Real-time quantitative PCR assay with Taqman® probe for rapid detection of MCR-1 plasmid-mediated colistin resistance

S. Chabou, T. Leangapichart, L. Okdah, S. Le Page, L. Hadjadj and J.-M. Rolain

URMITE UM 63 CNRS 7278 IRD 198 INSERM U1905, IHU Méditerranée Infection, Faculté de Médecine et de Pharmacie, Aix-Marseille Université, Marseille, France

Abstract

Here we report the development of two rapid real-time quantitative PCR assays with TaqMan® probes to detect the MCR-1 plasmid-mediated colistin resistance gene from bacterial isolates and faecal samples from chickens. Specificity and sensitivity of the assay were 100% on bacterial isolates including 18 colistin-resistant isolates carrying the *mcr-1* gene (six *Klebsiella pneumoniae* and 12 *Escherichia coli*) with a calibration curve that was linear from 10^1 to 10^8 DNA copies. Five out of 833 faecal samples from chickens from Algeria were positive, from which three *E. coli* strains were isolated and confirmed to harbour the *mcr-1* gene by standard PCR and sequencing.

Keywords: Antibiotic resistance surveillance, colistin resistance, *mcr-1* gene, PCR detection, Taqman probe
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Corresponding author: J.-M. Rolain, URMITE UM 63 CNRS 7278 IRD 198 INSERM U1905, IHU Méditerranée Infection, Faculté de Médecine et de Pharmacie, Aix-Marseille Université, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France
E-mail: jean-marc.rolain@univ-amu.fr

Introduction

The increasing prevalence of infections caused by multidrug-resistant Gram-negative bacteria combined with few antimicrobial agents being in development has led to a resurgence in interest in colistin as a last-line therapy with the inevitable risk of emerging resistance [1–3]. MCR-1 plasmid-mediated colistin resistance is a member of the phosphoethanolamine transferase enzyme family, with expression in *Escherichia coli* resulting in the addition of phosphoethanolamine to lipid A and resistance to colistin [4]. This plasmid-mediated colistin resistance is an emerging concern that has already spread worldwide [5] in *E. coli* and *Klebsiella pneumoniae* from pigs, chicken, retail meat (pork, chicken), humans [4]. In animal health, colistin is used to prevent infections from *E. coli* isolates that are known to cause serious adverse effects such as diarrhoea, sepsis and colibacillosis, which result in huge economic losses [6]. The extensive use of antibiotics in food-animal production has been shown to increase the risk of transferring resistant bacteria to humans [7].

There is a need to screen for colistin resistance even in patients without a history of colistin usage for the timely detection and isolation of patients harbouring such resistant strains to prevent clonal transmission [8]. For this reason the aim of this study was to develop rapid real-time quantitative PCR (qPCR) to detect the MCR-1 plasmid-mediated colistin resistance and to evaluate its sensitivity and specificity both from strains and stool samples.

Materials and methods

Specific primers and probes design

Primers and probes design. We designed specific primers and probes to develop two real-time qPCR assays (PE1 and PE2) for the detection of MCR-1-encoding gene (Table 1). Specificity of the primers and probes were verified *in silico* by BLASTN analysis on the National Center for Biotechnology Information (NCBI) database.

Sample collection

Bacterial strains. A total of 100 strains from humans and animals were used in this study including 18 colistin-resistant isolates carrying the *mcr-1* gene (six *K. pneumoniae* and 12 *E. coli*).

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TABLE 1. Primers and probe designed to target the plasmid-mediated colistin resistance (MCR-1)

Primer/probe name	Sequence	PCR product size (bp)	References
Real-time PCR			
PE_F1	GCAGCATACTTCTGTGTGGTAC	145	This study
PE_R1	ACAAAGCCGAGATTGTCCGCG		
PE_Probe 1	6 FAM –GACCGCGACCGCCAATCTTACC-TAMRA		
PE_F2	GGGTGTGCTACCAAGTTTGCTT		
PE_R3	TATGCACGCGAAAGAAACTGGC		
PE_Probe	6 FAM –GCGCTGATTTTACTGCCTGTGGTG-TAMRA		
Standard PCR			
PE_F1	GCAGCATACTTCTGTGTGGTAC	554	This study
PE_R3	TATGCACGCGAAAGAAACTGGC		
CLR5-F	5′-CGGTCAGTCCGTTTGTTC-3′		[4]
CLR5-R	5′-CTTGGTCGGTCTGTA GGG-3′		

