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ORIGINAL RESEARCH

High incidence of multidrug-resistant *Escherichia coli* coharboring *mcr-1* and $bla_{CTX-M-15}$ recovered from pigs

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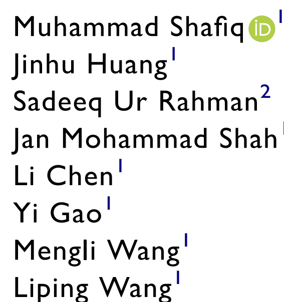

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Purpose: The coexistence of mobile colistin (COL)-resistant gene *mcr-1* with extended-spectrum beta-lactamase (ESBL) gene in *Escherichia coli* has become a serious threat globally. The aim of this study was to investigate the increasing resistance to COL and in particular its coexistence with ESBL-producing *E. coli* recovered from pig farms in China.
Materials and methods: *E. coli* were isolated from 14 pig farms in Jiangsu China. Susceptibility testing was identified by micro-dilution method. PCR assay and nucleotide sequencing were used to detect COL-resistant genes, *mcr*-1 to −5, as well as ESBL genes, bla_{CTX-M}, bla_{SHV} and bla_{TEM}. Conjugation experiment, plasmid replicon typing of the multidrug resistance (MDR), S1-PFGE and DNA southern hybridization were performed to study the transferability of these genes.
Results: Overall, 275 *E. coli* isolates were recovered from a total of 432 cloacal and nasal swabs. More than 90% of the isolates were MDR, of which 70.18% were resistant to COL. Of these 275 isolates, *mcr-1* was identified as the most predominant gene carried by 71.63% (197/275) of isolates, 39.59% (78/197) of the isolates were harboring both *mcr-1* and ESBL genes (bla_{CTX-M}, bla_{SHV} and bla_{TEM}). ESBL genotyping showed that bla_{CTX-M} was the most predominant ESBL (68.49%) followed by bla_{SHV} (16.4%) and bla_{TEM} (15%). Sequencing revealed that the most common variants of bla_{CTX-M} identified were, $bla_{CTX-M-15}$ (69%), $bla_{CTX-M-55}$ (29%) and $bla_{CTX-M-1}$ (1.8%). IncHI2, IncFIB, IncFIC, IncN and IncX4 were found to be the most common Inc-types found both in donors and in transconjugants and were associated with the transfer of the *mcr-1* and ESBL encoding genes. Six strains carried a total of five different plasmids: approximately 97-, 130-, 160-, 227- and 242-kb plasmids.
Conclusion: The coexistence of the *mcr-1*- and $bla_{CTX-M-15}$-carrying isolates displaying high MDR, recovered from *E. coli* of pig origin, is a major concern for both humans and veterinary medicine.
Keywords: *E. coli*, colistin, *mcr-1*, ESBL, coexistence

Introduction

Antimicrobial resistance (AMR) has now been widely recognized as a crucial threat to human and animal health as the extensive use of antimicrobials in humans as well as in food-producing animals.[1] Global consumption of antimicrobials in animal settings may rise up to 67% by 2030, determined predominantly by BRICS (Brazil, Russia, India, China and South Africa) countries, as large-scale and intensive farming operations are greatly in demand with the upsurge in revenue and animal protein consumption.[2,3] This heavy antimicrobial practice creates a selective pressure that contributes to the emergence and spread of bacterial resistance. One of the major concerns is the rapid increase

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of the multidrug-resistant (MDR) *Escherichia coli* in animal settings and clinical medicine.[4–6] This is not only because of the lessened number of useful antimicrobials for curing MDR *E. coli* infections, but also due to the potential transfer of MDR *E. coli* strains from animals to humans, especially which producing extended-spectrum β-lactamases (ESBLs) and carbapenemases, and display resistance to colistin (COL).[7]

ESBLs are β-lactamases that confer resistance to oxyimino "second- and third-generation" cephalosporin's (eg, cefotaxime (CTX), ceftriaxone and ceftazidime) and aztreonam.[8,9] ESBL-producing bacteria were first reported in 1980, soon after the introduction of the third-generation cephalosporin's (CTX and ceftiofur (CEF) into clinical settings.[10] Currently, there are more than 350 ESBL genes that have been reported, and these genes are commonly developed through point mutations of the classical SHV-1 and TEM-1 β-lactamases and more increasingly prominent the CTX-M types.[11–13] Among the CTX-M enzymes, $bla_{\text{CTX-M-55}}$ has become the leading CTX-M type in ESBL-producing *E. coli* isolates of animal origin during the last decade.[14,15] In contrast, $bla_{\text{CTX-M-15}}$ seems to be the most extensive types in isolates of human origin.[16] ESBL-producing *E. coli* are highly linked with multiple plasmids and studies have reported that ESBL genes are often carried on IncF, IncI1, IncN, IncHI1 and IncHI2 in food-producing animals worldwide.[13,17–19] There is potential for ESBL genes/plasmid spreading between *E. coli* from animals, food and humans.[20,21]

The co-occurrence of ESBL genes and *mcr-1* in *E. coli* was reported from China in 2016.[22] Rhouma and Letellier assumed that a historic relation existed between ESBL genes, carbapenemase genes and *mcr-1*.[23] A recent study proposed that cephalosporin resistance is commonly spread in animals and humans through distinct plasmids.[24] It is highly expected that food-producing animals have become the most significant reservoirs in disseminating these resistance genes in the community through horizontal gene transfer. To assess the co-occurrence and emergence of *mcr* and *ESBL* genes in *E. coli* of pig source, we examined 14 pig farms of Jiangsu province in China to evaluate the current scenario of these resistant genes in pigs and further clarified the predominant genotype and plasmids diversity of *mcr* and ESBL genes.

Materials and methods

Collection of samples

A total of 432 samples (400 from healthy and 32 from dead pigs) were collected from 14 commercial pig farms in Jiangsu, China (Figure S1), during the period of August 2016 until December 2017. From each farm, samples were randomly collected. The anal swabs were collected by inserting the swab into the rectum and being rotated. To collect nasal swabs from swine, the nose was wiped with a piece of paper and a sterile swab was inserted into the nasal cavity and rotated for 3 s at 90°. From 32 dead pigs, all samples were aseptically obtained from different organs. All collected samples were immediately transported at 4°C to the laboratory for microbial examination and processed within 4 hrs.

