

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

S  verine Bontron¹, Laurent Poirel^{1*} and Patrice Nordmann^{1,2}

¹Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Department of Medicine, Faculty of Science, University of Fribourg, Fribourg, Switzerland; ²H  pital Cantonal HFR, Fribourg, Switzerland

*Corresponding author. Tel: +41-26-300-9582; E-mail: laurent.poirel@unifr.ch

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 10² 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'-ACACTTATGGCAGGCTCATG-3') and *mcr-1*-qR1 (5'-GCACACCAACCAATGATAC-3') internal to the *mcr-1* gene (designed using the PrimerQuest program, Integrated DNA Technologies) and with universal primers 27F (5'-AGAGTTTGTATCCTGGCTCAG-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 10⁶ 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   10⁶ 16S rRNA copies or 10⁶ cells, the RNA operon being present at 7 copies per genome in *E. coli*.¹⁰ A total of 15  L 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  L 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  L loop of bacteria (corresponding to 1   10⁸ to 2   10⁸ bacteria or 0.2–0.4 OD₆₀₀) being resuspended in 20  L of 10 mM Tris-EDTA pH8 buffer, and incubated for 10 min at 95  C. After centrifugation for 2 min at 20 000 g, 20  L of supernatant was added to 80  L of H₂O.

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

Isolate	<i>mcr-1</i> ^a	Template dilution ^b	qPCR: <i>mcr-1</i> copies ^c	qPCR: 16S rRNA copies ^c	<i>mcr-1</i> copy number ^d
Ec1	+	1	3 600 000	8 400 000	3.0
Ec1	+	10 ⁻²	40 000	98 000	2.9
Ec1	+	10 ⁻⁴	350	980	2.5
KRI	+	1	2 200 000	9 800 000	1.6
KRI	+	10 ⁻²	26 000	126 000	1.4
KRI	+	10 ⁻⁴	270	980	1.9
Ec8	-	1	0	5 200 000	0
Ec8	-	10 ⁻⁴	0	630	0
TOP10	-	1	0	7 700 000	0
TOP10	-	10 ⁻⁴	0	1000	0

^aIsolates were previously characterized for the presence of the *mcr-1* gene by conventional PCR and sequencing.

^bGenomic DNA was extracted from 1×10⁸ to 2×10⁸ 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.

^dEstimate 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	<i>mcr-1</i> ^a	Associated β-lactamase	qPCR: <i>mcr-1</i> copies ^b	<i>mcr-1</i> copy number ^c	Origin	Reference
KRI	<i>E. coli</i>	+	bla _{VIM-1}	2 000 000	1.6	Switzerland	7
Ec1	<i>E. coli</i>	+	bla _{CMY-2}	3 200 000	2.5	South Africa	unpublished data
Ec2	<i>E. coli</i>	+	none	600 000	1.3	South Africa	unpublished data
Ec3	<i>E. coli</i>	+	none	890 000	1.9	South Africa	unpublished data
Ec4	<i>E. coli</i>	+	none	1 100 000	1.8	South Africa	unpublished data
Ec5	<i>E. coli</i>	+	bla _{CTX-M-55}	610 000	2.4	South Africa	unpublished data
Ec6	<i>E. coli</i>	+	none	1 300 000	2.2	South Africa	unpublished data
Ec7	<i>E. coli</i>	+	bla _{CTX-M-55}	940 000	3.3	South Africa	unpublished data
Ec8 ^d	<i>E. coli</i>	-	bla _{CTX-M-15}	0	0	South Africa	unpublished data
Ec9	<i>E. coli</i>	-	bla _{OXA-48}	0	0	France	unpublished data
Ec10	<i>E. coli</i>	-	bla _{OXA-48}	0	0	France	unpublished data
Ec11	<i>E. coli</i>	-	bla _{NDM-1}	0	0	France	unpublished data
Ec12	<i>E. coli</i>	-	bla _{VIM-1}	0	0	France	unpublished data
TOP10	<i>E. coli</i>	-	none	0	0	reference	Life Technologies
Cf1	<i>C. freundii</i>	-	bla _{OXA-48}	0	0	France	unpublished data
Kp1	<i>K. pneumoniae</i>	-	bla _{OXA-48}	0	0	France	unpublished data
NK34373 ^e	<i>K. pneumoniae</i>	-	none	0	0	France	11
Ec1	<i>E. cloacae</i>	-	bla _{OXA-48}	0	0	France	unpublished data
Ec2	<i>E. cloacae</i>	-	bla _{NDM-1}	0	0	France	unpublished data
Ko1	<i>K. oxytoca</i>	-	bla _{VIM-1}	0	0	France	unpublished data

