



Multiple Cross Displacement Amplification Coupled With Gold Nanoparticles-Based Lateral Flow Biosensor for Detection of the Mobilized Colistin Resistance Gene *mcr-1*

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Fast dissemination of the mobilized colistin resistance (*mcr*) gene *mcr-1* in *Enterobacteriaceae* causes a huge threat to the treatment of severe infection. In the current report, a multiple cross displacement amplification (MCDA) coupled with the detection of amplified products by gold nanoparticles-based lateral flow biosensor (LFB) assay (MCDA-LFB) was established to identify the *mcr-1* gene with simpleness, rapidity, specificity, and sensitivity. The MCDA-LFB assay was performed at a isothermal temperature (63°C) for only 30 min during the amplification stage, and the reaction products were directly identified by using LFB which obtained the result within 2 min. The entire process of experiments, from templates extraction to result judging, was accomplished in <60 min. For the analytical specificity of this method, all of the 16 *mcr-1*-producing strains were positive, and all of the non-*mcr-1* isolates produced the negative results. The sensitivity of *mcr-1*-MCDA-LFB assay was as little as 600 fg of plasmid DNA per reaction in pure culture, and approximately 4.5×10^3 CFU/mL (~ 4.5 CFU/reaction) in spiked fecal samples. Therefore, this technique established in the present study is suitable for the surveillance of *mcr-1* gene in clinic and livestock industry.

Keywords: *mcr-1*, colistin resistance, multiple cross displacement amplification, lateral flow biosensor, detection assay

INTRODUCTION

The rapid increase of carbapenem-resistant *Enterobacteriaceae* (CRE) expressing *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo- β -lactamase (NDM) and oxacillinase (OXA) OXA-48 has risen serious concerns in clinic. Colistin, a “last resort” antibiotic, has a crucial role for treating the infection caused by those species (Nation and Li, 2009). Therefore, the number of colistin consumptions increasing along with the global augment of CRE will bring about the risk of emerging resistance (Gelband et al., 2015).

Resistance to colistin was linked with chromosomal resistance mechanisms in varieties of strains in the past (Olaitan et al., 2014). Since a new mobilized colistin resistance gene, *mcr-1*, carried by plasmid in an *Escherichia coli* was first reported in China in 2015 (Liu et al., 2016), which has been identified in numerous countries. China, Germany and Vietnam carry an important proportion of positive samples (Wang et al., 2018a). The *mcr-1*-positive bacterial species include *Salmonella enterica*, *E. coli*, *Escherichia fergusonii*, *Enterobacter aerogenes*, *K. pneumoniae*, *Citrobacter braaki*, and *Klebsiella aerogenes* (Doumith et al., 2016; Li et al., 2016; Stoesser et al., 2016; Zeng et al., 2016; Sennati et al., 2017; Wang et al., 2017a, 2018a). Besides discovered in Clinical samples, *mcr-1* is also detected from environmental settings: meat and vegetable products purchased from markets, Animal feces collected from farms, fecal samples of pets gathered from pet hospital, river water, and seawater (Chen et al., 2017). The wide dissemination of *mcr-1* across diversified species is benefited from many types of *mcr-1*-bearing plasmids covering IncHI2, IncI2, IncFI, IncX4, and IncX1-X2 hybrid type (McGann et al., 2016; Sun et al., 2016, 2017; Yang et al., 2016; Guo et al., 2017). Similarly, the genetic environments of *mcr-1* gene also impact its transmission. A global data set of roughly 500 isolates producing *mcr-1* analyzed by whole-genome sequencing (WGS) has revealed that an initial mobilized event of *mcr-1* is mediated by an ISApI1-*mcr-1*-orf-ISApI1 transposon around 2006 (Wang et al., 2018a). The horizontal transfer of *mcr-1* gene causing inflation of colistin-resistant isolates will lead to the shortage of effective measures for treating infections with multidrug-resistant bacteria. Therefore, a rapid, sensitive and specific diagnostic assay for *mcr-1* detection is imperative to devise.

Currently, several categories of molecular diagnostic methodologies including conventional polymerase chain reaction (PCR) and real-time PCR methods have been devised to identify *mcr-1* gene (Bontron et al., 2016). Nevertheless, the requirements of highly sophisticated devices, strictly experimental environments and well-trained personnel restrict those techniques to apply in resource-challenged areas and “on-site” detection (Niu et al., 2018). Recently, multiple cross displacement amplification (MCDA), a novel nucleic acid amplification technique, has been utilized in detection of bacterial agents, such as *Listeria monocytogenes* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Wang et al., 2017b, 2018c). The approach was based on isothermal strand-displacement polymerization reaction, five pairs of primers were designed to amplify the corresponding regions of target sequence. Without the denaturing step, the primers of high concentration could bind to the template at a isothermal temperature. Several single stem-loop DNA structures and single-stranded DNAs were yielded with the primers binding and the reaction chain extending. Then, those DNA products were exponentially amplified as the reaction progressed (Wang et al., 2015). With the advantages of rapidity, specificity and sensitivity, MCDA operated in a simple heater can yield amplicons from a few colonies (Wang et al., 2017b, 2018b), the amplicons are identified by a gold nanoparticles-based lateral flow biosensor (LFB) subsequently.

In this study, a MCDA-LFB assay for the rapid detection of *mcr-1* was established, and the sensitivity and specificity of above method in pure culture and in spiked fecal samples were analyzed.