Isolation and screening of ESBL-producing and COL-resistant *E. coli*

All samples were directly streaked onto MacConkey agar (Binhe Microorganism Reagent Co. Ltd., Hangzhou, China) supplemented with CTX (1 μg/mL) and COL (2 μg/mL) for the screening of possible ESBL-producing and COL nonsensitive *E. coli* as previously described.[25] Plates were incubated at 37°C for 18–24 hrs. Presumptive *E. coli* colonies with dark pink to red colors were confirmed microscopically and further verified by species-specific PCR as described previously.[26] Confirmed *E. coli* strains were stored in Luria–Bertani medium (Oxoid, United Kingdom) containing 40% (vol/vol) glycerol in aliquots at −80°C until further use.

ESBL-producing *E. coli* were further confirmed by double-disk synergy (DDS) testing as recommended by the Clinical and Laboratory Standards Institute (CLSI) guideline, using antibiotic discs of ceftazidime (30 μg), ceftazidime plus clavulanic acid (30/10 μg), CTX (30 μg) and CTX plus clavulanic acid (30/10 μg). DDS test was performed for phenotypic detection of ESBLs. The test result is considered as positive if the zone of inhibition is ≥5 mm larger with clavulanic acid than without.[27]

Antimicrobial susceptibility testing

Antimicrobial susceptibility was performed by minimum inhibitory concentration (MIC) determination using broth microdilution method against 11 antibiotics for all 275 isolates and 17 antibiotics for transconjugants. The MIC data was interpreted according to the CLSI recommendations.[27] Antibiotics used in this study, comprised of 5 β-lactams – ampicillin (AMP), CTX, cefoxitin (CFX), CEF and meropenem (MEM) – and 12 non-β-lactams – COL, ciprofloxacin (CIP), chloramphenicol (CHL), enrofloxacin (ENR), gentamycin (GEN), kanamycin, nalidixic acid, polymyxin-B (POL-B), tetracycline (TET), trimethoprim, streptomycin and sulfamethoxazole. The MIC of COL was determined by broth

micro-dilution method recommended by the joint CLSI-EUCAST polymyxin breakpoints working group (www.EUCAST.org), CLSI VET01-A4 is used for CEF and ENR which are missing in the human CLSI M100-S27. *E. coli* ATCC 25922 was used as a quality control in antimicrobial susceptibility testing. Isolates that exhibited resistance to more than 3 antimicrobial agents were classified as MDR.[28]

PCR assays for detection of *mcr* and ESBL genes

PCR assay was used to detect COL-resistant genes *mcr-1* to *mcr-5* as well as ESBL genes ($bla_{CTX\text{-}M}$, bla_{SHV} and bla_{TEM}). Total DNA was isolated by conventional boiling method. All these resistant genes were screened via PCR-based diagnostics with specific primers, as previously described. All the primers and PCR conditions used in this study are listed in Table 1. All PCR positive amplicons of these targeted genes were sequenced by Sanger sequencing in TSINGKE Corporation (Nanjing, PR China).

Conjugation experiment

To determine the transferability of resistance genes, 15 COL-resistant *E. coli* isolates were selected as donors for conjugation. *E. coli* EC-600 (Nal^R, Rif^R) was used as recipient bacteria. Conjugation experiments were performed as previously described.[29] These putative transconjugants were further confirmed using antibiotic susceptibility testing, PCR detection and plasmid incompatibility (Inc) groups typing carried by the transconjugants.

Plasmid replicon typing

Plasmid DNA was extracted from both donors and transconjugants using the Wizard Genomic DNA Purification kit (Promega) and was characterized by PCR-based replicon typing method (PBRT). Eighteen pairs of primers were designed to perform 5 multiplex and 3 simples PCRs targeting the FIA, FIB, FIC, HI1, HI2, IncI1, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA replicons as previously mentioned.[30] While primers for two other plasmids IncI2 and IncX4 were designed

Table 1 PCR primers and conditions used in this study

Primer name	PCR target	Sequence (5′- 3′)	Annealing temperature (°C)	Product size (bp)	Reference
***E. coli*-specific**					
UAL UAR	*uidA*	TGGTAATTACCGACGAAAACG GC ACG CGT GGT TAC AGT CTT GCG	62	147	58
ESBL genes					
CTX-MA CTX-MB	$bla_{CTX\text{-}M}$	CGC TTT GCG ATG TGC AG ACC GCG ATA TCG TTG GT	54	550	58
SHV-F SHV-R	bla_{SHV}	GGG TTA TTC TTA TTT GTC GC TTA GCG TTG CCA GTG CTC	58	930	58
TEM-F TEM-R	bla_{TEM}	ATA AAA TTC TTG AAG ACG AAA GAC AGT TAC CAA TGC TTA ATC	56	1086	58
MCR genes					
CLR5-F CLR5-R	*mcr-1*	CGG TCA GTC CGT TTG TTC CTT GGT CGG TCT GTA GGG	58	309	41
MCR-2-F MCR-2-R	*mcr-2*	TGT TGC TTG TGC CGA TTG GA AGA TGG TAT TGT TGG TTG CTG	58	567	42
MCR-3-F MCR-3-R	*mcr-3*	TTG GCA CTG TAT TTT GCA TTT TTA ACG AAA TTG GCT GGA ACA	50	542	43
MCR-4F MCR-4R	*mcr-4*	ATT GGG ATA GTC GCC TTT TT TTA CAG CCA GAA TCA TTA TCA	58	487	44
MCR-5-F MCR-5-R	*mcr-5*	ATG CGG TTG TCT GCA TTT ATC TCA TTG TGG TTG TCC TTT TCT G	50	1644	45

separately which were missing in the previous replicon typing. All the PBRT primers and PCR conditions used in this study are listed in the Supplementary Table S1. PCR amplicons of plasmids were sequenced by Sanger sequencing (TSINGKE Corporation, Nanjing, PR China) and retrieved sequences were used to confirm replicon types by using BLAST tool available at NCBI web (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Pulsed field gel electrophoresis (PFGE) and Southern hybridization

To determine the genetic relatedness and location of transmissible *mcr-1*-positive elements, the six conjugative *E. coli* strains were characterized by S1-PFGE and Southern hybridization using a probe specific for *mcr-1*. Genomic DNA from each of the isolate was digested with S1 nuclease (Thermo scientific) and was examined by PFGE as previously described.[31]

Southern hybridizations of plasmid DNA were performed with a digoxin-labeled *mcr-1*-specific probe according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany) as previously described.[32]

Statistical analysis

Differences in the AMR profiles of *E. coli* isolates with or without *mcr-1* were assessed by a two-tailed Chi-square test or Fisher's exact test using the Statistical Packages of Social Sciences software for Windows, version 20.0 (IBM Corp., Armonk, NY), with $P<0.05$ set as the level of significant differences.

