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Research Letter

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Identification and characterization of *mcr* mediated colistin resistance in extraintestinal *Escherichia coli* from poultry and livestock in China

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One sentence summary: This study indicates the presence of *mcr-1* in extraintestinal *Escherichia coli* in livestock in China, and the *mcr-1*-positive bacteria do not appear to be readily lost after colistin withdrawal.
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ABSTRACT

Antimicrobial resistance to colistin has emerged worldwide threatening the efficacy of one of the last-resort antimicrobials used for the treatment of multidrug-resistant *Enterobacteriaceae* infection in humans. In this study, we investigated the presence of colistin resistance genes (*mcr-1*, *mcr-2*, *mcr-3*) in *Escherichia coli* strains isolated from poultry and livestock collected between 2004 and 2012 in China. Furthermore, we studied the maintenance and transfer of the *mcr-1* gene in *E. coli* after serial passages. Overall, 2.7% (17/624) of the *E. coli* isolates were positive for the *mcr-1* gene while none were positive for the *mcr-2* and *mcr-3* genes. The prevalences of *mcr-1* were similar in *E. coli* isolates from chickens (3.2%; 13/404), pigs (0.9%; 1/113) and ducks (6.8%; 3/44) but were absent in isolates from cattle (0/63). The *mcr-1* gene was maintained in the

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E. coli after six passages (equivalent to 60 generations). *In vitro* transfer of *mcr-1* was evident even without colistin selection. Our data indicate the presence of *mcr-1* in extraintestinal *E. coli* from food-producing animals in China, and suggest that high numbers of the *mcr-1*-positive bacteria in poultry and livestock do not appear to be readily lost after withdrawal of colistin as a food additive.

Keywords: *mcr-1*; *mcr-2*; *mcr-3*; poultry; livestock; colistin resistance

INTRODUCTION

Antimicrobial resistance is recognized as one of the most serious global threats to human health. These concerns are currently exemplified by the rapid increase in carbapenem-resistant *Enterobacteriaceae* (Kumarasamy *et al.* 2010; Munoz-Price *et al.* 2013) leading to a further limitation of the treatment options for infections caused by these bacteria. As a consequence, older and inconvenient antibiotics such as colistin are being used again in human medicine (Temkin *et al.* 2014). However, recently the first plasmid-mediated colistin resistance gene (designated *mcr-1*) was discovered and has now been shown to occur worldwide (Doumith *et al.* 2016; Fernandes *et al.* 2016; Haenni *et al.* 2016; Irrgang *et al.* 2016; Liu *et al.* 2016; Nordmann *et al.* 2016; Teo *et al.* 2016; Veldman *et al.* 2016; El Garch *et al.* 2017; Lei *et al.* 2017). Subsequently, three other colistin resistance genes (*mcr-2*, *mcr-3* and *mcr-4*) have been reported, further compromising the usefulness of colistin (Xavier *et al.* 2016b; Carattoli *et al.* 2017; Yin *et al.* 2017).

Although colistin is now an important 'last-line' drug for the treatment of multiple drug-resistant Gram-negative bacterial infections, its major use remains agricultural in pigs and chickens to treat and prevent disease and in some countries also as a growth promotor (Nguyen *et al.* 2016). Until its use was discontinued in April 2017, around 8000 tons of colistin was used each year for agriculture in China (Wang *et al.* 2017).

Little data are available on colistin resistance in pathogenic *Escherichia coli* from food-producing animals. In 2016, a study from Belgium showed a high prevalence of *mcr-1* and *mcr-2* (Xavier *et al.* 2016a,bbib31), but no data are available on *mcr-3* and *mcr-4* in pathogenic *E. coli* from Belgium (Carattoli *et al.* 2017). Here, we investigated the presence of colistin resistance genes (*mcr-1*, *mcr-2*, *mcr-3*) in extraintestinal *E. coli* from poultry and livestock in China. The sequence for *mcr-4* was published following the completion of this study and therefore was not available for our analyses. In addition, we investigated the *in vitro* maintenance and transfer of *mcr-1* between *E. coli* strains in the absence of colistin.

