Supplementary materials

Wastewater surveillance reveals the prevalence of last-resort antibiotic-resistant *Escherichia coli* and their multidrug resistance characteristics

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Summary

Number of Texts: 5

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Text S1 Concentration of antibiotics chosen to select the resistant *Escherichia Coli.*

To select antibiotic-resistant *Escherichia coli* (*E. coli*), specific antibiotics were added to the culture medium at concentrations corresponding to their non-susceptible minimum inhibitory concentration (MIC) breakpoints defined by the CLSI. The final concentrations were as follows: colistin (CT) at 2 mg/L, cefotaxime (CTX) at 2 mg/L, ciprofloxacin (CIP) at 0.2 mg/L, trimethoprim-sulfamethoxazole (SXT) at 3 mg/L + 57 mg/L, and amikacin (AMI) at 32 mg/L. For tigecycline (TIG), the CLSI does not define a non-susceptible breakpoint. The FDA and EUCAST criteria differ substantially: the FDA defines MIC ≥ 4 mg/L as intermediate, whereas the EUCAST defines MIC ≥ 0.5 mg/L as resistant. Although the FDA standard is widely used, its intermediate threshold is relatively high and may impede the growth of TIG-resistant *E. coli* under experimental conditions. Therefore, a TIG concentration of 2 mg/L was selected for this study based on previous findings (Zhai et al. 2022). For meropenem (MEM), preliminary experiments used culture medium supplemented with 2.5 mg/L MEM and 70 mg/L $ZnSO_4$, where $ZnSO_4$ was added to support the growth of *E. coli* carrying *bla*IMP (Yamamoto et al. 2017). Under these conditions, MEM-resistant *E. coli* failed to grow, likely due to complex env*iro*nmental mechanisms. The MEM concentration was then gradually reduced to 0.5 mg/L, at which point the resistant strains grew normally. Subsequent broth microdilution susceptibility testing confirmed that these resistant strains generally exhibited MIC values ≥ 2 mg/L, which corresponds to the intermediate breakpoint (2 mg/L) defined by the CHINET antimicrobial resistance surveillance guidelines. Accordingly, a final concentration of 0.5 mg/L MEM with 70 mg/L $ZnSO_4$ was selected for this experiment.

Text S2 DNA extraction from Isolates for PCR, qPCR and Illumina sequencing.

The DNA used for PCR/qPCR identification of AMR genes and Illumina short-read sequencing was extracted using the TGuide Smart Universal DNA Kit (DP605, Tiangen, Beijing, China) coupled with TGuide S16 Nucleic Acid Extractor (Tiangen, Beijing, China) with some modifications. Briefly, 200 μL overnight culture of bacteria was centrifuged at 15000 g for 1min at resuspended in 150 μL Lysis Buffer (250 mM Tris-HCl, 25 mM EDTA, 12.5% Triton X-100, pH=8.0) with 25 μL 100 mg/mL Lysozyme (10402ES08, Yeasen, Shanghai, China) and 5 μL 100 mg/mL RNase A solution (RT405-12, Tiangen, Beijing, China). After incubating at 37℃ for 15 min, 130 μL of GHA and 20 μL of 20 mg/mL proteinase K (HBP800001, New Enzyme Biotechnology, Wuhan, China) were added and the total volume of solution was transferred into the lysis/binding wells of the prepackaged reagents plate. After incubating at 56℃ on the extractor for 15 min, the program was paused and 350 μL of isopropanol (I112018, aladdin, Shanghai, China) was added to the lysis/binding well. The residual steps of binding, washing and elution steps were completed by the extractor based on the default program.

Text S3 PCR and qPCR identification of last-line antibiotic resistance-related genes.

Multiplex PCR was applied to identify the genes related to the resistance of TIG (*tet*(X_1) to *tet*(X_5)), carbapenem (*bla*KPC, *bla*NDM, *bla*OXA48, *bla*VIM) and colistin (*mcr*-1 to *mcr*-5) as previous studies described. Briefly, 20 μL PCR reaction was set up with 1×Taq Buffer (3 mM Mg^{2+} plus), 0.5 U Takara Taq Hot Start (R007, Takara Bio Inc., Shiga, Japan), 0.2 mM dNTPs, 5 μL bacteria nucleic acid extraction, and primers with their different working concentrations (Shown in Table S13). To avoid the false negative results caused by PCR inhibition, primers targeting the 16S rDNA (Table S13) were added to each PCR reaction as an internal quality control. After the amplification, 5 μL PCR products were mixed with 6× DNA Loading Buffer (Solarbio, Beijing, China) and visualized by 2% agarose gel electrophoresis in 1× TAE buffer.

For those isolates that showed positive results for resistance-related genes, the Taqman quantitative PCR was used for further validation. The primers and probes targeting *tet*(X_4), *bla*KPC, *bla*NDM, *bla*OXA48, *mcr*-1 and *mcr*-3 are shown in Table S6. The reaction systems for the detection of carbapenem and colistin resistance-related genes were set up in a 20 μL volume with 0.1 U/μL Hieff Unicon® Hotstart Direct Taq DNA Polymerase (10717, Yeasen, Shanghai, China), 1×Taq buffer, 6 mM Mg^{2+}, 0.5 mM dNTPs, 100 ng/μL bovine serum albumin, 0.3 M trehalose dihydrate, 400 nM of each primer, 200 nM of each probe and 5 μL 100 times diluted bacteria nucleic acid. For the detection system of *tet*(X_4) , to avoid the potential interference of *tet*(X_5), the 20 μL reaction volume was modified as 0.1 U/μL Hieff Unicon® Hotstart Direct Taq DNA Polymerase, 1×Taq buffer, 4 mM Mg^{2+}, 0.2 mM dNTPs, 100 ng/μL bovine serum albumin, 0.3 M trehalose dihydrate, 200 nM of each primer, 100 nM of each probe and 5 μL 100 times diluted bacteria nucleic acid. The qPCR reaction program was set as initial denaturation at 95℃ for 5 min, denaturation at 95℃ for 15 s, annealing at 60℃ (for carbapenem and colistin resistance-related genes) or 63℃ (for *tet*(X_4)) for 30s, and repeated for 40 cycles. Detailed information about the standard curve, limit of detection and specificity of each qPCR reaction was listed in the attached MIQE checklist.

Text S4 DNA extraction and Library Preparation for ONT sequencing.

To maintain the genomic DNA as complete as possible, the DNA used for the ONT sequencing was extracted using a sedimentation-based method. Briefly, 20 mL overnight culture of bacteria was centrifuged at 5000 g for 5min. The sediment at the bottom was softly resuspended using 1 mL Lysis buffer (250 mM Tris-HCl, 25 mM EDTA, 12.5% Triton X-100, pH=8.0) and transferred into a new 1.5 mL tube. Then, 100 mg/mL lysozyme (10402ES08, Yeasen, Shanghai, China) and RNase A (RT405-12, Tiangen, Beijing, China) were added into the suspension with the final concentration of 2.5 and 2 mg/mL, respectively. After digesting for 30 min, 1/10 volume of 20 mg/mL proteinase K (HBP800001, New Enzyme Biotechnology, Wuhan, China) and 1/10 volume of 10% sodium dodecyl sulfate (D885207, Macklin, Shanghai, China) were further added into the lysis and incubated at room temperature. For each 5 min, the lysis was inverted gently 3 times until it became nearly transparent. The total volume of transparent lysis was transferred into a new 15 mL tube and mixed with 4 mL pre-cooled nuclease-free water and 1.5 mL 5 M NaCl solution on ice. Then, 7 mL Phenol-Chloroform-Isoamyl alcohol (25:24:1, pH > 7.8, AC13308, Acmec, Shanghai, China) was added and lightly inverted 50 times. The mixture solution was centrifuged at 5000 g for 45 min, with the acceleration and deceleration set at four. The upper aqueous phase was slowly transferred into a new 15 mL without disturbing the white interphase and extracted using an equal volume of Chloroform-Isoamyl alcohol (24:1, M76752, Meryer, Shanghai, China) for an additional 2 times to remove the residual protein and phenol. All the extraction and centrifugation conditions were the same as above when using the Chloroform-Isoamyl alcohol for extraction. The aqueous phase after the second extraction was mixed with 1/10 volume of 3 M sodium acetate solution (pH=5.2, A1070, Solarbio, Beijing, China) and 2 times the volume of pre-cooled absolute alcohol in a new 50 mL tube on ice. The mixed solution was inverted gently 10 times on ice, and stored at -20 ℃ for 1 h to precipitate the DNA. The white chains formed during the cold precipitation were collected using a new 1 mL pipette tip by clockwise rotation and transferred to a new 50 mL tube with 10 mL 70% ethanol. After being softly washed for several rounds, the DNA chains were again collected using the pipette tip, leaned against the tube's inner wall several times to remove extra alcohol, and transferred into a new 1.5 mL tube with 1.5 mL 70% alcohol. The DNA chains were

carefully washed again and released from the pipette tip into the alcohol, and then centrifuged at 10000 g for 2 min for precipitation. After removing the alcohol, the DNA sediment was air dried for approximately 3 min until the edge of the sediments became transparent. 200 μL 10 mM Tris-HCl (pH = 8.0) was eventually added to dissolve the DNA on ice for 2-3 hours with the tube lid open (to evaporate residual alcohol), and the final obtained transparent and viscous DNA solution was used for ONT-based library preparation and sequencing. Based on the agarose gel electrophoresis results, the obtained DNA length was about 40 kbp.

The obtained High molecular bacteria DNA was firstly sheared to 10-20 kbp size using the g-TUBE (520079, Covaris Inc., Woburn, MA) according to the user manual. The ONT library preparation was conducted according to the SQK-NBD114.24 protocol using the Ligation sequencing gDNA - Native Barcoding Kit 24 V14 (SQK-NBD114.24, ONT, UK), NEBNext® FFPE DNA Repair v2 Module, NEBNext® Ultra™ II End Repair/dA-Tailing Module, NEB Blunt/TA Ligase Master Mix and NEBNext® Quick Ligation Module (M6630S, M0367S, E6056S, New England Biolabs, Ipswich, MA, USA). The final obtained library was loaded on the PromethION R10.4.1 (FLO-PRO114M, Oxford Nanopore Technologies, Oxford, UK) flow cell and sequenced on the PromethION 24/48 platform.

Text S5 Bioinformatic analysis.

For the Illumina short-read sequencing data, the low-quality reads and adaptor contaminations were first trimmed using the Fastp (version 0.23.0) (Price et al. 2010)with default parameters. The clean reads were assembled into contigs and scaffolds using the Spades (version 3.12) (Bankevich et al. 2012) with k-mers of "21, 33,55,77,99,127" by the "--careful" mode. The genomic size and characteristics, including total length, GC content, N50 and L50 were calculated using the QUAST (version 5.1.0) (Gurevich et al. 2013), and the genomic completeness and contamination were estimated using the CheckM(Parks et al. 2015). The Mutiple sequence typing of the isolates was determined using mlst(version 2.23.0) (De La Cruz 2020), and the phenotyping of *E. coli* was determined using ClermonTyping (version 24.02) (Beghain et al. 2018). The ARGs were identified using ResFinder (version 4.6.0) (Bortolaia et al. 2020) with the default parameters (identity > 90%, coverage > 60%).Virulence factor genes were identified against VFDB (version 2025) (Zhou et al. 2025)using predefined parameters (identity > 98%, coverage = 100%). Mobile genetic elements were detected using mobileOG-db (version 2.0) (Brown et al. 2022)with default parameters (e-value > 10^{-5}). Core single nucleotide polymorphisms (SNPs) were identified using Snippy (version 4.6.0) (https://github.com/tseemann/snippy)with ASM584v2 as the reference genome. A SNP-based phylogenetic tree was constructed with FastTree (version 2.1.11), (Price et al. 2010)and the corresponding characteristics of each isolate were illustrated using the online tool TvBOT (Xie et al. 2023).

For the ONT long-read sequencing data, the circular genome of WWB-CT-1 was assembled using Unicycler with default parameters. For WWE_MEM_4, since Unicycler (version 0.5.1) (Wick et al. 2017) failed to assemble the circular genome, another tool named Trycycler (version 0.5.5) (Wick et al. 2021)was used for assembly, and the results were validated using PCR (Figure S3). The plasmid replicon typing were determined using PlasmidFinder (version 2.0.1) (Carattoli et al. 2014). And the plasmid circos map was visualized using Proksee (Grant et al. 2023).

Text S6 Clinical surveillance data

The clinical surveillance data used in this study were obtained from the 2024 annual report of the China Antimicrobial Resistance Surveillance Network (CHINET) . Specifically, the resistance data for *E. coli* were derived from 55,409 clinical isolates collected from participating hospitals across China between January and December 2024. The time period is thus consistent with our wastewater sampling period. Meanwhile, it should be noted that the CHINET data represent the results on the national level. Although we cannot provide Beijing-specific resistance rates owing to data access constraints, we are confident that the national data serve as a meaningful reference for our analysis.

Figure

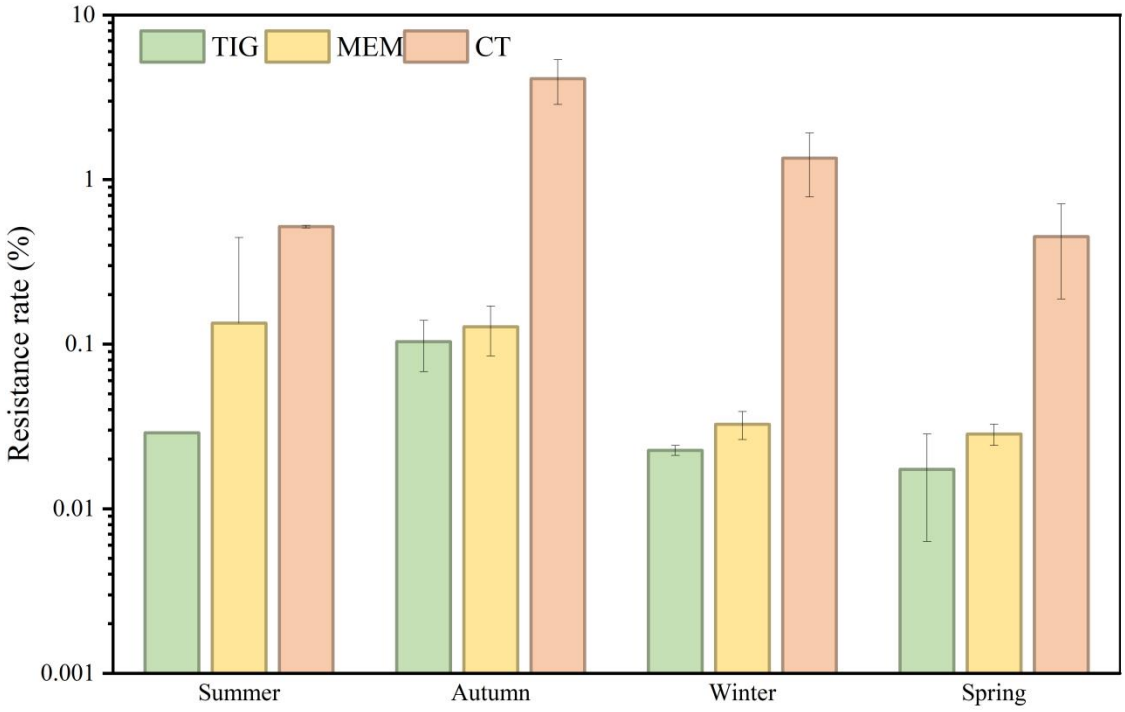


Figure S1 Resistance rates of *E.coli* in a WWTP to different LRAs during different seasons.

