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Housefly (*Musca domestica*) and Blow Fly (*Protophormia terraenovae*) as Vectors of Bacteria Carrying Colistin Resistance Genes

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ABSTRACT Flies have the capacity to transfer pathogens between different environments, acting as one of the most important vectors of human diseases worldwide. In this study, we trapped flies on a university campus and tested them for mobile resistance genes against colistin, a last-resort antibiotic in human medicine for treating clinical infections caused by multidrug-resistant Gram-negative bacteria. Quantitative PCR assays we developed showed that 34.1% of *Musca domestica* (86/252) and 51.1% of *Protophormia terraenovae* (23/45) isolates were positive for the *mcr-1* gene, 1.2% of *M. domestica* (3/252) and 2.2% of *P. terraenovae* (2.2%, 1/45) isolates were positive for *mcr-2*, and 5.2% of *M. domestica* (13/252) and 44.4% of *P. terraenovae* (20/45) isolates were positive for *mcr-3*. Overall, 4.8% (9/189) of bacteria isolated from the flies were positive for the *mcr-1* gene (*Escherichia coli*: 8.3%, 4/48; *Enterobacter cloacae*: 12.5%, 1/8; *Providencia alcalifaciens*: 11.8%, 2/17; *Providencia stuartii*: 4.9%, 2/41), while none were positive for *mcr-2* and *mcr-3*. Four *mcr-1*-positive isolates (two *P. stuartii* and two *P. alcalifaciens*) from blow flies trapped near a dumpster had a MIC for colistin above 4 mg/ml. This study reports *mcr-1* carriage in *Providencia* spp. and detection of *mcr-2* and *mcr-3* after their initial identification in Belgium and China, respectively. This study suggests that flies might contribute significantly to the dissemination of bacteria, carrying these genes into a large variety of ecological niches. Further studies are warranted to explore the roles that flies might play in the spread of colistin resistance genes.

IMPORTANCE Antimicrobial resistance is recognized as one of the most serious global threats to human health. An option for treatment of the Gram-negative ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) bacteria with multiple drug resistance was the reintroduction of the older antibiotic colistin. However, a mobile colistin resistance gene (*mcr-1*) has recently been found to occur widely; very recently, two other colistin resistance genes (*mcr-2* and *mcr-3*) have been identified in Belgium and China, respectively. In this study, we report the presence of colistin resistance genes in flies. This study also reports the carriage of colistin resistance genes in the genus *Providencia* and detection of *mcr-2* and *mcr-3* after

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their initial identification. This study will stimulate more in-depth studies to fully elucidate the transmission mechanisms of the colistin resistance genes and their interaction.

KEYWORDS colistin, *Musca domestica*, *Protophormia terraenovae*, *mcr-1*, *mcr-2*, *mcr-3*, resistance genes

Antimicrobial resistance is recognized as one of the most serious global threats to human health, with antimicrobial resistance genes spreading with remarkable speed under the selective evolutionary pressure imposed by widespread antibiotic use (1). A variety of insects that are commonly associated with food animals could be important vectors of resistance genes to people. Flies appear to be particularly important (2–6); several studies have demonstrated that flies carry multidrug-resistant bacteria, including human pathogens, in hospital environments (7). Also, a recent study showed that flies can carry multidrug-resistant bacteria belonging to specific clonal lineages identical to those found in animal manure (3).

Colistin is considered a last-resort antimicrobial for treating patients infected with multidrug-resistant Gram-negative bacteria. Recently, the usefulness of the drug has been compromised by the emergence of mobile colistin resistance genes (*mcr-1*, *mcr-2*, and *mcr-3*) (8–13). To be able to develop strategies to limit the spread of these resistance genes, it is important to better understand their epidemiology and transmission mechanisms. To this end, we investigated the presence of *mcr-1*, *mcr-2*, and *mcr-3* in flies captured on a university campus in an urban center of a city in China.