MATERIALS AND METHODS

Bacterial isolates

We studied 862 distinct clinical isolates of *Escherichia coli* archived in the Veterinary Microbiology Laboratory of the College of Veterinary Medicine, Yangzhou University. These isolates were recovered between 2004 and 2012 from lung and liver of chickens (n = 644), lung, spleen and liver of pigs (n = 113), milk of cows (n = 61), and liver of ducks (n = 44) from various areas in Jiangsu Province.

DNA extraction

The DNA was extracted from the isolates using the High Pure PCR Template Preparation Kit (Roche Diagnostic, USA) following the manufacturer's instructions.

mcr-1, *mcr-2* and *mcr-3* qPCRs

The nucleotide sequences of the *mcr-1* (*E. coli* SHP45, KP347127; *E. coli* KX276657; *E. albertii*, KX765477; *Citrobacter braakii*, NZ_MTCP01000048; *Cronobacter sakazakii*, KX505142; *Klebsiella pneumoniae*, KU761327; *Kluyvera ascorbata*, KU922754; *Salmonella enterica* SC23, KU934209; *S. enterica* Z319S, KX257482), *mcr-2* (*E. coli*, LT598652; *Moraxella pluranimalium*, MF176239; *Moraxella* sp., MF176240) and *mcr-3* (*E. coli*, KY924928) were obtained from NCBI (www.ncbi.nlm.nih.gov). Using the Clustal Multiple Alignment Algorithm, we identified conserved 342-bp and 1497-bp regions as targets for the two *mcr-1* PCRs we developed (Table 1). Similarly, we identified highly conserved 282-bp and 576-bp targets for the two *mcr-2* PCRs, and 267-bp and 1063-bp targets for the two *mcr-3* PCRs we developed (Table 1). For each gene, the primers we identified for the short target were used in a qPCR while the primers for the longer targets were used in standard PCR.

All PCRs were performed in the LightCycler 480II PCR platform with 10 μl of extracted DNA tested in a 20 μl final volume of reaction mixture. The thermal cycling for the three qPCRs we developed consisted of one activation cycle of 5 min at 95°C followed by18 high-stringency stepdown cycles, and 35 relaxed-stringency fluorescence acquisition cycles. The 18 high-stringency stepdown thermal cycles were 6 × 1 s with a temperature of 95°C, 12 s at 70°C, 8 s at 72°C; 9 × 1 s at 95°C, 12 s at 68°C, 8 s at 72°C; 3 × 1 s at 95°C, 12 s at 66°C, 8 s at 72°C. The relaxed-stringency cycling conditions consisted of 35 × 1 s at 95°C, 8 s at 57°C, followed by fluorescence acquisition of 30 s at 72°C. Melting curve analysis (T_m) was performed by monitoring fluorescence between 60°C and 95°C after 30 s at 95°C. The relaxed-stringency cycles for three conventional PCRs consisted of one activation cycle of 3 min at 93°C followed by 35 cycles consisting of 15 s at 93°C, 30 s at 57°C and 70 s at 68°C, one extension cycle at 72°C for 5 min.

The specificities of the primers in the qPCRs for the *mcr-1*, *mcr-2* and *mcr-3* were verified by BLASTN and also by the size of the PCR products on gel electrophoresis and DNA sequencing of PCR products. The TA Cloning® Kit with pCR2.1 vector (ThermoFisher Scientific) was used to clone the representative PCR products.

The sensitivities of the *mcr-1*-qPCR, *mcr-2*-qPCR and *mcr-3*-qPCR were determined by amplifying dilutions of synthesized plasmids containing portions of the *mcr-1*, *mcr-2* and *mcr-3* targets (GenScript, Nanjing, China). The plasmids were linearized with *Sac* I (Takara Biotechnology, Dalian, China) and quantified using the PicoGreen DNA fluorescence assay (Molecular Probes, Eugene, OR, USA) for preparation of quantitative standards (10^4, 10^3, 10^2, 10^1, 1 copies/reaction).

