International Journal of Antimicrobial Agents 53 (2019) 302–309

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Development and validation of a multiplex polymerase chain reaction assay for detection of the five families of plasmid-encoded colistin resistance

Agnès B. Jousset[a,b,c], Sandrine Bernabeu[a,b,c], Remy A. Bonnin[b,c], Elodie Creton[b], Garance Cotellon[b], Aimie Sauvadet[b], Thierry Naas[a,b,c], Laurent Dortet[a,b,c,*]

[a] Department of Bacteriology-Hygiene, Hôpital de Bicêtre, Assistance Publique - Hôpitaux de Paris, Le Kremlin-Bicêtre, France
[b] Associated French National Reference Centre for Antibiotic Resistance, Le Kremlin-Bicêtre, France
[c] Research Unit EA 7361 'Structure, Dynamic, Function and Expression of Broad-spectrum β-lactamases', Faculty of Medicine, University Paris-Sud, Le Kremlin-Bicêtre, France

ARTICLE INFO

Article history:
Received 14 May 2018
Accepted 30 October 2018

Editor: Po-Ren Hsueh

Keywords:
MCR
Polymyxin resistance
Molecular diagnostic

ABSTRACT

Plasmid-mediated colistin resistance is increasingly described worldwide in Enterobacteriaceae from animal and human isolates. Diffusion of these resistance traits among carbapenem-resistant enterobacterial isolates is of particular concern as colistin has become the last resort antibiotic for treating human infections with these organisms. Therefore, being able to monitor the presence of these transferable colistin resistance genes (*mcr-1* to *mcr-5*-variants) is crucial. This paper describes the development of a multiplex polymerase chain reaction (PCR) protocol for detection of all currently known transferable colistin resistance genes in Enterobacteriaceae. Five primer pairs were designed to amplify *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr*-5 gene products in a multiplex PCR. This assay was validated retrospectively on colonies of 50 *Escherichia coli*, 44 *Klebsiella pneumoniae* and 12 *Salmonella enterica* isolates of animal and human origin, all well characterized, and validated prospectively on 450 carbapenem-resistant enterobacterial isolates received by the French National Reference Centre. In addition, 82 *Aeromonas* spp. and 10 *Shewanella* spp. known to be the progenitors of *mcr-3* and *mcr-4* alleles, respectively, were screened. Mcr-multiplex PCR assay displayed 100% specificity, sensitivity, negative predictive value and positive predictive value. The assay was able to detect all variants of the different *mcr* alleles, and was able to detect chromosomally encoded *mcr-4*-like variants present in two *Shewanella bicestrii* JAB-1 and *Shewanella woodyi* S539. In conclusion, a rapid and robust multiplex PCR assay able to detect all known *mcr* gene families described in Enterobacteriaceae was developed and validated. This type of test is critical for the epidemiological surveillance of plasmid-encoded resistance, especially in carbapenem-resistant bacteria.

1. Introduction

Currently, one of the most threatening issues for human health is the rise of antimicrobial resistance. The increased prevalence of multi-drug-resistant (MDR) bacteria is of particular concern in Gram-negative bacteria, for which the pipeline of new antimicrobials is extremely limited. Among these MDR Gram-negative bacteria, carbapenemase-producing Enterobacteriaceae (CPE) emerged in the 2000s and disseminated worldwide; they are now endemic in some countries [e.g. *Klebsiella pneumoniae* carbapenemase (KPC) producing *K. pneumoniae* in Greece and Italy, and New Delhi metallo-β-lactamase (NDM) producers in India]. Of note, these CPE remain susceptible to very few antimicrobials, including polymyxin (colistin and polymyxin B) and – sometimes – fosfomycin or tigecycline. Accordingly, colistin has become the last resort therapy for the treatment of infections caused by CPE [1]. Unfortunately, the accelerated use of colistin in CPE-endemic countries led to the development of resistance [2,3].

