**Multiplex****loop-mediated isothermal amplification (multi-LAMP) assay for rapid detection of *mcr-1* to *mcr-5* in colistin-resistant bacteria**

Lan-Lan Zhong1,2**\***,QianZhou3**\***,Cui-yan Tan3**\***,Adam P. Roberts4,5,Mohamed Abd El-Gawad El-Sayed Ahmed1,2,6,Guanping Chen1,2,Min Dai7,Fan Yang8, Yong Xia9,Kang Liao10, Yingjian Liang3, Yongqiang Yang1,11, Siyuan Feng1,2, Xiaobin Zheng3†, Guo-Bao Tian1,2†

1Program in Pathobiology, the Fifth Affiliated Hospital, Zhongshan School of Medicine, Sun Yat-Sen University, Guangdong519000, China.

2Key Laboratory of Tropical Diseases Control (Sun Yat-sen University), Ministry of Education, Guangzhou 510080, China

3Department of Respiratory Medicine, the Fifth Affiliated Hospital of Sun Yat-sen University, Zhuhai 519000, China

4Department of Tropical Disease Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA, UK

5Centre for Drugs and Diagnostics, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA, UK

6Department of Microbiology and Immunology,Faculty of Pharmaceutical Sciences and Drug Manufacturing, Misr University for Science and Technology (MUST), Cairo, 6th of October City, Egypt

7School of Laboratory Medicine, Chengdu Medical College, Chengdu 610500, China

8Department of Microbiology, School of Basic Medical Science, Xinxiang Medical University, Xinxiang453003, China

9Department of Clinical Laboratory Medicine, the Third Affiliated Hospital of Guangzhou Medical University, Guangzhou 510150, China

10Department of Clinical Laboratory, the First Affiliated Hospital of Sun Yat-SenUniversity, Guangzhou 510080, China

11School of Pharmaceutical Sciences (Shenzhen), Sun Yat-sen University, Guangzhou510006, China

**\***These authors contributed equally to this article.

†These senior authors contributed equally to this article.

†**Corresponding author:**

Guo-BaoTian, Ph.D., Professor

Mailing address: Zhongshan School of Medicine, Sun Yat-sen University, 74 Zhongshan 2nd Road, Guangzhou 510080, China. Phone: +86 (0)20 87335387; Fax: +86 (0)20 87335387. Email: tiangb@mail.sysu.edu.cn, guobaotian@gmail.com.

**Running title:**Rapid detection of *mcr* genes using multi-LAMP

**ABSTRACT**

**Purpose:**

The discovery of the plasmid-mediated colistin resistancegenes, *mcr*,revealed a mechanism of transmission of colistin resistance, which is a major, globalpublic health concern especially among individuals infected with carbapenem-resistant Gram-negative bacteria. To monitor the spread and epidemiology of *mcr* genes, a convenient and reliable method to detect *mcr* genes in clinical isolates is needed, especially in the primary care institutions. This study aimed to establish a restriction endonuclease-based multiplex loop-mediated isothermal amplification (multi-LAMP) assay to detect *mcr* genes (*mcr*-*1* to *mcr-5*) harbored by colistin-resistant bacteria.

**Methods:**

A triple-LAMP assay for *mcr-1*, *mcr-3*, and *mcr-4* and a double-LAMP assay for *mcr-2*and *mcr-5* were established. The sensitivity and specificity of the LAMP reactions were determined via electrophoresis and visual detection.

**Results:**

The sensitivity of the LAMP assay was 10-fold greater than that of PCR, with high specificity among the screened primers. Specific *mcr* genes were distinguished in accordance with band numbers and the fragment length of the digested LAMP amplification products. Furthermore, the LAMP assay was confirmed as a rapid and reliable diagnostic technique upon application for clinical samples, and the results were consistent with those of conventional PCR assay.