Escherichia coli FRET-qPCR

The *E. coli* FRET-qPCR targeting the *gyrA* gene was performed as described (Shaheen *et al.* 2009) to differentiate strains based

Table 1. Primers for the PCRs used in this study.

PCR	Primer	Nucleotide sequence	Gene	Amplicon	Reference
mcr-1-qPCR	Forward	5′-TCTTGTGGCGAGTGTTGCCGT-3′	*mcr*-1	342	This study
	Reverse	5′-CCAATGATACGCATGATAAACGCTG-3′			
mcr-1-PCR	Forward	5′-GCTCGGTCAGTCCGTTTGTTCTTG-3′	*mcr*-1	1497	This study
	Reverse	5′-GGATGAATGCGGTGCGGTCTT-3′			
mcr-2-qPCR	Forward	5′-CTGTTGCTTGTGCCGATTGGACTA-3′	*mcr*-2	282	This study
	Reverse	5′-ACGGCCATAGCCATTGAACTGC-3′			
mcr-2-PCR	Forward	5′-AGCCGAGTCTAAGGACTTGATGAATTTG-3′	*mcr*-2	576	This study
	Reverse	5′-GCGGTATCGACATCATAGTCATCTTG-3′			
mcr-3-qPCR	Forward	5′-CCAATCAAAATGAGGCGTTAGCATAT-3′	*mcr*-3	267	This study
	Reverse	5′-TAACGAAATTGGCTGGAACAATCTC-3′			
mcr-3-PCR	Forward	5′-CGCTTATGTTCTTTTTGGCACTGTATT-3′	*mcr*-3	1067	This study
	Reverse	5′- TGAGCAATTTCACTATCGAGGTCTTG-3′			
gyrA FRET-PCR	Forward	5′-CCATGAACGT ACTAGGCAAT GACTG-3′	*gyrA*	310	Shaheen *et al.* (2009)
	Reverse	5′-TTTTCCGTGCCGTCATAGTTATCAAC-3′			
	Fluorescein probe	5′-GTTGGTGACGTAATCGGTAAATACCATCCCC-6-fam			
	Bodipy 630/650 probe	5′- Bodipy 630/650 -TGGTGACTCGGCGGTTTATGACACGA-3′			

on mutations in the *gyrA* detected by melting curve analysis (T_m) performed by monitoring fluorescence between 60°C and 95°C after 30 s at 95°C. The differentiation of *E. coli* FRET-qPCR by melting curve analysis was confirmed by DNA sequencing.

Colistin susceptibility testing

A custom-made microdilution susceptibility test was performed in triplicate according to CLSI guidelines and interpretive standards (CLSI 2010). The ATCC 25922 *E. coli* reference strain (American Tissue Cell Culture, Manassas, VA) was used as control. An isolate was designated as resistant (MIC $\geq$ 4 μg/ml) or susceptible (MIC $\leq$ 2 μg/ml) using the guidelines described previously (Hindler and Humphries 2013) (Table 2).

Determination of *in vitro* maintenance and transfer of *mcr-1* in the presence and absence of colistin

We co-cultured a 1:99 mixture of *mcr*-1-positive *E. coli* (E249) and *mcr*-negative *E. coli* (E254) in Luria-Bertani broth with or without colistin (2 μg/ml) for six passages of 3 h each with aeration. For each passage, a 100 μl of the culture was washed three times in 800 μl of PBS and inoculated into a fresh Luria-Bertani broth. Each passage was incubated for 3 h, representing thus each time approximately 10 generations.

After each passage, an aliquot was streaked onto agar plates in triplicate, and 30 colonies were randomly selected from each plate for DNA extraction. The extracted DNA from each bacterial colony was analyzed with the *mcr-1* PCR and by *gyrA*-based FRET-qPCR.

Statistical analysis

Confidence intervals were calculated based on the exact binomial distribution using an Excel macro. Prevalence between the different animal species was calculated based on the Chi square test of the Fisher exact test where appropriate. Level of significance was $P \leq 0.05$.