MATERIALS AND METHODS

Reagents and Instruments

Bacterial genomes extraction kits were obtained from Beijing ComWin Biotech Co., Ltd. (Beijing, China). QIAGEN plasmid kits and QIAamp fast DNA stool mini kits were purchased from Qiagen Co., Ltd. (Beijing, China). Isothermal amplification kits (including reaction buffer and Bst DNA polymerase 2.0), colorimetric indicator (Malachite Green), and disposable LFB were provided by Beijing-HaiTaiZhengYuan Technology Co., Ltd. (Beijing, China). The heating thermostat (MTH-100) was purchased from Hangzhou MiU Instruments Co., Ltd. (Hangzhou, China). The UV transilluminator (UVsolo touch) was obtained from Analytik Jena (Jena, Germany). Nanodrop instrument (ND-2000) was purchased from Thermo Fisher Scientific Co., Ltd. (Massachusetts, America).

Bacterial Isolates and Genomic Template Preparation

A total of 59 organisms consisting of 16 *mcr-1*-positive isolates and 43 non-*mcr-1* bacteria were used in this study (Table 1). The

TABLE 1 | Strains used in this study.

| Genotype ^a | Bacteria species | The source of strains ^b | No. of isolates |
|-----------------------|--------------------------------|------------------------------------|-----------------|
| <i>mcr-1</i> | <i>Escherichia fergusonii</i> | ICDC (ZG2016M34-3) | 1 |
| | <i>Escherichia fergusonii</i> | ICDC | 3 |
| | <i>Escherichia fergusonii</i> | WHCDC | 1 |
| | <i>Escherichia coli</i> | ICDC | 8 |
| | <i>Escherichia coli</i> | WHCDC | 2 |
| | <i>Salmonella enteritidis</i> | ICDC | 1 |
| KPC-2 | <i>Klebsiella pneumoniae</i> | WHCDC | 10 |
| | <i>Pseudomonas aeruginosa</i> | WHCDC | 1 |
| NDM-1 | <i>Escherichia coli</i> | WHCDC | 4 |
| | <i>Enterobacter cloacae</i> | WHCDC | 2 |
| | <i>Klebsiella pneumoniae</i> | WHCDC | 4 |
| NDM-5 | <i>Escherichia coli</i> | WHCDC | 2 |
| IMP-4 | <i>Escherichia coli</i> | WHCDC | 2 |
| NO | <i>Acinetobacter baumannii</i> | WHCDC | 3 |
| | <i>Pseudomonas aeruginosa</i> | WHCDC | 3 |
| | <i>Serratia marcescens</i> | WHCDC | 2 |
| | <i>Escherichia coli</i> | WHCDC | 2 |
| | <i>Staphylococcus aureus</i> | WHCDC | 4 |
| | <i>Enterococcus faecalis</i> | WHCDC | 2 |
| | <i>Enterococcus faecium</i> | WHCDC | 2 |

^aNO, the strains did not carry above genes.

^bICDC, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. WHCDC, Wuhan Centers for Disease Prevention and Control.

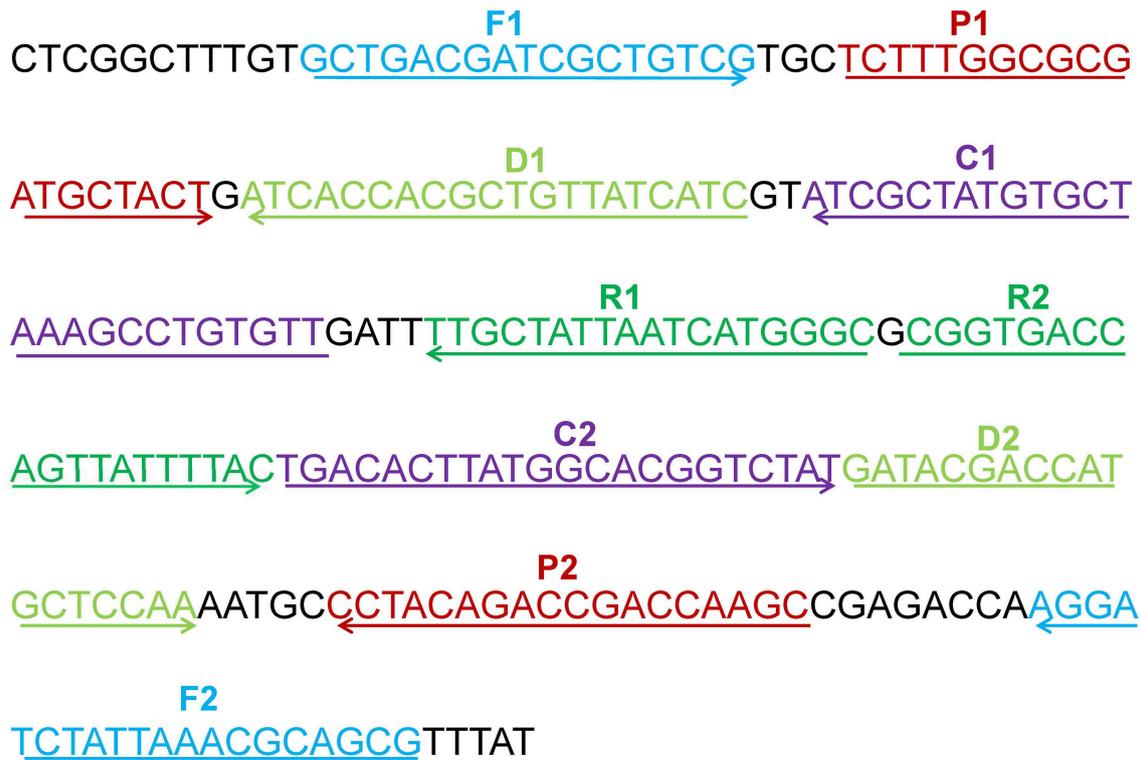


FIGURE 1 | The location of the five primers pairs on the partial *mcr-1* gene sequence. Different pairs of primers were represented by various of colors. The arrowed lines showed the amplification direction of primers.