**Conclusion:**

The multi-LAMP assay is a potentially promising method to detect *mcr* genes and will, if implemented,help prevent infections by drug-resistant bacteria in primary-care hospitals due to rapid and reliable surveillance.To our knowledge, this is the first study to report the application of LAMP to detect *mcr-2* to *mcr-5*genes and the first time that multi-LAMP has been applied to detect *mcr*genes.

**Key words:***mcr* genes, colistinresistance, Multi-LAMP, rapid detection, enzyme digestion.

**INTRODUCTION**

The misuse of antibioticsis the primary factor selecting forantibiotic resistance, followed by the unnecessary supplementation of animal feed with antibiotics.1Among the various types of antibiotics, colistin, a lastresort chemotherapeutic optionagainst carbapenem-resistant bacteria, is faced with a growing clinical challenge of antibiotic resistance.2Until recently, colistin resistance was believed to be chromosomally mediated; however, the discovery of plasmid-mediated colistin resistance via *mcr-1* in 2015 revealed colistin resistance was capable ofhorizontal transmission.3,4Subsequent studies reported that *mcr-1*is expressed in many bacteria worldwide.3,5-7 Moreover, new *mcr*genotypes were reported, and seven more *mcr* genes (*mcr-2* to *mcr-8*) have been reported since the discovery of *mcr-1*.8-14 Currently, PCR-based methods are the most widely adopted to detect *mcr*genes,15however, owing to the presence of several genotypes of *mcr* genes and their ability to undergo horizontal gene transfer;PCR assays are limited in clinical practice, including theprimary-care hospitals and the basic quarantine stations. Therefore, a rapid, efficient, reliable, and economical method to detect *mcr* genes is urgently required.

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method conducted at a constant temperature, based on auto-cycling strand displacement of DNA synthesis by Bst DNA polymerase.16 It is a promising method to detect nucleic acids, with advantages including the non-requirement of thermo-cycling instruments, higher sensitivity, and less time-consumption. With the rapid development of the LAMP method, studies have reported increasing applications of LAMP, including nucleic acid detection frombacteria,17,18 viruses,19 and parasites.20-22 Zou *et al*. first reported the application of LAMP to detect *mcr-1*, achieving higher sensitivity than traditional PCR.23Imirzalioglu*et al*.used the eazyplexSuperBug*mcr-1* kit, developed to rapidly detect *mcr-1*.24However, owing to the multiple *mcr*genotypes, a single LAMP cannot detect all potential target genes, thereby yielding incomplete information for nucleic acid detection.

In this study, we aimed to develop a restriction endonuclease-based multi-LAMP method to detect multiple *mcr* genes. We established a triple-LAMP system for the most extensively propagated*mcr* genes(*mcr-1*, *mcr-3*, and *mcr-4*) in China. A double-LAMP system for *mcr-2* and *mcr-5*has also been established to detect all *mcr*genes (*mcr-6* to *mcr-8*had not been discovered when this study started). We designed and screened 5 sets of primers for each *mcr-1* to *mcr-5*genes and assessed the sensitivity and specificity of the assay through electrophoresis and visual detection, using clinical samples from hospitals in Guangzhou, China. Primers for this assay were modified with restriction endonucleases and were used combinatorially for the multi-LAMP assay, and specific *mcr* genes were detected from the band numbers and fragment lengthsof the digested LAMP-amplified products.

**MATERIALS AND METHODS**

**Primers Design**

Sequences of *mcr-1* to *mcr*-*5* genes were downloaded from GenBank database: *mcr-1*(accession number: KX886345.1); *mcr-2*(accession number: NG\_051171.1); *mcr-3*(accession number: MG026622.1);*mcr-4* (accession number: MG026621.1); and*mcr-5* (accession number: MG241339.1). To determine the optimum primers,3 primer sets for each gene were designed by using Primer Explorer (version 5, <http://primerexplorer.jp/lampv5e/index.html>) (Table S1). Each primer set comprises 6 primers: a forward inner primer (FIP), backward inner primer (BIP), outer forward primer (F3), outer backward primer (B3), and two loop primers (LF and LB) to accelerate the LAMP reaction.16 All primers were synthesized by Tianyi Biotech Co.(Dongguan, China).

