Published in "Journal of Antimicrobial Chemotherapy
doi: 10.1093/jac/dkw139, 2016"
which should be cited to refer to this work.

# Real-time PCR for detection of plasmid-mediated polymyxin resistance (*mcr-1*) from cultured bacteria and stools

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*Received 19 January 2016; returned 8 March 2016; revised 16 March 2016; accepted 24 March 2016*

**Objectives:** The aim of the study was to develop a simple assay for rapid detection of the *mcr-1* gene, recently identified as a source of plasmid-mediated acquired resistance to polymyxins in Enterobacteriaceae.

**Methods:** A SYBR Green-based real-time PCR assay was designed for detection of the *mcr-1* gene. This assay was applied to cultured bacteria and to spiked human and cattle stools.

**Results:** The *mcr-1* gene could be detected with a lower limit of $10^2$ cultured bacteria. This test was highly sensitive and specific, and generated no false-positive results. The assay was also conclusive when applied to stools spiked with *mcr-1*-positive *Escherichia coli*.

**Conclusions:** This simple, rapid, sensitive and specific assay will be useful for rapid screening of this resistance trait in both human medicine and veterinary medicine.



## Introduction

Colistin is a polymyxin antibiotic widely used in animal production and currently increasingly prescribed for therapeutic usage in human medicine, as a consequence of the spread of MDR Gram-negatives. So far, acquired resistance to colistin has involved chromosomal mutations in genes encoding proteins involved in the lipopolysaccharide biosynthesis pathway.[1] Recently, the first plasmid-mediated colistin resistance determinant, MCR-1, has been identified in Enterobacteriaceae.[2] The *mcr-1* gene, encoding a phosphoethanolamine transferase, was first identified among Chinese enterobacterial isolates of human and animal origin, and then worldwide, mainly in *Escherichia coli*.[2–9] Here, a SYBR Green-based real-time PCR assay is proposed, for rapid, sensitive and specific detection of the *mcr-1* gene from cultured bacteria and stools.

## Materials and methods

### *Quantitative PCR*

The SYBR Green quantitative PCR (qPCR) assay for detection of *mcr-1* was performed with primers mcr-1-qF1 (5′-ACACTTATGGCACGGTCTATG-3′) and mcr-1-qR1 (5′-GCACACCCAAACCAATGATAC-3′) internal to the *mcr-1* gene (designed using the PrimerQuest program, Integrated DNA Technologies) and with universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 338R (5′-GCTGCCTCCCGTAGGAGT-3′) for the 16S rRNA gene used as a control. The product sizes of the amplicons were 120 and 350 bp, and the melting temperatures were 82.5 and 86.7°C, respectively. Similar results were obtained for *mcr-1* with primers mcr-1-qF2 (5′-TGGCGTTCAGCAGTCATTAT-3′) and mcr-1-qR2 (5′-AGCTTACCCACCGAGTAGAT-3′). This pair of primers may detect *mcr-1* variants to which the mcr-1-qF1 and mcr-1-qR1 primers would not anneal. Standard control for the *mcr-1* qPCR was a purified 1646 bp PCR product of the *mcr-1* gene (1.8 pg of DNA corresponding to $10^6$ copies) generated with primers mcr-1-F (5′-ATGATGCAGCATACTTCTGTGTG-3′) and mcr-1-R (5′-TCAGCGGATGAATGCGGTGC-3′). Standard control for the 16S rRNA gene qPCR was *E. coli* TOP10 (Life Technologies, Carlsbad, CA, USA) total genomic DNA, with 5 ng corresponding to $7\times10^6$ 16S rRNA copies or $10^6$ cells, the RNA operon being present at 7 copies per genome in *E. coli*.[10] A total of 15 μL qPCRs were processed in a Rotor-Gene Q (Qiagen, Hilden, Germany) with a KAPA SYBR FAST qPCR Kit (Kapabiosystems, Wilmington, MA, USA), according to the manufacturer's instructions. Cycling conditions were 95°C for 2 min and 40 cycles of 95°C for 3 s, 60°C for 20 s and 72°C for 7 s, followed by a ramp from 72°C to 95°C for melting analysis. The copy numbers of the 16S rRNA and *mcr-1* genes were calculated according to the values obtained with the standard curves. For DNA extracted from spiked stools, qPCRs were performed with 1 μL of template with the same cycling protocol, except for spiked human stools, for which the annealing temperature was revised to 63°C.

### *Boiled-lysate DNA extraction*

Both *mcr-1*-positive and *mcr-1*-negative strains of human and animal origins were collected in France, Switzerland and South Africa (see Table 2). DNA was extracted from cultured bacteria with the boiled-lysate protocol, with one 1 μL loop of bacteria (corresponding to $1\times10^8$ to $2\times10^8$ bacteria or 0.2–0.4 $OD_{600}$) being resuspended in 20 μL of 10 mM Tris-EDTA pH8 buffer, and incubated for 10 min at 95°C. After centrifugation for 2 min at 20000 **g**, 20 μL of supernatant was added to 80 μL of $H_2O$.