confirmation of all genes used conventional PCR and sequencing. The *mcr-1*-positive bacterial species included 5 *E. fergusonii*, 10 *E. coli*, and 1 *Salmonella enteritidis*. The *mcr-1*-negative isolates involved KPC-2-positive stains (*K. pneumoniae* and *Pseudomonas aeruginosa*), NDM-1-positive isolates (*E. coli*, *Enterobacter cloacae*, and *K. pneumoniae*), NDM-5-producing *E. coli*, IMP-4-producing *E. coli*, and *mcr-1*/carbapenemase-negative species (*Acinetobacter baumannii*, *P. aeruginosa*, *Serratia marcescens*, *E. coli*, *S. aureus*, *Enterococcus faecalis*, and *Enterococcus faecium*). According to the handling instruction, the genomic DNA of all strains was extracted by bacterial genomes extraction kits, the plasmid DNA of *mcr-1*-producing *E. fergusonii* (ICDC-ZG2016M34-3) acted as a representative sample for optimization of reaction condition and sensitivity detection was acquired by QIAGEN Plasmid Kits, and quantified by a Nanodrop ND-2000 instrument.

Primers Design of MCDA Assay

A set of five pairs of primers, including 2 displacement primers (F1 and F2), 2 cross primers (CP1 and CP2), and 3 pairs of amplification primers (C1, C2, D1, D2, R1, and R2), targeted 10 distinct regions more than 150 bp on *mcr-1* gene (Wang et al., 2015). Two softwares named Primer Premier 6.0 and PrimerExplorer V4 were used to design the 10 MCDA primers based on *mcr-1* gene (GenBank accession number: KX886345). The dimer and hairpin structures of all primers were detected

TABLE 2 | Primers of the MCDA assay to identify the *mcr-1* gene.

| Primers ^a | Sequences and modifications (5'-3') | Length ^b |
|----------------------|---|---------------------|
| F1 | GCTGACGATCGCTGTCTG | 17 nt |
| F2 | CGCTGCGTTTAATAGATCCT | 20 nt |
| CP1 | AACACAGGCTTTAGCACATAGCGATCTTTGGCGCGATGCTACT | 43 mer |
| CP2 | TGACACTTATGGCACGGTCTATGCTTGGTCGGTCTGTAGG | 40 mer |
| C1* | FITC-AACACAGGCTTTAGCACATAGCGAT | 25 nt |
| C2 | TGACACTTATGGCACGGTCTAT | 22 nt |
| D1* | biotin-GATGATAACAGCGTGGTGAT | 20 nt |
| D2 | GATACGACCATGCTCCAA | 18 nt |
| R1 | GCCCATGATTAATAGCAA | 18 nt |
| R2 | CGGTGACCAGTTATTTTAC | 19 nt |

^aC1*, 5' end of C1 was labeled with FITC; D1*, 5' end of D1 was labeled with biotin
^bnt meant the nucleotide; mer referred to monomeric.

by Integrated DNA Technologies design tools (Wang et al., 2016), and the specificity of which was analyzed by using Basic Local Alignment Search Tool (Blast). The relevant information of primers pairs regarding positions and sequences was displayed in **Figure 1** and **Table 2**. Furthermore, The FITC (Fluorescein isothiocyanate) and biotin labeled at 5' end of the C1 and D1 primers, respectively, and the labeled primers were named as C1* and D1*. All of the primers were synthesized and purified

by Sangon Biotechnology Co., Ltd. (Shanghai, China) at HPLC purification grade.

The Standard MCDA Assay

The MCDA reaction systems were performed according to the previous studies (Wang et al., 2016). Each reaction, the total volumes of 25 μ L, included reaction buffer (12.5 μ L), Bst DNA polymerase 2.0 (1 μ L), colorimetric indicator (1 μ L), cross primers (1.6 μ M each), displacement primers (0.4 μ M each), amplification primers (0.4 μ M each), and 1 μ L of DNA template. The NDM-1-positive *E. coli* (WHCDC-WH67) and KPC-2-producing *K. pneumoniae* (WHCDC-WH108) were regarded as the negative controls, and the distilled water was served as the blank control. To assess the optimal reaction temperature of *mcr-1*-MCDA, the MCDA amplification systems were executed with a constant temperature in the range of 60–67°C for 40 min.

The MCDA reaction products were analyzed by three detection methods including 2% agarose gel electrophoresis, colorimetric indicator, and LFB (Wang et al., 2017b). When employing gel electrophoresis, 3 μ L of reaction mixtures were run at 110 volts for 60 min. A ladder of multiple bands could be observed in the positive reactions, but not in the negative and blank controls. Reaction products were detected by using colorimetric indicator, the color of amplified products remained unchanged. Nevertheless, the negative and blank controls reactions changed from blue to colorless. The material, theory and operation procedure of LFB were previously described by Wang et al. (2017b). 0.2 μ L of amplicons followed by three drops of the running buffer consisting of 1% Tween 20 and 0.01 mol/L phosphate-buffered saline were added to the well of sample pad (Niu et al., 2018). After 1–2 min, two red lines named test line (TL) and control line (CL), respectively, could be visualized in positive products, while only the CL was observed for the negative and blank control.