**Samples Preparation**

In our study, five positive controls harboring *mcr-1* to *mcr*-*5* genes were used, wherein *mcr-1*, *mcr-3*, and *mcr-4* were screened in our laboratory, *mcr-2* was obtained from South China Agricultural University, and *mcr-5* was obtained from China Agricultural University (Table 1). Three multidrug-resistant bacterial strains (two *Escherichia coli*and one *Klebsiellapneumonia*) devoid of *mcr* genes, identified via PCR in our previous studies,25 were used as negative controls. All strains were stored in 30% (w/v) glycerol broth at -80°C. The strains were cultured in Luria-Bertani culture medium (OXOID, Hampshire, UK) supplemented with 2% colistin at 37°C overnight. Bacterial genomic DNA was extracted using the boiling method and recovered in 200μLRNase-free ddH2O. pMD19-T vector containing *mcr-1* to *mcr-5*DNA fragmentswas constructedseparately, as previously described.26,27 The recombinant plasmids were diluted 10-fold serially to yield 108copies/μLto 102 copies/μL.

**LAMP Reaction**

The LAMP reaction was carried out in a 25μL reaction mixture that contained12.5 µL LAMP-ReactionMix[20 mMTris-HCl (pH 8.8), 10 mM (NH4)2SO4, 8 mM MgSO4, 10 mMKCl, 0.8 M betaine, 0.1% Tween-20, 1.4 mMdeoxy-ribonucleotidetriphosphates (dNTP)], 1 µLBst 2.0 polymerase(New England Biolabs, 8,000 units/ml),1.25 µLprimermix(2μM each of FIP and BIP, 0.25 µM each of F3 and B3, and 1 µM each of LF and LB) (Table 2), 8.25 µL nuclease-free water, and 2 µL DNA lysate. The mixture was incubated for 60 min at 64°C in a heated, thermostatically controlledwater bath.

**PCR Assay**

A PCR assay was performed to compare its sensitivity and the clinical detection rates with those of the LAMP assay. Each plasmid sample was amplified in 20µL reaction mixtures containing 10µL PCR MasterMix (Tiangen Biotech Co., Ltd., Beijing, China), 400 pM primers (Table 2), and 1µL DNA template. The cycling conditions were as follows: 3min at 95°C; 30 cycles of 30s at 95°C, 30s at 55°C, and 30s at 72°C; 5min at 72°C. The PCR products were analyzed electrophoretically on a 2% agarose gel, followed by ethidium bromide staining. Images were obtained using the (Bio-Rad, Hercules, CA, USA).

**Detection of LAMP products**

LAMP products were detected using two methods: visual detection28 and electrophoresis. For visual detection, SYBR Green I was added into the LAMP products, where the positive reactionsyielded green coloration; whilethe negative ones gave yellow. For electrophoresis, the LAMP products were stained with GoldView TM, analyzed electrophoretically on a 2% agarose gel, and photographed.

**Multi-LAMP detection**

The triple-LAMP assay was performed using a set of three primer pairs each, for *mcr-1*, *mcr-3*, and *mcr-4*.The double-LAMP assay was performed using a set of three primer pairs each, for *mcr-2* and *mcr-5.*The amplification was carried out in a 25μL reaction mixtureas the LAMP reaction.Both of the amplification products of the triple-LAMP and double-LAMP were diluted by 2-fold,digested usingThermo Scientific FastDigestHindⅢ at 37°C for 15 minand then analyzed electrophoretically on a 2% agarose gel. Through the electrophoresis results, differenttypes of *mcr*genes were distinguished based on the band numbers and band locations on electropherograms.

**RESULTS**

**Primer selection and modification**

Fifteen primer sets were designed to detect *mcr-1* to *mcr-5*(3 sets for each gene) as shown in Table S1. To determine the optimum primers, LAMP reactions using different primer setswere conducted under the same conditions,and electropherogramsof the LAMP productswere compared.According to the electrophoretic analysis, the optimum primer sets were selected. The locations and sequences of selected optimal primer sets were shown in Figure 1.Additionally, for the multi-LAMP, we modified the selected optimal FIPs and BIPs by inserting the HindⅢ restriction sites between F1c and F2 and between B1c and B2, respectively (Table 2).

