Original Article

A New High-throughput Real-time PCR Assay for the Screening of Multiple Antimicrobial Resistance Genes in Broiler Fecal Samples from China*

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Abstract

Objective Antimicrobial resistance (AMR) has become a global concern and is especially severe in China. To effectively and reliably provide AMR data, we developed a new high-throughput real-time PCR assay based on microfluidic dynamic technology, and screened multiple AMR genes in broiler fecal samples.

Methods A high-throughput real-time PCR system with an new designed integrated fluidic circuit assay were performed AMR gene detection. A total of 273 broiler fecal samples collected from two geographically separated farms were screened AMR genes.

Results The new assay with limits of detection ranging from 40.9 to 8,000 copies/reaction. The sensitivity rate, specificity rate, positive predictive value, negative predictive value and correct indices were 99.30%, 98.08%, 95.31%, 99.79%, and 0.9755, respectively. Utilizing this assay, we demonstrate that AMR genes are widely spread, with positive detection rates ranging from 0 to 97.07% in 273 broiler fecal samples. bla_{CTX-M}, bla_{TEM}, *mcr*-1, *fex*A, *cfr*, *optr*A, and *int*I1 showed over 80% prevalence. The dissemination of AMR genes was distinct between the two farms.

Conclusions We successfully established a new high-throughput real-time PCR assay applicable to AMR gene surveillance from fecal samples. The widespread existence of AMR genes detected in broiler farms highlights the current and severe problem of AMR.

Key words: Antimicrobial resistance; Antimicrobial resistance gene; High-throughput real-time PCR array; Broiler fecal sample

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INTRODUCTION

Antimicrobial resistance (AMR) is a fast-growing global threat. Infections associated with AMR bacteria lead to over 23,000 deaths in the United States and 25,000 deaths in Europe annually, and the number is much higher in Asian countries[1,2]. It is estimated that up to 300 million premature deaths could occur from AMR by 2050, with 100 trillion U.S. dollars lost to the global economy, unless effective action is taken[3]. AMR is particularly severe in China, which is one of

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the largest producers and users of antimicrobial agents[4]. Bacterial AMR is a complicated and active process, whereby numerous AMR genes can be rapidly selected for and transmitted in clinical, veterinary, food production and transportation, and environmental settings. AMR originating from the use of antimicrobials in animals plays an important role in the development and spreading of antibiotic resistant bacteria. The AMR genes could be widely spread via transmission cycles, such as food or ecological chains, and broiler feces was considered as a good storage of AMR genes[5-7]. To investigate AMR genes in broiler fecal samples will give important information to AMR surveillance of 'One Health' in China.

Conventional AMR surveillance often focuses on bacterial resistant phenotypes (e.g. minimum inhibitory concentration values of antimicrobial agents), and/or the screening of a limited number of AMR genes. Such methods restrict the collection of comprehensive AMR data in a timely manner[8-11]. Therefore, a more direct, rapid and high-throughput AMR surveillance technique is essential for monitoring and controlling the increase of AMR genes. To address this issue, we developed a new high-throughput microfluidic dynamic assay and applied it to monitor AMR gene presence. Microfluidic dynamic technology has recently been introduced into biological automation applications and widely adopted due to its advantages of rapid, high-throughput screening, with high repeatability and low cross reaction[12-15]. It uses microfluidics and high-resolution imaging to perform *q*PCR with fluorescence detection in nanoliter reaction volumes. Here, we show that our microfluidic dynamic assay can be applied to perform screening for the detection of multiple AMR genes simultaneously. A total of 45 groups AMR genes and 2 AMR gene spread related elements were screened in the Fluidigm, selected according to previous research descriptions and frequency of antimicrobial agent usage in China. The AMR gene dissemination status in fecal samples collected from two broiler farms was analyzed and compared.

METHODS

Sample Collection and DNA Extraction

A total of 273 broiler fecal samples were collected from two geographically distinct farms in March 2017, with each farm containing approximately 20,000 broilers simultaneously. The farms were divided into five sampling zones (north, south, east, west, and middle). The samples were selected randomly at a ratio of 1:150 for each farm. In total, 145 and 128 broiler fecal samples were obtained from farm A and B, respectively. Samples were shipped to the laboratory at 4 °C within six hours. The antimicrobial agent usage was investigated by questionnaire during the sample collection period. Amoxicillin, ampicillin, ceftiofur sodium, enrofloxacin, florfenicol, gentamicin, kanamycin, tylosin, and terramycin were commonly used in both farms during broiler production.

Bacterial DNA was extracted directly from sample using Automated Nucleic Acid Extraction System-96 (BioTeke, Beijing, China). The bacterial DNA was eluted into 100 μL of TE buffer and stored at −80 °C for further study.

Choose Target AMR Gene

In this study, a total of 47 gene groups, containing 688 unique variants, were included in screening (Table 1 and Supplementary Table S1 available in www.besjournal.com). The 47 gene groups were subdivided into eight classes in this study, based on antimicrobial agent. Among these 47 gene groups, 42 could mediate resistance to a single class of antimicrobial agent, 3 could mediate multidrug resistance, and 2 were related with AMR gene transmission. All AMR gene sequences were downloaded from the GenBank database. Genbank accession numbers of reference gene sequences for each AMR gene group were list in Table 1. Due to the numerous variants existing among carbapenem-resistance, fluoroquinolone-resistance and β-lactam-resistance genes, DNAMAN version 5.0 and MEGA version 5.0 were used to analyze and classify variants. The AMR gene group classification used was neither based on the gene variant-mediated resistance phenotype nor order of discovery, but on gene sequence identity and evolutionary relationship. Therefore, the primers and probe design for each group in this study could potentially target many more AMR gene variants. Gene variants with high sequence similarity were classified together into one AMR gene group according to phylogenetic trees performed by MEGA software. The conserved sequences intergroup were identified and analyzed by DNAMAN software, and the probes/primers were assessed and chosen by Oligo version 6.0. All conserved sequences identified from carbapenem-resistance, fluoroquinolone-resistance, and β-lactam-resistance genes, as well as sequences from

AMR genes without variants, were used as target sequences for primer design using Primer version 6.0. All of the primers and probes were tested by BLAST analysis. In total, 688 unique AMR gene variants could be detected. The primers and minor groove binder (MGB) probes were specifically designed for this study, except for the genes *arm*A, *mcr*-1, *int*I1, and element IS*CR*1[9,16,17]. Additionally, the bacterial *16S* rDNA locus was used as an internal processing control.

Standard Curve Plot of Recombinant Plasmids

A unique recombinant plasmid was constructed for each target gene. Due to high similarity of AMR gene sequences with selectable markers encoding genes on vectors, different types of vectors were chosen to avoid inaccurate results in this study. The vectors T1, pUC19, pUC57 were used to generate plasmids harboring various target-specific fragments (using either the primers described in this study or isolated DNA containing AMR genes) to measure the copy number. A total of 43 novel recombinant plasmids were generated in this study (Supplementary Table S2, available in www.besjournal.com). Standard curves were generated for each recombinant plasmid by conventional real-time PCR using a LightCycler 480II (Roche Diagnostics)[9].