RESULTS

Flies. A total of 297 flies were trapped for the study, comprising 252 individuals of *Musca domestica* and 45 individuals of *Protophormia terraenovae* (Table 1). *Musca domestica* flies were trapped at all three locations, while *P. terraenovae* flies were only trapped near a dumpster (location 3).

Bacterial isolates from flies. Overall, 11 bacterial species were isolated from the flies, with *Escherichia coli* and *Providencia stuartii* being isolated most frequently (41 isolates each) and at all sampling locations (Table 2).

Development of *mcr-1* qPCR, *mcr-2* qPCR, and *mcr-3* qPCR assays. The short *mcr* sequences were obtained from quantitative real-time PCR (qPCR) assays, while the long sequences were obtained using conventional PCR assays. Our *mcr-1* qPCR (342-bp amplicon), *mcr-2* qPCR (282 bp) and *mcr-3* qPCR (267 bp) (Table 3) detected the positive-control plasmids containing their target gene sequences with a detection limit of one gene copy per reaction. Each qPCR amplified the plasmids containing its own *mcr* gene but did not amplify plasmids containing the other *mcr* genes. Sequences of amplified amplicons were as expected in each qPCR. The *mcr-1* PCR (1,497-bp amplicon), *mcr-2* PCR (576-bp amplicon) and *mcr-3*-PCR (1,063-bp amplicon) were also highly specific and had a detection limit of 50 gene copies per reaction.

Prevalence of *mcr-1*, *mcr-2*, and *mcr-3* in flies. In total, 109 (36.7%, 109/297) of the homogenized flies were found to be positive by *mcr-1* qPCR, with positive flies identified at each of the three trapping sites (location 1: 49.4%, 40/81; location 2: 24.2%, 38/157; location 3: 52.5%, 31/59) (Fig. 1, Table 2). The *mcr-1* gene was found in both fly species, *M. domestica* (34.1%, 86/252) and *P. terraenovae* (51.1%, 23/45) (Table 2).

The *mcr-2* qPCR assay was positive with four of the homogenized flies (1.3%, 4/297); three *M. domestica* (1.2%, 3/252) from location 1 (2.5%, 2/81) and location 2 (0.6%, 1/157) were positive and one *P. terraenovae* (2.2%, 1/45) from location 3 (1.7%, 1/59).

In total, 33 of the homogenized flies (11.1%, 33/297) from location 1 (9.9%, 8/81), location 2 (3.8%, 6/157), and location 3 (32.2%, 19/59), including 13 *M. domestica* (5.2%, 13/252) and 20 *P. terraenovae* (44.4%, 20/45), were positive by *mcr-3* qPCR.

TABLE 1 Colistin resistance genes in flies and their bacteria

Source location	Species of fly (no. of isolates)	No. of flies used for isolation	Gene presence in fly homogenates (% [no. of isolates/total])			Species of bacteria (no. of isolates)	Gene presence (no. of strains)[a]		
			mcr-1	*mcr-2*	*mcr-3*		*mcr-1*	*mcr-2*	*mcr-3*
1	*M. domestica* (81)	19	49.4% (40/81)	2.5% (2/81)	9.9% (8/81)	*E. coli* (10) *P. stuartii* (10) *K. pneumoniae* (7)	3	0	0
2	*M. domestica* (157)	40	24.2% (38/157)	0.6% (1/157)	3.8% (6/157)	*C. freundii* (15) *P. aeruginosa* (9) *E. coli* (10) *P. stuartii* (12) *E. ludwigii* (2)	1	0	0
3	*M. domestica* (14) *P. terraenovae* (45)	15	57.1% (8/14) 51.1% (23/45)	2.2% (1/45)	42.7% (6/14) 28.9% (13/45)	*E. coli* (21) *P. rettgeri* (16) *P. stuartii* (19) *P. alcalifaciens* (17) *K. pneumoniae* (8) *K. variicola* (9) *E. ludwigii* (5) *E. cloacae* (8)	5	0	0

[a]The numbers under *mcr* genes indicate number of bacterial strains positive for the *mcr* genes.