Sensitivity and Specificity of the *mcr-1*-MCDA-LFB Assay

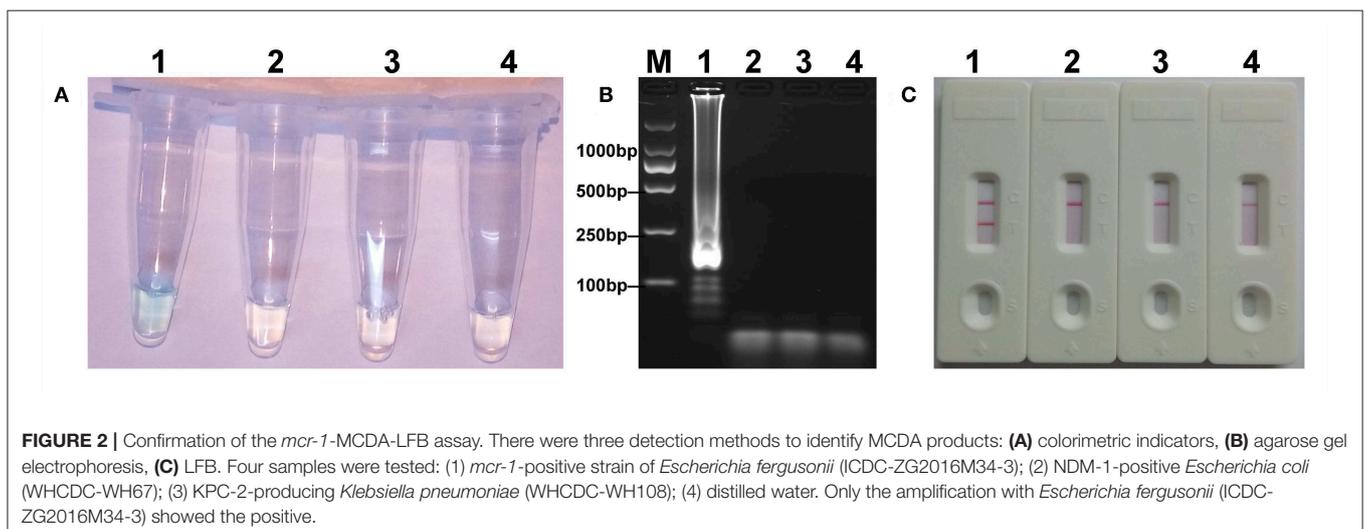
The plasmid DNA of *E. fergusonii* ICDC-ZG2016M34-3 was serially diluted (6 ng, 60 pg, 600 fg, 60 fg, and 6 fg per μ L) for sensitivity analysis of *mcr-1*-MCDA-LFB detection. The colorimetric indicator and agarose gel electrophoresis were carried out simultaneously. Each test was repeated three times. The specificity of *mcr-1*-MCDA-LFB assay was evaluated with the DNA templates of 16 *mcr-1*-producing strains and 43 non-*mcr-1* strains (Table 1). The specificity evaluations were confirmed twice.

The Optimal Amplification Time

In order to screen the optimal time for the *mcr-1*-MCDA-LFB assay, The MCDA mixture was completed at the reaction temperature in the range from 10 to 40 min at 10 min intervals. Subsequently, the MCDA products were detected by LFB detection. Each amplification time was operated at two times.

mcr-1-MCDA-LFB Detection in Spiked Fecal Samples

The fecal samples were obtained from a healthy man in Wuhan, China. *Mcr-1* gene was not detected in those samples according to the microbial culture and PCR identification. The volumes of 100 μ L were taken out from the *mcr-1*-positive *E. fergusonii* ICDC-ZG2016M34-3 cultures when the optical density (OD) of that reached to 0.6. The suspensions were serially diluted (10^{-1} – 10^{-8}), and the aliquots of 100 μ L dilutions (10^{-3} – 10^{-6}) were incubated on nutrient agar plates with three replicates, colony forming units (CFUs) were counted subsequently. One hundred microliter of diluted *mcr-1*-producing cultures (10^{-2} – 10^{-7}) with known amounts (4.5×10^6 – 4.5×10^1 CFU/ mL) was, respectively, added to 0.2 g of fecal sample and mixed well. DNA templates were extracted with the manufacturer's protocol by using QIAamp fast DNA stool mini kits. The extracted genomic DNA was dissolved in 100 μ L



of elution buffer, and 1 μ l of which was used for MCDA-LFB detection as templates. A non-spiked feces sample was tested as negative control. The products of MCDA were also detected by colorimetric indicator and 2% agarose gel electrophoresis. The evaluation assay for limit of detection in fecal samples was conducted triplicate.

Conventional PCR

Mcr-1 gene was amplified by conventional PCR, primers: *mcr-1*-F, 5'-CGG TCA GTC CGT TTG TTC-3'; *mcr-1*-R, 5'-CGG TCA GTC CGT TTG TTC-3'. And amplicons were subsequently sequenced (Liu et al., 2016). The LOD of *mcr-1*-MCDA-LFB and

conventional PCR in pure culture and in fecal samples were compared, respectively.