**Sensitivity of the LAMP assay**

To determine the sensitivity of the LAMP assay, purified genomic DNA templates were detected via 10-fold serial dilution ofknown copiesnumbersof DNA template molecules. Comparative PCR assays were also conducted using the same DNA templates. LAMP products were further analyzed visually (Figure 2 A-E) and via agarose gel electrophoresis (Figure 2 F-O). These results indicated that the detection limit of the LAMP assay was 104copies/µL for *mcr-1*,*mcr-2*,*mcr-4* and *mcr-5*, and 105 copies/µL for *mcr-3*. Comparatively, agarose gel electrophoresis was conducted for the PCR products, wherein the detection limits were 105 copies/µL for *mcr-1* to *mcr-5* genes. All reactions were carried out at least in triplicate.

**Specificity of the LAMP assay**

Assay specificity was assessed via direct visual detection through the addition of SYBR Green I during the reaction, followed by confirmatory evaluation via agarose gel electrophoresis. Three non-*mcr* genes (*bla*KPC-2, *bla*NDM-1, and *bla*CTX-M-9)were analyzed in addition to *mcr-1* to *mcr-5* genes. Our results revealed that *mcr-1* was positively identified via LAMP only when amplification was performed using the *mcr-1* primer set, while all non-*mcr-1*genes tested negative (Figure 3A, F). The *mcr-2* to *mcr-5* genes were similarly analyzed, indicating that this LAMP method was specific for *mcr-1* to *mcr-5*genes (Figure 3B-E, G-J). All reactions were carried out at least in triplicate.

**Multi-LAMP detection of *mcr* genes**

To generate the multi-LAMP system, endonuclease analysis was applied. For cleavage sites in *mcr-1* to *mcr-5* genes, enzyme digestion was performed using HindⅢrestriction enzyme. For the triple-LAMP, the target sequence of *mcr-1*was cleaved into 2 segments,*mcr-3* into 2 segments and *mcr-4* into 3 segments (Figure 4A).Forthe double-LAMP, the target sequence of *mcr-2*was cleaved into 3 segmentsand*mcr-5* into 2 segments (Figure 4B).The sizes of the digested products were consistent with those predicted for *mcr-1* to *mcr-5*genes. The DNA sequencing of the digested products confirmed the specificity of the amplification (data not shown).Through restriction endonuclease analysis of the amplification products, *mcr-1* to *mcr-5* genes were successfully distinguished (Figure4A,*mcr-1*,*mcr-3*, and *mcr-4*;Figure 4B,*mcr-2* and *mcr-5*).

**Multi-LAMP detection of clinical samples**

To further evaluate the accuracy of multi-LAMP, 58 clinical bacterial samplesfrom a previous study25 were subjected to the present assay. Among the 58 clinical samples, 12 were positive for *mcr-1*, five for *mcr-3*, andone for *mcr-4*,while the remaining40 samples were *mcr*negative. The clinical bacterialsamples were also subjected to traditional PCR analysis, which also revealed twelve*mcr-1*-positive samples, five*mcr-3-*positive samples, one*mcr-4*-positive sample, and forty negative samples, consistent with the results of multi-LAMP. Therefore, these findings reveal that multi-LAMP described hereshowed good consistency with conventional PCR analyses.