Table 2. Antimicrobial susceptibility testing (MIC).

Organism	Sources	*mcr*-1 gene	Colistin	
			MIC	Results
ATCC 25922		Negative	2 μg/ml	S*
E. coli E99	Chicken	Negative	1 μg/ml	S
E. coli E254	Chicken	Negative	0.5 μg/ml	S
E. coli E238	Pig	Negative	2 μg/ml	S
E. coli E1	Chicken	Positive	4 μg/ml	R
E. coli E17	Chicken	Positive	8 μg/ml	R
E. coli E97	Chicken	Positive	4 μg/ml	R
E. coli E103	Chicken	Positive	4 μg/ml	R
E. coli E111	Chicken	Positive	4 μg/ml	R
E. coli E117	Chicken	Positive	4 μg/ml	R
E. coli E118	Chicken	Positive	4 μg/ml	R
E. coli E122	Chicken	Positive	4 μg/ml	R
E. coli E123	Chicken	Positive	4 μg/ml	R
E. coli E249	Chicken	Positive	8 μg/ml	R
E. coli E251	Chicken	Positive	4 μg/ml	R
E. coli E270	Chicken	Positive	4 μg/ml	R
E. coli E278	Chicken	Positive	4 μg/ml	R
E. coli E336	Pig	Positive	4 μg/ml	R
E. coli E819	Duck	Positive	8 μg/ml	R
E. coli E859	Duck	Positive	8 μg/ml	R
E. coli E872	Duck	Positive	4 μg/ml	R

*The isolates, determined to be sensitive (S) or resistant (R), is based on: Wayne P A. Clinical and laboratory standards institute. Performance standards for antimicrobial susceptibility testing, 2007, 17.

RESULTS

Development of *mcr*-1-qPCR, *mcr*-2-qPCR and *mcr*-3-qPCR

The short targets we detected with our *mcr*-1-qPCR (342 bp amplicon), *mcr*-2-qPCR (282 bp) and *mcr*-3-qPCR (267 bp) (Table 1) were found with the appropriate positive control plasmids at a detection limit of one gene copy per reaction. Each qPCR amplified the plasmids containing its own *mcr* but did not amplify plasmids containing the other mcr genes. Sequences of amplified amplicons were as expected in each qPCR. The longer

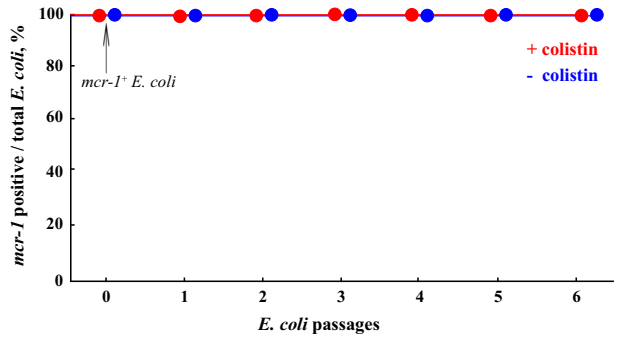

Figure 1. Passage of *mcr*-1-positive *E. coli* in the presence or absence of colistin. The *mcr*-1-positive *E. coli* (strain 249) was cultured in broth with or without colistin, for six passages. At the end of each passage, aliquots were streaked onto agar plates in triplicate, and 30 colonies were randomly selected from each plate for DNA extraction and *mcr*-1 detection by PCR. All bacterial colonies remained *mcr*-1 positive over six passages, irrespective of whether colistin was present or absent in the culture media.

