A SYBRGreen-Based Real-time PCR Method for Improved Detection of *Mcr-1*-mediated Colistin Resistance in Human Stool Samples

Valentina Donà,¹ Odette J. Bernasconi,^{1,2} Sara Kasraian,¹, Regula Tinguely,¹,Andrea Endimiani ^{1*}

¹ Institute for Infectious Diseases, University of Bern, Bern, Switzerland

²Graduate School of Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland

*Corresponding Author

Prof. Andrea Endimiani MD, PhD

Institute for Infectious Diseases, University of Bern

Friedbühlstrasse 51.

CH-3001, Bern,

Switzerland

Phone: +41-31-632 8 632; Fax: +41-31-632 8 766

Emails: andrea.endimiani@ifik.unibe.ch; aendimiani@gmail.com

Running Title: Real-time PCR for *mcr-1* detection in stools

ABSTRACT

Background: The aim of this study was to design a rapid and sensitive real-time PCR (RT-PCR) method for colistin resistance *mcr-I* gene detection in human fecal samples.

Methods: Stools (n=88) from 36 volunteers were analyzed. To isolate *mcr-I*-producing *Enterobacteriaceae* samples were enriched overnight in LB broth containing 2 mg/L colistin and then plated in selective agar plates with 4 mg/L colistin. We then designed a SYBRGreen-based RT-PCR targeting the *mcr-I*. For method validation and to establish the limit of detection (LOD), total DNA was extracted from *mcr-I*-negative and *mcr-I*-positive *E. coli*. RT-PCR was also performed with DNA extracted from the 88 native stools and after enriching them in LB broth containing colistin.

Results: Based on the culture approach, 3 unique volunteers resulted colonized with *mcr-I*-harboring *E. coli* strains. For culture isolates, our RT-PCR exhibited a LOD of 10 genomic copies/reaction with both sensitivity and specificity of 100%. Nevertheless, when testing native stools, only two out of the three *mcr-I*-positive specimens were detected. However, after enrichment in LB containing colistin, the RT-PCR was strongly positive for all culture-positive samples. The average cycle threshold was 22, granting rapid and confident detection of positive specimens within 30 cycles. No false-positives were observed for the remaining 85 culture-negative specimens.

Conclusions: We developed a rapid RT-PCR for the detection of *mcr-I* from stool specimens. We increased the detection rate by testing selective broth enrichments. This approach has also the advantage of concomitant isolation of the *mcr-I*-harboring strains for further antimicrobial susceptibility and genetic tests.

KEYWORDS: Real-time PCR, colistin, *mcr-I*, stools, *Enterobacteriaceae*, polymyxins

1. INTRODUCTION

The recent discovery of *mcr-1*-mediated colistin resistance has raised considerable public health concern [1]. This antimicrobial resistance determinant has already been detected in many *Enterobacteriaceae* species (particularly *Escherichia coli*) isolated in different parts of the world and from several reservoirs, including humans, environment, food and food-producing animals [2]. Notably, *mcr-1* possesses the ability to be transferred both horizontally and vertically following integration into plasmids or the bacterial chromosome, respectively [1-3].

To prevent further dissemination of *mcr-1*-possessing strains, as well as to elucidate their prevalence in surveillance studies, the need for a rapid, sensitive, and routine-oriented test has soon emerged. To date, this issue has been addressed only by few researchers, leading to the publication of three real-time (RT)-PCR assays for *mcr-1* detection in fecal samples. In particular, Bontron *et al.* published a SYBRGreen-based RT-PCR with a limit of detection (LOD) of approximately 10 bacterial colony forming unit (CFU) per reaction, but exclusively evaluating spiked stools [4]. Nijhuis and coworkers reported a similar LOD for their TaqMan-based assay, being able to confirm two out of two *mcr-1*-positive fecal samples [5]. Finally, Chabou *et al.* developed two TaqMan probe-based assays with comparable LODs (i.e., 750 CFU per reaction) and able to detect 5 positive broiler cloacal samples [6].