Table 1. Target AMR gene information used in this study

Antimicrobial agent	No.[a]	Gene group	Number[b]	Accession no. of reference sequences	Antimicrobial agent	No.	Gene group	Number	Accession no. of reference sequences
Carbapenems	1	*bla*$_{OXA-48}$-like	14	AY236073	other β-lactams	25	*bla*$_{SHV}$ D	2	JQ029959
	2	*bla*$_{IMP}$ A	13	S71932		26	*bla*$_{TEM}$ A	157	AF093512
	3	*bla*$_{IMP}$ B	5	AF290912		27	*bla*$_{TEM}$ B	4	J01749
	4	*bla*$_{IMP}$ C	4	AB074436		28	*bla*$_{OXA}$	7	JN596991
	5	*bla*$_{IMP}$ D	5	AY553332		29	*bla*$_{PER}$	6	AY740681
	6	*bla*$_{IMP}$ E	9	EF118171		30	*bla*$_{CARB}$ A	22	KJ934265
	7	*bla*$_{IMP}$ F	2	KF148593		31	*bla*$_{CARB}$ B	4	AF313471
	8	*bla*$_{NDM}$	15	FN396876		32	*bla*$_{CARB}$ C	3	AF030945
	9	*bla*$_{KPC}$	21	AF297554		33	*bla*$_{CARB}$ D	5	AF135373
	10	*bla*$_{VIM}$	38	JN982330	Fluoroquinolones	34	*qnr*A	8	GU295952
	11	*bla*$_{IMI}$	5	DQ173429		35	*qnr*C	1	EU917444
	12	*bla*$_{IND}$ A	6	AF219131		36	*qnr*S	4	FJ167861
	13	*bla*$_{IND}$ B	2	AF099139	Colistin	37	*mcr*-1	1	KP347127
	14	*bla*$_{IND}$ C	2	AF219127		38	*mcr*-3	1	KY924928
	15	*bla*$_{IND}$ D	2	GU186044	Aminoglycosides	39	*arm*A	1	[16]
Other β-lactams	16	*bla*$_{GES}$	22	AF156486		40	*aac*(6′)-*Ie*-*aph*(2″)-*Ia*	1	HQ015159
	17	*bla*$_{CTX-M}$ A	58	AF255298	Phenicols	41	*fex*A	1	KC222021
	18	*bla*$_{CTX-M}$ B	22	AJ416344		42	*fex*B	1	JN201336
	19	*bla*$_{CTX-M}$ C	11	FR682582	Multidrug	43	*cfr*	1	JF969273
	20	*bla*$_{CTX-M}$ D	48	HQ833652		44	*optr*A	1	KP396637
	21	*bla*$_{CTX-M}$ E	3	AY238472		45	*aac*(6′)-*Ib*-cr	1	GU189577
	22	*bla*$_{SHV}$ A	132	AF148850	Gene spread related	46	*int*I1	1	[17]
	23	*bla*$_{SHV}$ B	4	LN515533		47	IS*CR*1	1	[17]
	24	*bla*$_{SHV}$ C	2	JQ341060	Internal processing control	48	*16Sr*DNA	1	[9]

Note. [a]The serial number for the assay arranged on 48.48 Dynamic Array™ IFC. [b]Number of variants including in this AMR gene group.

High-throughput Real Time PCR Assay

An integrated fluidic circuit (IFC), commonly referred to as a 'chip', was used for AMR gene screening. The BioMark™ real-time PCR system (Fluidigm, USA) was used for high-throughput microfluidic real-time PCR amplification using 48.48 Dynamic Array™ IFC kit (Fluidigm, USA). 2,304 (48 target genes×48 samples) individual PCR reactions were conducted simultaneously[14].

Amplification was performed using 6-carboxyfluorescein (FAM)- and MGB-labeled probes with TaqMan® Gene Expression Master Mix, in accordance with the manufacturer's instructions (Applied Biosystems, USA). The high-throughput real time assay chip was prepared according to Fluidigm 48.48 Dynamic Array™ IFC kit manufacturer protocol. The assay conditions were: 2 min at 50 °C, 1 min at 95 °C, followed by 40 cycles of two-step amplification of 15 s at 95 °C, 1 min at 60 °C. Data were automatically collected using Biomark™ HD Data Collection Software.

Detection Limit Estimation for the High-throughput Real-time PCR Array

A detection limit estimation for the high-throughput BioMark™ real time PCR was carried out using a mixture of the 47 recombinant plasmids previously generated and harbouring different AMR target genes. The mixture was 10-fold serially diluted from 10^6 to 10^0 order of magnitude of copies/μL. Three independent dilution mixtures containing the AMR genes mentioned above were loaded into the sample inlets of one chip simultaneously to estimate the sensitivity and specificity of the high-throughput BioMark™ real-time PCR array.

Validation of the High-throughput BioMark™ Real-time PCR Array Results

In order to validate the detection sensitivity, capability, and specificity of the novel high-throughput real-time PCR assay, all recombinant plasmids harbouring AMR target genes were randomly combined into groups, serially diluted and then subjected to the high-throughput BioMark™ real-time PCR assay. Briefly, plasmids were divided into 8 groups each containing 6 target genes, or 4 groups each containing 12 target genes, or 3 groups each containing 16 target genes, or 2 groups each containing 24 target genes, or 1 group containing all target genes. Starting with 10^8 copies/μL, each plasmid mixture underwent a 10-fold serial dilution, as described above, followed by the high-throughput BioMark™ real-time PCR assay, run in triplicate. The formulas for sensitivity rate, specificity rate, positive predictive value, negative predictive value and correct indices were list in the notes of Table 2.

AMR Gene Dissemination Sreening among Broiler Fecal Samples

45 groups AMR genes and 2 AMR gene spread related elements were detected in a total of 273 broiler fecal samples using the BioMark™ real-time PCR system. The results were analyzed using Microsoft® Office Excel 2007 and IBM SPSS® Statistics version 19.0. The Chi-square test was used to estimate the difference in AMR gene dissemination between the two farms, and $P < 0.05$ was used as the cutoff for significance. The Pearson Chi-square test, correction for continuity, and Fisher's exact test were used to test for statistical significance when $n \geq 5$, $1 \leq n < 5$, and $n < 1$, respectively. The relative abundance values for AMR genes were calculated using the ratio of AMR gene copies and 16S rDNA copies. Software R version 3.6.1 was used to draw a heat-map of AMR gene dissemination from the two farms.

RESULTS

Standard Curve Parameters for AMR Genes from Conventional Real-time PCR Assays

A total of 43 new recombinant plasmids harbouring AMR genes were constructed in this study. Conventional real-time PCR assays performed on these plasmids gave a limit of detection (LOD), quantification of detection (LOQ), and the unique linear dynamic range for each AMR gene and standard curve parameters were list in Supplementary Table S3, available in www.besjournal.com. The standard curve parameter showed good relationship between logarithmic value of recombinant plasmid diluted concentration and Ct value and excellent LOQs in linear dynamic range of all the estimated plasmids. The LODs ranged from 12.2 to 89.2 copies/reaction suggested good detectability of this study.

LODs for High-throughput Real-time PCR Assays

The LODs for the high-throughput real-time PCR assays established here were verified using a 10-fold serially diluted mixture (10^6–10^0 order of magnitude copies/μL) of the 48 recombinant plasmids harbouring different AMR target genes/spread related elements/internal processing control gene.

As expected, all of tested genes were successfully detected and displayed no cross-detection, even among the AMR gene groups that shared a high level of sequence similarity. The amplification signals remained specific to the target gene until at least cycle 25. The LODs ranged from 40.9 to 8,470 copies/reaction, and are shown in Table 3.

Validation of the High-throughput BioMark™ Real-time PCR Array Results

A total of 34,560 microfluidic reactions were performed in this study to evaluate the sensitivity, specificity, and detection capability of the new array. A comparison between the novel high-throughput real-time PCR array results and the cloned target AMR genes contained in the plasmid mixtures is given in Table 2. The sensitivity rate, specificity rate, positive predictive value, negative predictive value and correct indices were 99.30%, 98.08%, 95.31%, 99.79%, and 0.9755, respectively.

Dissemination of AMR Genes among Broiler Fecal Samples

Among 273 broiler fecal samples, the AMR gene positive detection rate ranged from 0 to 97.07% (Table 4). Eight groups of the AMR gene positive detection rates were over 80%, while 28 of them were below 5%. Genes *bla*$_{IMP}$ F, *bla*$_{IMI}$, and *qnr*C were not identified in any fecal sample tested. The most common AMR genes related with carbapenem-

Table 2. Validation of the new high-throughput real-time PCR array for the detection of AMR target genes contained within a plasmid mixture

High-throughput assay	AMR target genes contained in plasmid mixture		Total
	Positive	Negative	
Positive reactions	9,670[a]	476[b]	10,146
Negative reactions	68[c]	24,346[d]	24,414
Total	9,738	24,822	34,560

Note. Sensitivity rate = $\frac{a}{a+c} \times 100\%$; specificity rate = $\frac{d}{b+d} \times 100\%$; positive predictive value = $\frac{a}{a+b} \times 100\%$; negative predictive value = $\frac{d}{c+d} \times 100\%$; correct indices = sensitivity rate + specificity rate-1.

