Detection of plasmid-mediated colistin-resistant and carbapenem-resistant genes by multiplex PCR

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A B S T R A C T
A multiplex PCR was described to simultaneously detect mcr-1 and frequently occurring carbapenem-resistant genes including blaKPC, blaNDM, blaIMP, and blaOXA-48-like in a single reaction. The PCR product sizes of these 4 carbapenem-resistant genes were 232 bp, 438 bp, 621 bp, and 798 bp for blaIMP, blaOXA-48-like, blaNDM, and blaKPC, respectively, whereas mcr-1 revealed 1126 bp of PCR product. This protocol accurately detected those resistant genes in agreement with the reference strains, 127 local carbapenem-resistant Enterobacteriaceae, 8 mcr-1 carrying Enterobacteriaceae, and 62 carbapenem-susceptible Enterobacteriaceae. This method will be useful for laboratory application and surveillance of carbapenem and/or colistin-resistant bacteria.

A R T I C L E I N F O
Protocol name: mPCR for mcr-1 and carbapenem-resistant genes
Keywords: PCR, mcr-1, blaNDM, blaKPC, blaIMP, blaOXA-48-like
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Specifications Table

| Subject area          | Immunology and Microbiology |
|-----------------------|-----------------------------|
| More specific subject area | Clinical Bacteriology |
| Protocol name         | mPCR for mcr-1 and carbapenem-resistant genes |
| Reagents/tools        | 1. JumpStart™ RED Taq; ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, USA) |
|                       | 2. Primers |
|                       | IMP-F = 5'-GGAAATAGAGTGCTAAYTCTC-3' |
|                       | IMP-R = 5'-GGTTAAYAAAACACCCGAC-3' |
|                       | OXA48-like-F = 5'-GGCCTGTTAAGGATGGAAC-3' |
|                       | OXA48-like-R = 5'-CATCAAGTCCAACCAACCG-3' |
|                       | NDM-F = 5'-GGTTTGGCGATCTGGTTTTC-3' |
|                       | NDM-R = 5'-GCGTGGTTAAGGATGAACAC-3' |
|                       | KPC-F = 5'-CGAATTTGAGTATCCTTCTGCTCAG-3' |
|                       | KPC-R = 5'-CTTTGCATGCTTGTGACAGGC-3' |
|                       | MCR1-F = 5'-GTTGTGTGCTACACCTGAGTGC-3' |
|                       | MCR1-R = 5'-CATGGCGGTGATGCCAGTTT-3' |
| Experimental design   | We modified a multiplex PCR for detection of acquired carbapenemase genes described by Poirel et al. [1] and added the primers to detect mcr-1 in the same PCR reaction. This method can simultaneously detect 4 prevalent carbapenem-resistant genes (bla<sub>IMP</sub>, bla<sub>OXA48-like</sub>, bla<sub>NDM</sub>, and bla<sub>KPC</sub>) and a colistin-resistant gene (mcr-1) in a single reaction and revealed different PCR product sizes that are easy to interpret. |
| Trial registration    | None |
| Ethics                | None |

Value of the protocol

* Simultaneous detection of four frequent clinically relevant carbapenem-resistant genes and mcr-1 by multiplex PCR in a single reaction.
* Rapid, simple, and reliable for detection of frequently clinically relevant carbapenem and colistin-resistant genes (mcr-1) from pure culture.
* Useful for laboratory application and surveillance of carbapenem-resistant and/or colistin-resistant bacteria.
* Useful for detection of isolates co-carry mcr-1 and carbapenemase genes such as mcr-1 and bla<sub>NDM</sub>.

Description of protocol

Carbapenem-resistant organisms such as bla<sub>KPC</sub>, bla<sub>NDM</sub>, bla<sub>IMP</sub>, bla<sub>OXA48-like</sub>, and the emergence of the mcr-1 gene, a plasmid-mediated gene that confers colistin resistance in Enterobacteriaceae, have both been increasingly recognized worldwide. The spread of mcr-1-encoding plasmids into carbapenem-resistant Enterobacteriaceae raises concerns about the emergence of untreatable bacteria and it poses a serious threat to public health worldwide.

Many PCR techniques have been described to detect these resistant genes; however, no PCR (especially multiplex PCR) procedure has been described for detecting both mcr-1 and carbapenem-resistant genes in a single reaction. This study describes a protocol to simultaneously detect mcr-1 and frequently occurring carbapenem-resistant genes (bla<sub>KPC</sub>, bla<sub>NDM</sub>, bla<sub>IMP</sub>, bla<sub>OXA48-like</sub>) as well as to detect co-existence of mcr-1 and carbapenem-resistant genes in a single reaction from Gram-negative bacteria.