In this study, we first aimed to design a rapid SYBRGreen-based RT-PCR for *mcr-1* detection in native human fecal samples. We further investigated the impact of testing selective broth enrichments on the sensitivity of the method. To the best of our knowledge, this latter approach has not yet been evaluated in this specific context.

2. MATERIAL AND METHODS

2.1 Stool specimens and isolation of *mcr-1*-positive *Enterobacteriaceae*

We tested the post-traveling stool samples (n=78) of 26 people living in Switzerland and traveling to India in the time period from January to August 2015 (i.e., 25 provided samples immediately after return, as well as after 3 and 6 months; one also after 12 months following return from additional traveling) [7, 8]. Furthermore, we also analyzed fecal specimens of 10 HIV-positive (HIV+) individuals monitored at the Department of Infectious Diseases of the Bern University Hospital, Switzerland (Table 1) [9].

As previously described [7, 8], to isolate *mcr-1*-producing *Enterobacteriaceae* ~20 µg stools were enriched overnight in 10 ml Luria-Bertani (LB) broth with 2 mg/L colistin (i.e., selective enrichment). Then, 100 µL were plated on four selective agar plates: MacConkey agar with or without colistin (4 mg/L) and CHROMagar Orientation plus colistin (4 mg/L) and vancomycin (8 mg/L) with or without cefotaxime (2 mg/L). Colonies (if any) were subjected to species identification using the MALDI-TOF MS (Bruker). Microdilution Trek panels (Thermo Scientific Microbiology) were used to obtain the antibiotic MICs for species not naturally resistant to polymyxins. In case of colistin MICs >2 mg/L, the presence of *mcr-1* was evaluated by PCR/Sequencing [7, 9].

2.2 SYBRGreen RT-PCR design

The SYBRGreen RT-PCR assay for *mcr-1* detection was performed with primers *mcr-1*-FW (5'-ACGCCATCTGCAACACCAA-3') and *mcr-1*-RV (5'-GCCAACGAGCATACCGACAT-3') which were designed with Oligo Primer Analysis software v4.0 (Molecular Biology Insights). The product size of the amplicon was 59 bp with a melting temperature of 78.4°C. Each 20 µL reaction mixture contained 0.2 µM of each

primer, 1X PowerUp master mix containing Uracil N-glycosylase (UNG) for carryover contamination control (Applied Biosystems) and 2 μ L of DNA template. Experiments were performed with the QuantStudio 7 Flex System and data were analyzed with the QuantStudio 6 & 7 Flex Real-Time PCR System Software v1.0 (Applied Biosystems). Cycling conditions included incubation at 50°C for 2 min for UNG and a first denaturation step (95°C for 10 min), followed by 30 or 40 cycles (see below) of denaturation (95°C for 15 sec), annealing (63°C for 10 sec), and extension (72°C for 10 sec).

2.3 DNA extraction

To establish the LOD and for method validation, genomic DNA (gDNA) was extracted (QiaAMP DNA Mini kit, Qiagen) from 8 *mcr-1*-negative and the three *mcr-1*-positive *E. coli*, as well as from 88 stools, both native (using 100 mg extracted with the QiaAMP Stool Mini kit, Qiagen, and eluted in 100 μ L) and after performing the selective enrichment in LB containing colistin (using 200 μ L extracted with the QiaAMP DNA Mini kit, Qiagen, and eluted in 100 μ L).

3. RESULTS AND DISCUSSION

Based on our selective culture approach, three *E. coli* strains isolated from stool specimens of three unique individuals resulted positive for the *mcr-I* gene (two from travelers and one from a HIV+ patient). Additionally, 6 persons carried colistin-resistant isolates (of which, *E. coli*, n=3; *Klebsiella pneumoniae*, n=1; *Providencia rettgeri*, n=1; *Enterobacter asburiae*, n=1), but none of them resulted *mcr-I*-positive by PCR (Table 1) [7, 9].