RESULTS

The Verification of *mcr-1* MCDA Products

The *mcr-1* MCDA assays were performed at a constant temperature (63°C) for 40 min to verify the availability of MCDA primers. Positive reaction appeared with DNA from *mcr-1*-producing *E. fergusonii* (ICDC-ZG2016M34-3), but not with NDM-1-positive *E. coli* (WHCDC-WH67), KPC-2-producing *K. pneumoniae* (WHCDC-WH108), and the blank control (Figure 2). Therefore, the primers of *mcr-1*-MCDA was suitable for establishment of the MCDA-LFB assay to detect *mcr-1* gene.

Temperature Optimization for *mcr-1*-MCDA-LFB Assay

To optimize the reaction temperature of MCDA-LFB assay during the amplification stage, the plasmid DNA of *E. fergusonii* (ICDC-ZG2016M34-3) at the level of 6 pg per reaction was used as the templates. A series of temperatures (ranging from 60 to 67°C, with 1°C intervals) was compared for amplifying efficiency of MCDA-LFB assay by employing 2% agarose gel electrophoresis. The result showed that 62 and 63°C were the better candidates for this method (Figure 3). Therefore, the reaction temperature of 63°C was performed for the subsequent MCDA-LFB experiments.

Sensitivity and Specificity of MCDA-LFB Assay for *mcr-1*

To acquire the detection limit of this assay, serial dilutions of *E. fergusonii* ICDC-ZG2016M34-3 plasmid DNA were used in *mcr-1*-MCDA-LFB assay. It indicated that the threshold was as

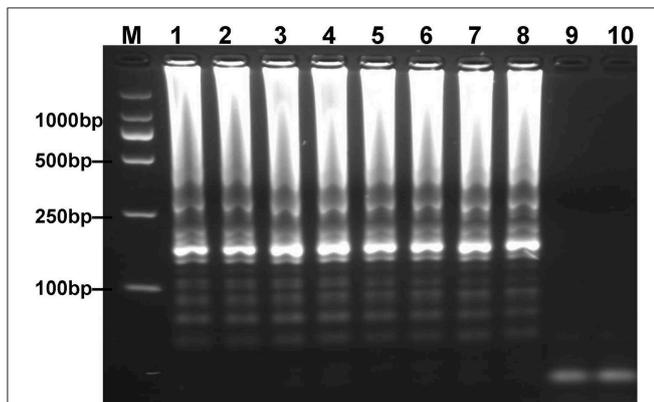


FIGURE 3 | Optimal reaction temperature for *mcr-1*-MCDA assay was determined by using agarose gel electrophoresis. The plasmid DNA of *Escherichia fergusonii* (ICDC-ZG2016M34-3) (6 pg) was amplified in different temperatures. Lane 1–8 represented the amplification temperature in the range from 60 to 67°C (1°C intervals); lane 9, negative control (6 pg of *Escherichia coli* WHCDC-WH67 genomic DNA); lane 10, blank control (DW).

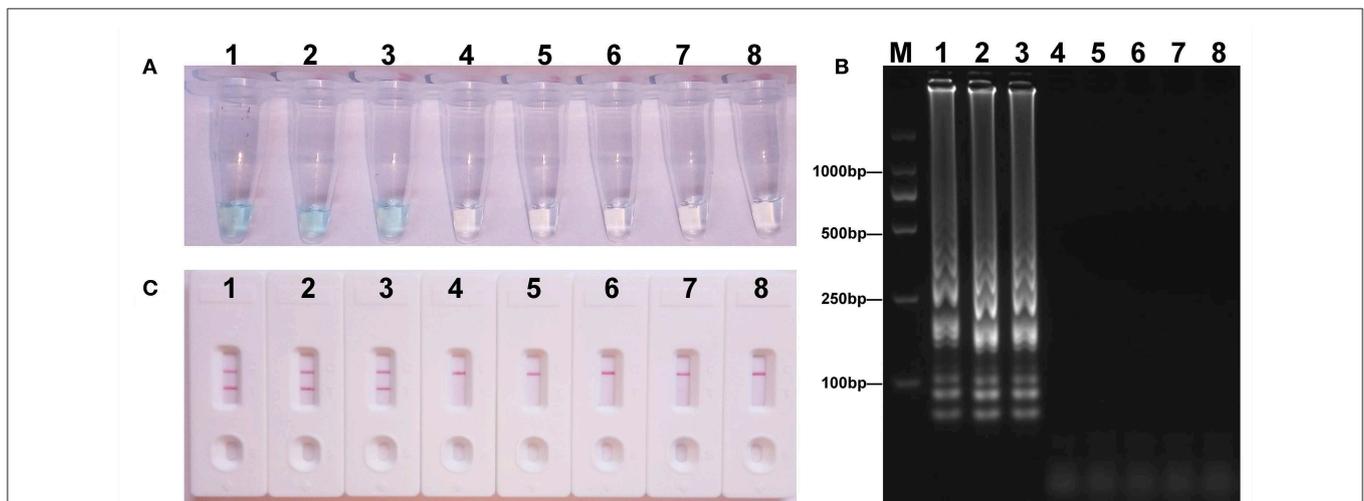


FIGURE 4 | Sensitivity of the *mcr-1*-MCDA assay using serially diluted plasmid DNA with *Escherichia fergusonii* ICDC-ZG2016M34-3. Three detection techniques, including colorimetric indicators (A), gel electrophoresis (B), and LFB (C), were applied for testing MCDA products. Tubes (A)/lanes (B)/biosensors (C) 1–8 represented the plasmid DNA levels of 6 ng, 60 pg, 600 fg, 60 fg, and 6 fg per reaction, negative control (NDM-1-positive *Escherichia coli* WHCDC-WH67 genomic DNA, 6 pg per reaction), negative control (KPC-2-producing *Klebsiella pneumoniae* WHCDC-WH108 genomic DNA, 6 pg per reaction), and blank control (DW).