Major equipment and supplies for PCR assay

* PCR thermal cycler (Takara, Japan or equivalent)
* PCR tubes (Nest Scientific, USA or equivalent)
* Sterile Eppendorf style microcentrifuge tubes (Nest Scientific, USA or equivalent)
- Sterile inoculating loops or needles (Nest Scientific, USA or equivalent)
- Ice bucket or bench top cooler
- Adjustable micropipettors (0.1–1000 μl)
- Aerosol-resistant micropipettor tips (0.1–1000 μl)
- Vortex Mixer (CAPP, Denmark or equivalent)
- Microcentrifuge (CAPP, Denmark or equivalent)

**Reagents for DNA extraction**
- Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, USA or equivalent)
- Sodium hydroxide (Sigma-Aldrich, USA or equivalent)

**Reagents for PCR assay**
- JumpStart™ REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, USA)
- PCR grade water (Omega, USA or equivalent)
- 10X Tris-Borate-EDTA buffer (TBE) (Omega, USA or equivalent)
- Agarose gel (Sigma-Aldrich, USA or equivalent)
- Primers (Sigma-Aldrich, USA or equivalent)

**Procedures**

**Bacteria and DNA extraction**

Bacteria were cultured on blood agar or McConkey agar at 37 °C for 18–24 h. DNA was prepared by heating one or two colonies from an overnight grown plate at 95 °C for 15 min in 30 μl of lysis buffer containing 0.25% (vol/vol) sodium dodecyl sulfate and 0.05 M NaOH. After lysis, 200 μl of sterile distilled water was added to the lysis buffer and the DNA solutions were stored at −20 °C until PCR analysis.

**Multiplex PCR analysis**

The multiplex PCR assay was performed in 15-μl reaction mixtures, containing 2 μl of template, 1.5 μl of deionized water, 1X JumpStart™ REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, USA) and 0.53 μM of each primer (Table 1). The composition of the reagents in the multiplex PCR is shown in the Table 2.

| Name            | Sequence (5′–3′) | PCR product size (bp) | Reference |
|-----------------|------------------|-----------------------|-----------|
| IMP-F           | GGAATAGAGTGGCTTAAYTCTC | 232                   | [1]       |
| IMP-R           | GGTTTAYAAAACAACACC |                       |           |
| OXA-48-like-F   | GGGTTGTTAAGGATGAACAC | 438                   | [1]       |
| OXA-48-like-R   | CATCAAGTCAAACCCAGC |                       |           |
| NDM-F           | GGGTGGCAGTCTGTCTTTC | 621                   | [1]       |
| NDM-R           | CCGAATGGCTCATCAGATC |                       |           |
| KPC-F           | CGTCTAGTCTGCTGCTTGC | 798                   | [1]       |
| KPC-R           | CTGTGTTCCGTCTTGCAGCG |               |           |
| MCR1-F          | GGGTGCTCAACAGTTTGC | 1126                  | [2]       |
| MCR1-R          | CATTGGCGTGATGCCAGTTC |                  |           |
Table 2
Contents of mPCR reaction.

| Reagents                                      | Final concentration | µl per reaction |
|-----------------------------------------------|---------------------|-----------------|
| Deionized water                              | -                   | 1.5             |
| 2X JumpStart™ REDTaq™ ReadyMix™ PCR reaction mix | 1X                  | 7.5             |
| 20 µM IMP-F                                   | 0.53                | 0.4             |
| 20 µM IMP-R                                   | 0.53                | 0.4             |
| 20 µM OXA-48-F                                | 0.53                | 0.4             |
| 20 µM OXA-48-R                                | 0.53                | 0.4             |
| 20 µM NDM-F                                   | 0.53                | 0.4             |
| 20 µM NDM-R                                   | 0.53                | 0.4             |
| 20 µM KPC-F                                   | 0.53                | 0.4             |
| 20 µM KPC-R                                   | 0.53                | 0.4             |
| 20 µM MCR1-F                                  | 0.53                | 0.4             |
| 20 µM MCR1-R                                  | 0.53                | 0.4             |
| DNA extracted                                 | -                   | 2               |
| **Total volume**                              | -                   | **15**          |

The following PCR thermal profile was used: initial activation of DNA polymerase at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 56 °C for 30 s and extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min. The PCR products were analyzed using gel electrophoresis for 30 min on 2% agarose gels in 0.5X TBE buffer. The gels were stained with ethidium bromide and visualized under ultraviolet light (GeneGenius Bioimaging System, SynGene). The sizes of the PCR products were determined by comparison with a molecular-sized standard (GeneRuler™ 100 bp Plus DNA ladder, Thermo Fisher Scientific).