**DISCUSSION**

Colistin is a polypeptide antibiotic, belonging to the family of polymyxins. Colistin was first reported to treat infections by Gram-negative bacteria in the late 1940s. Owing to its risk of nephrotoxicity and neurotoxicity, colistin was not popularized in clinical treatment. Later, antibiotics acting on emerging carbapenem-resistant superbugs were deemed critical because of their high morbidity and mortality, framingcolistin as a significant therapeutic alternative.2 Emerging colistin resistance, therefore, has confounding implications in patient care. Before 2015, the mechanismsunderlying colistin were known only to involvechromosomal mutations; hence, its spread was expected to be limited to vertical transmission, which was usually stable, incapable of spreading to other bacteria and imposing a fitness cost upon the bacteria. The discovery of *mcr* genes signifies plasmid-meditated colistin resistance, implying a new horizontal transmission channel for the propagation of the *mcr* genes, increasing their rapid transmission risk and range. Moreover, colistin abuse via its supplementation in animal feed contributed to its disseminationthrough horizontal gene transfer.4 Therefore, analysis of *mcr*genes is necessary to identify colistin resistance and control its horizontal transmission.

This study established a novel multi-LAMP system to detect multiple *mcr* genes, with the establishment of a triple-LAMP for *mcr-1*, *mcr-3*, and*mcr-4*,as well as a double-LAMP for *mcr-2* and *mcr-5*. We designed 15 primer pairs for the conserved sequences of *mcr-1* to *mcr-5*genes (3 for each gene). Because *mcr*genes share significant homology, we screened the best primer sets by considering *mcr* genes as positive controls. Five primer sets each for*mcr-1* to *mcr-5* geneswere screened out and determined to be the optimal primers for the LAMP assay. Subsequent specificity analyses confirmed the optimal nature of these primer sets. We compared the sensitivity of LAMP with that of traditional PCR analysis, reporting that the detection limit of the LAMP assay was 10-fold that of conventional PCR analysis. This high sensitivity and specificity rendered LAMP suitable for early screening in clinical settings, especially in the primary medical institutions. Through restriction digestion of the LAMP products based on band numbers and fragment lengths, we successfully distinguished *mcr-1*, *mcr-3*, and *mcr-4*,as well as*mcr-2* and *mcr-5*, thereby enabling multiplex detections of *mcr* genes. Additionally, we applied this multi-LAMP method to clinical samples to assess the reliability of our methods, with results being consistent with those of conventional PCR analyses.

Compared with traditional PCR, the present method exhibited the following advantages: (a) high specificity and sensitivity and ease of operation; (b) multiplex detections of *mcr* genes using the same detection system, thus reducing manual operation; (c) a total operating time of less than 60 min, as opposed to 90 min to detect *mcr* genes via conventional PCR analysis;(d)greater user-friendliness than conventional PCR analysis, with no requirement of specialized instruments and complicated operations. These advantages render the multi-LAMP assay promising for clinical application, especially in resource limitedmedical institutions.

This study has some limitations;the sensitivity and specificity of the multi-LAMP assay are relatively poorer when applied to samples containingmore than one*mcr* genes. Nonetheless, according to other previous studies and data from our on-going experiments, it is rare for a single strain to contain multiple*mcr* genes in clinical samples (0.01% is according to our data; unpublished data). Therefore, our approach is applicable in most practical situations. Moreover, gene sets (*mcr-1*, *mcr-3*,and *mcr-4*)andgene sets (*mcr-2* and*mcr-5)* were successfully detected,where*mcr-1*, *mcr-3*, and *mcr-4*are the most prevalent*mcr* genes in China and *mcr-2* and*mcr-5*,not yet detected in humans,complemented the whole *mcr* gene family. With newer *mcr* genes being reported (*mcr-6* to *mcr-8*genes during our study in 2018) in future studies, continuous efforts are needed to track newly reported *mcr* genes and to establish a multi-LAMP system encompassing all *mcr* genes.

In our future studies, we intend to investigate the LAMP-based methods for drug resistance genes of these *mcr* genes and to continuously track the newly reported *mcr*genes. Upon analyzing more *mcr* genes and using larger sample sizes, we intend to further assess the potential of multi-LAMP and further validate its diagnostic power. These results provide critical insights into the efficacy of this method for future clinical applications related to antibiotic resistance. Our results potentially contribute to the prevention of antibiotic resistance in healthcare settings by providing a system of early detection of antibiotic resistance and prescribing patterns at national, regional, and local levels.