In Gram-negative bacteria, acquired resistance to colistin results mainly from modifications of lipid A, the part of the lipopolysaccharide anchored to the outer membrane [4,5]. These modifications consist of the addition of cationic groups such as phosphoethanolamine (pETN) and/or 4-amino-L-arabinose (L-Ara4N). They cause a decrease in the electronegative charge of lipid A, which leads to electrostatic repulsion of the positively

* Corresponding author. Address: Service de Bactériologie-Hygiène, Hôpital de Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin-Bicêtre, France. Tel.: +33 1 45 21 36 24; fax: +33 1 45 21 63 40.
E-mail address: laurent.dortet@aphp.fr (L. Dortet).

https://doi.org/10.1016/j.ijantimicag.2018.10.022
0924-8579/

charged polymyxin. The addition of L-Ara4N and pETN is triggered by chromosome-encoded mechanisms such as mutations on genes encoding the PmrA/PmrB or PhoP/PhoQ two-component systems, or through alterations of the master regulator MgrB. More recently, it has been reported that addition of pETN can also occur through the expression of a plasmid-encoded phosphoethanolamine transferase, MCR-1 [6]. Since the initial description of this plasmid-encoded resistance to polymyxin in 2016, the *mcr-1* gene has been reported worldwide, mainly in *Escherichia coli* and *Salmonella* spp. [7]. These *mcr-1*-positive strains are often isolated from animals or food samples, but some have been found in humans [7]. To date, 10 distinct MCR-1 variants have been assigned. The 10 variants differ from MCR-1 by one or a few amino acids, and thus share high nucleotide and amino acid identity (∼99%). Furthermore, four other families of *mcr* genes (*mcr-2, mcr-3*-like, *mcr-4* and *mcr-5* genes) have been reported in Enterobacteriaceae. MCR-2, MCR-3-like, MCR-4 and MCR-5 share 81%, 34%, 33% and 31% amino acid identity with MCR-1, respectively [7]. Currently, the detection of plasmid-encoded polymyxin resistance relies on molecular tests dedicated to the detection of *mcr-1* alone, or *mcr-1* and *mcr-2*. These test are based on real-time polymerase chain reaction (PCR) [8–10], loop-mediated isothermal amplification [11] and microarray [12]. Due to the high diversity of *mcr* genes (Fig. S1, see online supplementary material), the design of molecular-based assays able to detect all *mcr* gene families is challenging. Unfortunately, all of these variants have already disseminated in Enterobacteriaceae of animal origin, and *mcr-3* and *mcr-4* – at least – have disseminated in Enterobacteriaceae of human origin [13–16].

This study developed a multiplex PCR assay for detection and discrimination of the five *mcr* gene families. It was validated on a collection of characterized strains. As colistin remains one of the last options to treat infections caused by carbapenem-resistant isolates, this novel assay was tested prospectively on carbapenem-resistant isolates sent to the French National Reference Centre for CPE over a 2-month period.

2. Materials and methods

2.1. Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) were determined by broth microdilution according to the guidelines of the Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing (EUCAST) joint subcommittee [17]. Results were interpreted using the EUCAST breakpoint as updated in 2018.

2.2. Multiplex PCR assay

Nucleotide sequences of reported MCR variants were aligned and primers were designed to specifically amplify each *mcr* family. Primer and amplicon sizes are listed in Table S1 (see online supplementary material).

DNA templates were obtained by boiling extract of one colony grown overnight on agar medium. Each PCR reaction consisted of 25 μL of DreamTaq Green PCR Master Mix (ThermoFisher, Waltham, MA, USA), 2.2 μL of each primer at 10 μM, 1 μL of water and 2 μL of the DNA template. Running conditions were as follow: one denaturation cycle at 94°C for 5 min, 25 denaturation cycles at 94°C for 30 s followed by annealing at 56°C for 1 min and elongation at 72°C for 1 min, and a final elongation cycle at 72°C for 5 min. The PCR products were loaded on a 2% agarose gel containing ethidium bromide and visualized after 30 min of electrophoresis at 120 V.