TABLE 2. Presentation of strains of the study with the genes specificity

Species	Presence of gene MCR-1	COL MIC (mg/L)	Genes specificity	CT value with PE1 system	CT value with PE2 system	Origins	References
Escherichia coli (*n* = 25)	+ (*n* = 12) − (*n* = 13)	4–16 <1–16	None	18–25 0	19–25 0	Thailand, Laos, Algeria, France, Nigeria.	[5,7] unpublished data
Klebsiella pneumoniae (*n* = 33)	+ (*n* = 6) − (*n* = 27)	4–32 <1–32	*mgrB** (*n* = 2) pmrB*(*n* = 1) *mgrB** (12)	18–24 0	19–25 0	Thailand, Laos, France, Nigeria, Algeria	[9] unpublished data
Klebsiella oxytoca (*n* = 2)	–	6–12	*mgrB** (*n* = 1)	0	0		[9]
Salmonella enterica subsp. *enterica* (*n* = 5)	–	0.125–16	pmrB* (*n* = 2) $bla_{CTX-M-2}$ (*n* = 5)	0	0	France	[10]
Pseudomonas aeruginosa (*n* = 10)	–	<1	bla_{VIM-2} (*n* = 10)	0	0	Lebanon	[11]
Acinetobacter baumannii (*n* = 10)	–	<1	bla_{OXA23} (*n* = 2), bla_{OXA24} (*n* = 2), bla_{OXA58} (*n* = 1), bla_{VEB} (*n* = 1)	0	0	Algeria	[12–14]
Providencia rettgeri (*n* = 2)	–	>256	bla_{NDM-1} (*n* = 1)	0	0	Israel	[15]
Morganella morganii (*n* = 2)	–	>256	bla_{NDM-1} (*n* = 1)	0	0	Israel	[16]
Enterobacter cloacae (*n* = 5)	–	<1	None	0	0	Laos, Nigeria.	unpublished data
Proteus mirabilis (*n* = 2)	–	>256	None	0	0	Algeria	unpublished data
Proteus vulgaris (*n* = 2)	–	>256	None	0	0	Algeria	unpublished data
Serratia marcescens (*n* = 2)	–	>256	None	0	0	Algeria	unpublished data

* Mutation; +, positive; −, negative.
COL MIC, minimum inhibitory concentration of colistin.

Phenotypic and genotypic features of these strains are summarized in Table 2.

Chicken stool collection. A total of 833 faeces samples from broilers were collected between August and February 2015 from eight regions in Algeria (El Tarf, Souk Ahras, Skikda, Setif, Jijel, Algiers, Biskra and Ourgla; *n* = 503) and in three slaughterhouses in Marseille (*n* = 330). All the extracted DNA from the 833 faeces of broilers was tested using our qPCR assay and positive samples were inoculated on agar for isolation of positive *mcr-1* isolates.

Molecular analysis

Strategy for PCR amplification and sequencing. Standard PCR amplification and sequencing of the MCR-1-encoding gene was used as the gold standard and performed as previously described [4]. Quantification of the MCR-1-encoding gene using our two sets of primers and probes was performed using a quantitative CFX96™ Real Time system C1000™ Touch thermal cycler (Bio-Rad, Singapore). The qPCR conditions were as follows: the reaction mixtures were kept at 95°C for 15 min and subsequently put through 35 cycles of 95°C for 30 s and 60°C for 1 min.

Specificity and reproducibility of the new system of real-time PCR. The specificity of the primers and probes were verified *in vitro* using our local collection of 100 strains (Table 2). The sensitivity of our assays was determined using ten-fold serial dilutions (between 10^8 and 10^1 DNA copies) of *E. coli* strain P10 by triplicate amplification, the number of *mcr-1* in each sample was calculated based on the DNA copy numbers. The obtained Ct values were used to generate the calibration curves compared with the number of bacteria quantified by standard bacterial count on agar plates. The standard curve was