**CONCLUSION**

In conclusion, we established a restriction digestion-based multi-LAMP method, which successfully detected *mcr-1*, *mcr-3*, and *mcr-4*via a triple-LAMP and *mcr-2* and *mcr-5* via a double-LAMP. We anticipate that this method could rapidlyhelp screen *mcr* genes and other drug-resistant genes in a variety ofclinical settings. To our knowledge, this is the first study reporting the application of LAMP in the detection of*mcr-2* to *mcr-5* genes and the first time that multi-LAMP has been applied to detect *mcr* genes.

**DISCLOSURE**

The authors report no conflicts of interest in this work. All authors have read and approved the manuscript.

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**Table 1** *mcr* genesand other resistant genes used in this study

|  |  |  |  |
| --- | --- | --- | --- |
| NO. | Genetic types | Source | Gene Role |
| 1 | *mcr-1* | *E. coli* | Positive control |
| 2 | *mcr-2* | Plasmid | Positive control |
| 3 | *mcr-3* | *E. coli* | Positive control |
| 4 | *mcr-4* | *E. coli* | Positive control |
| 5 | *mcr-5* | Plasmid | Positive control |
| 6 | *bla*KPC-2 | *K. pneumonia* | Negative control |
| 7 | *bla*NDM-1 | *E. coli* | Negative control |
| 8 | *bla*CTX-M-9 | *E. coli* | Negative control |

**Table 2** The primer sequences of multi-LAMP and PCR for *mcr-1* to *mcr-5* genes

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Target genes | multi-LAMP | Reference | PCR | Reference |
| **Primers** | **Sequences(5'-3')**a |  | **Primers** | **Sequences(5'-3')** | 15 |
| *mcr-1* | F3 | TGATGCAGCATACTTCTGTG | This study | F | AGTCCGTTTGTTCTTGTGGC |
| B3 | GACCGTGCCATAAGTGTC |
| FIP | GCGATGGGATAGGTTTGGCT*AAGCTT*GTGTTGCCGTTTTCTTGAC |
| BIP | TGCTGACGATCGCTGTCG*AAGCTT*GCACATAGCGATACGATGAT | R | AGATCCTTGGTCTCGGCTTG | 15 |
| LF | AAGGTAAGATTGGCGGTCG |
| LB | CTACTGATCACCACGCTGTT |
| *mcr-2* | F3 | CAAAGACGCCGTGCAGAC | This study | F | CAAGTGTGTTGGTCGCAGTT | 15 |
| B3 | CAGAATACGCCGTCGATGT |
| FIP | ACTGCACATGGTCAGCACGC*AAGCTT*GAGCGTAAGCCACGCCTA |
| BIP | GGCTATGGCCGTGAGACTTTCC*AAGCTT*GCCACACGATGTCACTTGG | R | TCTAGCCCGACAAGCATACC | 15 |
| LF | ACCGACGACGAACACCAC |  |  |
| LB | CTTGCCAAAGTTGATGGCTTG |
| *mcr-3* | F3 | CATTACCAATATTGCTTGTTGC | This study | F | AAATAAAAATTGTTCCGCTTATG | 15 |
| B3 | TTGGCTGGAACAATCTCAC |
| FIP | GCTAACGCCTCATTTTGATTGG*AAGCTT*GCACTTCTTATCGCACTTAG |
| BIP | TCAAAGGGATTCTAACTCGTGC*AAGCTT*GCAATAACCGCAATCACTAT | R | AATGGAGATCCCCGTTTTT | 15 |
| LF | TCATTGTGTAACTAACGATTGC |
| LB | CCTATCGATGTTTGCATCACTT |
| *mcr-4* | F3 | TGAGTTAAGGCGTTACATTGT | This study | F | TCACTTTCATCACTGCGTTG | 15 |
| B3 | CGCATGAGCTAGTATCGTTAA |
| FIP | TTACGACTGGCATTCTTCGCA*AAGCTT*CTATTTGCAGACGCCCAT |
| BIP | AGTGGTTGTTGTGGGTGAAACT*AAGCTT*AGCATTGGTTGGCTTGTTA | R | TTGGTCCATGACTACCAATG | 15 |
| LF | TCTAGGCCAAGTTGTTGGTATT |
| LB | CGCGCTCAATGAGCTATCA |  |  |
| *mcr-5* | F3 | CAATGGAGAATGCTGCCCTA | This study | F | ATGCGGTTGTCTGCATTTATC | 15 |
| B3 | GCGTGGGTATCAGCACATC |
| FIP | AGCCCGTTCGTAAAACCCTGAC*AAGCTT*CTTGTTGGTTGCAGCCGT |
| BIP | AGCGGTAATGATGCGCAGCG*AAGCTT*CATGACTGGCCACAGACC | R | TCATTGTGGTTGTCCTTTTCTG | 15 |
| LF | CTCGCAATCCACCACACGGAT |
| LB | TGGCGCTCTCGCCATGA |