In the efforts to rapidly detect positive fecal specimens, we designed a SYBRGreen-based RT-PCR targeting the *mcr-I*. When testing different dilutions of gDNA extracted from *mcr-I*-positive culture isolates, the standard curve was linear throughout all concentrations tested (i.e., from 10^7 to 10^1 gDNA copies/reaction), exhibiting an estimated PCR efficiency of approximately 106% (Figure 1A). The LOD of our method was 10 gDNA copies/reaction using 40 cycles, which is comparable to the previously described methods (Figure 1B) [4-6]. Notably, our RT-PCR correctly identified all *mcr-I*-harboring (n=3) and *mcr-I*-negative (n=8) *E. coli* isolates, showing high sensitivity and specificity (data not shown). Nevertheless, when testing DNA extracted from native stools, only two out of the three samples containing *mcr-I*-positive strains were successfully detected by the RT-PCR (Table 1). In particular, the average cycle threshold (Ct) was of 34.37 (sample 100-R) and 36.77 (sample 31349), whereas for sample 19-M12 resulted negative (Ct>40). However, our method was highly specific and correctly reported all *mcr-I* culture-negative specimens (n=85). It can be speculated that the observed low sensitivity was due to the presence of PCR inhibitors, as well as too little amounts of *mcr-I*-carrying bacteria present in the stools. Additionally, this study was conducted retrospectively on previously collected samples [7, 8], so we cannot exclude a possible negative effect of long-term storage (i.e., at -80°C without addition of cryoprotectant) of the stool specimens.

Since direct screening of fecal samples using our RT-PCR could lead to false-negative results, we decided to improve the sensitivity testing the DNA extracts from the selective LB broth enrichments (Table 1). As speculated, all three samples displayed strong positivity (i.e., Ct of 23.00, 21.97, and 21.05 for 100-R, 19-M12, and 31349, respectively). No additional positives were observed for the remaining 85 *mcr-1* culture-negative specimens, confirming the high specificity of the method. Moreover, this also allowed us to confidently shorten the method to 30 cycles in order to save about 15 minutes.

Our results are consistent with previous observations made by Helmy O *et al.* who already evaluated the extent to which an enrichment step is able to increase the sensitivity of a PCR-based assay. Notably, in such work authors developed a standard multiplex PCR for the detection of *E. coli* and *Shigella* spp. from stool samples reporting a 10⁵-fold increase in the LOD following enrichment (from 1.8 x10⁶ CFU/ml to 1.5x10¹ CFU/ml) [10].

A limitation of the present RT-PCR is that *in silico* sequence analysis suggests that our RT-PCR may not be able to detect the *mcr-2* [11]. Unfortunately, such genetic variant of *mcr-1* was only recently published, well after our method was designed. Nevertheless, the prevalence of *mcr-2* seems to be low (i.e., it has never been reported again in other studies). Furthermore, our enrichment approach could be applied to improve the sensitivity of other previously published RT-PCR methods as well [5, 6], including the one that is able to detect also such genetic variant [4]. Finally, broth enrichments resulted positive with the RT-PCR can easily be further processed by plating on selective agar plates in order to obtain the *mcr-1*-carrying strains for further phenotypic and molecular characterizations.

4. CONCLUSIONS

We developed a RT-PCR for *mcr-I* detection in human fecal specimens. Since the sensitivity for native stools was relatively low, we increased the detection rate by testing selective broth enrichments. This additional step promotes the growth of *mcr-I*-carrying strains that are probably underrepresented in the original fecal specimens and contributes to eliminate potential PCR inhibitors which may be present in stools. It also displays the major advantage of simple concomitant isolation of the *mcr-I*-harboring strains for antimicrobial susceptibility and genetic tests. Furthermore, the selective enrichment strategy is valuable information for the other already developed *mcr-I* RT-PCRs and may be used as a complement step to enhance their sensitivities. Thus, we believe that this method could play an essential role averting the treat of the difficult to detect *mcr-I*-mediated colistin resistance, as well as the risk to underestimate such major emerging epidemiological issue.

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DECLARATIONS

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Competing Interests: None declared

Ethical Approval: The study was approved by Kantonale Ethikkommission Bern (KEK): *Schweizerische HIV Kohortenstudie* (No. 21/88) and by the Ethikkommission Nordwest- und Zentralschweiz (EKNZ 239/12).