Table 3. LODs of high-throughput real-time pcr assay in this study

No.	Gene group	LOD (copies/reaction)	No.	Gene group	LOD (copies/reaction)	No.	Gene group	LOD (copies/reaction)
1	*bla*$_{OXA-48}$-like	5,750	17	*bla*$_{CTX-M}$ E	442	33	*bla*$_{CARB}$ D	2,530
2	*bla*$_{IMP}$ A	5,250	18	*bla*$_{SHV}$ A	294	38	*qnr*A	124
3	*bla*$_{IMP}$ B	1,610	19	*bla*$_{SHV}$ B	1,220	39	*qnr*C	892
4	*bla*$_{IMP}$ C	153	20	*bla*$_{SHV}$ C	615	40	*qnr*S	8,470
5	*bla*$_{IMP}$ D	689	21	*bla*$_{SHV}$ D	711	13	*mcr*-1	100
6	*bla*$_{IMP}$ E	496	22	*bla*$_{TEM}$ A	59.7	14	*mcr*-3	2,730
7	*bla*$_{IMP}$ F	382	23	*bla*$_{TEM}$ B	54.5	15	*arm*A	4,070
8	*bla*$_{NDM}$	223	24	*bla*$_{CTX-M}$ A	3,330	16	*aac*(6')-*Ie*-*aph*(2")-*Ia*	3,240
9	*bla*$_{KPC}$	423	25	*bla*$_{CTX-M}$ B	4,100	41	*fex*A	4,410
10	*bla*$_{VIM}$	452	26	*bla*$_{CTX-M}$ C	4,620	42	*fex*B	488
11	*bla*$_{IMI}$	4,470	27	*bla*$_{CTX-M}$ D	40.9	43	*cfr*	8,000
12	*bla*$_{IND}$ A	4,440	28	*bla*$_{OXA}$	2,120	44	*optr*A	393
13	*bla*$_{IND}$ B	4,440	29	*bla*$_{PER}$	3,770	45	*aac*(6')-*Ib*-cr	475
14	*bla*$_{IND}$ C	780	30	*bla*$_{CARB}$ A	3,430	46	*int*I1	589
15	*bla*$_{IND}$ D	581	31	*bla*$_{CARB}$ B	322	47	IS*CR*1	413
16	*bla*$_{GES}$	276	32	*bla*$_{CARB}$ C	2,000	48	*16Sr*DNA	321

Table 4. AMR genes dissemination and statistical differences between two broiler farms

Antimicrobial agent	No.	Gene group	Frequency of AMR gene (%)			Statistical analysis	
			Total (%)	Farm A (%)	Farm B (%)	χ^2 value	*P* value
Carbapenems	1	*bla*$_{\text{OXA-48}}$-like	1 (0.37)	1 (0.37)	0 (0)	0.000	1.000
	2	*bla*$_{\text{IMP}}$ A	7 (2.56)	3 (1.10)	4 (1.47)	0.000	1.000
	3	*bla*$_{\text{IMP}}$ B	7 (2.56)	0 (0)	7 (2.56)	5.210	0.015
	4	*bla*$_{\text{IMP}}$ C	1 (0.37)	0 (0)	1 (0.37)	0.000	1.000
	5	*bla*$_{\text{IMP}}$ D	2 (0.73)	2 (0.73)	0 (0)	0.502	0.499
	6	*bla*$_{\text{IMP}}$ E	3 (0.73)	1 (0.37)	2 (0.73)	0.000	1.000
	7	*bla*$_{\text{IMP}}$ F	0 (0)	0 (0)	0 (0)	–	–
	8	*bla*$_{\text{NDM}}$	14 (5.13)	5 (1.83)	9 (3.30)	1.173	0.418
	9	*bla*$_{\text{KPC}}$	2 (0.73)	2 (0.73)	0 (0)	0.502	0.499
	10	*bla*$_{\text{VIM}}$	7 (2.56)	2 (0.73)	5 (1.83)	0.579	0.447
	11	*bla*$_{\text{IMI}}$	0 (0)	0 (0)	0 (0)	–	–
	12	*bla*$_{\text{IND}}$ A	1 (0.37)	1 (0.37)	0 (0)	0.000	1.000
	13	*bla*$_{\text{IND}}$ B	2 (0.73)	2 (0.73)	0 (0)	0.502	0.499
	14	*bla*$_{\text{IND}}$ C	2 (0.73)	2 (0.73)	0 (0)	0.502	0.499
	25	*bla*$_{\text{IND}}$ D	1 (0.37)	1 (0.37)	0 (0)	0.000	1.000
Other β-lactams	16	*bla*$_{\text{GES}}$	8 (2.93)	0 (0)	8 (2.93)	6.216	0.007
	17	*bla*$_{\text{CTX-M}}$ A	254 (93.04)	143 (52.38)	111 (40.66)	7.538	0.006
	18	*bla*$_{\text{CTX-M}}$ B	6 (2.20)	5 (1.83)	1 (0.37)	1.517	0.218
	19	*bla*$_{\text{CTX-M}}$ C	1 (0.37)	1 (0.37)	0 (0)	0.000	1.000
	20	*bla*$_{\text{CTX-M}}$ D	205 (75.10)	135 (49.45)	70 (25.64)	33.000	0.000
	21	*bla*$_{\text{CTX-M}}$ E	260 (95.24)	144 (52.75)	116 (42.49)	5.757	0.016
	22	*bla*$_{\text{SHV}}$ A	17 (6.23)	9 (3.30)	8 (2.93)	0.061	1.000
	23	*bla*$_{\text{SHV}}$ B	12 (4.40)	8 (2.93)	4 (1.47)	0.767	0.381
	24	*bla*$_{\text{SHV}}$ C	7 (2.56)	4 (1.47)	3 (1.10)	0.000	1.000
	25	*bla*$_{\text{SHV}}$ D	8 (2.93)	6 (2.20)	2 (0.73)	1.142	0.285
	26	*bla*$_{\text{TEM}}$ A	230 (84.25)	142 (52.01)	88 (32.23)	21.906	0.000
	27	*bla*$_{\text{TEM}}$ B	260 (95.24)	145 (53.11)	115 (42.12)	6.608	0.013
	28	*bla*$_{\text{OXA}}$	140 (51.28)	108 (39.56)	32 (11.72)	55.484	0.000
	29	*bla*$_{\text{PER}}$	12 (4.03)	4 (1.47)	8 (2.93)	0.767	0.381
	30	*bla*$_{\text{CARB}}$ A	1 (0.37)	1 (0.37)	0 (0)	0.000	1.000
	31	*bla*$_{\text{CARB}}$ B	23 (8.42)	12 (4.40)	11 (4.03)	0.045	1.000
	32	*bla*$_{\text{CARB}}$ C	2 (0.73)	2 (0.73)	0 (0)	0.502	0.499
	33	*bla*$_{\text{CARB}}$ D	65 (23.81)	51 (18.68)	14 (5.13)	23.908	0.000
Fluoroquinolones	34	*qnr*A	5 (1.83)	0 (0)	5 (1.83)	3.230	0.061
	35	*qnr*C	0 (0)	0 (0)	0 (0)	–	–
	36	*qnr*S	113 (41.39)	94 (34.43)	19 (6.96)	62.770	0.000
Colistin	37	*mcr*-1	217 (79.49)	105 (38.46)	112 (41.03)	0.375	0.540
	38	*mcr*-3	10 (3.66)	6 (2.20)	4 (1.47)	0.102	0.750