In total, 41.4% of the trapped flies (123/297) carried at least one of the colistin resistance genes. One fly (*P. terraenovae*) carried *mcr-1* and *mcr-2* genes, and 24 flies (14 *M. domestica* and 10 *P. terraenovae*) were positive for both *mcr-1* and *mcr-3*.

Prevalence of *mcr-1*, *mcr-2*, and *mcr-3* in bacterial isolates. Overall, 4.8% (9/189) of the bacterial isolates we obtained from the flies were *mcr-1*-positive: *E. coli* (8.3%, 4/48), *P. stuartii* (4.9%, 2/41), *P. alcalifaciens* (11.8%, 2/17), and *E. cloacae* (12.5%, 1/8) (Table 3). None of the bacterial isolates we obtained were positive by *mcr-2* qPCR or *mcr-3* qPCR.

Susceptibility testing. Microdilution susceptibility testing showed that 7 of the 9 (77.8%) isolates positive by *mcr-1* qPCR were resistant to colistin (Table 3). Four of these isolates (two *P. stuartii* and two *P. alcalifaciens* from *P. terraenovae* trapped near a dumpster) had a MIC of ≥4 mg colistin/ml.

Phylogenetic comparison. The DNA from the homogenized flies and bacterial isolates that were positive by short-amplicon qPCR were also positive by long-amplicon PCR (*mcr-1*, 1,497 bp; *mcr-2*, 576 bp; *mcr-3*, 1,063 bp). Overall, all *mcr-2* amplicons were sequenced, along with 43% of *mcr-1* amplicons and 80% of *mcr-3* amplicons derived from qPCR (short amplicons) and standard PCR (long amplicons) assays of the fly homogenates and bacterial isolates.

The 1,497-bp nucleotide sequences for *mcr-1* were all identical to each other (*Escherichia coli*, MF069152; *Providencia stuartii*, MF598564; *Enterobacter cloacae*, MF598565; *Providencia alcalifaciens*, MF598566) and to a strain from GenBank (*Esche-*

TABLE 2 Antimicrobial susceptibility testing against colistin

Organism	*mcr-1* status	MIC against colistin (resistance status)[a]
E. coli ATCC 25922	Negative	2 μg/ml (S)
K. pneumoniae	Negative	2 μg/ml (S)
E. cloacae	Negative	2 μg/ml (S)
Plesiomonas shigelloides	Negative	0.5 μg/ml (S)
E. coli	Positive	2 μg/ml (S)[b] 4 μg/ml (R)[b]
E. cloacae	Positive	4 μg/ml (R)
P. alcalifaciens	Positive	>4 mg/ml (R)
P. stuartii	Positive	>4 mg/ml (R)

[a]S, susceptible; R, resistant.
[b]$n = 2$ isolates.

TABLE 3 Primers for PCRs used in this study

PCR	Primer	Nucleotide sequence	Gene	Amplicon	Reference or source
mcr-1 qPCR	Forward Reverse	5′-TCTTGTGGCGAGTGTTGCCGT-3′ 5′-CCAATGATACGCATGATAAACGCTG-3′	*mcr-1*	342	This study
mcr-1 PCR	Forward Reverse	5′-GCTCGGTCAGTCCGTTTGTTCTTG-3′ 5′-GGATGAATGCGGTGCGGTCTT-3′	*mcr-1*	1,497	This study
mcr-2 qPCR	Forward Reverse	5′-CTGTTGCTTGTGCCGATTGGACTA-3′ 5′-ACGGCCATAGCCATTGAACTGC-3′	*mcr-2*	282	This study
mcr-2 PCR	Forward Reverse	5′-AGCCGAGTCTAAGGACTTGATGAATTTG-3′ 5′-GCGGTATCGACATCATAGTCATCTTG-3′	*mcr-2*	576	This study
mcr-3 qPCR	Forward Reverse	5′-CCAATCAAAATGAGGCGTTAGCATAT-3′ 5′-TAACGAAATTGGCTGGAACAATCTC-3′	*mcr-3*	267	This study
mcr-3 PCR	Forward Reverse	5′-CGCTTATGTTCTTTTTGGCACTGTATT-3′ 5′-TGAGCAATTTCACTATCGAGGTCTTG-3′	*mcr-3*	1,063	This study
Bacterial identification PCR	Forward Reverse	5′-AGAGTTTGATCCTGGCTCAG-3′ 5′-TACGGTTACCTTGTTACGACTT-3′	16S rRNA	1,400	27

richia coli strain SHP45, KP347127, isolated from swine in China) (Fig. 2; see also Fig. S1 in the supplemental material).