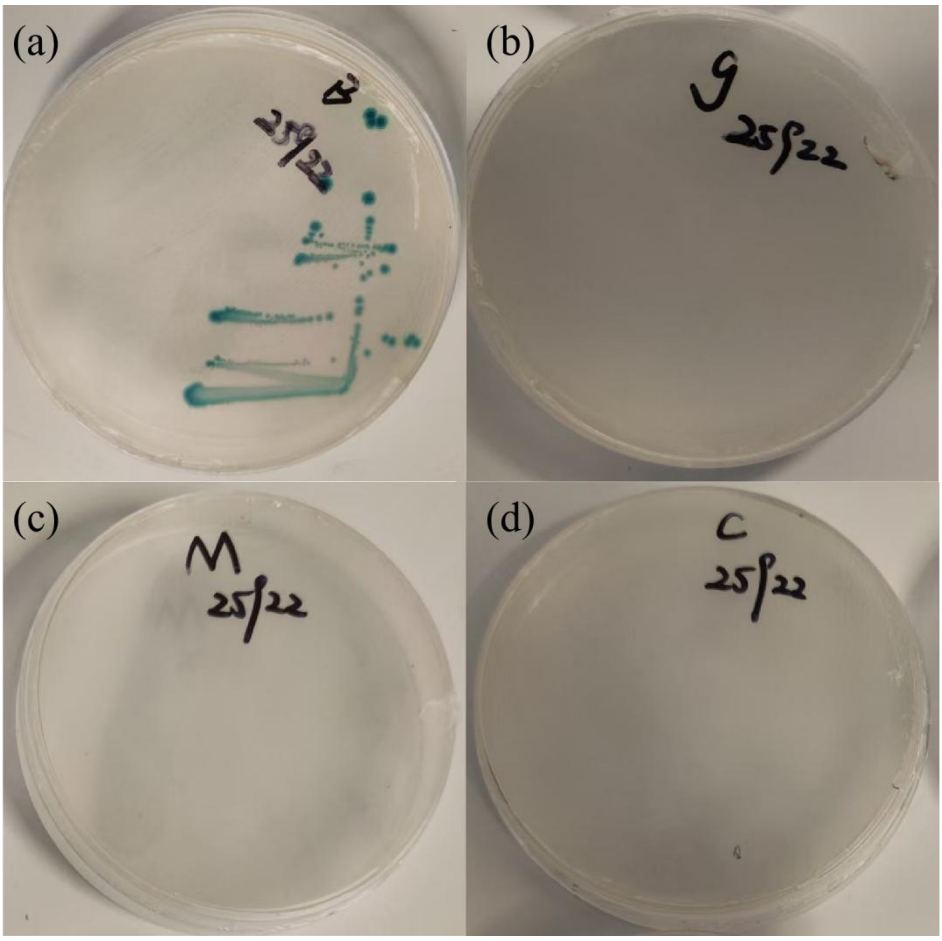
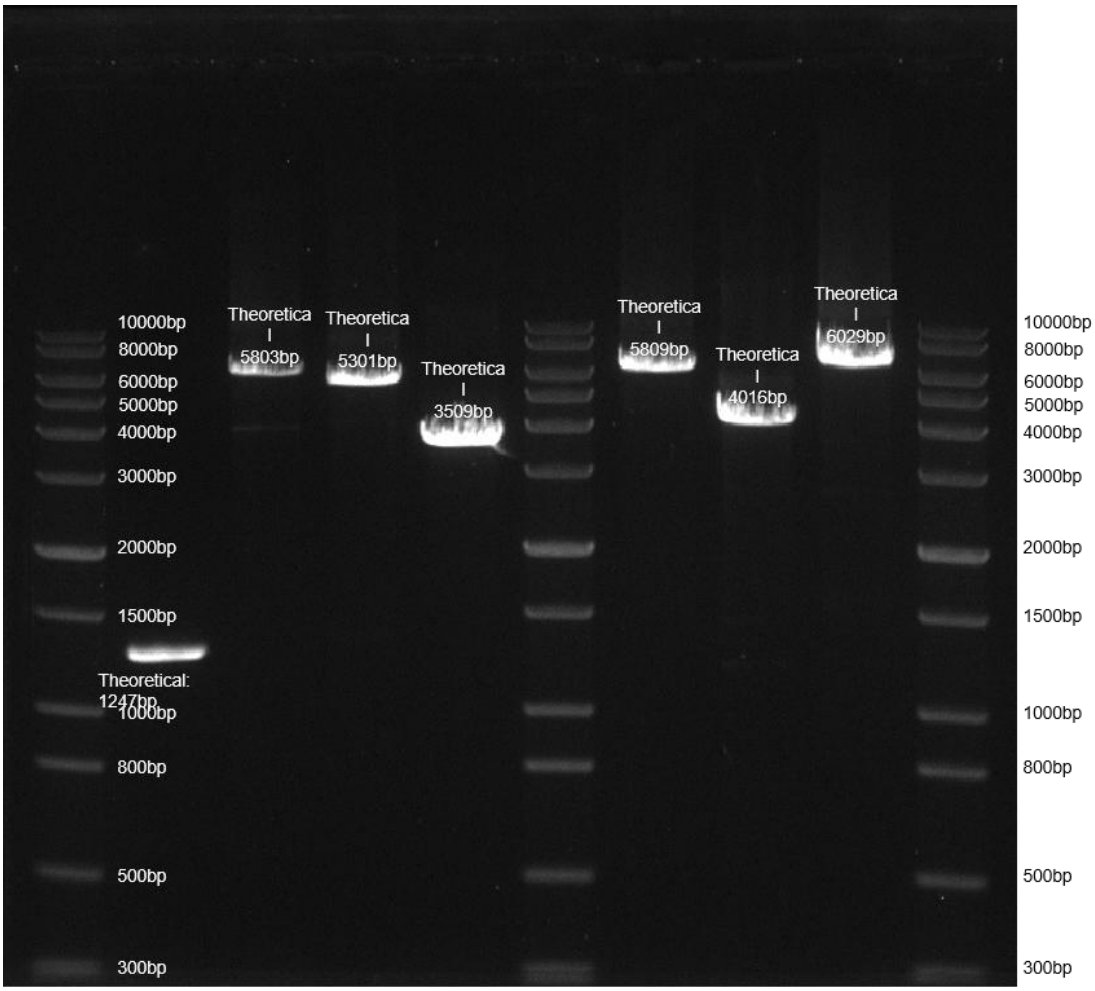


Figure S2 Validation of the reliability of the enumeration methods for *E. coli* using the standard strain (ATCC 25922): (a) Chromagar™ ECC agar; (b) with tigecycline; (c) with meropenem and $ZnSO_4$; (d) with colistin.



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224 **Figure S3 Validation of the WWE_MEM_4 genome assembly by long-template PCR**
225 **and agarose gel electrophoresis.**

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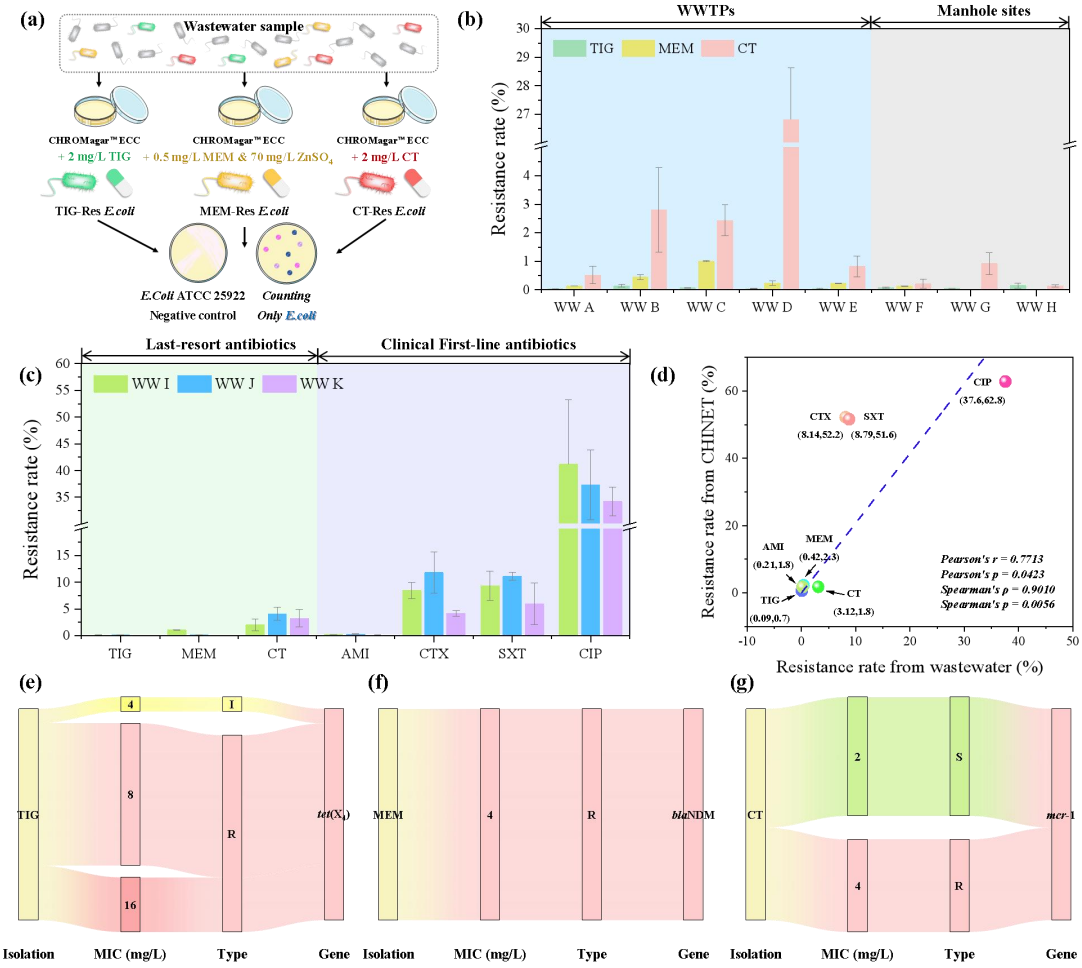
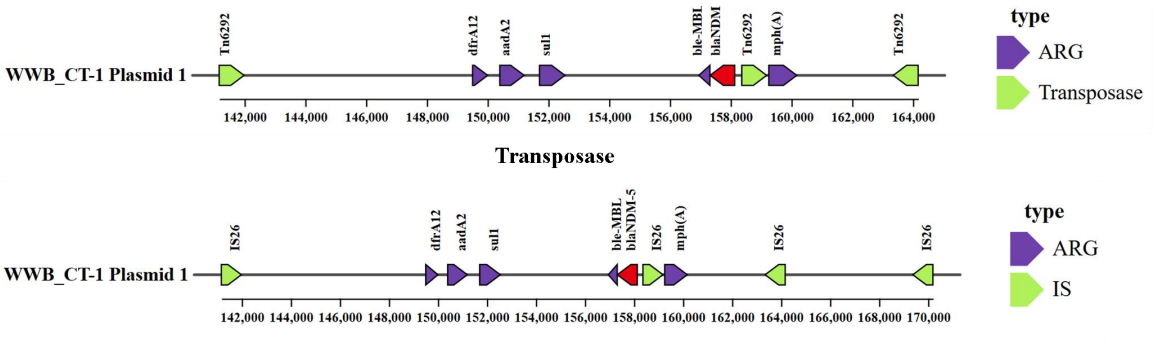


Figure S4. Resistance rates shown on a linear scale (corresponding to the logarithmic-scale presentation in Figure 1).

(a) Schematic diagram of the simultaneous species-resistance selection strategy for isolating LRA-resistant E. coli from wastewater. (b) Resistance rates of E. coli in wastewater to different LRAs. (c) Comparison of resistance rates to LRAs versus clinical first-line antibiotics. (d) Correlation between resistance rate in wastewater and clinical resistance data. The clinical data were derived from the 2024 annual report of CHINET. (e~g) Antimicrobial susceptibility testing results and ARG carriage of isolates from TIG (e), MEM (f), and CT (g) plates, respectively



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238 **Figure S5. Flanking relationship of *bla*NDM with transposase (Tn6292) and insertion**

239 **sequence IS26 on IncHI2 plasmid 1 of WWB_CT_1.**

Table

Table S1 Chemicals and Reagents.

Chemicals and Reagents	Source	Identifier
Tigecycline	Macklin Biochemical Technology Co., Ltd., Shanghai, China	T830543
Meropenem		M861173
Colistin		C805491
Cefotaxime sodium salt		C804340
Ciprofloxacin		C824343
Amikacin Sulfate		A837351
Trimethoprim		T832374
Sulfamethoxazole		S861458
Azithromycin		A796580
Zinc Sulfate		Z887856
CHROMagar™ ECC	CHROMagar, France	EF323
Cation-adjusted Mueller-Hinton Broth	Hope Bio-Technology Co., Ltd., Qingdao, China	HB6231-1
Mueller-Hinton Broth	Hope Bio-Technology Co., Ltd., Qingdao, China	HB6231
Agar Powder	Beijing Solarbio Science & Technology Co., Ltd., Beijing, China	A8190
Susceptibility-Testing Disks (Ampicillin, 10 μg)	BKMAM Biotechnology Co.,Ltd., Changde, China	110705007
Susceptibility-Testing Disks (Piperacillin, 100 μg)		110705034
Susceptibility-Testing Disks (Cefalexin, 30 μg)		110705043
Susceptibility-Testing Disks (Cefazolin, 30 μg)		110705054
Susceptibility-Testing Disks (Cefuroxime Sodium, 30 μg)		110705045
Susceptibility-Testing Disks (Ceftazidime, 30 μg)		110705052
Susceptibility-Testing Disks (Ceftriaxone, 30 μg)		110705049
Susceptibility-Testing Disks (Cefoperazone, 75 μg)		110705048
Susceptibility-Testing Disks (Amikacin, 30 μg)		110705013
Susceptibility-Testing Disks (Gentamicin, 10 μg)		110705038
Susceptibility-Testing Disks (Kanamycin, 30 μg)		110705022

Susceptibility-Testing Disks (Streptomycin, 10 μg)		110705026
Susceptibility-Testing Disks (Tetracycline, 30 μg)		110705039
Susceptibility-Testing Disks (Minocycline, 30 μg)		110705032
Susceptibility-Testing Disks (Azithromycin, 15 μg)		110705006
Susceptibility-Testing Disks (Norfloxacin, 10 μg)		110705033
Susceptibility-Testing Disks (Ciprofloxacin, 5 μg)		110705020
Susceptibility-Testing Disks (Lincomycin, 2 μg)		110705027
Susceptibility-Testing Disks (Vancomycin, 30 μg)		110705055
Susceptibility-Testing Disks (Polymyxin B, 300 μg)		110705014
Susceptibility-Testing Disks (Trimethoprim sulfamethoxazole, 25 μg)		110705017
Susceptibility-Testing Disks (Chloramphenicol, 30 μg)		110705030
Susceptibility-Testing Disks (Clindamycin, 2 μg)		110705024
Susceptibility-Testing Disks (Levofloxacin, 5 μg)		110705060
Susceptibility-Testing Disks (Imipenem, 10 μg)		110705058
Susceptibility-Testing Disks (Doxycycline, 30 μg)		110705047
Susceptibility-Testing Disks (Florfenicol, 30 μg)		110705016
Susceptibility-Testing Disks (MEM, 10 μg)	Kangtai Biotechnology Co., Ltd, Wenzhou China	Z21050
Susceptibility-Testing Disks (Fosfomycin, 200 μg)		Z21034
Susceptibility-Testing Disks (Rifampicin, 5μg)		Z21037

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Table S2 Summary of the detection rates of LRA-resistant *E.coli* from wastewater.

Country	LRAs	Isolation Strategy	Sample size	Positive rate (%)	Reference
China	Colistin	Species(First); Resistance(Second)	306	0.33	(Feng et al. 2024a)
China	Colistin	Resistance(First); Species(Second)	3574	2.43	(Feng et al. 2024b)
Vietnam	Colistin	Species(First); Resistance(Second)	10	32*	(Thanh et al. 2024)
China	Colistin	Resistance(First); Species(Second)	10	4	(Lin et al. 2026)
South Korea	Colistin	Species(First); Resistance(Second)	33	9.09	(Park et al. 2024a)
Iran	Imipenem	Species(First); Resistance(Second)	36	11.11**	(Zomorodi et al. 2025)
USA	Imipenem	Species(First); Resistance(Second)	6	0.0	(Mukherjee et al. 2021)
Egypt	Imipenem	Species(First); Resistance(Second)	120 (92)	6.5	(Abdelgalel et al. 2025)
Iran	MEM	Species(First); Resistance(Second)	3	0.0	(Afsharnia et al. 2025)
South Africa	MEM	Species(First); Resistance(Second)	60	0.7*	(Mbanga et al. 2021)
South Korea	Colistin	Species(First); Resistance(Second)	33	0.0	(Park et al. 2024b)
Turkey	TIG	Not mentioned	20	20**	(Kürekci et al. 2022)
Norway	TIG	Species(First); Resistance(Second)	Not mentioned	0.15*	(Marathe et al. 2021)

*: The reference did not report the positive rate on the sample level, but only picked a few isolates per sample, so we use the positive rate at the isolate level.

