prevention, and good management/hygiene of backyard farming, should be taken into account to limit the spread of *mcr* genes, including *mcr-3* and its variants.

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Transparency declarations

None to declare.

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