The nucleotide sequence of the *mcr-2* gene (LT598652) in *Escherichia coli* strain KP37 isolated from pigs and cattle in Belgium was very similar to that of the *mcr-2* gene we identified in flies in our study (*Protophormia terraenovae*, MF580947, 561/576 bp similarity; *Musca domestica*, MF580948, 563/576 bp similarity) (Fig. 2; see also Fig. S2 in the supplemental material). At the amino acid level, our *mcr-2* sequences were 98.4% identical (MF580947 and MF580948; 187/190 bp similarity) to the *mcr-2* gene in *E. coli* strain KP37 (LT598652) (see Fig. S3 in the supplemental material).

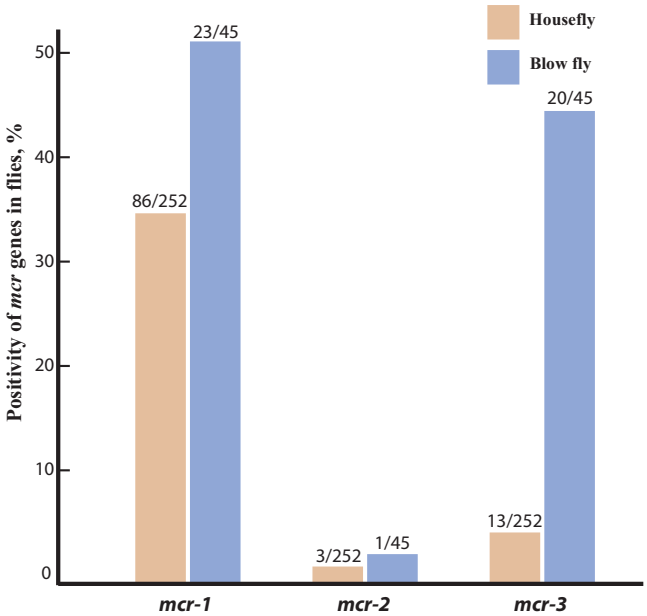

FIG 1 Flies positive for the *mcr* colistin resistance genes. In total, 34.1% (95% confidence interval [CI]: 28.3, 40.0) of *M. domestica* (86/252) and 51.1% (95% CI: 36.5, 65.7) of *P. terraenovae* (23/45) isolates were positive for *mcr-1*, 1.2% (95% CI: 0.001, 2.5) of *M. domestica* (3/252) and 2.2% (95% CI: 0.001, 6.5) of *P. terraenovae* (1/45) isolates were positive for *mcr-2*, and 5.2% (95% CI: 2.4%, 7.9%) of *M. domestica* (13/252) and 44.4% (95% CI: 29.9%, 59.0%) of *P. terraenovae* (20/45) isolates were positive for *mcr-3*.