^aIsolates 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.

^cEstimate 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*).

^dIsolate resistant to colistin, unknown mechanism.

^eIsolate resistant to colistin, disruption of the *mgrB* gene.¹¹

Spiked stools and DNA extraction

An amount of 10⁸ bacteria (corresponding to 0.2 OD₆₀₀) 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, Retsch, 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	<i>mcr-1</i> ^a	Bacteria/75 mg of faeces	Faeces	qPCR: <i>mcr-1</i> 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

^aIsolates 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 10⁶ to 10² copies of *mcr-1* and 7 × 10⁶ to 7 × 10² copies of 16S rRNA, corresponding to 10⁶–10² *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 KRI, the *mcr-1* gene was detected using 1 µL of DNA template, corresponding to a starting amount of 1 × 10⁶ to 2 × 10⁶ 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 ANIWAH ERA-NET project (No. 1.15.08) (Switzerland).

Transparency declarations

None to declare.

References

- 1 Falagas ME, Rafailidis PI, Matthaiou DK. Resistance to polymyxins: mechanisms, frequency and treatment options. *Drug Resist Updat* 2010; **13**: 132–8.
- 2 Liu YY, Wang Y, Walsh TR et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 2016; **16**: 161–8.
- 3 Nordmann P, Lienhard R, Kieffer N et al. Plasmid-mediated colistin-resistant *Escherichia coli* in bacteremia in Switzerland. *Clin Infect Dis* 2016; doi:10.1093/cid/ciw124.
- 4 Hasman H, Hammerum AM, Hansen F et al. Detection of *mcr-1* encoding plasmid-mediated colistin-resistant *Escherichia coli* isolates from human bloodstream infection and imported chicken meat, Denmark 2015. *Euro Surveill* 2015; **20**: pii=30085.
- 5 Hu Y, Liu F, Lin IY et al. Dissemination of the *mcr-1* colistin resistance gene. *Lancet Infect Dis* 2016; **16**: 146–7.
- 6 Olaitan AO, Chabou S, Okdah L et al. Dissemination of the *mcr-1* colistin resistance gene. *Lancet Infect Dis* 2016; **16**: 147.
- 7 Poirel L, Kieffer N, Liassine N et al. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*. *Lancet Infect Dis* 2016; **16**: 281.
- 8 Zurfluh K, Poirel L, Nordmann P et al. Occurrence of the plasmid-borne *mcr-1* colistin resistance gene in ESBL-producing Enterobacteriaceae in river water and imported vegetable samples in Switzerland. *Antimicrob Agents Chemother* 2016; **60**: 2594–5.
- 9 Webb HE, Granier SA, Marault M et al. Dissemination of the *mcr-1* colistin resistance gene. *Lancet Infect Dis* 2016; **16**: 144–5.
- 10 Stoddard SF, Smith BJ, Hein R et al. *rrnDB*: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. *Nucleic Acids Res* 2015; **43**: D593–8.
- 11 Kieffer N, Poirel L, Nordmann P et al. Emergence of colistin resistance in *Klebsiella pneumoniae* from veterinary medicine. *J Antimicrob Chemother* 2015; **70**: 1265–7.