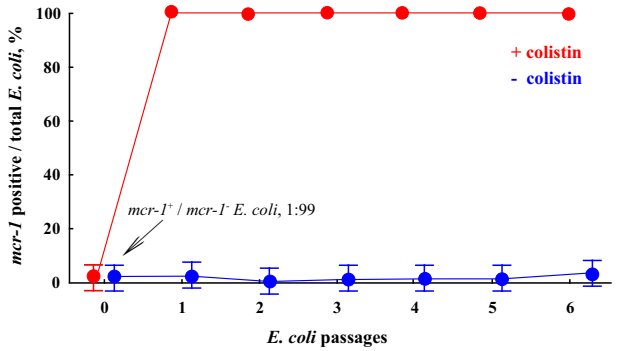

Figure 2. Passage of a 1:99 mixture of *mcr*-1-positive and *mcr*-1-negative *E. coli* in the presence or absence of colistin. The *mcr*-1-positive *E. coli* (strain 249) or 1:99 mixture of *mcr*-1$^+$ (strain 249) and *mcr*-1$^-$ (strain 254) *E. coli* was cultured in broth with or without colistin, for six passages. At the end of each passage (3 h, about 10 generations), aliquots were streaked onto agar plates in triplicate, and 30 colonies were randomly selected from each plate for DNA extraction and *mcr*-1 detection by PCR.

targets detected in our *mcr*-1-PCR (1497 bp amplicon), *mcr*-2-PCR (576 bp amplicon) and *mcr*-3-PCR (1063 bp amplicon) were also found in the appropriate positive control plasmids with a detection limit of 50 gene copies per reaction.

Prevalence of the *mcr*-1 in extraintestinal *Escherichia coli*

Overall, 2.7% (17/624) of the *E. coli* isolates were positive for the *mcr*-1. The prevalences did not significantly differ between the different animal species with isolates from chickens (3.2%, CI 1.7–5; 13/404), pigs (0.9%, CI 0–5; 1/113) and ducks (6.8%, CI 1.4–19; 3/44), but no positive isolations from cattle (0/63, CI 0–6). Neither *mcr*-2 nor *mcr*-3 was detected in any of the isolates.

Susceptibility testing

While *E. coli* ATCC strain 25922 and three randomly selected *mcr*-1-negative isolates from the study were susceptible to colistin, the 17 *mcr*-1-positive isolates recovered here were resistant to colistin (Table 2).

Differentiation of *Escherichia coli* strain E249 (*mcr*-1 positive) and E254 (*mcr*-1 negative) with a *gyrA* FRET-qPCR

Using FRET-qPCR to amplify the *E. coli gyrA* in our isolates, we identified a *mcr*-1-positive *E. coli* (strain E249) that had a lower T_m value (~60.50°C) than that of a *mcr*-1-negative *E. coli* (strain E254) (~69.30°C). Sequencing showed that the *mcr*-1-positive *E. coli* strain E249 had two nucleotide mismatches in the *gyrA* (positions 259 and 264) which resulted in a nine-degree reduction in melting temperature compared to the *mcr*-1-negative *E. coli* strain E254 (no *gyrA* mismatch) and this enabled the ready differentiation of the two strains grown together.

Determination of *in vitro* maintenance and transfer of *mcr*-1 in the presence and absence of colistin

After six passages, corresponding to approximately 60 generations of the *E. coli* strains, all the *mcr*-1-positive *E. coli* remained positive for the *mcr*-1, irrespective of whether colistin was present in the culture media or not (Fig. 1).

In the presence of colistin in the culture media, the percentage of *mcr*-1-positive *E. coli* relative to *mcr*-1-negative *E. coli* rose rapidly from 1% in generation 0 to 100% in all subsequent passages. In all passages, *mcr*-1-positive isolates were found, confirming maintenance of the resistance in the presence of MIC concentrations of colistin (Fig. 2).

In the absence of colistin in the media, the percentage of colonies of *mcr*-1-positive *E. coli* relative to *mcr*-1-negative *E. coli* did not increase and remained low (under 3%) through all the passages studied. The 3% of the *E. coli* were *mcr*-1 positive in each passage, compared to the initial 1% (Fig. 2). The *mcr*-1 qPCR followed by melting temperature analysis and DNA sequencing identified eight *mcr*-1-positive colonies: four were the *E. coli* strain 249 that was originally positive for the *mcr*-1 and four (50%) were the *E. coli* strain 254 that did not contain the *mcr*-1 at the beginning of the experiment. This indicates that there was a transfer of the *mcr*-1 containing plasmid from *E. coli* strain 249 to *E. coli* strain 254 while they were co-cultured in the absence of colistin.