**: Not directly mentioned in the reference, calculated by the reporting data.

Table S3 Information and related parameters of wastewater sampling sites.

Sample	Site	Longitude	Latitude	Date	Average Flow (m^3/d)
WWA	WWTP A	116.70 E	39.89 N	2024.5.31	180000
WWB	WWTP B	116.15 E	39.90 N	2024.5.31	40000
WWC	WWTP C	116.31 E	39.69 N	2024.5.31	80000
WWD	WWTP D	116.37 E	39.54 N	2024.5.31	50000
WWE	WWTP E	116.37 E	39.79 N	2024.5.31	40000
WWF	Manhole A	116.35 E	40.04 N	2024.5.31	-
WWG	Manhole B	116.35 E	39.98 N	2024.5.31	-
WWH	Manhole C	116.33 E	40.01 N	2024.5.31	-
WWI	WWTP E	116.37 E	39.79 N	2024.7.1	40000
WWJ	WWTP A	116.70 E	39.89 N	2024.7.1	180000
WWK	WWTP F	116.77 E	39.82 N	2024.7.1	4000
WWL	WWTP A	116.70 E	39.89 N	2025.1.15	180000
WWM	WWTP A	116.70 E	39.89 N	2025.4.16	180000

Table S4 The performance of CHROMagar^{TM} ECC agar for selective culturing of *E. coli* from wastewater.

Colony types	Blue colonies (identified *E.Coli*)	Pink/purple colonies (identified non-*E.Coli*)
uidA positive rate (%)	98.2 (55/56)	0.0 (0/17)
uidA negative rate (%)	1.8 (1/56)	100.0 (17/17)

252 **Table S5 The Breakpoints for different antibiotics used in this study.**

Antibiotics	MIC breakpoints (mg/L)			Disk Content	Zone diameter breakpoints (mm)			Reference
	R	I	S	(μg)	R	I	S	
TIG	≥8	-	≤4	15	≤12	13-15	≥16	FDA
MEM	≥4	2	≤1	10	≤19	20-22	≥23	CLSI
Colistin	≥4	-	22	-	-	-	-	CLSI
Cefotaxime	≥4	2	≤1	5	≤17		≥20	EU
Ciprofloxacin	≥1	0.5	≤ 0.25	5	≤21	22-25	≥26	CLSI
Azithromycin	≥32		≤16	15	≤12		≥13	CLSI
Trimethoprim sulfamethoxazole	≥76/4	-	≤ 38/2	25	≤10	11-15	≥16	CLSI
Amikacin	≥16	8	≤4	30	≤14	15-16	≥17	CLSI
Gentamicin	≥8	4	≤2	10	≤12	13-14	≥15	CLSI
Kanamycin	≥64	32	≤16	30	≤13	14-17	≥18	CLSI
Streptomycin	-	-	-	10	≤11	12-14	≥15	CLSI
Ampicillin	≥32	16	≤8	10	≤13	14-16	≥17	CLSI
Piperacillin	≥32	16	≤8	100	≤17	18-20	≥21	CLSI
Cefalexin	≥16	-	≤16	30	≤14	-	≥14	EU
Cefazolin	≥8	4	≤2	30	≤19	20-22	≥23	CLSI
Cefuroxime	≥32	16	≤8	30	≤14	15-17	≥18	CLSI
Ceftazidime	≥16	8	≤4	30	≤17	18-20	≥21	CLSI
Cefoperazone	≥64	32	≤16	75	≤15	16-20	≥21	CLSI
Ceftriaxone	≥4	2	≤1	30	≤19	20-22	≥23	CLSI
Imipenem	≥4	2	≤1	10	≤19	20-22	≥23	CLSI
Chloramphenicol	≥32	16	≤8	30	≤12	13-17	≥18	CLSI
Florfenicol	≥32	16	≤8	30	≤14	-	≥19	CLSI
Doxycycline	≥16	8	≤4	30	≤10	11-13	≥14	CLSI
Minocycline	≥16	8	≤4	30	≤12	13-15	≥16	CLSI
Tetracycline	≥16	8	≤4	30	≤11	12-14	≥15	CLSI
Levofloxacin	≥2	1	≤0.5	5	≤16	17-20	≥21	CLSI
Norfloxacin	≥16	8	≤4	10	≤12	13-16	≥17	CLSI
Fosfomycin	≥256	128	≤64	200	≤24		≥25	CLSI
Rifampicin	-	-	-	-	-	-	-	-

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Table S6 The set of antimicrobial susceptibility plates for MIC determination.

Antibiotics	Gradient Con.1	Gradient Con.2	Gradient Con.3	Gradient Con.4	Gradient Con.5
TIG	16	8	4	2	1
MEM	4	2	1	0.5	0.25
Colistin	8	4	2	1	0.5
Cefotaxime	16	8	4	2	1
Ciprofloxacin	1	0.5	0.25	0.125	0.0625
Azithromycin	64	32	16	8	4
Trimethoprim sulfamethoxazole	8/152	4/76	2/38	1/19	0.5/9.5
Amikacin	64	32	16	8	4

Table S7 Detection rate of LRA-resistant *E. coli* in wastewater using the conventional "isolation-first and resistance-second" strategy.

Wastewater Samples	Total Isolates	*E.coli* Isolates	Antimicrobial susceptibility test resistant number			Strain-level resistance rates		
			TIG	MEM	CT	TIG (%)	MEM (%)	CT (%)
WWI	64	62	0	1	3	0.0	1.6	4.8
WWJ	64	64	0	0	0	0.0	0.0	0.0
WWK	64	63	0	0	0	0.0	0.0	0.0
Sample-level positive rates (%)			0	33.3	33.3		-	

Table S8 Meta-analysis of resistance rates of LRA-resistant *E. coli* to other antibiotics.

Sample type	Isolates Number	year	Source	Resistance rate (%)								
				TIG	MEM	CT	CTX/CRO	SXT	CIP	AZM	AMI	Ref.
TIG-resistance *E. coli*	**44**	**2024**	**Wastewater**	**93.2**	**0.0**	**0.0**	**54.6**	**86.4**	**100.0**	**18.2**	**0.0**	**This study**
TIG-resistance *E. coli*	20	2023	Fly	100.0	0.0	NA[a]	42.9	64.3	56.1	NA	0.0	(Yan et al. 2024)
TIG-resistance *E. coli*	25	Not mentioned	Meat	100.0	NA	NA	16.0	84.0	28.0	NA	NA	(Sun et al. 2021)
TIG-resistance *E. coli*	149	2021	Human feces	100.0	0.0	NA	18.3	NA	13.6	NA	0.0	(Zeng et al. 2022)
TIG-resistance *E. coli* (*tet*(X4)-positive)	48	2019	Feces	100.0	0.0	0.0	10.4	85.4	14.6	NA	0.0	(Cui et al. 2022)
TIG-resistance *E. coli* (*tet*(X4)-positive)	24	2021	Pig feces	100.0	0.0	0.0	45.83	79.2	20.8	NA	12.5	(Wang et al. 2022a)

TIG-resistance *E. coli* (*tet*(X4)-positive)	7	Not mentioned	Pig	100.0	0.0	NA	28.6	85.7	28.6	NA	NA	(Bai et al. 2019)
TIG-resistance *E. coli* (*tet*(X4)-positive)	13	2017-2019	Farm	100.0	0.0	0.0	68.8	93.8	100.0	NA	0.0	(Yu et al. 2021a)
TIG-resistance *E. coli* (*tet*(X4)-positive)	21	2020-2022	Avain samples	100.0	NA	NA	NA	100.0	NA	33.3	NA	(Zhang et al. 2023b)
TIG-resistance *E. coli* (*tet*(X4)-positive)	22	2021	Pig	100.0	0.0	0.0	NA	NA	22.7	NA	NA	(Li et al. 2023)
TIG-resistance *E. coli* (*tet*(X4)-positive)	6	2021	Feces	100.0	0.0	0.0	0.0	100.0	NA	NA	0.0	(Yang et al. 2023)
TIG-resistance *E. coli* (*tet*(X4)-positive)	10	2021	Vegetable	100.0	0.0	NA	30.0	50.0	60.0	NA	0.0	(Yu et al. 2021b)
MEM-resistance *E. coli*	**45**	**2024**	**Wastewater**	**2.2**	**100.0**	**11.1**	**100.0**	**100.0**	**100.0**	**46.7**	**17.8**	**This study**

Carbapenem-resistance *E. coli*	181	2018-2019	Human	1.1	67.4	0.6	96.1	NA[a]	86.7	NA	27.1	(Wang et al. 2022b)
Carbapenem-resistance *E. coli*	231	2023	Fly	8.3	100.0	NA	100.0	NA	55.8	NA	12.1	(Zhou et al. 2024)
Carbapenem-resistance *E. coli*	134	2013-2020	Human	NA	NA	NA	100.0	85.8	100.0	NA	28.4	(Kong et al. 2024)
Carbapenem-resistance *E. coli*	158	Not mentioned	Feces	0.0	100.0	19.6	100.0	NA	93.7	NA	13.9	(Guo et al. 2024)
Carbapenem-resistance *E. coli*	45	Not mentioned	Meat	0.0	100.0	15.6	100.0	NA	77.8	NA	4.4	(Guo et al. 2024)
Carbapenem-resistance *E. coli*	43	2012-2016	Meat, pig and human	NA	100.0	11.6	100.0	88.4	90.7	NA	25.6	(Feng et al. 2021)
Carbapenem-resistance *E. coli*	631	2016	Human	3.3	77.2	1.7	95.7	65.6	76.4	NA	16.8	(Shen et al. 2022)

Carbapenem-resistance *E. coli*	117	2012-2019	Human	NA	87.2	NA	92.3	72.7	90.6	NA	32.5	(Liu et al. 2022)
Carbapenem-resistance *E. coli*	113	2018-202	Human	0.9	83.2	0.0	95.58	NA	89.4	74.3	10.6	(Zhang et al. 2023a)
Carbapenem-resistance *E. coli*	88	2018	Chicken farm	0.0	100.0	15.9	100.0	96.6	89.8	NA	12.5	(Ma et al. 2024)
Carbapenem-resistance *E. coli*	17	2017-2020	Human	0.0	100.0	5.9	100.0	100.0	100.0	NA	64.7	(Chen et al. 2023)
Colistin-resistance *E. coli*	**23**	**2024**	**Wastewater**	**0.0**	**13.0**	**34.8**	**69.6**	**82.6**	**100.0**	**39.1**	**17.4**	**This study**
Colistin-resistance *E. coli*	92	2023	Wastewater	0.0	4.35	75.0	67.4	90.2	54.35	NA	13.04	(Feng et al. 2024c)
Colistin-resistance *E. coli*	360	Not mentioned	Duck farm	6.9	0.6	100.0	58.1	100.0	74.7	16.4	5.8	(Liu et al. 2023)

Colistin-resistance *E. coli* (*mcr*-1-negative)	80	2014-2019	Chicken	NA	2.2	43.5	26.1	78.3	37.0	23.9	8.7	(Zhang et al. 2022)
Colistin-resistance *E. coli* (*mcr*-1-positive)	51	2014-2019	Chicken	NA	0.0	43.8	33.3	85.4	60.4	27.1	14.6	(Zhang et al. 2022)
Colistin-resistance *E. coli* (ESBL-producing)	25	2012	Human feces	0.0	0.0	100.0	100.0	96.0	80.0	NA	32.0	(Bi et al. 2017)
Colistin-resistance *E. coli*	21	2018-2019	Human feces	0.0	0.0	100.0	NA	NA	38.1	NA	4.8	(Mai et al. 2023)
Colistin-resistance *E. coli*	101	2016-2018	Feces	0.0	NA	100.0	15.8	NA	5.9	NA	0.0	(Li et al. 2022)
Colistin-resistance *E. coli*	13	2019-2020	Meat	NA	NA	NA	69.2	77.0	77.0	NA	7.7	(Mei et al. 2024)
Colistin-resistance *E. coli* (*mcr*-1-positive)	86	2017-2018	Feces	0.0	5.8	NA	NA	97.7	87.0	NA	8.7	(Cheng et al. 2020)

Colistin-resistance *E. coli* (*mcr*-1-positive)	58	2010-2015	Chicken	NA	NA	100.0	53.4	84.5	72.4	NA	56.9	(Yang et al. 2017)

a. NA means the resistance rate of this antibiotic was not measured in the related study

Table S9 Prevalence of virulence genes in representative isolates.

Isolates	VFs
WWA_MEM_2	*fim*C(1),*csg*C(1),*fim*I(1),*fim*D(1),*csg*B(1),*cgs*E(1),*cgs*D(1),*yag*W/*ecp*D(1), *yag*X/*ecp*C(1),*yag*Z/*ecp*A(1),*yag*Y/*ecp*B(1),*fim*E(1),*cgs*F(1),*hcp*1/*tss*D1(1), *fim*G(1),*iut*A(1),*cfa*B(1),*cfa*C(1),*cfa*D/*cfa*E(1),*cfa*A(1),east1(1),*tss*A(1), VF0579(1),*tss*J(1),*fha*(1),*tss*B(1),*tss*L(1),*fim*B(1),*hcp*2/*tss*D2(1),*tss*G(1)
WWA_MEM_4	*fim*C(1),*csg*C(1),*fim*I(1),*fim*D(1),*csg*B(1),*cgs*E(1),*cgs*D(1),*yag*W/*ecp*D(1), *yag*X/*ecp*C(1),*yag*Z/*ecp*A(1),*yag*Y/*ecp*B(1),*fim*E(1),*cgs*F(1),*hcp*1/*tss*D1(1), *fim*G(1),*iut*A(1),*cfa*B(1),*cfa*C(1),*cfa*D/*cfa*E(1),*cfa*A(1),*tss*A(1),*iro*C(1), *iro*D(1),*iro*E(1),*iro*N(1)
WWB_CT_1	*fim*C(1),*csg*C(1),*fim*I(1),*fim*D(1),*csg*B(1),*cgs*E(1),*cgs*D(1),*yag*W/*ecp*D(1), *yag*X/*ecp*C(1),*yag*Y/*ecp*B(1),*fim*E(1),*cgs*F(1),*hcp*1/*tss*D1(1),*cfa*B(1), *cfa*C(1),*cfa*D/*cfa*E(1),*cfa*A(1),*tss*A(1),*iro*C(1),*iro*D(1),*iro*E(1),*iro*N(1), *fim*F(1)
WWH_CT_21	*fim*C(1),*csg*C(1),*fim*I(1),*fim*D(1),*csg*B(1),*cgs*E(1),*cgs*D(1),*yag*W/*ecp*D(1), *yag*X/*ecp*C(1),*yag*Z/*ecp*A(1),*yag*Y/*ecp*B(1),*cgs*F(1),*hcp*1/*tss*D1(1),*fim*G(1), *tss*A(1),VF0579(1),*tss*J(1),ompA(1),*fha*(1),*tss*B(1),*tss*L(1),*hcp*2/*tss*D2(1), *tss*F(1),*tss*G(1),*ent*B(1),*vgr*G/*tss*I(1)
WWH_MEM_3	*fim*C(1),*csg*C(1),*fim*I(1),*fim*D(1),*csg*B(1),*cgs*E(1),*cgs*D(1),*yag*W/*ecp*D(1), *yag*X/*ecp*C(1),*yag*Z/*ecp*A(1),*hcp*1/*tss*D1(1),*fim*G(1),*iut*A(1),*east*1(1), VF0579(1),*iro*C(1),*iro*D(1),*tss*J(1),*iro*E(1),*iro*N(1),*fim*B(1),*chu*U(1), *chu*T(1),*chu*A(1),*ykg*K/*ecp*R(1),*kps*F(1),*kps*E(1),*ent*C(1),*cgs*G(1),gspG(1), *csg*A(1),*neu*E(1),*kps*S(1),*kps*T(1),*shu*V(1),*neu*A(1),*neu*B(1),*neu*C(1), *neu*D(1)
WWC_MEM_1	*fim*C(1),*csg*C(1),*fim*I(1),*fim*D(1),*csg*B(1),*yag*W/*ecp*D(1),*yag*X/*ecp*C(1), *yag*Z/*ecp*A(1),*yag*Y/*ecp*B(1),*hcp*1/*tss*D1(1),*fim*G(1),east1(1),*tss*A(1), VF0579(1),*tss*J(1),ompA(1),*fha*(1),*tss*B(1),*tss*L(1),*hcp*2/*tss*D2(1),*tss*F(1), *tss*G(1),*ent*B(1),*pap*G(1),*pap*K(1)
WWB_MEM_13	*fim*C(1),*csg*C(1),*fim*I(1),*fim*D(1),*csg*B(1),*cgs*E(1),*cgs*D(1),*yag*W/*ecp*D(1), *yag*X/*ecp*C(1),*yag*Z/*ecp*A(1),*yag*Y/*ecp*B(1),*fim*E(1),*fim*G(1),*fim*F(1), *fim*B(1),*chu*U(1),*chu*T(1),*chu*A(1),*ykg*K/*ecp*R(1),*chu*W(1),*chu*V(1),*ent*C(1), *ibe*B(1)