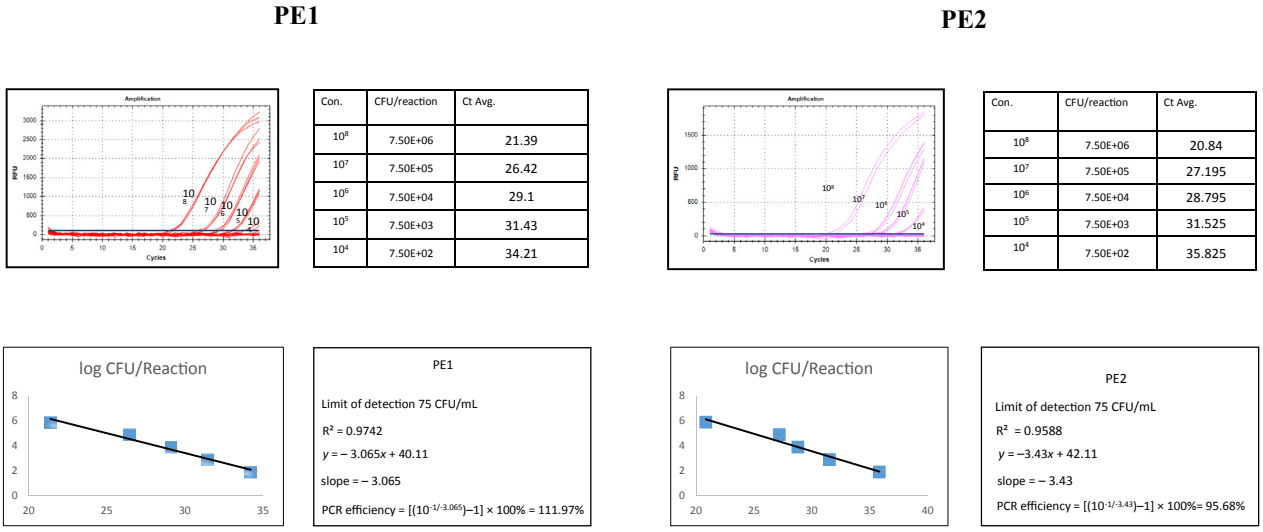

FIG. 1. Real-time PCR sensitivity test to detect -encoding plasmid-mediated colistin resistance (MCR-1) encoding gene from *Escherichia coli* strain P10. PE1 and PE2 are two quantitative PCR assays developed.

constructed on the basis of the concordance between Ct values and number of log CFU/mL. The limit of detection was based on the final dilution detected by PCR. Efficacy of qPCR was calculated from a standard curve according to Rutledge and Cote [17].

Results

Specificity and technical sensitivity of the qPCR

BLASTN analysis of the primers and probe designed for the development of the real-time PCR assay showed *in silico* a 100% homology with the MCR-1-encoding gene only. The sensitivity of the real-time PCR using the 18 *mcr-1*-positive strains using serial ten-fold dilutions of a calibrated inoculum was excellent with a calibration curve that was linear from 10^1–10^8 DNA copies corresponding to 35–21 Ct (Fig. 1). Regression formulae and PCR efficiency of the two real-time PCR assays are shown in Fig. 1. The reproducibility of the two qPCR assays was excellent, with a positive PCR at 21.4 ± 0.4 Ct for PE1 and 20.8 × 0.4 Ct for PE2 when testing one colony re-suspended in 200 µL of sterile water (Table 2). The specificity of the two qPCR assays *in vitro* against a panel of 82 clinically relevant bacteria negative for *mcr-1* gene was 100% (all real-time PCR were negative, Table 2).

Screening of faeces from broilers

Five of the 503 faecal samples from chickens from Algeria were positive, from which three *E. coli* strains were isolated and confirmed to harbour the *mcr-1* gene by standard PCR and sequencing. None of the 330 samples from France were positive.

Discussion

The recent description and emergence of MCR-1 plasmid-mediated resistance to colistin in humans and animals is a major concern worldwide [5]. In this study, two new qPCR assays using Taqman probes were developed that demonstrate high sensitivity and specificity for confirmation of the presence of this gene in colistin-resistant bacterial isolates as well as for screening directly from stool samples. Indeed both systems have the same performance to screen for the presence of MCR-1-containing isolates and in stools. We recommend the use of PE1 as a first set of primers for the rapid screening of *mcr-1* and PE2 system to confirm the positive results. Recently, Bontron *et al.* have reported a real-time PCR assay using SYBR green as fluorescent marker with similar sensitivity [18]. However it is well known that Taqman probes enhance specificity, which is a critical point when testing directly from biological samples. Our real-time PCR assays had advantages including sensitivity, specificity and the possibility of detecting MCR-1 plasmid-mediated colistin resistance very quickly (<2 h). We believe that these real-time PCR assays would be important and powerful tools that could be implemented easily in clinical microbiological laboratories that have molecular facilities, including at point of care, for identification of MCR-1 and implementation of healthcare policies.

Conflict of interest and financial disclosure

There is no potential conflict of interest or financial disclosure for all authors.

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