2.3. Strain collection

Fifty *E. coli*, 41 *K. pneumoniae* and 12 *Salmonella enterica* were used to develop and validate the multiplex PCR (Table 1). This collection was composed of 31 polymyxin-resistant *E. coli* including 29 MCR producers (17 MCR-1, two MCR-1.5, three MCR-2, two MCR-3, one MCR-3.2, four MCR-5), 25 polymyxin-resistant *K. pneumoniae* including three MCR-1 producers, and eight MCR-producing *S. enterica* isolates (six MCR-1, one MCR-4, one MCR-5). Nineteen *E. coli*, 16 *K. pneumoniae* and four *S. enterica* were polymyxin susceptible [18].

This multiplex PCR was also performed on a collection of 82 *Aeromonas* spp. and 10 *Shewanella* spp. described as potential progenitors of *mcr-3* and *mcr-4* genes, respectively [7,14].

Finally, since 1 January 2018, this multiplex PCR has been performed on all enterobacterial isolates received each day at the French Associated National Reference Centre for Antibiotic Resistance (NRC) to identify carbapenemase activity from strains selected for their reduced susceptibility to carbapenems (ertapenem, meropenem or imipenem) according to the Committee of the Antibiogram of the French Society of Microbiology. As described previously [19], all of these carbapenem-resistant enterobacterial isolates were recovered from screening samples and clinical samples (mainly urine, blood culture and pulmonary samples) of hospitalized patients (∼75%) and outpatients (∼25%). Indeed, as colistin is one of the last therapeutic options to treat carbapenem-resistant Enterobacteriaceae, looking for plasmid-encoded resistance in this group of isolates might be crucial for the management of patients and epidemiological purposes.

2.4. Whole-genome sequencing and analysis

Total DNA was extracted from colonies using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Ozyme, Saint-Quentin, France) following the manufacturer's instructions. The DNA concentration and purity were controlled by a Qubit 2.0 Fluorometer using the dsDNA HS and/or BR assay kit (Life Technologies, Waltham, MA, USA). The DNA library was prepared using the Nextera XT-v2 kit (Illumina, Paris, France), and then run on the NextSeq 500 Illumina system (2 × 150-bp paired end). De-novo assembly was performed by CLC Genomics Workbench v10.0.1 (Qiagen, Les Ulis, France). Whole genome sequencing facilities were provided by Pasteur International Bioressources Networking (PibNet, Paris, France). A search for colistin resistance gene was performed by: (i) an automatic search using Resfinder webtool (http://www.genomicepidemiology.org/); and (ii) a manual search within the genome. Genomes were annotated using the RAST server (www. http://rast.nmpdr.org). The manual search was based on the detection of phosphoethanolamine transferase within the genome and detection of sequence homology.

2.5. Genbank accession number

mcr-4.6 and *mcr-4.7* were deposited in the Genbank database under accession numbers MH323443 and MH323444, respectively.

2.6. Statistical analysis

The sensitivity and specificity values were calculated with their respective 95% confidence intervals (CI) using vassarStats (http://vassarstats.net/).

Table 1
Results of the multiplex polymerase chain reaction (PCR) for the detection of *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* genes in 42 *Escherichia coli*, 41 *Klebsiella pneumoniae* and eight *Salmonella* spp.