**Table 1.** Quantitative detection of the *mcr-1* gene in *E. coli*

| Isolate | *mcr-1*[a] | Template dilution[b] | qPCR: *mcr-1* copies[c] | qPCR: 16S rRNA copies[c] | *mcr-1* copy number[d] |
|---|---|---|---|---|---|
| Ec1 | + | 1 | 3600000 | 8400000 | 3.0 |
| Ec1 | + | $10^{-2}$ | 40000 | 98000 | 2.9 |
| Ec1 | + | $10^{-4}$ | 350 | 980 | 2.5 |
| KRI | + | 1 | 2200000 | 9800000 | 1.6 |
| KRI | + | $10^{-2}$ | 26000 | 126000 | 1.4 |
| KRI | + | $10^{-4}$ | 270 | 980 | 1.9 |
| Ec8 | − | 1 | 0 | 5200000 | 0 |
| Ec8 | − | $10^{-4}$ | 0 | 630 | 0 |
| TOP10 | − | 1 | 0 | 7700000 | 0 |
| TOP10 | − | $10^{-4}$ | 0 | 1000 | 0 |

[a]Isolates were previously characterized for the presence of the *mcr-1* gene by conventional PCR and sequencing.
[b]Genomic DNA was extracted from $1\times10^8$ to $2\times10^8$ bacteria, resuspended in 100 μL. qPCRs were performed with 1 μL of genomic DNA or with the indicated dilution.
[c]*mcr-1* or 16S rRNA copies detected in the qPCR.
[d]Estimate of the *mcr-1* copy number per bacterium, obtained by dividing number of *mcr-1* copies by the number of 16S rRNA copies multiplied by 7 (the RNA operon being present at 7 copies per genome in *E. coli*).



**Table 2.** Specificity of the qPCR assay for detection of the *mcr-1* gene

| Isolate | Species | *mcr-1*[a] | Associated β-lactamase | qPCR: *mcr-1* copies[b] | *mcr-1* copy number[c] | Origin | Reference |
|---|---|---|---|---|---|---|---|
| KRI | *E. coli* | + | $bla_{VIM-1}$ | 2000000 | 1.6 | Switzerland | 7 |
| Ec1 | *E. coli* | + | $bla_{CMY-2}$ | 3200000 | 2.5 | South Africa | unpublished data |
| Ec2 | *E. coli* | + | none | 600000 | 1.3 | South Africa | unpublished data |
| Ec3 | *E. coli* | + | none | 890000 | 1.9 | South Africa | unpublished data |
| Ec4 | *E. coli* | + | none | 1100000 | 1.8 | South Africa | unpublished data |
| Ec5 | *E. coli* | + | $bla_{CTX-M-55}$ | 610000 | 2.4 | South Africa | unpublished data |
| Ec6 | *E. coli* | + | none | 1300000 | 2.2 | South Africa | unpublished data |
| Ec7 | *E. coli* | + | $bla_{CTX-M-55}$ | 940000 | 3.3 | South Africa | unpublished data |
| Ec8[d] | *E. coli* | − | $bla_{CTX-M-15}$ | 0 | 0 | South Africa | unpublished data |
| Ec9 | *E. coli* | − | $bla_{OXA-48}$ | 0 | 0 | France | unpublished data |
| Ec10 | *E. coli* | − | $bla_{OXA-48}$ | 0 | 0 | France | unpublished data |
| Ec11 | *E. coli* | − | $bla_{NDM-1}$ | 0 | 0 | France | unpublished data |
| Ec12 | *E. coli* | − | $bla_{VIM-1}$ | 0 | 0 | France | unpublished data |
| TOP10 | *E. coli* | − | none | 0 | 0 | reference | Life Technologies |
| Cf1 | *C. freundii* | − | $bla_{OXA-48}$ | 0 | 0 | France | unpublished data |
| Kp1 | *K. pneumoniae* | − | $bla_{OXA-48}$ | 0 | 0 | France | unpublished data |
| NK34373[e] | *K. pneumoniae* | − | none | 0 | 0 | France | 11 |
| Ecl1 | *E. cloacae* | − | $bla_{OXA-48}$ | 0 | 0 | France | unpublished data |
| Ecl2 | *E. cloacae* | − | $bla_{NDM-1}$ | 0 | 0 | France | unpublished data |
| Ko1 | *K. oxytoca* | − | $bla_{VIM-1}$ | 0 | 0 | France | unpublished data |

[a]Isolates were previously characterized for the presence of the *mcr-1* gene by conventional PCR and sequencing.
[b]*mcr-1* copies detected in the qPCR performed with 1 μL of genomic DNA.
[c]Estimate of the *mcr-1* copy number per bacterium, obtained by dividing number of *mcr-1* copies by the number of 16S rRNA copies (not shown here) multiplied by 7 (the RNA operon being present at 7 copies per genome in *E. coli*).
[d]Isolate resistant to colistin, unknown mechanism.
[e]Isolate resistant to colistin, disruption of the *mgrB* gene.[11]