Continued

Antimicrobial agent	No.	Gene group	Frequency of AMR gene (%)			Statistical analysis	
			Total (%)	Farm A (%)	Farm B (%)	χ^2 value	*P* value
Aminoglycosides	39	*arm*A	11 (4.03)	2 (0.73)	9 (3.30)	3.340	0.068
	40	*aac*(6′)-*Ie-aph*(2″)-*Ia*	257 (94.14)	136 (49.81)	121 (44.32)	1.654	0.198
Phenicols	41	*fex*A	256 (93.77)	140 (51.28)	116 (42.49)	4.774	0.029
	42	*fex*B	4 (1.47)	3 (1.10)	1 (0.37)	0.252	0.616
Multidrug	43	*cfr*	134 (49.08)	123 (45.05)	11 (4.03)	124.059	0.000
	44	*optr*A	223 (81.68)	128 (46.89)	95 (34.80)	8.255	0.004
	45	*aac*(6′)-*Ib*-cr	199 (72.89)	135 (49.45)	64 (23.44)	55.956	0.000
Gene spread related	46	*int*I1	265 (97.07)	145 (53.11)	120 (43.96)	4.583	0.040
	47	IS*CR*1	128 (46.89)	96 (35.16)	32 (11.72)	41.799	0.000

resistance, alternative β-lactam-resistance, fluoroquinolone-resistance, colistin-resistance, aminoglycoside-resistance, phenicol and oxazolidinone-resistance, multidrug resistance, and AMR gene spread were bla_{NDM}, bla_{TEM} B, and bla_{CTX-M} E, *qnr*S, *mcr*-1, *aac*(6′)-*Ie-aph*(2″)-*Ia*, *fex*A, *optr*A, and *int*I1, respectively.

The AMR gene positive detection rates are shown in detail in Table 4. AMR genes were found to be widely disseminated and commonly co-existed in fecal samples using the high-throughput real-time PCR array. Except for 4 fecal samples in which no AMR genes were detected, 24 groups of distinct AMR genes were identified in 2 fecal samples (0.73%), 23 groups in 1 sample (0.37%), 21 groups in 1 (0.37%), 20 groups in 1 (0.37%), 19 groups in 6 (2.20%), 18 groups in 3 (1.10%), 17 groups in 10 (3.66%), 16 groups in 28 (10.26%), 15 groups in 25 (9.16%), 14 groups in 31 (11.36%), 13 groups in 33 (12.09%), 12 groups in 31 (11.36%), 11 groups in 30 (10.99%), 10 groups in 18 (6.59%), 9 groups in 20 (7.33%), 8 groups in 12 (4.40%), 7 groups in 4 (1.47%), 6 groups in 5 (1.83%), 5 groups in 4 (1.47%), 4 groups in 3 (1.10%) and 1 groups in 1 (0.73%).

Statistical Differences in AMR Gene Dissemination between the Two Farms

The frequencies (n) of AMR gene dissemination are shown in Table 4, Figures 1–2. The AMR gene groups bla_{IMP} B, bla_{GES}, bla_{CTX-M} A, bla_{CTX-M} D, bla_{CTX-M} E, bla_{TEM} A, bla_{TEM} B, bla_{OXA}, bla_{CARB} D, *qnr*S, *fex*A, *cfr*, *optr*A, *aac*(6′)-*Ib*-cr, *int*I1, and IS*CR*1 showed statistically significant differences ($P < 0.05$) in dissemination between the two broiler farms, while all other AMR gene groups showed no statistically significant differences in dissemination. Compared with farm B, farm A exhibited a significantly higher prevalence of 14 AMR gene groups. In contrast, farm B only exhibited a significantly higher prevalence of the AMR gene groups bla_{IMP} B and bla_{GES} than farm A. Heat-map analysis showed that the majority of broiler fecal samples exhibited low relative abundance values for AMR genes, except for IS*CR*1 in farm A, and *aac*(6′)-*Ie-aph*(2″)-*Ia* and *fex*A in farm B. Farm A had a greater number of fecal samples that contained multiple AMR genes compared to farm B.

DISCUSSION

AMR genes play a pivotal role in the occurrence, development and dissemination of antibiotic resistance worldwide, accelerating the progression of bacteria from being multi-drug resistant, to extensively-drug resistant to pan-drug resistant[18]. The AMR genes could be widely disseminated *via* transmission cycles, such as food or ecological chains, and animal is often considered as potential origin of AMR genes spread. Due to the numerous AMR genes already described, a rapid, convenient, accurate, and high-throughput screening method is urgently needed for AMR surveillance. Conventional PCR and real-time PCR methods have been widely applied to AMR gene detection for a decade, but high-throughput arrays have not. Here, we introduced a new high-throughput real-time PCR array based on integrated fluidic circuit, which could simultaneously analyze and detect 45 groups AMR genes and 2 AMR gene spread related elements. Compared with conventional PCR and real-time PCR assays, the new array exhibited higher fluxes and avoided cross-reaction in the detection of multiple AMR genes. Through one high-throughput real-time array, we could obtain a large amount of information on the presence and prevalence of AMR genes

including specific AMR gene variants.

The new high-throughput real-time PCR array was based on conventional real-time PCR. The recombinant plasmid standard curves of 43 AMR genes (others were constructed in a previous study) established by conventional real-time PCR displayed good linear relationship, sensitivity, and specificity. Compared with the low LODs of conventional real-time PCR, high-throughput real-time PCR showed a wide range of LODs. This phenomenon could be due to the requirement of restricting the annealing temperature of the high-throughput real-time PCR reaction to one value.