Species	Name[a]	Colistin MIC (mg/L)	Resistance mechanism to polymyxins	Multiplex PCR results
E. coli	41489	4	*mcr-1*	*mcr-1*
E. coli	J53 + *mcr-1*[*]	8	*mcr-1*	*mcr-1*
E. coli	CNR20140385	4	*mcr-1*	*mcr-1*
E. coli	S08-056	4	*mcr-1*	*mcr-1*
E. coli	CNR 117 G7	4	*mcr-1*	*mcr-1*
E. coli	CNR 121 G9	4	*mcr-1*	*mcr-1*
E. coli	CNR 1745	4	*mcr-1*	*mcr-1*
E. coli	CNR 1604	4	*mcr-1*	*mcr-1*
E. coli	CNR 1790	4	*mcr-1*	*mcr-1*
E. coli	CNR 1859	4	*mcr-1*	*mcr-1*
E. coli	CNR 1886	4	*mcr-1*	*mcr-1*
E. coli	4222	4	*mcr-1*	*mcr-1*
E. coli	4070	4	*mcr-1*	*mcr-1*
E. coli	979	4	*mcr-1*	*mcr-1*
E. coli	1724	4	*mcr-1*	*mcr-1*
E. coli	DH5α + pDM1-*mcr-1*[**]	4	*mcr-1*	*mcr-1*
E. coli	MC1000 + pDM1- *mcr-1*[**]	4	*mcr-1*	*mcr-1*
E. coli	6383	4	*mcr-1.5*	*mcr-1*
E. coli	1670	4	*mcr-1.5*	*mcr-1*
E. coli	R12 F5[b]	4	*mcr-2*	*mcr-2*
E. coli	DH5α + pDM1-*mcr-2*[**]	4	*mcr-2*	*mcr-2*
E. coli	MC1000 + pDM1- *mcr-2*[**]	4	*mcr-2*	*mcr-2*
E. coli	DH5α + pDM1-*mcr-3*[**]	4	*mcr-3*	*mcr-3*
E. coli	MC1000 + pDM1- *mcr-3*[*]	4	*mcr-3*	*mcr-3*
E. coli	37922	8	*mcr-3.2*[c]	*mcr-3*
E. coli	DH5α + pDM1-*mcr-5*[**]	4	*mcr-5*	*mcr-5*
E. coli	MC1000 + pDM1- *mcr-5*[*]	4	*mcr-5*	*mcr-5*
E. coli	TOP 10 + *mcr-5*[*]	8	*mcr-5*	*mcr-5*
E. coli	J53 + *mcr-5*[*]	8	*mcr-5*	*mcr-5*
E. coli	CNR 1728	8	PmrB mutation (G160E)	-
E. coli	CNR 111 J7	8	PmrB mutations (D14N, S71C et V83A)	-
K. pneumoniae	CNR 1732	4	*mcr-1*	*mcr-1*
K. pneumoniae	CNR 1853	4	*mcr-1*	*mcr-1*
K. pneumoniae	CNR 1601	32	*mcr-1* + *mgrB* truncated in orf by IS5	*mcr-1*
K. pneumoniae	CNR 20150309	64	MgrB frame shift after AA 37 (V)	-
K. pneumoniae	CNR 20150944	64	MgrB frame shift after AA 42	-
K. pneumoniae	CNR 20151119	64	MgrB L4 stop	-
K. pneumoniae	CNR 20140042	16	MgrB N42Y and K43I	-
K. pneumoniae	CNR 20140661	64	MgrB Q30 stop	-
K. pneumoniae	CNR 20150675	64	*mgrB* truncated in orf by IS*10*	-
K. pneumoniae	CNR 20140483	32	*mgrB* truncated in orf by IS*1F*-like	-
K. pneumoniae	CNR 20140563	64	*mgrB* truncated in orf by IS*1R*	-
K. pneumoniae	CNR 1630	32	*mgrB* truncated in orf by IS*5*	-
K. pneumoniae	CNR 20140591	64	*mgrB* truncated in orf by IS*5*-like	-
K. pneumoniae	CNR 20151285	32	*mgrB* truncated in orf by IS*903*-like	-
K. pneumoniae	SAG	64	*mgrB* truncated in orf by IS*Kpn25*	-
K. pneumoniae	CNR 20150050	32	*mgrB* truncated in promoter by IS*1R*	-
K. pneumoniae	CNR 20140550	32	*mgrB* truncated in promoter by IS*903D*	-
K. pneumoniae	S14-002	64	*mgrB* truncated in promoter by IS*Kpn14*	-
K. pneumoniae	CNR 20150622	64	MgrB Y41 stop	-
K. pneumoniae	CNR 20150777	128	MgrB Y41 stop	-
K. pneumoniae	CNR 1861	16	Mutated PmrB (T157P)	-
K. pneumoniae	CNR 20140101	32	Δ*mgrB*	-
K. pneumoniae	CNR 20150078	32	Δ*mgrB*	-
K. pneumoniae	CNR 20150066	16	Δ*mgrB*	-
K. pneumoniae	CNR 20151223	32	Δ*mgrB*	-
S. enterica serovar 4,12:i:- (monophasic)	201607059	4	*mcr-1*	*mcr-1*
S. enterica serovar 4,12:i:- (monophasic)	201606765	8	*mcr-1*	*mcr-1*
S. enterica serovar 4,12:i:- (monophasic)	201609932	8	*mcr-1*	*mcr-1*
S. enterica serovar 4,12:i:- (monophasic)	201610655	8	*mcr-1*	*mcr-1*
S. enterica serovar Paratyphi B	201610686	8	*mcr-1*	*mcr-1*
S. enterica serovar Typhimurium	CNR 1776	8	*mcr-1*	*mcr-1*
S. enterica serovar Typhimurium	R3445[d]	8	*mcr-4*	*mcr-4*
S. enterica serovar Paratyphi B	13-SA01718[e]	8	*mcr-5*	*mcr-5*
Polymyxin susceptible isolates				
E. coli	TOP 10	0.25	-	-
E. coli	1608071881	0.25	-	-
E. coli	1608072264	0.25	-	-
E. coli	1608073733	0.5	-	-
E. coli	1608073228	0.25	-	-
E. coli	1608078635	0.25	-	-