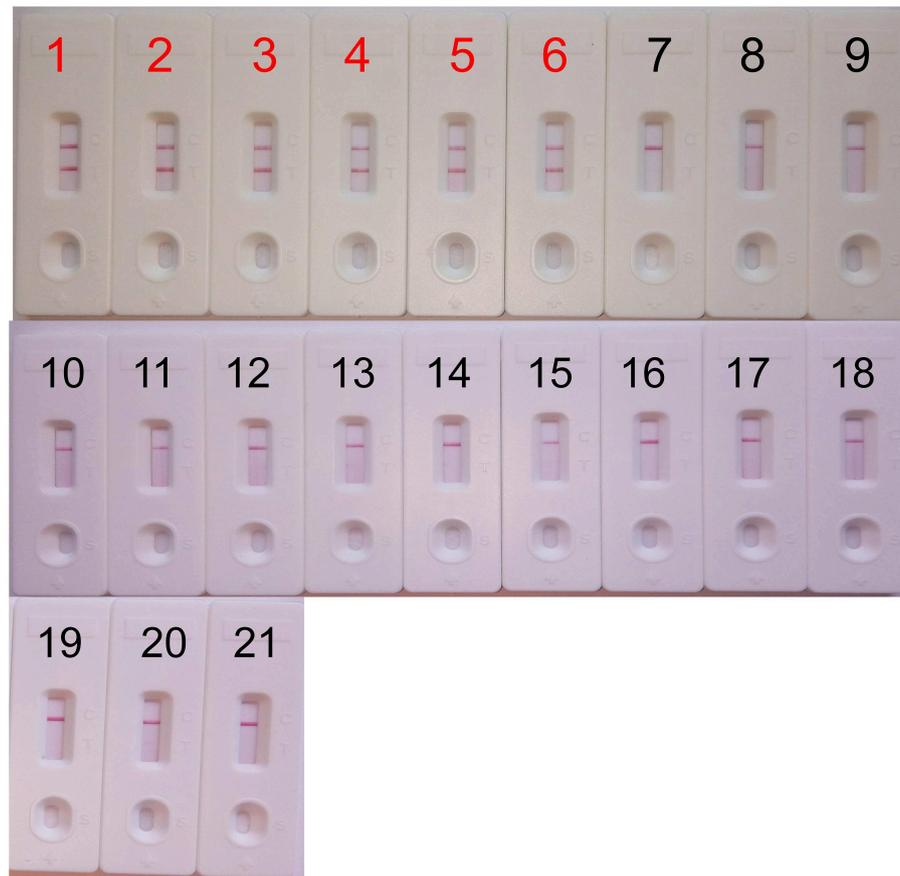


FIGURE 5 | Specificity of the *mcr-1*-MCDA-LFB assay. Biosensor 1, *mcr-1*-positive *Escherichia fergusonii* ICDC-ZG2016M34-3. Biosensor 2, *mcr-1*-positive *Escherichia fergusonii* from ICDC. Biosensor 3, *mcr-1*-positive *Escherichia fergusonii* from WHCDC. Biosensor 4, *mcr-1*-producing *Escherichia coli* from ICDC. Biosensor 5, *mcr-1*-producing *Escherichia coli* from WHCDC. Biosensor 6, *mcr-1*-producing *Salmonella enteritidis* from ICDC. Biosensors 7–17, KPC-2-positive *Klebsiella pneumoniae*, KPC-2-positive *Pseudomonas aeruginosa*, NDM-1-positive *Escherichia coli*, NDM-1-positive *Enterobacter cloacae*, NDM-1-positive *Klebsiella pneumoniae*, NDM-5-positive *Escherichia coli*, IMP-4-positive *Escherichia coli*, *Acinetobacter baumannii* carrying non-*mcr-1*/non-carbapenemase gene, *Pseudomonas aeruginosa* carrying non-*mcr-1*/non carbapenemase gene, *mcr-1*/carbapenemase-negative *Serratia marcescens*, *mcr-1*/carbapenemase-negative *Escherichia coli*, all of the stains derived from WHCDC. Biosensor 18, blank control (DW). Biosensors 19–21, *Staphylococcus aureus* carrying non-*mcr-1*/non-carbapenemase gene, *Enterococcus faecalis* carrying non-*mcr-1*/non carbapenemase gene, *mcr-1*/carbapenemase-negative *Enterococcus faecium*, those stains were isolated from WHCDC.

little as 600 fg of plasmid DNA (**Figure 4**). The same results were observed by using colorimetric indicator and agarose gel electrophoresis analysis.

The analytical specificity of the *mcr-1*-MCDA-LFB assay was assessed with genomic DNA extracted from 16 *mcr-1*-producing strains and 43 non-*mcr-1* isolates. As shown in **Figure 5**, all products derived from the strains carrying *mcr-1* gene exhibited two red bands (TL and CL) in the LFB, but each sample from the *mcr-1*-negative organisms and blank control yielded only one red band. The results certified that the MCDA-LFB assay had a complete specificity for *mcr-1* detection.