a Underlining indicates the restriction enzyme sites of HindⅢ.

**Figure 1** Locations and sequences of *mcr* genes used to design multi-LAMP primers. (A–E): The nucleotide sequences of the target strands of *mcr-1* to *mcr-5* genes. Right arrows indicate the original sequences and left arrows indicate the complementary sequences.

**Figure 2** Sensitivity of the loop-mediated isothermal amplification (LAMP) and polymerase chain reaction (PCR) assays. (A-E): Visual detection of the LAMP amplification products of *mcr-1* to *mcr-5* genes with SYBR Green I. (F-J): Agarose gel electrophoresis was conducted for the LAMP products of *mcr-1* to *mcr-5* genes. (K-O): Comparative agarose gel electrophoresis analysis of products of the PCR assay and the corresponding LAMP assay. Lane M, Trans 2K plus II DNA marker; Lanes 1–7, serial 10-fold dilutions of templates from 108 copies/µL to 102 copies/µL; Lane 8, negative (water).

**Figure 3** Specificity of the loop-mediated isothermal amplification (LAMP) assays. (A–E): Visual detection of the LAMP amplification products with SYBR Green I. (F–J): Agarose gel electrophoresis of the LAMP products. Lane M: Trans 2K plus II DNA marker; Lanes 1–5: *mcr-1* to *mcr-5* genes; Lane 6: *bla*KPC-2; Lane 7: *bla*NDM-1; Lane 8: *bla*CTX-M-9; Lane 9: negative (water).

**Figure 4** Multiplex loop-mediated isothermal amplification (multi-LAMP) detection. Agarose gel electrophoresis and enzyme digestion analysis of *mcr* genes was performed for the multi-LAMP products on 2% agarose gel. (A) Lane M, Trans 2K plus II DNA marker; Lane 1, restriction enzyme digestion of *mcr-1* multi-LAMP products, 170 bp, 115 bp respectively; Lane 2, restriction enzyme digestion of *mcr-3* multi-LAMP products, 260 bp, 155 bp respectively; Lane 3, restriction enzyme digestion of *mcr-4* multi-LAMP product, 270 bp, 230 bp, and 185 bp respectively; Lane 4, restriction enzyme digestion of mixed *mcr-1*, *mcr-3*, and *mcr-4* multi-LAMP products, 260 bp, 225 bp, 170 bp, 120 bp, and 90 bp; Lane 5, negative (water). (B) Lane M, Trans 2K plus II DNA marker; Lane 1, restriction enzyme digestion of *mcr-2* multi-LAMP products, 220 bp, 175 bp, and 140 bp respectively; Lane 2, restriction enzyme digestion of *mcr-5* multi-LAMP products, 120 bp, 90 bp respectively; Lane 3, restriction enzyme digestion of mixed *mcr-2* and *mcr-5* multi-LAMP products, 215 bp, 170 bp, 140 bp, 120 bp, and 90 bp, respectively; Lane 4: negative (water).

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