(*continued on next page*)

Table 1 (*continued*)

Species	Name[a]	Colistin MIC (mg/L)	Resistance mechanism to polymyxins	Multiplex PCR results
E. coli	1608078858	0.25	-	-
E. coli	1608062671	0.25	-	-
E. coli	1608064819	0.25	-	-
E. coli	2H6	0.25	-	-
E. coli	LAN 10.48	0.25	-	-
E. coli	VER 9.39	0.25	-	-
E. coli	1F1	0.25	-	-
E. coli	1A6	0.25	-	-
E. coli	1A8	0.25	-	-
E. coli	2A1	0.25	-	-
E. coli	2D9	0.5	-	-
E. coli	2C4	0.25	-	-
E. coli	2D5	0.25	-	-
K. pneumoniae	1609056413	0.5	-	-
K. pneumoniae	1609061149	1	-	-
K. pneumoniae	2 E8	0.5	-	-
K. pneumoniae	2 F1	0.5	-	-
K. pneumoniae	2 F4	0.5	-	-
K. pneumoniae	2 I5	0.5	-	-
K. pneumoniae	3 D6	0.5	-	-
K. pneumoniae	3 D7	0.5	-	-
K. pneumoniae	3 B4	0.5	-	-
K. pneumoniae	3 B7	0.5	-	-
K. pneumoniae	1 B6	0.5	-	-
K. pneumoniae	1 C9	1	-	-
K. pneumoniae	1 E3	1	-	-
K. pneumoniae	2 B1	1	-	-
K. pneumoniae	2 C6	0.5	-	-
K. pneumoniae	2 D2	0.5	-	-
S. enterica serovar Enteritidis	201607559	0.5	-	-
S. enterica serovar Veneziana	201610299	0.5	-	-
S. enterica serovar Typhimurium	201606509	1	-	-
S. enterica serovar Enteritidis	201608919	1	-	-