WWD_MEM_18	*fim*C(1),*csg*C(1),*fim*I(1),*fim*D(1),*csg*B(1),*cgs*E(1),*cgs*D(1),*yag*W/*ecp*D(1), *yag*X/*ecp*C(1),*yag*Z/*ecp*A(1),*yag*Y/*ecp*B(1),*fim*E(1),*cgs*F(1),*hcp*1/*tss*D1(1), *cfa*B(1),*cfa*C(1),*cfa*D/*cfa*E(1),*cfa*A(1),east1(1),*tss*A(1),VF0579(1),*tss*J(1), *omp*A(1),*fha*(1),*tss*B(1),*fim*F(1),*tss*L(1),*tss*F(1)
WWA_MEM_11	*fim*C(1),*csg*C(1),*fim*I(1),*fim*D(1),*csg*B(1),*cgs*E(1),*cgs*D(1),*yag*W/*ecp*D(1), *yag*X/*ecp*C(1),*yag*Z/*ecp*A(1),*yag*Y/*ecp*B(1),*fim*E(1),*cgs*F(1), *hcp*1/*tss*D1(1),*east*1(1),*tss*A(1),VF0579(1),*tss*J(1),ompA(1),*fha*(1), *tss*B(1),*fim*F(1),*tss*L(1),*hcp*2/*tss*D2(1),*tss*F(1),*tss*G(1),*ent*B(1),VF1111(1)
WWA_MEM_14	*fim*C(1),*csg*C(1),*fim*I(1),*fim*D(1),*csg*B(1),*cgs*E(1),*cgs*D(1),*fim*E(1), *cgs*F(1),*hcp*1/*tss*D1(1),*iut*A(1),*cfa*B(1),*cfa*C(1),*cfa*D/*cfa*E(1),*cfa*A(1), *east*1(1),*omp*A(1),*ybt*U(1),*ybt*T(1),*irp*1(1),*irp*2(1),*fyu*A/*psn*(1),*ybt*Q(1), *ybt*P(1),*ybt*X(1),*ybt*E(1),*ybt*S(1),*ybt*A(1)
WWE_MEM_2	*fim*C(1),*csg*C(1),*fim*I(1),*fim*D(1),*csg*B(1),*cgs*E(1),*cgs*D(1),*yag*W/*ecp*D(1), *yag*X/*ecp*C(1),*yag*Z/*ecp*A(1),*yag*Y/*ecp*B(1),*fim*E(1),*iut*A(1),*iro*C(1), *iro*D(1),*iro*E(1),*iro*N(1),*fim*F(1),*fim*B(1),*ent*B(1),*chu*U(1),*chu*T(1), *chu*A(1),*ykg*K/*ecp*R(1),*chu*W(1),*ybt*U(1),ybtT(1),*chu*V(1),*irp*1(1),*irp*2(1), *fyu*A/*psn*(1),*ybt*Q(1),*ybt*P(1),*ybt*X(1),*kps*F(1),*ybt*E(1),*ybt*S(1),*ybt*A(1), *kps*E(1),*ent*E(1),*fep*B(1),*fes*(1),*kps*D(1),*fep*A(1)
WWE_MEM_4	*fim*C(1),*csg*C(1),*fim*I(1),*fim*D(1),*csg*B(1),*cgs*E(1),*cgs*D(1),*yag*W/*ecp*D(1), *yag*Z/*ecp*A(1),*yag*Y/*ecp*B(1),*fim*E(1),*cgs*F(1),*fim*G(1),*iut*A(1),*cfa*B(1), *cfa*C(1),*cfa*D/*cfa*E(1),*cfa*A(1),*east*1(1),*iro*C(1),*iro*D(1),*iro*E(1),*iro*N(1)
WWE_MEM_10	*fim*C(1),*csg*C(1),*fim*I(1),*fim*D(1),*csg*B(1),*cgs*E(1),*cgs*D(1),*yag*W/*ecp*D(1), *yag*X/*ecp*C(1),*yag*Z/*ecp*A(1),*yag*Y/*ecp*B(1),*fim*E(1),*cgs*F(1),*fim*G(1), *iut*A(1),*cfa*B(1),*cfa*C(1),*cfa*D/*cfa*E(1),*cfa*A(1),*iro*C(1),*iro*D(1),*iro*E(1), *iro*N(1)

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Table S10 Prevalence of MGEs in representative isolates.

	MGEs
WWB_CT_1	*tnp*A(20),*tnp*B(3),*int*(4),*tns*3(5),*tnp*R(5),*ins*K(1),*xer*C(1),*int*M(1),*tnp*(3), *xer*D(1),*tnp*M(2),*ist*A(1),*ins*J(1),*ins*Z(2),*pin*R(1),*int*D(1),*par*A(1),*ins*A(1), *uvp*1(1),*int*R(1),*gin*(1),*pin*E(1)
WWH_CT_21	*tnp*A(24),*tnp*B(7),*int*(1),*tnp*C(4),*tns*3(4),*tnp*R(1),*ins*K(8),*xer*C(1),*int*M(1), *tnp*(1),*ins*D(1),*xer*D(1),IS26(3),*ins*J(1),*ins*Z(2),*ray*T(1),*par*A(1),*ins*N(1),*ref*(1), *int*E(1)
WWH_MEM_3	*tnp*A(33),*tnp*B(10),*int*(5),*tnp*C(18),tns3(2),*tnp*R(6),*ins*K(7),*xer*C(1),*int*M(3), *tnp*(4),*ins*D(7),*xer*D(1),*tnp*M(2),*ist*A(2),rci(2),*ins*Z(1),*pin*R(3),*ins*G(1),*ins*A(1), *ist*B(3),*ins*N(1),*uvp*1(1),*int*I(1),*ref*(1),*tISRso16a*(1)
WWC_MEM_1	*tnp*A(36),*tnp*B(3),*int*(5),*tnp*C(1),*tns*3(5),*tnp*R(7),*ins*K(3),*xer*C(1),*tnp*(1), *ins*D(8),*xer*D(1),*ins*J(1),*ins*Z(2),*pin*R(2),*ray*T(1),*ins*N(1),*uvp*1(1),*ltr*A(2),*int*I(1) ,*ins*B(2),*ref*(1),*gin*(1),*tpase*(1)
WWB_MEM_13	*tnp*A(24),*tnp*B(14),*int*(2),*tnp*C(8),*tnp*R(1),*ins*K(3),xerC(1),*tnp*(2),*ins*D(1), *xer*D(1),*tnp*M(3),*ins*J(1),*rci*(1),*alp*A(1),*ray*T(1),*int*D(1),*par*A(4),*ins*G(3), *ins*N(1),*ltr*A(1),*int*S(1),*int*2(1),*mob*I(1)
WWD_MEM_18	*tnp*A(29),*tnp*B(2),*int*(7),*tnp*C(1),*tns*3(7),*tnp*R(4),*ins*K(1),*xer*C(1),*int*M(2), *tnp*(3),*xer*D(1),*tnp*M(1),*ins*J(1),*ins*Z(1),*alp*A(1),*int*D(1),*par*A(1),*ins*A(1),*int*I(1) ,*pin*H(1)
WWA_MEM_11	*tnp*A(22),*tnp*B(6),*int*(3),*tnp*C(3),*tns*3(4),*tnp*R(2),*ins*K(2),*xer*C(1),*int*M(4), *tnp*(1),*xer*D(1),*tnp*M(2),*ist*A(2),*ins*J(1),*rci*(3),*pin*R(1),*alp*A(1),*ray*T(1),*int*D(1), *ins*A(1),*ist*B(1),*uvp*1(2),*int*E(1)
WWA_MEM_14	*tnp*A(20),*tnp*B(4),*int*(6),*tnp*C(2),*tns*3(4),*tnp*R(3),*xer*C(1),*int*M(2),*xer*D(1), *tnp*M(2),*ist*A(1),*rci*(2),*ins*Z(2),*int*D(1),*ins*A(1),*ist*B(2),*ltr*A(2),*tns*B(1),*int*R(1), *tns*D(1),*tns*A(1),*tns*C(1)
WWE_MEM_2	*tnp*A(43),*tnp*B(8),*int*(8),*tnp*C(4),*tnp*R(3),*ins*K(4),*xer*C(1),*int*M(2),*tnp*(1), *xer*D(1),*ist*A(2),*IS*26(2),*ins*J(2),*alp*A(1),rayT(1),*ins*G(3),*uvp*1(1),*int*I(1), *IS*1501(2),*pin*H(1)
WWE_MEM_4	*tnp*A(25),*tnp*B(4),*int*(3),*tnp*C(2),tns3(3),*tnp*R(3),*ins*K(3),*xer*C(8),*int*M(5), *tnp*(1),*xer*D(1),*ist*A(1),IS26(1),*ins*J(1),*rci*(1),*ins*Z(1),*alp*A(2),*ray*T(1),*par*A(1), *int*S(1),*hin*(1),*tnp*I(1)
WWE_MEM_10	*tnp*A(32),*tnp*B(3),*int*(1),*tnp*C(2),*tns*3(4),*tnp*R(7),*ins*K(3),*xer*C(8),*int*M(3), *tnp*(4),*xer*D(1),*tnp*M(1),*ist*A(1),*IS*26(2),*ins*J(1),*pin*R(2),*alp*A(2),*ray*T(1), *int*D(1),*ins*N(1),*int*S(1),*int*R(1),*tnp*1(1)

Table S11 Genomic features and the distribution of ARGs in WWB_CT_1.

Components	Length	Inc group	ARGs
Chromosome	4.78 Mbp	-	None
Plasmid1	256.9 kbp	IncHI2	*aph*(3')-Ia, *aph*(4)-Ia, *aad*A2, *aad*A1, *aac*(3)-IV, *bla*CTX-M-14, *bla*NDM-5, *mcr*-1.1, *fos*A3, *mph*(A), *cml*A1, *Oqx*B, *Oqx*A, *sul*1, *sul*2, *sul*3, *dfr*A12
Plasmid2	127.5 kbp	IncFIB	*bla*TEM-32, *bla*TEM-135, *bla*TEM-20, *bla*TEM-1C, *flo*R, *qnr*S1, *tet*(A), *dfr*A14

Table S12 Genomic features and the distribution of ARGs in WWE_MEM_4.

Components	Length	Inc group	ARGs
Chromosome	4.91 Mbp	-	None
Plasmid1	184.7 kbp	IncX1	*aac*(3)-IId, *aph*(3')-Ia, *bla*TEM-1B, *mph*(A), *dfr*A17
Plasmid2	112.6 kbp	IncI1-I	*aad*A22, *bla*NDM-5, *erm*(B)
Plasmid3	66.8 kbp	IncFIB(K)	*erm*(42), *mph*(A), *flo*R, *tet*(X_4)
Plasmid4	58.6 kbp	IncM2	None
Plasmid5	6.65 kbp	ColRNA1	None

Table S13 The primers and probes used in this study.

Primers /Probes	Sequence (5'-3')	Use	Con. (nM)	Ref.
UidA-F	ATGGAATTTCGCCGATTTTGC	Colony PCR identification of *E.Coli*	500	(Heijnen and Medema 2006)
UidA-R	ATTGTTTGCCTCCCTGCTGC		500	
27 F	AGRGTTYGATYMTGGCTCAG	Colony PCR Confirmation of Isolates	500	(Frank et al. 2008)
1492 R	RGYTACCTTGTTACGACTT		500	
tet(X_1)_F	CGAAAAATGTTGCTTGGCAGCTT	Multiplex PCR Identification for *tet*(X) variants genes in isloates	100	(Ji et al. 2020)
tet(X_1)_R	AGTTGTTGAACGAATTAACTCC		100	
tet(X_2)_F	CGGGATGTCCAAGGTAAGAAAA		600	
tet(X_2)_R	TGACAACGTCGTATGAATCAA		600	
tet(X_3)_F	GACACTTGATCTGCACAGGGATT		300	
tet(X_3)_R	CCCTACAAAAGATGATGTCAAAC		300	
tet(X_4)_F	CTGATTCGTGTGACATCATCTTTTG		600	
tet(X_4)_R	GTTAAATTTCCCATTGGTCAGATTA		600	
tet(X_5)_F	GGTATCAACATTTCAATGCTTG		100	
tet(X_5)_R	CGATTCGTCCTGCGTATCTTTTG		100	
*bla*IMP-F	GGAATAGAGTGGCTTAAYTCTC	Multiplex PCR Identification for Carbapenem-resistance genes	530	(Bej et al. 1991)
*bla*IMP-R	GGTTTAAYAAAACAACCACC		530	
*bla*OXA48-F	GCGTGGTTAAGGATGAACAC		530	
*bla*OXA48-R	CATCAAGTTCAACCCAACCG		530	
*bla*NDM-F	GGTTTGGCGATCTGGTTTTC		530	
*bla*NDM-R	CGGAATGGCTCATCACGATC		530	
*bla*KPC-F	CGTCTAGTTCTGCTGTCTTG		530	
*bla*KPC-R	CTTGTCATCCTTGTTAGGCG		530	

*mcr*1-F	ATGCCAGTTTCTTTCGCGTG	Multiplex PCR Identification for *mcr* variants genes in isloates	1000	(Lescat et al. 2018)
*mcr*1-R	TCGGCAAATTGCGCTTTTGGC		1000	
*mcr*2-F	GATGGCGGTCTATCCTGTAT		1000	
*mcr*2-R	AAGGCTGACACCCCATGTCAT		1000	
*mcr*3-F	ACCAGTAAATCTGGTGGCGT		1000	
*mcr*3-R	AGGACAACCTCGTCATAGCA		1000	
*mcr*4-F	TTGCAGACGCCCATGGAATA		500	
*mcr*4-R	GCCGCATGAGCTAGTATCGT		500	
*mcr*5-F	GGACGCGACTCCCTAACTTC		500	
*mcr*5-R	ACAACCAGTACGAGAGCACG		500	
16S-IS-F	GTGCAATATTCCCCACTGCT	Inner Standard used in multiplex PCR	250	(Lescat et al. 2018)
16S-IS-R	CGATCCCTAGCTGGTCTGAG		250	
tet(X_4)qP-F	TCGTGTCGCTAACCCTACAAAAG	qPCR validation for *tet*(X4)	200	This study
tet(X_4)qP-R	GAATTTTCCGATTGGGACGAA		200	
tet(X_4)qP-P	TGATGTCACACGAATC (5'-FAM, 3'-MGB)		100	
*mcr*1qP-F	TTGTGGCGTGATAATAATTCG	qPCR validation for *mcr*-1	400	This study
*mcr*1qP-R	TGGTCGCGGATTTATAATCG		400	
*mcr*1qP-P	CTGCCAAAAGCGCA (5'-FAM, 3'-MGB)		200	
*mcr*3qP-F	ATGATGCAAAACGGGATACTAA		400	
*mcr*3qP-R	TCATAGCCATTCATCGAGAAAT		400	
*mcr*3qP-P	AGTAAGCCCACGTTGAT (5'-VIC, 3'-MGB)		200	
*bla*KPCqP-F	CTGTATCGCCGTCTAGTTCTG	qPCR validation for *bla*NDM	400	
*bla*KPCqP-R	AGTTTAGCGAATGGTTCCG		400	
*bla*KPCqP-P	TGTCTTGTCTCTCATGGCCGCTGG (5'-FAM, 3'-BHQ1)		200	

*bla*NDMqP-F	GCATTAGCCGCTGCATT	400	(Weiß
*bla*NDMqP-R	GATCGCCAAACCGTTGG	400	et al.
*bla*NDMqP-P	ACGATTGGCCAGCAAATGGAAACT GG (5'-ROX, 3'-BHQ2)	200	2017)
*bla*OXA48qP-F	TGTCTTGTCTCTCATGGCCGCTGG	400	
*bla*OXA48qP-R	ACGATTGGCCAGCAAATGGAAACT GG	400	
*bla*OXA48qP-P	(5'-VIC, 3'-BHQ1)	200	

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Table S14 Genome assembly quality assessment of representative isolates.

