J Antimicrob Chemother 2016; **71**: 2318–2320
doi:10.1093/jac/dkw139 Advance Access publication 27 April 2016

Journal of Antimicrobial Chemotherapy

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 7×10^6 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 1×10^8 to 2×10^8 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 1×10^8 to 2×10^8 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 7×10^6 to 7×10^2 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 1×10^6 to 2×10^6 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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