DISCUSSION

Few investigations have looked for colistin resistance in pathogenic *Escherichia coli* from animals, and even less data are available on their colistin susceptibility, as regular disk diffusion tests, used in many clinical veterinary laboratories, do not allow accurate detection of resistance. This report provides comprehensive data on the prevalence of colistin resistance genes in extraintestinal *E. coli* isolates from poultry and livestock in China. We found no differences in *mcr* prevalence between different animal species; however, this may be due to the small sample size for some animal species. The *mcr*-2 plasmid-mediated colistin resistance determinant was identified for the first time in *E. coli* from pigs and cattle in Belgium (Xavier *et al.* 2016b). In a subsequent retrospective survey of 58 German pig-fattening farms (Roschanski *et al.* 2017), the *mcr*-1 was found in 9.9% of 436 samples and 25.9% of the farms while the *mcr*-2 was not detected (Roschanski *et al.* 2017). Also, the *mcr*-2 could not be identified in almost 10 000 animal/clinical samples from China (Sun *et al.* 2017). We could also not identify the *mcr*-2 in this study in China.

The third mobile colistin resistance gene, *mcr*-3, was first described in *E. coli* from pigs in China (Doumith *et al.* 2016). Then, *mcr*-3 or *mcr*-3 variants were detected in low prevalence from *E. coli* recovered from bloodstream infections in Denmark(Roer

et al. 2017), human *Salmonella* from Denmark (Litrup *et al.* 2017), bovine *E. coli* in Spain (Hernández *et al.* 2017) and *Aeromonas veronii* from chickens in China (Ling *et al.* 2017). We did not find the *mcr-3* gene in our extraintestinal *E. coli* isolates that indicates a very low prevalence or absence altogether.

Since 1 April 2017, the Chinese government has implemented the withdrawal of colistin as a food additive for growth promotion in food animal (Wang *et al.* 2017). This appears appropriate as studies have demonstrated high levels of the *mcr-1* in China (Liu *et al.* 2016; Huang *et al.* 2017; Wang *et al.* 2017; Wang *et al.* 2017a,b). These high prevalences may be explained by the strong selection pressure for the *mcr-1* we identified in our *E. coli* passage experiments. The percentage of *E. coli* with the *mcr-1* relative to those without the gene rose from an initial 1% to 100% in one passage.

Following the withdrawal of colistin, we were interested in how long *mcr* may remain in the population. There is currently no data on the fate of the *mcr-1*-positive bacteria once there is no longer selection pressure from colistin. Therefore, we performed *in vitro* stability and horizontal gene transfer studies of mcr-1 in *E. coli*. We chose shorter incubation periods with a lower concentration of resistant strains compared with many other studies to better reflect the actual environment with a low *mcr* prevalence. Our findings demonstrate that the colistin resistance gene is maintained at an unchanged level through 60 generations in the absence of colistin, and that transfer occurs even in the absence of selective pressure by colistin. The latter has also been demonstrated for extended-spectrum beta-lactamases (ESBLs) (Smet *et al.* 2011). A 2% increase in *mcr*-1-positive *E. coli* in the absence of colistin may suggest that the resistance is stable and may still transfer even when colistin removed from food animal production (Jørgensen *et al.* 2017). The viability of the strains compared to the other strains could not be determined, though the transfer experiment shows that there is likely no competition between those two strains. Further close monitoring of the *mcr* gene in bacteria as the colistin withdrawal is completed in China is indicated to see if this will be the case and provide further data on the effects of lessened antimicrobial use and its effects on antibiotic resistance in bacteria in animals and the environment.

In conclusion, our data show the prevalence, albeit low, of *mcr* in animal extraintestinal *E. coli*. Moreover, our data demonstrate that *mcr-1* is readily transmitted between bacteria and is well maintained *in vitro*, even without the selective pressure of colistin.

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Conflict of interest. None declared.

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