Results

Bacterial isolation and antimicrobial susceptibility

Overall, 275 *E. coli* isolates (243 from healthy and 32 from dead pigs) were recovered from 432 samples of 14 different pig farms. All isolates were observed to be ESBL-producing and COL nonsensitive as determined by phenotypic approaches, giving a carriage rate of 63.6% (275/432). Of these 275 *E. coli* isolates, 174 (63.27%) were from feces, 69 (25.09%) were from nasal and remaining 32 (11.63%) were from diseased and dead pigs.

The MICs were obtained from the antibiotic susceptibility testing for all isolates. To determine the resistance profiles of 275 representative *E. coli* strains, susceptibility of 11 antibiotics were used (Table 2). Of the 275 *E. coli* isolates, the resistant rate to TET was 97.81%, followed by AMP (96.72%), CHL (94.54%), CFX (86.18%), CTX (78.18%), CEF (77.81%), CIP (73.81%), POL-B (71.27%), GEN (70.54%), and COL (70.18%). In contrast, the most effective antibiotic against these isolates was MEM with 99.6% susceptibility. Majority of the *E. coli* strains showed considerable MDR to β-lactams and several non-β-lactams groups, including polypeptides group, fluoroquinolones, aminoglycosides, amphenicol, quinolone group, sulfonamides and TET.

mcr-1 and ESBL genes are prevalent among *E. coli* strains

Although 70.18% (193 of 275) swine *E. coli* isolates conferred resistance to COL, the *mcr-1* carriage rate was 71.63% (197/275) (Table 3) and only *mcr-1* gene was detected in these COL-resistant *E. coli* isolates. No other colistin-resistant gene (*mcr-2 to mcr-5*) could be detected in the study population of *E. coli* isolates. The *mcr-1* gene was detected in all farms, and the prevalence rate was enormously high 71.6%, ranging from 47.8% to 100% in different farms, while 40.6% in diseased isolates (Table 3).

We recovered a total of 146 (53.09%) ESBL-producing *E. coli* strains from 275 samples collected from 14 different farms of pigs (Figure S2 and Table 5). Among the 146 ESBL-producing isolates, 68.49% (100/146) harbored $bla_{\text{CTX-M}}$ genes, 22.60% (33/146) harbored bla_{SHV}, while 18.49% (27/146) were carrying bla_{TEM} genes. Among them, 6 isolates carried $bla_{\text{CTX-M}}$ and bla_{SHV}, 3 isolates contained $bla_{\text{CTX-M}}$ and bla_{TEM}, and one isolate had three genes of $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM}.

The resistance patterns of *E. coli* are different in *mcr-1*-positive and *mcr-1*-negative isolates

The *mcr-1*-positive *E. coli* isolates displayed more resistance to other antimicrobials than those of *mcr-1*-negative isolates (Table 4). For *mcr-1*-positive *E. coli*, all isolates, 100% (197/197), possessed not less than 3 antibiotics resistance pattern, while about 96.15% (75/78) of COL-negative isolates did (P=0.006), with 3 COL-sensitive isolates which displayed resistance to not more than 2 antibiotics. In addition, about two-thirds of *mcr-1*-positive *E. coli* isolates, 68.52% (135/197), showed resistance to at least 9 drugs, but only 3.84% (3/78) of *mcr-1*-negative isolates did (P=0.000). Interestingly, 68.02% (134/197) of *mcr-1*-positive strains presented resistance profiles to 10 drugs, but no COL-negative *E. coli* did (P=0.000).

Table 2 Distribution of MICs of 11 antibiotics for 275 MDR *Escherichia coli* isolates

Antibiotics	MIC (mg/L)																			
	0.008	**0.016**	**0.032**	**0.063**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**>256**	**MIC_{50}**	**MIC_{90}**	**Resistance**
Ampicillin	-	-	-	-	-	-	-	0	1	1	1	6	4	12	23	38	189	>256	>256	96.72%
Cefotaxime	-	-	-	0	8	9	11	14	18	15	14	19	31	45	44	33	14	32	256	78.18%
Cefoxitin	-	-	-	-	0	3	2	11	3	1	18	72	51	56	30	9	19	32	128	86.18%
Ceftiofur	-	-	-	-	0	3	21	23	14	12	26	19	28	33	56	31	9	32	256	77.81%
Chloramphenicol	-	-	-	-	-	-	0	0	2	1	7	5	17	34	35	61	113	256	>256	94.54%
Ciprofloxacin	-	0	15	19	5	6	3	14	10	25	26	42	47	38	23	2	-	16	64	73.81%
Colistin	-	-	0	9	29	8	14	22	29	110	41	7	4	2	0	-	-	4	8	70.18%
Gentamycin	-	-	-	0	12	14	9	17	13	4	12	25	54	56	27	16	16	32	256	70.54%
Meropenem	0	16	158	75	10	4	3	2	3	1	0	0	0	-	-	-	-	0.032	0.0625	0.36%
Polymyxin-B	-	-	-	3	12	12	15	37	41	87	49	16	2	1	0	-	-	4	8	71.27%
Tetracycline	-	-	-	-	-	-	0	3	0	3	0	1	16	41	47	69	96	256	>256	97.81%

Notes: Red vertical lines indicate the breakpoints between intermediate and resistant values. White areas indicate range of tested dilutions for each antibiotic; the MIC_{50} and MIC_{90} values are concentrations at which ≥50% and ≥90% of isolates are inhibited.
Abbreviations: MIC, minimum inhibitory concentration.

Table 3 Prevalence of *mcr-1* & or ESBL-producing *E. coli* in swine samples collected from different farms of Jiangsu China

Farm numbers	No. of positive *E. coli* samples	No. of *mcr-1* positive *E. coli* isolates (%)	No. of *mcr-1* & ESBL producing *E. coli* isolates (%)
Farm 1	32	22 (68.7)	10 (31.2)
Farm 2	11	7 (63.6)	3 (27.2)
Farm 3	10	8 (80.0)	4 (40.0)
Farm 4	26	18 (69.2)	4 (15.3)
Farm 5	14	14 (100)	2 (14.2)
Farm 6	14	13 (92.8)	2 (14.2)
Farm 7	7	5 (71.4)	N.D.
Farm 8	14	12 (85.7)	6 (42.8)
Farm 9	16	14 (87.5)	9 (56.2)
Farm 10	43	37 (86.0)	11 (25.5)
Farm 11	23	11 (47.8)	5 (21.7)
Farm 12	12	9 (75.0)	6 (50)
Farm 13	14	7 (50.0)	4 (28.5)
Farm 14	7	7 (100.)	5 (71.4)
Diseased	32	13 (40.6)	7 (21.8)
Total	275	197	78

Abbreviations: ESBL, extended-spectrum β-lactamase; N.D., not determined.