[a] Laboratory strains are underlined; all other strains are clinical isolates.
[b] The MCR-2-producing *E. coli* R12 F5 has been described previously by Xavier et al. [16].
[c] The MCR-3.2-producing *E. coli* 37922 has been described previously by Haenni et al. [25].
[d] The MCR-4-producing *S. enterica* R3445 has been described previously by Carattoli et al. [14].
[e] The MCR-5-producing *S. enterica* 13-SA01718 has been described previously by Borowiak et al. [13].
* Natural plasmids; the natural *mcr-5* carrying plasmid (from *S. enterica* 13-SA01718) has been described previously by Borowiak et al. [13].
** Functional *mcr-like* gene cloned into pDM1, an IPTG-inducible (final concentration 0.5 mM), Tet^R derivative of pACYC184 [26]. The genes *mcr-1, mcr-2* and *mcr-5* were cloned into the SacI/XmaI sites of the vector, while for *mcr-3*, the NdeI/XmaI sites were used.

3. Results

3.1. Validation of the multiplex PCR

The multiplex PCR was designed to specifically amplify *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* (Table S1, see online supplementary material). As shown in Fig. 1A, specific amplicons of 1139 bp, 816 bp, 676 bp, 405 bp and 207 bp were observed for MCR-1, MCR-2, MCR-3, MCR-4 and MCR-5 producers. In *E. coli* and salmonella isolates, *mcr* variants *mcr-1.5* and *mcr-3.2* were correctly assigned in their respective families (Fig. 1C, Table 1). No non-specific product or interference could be observed with polymyxin-resistant or polymyxin-susceptible isolates (data not shown). Using this collection of 50 *E. coli*, 41 *K. pneumoniae* and 12 *S. enterica*, the multiplex PCR assay was 100% (95% CI 89.1–100%) sensitive and 100% (95% CI 92.8–100%) specific for the detection and classification of MCR producers.

3.2. Analysis of Aeromonas spp. and Shewanella spp. collections

As the progenitors of *mcr-3* and *mcr-4* genes are suspected to be *Aeromonas* spp. and *Shewanella* spp., respectively, 82 *Aeromonas* spp. isolates belonging to nine species and 10 different species of *Shewanella* spp. were screened (Table 2). Although no positive result was obtained with all *Aeromonas* spp. isolates tested, two *Shewanella* spp. isolates, including *S. bicestrii* JAB-1 strain [20] and *S. woodyi* S539, gave a positive result for *mcr-4*-like gene. Both *Shewanella* spp. isolates remained susceptible to colistin with MICs of 0.25 mg/L and <0.12 mg/L, respectively. Whole-genome sequencing of these two isolates led to the identification of an *mcr-4*-like gene encoding putative phosphoethanolamine transferase (Fig. S2, see online supplementary material). The *mcr-4*-like genes from *S. bicestrii* JAB-1 strain (*mcr-4.6*) and *S. woodyi* S539 (*mcr-4.7*) shared 74% and 74% nucleotide identity with *mcr-4.1*, respectively (Fig. S2, see online supplementary material). Deduced protein sequences shared 81% and 78% amino acid identity with MCR-4.1, respectively. The *mcr-4.6* gene from *S. bicestrii* JAB-1 was cloned into *E. coli* TOP10. This clone also gave a positive result for *mcr-4*, and the MCR-4-expressing *E. coli* was resistant to colistin with MIC of 4 mg/L, confirming the specificity of the multiplex PCR.