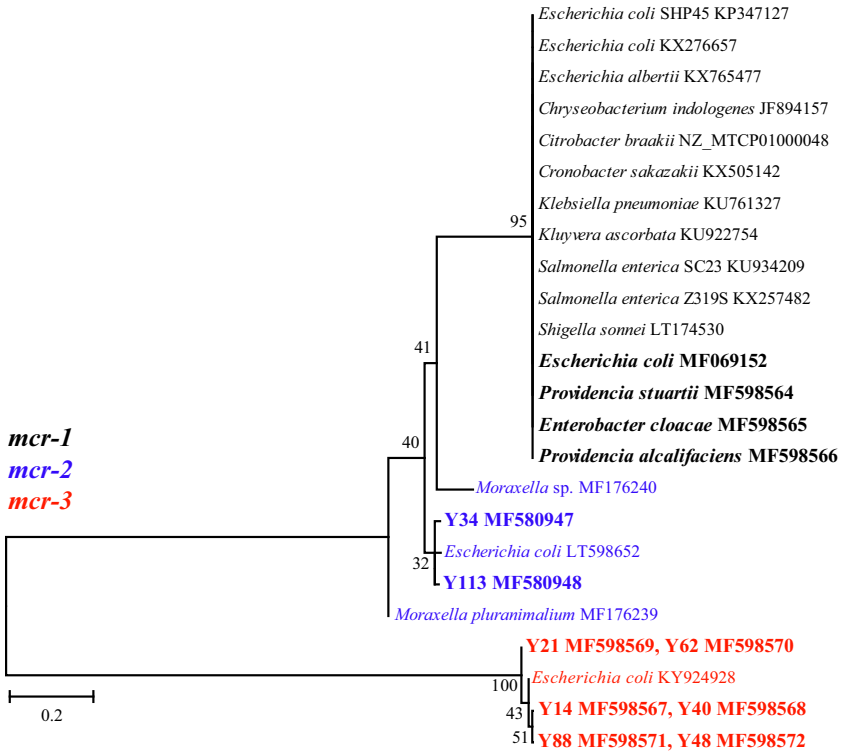

FIG 2 Phylogenetic analysis of colistin resistance genes. The nucleotide sequences of colistin resistance genes (*mcr-1* in black, *mcr-2* in blue, and *mcr-3* in red) identified in this study (in boldface) are compared with representative sequences from NCBI. The evolutionary history was inferred using the neighbor-joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

The *mcr-3* gene sequences obtained in this study (*Musca domestica* MF598567 and *Protophormia terraenovae* MF598568, 1,046/1,063 bp identical; *Musca domestica* MF598569 and *Protophormia terraenovae* MF598570, 1,047/1,063 bp identical; *Musca domestica* MF598571 and *Protophormia terraenovae* MF598572, 1,046/1,063 bp identical) were very similar to the *mcr-3* sequence in plasmid pWT1 and from *E. coli* strain (KY924928) isolated from the feces of an apparently healthy pig in China (14) (Fig. 2; see also Fig. S4 in the supplemental material). At the amino acid level, the sequences of our *mcr-3*-positive isolates were 98.5% to 98.8% similar to that of *mcr-3* in *Escherichia coli* strain pWT1 (see Fig. S5 in the supplemental material).

DISCUSSION

The *mcr-1* gene was first described in *E. coli* from a pig in China and has now also been found in numerous bacterial species worldwide (15). Our study shows the presence of mobile colistin resistance genes in samples from flies and also identifies an additional genus (*Providencia*) and two species (*P. stuartii* and *P. alcalifaciens*) of bacteria that carry *mcr-1*. *Providencia* (family *Enterobacteriaceae*) is a genus of ubiquitous Gram-negative bacteria that has been found in a variety of animals and is a part of the normal human gut flora (16, 17). It is also a prominent component of the gut bacteria of houseflies (18) and has been found in blow, fruit, and stable flies (17). The genus has six recognized species (16), including the pathogens *P. stuartii*, commonly associated with urinary tract infections in people with indwelling catheters, and *P. alcalifaciens*, a cause of traveler's diarrhea and gastroenteritis (18). It was beyond the scope of our study to determine the carrier (transposon or plasmid) of the *mcr* gene, but this should be considered in other studies designed to identify bacteria that can spread resistance genes.

The *mcr-2* mobile colistin resistance determinant was identified for the first time in

E. coli from pigs and cattle in Belgium (19). In a subsequent study, however, the *mcr-2* gene could not be identified in almost 10,000 animal/clinical samples from China (20). Also, in a retrospective survey of 58 German pig-fattening farms (21), *mcr-1* was found in 9.9% of 436 samples and on 25.9% of the farms, whereas *mcr-2* was not detected (21). Our use of qPCR analysis of DNA extracts rather than colony isolation appears to be a more sensitive method for detection of *mcr* genes and our study also reports the detection of *mcr-2*. Further studies are underway in our laboratories to isolate *mcr-2*-postive bacteria and further evaluate the mechanisms of resistance to colistin. Our sequences deviated little from the original sequence and may thus be named subtypes of *mcr-2*.