### *Spiked stools and DNA extraction*

An amount of $10^8$ bacteria (corresponding to 0.2 $OD_{600}$) or dilution was resuspended in 50 μL of 0.85% NaCl and spiked in 200 μL of human or cattle faecal suspension (75 mg of fresh faeces per 200 μL). DNA was extracted from spiked stool samples with a DNA extraction ZR Fecal DNA MiniPrep (Zymo Research, Freiburg im Breisgau, Germany) according to the manufacturer's instructions. The samples were lysed with a mixer mill (MM 400, Rentsch, Haan, Germany) for 3 min at a frequency of 30 Hz. DNA was

**Table 3.** Detection of the *mcr-1* gene in spiked human and cattle stools

| Isolate | *mcr-1*[a] | Bacteria/75 mg of faeces | Faeces | qPCR: *mcr-1* copies[b] |
|---|---|---|---|---|
| Ec1 | + | $10^8$ | cattle | 970000 |
| Ec1 | + | $10^7$ | cattle | 84000 |
| Ec1 | + | $10^6$ | cattle | 12000 |
| Ec1 | + | $10^5$ | cattle | 1700 |
| Ec1 | + | $10^4$ | cattle | 170 |
| Ec1 | + | $10^3$ | cattle | 30 |
| KRI | + | $10^7$ | cattle | 19000 |
| Ec8 | − | $10^7$ | cattle | 0 |
| TOP10 | − | $10^7$ | cattle | 0 |
| Ec1 | + | $10^8$ | human | 950000 |
| Ec1 | + | $10^7$ | human | 120000 |
| Ec1 | + | $10^6$ | human | 5700 |
| Ec1 | + | $10^5$ | human | 990 |
| Ec1 | + | $10^4$ | human | 80 |
| Ec1 | + | $10^3$ | human | 10 |
| KRI | + | $10^7$ | human | 8100 |
| Ec8 | − | $10^7$ | human | 0 |
| TOP10 | − | $10^7$ | human | 0 |

[a]Isolates were previously characterized for the presence of the *mcr-1* gene by conventional PCR and sequencing.
[b]*mcr-1* copies detected in the qPCR; total DNA was extracted from 75 mg of faeces spiked with the indicated cfu of bacteria and qPCRs were performed with 1/80th of the extracted DNA.

recovered in 80 μL of elution buffer and qPCRs were performed with 1 μL of DNA template.

## Results and discussion

The SYBR Green-based real-time PCR assay could reproducibly and quantitatively detect $10^6$ to $10^2$ copies of *mcr-1* and $7\times10^6$ to $7\times10^2$ copies of 16S rRNA, corresponding to $10^6$–$10^2$ *E. coli* bacteria. The $r^2$ value was >0.999, the amplification efficiency was ≥0.9 and the slope was between −3.59 and −3.40. This assay could also detect the *mcr-1* and 16S rRNA genes at higher concentrations, but not in the linear range of the qPCR. This assay was applied to two *mcr-1*-positive *E. coli* isolates of human origin, Ec1 and KRI, with Ec8 and TOP10 as negative controls. For Ec1 and KIR, the *mcr-1* gene was detected using 1 μL of DNA template, corresponding to a starting amount of $1\times10^6$ to $2\times10^6$ bacteria, and with $10^{-2}$ and $10^{-4}$ dilutions of the DNA template. Normalization for amplification of the 16S rRNA gene showed that the *mcr-1* gene was present in isolates Ec1 and KRI at ∼3 and 1.5 copies per bacterium, respectively (Table 1).

In order to further validate the sensitivity and specificity of this test, it was applied to a larger set of isolates, i.e. eight *mcr-1*-positive and six *mcr-1*-negative *E. coli*, and six *mcr-1*-negative isolates of other species (*Klebsiella pneumoniae*, *Enterobacter cloacae*, *Citrobacter freundii* and *Klebsiella oxytoca*). The assay was sensitive, as it allowed detection of the *mcr-1* gene in all cases. It was also specific, as it did not generate any false-positive signal, in contrast to the previously published conventional PCR assay with the CLR5-F and CLR5-R primers,[2] which generated false-positive signals for two strains. Normalization for the amplification of the 16S rRNA gene indicated that the *mcr-1* gene was present at 1.3–3.3 copies per bacterial cell (Table 2).

Then, the real-time qPCR assay was applied to cattle and human stools spiked with different concentrations of *mcr-1*-positive bacteria, the rationale for detecting *mcr-1* in stools being that this gene has been frequently identified in animal stools.[2] The *mcr-1* gene was efficiently detected in human and cattle faeces (Table 3).

Overall, this SYBR Green-based real-time PCR assay is a rapid, sensitive and highly specific detection assay for the *mcr-1* gene either from cultured bacteria or from cattle or human stools. It is easy to perform in any laboratory having at its disposal a qPCR machine. This rapid technique may be used for the evaluation of the prevalence of this resistance trait in humans and animals (surveillance studies). In addition, it will be a valuable tool for following up outbreaks in order to promptly isolate colonized patients and assign them to a cohort.


## Funding

This work was supported by the University of Fribourg and by a grant of the ANIWHA ERA-NET project (No. 1.15.08) (Switzerland).


## Transparency declarations

None to declare.

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