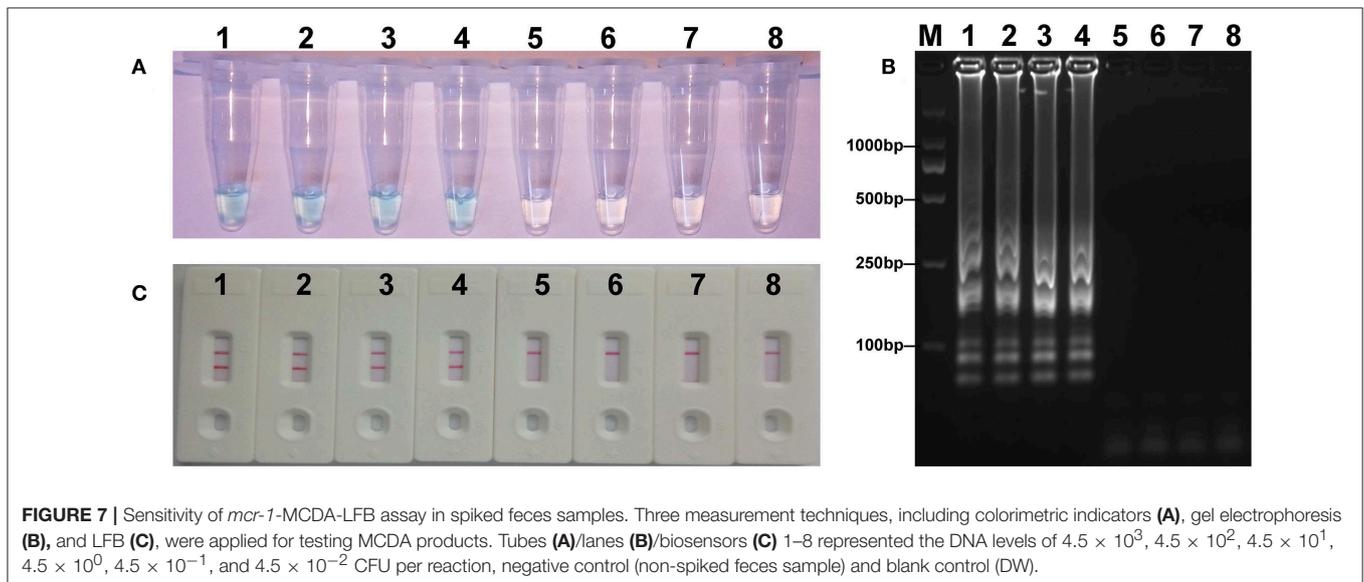
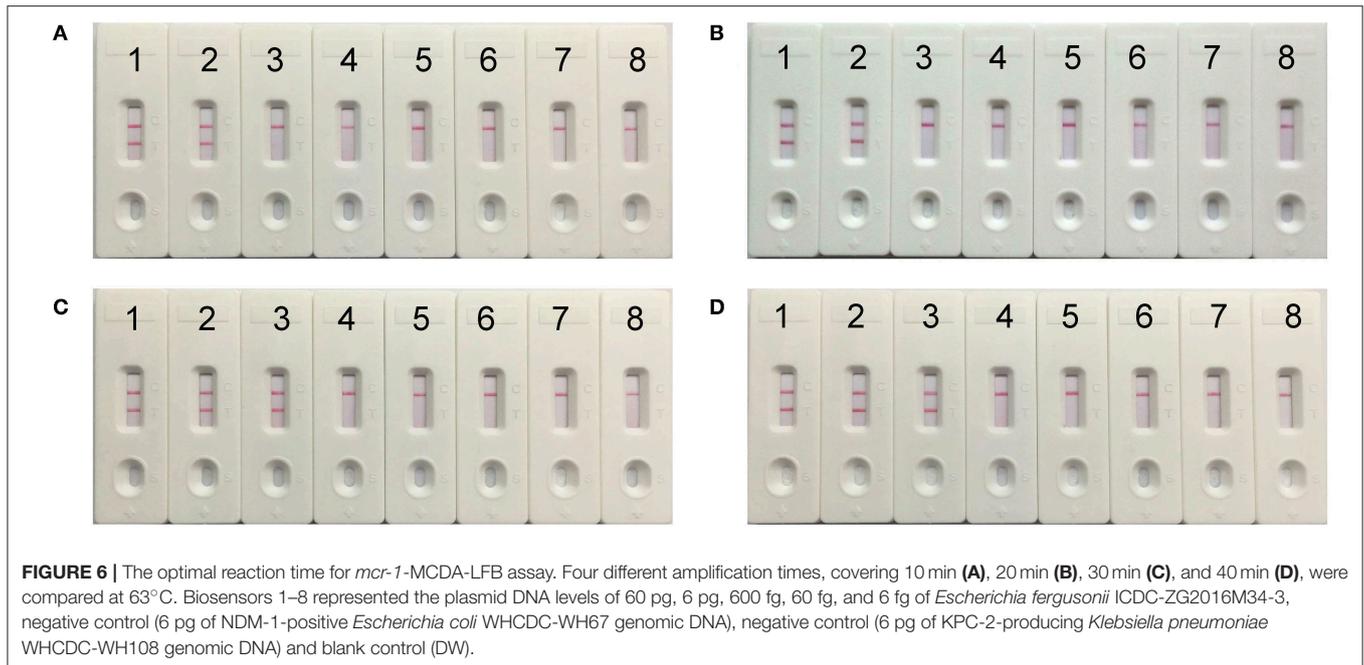
Optimization of the Time for *mcr-1*-MCDA-LFB Assay

To evaluate the optimum time, four reaction times (10–40 min at 10 min intervals) were tested for the *mcr-1*-MCDA-LFB assay

during the amplification stage. The *mcr-1*-producing *E. fergusonii* (ICDC-ZG2016M34-3) plasmid DNA, 600 fg/ μ l (the LOD of the method), did not gain the positive results until the reaction had operated for 30 min (**Figure 6**). Hence, the amplification time of 30 min at 63°C was considered as an optimal reaction condition for the current assay.