Coexistence of *mcr-1* and ESBL genes screened among the strains

Based on the results of this study, among 197 *mcr-1* positive isolates, 39.59% (78/197) were identified as carrying both *mcr-1* and ESBL genes. Distribution of all *mcr-1* positive *E. coli* isolates (n=78) harboring ESBL genes are analyzed and presented in Figure 1 and Table 5. Our findings indicate that the combination of *mcr-1* with bla_{CTX-M} was the most prevalent with the rate of 70.51% (55/78) followed by the combination of *mcr-1* and bla_{SHV} (14.10%, 11/78). Finally, 7.69% (6/78) isolates were identified carrying both *mcr-1* and $bla_{TEM.}$ Furthermore, combination of *mcr-1* with two or more than two ESBL genes was also identified. Results showed that a total of 5.12% (4/78) of *mcr-1*-positive isolates also carried bla_{CTX-M} *and* bla_{SHV}, while 1.28% (1/78) of *mcr-1*-positive isolates carried bla_{CTX-M} and bla_{TEM}. Interestingly, a single isolate was carrying (*mcr-1*+ bla_{CTX-M} + bla_{SHV}+ bla_{TEM}). In this study, *mcr-1* and bla_{CTX-M} were identified as the dominant genes (Table 5).

As *mcr-1* in combination with bla_{CTX-M} were identified as the most prevalent (70.51%, 55/78), we further sequenced the bla_{CTX-M} genes to explore the subtypes. Sequencing analysis of these 55 bla_{CTX-M} isolates showed that all these *mcr-1*-positive isolates were harboring $bla_{CTX-M-1}$ group. The most prevalent variants identified in these 55 isolates belonged to this group were $bla_{CTX-M-15}$ in 38/55 (69%) isolates, followed by $bla_{CTX-M-55}$ in 16/55 (29%) isolates and $bla_{CTX-M-1}$ in one isolate (1.8%).

Table 4 The resistance patterns of *E. coli* with or without *mcr-1*

Resistance profiles	*mcr-1* positive (n=197)	*mcr-1* negative (n=78)	*Chi-square (P-value)*
≥11 drugs	0 (0)	0.0 (0)	-
≥10 drugs	68.02 (134)	0.0 (0)	0.000
≥9 drugs	68.52 (135)	3.84 (3)	0.000
≥8 drugs	81.21 (160)	58.97 (46)	0.000
≥7 drugs	89.84 (177)	76.92 (60)	0.005
≥6 drugs	93.90 (185)	88.46 (69)	0.125
≥5 drugs	99.49 (196)	91.02 (71)	0.000
≥4 drugs	100.0 (197)	93.58 (73)	0.000
≥3 drugs	100.0 (197)	96.15 (75)	0.006
≥2 drugs	100.0 (197)	98.71 (77)	0.111

mcr-1 and ESBL genes could be conjugative transfer by plasmids with different replicon type

Conjugation experiments were performed on random 15 *mcr-1* positive isolates. Of the 15 *mcr-1* resistant isolates, 10 isolates were carrying additional $bla_{CTX-M,}$ while a single isolate was harboring $bla_{TEM.}$ Of these 15 isolates, 12 were successfully transferred to *E. coli* EC-600. The resistance profiles of the 12 transconjugants were identical to those of the *mcr-1* and bla_{CTX-M} carrying *E. coli* donor isolates, indicating the transfer of antibiotic resistance. In addition, resistant to several non-β-lactam antibiotics, such as aminoglycosides, fluoroquinolones, TET, macrolides and sulfonamides, were also co-transferred along with COL and β-lactam resistance. MICs of COL of these transconjugants revealed 4- to 8-fold increase as compared with the recipient EC-600 (0.125 μg/mL).

PCR-based replicon typing (PBRT) showed that in the *E. coli* isolates carrying *mcr-1* and $bla_{CTX-M,}$ the plasmids with different replicons, including IncHI2 (n=7), IncFIB (n=7), IncFIC (n=4), IncP (n=4), IncFrepB (n=4), IncN (n=3), IncX4 (n=2) IncY (n=2) and IncI1 (n=1), were detected in the donor strains (Figure 2 and Table 6). However, PBRT of the transconjugants confirmed only five replicons, IncHI2, IncFIB, IncFIC, IncN and IncX4, which were present in both donors and transconjugants and were associated with the transfer of the *mcr-1* and ESBL genes.

S1-PFGE analysis demonstrated that these six strains carried multiple plasmids varying in sizes ranging from

Table 5 Distribution of various resistance genes among 275 MDR *Escherichia coli* isolates from pigs

Colistin resistant gene	Extended-spectrum beta-lactamase genes			*No. of isolates*
mcr-1	bla_{CTX}	bla_{SHV}	bla_{TEM}	
+				119
				1
	+			34
		+		13
			+	15
+	+			55
+		+		11
+			+	6
+	+	+	+	1
+	+	+		4
+	+		+	1
	+	+	+	1
	+	+		2
	+		+	2
		+	+	1
N.D.	N.D.	N.D.	N.D.	9
197	100	33	27	275

Abbreviations: +, positive; N.D., not determined.

~97 kb to 242 kb (Figure 3A). Southern hybridization assay confirmed that the *mcr-1* gene recovered from these six strains was positioned on the following five different types of plasmids: with the size of approximately 97, 130, 160, 227 and 242 kb, respectively (Figure 3B).