REFERENCES

- [1] Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, 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.
- [2] Nordmann P, Poirel L. Plasmid-mediated colistin resistance: an additional antibiotic resistance menace. *Clin Microbiol Infect*. 2016;22:398-400.
- [3] Zurfluh K, Tasara T, Poirel L, Nordmann P, Stephan R. Draft Genome Sequence of *Escherichia coli* S51, a Chicken Isolate Harboring a Chromosomally Encoded *mcr-1* Gene. *Genome Announc*. 2016;4.
- [4] Bontron S, Poirel L, Nordmann P. Real-time PCR for detection of plasmid-mediated polymyxin resistance (*mcr-1*) from cultured bacteria and stools. *J Antimicrob Chemother*. 2016;71:2318-20.
- [5] Nijhuis RH, Veldman KT, Schelfaut J, Van Essen-Zandbergen A, Wessels E, Claas EC, et al. Detection of the plasmid-mediated colistin-resistance gene *mcr-1* in clinical isolates and stool specimens obtained from hospitalized patients using a newly developed real-time PCR assay. *J Antimicrob Chemother*. 2016;71:2344-6.
- [6] Chabou S, Leangapichart T, Okdah L, Le Page S, Hadjadj L, Rolain JM. Real-time quantitative PCR assay with Taqman((R)) probe for rapid detection of MCR-1 plasmid-mediated colistin resistance. *New Microbes New Infect*. 2016;13:71-4.
- [7] Bernasconi OJ, Kuenzli E, Pires J, Tinguely R, Carattoli A, Hatz C, et al. Travelers Can Import Colistin-Resistant *Enterobacteriaceae*, Including Those Possessing the Plasmid-Mediated *mcr-1* Gene. *Antimicrob Agents Chemother*. 2016;60:5080-4.
- [8] Pires J, Kuenzli E, Kasraian S, Tinguely R, Furrer H, Hilty M, et al. Polyclonal Intestinal Colonization with Extended-Spectrum Cephalosporin-Resistant *Enterobacteriaceae* upon Traveling to India. *Front Microbiol*. 2016;7:1069.

217 [9] Pires J, Kasraian S, Tinguely R, Hauser C, Rauch A, Furrer H, et al. HIV-positive
218 individuals in Switzerland and intestinal colonization with extended-spectrum cephalosporin-
219 resistant *E. coli* strains. Poster at ASM Microbe, Boston, June 16-20. 2016.

220 [10] Helmy O. M. RYM, Hussein M. M. M. Multiplex polymerase chain reaction (PCR) for
221 the detection of diarrheagenic *Escherichia coli* and *Shigella* directly from stool. African
222 Journal of Microbiology Research. 2013;7 (34):4368-72.

223 [11] Xavier BB, Lammens C, Ruhel R, Kumar-Singh S, Butaye P, Goossens H, et al.
224 Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia*
225 *coli*, Belgium, June 2016. Euro Surveill. 2016;21.

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228   **LEGEND TO THE FIGURE**

229   **Figure 1.** Linear regression and PCR efficiency (**A**), as well as amplification plot (**B**)
230   obtained when testing different dilutions of genomic DNA (gDNA) extracted from an *E. coli*
231   *mcr-I*-harboring strain by real-time-PCR. NTC, no template control.

Table 1. Results of selective culture- and *mcr-1* RT-PCR screening for stool specimens and selective enrichments of 26 travelers and 10 HIV-positive (HIV+) patients