As such, the amplification efficiency may be

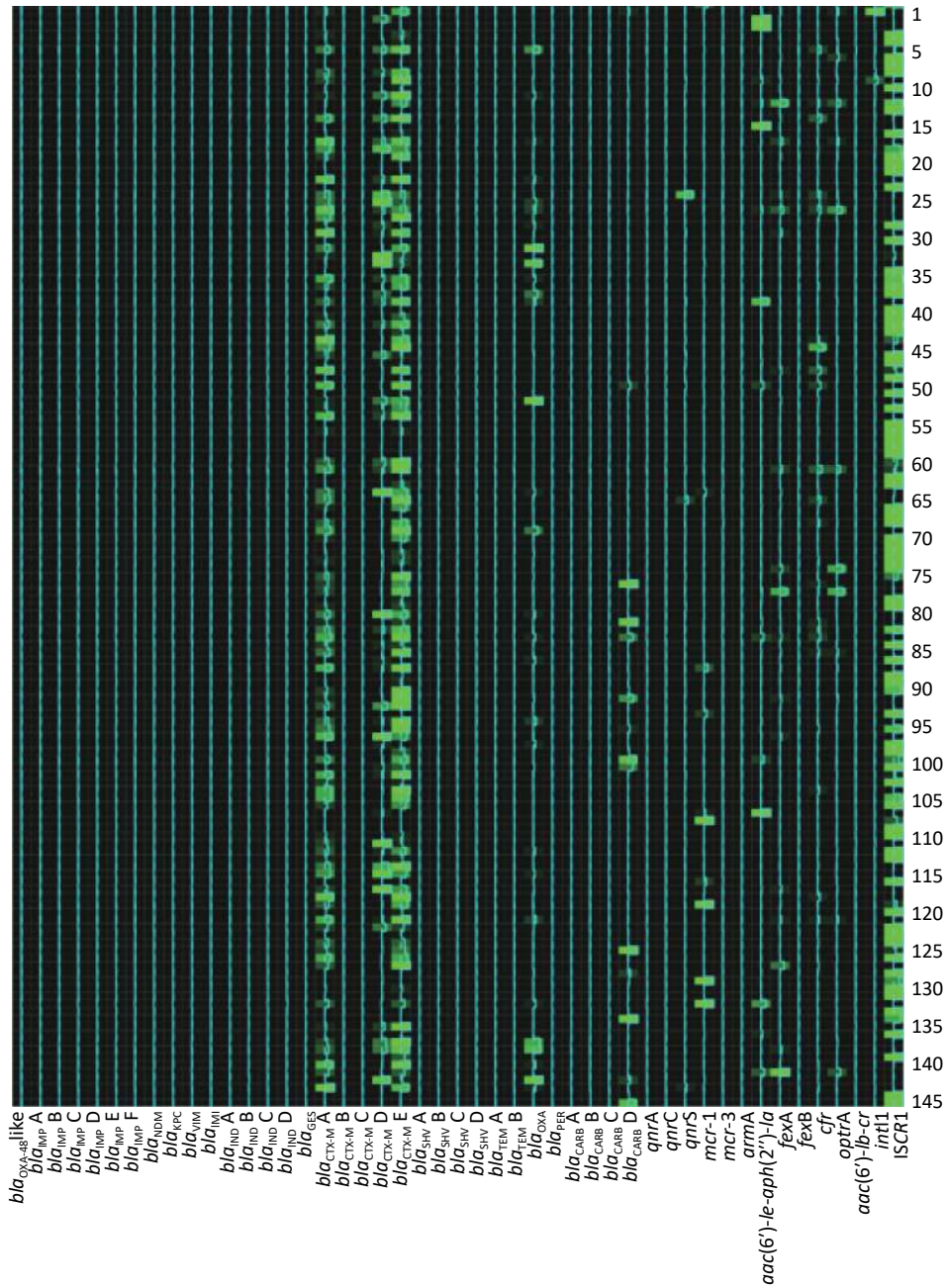

Figure 1. Heat-map of AMR gene dissemination of 145 broiler fecal samples obtained from farm A. Black block: AMR gene negative; Green block: AMR gene positive, the deeper of the green color, the higher of the gene relative abundance.

reduced in certain AMR gene PCR reactions. Previous research suggests that 14 cycles of specific target amplification before initiating the microfluidic reaction could increase the target DNA by 2^{10} to 2^{14} times without bias[19]. Such an additional reaction could easily be introduced into the current AMR gene screening using high-throughput real-time PCR array.

The validation of the high-throughput real-time PCR array results showed that the primers and probes designed here were highly specific (specificity rate 98.09%) to target genes. The high positive predictive value, negative predictive value, and correct indices value indicated that the new high-

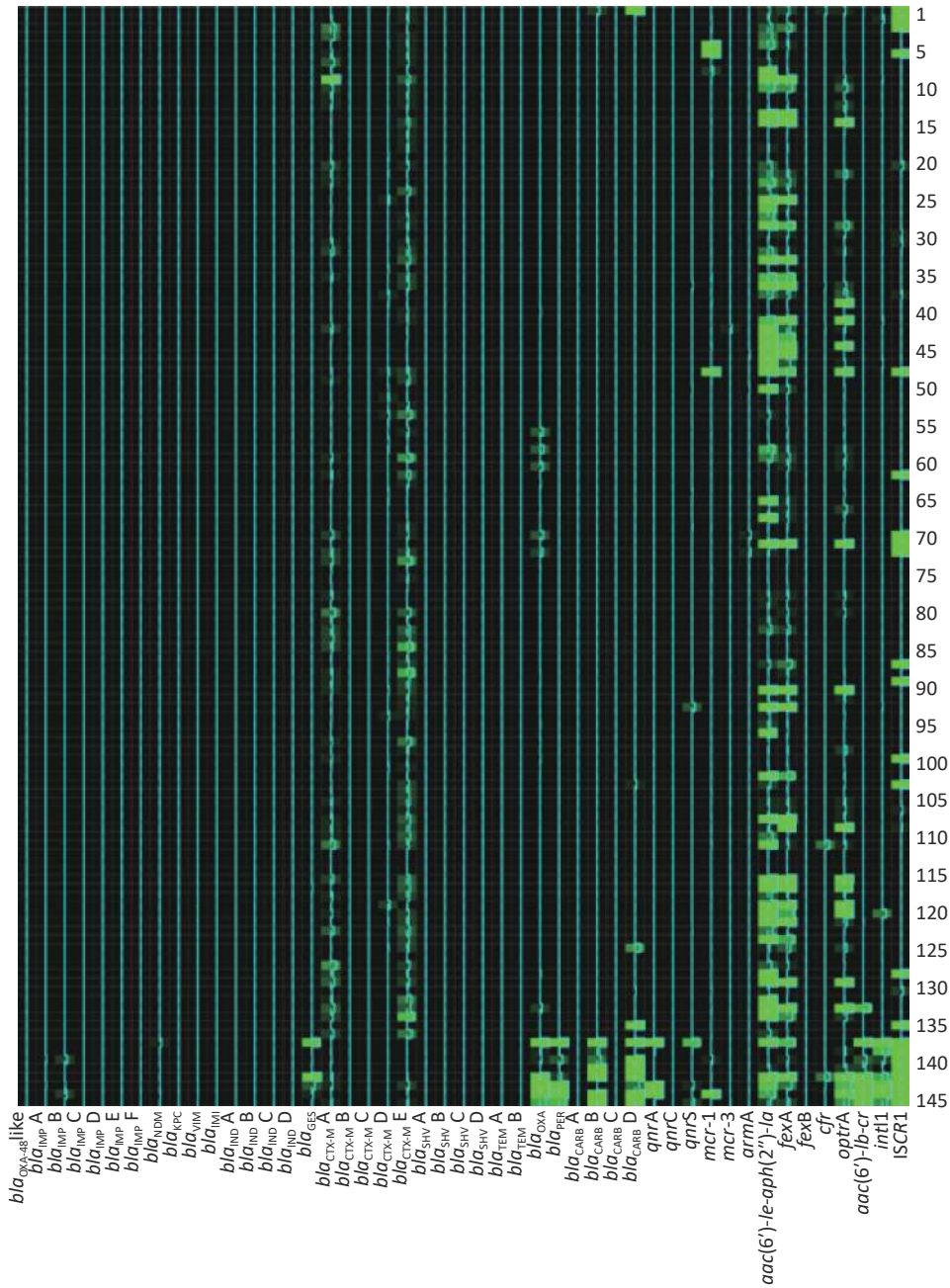

Figure 2. Heat-map of AMR gene dissemination of 128 broiler fecal samples obtained from farm B. Black block: AMR gene negative; Green block: AMR gene positive, the deeper of the green color, the higher of the gene relative abundance.

throughput real-time PCR array was credible and valuable in the detection of multiple AMR genes, even when up to 45 groups AMR genes and 2 AMR gene spread related elements were simultaneously present in one recombinant plasmid mixture. The results of this study suggested that the high-throughput real-time PCR array was exactly stabled and sensitive. The novel designed assay could be widely used in large scale screening of AMR gene, and more AMR target genes could be involved in the 'chip' assay design in further.

The novel high-throughput method established in this study was applied to detect the AMR genes disseminated in 273 broiler fecal samples. A total of 45 groups AMR genes and 2 AMR gene spread related elements could be detected in 47 fecal samples simultaneously using the array, along with one internal processing control for the detection arrays and one negative control in the sample arrays. Compared with conventional culturing and antimicrobial susceptibility tests, the novel array was successfully used on broiler fecal samples to obtain greater detail on the presence and prevalence of AMR genes, saving both time and reagents. The AMR gene dissemination patterns identified in this study indicated that a wide range of antimicrobial resistance existed among the 273 broiler fecal samples. All eight groups of AMR genes were detected, some with high prevalence. Gene *int*I1 is commonly involved in the of many AMR genes transmission, and is closely associated with multidrug resistance[20-22]. Our results showed a high positive detection rate for *int*I1 indicating that the potential for multiple AMR genes movement and widespread in the two broiler farms studied. The β-lactam-resistance genes $bla_{\text{CTX-M}}$ A, $bla_{\text{CTX-M}}$ B, $bla_{\text{CTX-M}}$ E, bla_{TEM} A, and bla_{TEM} B also showed high prevalence in this study, which suggested that bacteria in the collected samples may show common resistance to cephalosporins including the third generation cephalosporins as previously described[7,9,23,24]. Although many AMR genes showed low relative abundance values, antimicrobial agent usage strictly controlling, reduce additives usage of antimicrobial agent rotational and mixed application, and non-pharmaceutical therapy should be performed to enhance broiler growth and reduce feed cost in broiler production in the farm. In this study, the patterns of AMR gene dissemination between the two broiler farms were distinct. Many researchers have found that these AMR genes are widely spread in livestock and poultry husbandry[4,6,20,24]. Our questionnaire showed that many kinds of antimicrobial agents were commonly used during broiler production. It suggested that the drugs may give selection pressure for AMR genes and multidrug resistance widely dissemination. A greater amount of information on AMR gene presence and prevalence could be collected through expansion of both the types of genes and numbers of primers used in AMR surveillance in further.