	Length (Mbp)	GC (%)	N50 (kbp)	L50	N90 (kbp)	L90	Completeness (%)	Contamination (%)
WWA_MEM_11	5.29	50.51	240.85	7	34.24	29	99.93	1.03
WWA_MEM_14	5.17	50.59	177.35	9	43.26	31	99.93	0.35
WWE_MEM_2	5.60	50.41	313.52	6	46.77	22	99.93	1.14
WWE_MEM_4	5.28	50.77	242.21	8	60.54	22	99.93	0.61
WWE_MEM_10	5.30	50.59	157.76	9	46.62	30	99.93	0.53
WWA_MEM_2	5.22	50.47	240.49	7	31.83	26	99.93	0.60
WWA_MEM_4	4.95	50.54	206.08	9	72.23	25	99.93	0.31
WWB_CT_1	5.09	50.40	223.89	8	51.08	26	99.93	0.31
WWH_CT_21	4.89	50.61	138.35	11	33.48	39	99.93	0.10
WWH_MEM_3	5.54	50.46	226.64	9	38.74	32	99.97	0.14
WWC_MEM_1	4.97	50.77	98.91	17	19.74	57	99.97	0.49
WWB_MEM_13	5.21	50.37	208.93	9	49.12	30	99.97	0.41
WWD_MEM_18	5.11	50.58	146.85	10	34.25	36	99.93	0.21

Table S15 The MLST and phylogroups of representative isolates.

	LRAs	ST	Group
WWA_MEM_11	MEM	410	C
WWA_MEM_14	MEM	354	F
WWE_MEM_2	MEM	162	B1
WWE_MEM_4	MEM	4980	A
WWE_MEM_10	CT	5229	B1
WWA_MEM_2	MEM	1196	B1
WWA_MEM_4	MEM	1196	B1
WWB_CT_1	CT	196	B1
WWH_CT_21	CT	617	A
WWH_MEM_3	MEM	349	D
WWC_MEM_1	MEM	746	A
WWB_MEM_13	MEM	457	F
WWD_MEM_18	MEM	156	B1

References:

Abdelgalel RR, Ibrahem RA, Mohamed DS, et al (2025) Multidrug-resistant E. Coli in wastewater sources: a comparative study and identification of resistance hotspots. BMC Microbiology 25: 498. https://doi.org/10.1186/s12866-025-04244-5.

Afsharnia M, Naraghi B, Biglari H, et al (2025) Detection of the phenotypic and genotypic characteristics of antibiotic and chlorine resistance Escherichia coli isolates from municipal and industrial wastewaters. Desalination and Water Treatment 324: 101561. https://doi.org/10.1016/j.dwt.2025.101561.

Bai L, Du P, Du Y, et al (2019) Detection of plasmid-mediated tigecycline-resistant gene tet(X4) in Escherichia coli from pork, Sichuan and Shandong Provinces, China, February 2019. Eurosurveillance 24. https://doi.org/10.2807/1560-7917.ES.2019.24.25.1900340.

Bankevich A, Nurk S, Antipov D, et al (2012) SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. Journal of Computational Biology 19: 455–477. https://doi.org/10.1089/cmb.2012.0021.

Beghain J, Bridier-Nahmias A, Le Nagard H, et al (2018) ClermonTyping: an easy-to-use and accurate in silico method for Escherichia genus strain phylotyping. Microbial Genomics 4. https://doi.org/10.1099/mgen.0.000192.

Bej AK, DiCesare JL, Haff L, Atlas RM (1991) Detection of Escherichia coli and Shigella spp. in water by using the polymerase chain reaction and gene probes for uid. Applied and Env*iro*nmental Microbiology 57: 1013–1017. https://doi.org/10.1128/aem.57.4.1013-1017.1991.

Bi Z, Berglund B, Sun Q, et al (2017) Prevalence of the mcr-1 colistin resistance gene in extended-spectrum β-lactamase-producing Escherichia coli from human faecal samples collected in 2012 in rural villages in Shandong Province, China. International Journal of Antimicrobial Agents 49: 493–497. https://doi.org/10.1016/j.ijantimicag.2016.12.018.

Bortolaia V, Kaas RS, Ruppe E, et al (2020) ResFinder 4.0 for predictions of phenotypes from genotypes. Journal of Antimicrobial Chemotherapy 75: 3491–3500. https://doi.org/10.1093/jac/dkaa345.

Brown CL, Mullet J, Hindi F, et al. (2022) mobileOG-db: a Manually Curated Database of Protein Families Mediating the Life Cycle of Bacterial Mobile Genetic Elements. Nojiri H (Ed.). Applied and Env*iro*nmental Microbiology 88: e00991-22. https://doi.org/10.1128/aem.00991-22.

Carattoli A, Zankari E, García-Fernández A, et al (2014) *In Silico* Detection and Typing of Plasmids using PlasmidFinder and Plasmid Multilocus Sequence Typing. Antimicrobial Agents and Chemotherapy 58: 3895–3903. https://doi.org/10.1128/AAC.02412-14.

Chen R, Wang G, Wang Q, et al (2023) Antimicrobial resistance and molecular epidemiology of carbapenem-resistant Escherichia coli from urinary tract infections in Shandong, China. International Microbiology 26: 1157–1166. https://doi.org/10.1007/s10123-023-00369-7.

Cheng P, Yang Y, Zhang J, et al (2020) Antimicrobial Resistance and Virulence Profiles of mcr-1–Positive Escherichia coli Isolated from Swine Farms in Heilongjiang Province of China. Journal of Food Protection 83: 2209–2215. https://doi.org/10.4315/JFP-20-190.

Cui C-Y, Li X-J, Chen C, et al (2022) Comprehensive analysis of plasmid-mediated tet(X4)-positive Escherichia coli isolates from clinical settings revealed a high correlation with animals and env*iro*nments-derived strains. Science of The Total Env*iro*nment 806: 150687. https://doi.org/10.1016/j.scitotenv.2021.150687.

De La Cruz F (Ed.) (2020) 2075 Horizontal Gene Transfer: Methods and Protocols. Springer US, New York, NY. https://doi.org/10.1007/978-1-4939-9877-7.

Feng J, Pan M, Zhuang Y, et al (2024a) Genetic epidemiology and plasmid-mediated transmission of *mcr-1* by *Escherichia coli* ST155 from wastewater of long-term care facilities. Lai Z (Ed.). Microbiology Spectrum 12: e03707-23. https://doi.org/10.1128/spectrum.03707-23.

Feng J, Xiang Q, Ma J,et al (2021) Characterization of Carbapenem-Resistant Enterobacteriaceae Cultured From Retail Meat Products, Patients, and Porcine Excrement in China. Frontiers in Microbiology 12: 743468. https://doi.org/10.3389/fmicb.2021.743468.

Feng J, Jia M, Zhuang Y, et al (2024b) Prevalence, transmission and genomic epidemiology of mcr-1-positive colistin-resistant Escherichia coli strains isolated from international airplane waste, local resident fecal and wastewater treatment plants. Science of The Total Env*iro*nment 957: 177556. https://doi.org/10.1016/j.scitotenv.2024.177556.

Feng J, Jia M, Zhuang Y, et al (2024c) Prevalence, transmission and genomic epidemiology of mcr-1-positive colistin-resistant Escherichia coli strains isolated from international airplane waste, local resident fecal and wastewater treatment plants. Science of The Total Env*iro*nment 957: 177556. https://doi.org/10.1016/j.scitotenv.2024.177556.

Frank JA, Reich CI, Sharma S, et al (2008) Critical Evaluation of Two Primers Commonly Used for Amplification of Bacterial 16S rRNA Genes. Applied and Env*iro*nmental Microbiology 74: 2461–2470. https://doi.org/10.1128/AEM.02272-07.

Grant JR, Enns E, Marinier E,et al. (2023) Proksee: in-depth characterization and visualization of bacterial genomes. Nucleic Acids Research 51: W484–W492. https://doi.org/10.1093/nar/gkad326.

Guo C-H, Chu M-J, Liu T, et al (2024) High prevalence and transmission of blaNDM-positive Escherichia coli between farmed ducks and slaughtered meats: An increasing threat to food safety. International Journal of Food Microbiology 424: 110850. https://doi.org/10.1016/j.ijfoodmicro.2024.110850.

Gurevich A, Saveliev V, Vyahhi N, Tesler G (2013) QUAST: quality assessment tool for genome assemblies. Bioinformatics 29: 1072–1075. https://doi.org/10.1093/bioinformatics/btt086.

Heijnen L, Medema G (2006) Quantitative detection of E. coli, E. coli O157 and other shiga toxin producing E. coli in water samples using a culture method combined with real-time PCR. Journal of Water and Health 4: 487–498. https://doi.org/10.2166/wh.2006.0032.

Ji K, Xu Y, Sun J, et al (2020) Harnessing efficient multiplex PCR methods to detect the

expanding Tet(X) family of tigecycline resistance genes. Virulence 11: 49–56. https://doi.org/10.1080/21505594.2019.1706913.

Kong H, Hu Z, Zhang L, et al (2024) Clinical risk factors and outcomes of carbapenem-resistant *Escherichia coli* nosocomial infections in a Chinese teaching hospital: a retrospective study from 2013 to 2020. She RC (Ed.). Microbiology Spectrum 12: e04228-23. https://doi.org/10.1128/spectrum.04228-23.

Kürekci C, Lu X, Celil BG, et al (2022) Emergence and Characterization of Tigecycline Resistance Gene *tet* (X4) in ST609 Escherichia coli Isolates from Wastewater in Turkey. Garcia-Solache MA (Ed.). Microbiology Spectrum 10: e00732-22. https://doi.org/10.1128/spectrum.00732-22.

Lescat M, Poirel L, Nordmann P (2018) Rapid multiplex polymerase chain reaction for detection of mcr-1 to mcr-5 genes. Diagnostic Microbiology and Infectious Disease 92: 267–269. https://doi.org/10.1016/j.diagmicrobio.2018.04.010.

Li F, Cheng P, Li X, et al (2022) Molecular Epidemiology and Colistin-Resistant Mechanism of mcr-Positive and mcr-Negative Escherichia coli Isolated From Animal in Sichuan Province, China. Frontiers in Microbiology 13: 818548. https://doi.org/10.3389/fmicb.2022.818548.

Li Y, Li Y, Bu K, et al (2023) Antimicrobial Resistance and Genomic Epidemiology of tet(X4)-Bearing Bacteria of Pork Origin in Jiangsu, China. Genes 14: 36. https://doi.org/10.3390/genes14010036.

Lin M, Ali RAA, Khan MN, et al (2026) Genomic insights into *mcr* -mediated colistin resistance in *Escherichia coli, Aeromonas veronii* , and *Enterobacter kobei* from wastewater. Journal of Applied Microbiology 137: lxaf307. https://doi.org/10.1093/jambio/lxaf307.

Liu K-D, Jin W-J, Li R-B, et al (2023) Prevalence and molecular characteristics of mcr-1-positive Escherichia coli isolated from duck farms and the surrounding env*iro*nments in coastal China. Microbiological Research 270: 127348. https://doi.org/10.1016/j.micres.2023.127348.

Liu M, Liu J, Ma J, et al (2022) Antimicrobial Resistance and Molecular Characterization of Gene Cassettes from class 1 Integrons in Carbapenem-resistant Escherichia coli strains. Microbial Pathogenesis 170: 105669. https://doi.org/10.1016/j.micpath.2022.105669.

Ma Z, Wang B, Zeng D, et al (2024) Rapid Dissemination of blaNDM-5 Gene among Carbapenem-Resistant Escherichia coli Isolates in a Yellow-Feather Broiler Farm via Multiple Plasmid Replicon. Pathogens 13: 387. https://doi.org/10.3390/pathogens13050387.

Mai J, Liang Z, Xiong Z, et al (2023) Fecal carriage and molecular epidemiology of mcr-1-harboring Escherichia coli from children in southern China. Journal of Infection and Public Health 16: 1057–1063. https://doi.org/10.1016/j.jiph.2023.05.005.

Marathe NP, Svanevik CS, Ghavidel FZ, Grevskott DH (2021) First report of mobile tigecycline resistance gene tet(X4)-harbouring multidrug-resistant Escherichia coli from wastewater in Norway. Journal of Global Antimicrobial Resistance 27: 37–40. https://doi.org/10.1016/j.jgar.2021.07.019.

Mbanga J, Abia ALK, Amoako DG, Essack SY (2021) Longitudinal Surveillance of

Antibiotic Resistance in *Escherichia coli* and *Enterococcus* spp. from a Wastewater Treatment Plant and Its Associated Waters in KwaZulu-Natal, South Africa. Microbial Drug Resistance 27: 904–918. https://doi.org/10.1089/mdr.2020.0380.

Mei C-Y, Jiang Y, Ma Q-C, et al (2024) Low prevalence of mcr-1 in Escherichia coli from food-producing animals and food products in China. BMC Veterinary Research 20: 40. https://doi.org/10.1186/s12917-024-03891-6.

Mukherjee M, Laird E, Gentry TJ, et al (2021) Increased Antimicrobial and Multidrug Resistance Downstream of Wastewater Treatment Plants in an Urban Watershed. Frontiers in Microbiology 12: 657353. https://doi.org/10.3389/fmicb.2021.657353.

Park J-H, Bae K-S, Kang J, et al (2024a) Comprehensive Assessment of Multidrug-Resistant and Extraintestinal Pathogenic Escherichia coli in Wastewater Treatment Plant Effluents. Microorganisms 12: 1119. https://doi.org/10.3390/microorganisms12061119.

Park J-H, Bae K-S, Kang J, et al (2024b) Comprehensive Assessment of Multidrug-Resistant and Extraintestinal Pathogenic Escherichia coli in Wastewater Treatment Plant Effluents. Microorganisms 12: 1119. https://doi.org/10.3390/microorganisms12061119.

Parks DH, Imelfort M, Skennerton CT, et al (2015) CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Research 25: 1043–1055. https://doi.org/10.1101/gr.186072.114.

Price MN, Dehal PS, Arkin AP (2010) FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments. Poon AFY (Ed.). PLoS ONE 5: e9490. https://doi.org/10.1371/journal.pone.0009490.

Shen Y, Hu F, Wang Y, et al (2022) Transmission of Carbapenem Resistance Between Human and Animal NDM-Positive Escherichia coli Strains. Engineering 15: 24–33. https://doi.org/10.1016/j.eng.2021.07.030.

Sun H, Wan Y, Du P, et al (2021) Investigation of tigecycline resistant Escherichia coli from raw meat reveals potential transmission among food-producing animals. Food Control 121: 107633. https://doi.org/10.1016/j.foodcont.2020.107633.

Thanh PN, Xuan PH, Van CD, et al (2024) Antibiotic resistance genes, colistin-resistant Escherichia coli, and physicochemicals in health care wastewater in Vinh Long General Hospital, Vietnam. Env*iro*nmental Monitoring and Assessment 196: 1187. https://doi.org/10.1007/s10661-024-13345-z.

Wang Q, Lei C, Cheng H, et al (2022a) Widespread Dissemination of Plasmid-Mediated Tigecycline Resistance Gene *tet* (X4) in *Enterobacterales* of Porcine Origin. Shen Z (Ed.). Microbiology Spectrum 10: e01615-22. https://doi.org/10.1128/spectrum.01615-22.

Wang Q, Jin L, Sun S, et al (2022b) Occurrence of High Levels of Cefiderocol Resistance in Carbapenem-Resistant Escherichia coli before Its Approval in China: a Report from China CRE-Network. Tyne DV (Ed.). Microbiology Spectrum 10: e02670-21. https://doi.org/10.1128/spectrum.02670-21.

Weiß D, Engelmann I, Braun SD, et al (2017) A multiplex real-time PCR for the direct, fast, economic and simultaneous detection of the carbapenemase genes blaKPC, blaNDM, blaVIM and blaOXA-48. Journal of Microbiological Methods 142: 20–26.

https://doi.org/10.1016/j.mimet.2017.08.017.

Wick RR, Judd LM, Gorrie CL, Holt KE (2017) Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. Phillippy AM (Ed.). PLOS Computational Biology 13: e1005595. https://doi.org/10.1371/journal.pcbi.1005595.