Discussion

China alone produces and consumes roughly half the planet's pigs, about 500 million annually, and has been the leading consumption of antibiotics in the world.[33] The increased usage of antibiotics may trigger the emergence of AMR. Reports on emergence of AMR particularly resistance of β-lactam and COL are increasing all over the world.[34–37] The prevalence of ESBL in animal origin has been rising since 2003, with slight variances amongst terrestrial regions and different animal species.[4,14,38] We report on the high incidence of *mcr-1*-carrying ESBL-producing *E. coli* recovered from pigs in Jiangsu, China. Our results indicated that all the *mcr-1* and ESBL-producing *E. coli* isolates showed MDR. The majority of these isolates (77–86%) showed resistance to cephalosporin (Table 2). In addition, high resistance was also observed to common β-lactam and non-β-lactam antimicrobials such as AMP, fluoroquinolones, aminoglycosides, amphenicol, quinolones, sulfonamides and TET which are commonly using in human as well in veterinary practice. Many recent studies have reported MDR ESBL-producing *E. coli* isolated from poultry,[3] pigs,[39] cattle[36] and humans.[40]

Recently, plasmid-mediated COL-resistant genes *mcr*-1 to *mcr-8* have been widely discovered around the world.[34,41–47] Herein, we screened 275 MDR *E. coli* isolated from 14 pig farms from Jiangsu province for the presence of *mcr*-1 to *mcr-5* genes. Only *mcr-1* gene was detected in isolates from every farm, and the carriage rate was extremely high in 71.6% (197/275). The high prevalence rate of the *mcr-1* found in this study

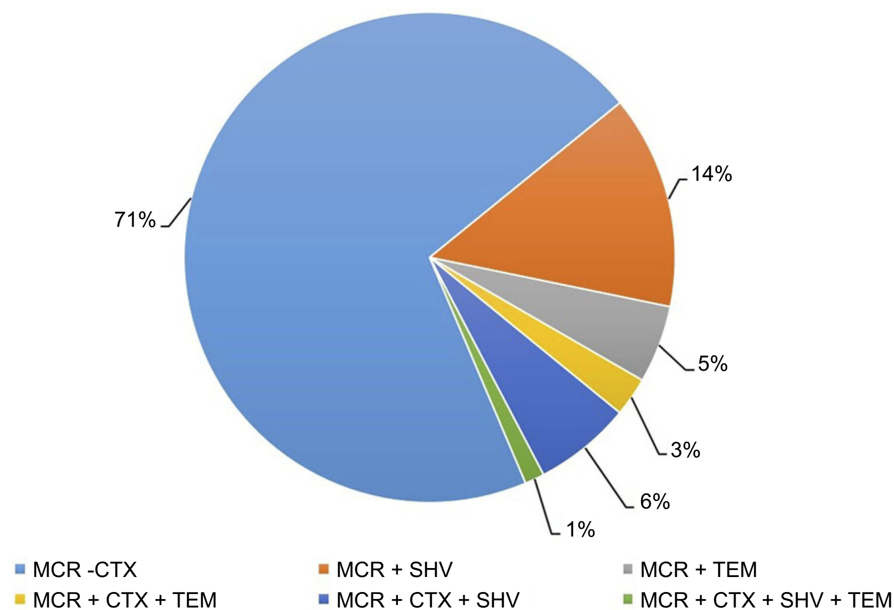

Figure 1 Distribution of various resistance genes in combination.

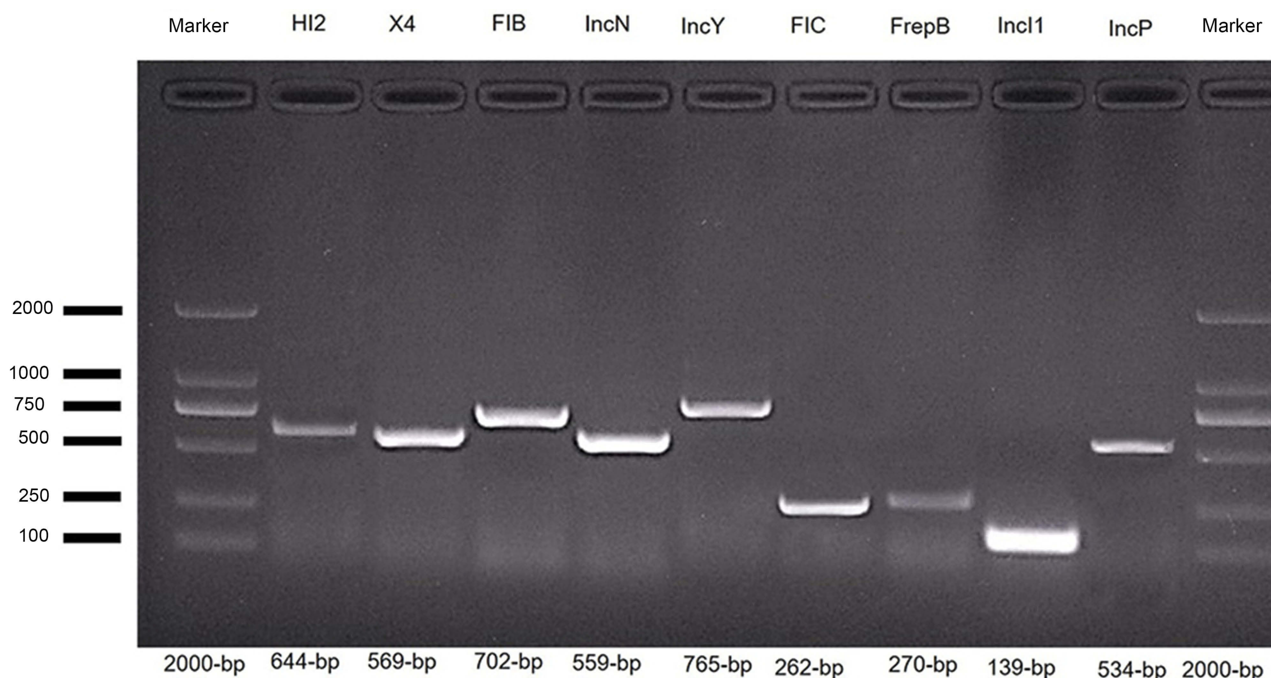

Figure 2 Detection of plasmid replicon types in multidrug-resistant *E. coli* using PCR assay.

from pigs in Jiangsu is consistent with very recent reports from China.[48,49] These recent studies in pigs reported similarly high *mcr-1*-positive carriage (79.2% and 76.2%), ranging from 45% to 100% in different provinces, while the *mcr-1* rate in Jiangsu province reported by[48] was 71.9% which is very similar to our findings 71.6%. The present study and, together with the previous studies, confirmed a surprisingly high rate of *mcr-1* in swine farms and is likely associated with the prolonged and extensive practice of COL as a growth promoter in pigs.

The coexistence of *mcr-1* with other resistance genes in an *E. coli* was reported in China.[50] One recent study also assumed that a historic bridge existed between *mcr-1* and ESBL.[23] However, there is scarcity in the incidence of the coexistence of *mcr-1* and ESBL in the pig origin. Herein, we screened 275 MDR *E. coli*–resistant strains from pig farms in Jiangsu during 2016–2017 and found a high occurrence of *mcr-1*-positive strains with ESBL in the swine 39.5% (78/197), which was very high from the previous report.[51] A very recent longitudinal study from China investigated the co-rising of *mcr-1* and ESBL in chicken isolates.[14] Among ESBL-positive strains, we found that $bla_{\text{CTX-M}}$ is the most predominant.