The prevalence of the carbapenem-resistance gene group was the lowest among the eight AMR gene groups, with bla_{NDM} showing the highest positive detection rate in the group (Table 4, 5.13%, assay 1 to assay 12). Although the use of carbapenems is forbidden in livestock and poultry husbandry, carbapenem-resistance AMR genes are being increasingly identified[25-27]. It has been suggested that plasmid-mediated AMR gene transfer might play an important role in the spread of bacterial resistance even with a lack of antimicrobial agent selective pressure. The *mcr* genes have been found giving a big contribution to high levels of colistin resistance[4,9,18,25,26]. Colistin was a common and legal antimicrobial agent used in livestock/poultry husbandry in China until April, 2017. The wide spread of *mcr*-1 in broiler farms indicates that colistin perhaps cannot be used effectively in disease prevention and control under these conditions.

IS*CR*1 is recognized as a powerful AMR gene capture and movement system element, and can widely transmit AMR genes among different species of bacteria[28,29]. The existence of the *int*I1- IS*CR*1 complex structure is responsible for both multidrug resistance and extensive resistance[30-32]. Our finding of over 95% prevalence for *int*I1 and nearly 50% prevalence for IS*CR*1 are suggestive of a large number of multidrug resistant and extensively resistant bacteria in broiler farms, which could seriously reduce the effectiveness of antimicrobial agents.

In this study, two farms underwent AMR gene screening. The results showed statistically significant differences in the dissemination and prevalence of 16 AMR gene groups between these two farms. These differences indicated that the predominant AMR gene types varied between the two geographically distinct farms. This observation is consistent with previous research[9,33]. Heat-map analysis further highlighted the different relative abundance levels in AMR genes between farm A and farm B. There were higher relative abundance values for AMR genes in fecal samples from farm B, suggesting higher copy numbers for these AMR

genes, which could contribute to a higher level of AMR and severe multidrug resistance. Compared with farm B, the AMR gene prevalence rates were more seriously in farm A, especially gene spread related element *int*I1 and IS*CR*1. This observation indicated that more strictly antimicrobial agent usage controlling should be given, and antimicrobial agent rotational and mixed application strategy should be considered urgently in farm A. From Heat-map analysis, the high relative abundance values of *aac*(6')-*Ie*-*aph*(2'')-*Ia* and *fex*A in farm B indicated that aminoglycosides and phenicols agents should be used cautiously in further.

CONCLUSIONS

In conclusion, we have established a new, reliable, convenient, rapid and high-throughput real-time PCR assay for the screening and identification of AMR genes in broiler fecal samples. Our method can provide a great amount of data on the presence and abundance of 47 groups of distinct AMR genes/AMR gene spread related elements, including 688 variants, from a total of 47 different samples directly and simultaneously. The new assay was successfully applied to AMR gene screening in 273 broiler fecal samples from two farms. Both qualitative and quantitative information obtained from this novel assay highlighted the high prevalence of AMR gene existence in broiler farms, with different AMR gene groups predominating between the two farms. To our best knowledge, this is the first report of the simultaneous screening of up to 47 groups of AMR genes/AMR gene spread related elements directly from broiler fecal samples using a high-throughput microfluidic dynamic real-time assay. This novel assay could be popularized and applied to AMR surveillance in situations other than livestock and poultry husbandry in the near future.

DECLARATIONS

The ethics approval and consent of participate isnot applicable.

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Supplementary Table S1. Target AMR gene primers and probes used in this study