Application of MCDA-LFB to *mcr-1*-spiked Fecal Samples

The LOD for strains expressing *mcr-1* in fecal samples was determined to assess the practical application of the established assay. The detected threshold of *mcr-1*-positive bacteria was approximately 4.5×10^3 CFU/mL (~ 4.5 CFU/reaction) in 0.2 g fecal samples spiked with 100 μ l of dilutions of strains (**Figure 7**). The results of other subjects including the lower suspensions concentrations, negative control, and blank control



were negative. As the same to the aforementioned experiments, detection of the amplicons with three methods got an equivalent conclusion.

The Detection Limit of Conventional PCR

The detection limit of the conventional PCR assay was 6 pg of plasmid DNA per microliter in pure culture and 4.5×10^4 CFU/mL (~ 45 CFU/reaction) in spiked fecal samples. *Mcr-1*-MCDA-LFB method was thus highly sensitive, 10-fold more so than that of PCR (Table 3).

DISCUSSION

Polymyxins (polymyxin B and colistin) have nearly become last-resort drugs for treating the severe infections caused by multidrug-resistant or pan-resistant *Enterobacteriaceae* (Wang et al., 2017a). The defensive line will be destroyed by the emergence of *mcr-1*-positive strains resisting to colistin. Moreover, the *mcr-1* gene mediated by plasmids or transposons can transfer in different species freely. Thus, the isolates carrying *mcr-1* gene will undoubtedly become a major issue for public health. Under the circumstances, a convenient and fast technique

TABLE 3 | Detection limit of the *mcr-1*-MCDA-LFB assay and conventional PCR.

| | Detection limit | |
|----------------------|------------------|------------------------------|
| | Conventional PCR | <i>Mcr-1</i> -MCDA-LFB assay |
| Purified plasmid DNA | 6 pg/μL | 600 fg/μL |
| DNA-spiked specimens | ~45 CFU/reaction | ~4.5 CFU/reaction |

for detection of *mcr-1* in various samples is of great importance. Here, an approach was reported to detect target gene by MCDA united with lateral flow biosensor (MCDA-LFB). In the MCDA-LFB assay, the high specificity was guaranteed, as a set of 10 primers was employed for specially amplifying the target sequence. The specificity of *mcr-1*-MCDA-LFB was successfully confirmed with the genomic templates extracted from *mcr-1*-producing strains and non-*mcr-1* organisms, and the results were positive for all *mcr-1*-positive isolates, but negative for non-*mcr-1* isolates and blank control. Therefore, the diagnostic test based on MCDA-LFB for the detection of *mcr-1* in bacteria identifies target gene with high selectivity.

The MCDA products can be analyzed with LFB, colorimetric indicator and agarose gel electrophoresis, respectively. LFB, as a detection technique by observing the number of red lines on sensor bar, is more objective than colorimetric indicator, which reports the result through color change. Maybe the latter is in trouble when the concentration of target gene is very low (Wang et al., 2017c). Likewise, LFB is more rapid and convenient than gel electrophoresis, which requires the use of an additional operation procedure and complex equipment. Hence, LFB will be a better candidate for the results' judge of MCDA products.

Besides specificity, the superb sensitivity is also very important for the newly established assay. The *mcr-1*-MCDA-LFB method sufficed to detect as little as 600 fg of *mcr-1*-positive plasmid DNA per microliter in pure culture and 4.5×10^3 CFU/mL (~4.5 CFU/reaction) in fecal samples spiked with 100 μl of strains. This technique has the same sensitivity to *mcr-1*-LAMP described in previously report (Zou et al., 2017), and is 10 times more sensitive than that of conventional PCR. Moreover, in spite of the equivalent specificity and sensitivity in both isothermal amplification assays, the *mcr-1*-MCDA-LFB method which expends less reaction time than the other will provide faster detection for *mcr-1*. Likewise, although the Real-time PCR and MALDI-TOF MS-based method could also test the *mcr-1*

gene in low limit of detection and less time, respectively (Bontron et al., 2016; Dortet et al., 2018), they needed expensive apparatus and immaculately experimental condition that were not well-equipped in resource-challenged fields, especially in the livestock industry where a large quantity of stains carrying *mcr* gene were identified (Dortet et al., 2018).

The *mcr-1*-MCDA-LFB assay only required a constant reaction temperature at 63°C. The entire process of experiments, including sample processing (25 min), isothermal amplification (30 min), and detection (2 min), could be accomplished in <60 min. Herein, this assay economizes the test time and device, and is suitable for timely identification on the spot particularly.

In conclusion, we devised a reliable MCDA-LFB assay for the detection of *mcr-1* with simplicity, rapidity, and low-cost facility. The LOD of this assay was only 600 fg per reaction with pure culture, and the specificity was 100% according to the trial results. From the above, the *mcr-1*-MCDA-LFB assay built in this study will greatly improve the detection efficiency for the monitor of target gene in practical application.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

LG and JiL designed the experiments. LG and EL carried out the experiments. JuL and JC contributed the partial isolates. XL and HX provided the materials and reagents. LG analyzed the related data and wrote the paper.

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