In this study, 39.5% *mcr-1*-positive *E. coli* strains were detected which coexist in different ESBL genes. Among them, $bla_{\text{CTX-M}}$ was the predominant one which was found in 55 *mcr-1*-positive *E. coli* 70.5% (Figure 1). On sequence-based analysis of these 55 $bla_{\text{CTX-M}}$ isolates, interestingly the $bla_{\text{CTX-M-15}}$ gene was found to be the most prevalent $bla_{\text{CTX-M}}$ gene (69%) followed by $bla_{\text{CTX-M-55}}$ (29%). The spread of the $bla_{\text{CTX-M-15}}$ gene is a common $bla_{\text{CTX-M}}$ enzyme and detected widely in Enterobacteriaceae of human origin.[19,52] Very few studies have reported the co-occurrence of *mcr-1* and $bla_{\text{CTX-M-15}}$ of human origin.[22,53] From China, one recent study in dairy cows also found $bla_{\text{CTX-M-15}}$ as the second prevalent ESBL gene 21.4% (62/275), but there was no *mcr-1* gene detected in those $bla_{\text{CTX-M-15}}$ isolates.[54] Another study also reported the coexistence of *mcr-1* and $bla_{\text{CTX-M-15}}$ in Turkey hen meat.[55] Herein, this is the first investigation that reveals the coexistence of *mcr-1* and $bla_{\text{CTX-M-15}}$ in *E. coli* strains of pig origin in a very high proportion.

A significant increase in the $bla_{\text{CTX-M-55}}$ was found in *E. coli* by over a period of ten years.[4] Many previous studies reported that $bla_{\text{CTX-M-55}}$ in human strains in China has become the second dominant $bla_{\text{CTX-M}}$ type and even the occurrence of $bla_{\text{CTX-M-55}}$ was higher than $bla_{\text{CTX-M-15}}$.[51] A high rate of *mcr-1* and $bla_{\text{CTX-M-55}}$ was recently detected from the chicken origin in China,[14] which was consistent with our results. Thus, the concurrent dissemination of the *mcr-1* harboring $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-55}}$ mediated by a single bacterial clone is existing which suggests that *mcr-1* is found in the diverse reservoirs.

Table 6 Conjugation experiments and plasmid replicon type detection for 15 *mcr-1*-positive *E. coli*

Strain	CTX MIC (mg/L)	COL MIC (mg/L)	Plasmid (Inc) types	Resistance genes	Resistance profile for non-beta-lactam antibiotics GEN ENR SUL CIP KEN CHL TET TRM STR POL-B
EC-9	128	16	HI2,I1,Y, FIC	MCR-1, CTX-M-15	GEN, ENR, NAL, SUL, CIP, KEN, CHL, TET, TRM, STR,POL-B
EC-9-T	128	4	HI2, FIC	MCR-1, CTX-M-15	GEN, ENR, NAL, SUL, KEN, CHL, TET, TRM, POL-B
EC-37	256	8	N, P	MCR-1, CTX-M-55	NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, STR, POL-B
EC-37-T	32	4	N	MCR-1, CTX-M-55	NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, STR, POL-B
EC-48	256	4	FIB, P, FrepB, N	MCR-1, CTX-M-55	GEN, ENR, NAL, SUL, KEN, CHL, TET, TRM, STR,POL-B
EC-48-T	32	2	FIB, N	MCR-1, CTX-M-55	GEN, ENR, NAL, SUL, KEN, CHL, TET, TRM, STR,POL-B
EC-52	128	4	FIB,N, P	MCR-1, CTX-M-55	ENR, NAL, SUL, CEF, KEN, CHL, TET, TRM, STR,POL-B
EC-52-T	256	2	FIB,N	MCR-1, CTX-M-55	ENR, NAL, SUL, CEF, KEN, CHL, TET, TRM, STR
EC-29	256	8	HI2, FIB	MCR-1, CTX-M-15	GEN, NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, STR,POL-B
EC-29-T	0.5	2	HI2, FIB	MCR-1, CTX-M-15	GEN, NAL, SUL, KEN, CHL, TET, TRM, POL-B
EC-34	256	8	FIB, HI2, FrepB	MCR-1, CTX-M-15	GEN, NAL, SUL, CEF, KEN, CHL, TET, TRM, STR,POL-B
EC-34-T	>256	8	HI2,FIB	MCR-1, CTX-M-15	GEN, NAL, SUL, CEF, KEN, CHL, TET, TRM, POL-B
EC-40	128	4	HI2, FIB, FrepB	MCR-1, CTX-M-15	ENR, NAL, SUL, CFX, CEF, CIP, KEN, CHL, TET, TRM, POL-B
EC-40-T	64	2	HI2, FIB	MCR-1, CTX-M-15	ENR, NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, POL-B
EC-1	256	4	X4, FIC	MCR-1, TEM	GEN, ENR, NAL, SUL, CIP, KEN, CHL, TET, TRM, STR, POL-B
EC-1-T	256	2	X4, FIC	MCR-1, TEM	GEN, NAL, SUL, CEF, CIP, KEN, CHL, TET, TRM, STR, POL-B
EC-2SF	256	2	HI2,FIB, P	MCR-1, CTX-M-15	GEN, ENR, NAL, SUL, CIP, KEN, CHL, TET, TRM, STR,POL-B
EC-2SF-T	>256	4	HI2, FIB	MCR-1, CTX-M-15	ENR, NAL, SUL, CIP, KEN, CHL, TET, TRM, STR,POL-B
EC-55	64	8	HI2,FIB, FIC,Y	MCR-1, CTX-M-55	ENR, NAL, SUL, CIP, KEN, CHL, TET, TRM, STR,POL-B
EC-55-T	64	4	HI2,FIC	MCR-1, CTX-M-55	ENR, NAL, SUL, CIP, KEN, CHL, TET, TRM, POL-B, STR
EC-20	2	4	X4, FIC, FrepB	MCR-1	GEN, ENR, NAL, SUL, CIP, KEN, CHL, TET, TRM, POL-B
EC-20-T	0.25	4	X4, FIC	MCR-1	GEN, NAL, SUL, KEN, CHL, TET, TRM, STR,POL-B
EC-19	128	32	HI2	MCR-1	GEN, NAL, SUL, CIP, KEN, CHL, TET, TRM, POL-B
EC-19-T	256	4	HI2	MCR-1	GEN, NAL, SUL, KEN, CHL, TET, TRM, POL-B
EC-600	0.25	0.125	ND	ND	

Abbreviations: COL, colistin; CTX, cefotaxime; GEN, gentamycin; ENR, enrofloxacin; NAL, nalidix-acid; SUL, sulfamethaxozole; CIP, ciprofloxacin; KEN, kenamycin; CHL, chloramphenicol; TET, tetracycline; TRM, trimethropim; STR, streptomycin; POL-B, polymyxin-B; ND, not determined.