No	Gene group	Forward primer (5′-3′)	Reverse primer (5′-3′)	Probe (5′-FAM-MGB-3′)
1	*bla*$_{\text{OXA-48}}$-like	GGG CGA ACC AAG CAT TTT T	GCG ATC AAG CTA TTG GGA ATT T	CCC GCA TCT ACC TTT
2	*bla*$_{\text{IMP}}$ A	CCG(A) GGA CAC ACT CCA GAT AAC	CCG TAC GGT TTA ATA AAA CAA CCA	TGC CTG AAA GGA AAA T
3	*bla*$_{\text{IMP}}$ B	CGC GGC TAT AAA ATA AAA GGC AG	ATA GAT TGA GAA TTA AGC CAC TCT ATT CC	TTC ATA GCG ACA GCA CG
4	*bla*$_{\text{IMP}}$ C	TGT GGA GCG CGG CTA TAA A	TGC TGT CGC TAT GGA AAT GTG	TCA AAG GCA GTA TTT CC
5	*bla*$_{\text{IMP}}$ D	CAG TAT TTC CAC ACA TTT CCA TGG	GTG GAG GAT AGA TTG AGA ATT AAG CCA CT	ACA GTA CGG CTG GAA TA
6	*bla*$_{\text{IMP}}$ E	ACA TTT CCA TAG CGA CAG CAC AG	CCA AAC CAC TAC GGT TAT CTT GAG TGT	CCC ACG TAT GCA TCT G
7	*bla*$_{\text{IMP}}$ F	GCC GGA CGG TCT TGG TTA	CGG ACT TAG GCC ATG CTT CT	TGG GAG ACG CAA ATC
8	*bla*$_{\text{NDM}}$	GCC CGC TCA AGG TAT TTT ACC	CGA TCC CAA CGG TGA TAT TGT	CCG GCC ACA CCA GT
9	*bla*$_{\text{KPC}}$	AAC CAT TCG CTA AAC TCG AAC AG	AAT GAG CTG CAC AGT GGG AAG	ACT TTG GCG GCT CC
10	*bla*$_{\text{VIM}}$	AAT GGT CTC ATT GTC CGT GAT G	TTC GCA CCC CAC GCT GTA	TGA TGA GTT GCT TTT GAT TG
11	*bla*$_{\text{IMI}}$	GGT GTC TAC GCT TTA GAC ACT GGC	CTG TGT TTA GAT CTA ACT CCC AAC GA	TGG TCC TGA GGG TAT G
12	*bla*$_{\text{IND}}$ A	CGA CCG CCA AGA CCA ATG	ATA ATT TCG GTT GAG GTT GCT TTT	ACT GTT GAA AAA AGA CG
13	*bla*$_{\text{IND}}$ B	TTG TTA CCC AAA AAG GAG TTG TCT T	GGT ATC CAT CAG GCT TTG GTA CTG	CCG TGG GAA AAG G
14	*bla*$_{\text{IND}}$ C	TCT TGA AAA AAG ACG GAA AAG CA	GGT ACG GTT TTC CGG TTT TGA	CAT CCA CAG AAA TC
15	*bla*$_{\text{IND}}$ D	GCC CAA GAC GAT GAA TAA ATT AAA A	TTC ATC ATG CCC GGG AAT	CCA AAT ATT CAA AAG CC
16	*bla*$_{\text{GES}}$	CAC YTC GAC CCA CAC CAT TG	CGC GTC TCC CGT TTG GT	AGG TGG CTG ATC GGA
17	*bla*$_{\text{CTX-M}}$ A	YGR ACG TAC AGC AAA AAC	CGC TCA TCW GCA CGA TAA	ATT AGA GCR GCA GTC GGG AG
18	*bla*$_{\text{CTX-M}}$ B	CAG TTG GTG ACG TGG CTT AAG G	GCC GGT TTT ATC GCC CAC T	CCG GTA GCG CGA GCA
19	*bla*$_{\text{CTX-M}}$ C	TGG CTG AAA GGC AAC ACC AC	GCG ATA TCA TTC GTC GTA CCA TAA	CAG GGC TAC CCA CAT C
20	*bla*$_{\text{CTX-M}}$ D	TAT CGC GGT GAT GAA CGC TT	AAG CAG CTG CTT AAT CAG CCT G	CCA ATG TGC AGT ACC AG
21	*bla*$_{\text{CTX-M}}$ E	GCC GCC GAC GCT AAT ACA T	TTA GGT TGA GGC TGG GTG AAG T	CGA CGG CTT TCT G
22	*bla*$_{\text{SHV}}$ A	TGC CTT TTT GCG CCA GAT	GCC TCA TTC AGT TCC GTT TCC	CAA CGT CAC CCG CCT T
23	*bla*$_{\text{SHV}}$ B	GAC GGT CGG CGA ACT CTG T	AGA TTG GCG GCG CTG TTA T	CGC CAT TAC CAT GAG C
24	*bla*$_{\text{SHV}}$ C	CCG TCG GCA AGG TGT TTT T	TCC ACT ATC GCC AGC AGG AT	ACC GGC GAG TAG TC
25	*bla*$_{\text{SHV}}$ D	ATT GTC GCC CTG CTT GGC	TTG TGG TGA TAT TAT CTG CGG GA	CGA ATA ACA AAG CAG AGC G
26	*bla*$_{\text{TEM}}$ A	GAT AAC ACT GCD GCC MAC	TGG TAY GGC YTC AKT CAG	TTC TGA CAA CGA TCG GAG GA
27	*bla*$_{\text{TEM}}$ B	TCG TCG TTT GGT ATG GCT	GCC AAC TTA CTT CTG ACA AC	TTC AGC TCC GGT TCC CAA CG
28	*bla*$_{\text{OXA}}$	GGA ACA GCA ATC ATA CAC CAA AGA C	TTG GGT TAT TTC TTG CGA AAC C	TGG ATG CAA TTT TC
29	*bla*$_{\text{PER}}$	GCC GAT GAT CAG GTG CAG TA	ACT TCC ATA ACA AAG CCT GCG A	CAA AAC TGG ACC TCG ATG
30	*bla*$_{\text{CARB}}$ A	CCT TGA TGG AAG ATA ACG CCC TA	CAG AGC GGT CTG CAA TCG A	CTT ACG AGT CAC GCA CAC A
31	*bla*$_{\text{CARB}}$ B	CGT CTA GAC CGT ATT GAG CCT GAT	TTG CCT TAG GAG TTG TCG TAT CC	AAA TGA AGG TAA GCT CG
32	*bla*$_{\text{CARB}}$ C	CCA GCA CGT TAA ATC AAT TAT TAT TTG	CAC TGG CAA TAC TGA CCT CAA TAA AT	TCC ACA TTA TCT GAA GCT AGT CA
33	*bla*$_{\text{CARB}}$ D	TCG TCA ACA ACT AGA ATC TTG GCT TA	GGA ACG CCT TTA CGA AAC AAT G	AGG TAA TGA GGT TGG CGA T
34	*qnr*A	TCG AGG ATT GCA GTT TCA TTG A	TCC CTG AAC TCT ATG CCA AAG C	CAC TTC AGC TAT GCC GAT
35	*qnr*C	CAT TTT CTG ATG ACT TTT GGG AGC	CTG CTC CAA AAG CTG CTC TTG T	TGG CTT AGA ACC TCG
36	*qnr*S	GCG GGY GCA TCA CTG AAA G	ACA ACA ATA CCC ARY GCT TCG AGA	CAC GCC GAA CTC G
37	*mcr*-1	TCG GCT TTG TGC TGA CGA T	AAA TCA ACA CAG GCT TTA GCA CAT A	CTG TCG TGC TCT TTG
38	*mcr*-3	AAT CGC GCT CGC AAT AGC	TTA GGT ACT CGG TCG CAG ACG	AGG GCC TGC TAG ATG
39	*arm*A	TCA AAA ACC TAT ACT TTA TCG TCG TCT T	TAT TTT AGA TTT TGG TTG TGG CTT CA	AAC TTC CCA ATA ATG CTA C
40	*aac*(6′)-*Ie*-*aph*(2″)-*Ia*	GCTAATGCAGTTATTTTAGACCCTCATA	ATA TTT CAT TGC CTT AAC ATT TGT GG	CCA AGA GCA ATA AGG

Continued

No	Gene group	Forward primer (5′-3′)	Reverse primer (5′-3′)	Probe (5′-FAM-MGB-3′)
41	*fex*A	GCA AAT AAC GCC GCA GTA AGT	TTA TCG GCT CAG TAG CAT CAC G	ACT CGA TGC AGA CAA G
42	*fex*B	TGA TTG AAC GCG GAG TCT GG	AAA TCA CCC AAG TCA ATG ACG TCT	AGT GTG GCT CTT GTC C
43	*cfr*	AAT AAG TGC ACC TGA GAT GTA TGG AG	CAT ATA ATT GAC CAC AAG CAG CG	CGA AGG GCA GGT AGA
44	*optr*A	AAG GGG ACA GAA GCT TGG AAT T	TCC ACT AAA CTG AAA TGA GCCAAG	TTG GTA AAT CCA CGT TGC T
45	*aac*(6′)-*Ib*-cr	AGCTCTGGTTGAGTTGCTGTTC	TAG GCA TCA CTG CGT GTT CG	CCG AGG TCA CCA AGA T
46	*int*I1	GGC AAC YTT GGG CAG CA	CTG AAG CCA GGG CAG ATC C	TTC GGT CTC CAC GCA TCG TCA GG
47	IS*CR*1	TAA CCG AAG CAC CAT GTA ATT GA	CCT CAG CCA TCG CAT CG	TCG TCT TCG CCC TCT TCC AGT GTC A
48	*16Sr*DNA	CCT GGA CGA AGA CTG ACG CTC	CTC AAG GGC ACA ACC TCC AAG	CAA ACA GGA TTA GAT ACC CTG GTA GT

Supplementary Table S2. 43 novel recombinant plasmids generated in this study

No.	Gene group	Vector type	Recombinant plasmid concentration (ng/μL)	Recombinant plasmid length (nt)	$\times 10^{10}$ Copies/μL
1	$bla_{\text{OXA-48}}$-like	pUC19	173.3	2,750	5.57
2	bla_{IMP} A	pUC57	160.8	2,794	5.25
3	bla_{IMP} B	pUC57	49.3	2,796	1.61
4	bla_{IMP} C	pUC57	46.3	2,769	1.53
5	bla_{IMP} D	pUC57	210.2	2,782	6.89
6	bla_{IMP} E	pUC57	159.2	2,924	4.96
7	bla_{IMP} F	pUC57	115.3	2,766	3.82
8	bla_{NDM}	T1	95.0	3,892	2.23
9	bla_{KPC}	T1	183.8	3,967	4.23
10	bla_{VIM}	T1	192.8	3,890	4.52
11	bla_{IMI}	pUC19	147.7	3,074	4.47
12	bla_{IND} A	pUC19	83.8	2,753	4.63
13	bla_{IND} B	pUC19	139.7	2,766	4.44
14	bla_{IND} C	pUC19	134.7	2,752	7.80
15	bla_{IND} D	pUC19	235.2	2,762	5.81
16	bla_{GES}	pUC57	83.8	2,766	2.76
17	$bla_{\text{CTX-M}}$ A	pUC57	93.2	2,776	3.33
18	$bla_{\text{CTX-M}}$ B	pUC57	126.1	2,803	4.10
19	$bla_{\text{CTX-M}}$ C	pUC57	142.9	2,823	4.62
20	$bla_{\text{CTX-M}}$ D	T1	176.3	3,934	4.09
21	$bla_{\text{CTX-M}}$ E	T1	188.3	3,886	4.42
22	bla_{SHV} A	T1	125.7	3,898	2.94
23	bla_{SHV} B	pUC57	370.6	2,770	12.21
24	bla_{SHV} C	T1	262.4	3,893	6.15
25	bla_{SHV} D	T1	303.1	3,889	7.11
26	bla_{TEM} A	T1	155.7	3,889	5.97
27	bla_{TEM} B	T1	70.8	3,890	5.54
28	bla_{OXA}	T1	90.6	3,899	2.12
29	bla_{PER}	pUC57	117.0	2,828	3.77