No.	Group	ID Sample	-R ^e stool culture	-R RT-PCR (or otherwise indicated)		-M3 stool culture	-M3 RT-PCR		M6 stool culture	-M6 RT-PCR	
				Stool ^a	LB ^b		Stool ^a	LB ^b		Stool ^a	LB ^b
1	Traveler	100	<i>mcr-1-Ec</i>	+	+	-	-	-	-	-	-
2	Traveler	71	-	-	-	-	-	-	-	-	-
3	Traveler	23	-	-	-	-	-	-	-	-	-
4	Traveler	80	-	-	-	-	-	-	-	-	-
5	Traveler	18	-	-	-	- (Col-R- <i>Ec</i>) ^d	-	-	-	-	-
6	Traveler	34	-	-	-	- (Col-R- <i>Ea</i>) ^d	-	-	-	-	-
7	Traveler	31	-	-	-	-	-	-	-	-	-
8	Traveler	45	-	-	-	-	-	-	-	-	-
9	Traveler	19	-	-	-	-	-	-	-	-	-
		19-M12 ^c	<i>mcr-1-Ec</i>	-	+	na	na	na	na	na	na
10	Traveler	29	- (Col-R- <i>Pr</i>) ^d	-	-	-	-	-	-	-	-
11	Traveler	97	-	-	-	-	-	-	-	-	-
12	Traveler	83	-	-	-	-	-	-	-	-	-
13	Traveler	41	-	-	-	-	-	-	-	-	-
14	Traveler	37	-	-	-	-	-	-	-	-	-
15	Traveler	56	-	-	-	- (Col-R- <i>Ec</i>) ^d	-	-	-	-	-
16	Traveler	50	-	-	-	-	-	-	-	-	-
17	Traveler	26	-	-	-	-	-	-	-	-	-
18	Traveler	59	-	-	-	-	-	-	-	-	-
19	Traveler	43	-	-	-	-	-	-	-	-	-
20	Traveler	68	-	-	-	-	-	-	-	-	-
21	Traveler	88	- (Col-R- <i>Ec</i>) ^d	-	-	-	-	-	-	na	na
22	Traveler	60	-	-	-	-	-	-	-	-	-
23	Traveler	33	-	-	-	-	-	-	-	-	-
24	Traveler	96	- (Col-R- <i>Kp</i>) ^d	-	-	-	-	-	-	-	-
25	Traveler	55	-	-	-	-	-	-	-	-	-
26	Traveler	51	-	-	-	-	-	-	-	-	-
27 ^e	HIV+	31349	<i>mcr-1-Ec</i>	+	+	na	na	na	na	na	na
28 ^e	HIV+	32143	-	-	-	na	na	na	na	na	na
29 ^e	HIV+	31454	-	-	-	na	na	na	na	na	na
30 ^e	HIV+	32339	-	-	-	na	na	na	na	na	na
31 ^e	HIV+	31171	-	-	-	na	na	na	na	na	na
32 ^e	HIV+	31998	-	-	-	na	na	na	na	na	na
33 ^e	HIV+	32192	-	-	-	na	na	na	na	na	na
34 ^e	HIV+	30940	-	-	-	na	na	na	na	na	na
35 ^e	HIV+	32388	-	-	-	na	na	na	na	na	na
36 ^e	HIV+	32013	-	-	-	na	na	na	na	na	na

Note. +, positive; -, negative; *mcr-1-Ec*, *mcr-1*-positive *E. coli* strain; na, not available or not applicable; -R, stools obtained immediately after return from travel; -M3, stools obtained three months after return from the trip; -M6, stools obtained six months after return from trip; Col-R, colistin-resistant; *Ec*, *E. coli*; *Kp*, *K. pneumoniae*; *Pr*, *P. rettgeri*; *Ea*, *E. asburiae*.

^a RT-PCR performed with 40 cycles for native stools

^b RT-PCR performed with 30 cycles for enrichment in LB containing 2 µg/ml colistin

^c This sample was obtained after 12 months immediately after returning from a second travel abroad (India)

^d Colistin-resistant *mcr-1*-negative *Enterobacteriaceae*

^e A single stool sample was collected for HIV+ patients

Table 1

Results of selective culture and *mcr-1* real-time PCR (rt-PCR) screening for stool specimens and selective enrichments of 26 travellers and 10 HIV-positive patients