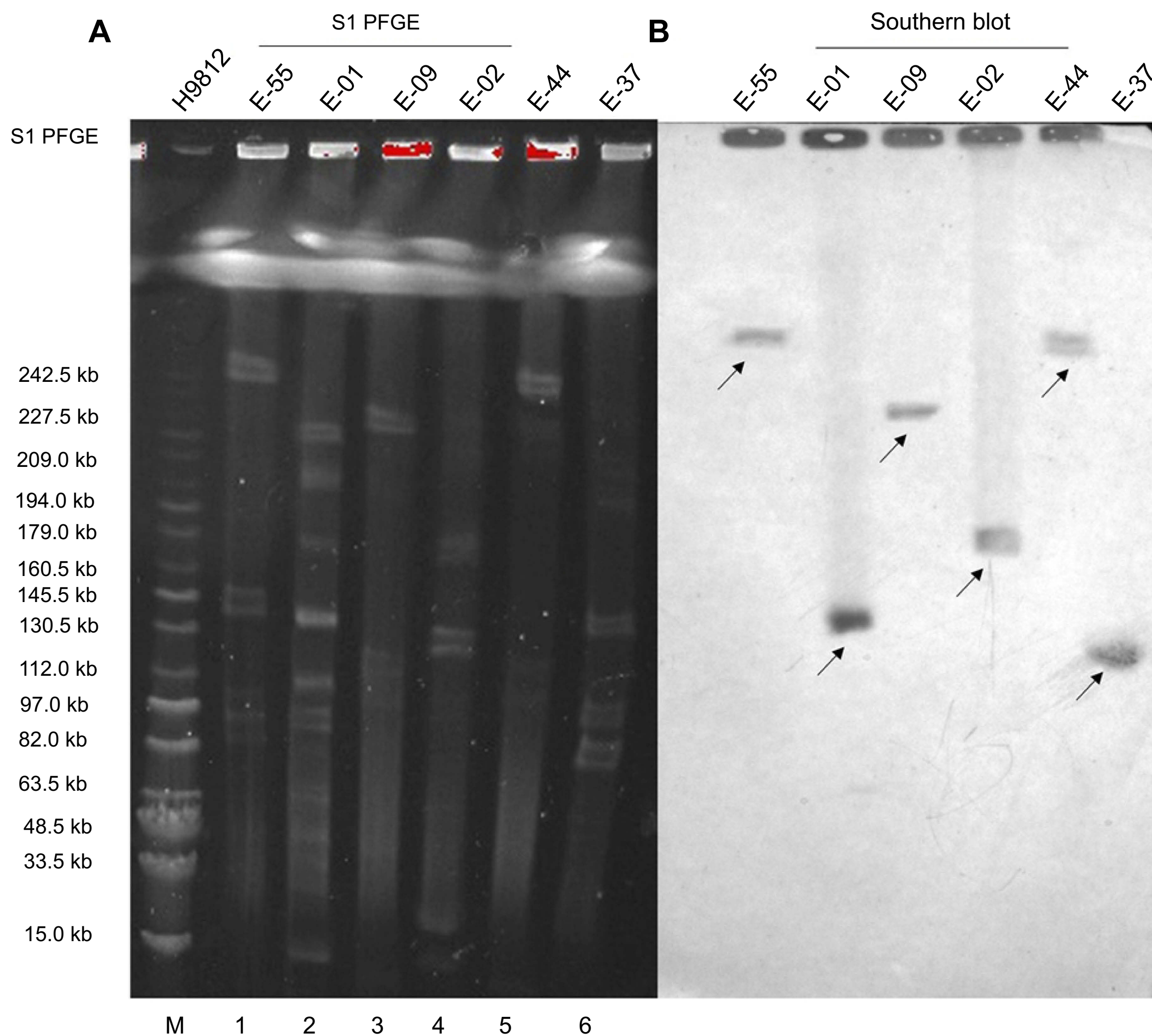

Figure 3 (**A**) S1-nuclease pulsed-field gel electrophoresis profiles of six *E. coli* and (**B**) Southern Blot hybridization of six *E. coli* carrying the *mcr-1* plasmid.
Notes: M – Salmonella H9812 (15.0–242.5 kb); 1: E-55; 2: E-01; 3: E-09; 4: E-02; 5: E-44; and 6: E-37. The arrows in the figure represent the position of the *mcr-1* plasmid.

In addition, $bla_{CTX-M-15}$ and $bla_{CTX-M-55}$ were previously reported on conjugative plasmids, ie, FIB, IncI1, IncHI2, IncK, IncP and IncN.[35,56] Therefore, we also detected these incompatibility types by PCR typing. While *mcr-1* gene was often found on conjugative plasmids like IncF, IncI2, IncHI2, IncN, IncP and X4, which exhibit an unexpected diversity.[57] In conjugation experiment, IncHI2, IncFIB, IncFIC, IncN and IncX4, were found in both donors and transconjugants and were associated with the transfer of the *mcr-1* and ESBL encoding genes.

Genetic representation of *mcr-1*-carrying plasmids demonstrated that this gene is located on different conjugative elements of ~97 kb and 242 kb in size. The fact that *mcr-1*-carrying *E. coli* isolates display divergent PFGE profiles suggests that these elements may play a vital role in *mcr-1* transmission. The incidence of closely related plasmids that carry *mcr-1* and ESBL resistance genes among genotypically varied *E. coli* strains from various origins is a threat for alarm as it indicates that plasmids can easily disseminate from animals to humans and the spread of these plasmids may be remarkably challenging to control.

Considering that bla_{CTX-M} has become the most prevalent ESBL type of animal origin in the last few years, this situation may suggest that *mcr-1* and bla_{CTX-M} emerged and arose due to the extensive use of antimicrobial practice in animal farming in the last decade. Our results also suggested that $bla_{CTX-M-15}$ and $bla_{CTX-M-55}$ and other β-lactamase genes coharboring with *mcr-1* positive isolates is a potential threat to public health as the pig carrying these genes may enter the food chain. It is recommended that we should pay high consideration in monitoring the incidence of ESBL-producing & COL-resistant *E. coli* in both clinical and food-producing animals.