Wick RR, Judd LM, Cerdeira LT, et al (2021) Trycycler: consensus long-read assemblies for bacterial genomes. Genome Biology 22: 266. https://doi.org/10.1186/s13059-021-02483-z.

Xie J, Chen Y, Cai G, et al (2023) Tree Visualization By One Table (tvBOT): a web application for visualizing, modifying and annotating phylogenetic trees. Nucleic Acids Research 51: W587–W592. https://doi.org/10.1093/nar/gkad359.

Yamamoto N, Kawahara R, Akeda Y, et al (2017) Development of selective medium for IMP-type carbapenemase-producing Enterobacteriaceae in stool specimens. BMC Infectious Diseases 17: 229. https://doi.org/10.1186/s12879-017-2312-1.

Yan Z, Wang P, Wang H, et al (2024) Emergence and genomic epidemiology of tigecycline resistant bacteria of fly origin across urban and rural China. Env*iro*nment International 193: 109099. https://doi.org/10.1016/j.envint.2024.109099.

Yang J, Xiao G, Xiao N, et al (2023) Characteristics of tet(X4)−Producing Escherichia coli in Chicken and Pig Farms in Hunan Province, China. Antibiotics 12: 147. https://doi.org/10.3390/antibiotics12010147.

Yang Y-Q, Li Y-X, Song T, et al (2017) Colistin Resistance Gene *mcr-1* and Its Variant in Escherichia coli Isolates from Chickens in China. Antimicrobial Agents and Chemotherapy 61: e01204-16. https://doi.org/10.1128/AAC.01204-16.

Yu Y, Cui C-Y, Kuang X, et al (2021a) Prevalence of tet(X4) in Escherichia coli From Duck Farms in Southeast China. Frontiers in Microbiology 12: 716393. https://doi.org/10.3389/fmicb.2021.716393.

Yu Y, Cui C-Y, Kuang X, et al (2021b) Prevalence of tet(X4) in Escherichia coli From Duck Farms in Southeast China. Frontiers in Microbiology 12: 716393. https://doi.org/10.3389/fmicb.2021.716393.

Zeng Y, Deng L, Zhou X, et al (2022) Prevalence and risk factors of tet(X4)-positive Enterobacteriaceae in human gut microbiota. Journal of Global Antimicrobial Resistance 31: 15–21. https://doi.org/10.1016/j.jgar.2022.07.014.

Zhai W, Wang T, Yang D, et al (2022) Clonal relationship of *tet* (X4)-positive *Escherichia coli* ST761 isolates between animals and humans. Journal of Antimicrobial Chemotherapy 77: 2153–2157. https://doi.org/10.1093/jac/dkac175.

Zhang R, Li Y, Chen J, et al (2023a) Population genomic analysis reveals the emergence of high-risk carbapenem-resistant Escherichia coli among ICU patients in China. Journal of Infection 86: 316–328. https://doi.org/10.1016/j.jinf.2023.02.004.

Zhang S, Wen J, Wang Y, et al (2023b) Decoding the enigma: unveiling the molecular transmission of avian-associated tet(X4)-positive E. coli in Sichuan Province, China. Poultry Science 102: 103142. https://doi.org/10.1016/j.psj.2023.103142.

Zhang W, Zhang T, Wang C, et al (2022) Prevalence of colistin resistance gene mcr-1 in Escherichia coli isolated from chickens in central China, 2014 to 2019. Journal of Global Antimicrobial Resistance 29: 241–246.

https://doi.org/10.1016/j.jgar.2022.03.024.

Zhou H, Wang H, Chen K, et al (2024) Epidemiological and genomic analysis revealed the significant role of flies in dissemination of carbapenem-resistant Enterobacteriaceae (CRE) in China. Journal of Hazardous Materials 480: 136374. https://doi.org/10.1016/j.jhazmat.2024.136374.

Zhou S, Liu B, Zheng D, et al (2025) VFDB 2025: an integrated resource for exploring anti-virulence compounds. Nucleic Acids Research 53: D871–D877. https://doi.org/10.1093/nar/gkae968.

Zomorodi AR, Samaei MR, Salimizand H, et al (2025) High prevalence of MDR and XDR Escherichia coli in hospital wastewater from Shiraz, Iran: ESBL and carbapenemase production. BMC Microbiology 25: 647. https://doi.org/10.1186/s12866-025-04398-2.

Wastewater Number	Drug resistance	Unique Number	Group	TIG resistance gene
WWA	TIG	WWA_TIG_1	B1	tet(X4)
WWA	TIG	WWA_TIG_2	B1	tet(X4)
WWA	TIG	WWA_TIG_3	A	tet(X4)
WWA	TIG	WWA_TIG_4	B1	tet(X4)
WWA	TIG	WWA_TIG_5	A	tet(X4)
WWA	TIG	WWA_TIG_6	D	tet(X4)
WWA	TIG	WWA_TIG_7	D	tet(X4)
WWA	TIG	WWA_TIG_9	A	tet(X4)
WWA	TIG	WWA_TIG_11	A	tet(X4)
WWA	TIG	WWA_TIG_13	E/clade I	tet(X4)
WWA	TIG	WWA_TIG_14	B1	tet(X4)
WWA	TIG	WWA_TIG_15	D	tet(X4)
WWA	MEM	WWA_MEM_1	B1	None
WWA	MEM	WWA_MEM_2	B1	None
WWA	MEM	WWA_MEM_3	C	None
WWA	MEM	WWA_MEM_4	C	None
WWA	MEM	WWA_MEM_5	F	None
WWA	MEM	WWA_MEM_6	A	None
WWA	MEM	WWA_MEM_7	A	None
WWA	MEM	WWA_MEM_8	F	None
WWA	MEM	WWA_MEM_9	C	None
WWA	MEM	WWA_MEM_10	D	None
WWA	MEM	WWA_MEM_11	A	None
WWA	MEM	WWA_MEM_12	B1	None
WWA	CT	WWA_CT_1	B1	None
WWA	CT	WWA_CT_2	B1	None
WWA	CT	WWA_CT_3	B1	None
WWA	CT	WWA_CT_4	A	None
WWA	CT	WWA_CT_5	B1	None
WWD	CT	WWD_CT_1	B1	None
WWA	MEM	WWA_MEM_13	B1	None
WWA	MEM	WWA_MEM_14	B1	None
WWA	CT	WWA_CT_6	B1	None
WWA	CT	WWA_CT_7	A	None
WWB	TIG	WWB_TIG_3	B1	tet(X4)
WWB	TIG	WWB_TIG_5	A	tet(X4)
WWB	TIG	WWB_TIG_7	B1	tet(X4)
WWB	TIG	WWB_TIG_16	A	tet(X4)
WWB	MEM	WWB_MEM_1	A	None
WWB	MEM	WWB_MEM_2	F	None
WWE	MEM	WWE_MEM_1	F	None
WWE	MEM	WWE_MEM_2	F	None
WWE	MEM	WWE_MEM_3	B1	None
WWE	MEM	WWE_MEM_4	B1	tet(X4)
WWE	MEM	WWE_MEM_5	F	None
WWE	MEM	WWE_MEM_6	F	None
WWB	MEM	WWB_MEM_11	A	None
WWE	MEM	WWE_MEM_9	B1	None
WWE	MEM	WWE_MEM_10	B1	None
WWH	TIG	WWH_TIG_1	B1	tet(X4)
WWH	TIG	WWH_TIG_3	A	tet(X4)
WWH	TIG	WWH_TIG_6	A	tet(X4)

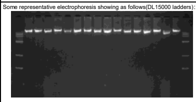
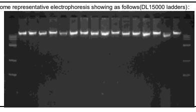
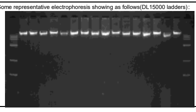
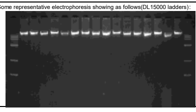
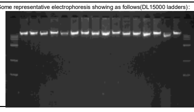
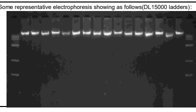
WWH	TIG	WWH_TIG_7	B1	tet(X4)
WWH	TIG	WWH_TIG_12	A	tet(X4)
WWH	TIG	WWH_TIG_15	A	tet(X4)
WWC	TIG	WWC_TIG_1	B1	tet(X4)
WWC	TIG	WWC_TIG_5	B1	tet(X4)
WWC	TIG	WWC_TIG_7	A	tet(X4)
WWC	TIG	WWC_TIG_9	D	tet(X4)
WWC	TIG	WWC_TIG_10	D	tet(X4)
WWC	TIG	WWC_TIG_12	C	tet(X4)
WWC	TIG	WWC_TIG_13	E/clade I	tet(X4)
WWC	TIG	WWC_TIG_14	A	tet(X4)
WWC	TIG	WWC_TIG_15	B1	tet(X4)
WWC	TIG	WWC_TIG_16	A	tet(X4)
WWD	TIG	WWD_TIG_3	A	tet(X4)
WWD	TIG	WWD_TIG_4	A	tet(X4)
WWD	TIG	WWD_TIG_7	B1	tet(X4)
WWD	TIG	WWD_TIG_8	A	tet(X4)
WWE	TIG	WWE_TIG_2	B1	tet(X4)
WWE	TIG	WWE_TIG_3	A	tet(X4)
WWE	TIG	WWE_TIG_4	B2	tet(X4)
WWD	TIG	WWD_TIG_14	B1	tet(X4)
WWD	TIG	WWD_TIG_15	B1	tet(X4)
WWE	TIG	WWE_TIG_7	C	tet(X4)
WWE	TIG	WWE_TIG_8	B1	tet(X4)
WWB	CT	WWB_CT_1	B1	None
WWB	CT	WWB_CT_2	A	None
WWF	TIG	WWF_TIG_1	B1	tet(X4)
WWF	TIG	WWF_TIG_4	A	tet(X4)
WWF	TIG	WWF_TIG_7	A	tet(X4)
WWF	TIG	WWF_TIG_9	B1	tet(X4)
WWF	TIG	WWF_TIG_12	A	tet(X4)
WWF	TIG	WWF_TIG_13	A	tet(X4)
WWF	TIG	WWF_TIG_15	A	tet(X4)
WWG	TIG	WWG_TIG_1	A	tet(X4)
WWG	TIG	WWG_TIG_2	A	tet(X4)
WWG	TIG	WWG_TIG_3	A	tet(X4)
WWG	TIG	WWG_TIG_4	A	tet(X4)
WWG	TIG	WWG_TIG_5	B1	tet(X4)
WWG	TIG	WWG_TIG_6	B1	tet(X4)
WWG	TIG	WWG_TIG_7	B1	tet(X4)
WWH	TIG	WWH_TIG_16	A	tet(X4)
WWH	CT	WWH_CT_17	A	None
WWH	CT	WWH_CT_19	A	None
WWH	CT	WWH_CT_20	B1	None
WWH	CT	WWH_CT_21	A	None
WWH	CT	WWH_CT_22	B1	None
WWH	CT	WWH_CT_23	A	None
WWH	CT	WWH_CT_24	B1	None
WWC	CT	WWC_CT_1	B1	None
WWE	CT	WWE_CT_9	A	None
WWE	CT	WWE_CT_10	B1	None
WWE	CT	WWE_CT_11	B1	None
WWF	CT	WWF_CT_1	A	None
WWF	CT	WWF_CT_3	B1	None

WWF	CT	WWF_CT_2	B1	None
WWF	CT	WWF_CT_4	A	None
WWG	CT	WWG_CT_1	B1	None
WWG	CT	WWG_CT_2	F	None
WWG	CT	WWG_CT_3	A	None
WWG	CT	WWG_CT_7	G	None
WWG	CT	WWG_CT_9	B1	None
WWG	CT	WWG_CT_12	B1	None
WWC	CT	WWC_CT_2	F	None
WWH	MEM	WWH_MEM_2	B1	None
WWH	MEM	WWH_MEM_3	D	None
WWC	MEM	WWC_MEM_1	A	None
WWC	MEM	WWC_MEM_2	A	None
WWC	MEM	WWC_MEM_3	A	None
WWC	MEM	WWC_MEM_4	C	None
WWC	MEM	WWC_MEM_7	D	None
WWF	MEM	WWF_MEM_3	A	None
WWF	MEM	WWF_MEM_5	F	None
WWG	MEM	WWG_MEM_1	B1	None
WWG	MEM	WWG_MEM_5	A	None
WWG	MEM	WWG_MEM_7	B1	None
WWG	MEM	WWG_MEM_8	F	None
WWF	MEM	WWF_MEM_5	A	None
WWF	MEM	WWF_MEM_6	C	None
WWF	MEM	WWF_MEM_7	A	None
WWF	MEM	WWF_MEM_8	A	None
WWF	MEM	WWF_MEM_9	B1	None
WWG	MEM	WWG_MEM_11	B1	None
WWG	MEM	WWG_MEM_12	B1	None
WWG	MEM	WWG_MEM_14	D	None
WWG	MEM	WWG_MEM_15	B1	None
WWB	MEM	WWB_MEM_13	F	None
WWB	MEM	WWB_MEM_14	B1	None
WWD	MEM	WWD_MEM_18	B1	None
WWG	MEM	WWG_MEM_16	D	None
WWG	MEM	WWG_MEM_17	B1	None

MEM resistance gene	CT resistance gene	MIC (μg/mL)							
		TIG	MEM	CT	CTX	CIP	AZM	SXT	AMI
None	None	8	0	0	16	1	0	160	4
None	None	8	0	0	0	1	64	160	4
None	None	8	0	0	0	1	0	160	0
None	None	8	0	0	16	1	16	160	0
None	None	4	0	0	0	1	0	160	0
None	None	16	0	0	0	1	0	160	4
None	None	8	0	0	4	1	0	160	0
None	None	8	0	0	16	1	4	160	0
None	None	8	0	0	0	1	4	160	0
None	None	4	0	0	16	1	0	160	0
None	None	4	0	0	16	1	4	160	0
None	None	8	0	0	0	1	64	160	4
blaNDM	None	0	4	0	16	1	0	160	4
blaNDM	None	4	4	0	16	1	0	160	0
blaNDM	None	2	4	0	16	1	64	160	0
blaNDM	None	2	4	0	16	1	32	160	64
blaNDM	None	1	4	0	16	1	32	160	64
blaNDM	None	0	4	0	16	1	4	160	4
blaNDM	None	2	4	0	16	1	64	160	8
blaNDM	None	0	4	0	16	1	16	160	8
blaNDM	None	0	4	0	16	1	64	160	64
blaNDM	None	0	4	0	16	1	0	160	0
blaNDM	mcr-1	1	4	4	16	1	64	160	0
blaNDM	None	2	4	0	16	1	4	160	64
None	mcr-1	0	0	4	0	1	4	160	0
None	mcr-1	0	0	4	16	1	0	160	0
None	mcr-1	1	0	4	16	1	4	160	64
None	mcr-1	0	0	2	16	1	32	160	0
None	mcr-1	0	0	4	0	1	16	160	8
None	mcr-1	0	0	2	16	1	32	160	0
blaNDM	None	0	4	0	16	1	32	160	0
blaNDM	mcr-1	0	4	2	16	1	64	160	64
None	mcr-1	0	0	2	16	1	4	80	4
None	mcr-1	0	0	2	16	1	32	160	4
None	None	16	0	0	16	1	0	160	0
None	None	8	0	0	16	1	0	160	0
None	None	8	0	0	16	1	0	160	8
None	None	8	0	0	16	1	0	20	0
blaNDM	None	0	4	0	16	1	64	160	8
blaNDM	None	1	4	0	16	1	64	160	64
blaNDM	None	0	4	1	16	1	64	160	64
blaNDM	mcr-1	0	4	4	16	1	0	160	8
blaNDM	None	0	4	0	16	1	4	160	0
blaNDM	None	8	4	0	16	1	64	160	0
blaNDM	None	0	4	0	16	1	16	160	0
blaNDM	None	0	4	1	16	1	16	160	4
blaNDM	None	0	4	0	16	1	64	160	0
blaNDM	None	0	4	0	16	1	64	160	0
blaNDM	mcr-1	0	4	4	16	1	64	160	4
None	None	16	0	0	16	1	16	160	4
None	None	8	0	0	0	1	4	160	16
None	None	8	0	0	16	1	32	160	0