The recently described *mcr-3* is the third mobile colistin resistance gene. To date, it has only been detected in *E. coli* from pigs in Shandong province, China (14), which is approximately 500 kilometers from our sampling site. Database searches have indicated that *mcr-3* might already be present in *Enterobacteriaceae* isolated from people, animals, and the environment in Malaysia, Thailand, and the United States (14) but our study also reports the detection of *mcr-3*. The fact that we found this gene in flies suggests that the gene may already be widely disseminated. It is of note that the nucleoside sequences and amino acid composition of the *mcr-3* gene we identified varied from those of the original description and there might thus be subtypes of *mcr-3*. Unfortunately, there is not yet agreement among the international scientific community on the naming and characterization of subtypes of the *mcr* gene.

Flies are important vectors of a number of important diseases because of their abundance, ability to move over relatively large distances, and close association with people, animals, and their waste. The houseflies and blow flies we studied are particularly important in disease transmission (22) and our finding that 41.4% are positive by our *mcr* qPCR assays indicates they are also likely to play an important role in the transmission of antibiotic resistance genes. Flies might also serve as a general indicator for regional antimicrobial resistance. Currently, antimicrobial resistance surveillance is mostly carried out using combined data from several domestic animal species and people, which can be confusing and is labor-intensive and costly. Recent studies have indicated that manure or sewage may serve as a good indicator of the general level of resistance in an area (23, 24) but optimal sample collection and processing methods need to be established. As flies can enter all ecosystems in an area and in the process be contaminated with a wide variety of bacteria, further studies appear to be indicated to establish whether they might be used as reliable and cost-effective indicators of regional antimicrobial resistance.

In conclusion, we report *mcr-1* carriage in *Providencia* spp. and the additional detection of *mcr-2* and *mcr-3* since their initial reports. Our nucleoside sequence and amino acid data provide further evidence of variation in the *mcr* gene and the need for an agreed nomenclature for these genes. The high prevalence of the colistin resistance genes in houseflies and blow flies is of great concern, as flies have the potential to rapidly and widely disseminate the genes. More in-depth epidemiological studies are needed to fully elucidate the epidemiology of the genes.

MATERIALS AND METHODS

Flies. Convenience samples of flies were trapped for an 8-week period in July and August 2013 on the campus of a university in Jiangsu province. Trapping was conducted at three sites: outside the Laboratory Animal Center (location 1), at the entrance to the Animal Clinic (location 2), and near an open dumpster (location 3). These locations were approximately 400 meters apart. A single commercial flypaper strip was placed at each location once a week and observed until flies had become attached to the paper (up to 40 flies per visit). The trapped flies were taken to the laboratory within 30 min and identified using standard morphological techniques (25, 26). The flies were individually washed three times in 1× phosphate-buffered saline (PBS) to reduce surface contaminants and homogenized individually with a tissue homogenizer (Bertin Technologies, MD, USA) at 5,000× rpm for 20 s in 800 μl of PBS. Aliquots of the resultant suspensions were used for bacterial isolation and DNA extraction.

Bacterial isolates from flies. Using no specific selection criteria, we selected around 25% of the flies ($n = 74$) captured at each location for bacterial isolation. Aliquots of the homogenates (100 μl) were streaked onto MacConkey agar plates and incubated at 37°C overnight. Representatives from one to three of the most dominant colonies from each plate were purified and subsequently grown in liquid

nutrient broth for 18 to 24 h on each MacConkey agar plate ($n = 189$ isolates) before DNA was extracted for species identification and detection of resistance genes.

DNA extraction. The High Pure PCR template preparation kit (Roche Diagnostic, USA) was used to extract DNA from aliquots of the fly homogenates (200 μl) and the bacterial isolates in liquid nutrient broth (10 μl) according the manufacturer's protocol.