**Interpretation**

As shown in Fig. 1, our multiplex PCR differentiated 4 prevalent carbapenemase genes and mcr-1. The PCR product sizes of these 4 carbapenemase genes were about 232 bp, 438 bp, 621 bp, and 798 bp for bla_IMP, bla_OXA-48-like, bla_NDM, and bla_KPC, respectively, whereas mcr-1 revealed 1126 bp of PCR product.

**Validation**

We have validated our protocol with reference strains including *Klebsiella pneumoniae* ATCC® BAA-1705™ (bla_KPC), *Escherichia coli* ATCC® BAA-2340™ (bla_KPC), *E. coli* ATCC® BAA-2452™ (bla_NDM-1), *E. coli* ATCC® BAA-2469™ (bla_NDM-1), *K. pneumoniae* ATCC® BAA-2524™ (bla_OXA-48), *Serratia marcescens* KU3838 (bla_IMP-6), *K. pneumoniae* strain 22 and strain 34 (bla_IMP-14A) [3], and *E. coli* A434-59 (mcr-1 and bla_NDM-1) [2]. The multiplex PCR revealed PCR product sizes of 232 bp for bla_IMP-6 and bla_IMP-14A in *S. marcescens* KU3838 and *K. pneumoniae* strain 22 and 34, 438 bp for bla_OXA-48 in *K. pneumoniae* ATCC® BAA-2524™, 621 bp for bla_NDM in *E. coli* ATCC® BAA-2452™, *E. coli* ATCC® BAA-2469™ and *E. coli* A434-59, 798 bp for bla_KPC in *K. pneumoniae* ATCC® BAA-1705™ and *E. coli* ATCC® BAA-2340™, and 1126 bp for mcr-1 in *E. coli* A434-59, respectively.

In total, testing with our multiplex PCR was undertaken on 127 carbapenem-resistant *Enterobacteriaceae* with known carbapenemase genes using either Sanger sequencing or next-generation sequencing. These 127 isolates consisted of 50 bla_NDM-1 harboring isolates, 19 bla_NDM-5 harboring isolates, 1 bla_NDM-7 harboring isolates, 32 bla_OXA-181 harboring isolates, 10 bla_OXA-232 harboring isolates, 5 isolates carrying bla_NDM-1 and bla_OXA-181, 4 isolates carrying bla_NDM-1 and bla_OXA-232, and 6 bla_IMP-14 harboring isolates, respectively. As expected, this multiplex PCR assay could detect these carbapenemase genes in agreement with either Sanger sequencing or next-generation sequencing results e.g., isolates containing bla_OXA-181 and bla_OXA-232 revealed 438 bp of bla_OXA-48-like PCR product, while isolates carrying bla_NDM-1, bla_NDM-5 and bla_NDM-7 showed about 621 bp of bla_NDM.
**Fig. 1.** Agarose gel electrophoresis of PCR-amplified products from the representative four carbapenem-resistant genes and mcr-1. Lane M = 100 bp DNA ladder, lane 1 = K. pneumoniae strain no.1385 (blaNDM), lane 2 = K. pneumoniae strain no. 1386 (blaOXA-48-like), lane 3 = E. coli strain no. A434-59 (blaNDM and mcr-1), lane 4 = E. coli strain no.98 (blaNDM and blaOXA-48-like), lane 5 = E. coli strain no.1387 (blaOXA-48-like), lane 6 = K. pneumoniae strain no.1263 (blaNDM), lane 7 = K. pneumoniae strain 22 (blaIMP-14a), lane 8 = K. pneumoniae ATCC® BAA-2524 (blaOXA-48B), lane 9 = E. coli ATCC® BAA-2452 (blaNDM-1), lane 10 = K. pneumoniae ATCC® BAA-1705 (blaKPC), lane 11 = E. coli AK1 (a strain carrying mcr-1 recombinant plasmid), and lane 12 = negative control (distilled water).

PCR product. Where isolates contained either blaNDM-1 and blaOXA-18 or blaNDM-1 and blaOXA-232, our PCR revealed 2 bands at 438 bp and 621 bp for blaOXA-48-like and blaNDM, respectively (Fig. 1).

We also tested the multiplex PCR with 8 known mcr-1 isolates using Sanger sequencing (6 E. coli and 2 K. pneumoniae). The PCR assay could accurately detect the 1126 bp of mcr-1 in 8 isolates. In addition, E. coli A434-59 a strain co-carry of mcr-1 and blaNDM-1 [2] revealed 2 bands of 1126 bp (mcr-1) and 621 bp (blaNDM-1) by this PCR (Fig. 1). Sixty-two carbapenem-resistant Enterobacteriaceae were tested using the multiplex PCR and the results revealed no PCR product bands.

This method has advance in case of easy to use and save cost and time to simultaneously detect 4 frequently occurring carbapenemase genes and mcr-1 in a single reaction.

**References**

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