None	None	16	0	0	0	1	4	160	0
None	None	16	0	0.5	16	1	0	160	0
None	None	8	0	0	16	1	0	160	4
None	None	8	0	0	0	1	0	160	0
None	None	8	0	0	16	1	0	160	0
None	None	8	0	0	0	1	0	160	4
None	None	16	0	0.5	0	1	0	160	0
None	None	8	0	1	16	1	0	160	8
None	None	8	0	0	2	1	0	160	0
None	None	16	0	0	0	1	64	160	0
None	None	8	0	0.5	2	1	4	80	0
None	None	8	0	0	0	1	64	160	16
None	None	16	0	0	16	1	16	160	0
None	None	16	0	0	0	1	4	10	8
None	None	16	0	0	16	1	64	160	0
None	None	16	0	0	16	1	0	160	4
None	None	16	0	0	16	1	0	160	4
None	None	16	0	0.5	4	1	0	160	32
None	None	16	0	0	16	1	4	160	0
None	None	16	0	0	0	1	4	40	0
None	None	8	0.5	0	16	1	16	160	8
None	None	8	0	0	0	1	0	160	0
None	None	8	0	0	16	1	0	160	0
None	None	8	0	0	16	1	8	160	4
blaNDM	mcr-1	2	4	4	16	1	4	160	0
None	mcr-1	1	0	4	16	1	64	160	4
None	None	8	0	0	16	1	0	160	4
None	None	8	0	0	8	1	0	160	4
None	None	8	0	0	16	1	4	160	0
None	None	4	0	0	0	1	0	0	0
None	None	8	0	0	0	1	64	160	0
None	None	8	0	0	16	1	64	160	0
None	None	8	0	0	0	1	0	160	0
None	None	8	0	0	16	1	4	160	4
None	None	8	0	0	0	1	4	160	4
None	None	8	0	0	0	1	0	160	0
None	None	8	0	0	16	1	64	160	0
None	None	8	0	0	0	1	0	10	0
None	None	8	0	0	16	1	0	160	4
None	None	8	0	0	16	1	64	160	4
None	None	8	0	0	0	1	4	10	0
None	mcr-1	1	0	2	16	1	64	160	64
None	mcr-1	0	0	2	16	1	64	160	64
None	mcr-1	2	0	4	16	1	64	160	8
None	mcr-1	2	0	4	16	1	64	160	64
None	mcr-1	0	0	4	16	1	0	160	0
None	mcr-1	1	0	4	16	1	16	160	0
None	mcr-1	4	0	2	0	1	4	160	32
None	mcr-1	0	0	2	0	1	0	160	0
None	mcr-1	0	0	2	16	1	0	160	0
None	mcr-1	0	0	2	16	1	0	160	0
None	mcr-1	0	0	4	0	1	0	0	0
blaNDM	mcr-1	0	0	2	0	1	4	160	0
None	mcr-1	0	0	4	16	1	16	20	0

blaNDM	mcr-1	0	4	4	16	1	64	160	0
None	mcr-1	0	0	2	0	1	0	40	0
None	mcr-1	0	0	2	0	1	0	160	0
None	mcr-1	0	0	4	16	1	8	160	4
None	mcr-1	0	0	2	16	1	4	160	4
None	mcr-1	0	0	2	0	1	0	160	0
None	mcr-1	0	0	2	16	1	0	160	8
None	mcr-1	0	0	2	16	1	0	160	0
None	mcr-1	0	0	2	16	1	64	160	4
blaNDM	None	1	4	0	16	1	64	160	4
blaNDM	mcr-1	0	4	4	16	1	0	160	4
blaNDM	None	2	4	0	16	1	64	160	64
blaNDM	None	0	4	0	16	1	4	160	8
blaNDM	None	0	4	0	16	1	64	160	4
blaNDM	None	0	4	0	16	1	16	160	4
blaNDM	None	0	4	0	16	1	4	160	4
blaNDM	None	0	4	0	16	1	16	160	0
blaNDM	None	0	4	0	16	1	64	160	0
blaNDM	None	0	4	0	16	1	64	160	0
blaNDM	None	0	4	0	16	1	4	160	4
blaNDM	None	4	4	0	16	1	64	160	4
blaNDM	None	4	4	0	16	1	4	160	0
blaNDM	None	0	4	0	16	1	4	160	4
blaNDM	None	1	4	0	16	1	4	160	4
blaNDM	None	0	4	0	16	1	16	160	4
blaNDM	None	1	4	0.5	16	1	32	160	0
blaNDM	None	1	4	0	16	1	8	160	0
blaNDM	None	1	4	0.5	16	1	4	160	0
blaNDM	None	1	4	0	16	1	4	160	4
blaNDM	None	1	4	0	16	1	64	160	4
blaNDM	None	1	4	0	16	1	4	160	0
blaNDM	None	4	4	0	16	1	64	160	64
blaNDM	mcr-1	0	4	2	16	1	4	160	0
blaNDM	mcr-1	0	4	4	16	1	8	160	8
blaNDM	None	0	4	0	16	1	64	160	4
blaNDM	None	0	4	1	16	1	8	160	8