Continued

No.	Gene group	Vector type	Recombinant plasmid concentration (ng/μL)	Recombinant plasmid length (nt)	×10^{10}Copies/μL
30	*bla*$_{CARB}$ A	pUC57	106.9	2,841	3.43
31	*bla*$_{CARB}$ B	pUC57	98.4	2,795	3.22
32	*bla*$_{CARB}$ C	pUC57	62.1	2,831	2.00
33	*bla*$_{CARB}$ D	pUC57	77.2	2,778	2.53
34	*qnr*A	pUC57	38.8	2,855	1.24
35	*qnr*C	pUC57	27.9	2,853	0.89
36	*qnr*S	pUC57	27.0	2,907	0.85
37	*mcr*-1		[9]		
38	*mcr*-3	T1	248.0	3,942	5.74
39	*arm*A		[16]		
40	*aac*(6′)-*Ie*-*aph*(2″)-*Ia*	pUC57	102.9	2,896	3.24
41	*fex*A	pUC57	138.3	2,861	4.41
42	*fex*B	pUC57	151.2	2,827	4.88
43	*cfr*	pUC57	25.0	2,853	0.80
44	*optr*A	pUC57	129.7	2,963	3.93
45	*aac*(6′)-*Ib*-cr	pUC57	151.3	2,908	4.75
46	*int*I1		[17]		
47	IS*CR*1		[17]		
48	*16Sr*DNA		[9]		

Supplementary Table S3. Standard curves parameters for 43 AMR genes from conventional real-time PCR assays

No.	Gene type group	linear dynamic range	r^2 (%)	Linear regression equation	LOD (copies/reaction)
1	*bla*$_{OXA-48}$-like	5.75×10^{9}–5.75×10^{2}	0.999	y = −3.519x + 45.50	57.5
2	*bla*$_{IMP}$ A	5.25×10^{9}–5.25×10^{2}	0.997	y = −3.551x + 46.85	52.5
3	*bla*$_{IMP}$ B	1.61×10^{9}–1.61×10^{2}	0.993	y = −3.321x + 45.49	16.1
4	*bla*$_{IMP}$ C	1.53×10^{9}–1.53×10^{1}	0.998	y = −3.592x + 45.09	15.3
5	*bla*$_{IMP}$ D	6.89×10^{9}–6.89×10^{1}	0.999	y = −3.653x + 46.44	68.9
6	*bla*$_{IMP}$ E	4.96×10^{9}–4.96×10^{1}	0.998	y = −3.568x + 45.16	49.6
7	*bla*$_{IMP}$ F	3.82×10^{9}–3.82×10^{2}	0.998	y = −3.506x + 44.56	38.2
8	*bla*$_{NDM}$	2.23×10^{9}–2.23×10^{1}	0.995	y = −3.470x + 43.73	22.3
9	*bla*$_{KPC}$	4.23×10^{9}–4.23×10^{1}	0.997	y = −3.343x + 43.77	42.3
10	*bla*$_{VIM}$	4.52×10^{9}–4.52×10^{2}	0.997	y = −3.534x + 46.05	45.2
11	*bla*$_{IMI}$	4.47×10^{9}–4.47×10^{2}	0.999	y = −3.802x + 49.85	44.7
12	*bla*$_{IND}$ A	4.44×10^{9}–4.44×10^{3}	0.986	y = −3.520x + 51.11	44.4
13	*bla*$_{IND}$ B	4.44×10^{7}–4.44×10^{2}	0.998	y = −3.410x + 42.51	44.4
14	*bla*$_{IND}$ C	7.80×10^{9}–7.80×10^{1}	0.998	y = −3.568x + 43.73	78.0
15	*bla*$_{IND}$ D	$5.81\times15.81\times10^{9}$–$5.81\times10^{1}$	0.997	y = −3.469x + 44.63	58.1
16	*bla*$_{GES}$	2.76×10^{9}–2.76×10^{1}	0.999	y = −3.477x + 44.03	27.6
17	*bla*$_{CTX-M}$ A	3.33×10^{9}–3.33×10^{3}	0.995	y = −3.753x + 42.96	33.3
18	*bla*$_{CTX-M}$ B	4.10×10^{9}–4.10×10^{3}	0.999	y = −3.748x + 47.70	41.0

Continued

No.	Gene type group	linear dynamic range	r^2 (%)	Linear regression equation	LOD (copies/reaction)
19	*bla*$_{CTX-M}$ C	4.62×10^{9}–4.62×10^{2}	0.996	y = −3.486x + 44.81	46.2
20	*bla*$_{CTX-M}$ D	4.09×10^{9}–4.09×10^{3}	0.984	y = −3.651x + 47.14	40.9
21	*bla*$_{CTX-M}$ E	4.42×10^{9}–4.42×10^{1}	0.983	y = −3.558x + 44.01	44.2
22	*bla*$_{SHV}$ A	2.94×10^{7}–2.94×10^{2}	0.998	y = −3.228x + 42.61	29.4
23	*bla*$_{SHV}$ B	1.22×10^{9}–1.22×10^{2}	0.998	y = −3.081x + 46.53	12.2
24	*bla*$_{SHV}$ C	6.15×10^{8}–6.15×10^{1}	0.998	y = −2.971x + 38.70	61.5
25	*bla*$_{SHV}$Ⅳ	7.11×10^{9}–7.11×10^{1}	0.998	y = −3.457x + 46.18	71.1
26	*bla*$_{TEM}$ A	5.97×10^{8}–5.97×10^{1}	0.991	y = −3.628x + 42.58	59.7
27	*bla*$_{TEM}$ B	5.45×10^{8}–5.45×10^{2}	0.995	y = −3.670x + 42.46	54.5
28	*bla*$_{OXA}$	2.12×10^{9}–2.12×10^{2}	0.991	y = −3.133x + 41.88	21.2
29	*bla*$_{PER}$	3.77×10^{9}–3.77×10^{2}	0.998	y = −3.464x + 48.88	37.7
30	*bla*$_{CARB}$ A	3.43×10^{9}–3.43×10^{2}	0.999	y = −3.502x + 47.06	34.3
31	*bla*$_{CARB}$ B	3.22×10^{9}–3.22×10^{2}	0.999	y = −3.400x + 46.71	32.2
32	*bla*$_{CARB}$ C	2.00×10^{9}–2.00×10^{3}	0.999	y = −3.763x + 50.30	20.0
33	*bla*$_{CARB}$ D	2.53×10^{9}–2.53×10^{2}	0.999	y = −3.494x + 46.60	25.3
34	*qnr*A	1.24×10^{9}–1.24×10^{1}	0.995	y = −3.294x + 44.77	12.4
35	*qnr*C	8.92×10^{8}–8.92×10^{1}	0.990	y = −3.125x + 42.66	89.2
36	*qnr*S	8.47×10^{9}–8.47×10^{3}	0.998	y = −3.692x + 52.81	84.7
37	*mcr*-3	2.70×10^{9}–2.70×10^{3}	0.994	y = −3.224x + 46.27	27.3
38	*aac*(6′)-*Ie*-*aph*(2″)-*Ia*	3.24×10^{9}–3.24×10^{3}	0.980	y = −2.858x + 46.50	32.4
39	*fex*A	4.41×10^{8}–4.41×10^{3}	0.999	y = −3.625x + 46.93	44.1
40	*fex*B	4.88×10^{9}–4.88×10^{2}	0.996	y = −3.766x + 50.58	48.8
41	*cfr*	8.00×10^{9}–8.00×10^{3}	0.995	y = −3.560x + 48.66	80.0
42	*optr*A	3.93×10^{9}–3.93×10^{2}	0.998	y = −3.663x + 46.63	39.3
43	*aac*(6′)-*Ib*-cr	4.75×10^{9}–4.75×10^{2}	0.999	y = −3.499x + 45.48	47.5