***mcr-1*, *mcr-2*, and *mcr-3* qPCR assays.** The nucleotide sequences of the *mcr-1* gene (*Escherichia coli* SHP45, KP347127; *Escherichia coli*, KX276657; *Escherichia albertii*, KX765477; *Citrobacter braakii*, NZ_MTCP01000048; *Cronobacter sakazakii*, KX505142; *Klebsiella pneumoniae*, KU761327; *Kluyvera ascorbata*, KU922754; *Salmonella enterica* SC23, KU934209; *Salmonella enterica* Z319S, KX257482), *mcr-2* (*Escherichia coli*, LT598652; *Moraxella pluranimalium*, MF176239; *Moraxella* sp., MF176240), and *mcr-3* (*Escherichia coli*, KY924928) were obtained from NCBI (https://www.ncbi.nlm.nih.gov). Using the Clustal Multiple Alignment Algorithm we identified conserved 342-bp and 1,497-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 1,063-bp targets for the two *mcr-3* PCRs we developed (Table 1).

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 1 activation cycle of 5 min at 95°C, followed by 18 high-stringency step-down cycles and 35 relaxed-stringency fluorescence acquisition cycles. The 18 high-stringency step-down 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, and 8 s at 72°C. The relaxed-stringency cycling conditions consisted of 35 × 1 s of 95°C and 8 s of 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, 70 s at 68°C, and one extension cycle at 72°C for 5 min. The short sequences for the *mcr* genes were obtained by qPCR in this study, while PCRs with the long amplicons were performed with conventional PCR.

The specificity of the primers for the *mcr-1*, *mcr-2*, and *mcr-3* qPCRs was 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 (Thermo Fisher 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 SacI (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, and 10^0 copies/reaction).

Bacterial identification PCR assays. A 16S rRNA-based PCR resulting in a 1,400-bp amplicon was used to identify the species of bacteria isolated from flies as described previously (27). The amplification was verified by gel electrophoresis through 1% agarose gels (Biowest, Hong Kong, China). The PCR product was then purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and the DNA on both strands was sequenced using the appropriate primers (GenScript, Nanjing, China). The TA cloning kit with pCR2.1 vector (Thermo Fisher Scientific) was used to clone the representative PCR products. The bacteria were identified using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Susceptibility testing. A custom-made microdilution susceptibility test was performed on the 9 *mcr-1*-positive isolates, 4 *mcr-1*-negative bacteria, and *E. coli* strain ATCC 25922, according to CLSI guidelines (28, 29) and interpretive standards were performed to determine MIC against colistin. The MIC results were recorded using the Sensititre1 Vizion system (TREK Diagnostic Systems, Cleveland, OH). The *E. coli* ATCC 25922 strain (American Type Culture Collection, Manassas, VA) was used for quality control purposes. Each isolate was designated resistant (MIC $\geq$ 4 μg/ml) or susceptible (MIC $\leq$ 2 μg/ml) using guidelines described previously (30). Susceptibility testing was performed in triplicate for each isolate.

Phylogenetic analysis. Sequences identified in this study and obtained from GenBank for *mcr-1*, *mcr-2*, and *mcr-3* genes were aligned using the MEGA 6.0 software. Based on these alignments, phylogenetic trees were constructed by the neighbor-joining method using the Kimura 2-parameter model with MEGA 6.0. Bootstrap values were calculated using 500 replicates (Fig. 2).

Accession number(s). All sequences described in this study have been deposited in GenBank. The 16S rRNA gene nucleotide sequences of the bacteria isolated from flies described in this study have been deposited under accession numbers MF370887 to MF370906. Described *mcr-1* sequences have been deposited under accession numbers MF069152 and MF598564 to MF598566. Described *mcr-2* sequences have been deposited under accession numbers MF580947 and MF580948. Described *mcr-3* sequences have been deposited under accession numbers MF598567 to MF598572.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01736-17.

SUPPLEMENTAL FILE 1, PDF file, 9.3 MB.

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