Conclusion

Our study reported a high incidence of the *mcr-1*-carrying ESBL-producing *E. coli* recovered from pigs in Jiangsu, China. The coexistence of the *mcr-1*- and $bla_{CTX\text{-}M\text{-}15}$-carrying isolates displaying MDR, recovered from pig origin is a major concern for both humans and veterinary medicine. The presence of these genes on the conjugative plasmids with the ability to transfer between similar strains which contain other drug resistance genes emphasizes on urgent intervention.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Figure S1 Map of sampling sites in Jiangsu province.

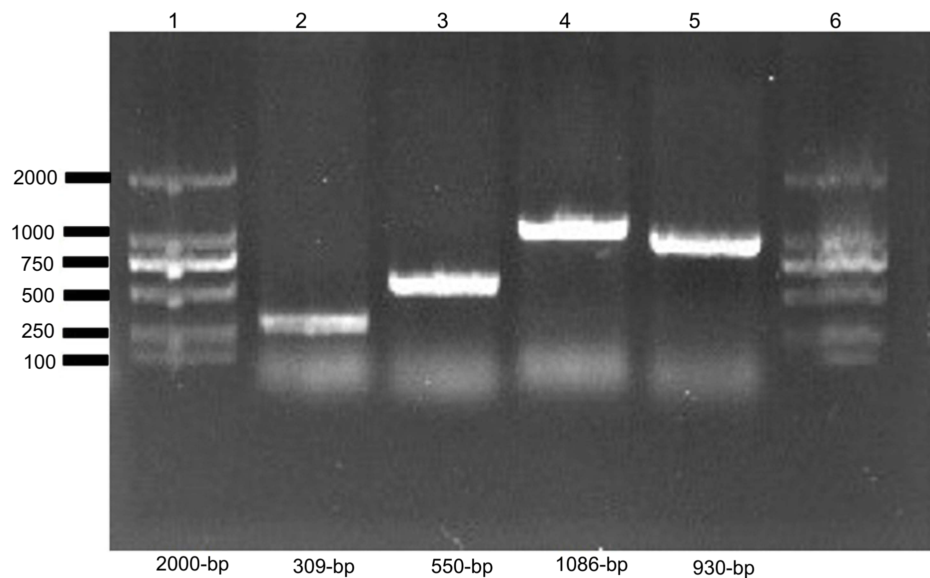

Figure S2 Screening of *mcr-1* and ESBL encoding genes in *E. coli.*

Notes: PCR product was separated on 1% agarose gel. Lane 1 and 6 shows 2000 bp molecular marker (Vazyme, Beijing, China); Lane 2, (*mcr-1*), Lane 3 (*blaCTX-M*) isolate; Lane 4, (*bla*TEM); and Lane 5 shows (*bla*SHV).

Table S1 List of primers used for plasmid replicons typing in this study

Primers	Sequence (5′ to 3′)	Target sites/ genes	Annealing temperature	Amplicons size	References
PBRT* primers					
HI1-F HI1-R	GGAGCGATGGATTACTTCAGTAC TGCCGTTTCACCTCGTGAGTA	parA-parB	58 °C	471-bp	1
HI2 -F HI2- R	GGCTCACTACCGTTGTCATCCT CGAAAGCCGGACGGCAGAA	RNAI	58 °C	644-bp	1
I1-F I1-F	CGAAAGCCGGACGGCAGAA TCGTCGTTCCGCCAAGTTCGT	iterons	58 °C	139-bp	1
X -F X -R	AACCTTAGAGGCTATTTAAGTTGCTGAT TGAGAGTCAATTTTTATCTCATGTTTTAGC	ori □	58 °C	376-bp	1
L/M-F L/M-F	GGATGAAAACTATCAGCATCTGAAG CTGCAGGGGCGATTCTTTAGG	repA, B, C	58 °C	785-bp	1
N-F N-R	GTCTAACGAGCTTACCGAAG GTTTCAACTCTGCCAAGTTC	repA	58 °C	559-bp	1
FIA- F FIA- R	CCATGCTGGTTCTAGAGAAGGTG GTATATCCTTACTGGCTTCCGCAG	iterons	58 °C	462-bp	1
FIB –F FIB -R	GGAGTTCTGACACACGATTTTCTG CTCCCGTCGCTTCAGGGCATT	repA	58 °C	702-bp	1
W-F W-R	CCTAAGAACAACAAAGCCCCCG GGTGCGCGGCATAGAACCGT	repA	58 °C	242-bp	1
Y-F Y-R	AATTCAAACAACACTGTGCAGCCTG GCGAGAATGGACGATTACAAAACTTT	repA	58 °C	765-bp	1
P-F *P*-R	CTATGGCCCTGCAAACGCGCCAGAAA TCACGCGCCAGGGCGCAGCC	iterons	58 °C	534-bp	1
FIC –F FIC -R	GTGAACTGGCAGATGAGGAAGG TTCTCCTCGTCGCCAAACTAGAT	repA2	58 °C	262-bp	1
A/C -F A/C -R	GAGAACCAAAGACAAAGACCTGGA ACGACAAACCTGAATTGCCTCCTT	repA	58 °C	465-bp	1
T-F T-R	TTGGCCTGTTTGTGCCTAAACCAT CGTTGATTACACTTAGCTTTGGAC	repA	58 °C	750-bp	1
FIIS –F FIIS -R	CTGTCGTAAGCTGATGGC CTCTGCCACAAACTTCAGC	repA	58 °C	270-bp	1
FrepB-F FrepB-R	TGATCGTTTAAGGAATTTTG GAAGATCAGTCACACCATCC	RNAI/repA	58 °C	270-bp	1
K/B -F K/B-R	GCGGTCCGGAAAGCCAGAAAAC TCTTTCACGAGCCCGCCAAA	RNAI	58 °C	160 bp	1

(*Continued*)

Table S1 (Continued).

Primers	Sequence (5′ to 3′)	Target sites/ genes	Annealing temperature	Amplicons size	References
B/O-F B/O-R	GCGGTCCGGAAAGCCAGAAAAC TCTGCGTTCCGCCAAGTTCGA	RNAI	58 °C	159 bp	[1]
X4- F X4-R	AGCAAACAGGGAAAGGAGAAGACT TACCCCAAATCGTAACCTG	-	62 °C	569 bp	[2]
IncI2-F IncI2-R	ATTGTTGCGTGGCTTCAT TGGAGAGGATTAAGGAGGAA	-	60 °C	353 bp	This study

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