No.	Group	Sample ID	R ^a stool culture	R ^a rt-PCR		M3 ^a stool culture	M3 ^a rt-PCR		M6 ^a stool culture	M6 ^a rt-PCR	
				Stool ^b	LB ^c		Stool ^b	LB ^c		Stool ^b	LB ^c
1	Traveller	100	<i>mcr-1-Ec</i>	+	+	–	–	–	–	–	–
2	Traveller	71	–	–	–	–	–	–	–	–	–
3	Traveller	23	–	–	–	–	–	–	–	–	–
4	Traveller	80	–	–	–	–	–	–	–	–	–
5	Traveller	18	–	–	–	– (Col-R- <i>Ec</i>) ^d	–	–	–	–	–
6	Traveller	34	–	–	–	– (Col-R- <i>Ea</i>) ^d	–	–	–	–	–
7	Traveller	31	–	–	–	–	–	–	–	–	–
8	Traveller	45	–	–	–	–	–	–	–	–	–
9	Traveller	19	–	–	–	–	–	–	–	–	–
		19-M12 ^e	<i>mcr-1-Ec</i> ^e	– ^e	+ ^e	na	na	na	na	na	na
10	Traveller	29	– (Col-R- <i>Pr</i>) ^d	–	–	–	–	–	–	–	–
11	Traveller	97	–	–	–	–	–	–	–	–	–
12	Traveller	83	–	–	–	–	–	–	–	–	–
13	Traveller	41	–	–	–	–	–	–	–	–	–
14	Traveller	37	–	–	–	–	–	–	–	–	–
15	Traveller	56	–	–	–	– (Col-R- <i>Ec</i>) ^d	–	–	–	–	–
16	Traveller	50	–	–	–	–	–	–	–	–	–

17	Traveller	26	–	–	–	–	–	–	–	–	–
18	Traveller	59	–	–	–	–	–	–	–	–	–
19	Traveller	43	–	–	–	–	–	–	–	–	–
20	Traveller	68	–	–	–	–	–	–	–	–	–
21	Traveller	88	– (Col-R- <i>Ec</i>) ^d	–	–	–	–	–	–	na	na
22	Traveller	60	–	–	–	–	–	–	–	–	–
23	Traveller	33	–	–	–	–	–	–	–	–	–
24	Traveller	96	– (Col-R- <i>Kp</i>) ^d	–	–	–	–	–	–	–	–
25	Traveller	55	–	–	–	–	–	–	–	–	–
26	Traveller	51	–	–	–	–	–	–	–		
27 ^f	HIV+	31349	<i>mcr-1-Ec</i>	+	+	na	na	na	na	na	na
28 ^f	HIV+	32143	–	–	–	na	na	na	na	na	na
29 ^f	HIV+	31454	–	–	–	na	na	na	na	na	na
30 ^f	HIV+	32339	–	–	–	na	na	na	na	na	na
31 ^f	HIV+	31171	–	–	–	na	na	na	na	na	na
32 ^f	HIV+	31998	–	–	–	na	na	na	na	na	na
33 ^f	HIV+	32192	–	–	–	na	na	na	na	na	na
34 ^f	HIV+	30940	–	–	–	na	na	na	na	na	na
35 ^f	HIV+	32388	–	–	–	na	na	na	na	na	na
36 ^f	HIV+	32013	–	–	–	na	na	na	na	na	na

HIV, human immunodeficiency virus; LB, Luria–Bertani; +, positive; –, negative; *mcr-1-Ec*, *mcr-1*-positive *Escherichia coli*; Col-R, colistin-resistant; *Ec*, *E. coli*; *Ea*, *Enterobacter asburiae*; *Pr*, *Providencia rettgeri*; na, not available or not applicable; *Kp*, *Klebsiella pneumoniae*.

^a R, stools obtained immediately after return from travel; M3, stools obtained 3 months after return from the trip; M6, stools obtained 6 months after return from trip.

^b rt-PCR performed with 40 cycles for native stools.

^c rt-PCR performed with 30 cycles for enrichment in LB broth containing 2 mg/L colistin.

^d Colistin-resistant *mcr-1*-negative Enterobacteriaceae.

^e This sample was obtained after 12 months immediately after returning from a second travel abroad (India).

^f A single stool sample was collected for HIV-positive patients.

A

B