Item to check	Importance	Remarks	Remarks	Remarks	Remarks	Remarks	Remarks
Experimental design		Bacteria samples for tet(X4)	Bacteria samples for mcr-1	Bacteria samples for mcr-3	Bacteria samples for blaNDM	Bacteria samples for blaKPC	Bacteria samples for blaOXA48
Definition of experimental and control groups	Essential	Bacteria isolated from wastewater samples that showed resistance to tigecycline	Bacteria isolated from wastewater samples that showed resistance to colistin	Bacteria isolated from wastewater samples that showed resistance to colistin	Bacteria isolated from wastewater samples that showed resistance to meropenem	Bacteria isolated from wastewater samples that showed resistance to meropenem	Bacteria isolated from wastewater samples that showed resistance to meropenem
Number within each group	Essential						
Assay carried out by the core or investigator's laboratory?	Desirable	Investigator's laboratory	Investigator's laboratory	Investigator's laboratory	Investigator's laboratory	Investigator's laboratory	Investigator's laboratory
Acknowledgment of authors' contributions	Desirable	Designed the primer and probe, experiment, data analysis	Designed the primer and probe, experiment, data analysis	Designed the primer and probe, experiment, data analysis	Experiment, data analysis	Experiment, data analysis	Experiment, data analysis
SAMPLE							
Description	Essential	Bacteria isolated from wastewater samples that showed resistance to tigecycline. Genomic DNA was extracted from bacterial culture for the identification of emerging AMR gene tet(X4)	Bacteria isolated from wastewater samples that showed resistance to tigecycline. Genomic DNA was extracted from bacterial culture for the identification of emerging AMR gene mcr-1	Bacteria isolated from wastewater samples that showed resistance to tigecycline. Genomic DNA was extracted from bacterial culture for the identification of emerging AMR gene mcr-3	Bacteria isolated from wastewater samples that showed resistance to tigecycline. Genomic DNA was extracted from bacterial culture for the identification of emerging AMR gene blaNDM	Bacteria isolated from wastewater samples that showed resistance to tigecycline. Genomic DNA was extracted from bacterial culture for the identification of emerging AMR gene blaKPC	Bacteria isolated from wastewater samples that showed resistance to tigecycline. Genomic DNA was extracted from bacterial culture for the identification of emerging AMR gene blaOXA48
Volume/mass of sample processed	Desirable	200 μL of overnight bacteria culture	200 μL of overnight bacteria culture	200 μL of overnight bacteria culture	200 μL of overnight bacteria culture	200 μL of overnight bacteria culture	200 μL of overnight bacteria culture
Microdissection or	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Processing procedure	Essential	Centrifuge at 15000g for 2 min to pellet the bacteria and discard the culture	Centrifuge at 15000g for 2 min to pellet the bacteria and discard the culture	Centrifuge at 15000g for 2 min to pellet the bacteria and discard the culture	Centrifuge at 15000g for 2 min to pellet the bacteria and discard the culture	Centrifuge at 15000g for 2 min to pellet the bacteria and discard the culture	Centrifuge at 15000g for 2 min to pellet the bacteria and discard the culture
If frozen, how and how quickly?	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
If fixed, with what and how quickly?	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Sample storage conditions and duration	Essential	All the bateria culture are fresh and free of storge before nucleic acid extraction	All the bateria culture are fresh and free of storge before nucleic acid extraction	All the bateria culture are fresh and free of storge before nucleic acid extraction	All the bateria culture are fresh and free of storge before nucleic acid extraction	All the bateria culture are fresh and free of storge before nucleic acid extraction	All the bateria culture are fresh and free of storge before nucleic acid extraction
Procedure and/or instrumentation	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Name of kit and details of any modifications	Essential	TGuide Smart Universal DNA Kit with lysozyme treatment	TGuide Smart Universal DNA Kit with lysozyme treatment	TGuide Smart Universal DNA Kit with lysozyme treatment	TGuide Smart Universal DNA Kit with lysozyme treatment	TGuide Smart Universal DNA Kit with lysozyme treatment	TGuide Smart Universal DNA Kit with lysozyme treatment
Source of additional reagents	Desirable	lysozyme (Sangon)	lysozyme (Sangon)	lysozyme (Sangon)	lysozyme (Sangon)	lysozyme (Sangon)	lysozyme (Sangon)
Details of DNase or RNase treatment	Essential	Using the RNase A treatment for the removal of RNA	Using the RNase A treatment for the removal of RNA	Using the RNase A treatment for the removal of RNA	Using the RNase A treatment for the removal of RNA	Using the RNase A treatment for the removal of RNA	Using the RNase A treatment for the removal of RNA
Contamination assessment (DNA or RNA)	Essential	A reagent blank was extracted as a negative control, which showed negative results for the tet(X4) detection	A reagent blank was extracted as a negative control, which showed negative results for the mcr-1 detection	A reagent blank was extracted as a negative control, which showed negative results for the mcr-3 detection	A reagent blank was extracted as a negative control, which showed negative results for the mcr-1 detection	A reagent blank was extracted as a negative control, which showed negative results for the mcr-3 detection	A reagent blank was extracted as a negative control, which showed negative results for the mcr-3 detection
Nucleic acid quantification	Essential	All the samples were quantified using the Qubit HS dsDNA Kit on the Qubit 4 platform, with all the DNA concentration ranging from 7.6 to 25.4 ng/μl	All the samples were quantified using the Qubit HS dsDNA Kit on the Qubit 4 platform, with all the DNA concentration ranging from 7.6 to 25.4 ng/μl	All the samples were quantified using the Qubit HS dsDNA Kit on the Qubit 4 platform, with all the DNA concentration ranging from 7.6 to 25.4 ng/μl	All the samples were quantified using the Qubit HS dsDNA Kit on the Qubit 4 platform, with all the DNA concentration ranging from 7.6 to 25.4 ng/μl	All the samples were quantified using the Qubit HS dsDNA Kit on the Qubit 4 platform, with all the DNA concentration ranging from 7.6 to 25.4 ng/μl	All the samples were quantified using the Qubit HS dsDNA Kit on the Qubit 4 platform, with all the DNA concentration ranging from 7.6 to 25.4 ng/μl
Instrument and method	Essential	Qubit HS dsDNA Assay/Qubit 3.0(instrument)	Qubit HS dsDNA Assay/Qubit 3.0(instrument)	Qubit HS dsDNA Assay/Qubit 3.0(instrument)	Qubit HS dsDNA Assay/Qubit 3.0(instrument)	Qubit HS dsDNA Assay/Qubit 3.0(instrument)	Qubit HS dsDNA Assay/Qubit 3.0(instrument)
Purity	Desirable						
Yield	Desirable	7.6 to 25.4 ng/μL. Measured by Qubit HS dsDNA Assay	7.6 to 25.4 ng/μL. Measured by Qubit HS dsDNA Assay	7.6 to 25.4 ng/μL. Measured by Qubit HS dsDNA Assay	7.6 to 25.4 ng/μL. Measured by Qubit HS dsDNA Assay	7.6 to 25.4 ng/μL. Measured by Qubit HS dsDNA Assay	7.6 to 25.4 ng/μL. Measured by Qubit HS dsDNA Assay
RNA integrity: method/instrument	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
RIN/RQI or Cq of 3' and 5' transcripts	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Electrophoresis traces	Desirable	Some representative electrophoresis showing as follows(DL15000 ladders): 	Some representative electrophoresis showing as follows(DL15000 ladders): 	Some representative electrophoresis showing as follows(DL15000 ladders): 	Some representative electrophoresis showing as follows(DL15000 ladders): 	Some representative electrophoresis showing as follows(DL15000 ladders): 	Some representative electrophoresis showing as follows(DL15000 ladders): 
Inhibition testing (Cq dilutions, spike, or other)	Essential	The inhibition testing was conducted through a series of 10-fold dilutions of the representative extracted DNA, which showed no significant changes in its quantitative value	The inhibition testing was conducted through a series of 10-fold dilutions of the representative extracted DNA, which showed no significant changes in its quantitative value	The inhibition testing was conducted through a series of 10-fold dilutions of the representative extracted DNA, which showed no significant changes in its quantitative value	The inhibition testing was conducted through a series of 10-fold dilutions of the representative extracted DNA, which showed no significant changes in its quantitative value	The inhibition testing was conducted through a series of 10-fold dilutions of the representative extracted DNA, which showed no significant changes in its quantitative value	The inhibition testing was conducted through a series of 10-fold dilutions of the representative extracted DNA, which showed no significant changes in its quantitative value
REVERSE TRANSCRIPTION							
Complete reaction	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Amount of RNA and reaction volume	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Priming oligonucleotide (if using GSP) and concentration	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Reverse transcriptase and concentration	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Temperature and time	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Manufacturer of reagents and catalogue	Desirable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Cqs with and without reverse transcription	Desirable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Storage conditions of	Desirable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
qPCR PROTOCOL							
Complete reaction conditions	Essential	For RT-qPCR, Hieff UNICON™ Hotstart Direct DNA Polymerase and self-prepared TaqMan Buffer was used with 5 μl of DNA template and standardized primers and probes with concentrations of 200 nM and 100 nM, respectively. PCR cycler conditions were initial denaturation for 5 mins at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 63 °C on the Applied Biosystems Quantstudio 5 qPCR machine (ThermoFisher). For every batch of RT-qPCR detection assays, a non-template control (NTC) and a series of standard samples were included	For RT-qPCR, Hieff UNICON™ Hotstart Direct DNA Polymerase and self-prepared TaqMan Buffer was used with 5 μl of DNA template and standardized primers and probes with concentrations of 400 nM and 200 nM, respectively. PCR cycler conditions were initial denaturation for 5 mins at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C on the Applied Biosystems Quantstudio 5 qPCR machine (ThermoFisher). For every batch of RT-qPCR detection assays, a non-template control (NTC) and a series of standard samples were included	For RT-qPCR, Hieff UNICON™ Hotstart Direct DNA Polymerase and self-prepared TaqMan Buffer was used with 5 μl of DNA template and standardized primers and probes with concentrations of 400 nM and 200 nM, respectively. PCR cycler conditions were initial denaturation for 5 mins at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C on the Applied Biosystems Quantstudio 5 qPCR machine (ThermoFisher). For every batch of RT-qPCR detection assays, a non-template control (NTC) and a series of standard samples were included	For RT-qPCR, Hieff UNICON™ Hotstart Direct DNA Polymerase and self-prepared TaqMan Buffer was used with 5 μl of DNA template and standardized primers and probes with concentrations of 400 nM and 200 nM, respectively. PCR cycler conditions were initial denaturation for 5 mins at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C on the Applied Biosystems Quantstudio 5 qPCR machine (ThermoFisher). For every batch of RT-qPCR detection assays, a non-template control (NTC) and a series of standard samples were included	For RT-qPCR, Hieff UNICON™ Hotstart Direct DNA Polymerase and self-prepared TaqMan Buffer was used with 5 μl of DNA template and standardized primers and probes with concentrations of 400 nM and 200 nM, respectively. PCR cycler conditions were initial denaturation for 5 mins at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C on the Applied Biosystems Quantstudio 5 qPCR machine (ThermoFisher). For every batch of RT-qPCR detection assays, a non-template control (NTC) and a series of standard samples were included	For RT-qPCR, Hieff UNICON™ Hotstart Direct DNA Polymerase and self-prepared TaqMan Buffer was used with 5 μl of DNA template and standardized primers and probes with concentrations of 400 nM and 200 nM, respectively. PCR cycler conditions were initial denaturation for 5 mins at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C on the Applied Biosystems Quantstudio 5 qPCR machine (ThermoFisher). For every batch of RT-qPCR detection assays, a non-template control (NTC) and a series of standard samples were included
Reaction volume and amount of cDNA/DNA	Essential	Final reaction volume of 20 μL, containing 5 μL of 100-fold diluted extracted bacteria DNA.	Final reaction volume of 20 μL, containing 5 μL of 100-fold diluted extracted bacteria DNA.	Final reaction volume of 20 μL, containing 5 μL of 100-fold diluted extracted bacteria DNA.	Final reaction volume of 20 μL, containing 5 μL of 100-fold diluted extracted bacteria DNA.	Final reaction volume of 20 μL, containing 5 μL of 100-fold diluted extracted bacteria DNA.	Final reaction volume of 20 μL, containing 5 μL of 100-fold diluted extracted bacteria DNA.
Primer, (probe), Mg2, and dNTP concentrations	Essential	The final concentration of the all primers were 200 nM and the probe was at 100 nM	The final concentration of the all primers were 400 nM and the probe was at 200 nM	The final concentration of the all primers were 400 nM and the probe was at 200 nM	The final concentration of the all primers were 400 nM and the probe was at 200 nM	The final concentration of the all primers were 400 nM and the probe was at 200 nM	The final concentration of the all primers were 400 nM and the probe was at 200 nM
Polymerase identity and concentration	Essential	Hieff UNICON™ Hotstart Direct DNA Polymerase, 2U/20 μL reaction	Hieff UNICON™ Hotstart Direct DNA Polymerase, 2U/20 μL reaction	Hieff UNICON™ Hotstart Direct DNA Polymerase, 2U/20 μL reaction	Hieff UNICON™ Hotstart Direct DNA Polymerase, 2U/20 μL reaction	Hieff UNICON™ Hotstart Direct DNA Polymerase, 2U/20 μL reaction	Hieff UNICON™ Hotstart Direct DNA Polymerase, 2U/20 μL reaction
Buffer/kit identity and manufacturer	Essential	Self prepared Taqman qPCR Buffer	Self prepared Taqman qPCR Buffer	Self prepared Taqman qPCR Buffer	Self prepared Taqman qPCR Buffer	Self prepared Taqman qPCR Buffer	Self prepared Taqman qPCR Buffer
Exact chemical composition of the buffer	Desirable	1×Taq Buffer, 4 mM MgCl2, 0.2 mM dNTPs(each), 100ng/μL BSA, 0.3 mol/L Trehalose	1×Taq Buffer, 6 mM MgCl2, 0.5 mM dNTPs(each), 100ng/μL BSA, 0.3 mol/L Trehalose	1×Taq Buffer, 6 mM MgCl2, 0.5 mM dNTPs(each), 100ng/μL BSA, 0.3 mol/L Trehalose	1×Taq Buffer, 6 mM MgCl2, 0.5 mM dNTPs(each), 100ng/μL BSA, 0.3 mol/L Trehalose	1×Taq Buffer, 6 mM MgCl2, 0.5 mM dNTPs(each), 100ng/μL BSA, 0.3 mol/L Trehalose	1×Taq Buffer, 6 mM MgCl2, 0.5 mM dNTPs(each), 100ng/μL BSA, 0.3 mol/L Trehalose
Additives (SYBR Green I, DMSO, and so	Essential	No additional addings	No additional addings	No additional addings	No additional addings	No additional addings	No additional addings
Manufacturer of plates/tubes and catalog	Desirable	Tubes：PCR-2CP-RT-C, Axygen	Tubes：PCR-2CP-RT-C, Axygen	Tubes：PCR-2CP-RT-C, Axygen	Tubes：PCR-2CP-RT-C, Axygen	Tubes：PCR-2CP-RT-C, Axygen	Tubes：PCR-2CP-RT-C, Axygen
Complete thermocycling parameters	Essential	5min at 95°C, followed by 40 cycles of 15s at 95°C and 30s 63°C	5min at 95°C, followed by 40 cycles of 15s at 95°C and 30s 60°C	5min at 95°C, followed by 40 cycles of 15s at 95°C and 30s 60°C	5min at 95°C, followed by 40 cycles of 15s at 95°C and 30s 60°C	5min at 95°C, followed by 40 cycles of 15s at 95°C and 30s 60°C	5min at 95°C, followed by 40 cycles of 15s at 95°C and 30s 60°C
Reaction setup (manual/robotic)	Desirable	manual	manual	manual	manual	manual	manual
Manufacturer of qPCR instrument	Desirable	Applied Biosystems Quantstudio 3 qPCR machine (ThermoFisher)	Applied Biosystems Quantstudio 3 qPCR machine (ThermoFisher)	Applied Biosystems Quantstudio 3 qPCR machine (ThermoFisher)	Applied Biosystems Quantstudio 3 qPCR machine (ThermoFisher)	Applied Biosystems Quantstudio 3 qPCR machine (ThermoFisher)	Applied Biosystems Quantstudio 3 qPCR machine (ThermoFisher)
qPCR VALIDATION							
Evidence of optimization (from	Desirable	No optimization was conducted since original result has been satisfactory	No optimization was conducted since original result has been satisfactory	No optimization was conducted since original result has been satisfactory	No optimization was conducted since original result has been satisfactory	No optimization was conducted since original result has been satisfactory	No optimization was conducted since original result has been satisfactory
Specificity (gel, sequence, melt, or digest)	Essential	Using other tet(X) variants to validate the specificity, with no cross-reaction to tet(X3) and tet(X5)<10^8 copies/mL	Using other mcr variants to validate the specificity, with no cross-reaction to mcr-2、mcr-3、mcr-10	Using other mcr variants to validate the specificity, with no cross-reaction to mcr-1、mcr-3、mcr-10	With no cross-reaction with blaKPC and blaOXA48. The specificity has also been validated in the previous study	With no cross-reaction with blaNDM and blaOXA48. The specificity has also been validated in the previous study	With no cross-reaction with blaNDM and blaKPC. The specificity has also been validated in the previous study
For SYBR Green I, Cq of the NTC	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Calibration curves with slope and y intercept	Essential	y=-3.469*x+48.696	y=-3.517*x+49.118	y=-3.444*x+49.228	y=-3.23*x+46.349	y=-3.446*x+48.217	y=-3.421*x+48.638
PCR efficiency (%) calculated from	Essential	0.94197	0.92468	0.95135	1.03985	0.95086	0.96025
CIs for PCR efficiency or SE	Desirable	-	-	-	-	-	-
r2 of calibration curve	Essential	0.999	0.999	0.999	0.997	0.999	0.998
Linear dynamic range	Essential	5~10^5 copies/uL	5~10^5 copies/uL	5~10^5 copies/uL	5~10^5 copies/uL	5~10^5 copies/uL	5~10^5 copies/uL
Cq variation at LOD	Essential	About 1.5 Cq value at LoD	About 1.5 Cq value at LoD	About 1.5 Cq value at LoD	About 1.5 Cq value at LoD	About 1.5 Cq value at LoD	About 1.5 Cq value at LoD
CIs throughout range	Desirable	-	-	-	-	-	-
Evidence for LOD	Essential	We conducted a series of 2-fold dilutions from 50 copies/ 20 μL reaction to 1.75 copies/ 20 μL reaction. For each gradient, 20 repetitions were tested, and 12.5 copies/20 μL reaction was the lowest gradient which showed positive rate over 95%	We conducted a series of 2-fold dilutions from 50 copies/ 20 μL reaction to 1.75 copies/ 20 μL reaction. For each gradient, 20 repetitions were tested, and 12.5 copies/20 μL reaction was the lowest gradient which showed positive rate over 95%	We conducted a series of 2-fold dilutions from 50 copies/ 20 μL reaction to 1.75 copies/ 20 μL reaction. For each gradient, 20 repetitions were tested, and 12.5 copies/20 μL reaction was the lowest gradient which showed positive rate over 95%	We conducted a series of 2-fold dilutions from 50 copies/ 20 μL reaction to 1.75 copies/ 20 μL reaction. For each gradient, 20 repetitions were tested, and 12.5 copies/20 μL reaction was the lowest gradient which showed positive rate over 95%	We conducted a series of 2-fold dilutions from 50 copies/ 20 μL reaction to 1.75 copies/ 20 μL reaction. For each gradient, 20 repetitions were tested, and 12.5 copies/20 μL reaction was the lowest gradient which showed positive rate over 95%	We conducted a series of 2-fold dilutions from 50 copies/ 20 μL reaction to 1.75 copies/ 20 μL reaction. For each gradient, 20 repetitions were tested, and 12.5 copies/20 μL reaction was the lowest gradient which showed positive rate over 95%
If multiplex, efficiency and LOD of each assay	Essential	No multiplex	Multiplex with mcr-3, The efficiency and LoD was determined under the mutiplex reaction	Multiplex with mcr-1, The efficiency and LoD was determined under the mutiplex reaction	Multiplex with mcr-3, The efficiency and LoD was determined under the mutiplex reaction	Multiplex with mcr-1, The efficiency and LoD was determined under the mutiplex reaction	Multiplex with mcr-1, The efficiency and LoD was determined under the mutiplex reaction
DATA ANALYSIS							
qPCR analysis program (source, version)	Essential	Applied Biosystems Quantstudio 3 qPCR machine (ThermoFisher)	Applied Biosystems Quantstudio 3 qPCR machine (ThermoFisher)	Applied Biosystems Quantstudio 3 qPCR machine (ThermoFisher)	Applied Biosystems Quantstudio 3 qPCR machine (ThermoFisher)	Applied Biosystems Quantstudio 3 qPCR machine (ThermoFisher)	Applied Biosystems Quantstudio 3 qPCR machine (ThermoFisher)
Method of Cq determination	Essential	thresholds were fixed at delta Rn equals to 30000	thresholds were fixed at delta Rn equals to 30000	thresholds were fixed at delta Rn equals to 30000	thresholds were fixed at delta Rn equals to 30000	thresholds were fixed at delta Rn equals to 30000	thresholds were fixed at delta Rn equals to 30000
Outlier identification and disposition	Essential	No outlier	No outlier	No outlier	No outlier	No outlier	No outlier
Results for NTCs	Essential	No amplification	No amplification	No amplification	No amplification	No amplification	No amplification
Justification of number and choice of reference genes	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Description of normalization method	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Number and concordance of biological	Desirable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Number and stage (RT or qPCR) of technical	Essential	Triplicate (n = 3)	Triplicate (n = 3)	Triplicate (n = 3)	Triplicate (n = 3)	Triplicate (n = 3)	Triplicate (n = 3)
Repeatability (intraassay variation)	Essential	All technical replicates were within 1 Cq of each other	All technical replicates were within 1 Cq of each other	All technical replicates were within 1 Cq of each other	All technical replicates were within 1 Cq of each other	All technical replicates were within 1 Cq of each other	All technical replicates were within 1 Cq of each other
Reproducibility (interassay variation, CV)	Desirable	We conducted the interassay variation test of the standard samples and the results showed that the variation was less than 1 Cq	We conducted the interassay variation test of the standard samples and the results showed that the variation was less than 1 Cq	We conducted the interassay variation test of the standard samples and the results showed that the variation was less than 1 Cq	We conducted the interassay variation test of the standard samples and the results showed that the variation was less than 1 Cq	We conducted the interassay variation test of the standard samples and the results showed that the variation was less than 1 Cq	We conducted the interassay variation test of the standard samples and the results showed that the variation was less than 1 Cq
Power analysis	Desirable	-	-	-	-	-	-
Statistical methods for results	Essential	T-test	T-test	T-test	T-test	T-test	T-test
Software (source, version)	Essential	SPSS	SPSS	SPSS	SPSS	SPSS	SPSS
Cq or raw data submission with RDML	Desirable	-	-	-	-	-	-
qPCR TARGET INFORMATION							
Gene symbol	Essential	tetracycline-inactivating monooxygenase Tet(X4)	Escherichia coli SHP45 pHNSHP45 mcr-1 gene	Escherichia coli WJ1 pWJ1 mcr-3 gene	Klebsiella pneumoniae pKpANDM-1 blaNDM gene for subclass B1 metallo-beta-lactamase NDM-1	Klebsiella pneumoniae blaKPC gene for carbapenem-hydrolyzing class A beta-lactamase KPC-2	Klebsiella pneumoniae 11978 blaOXA gene for OXA-48 family carbapenem-hydrolyzing class D beta-lactamase OXA-48
Sequence accession	Essential	NG_065852.1	NG_050417.1	NG_055505.1	NG_049326.1	NG_049253.1	NG_049762.1
Location of	Desirable	-	-	-	-	-	-
Amplicon length	Essential	75	85	100	100	101	70
In silico specificity screen (BLAST, and so	Essential	nBlast and primer-blast were used to check the specificity	nBlast and primer-blast were used to check the specificity	nBlast and primer-blast were used to check the specificity	nBlast and primer-blast were used to check the specificity	nBlast and primer-blast were used to check the specificity	nBlast and primer-blast were used to check the specificity
Pseudogenes, retropseudogenes, or other homologs?	Desirable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Sequence	Desirable	GAATTTTCCGATTGGGACGAACGCTACAAAGAACTGATTCGTGTGACATCATCTTTTGTAGGGTTAGCGACACGA	TTGTGGCGTGATAATAATTCGGACTCAAAAGGCGTGATGGATAAGCTGCCAAAAGCGCAATTTGCCGATTATAAATCCGCGACCA	ATGATGCAAAACGGGATACTAATCAAAGTAAGCCCACGTTGATGTTTCTGGTCGTTGGTGAAACCGCTCGTGGTAAAAATTTCTCGATGAATGGCTATGA	GCATTAGCCGCTGCATTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAAACTGGCGACCAACGGTTTGGCGATC	CTGTATCGCCGTCTAGTTCTGCTGTCTTGTCTCTCATGGCCGCTGGCTGGCTTTTCTGCCACCGCGCTGACCAACCTCGTCGCGGAACCATTCGCTAAACT	TTCCCAATAGCTTGATCGCCCTCGATTTGGGCGTGGTTAAGGATGAACACCAAGTCTTTAAGTGGGATGG
Secondary structure analysis of amplicon	Desirable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Location of each primer by exon or intron (if applicable)	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
What splice variants are targeted?	Essential	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
qPCR OLIGONUCLEOTIDES							
Primer sequences	Essential	tet(X4) _qPCR _F: TCGTGTCGCTAACCCTACAAAAG tet(X4) _qPCR _R: GAATTTTCCGATTGGGACGAA	mcr-1_ qPCR _F: TTGTGGCGTGATAATAATTCG mcr-1_ qPCR _R: TGGTCGCGGATTTATAATCG	mcr-3_ qPCR _F: ATGATGCAAAACGGGATACTAA mcr-3_ qPCR _R: TCATAGCCATTCATCGAGAAAT	blaNDM_qPCR_F: GCATTAGCCGCTGCATT blaNDM_qPCR_R: GATCGCCAAACCGTTGG	blaKPC_qPCR_F: CTGTATCGCCGTCTAGTTCTG blaKPC_qPCR_R: AGTTTAGCGAATGGTTCCG	blaOXA-48_qPCR_F: TTCCCAATAGCTTGATCGC blaOXA-48_qPCR_R: CCATCCCACTTAAAGACTTGG
RTPrimerDB identification number	Desirable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Probe sequences	Desirable	tet(X4) _qPCR _P: FAM-TGATGTCACACGAATC-MGB	mcr-1_qPCR_P: FAM-CTGCCAAAAGCGCA-MGB	mcr-3_qPCR_P: ROX-AGTAAGCCCACGTTGAT-MGB	blaNDM_qPCR_P: ROX-ACGATTGGCCAGCAAATGGAAACTGG-BHQ2	blaKPC_qPCR_P: FAM-TGTCTTGTCTCTCATGGCCGCTGG-BHQ1	blaOXA-48_qPCR_P: VIC-TCGATTTGGGCGTGGTTAAGGATGAAC-BHQ1
Location and identity of any modifications	Essential	5' FAM reporter and 3' NFQ-MGB quencher for probes	5' FAM reporter and 3' NFQ-MGB quencher for probes	5' ROX reporter and 3' NFQ-MGB quencher for probes	5' ROX reporter and 3'BHQ2 quencher for probes	5' FAM reporter and 3' BHQ1 quencher for probes	5' VIC reporter and 3' BHQ1 quencher for probes
Manufacturer of oligonucleotides	Desirable	Sangon	Sangon	Sangon	Sangon	Sangon	Sangon
Purification	Desirable	HAP for primers, HPLC for probes	HAP for primers, HPLC for probes	HAP for primers, HPLC for probes	HAP for primers, HPLC for probes	HAP for primers, HPLC for probes	HAP for primers, HPLC for probes