3.3. Prospective evaluation of the multiplex PCR

This multiplex PCR has been implemented since January 2018 in the French NRC for Antimicrobial Resistance, dedicated to the identification of carbapenemase-producing Enterobacteriaceae. From 1 January 2018 to 28 February 2018, 548 enterobacterial isolates (all with reduced susceptibility to carbapenems) were tested each day for colistin resistance using broth microdilution, and for the presence of *mcr* genes using the developed multiplex. All colistin-resistant isolates were subjected to whole-genome sequencing to assess the presence of *mcr*-like genes. During this 2-month period, 29 colistin-resistant isolates (5.3%), including four *Proteus mirabilis*, one *Serratia marcescens*, two *Morganella morganii* and three *Haf-*

Table 2
Results of the multiplex polymerase chain reaction (PCR) for the detection of *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* genes in 10 *Shewanella* spp. and 82 *Aeromonas* spp.

Name	Species	Isolation location	Multiplex PCR results
Shewanella spp.			
ATCC700550	*S. oneidensis*	France (Marseille)	Neg
KB2	*S. algae*	-	Neg
S12	*S. xiamenensis*[a]	India	Neg
CN32	*S. putrefaciens*	-	Neg
NCTC10735	*S. baltica*	-	Neg
ATCC 1417	*S. morhuae*	-	Neg
HSOC2	*S. baltica*	-	Neg
116-17-9	*S. putrefaciens*	UK	Neg
JAB-1	*S. bicestrii*[b]	France (Paris)	*mcr-4*-like
S539	*S. woodyi*	France (Paris)	*mcr-4*-like
Aeromonas spp.			
CIP102629	*A. caviae*	-	Neg
CIP104171T	*A. jandaei*	-	Neg
R10 B7	*A. allosaccharophila*	France (Paris)	Neg
R10 B8	*A. punctata*	France (Paris)	Neg
R10 C1	*A. veronii*	France (Paris)	Neg
R10 C2	*A. allosaccharophila*	France (Paris)	Neg
R10C4	*A. veronii*	France (Paris)	Neg
R10C5	*A. punctata*	France (Paris)	Neg
R10C6	*A. media*	France (Paris)	Neg
R10C7	*A. allosaccharophila*	France (Paris)	Neg
R10C8	*A. allosaccharophila*	France (Paris)	Neg
R10C9	*A. veronii*	France (Paris)	Neg
ATCC 49847	*A. hydrophila*	-	Neg
ATCC 85623	*A. veronii*	-	Neg
ATCC 13137	*A. caviae*	-	Neg
107-5-76	*A. caviae*	UK	Neg
437-STAB	*A. veronii*	UK	Neg
O33C3	*Aeromonas* spp.	Switzerland	Neg
O33C4	*Aeromonas* spp.	Switzerland	Neg
O33C5	*Aeromonas* spp.	Switzerland	Neg
O33C8	*Aeromonas* spp.	Switzerland	Neg
O33D2	*Aeromonas* spp.	Switzerland	Neg
O33D3	*Aeromonas* spp.	Switzerland	Neg
O33D5	*Aeromonas* spp.	Switzerland	Neg
O33D6	*Aeromonas* spp.	Switzerland	Neg
O33D7	*Aeromonas* spp.	Switzerland	Neg
O33E1	*Aeromonas* spp.	Switzerland	Neg
O33E2	*Aeromonas* spp.	Switzerland	Neg
O33E3	*Aeromonas* spp.	Switzerland	Neg
O33E4	*Aeromonas* spp.	Switzerland	Neg
O33E5	*Aeromonas* spp.	Switzerland	Neg
O33E6	*Aeromonas* spp.	Switzerland	Neg
O33E7	*Aeromonas* spp.	Switzerland	Neg
O33E8	*A. allosaccharophila*	Switzerland	Neg
O33F1	*Aeromonas* spp.	Switzerland	Neg
O33F4	*Aeromonas* spp.	Switzerland	Neg
O33F5	*Aeromonas* spp.	Switzerland	Neg
O33F6	*Aeromonas* spp.	Switzerland	Neg
O33F7	*Aeromonas* spp.	Switzerland	Neg
O33F8	*Aeromonas* spp.	Switzerland	Neg
O33G2	*Aeromonas* spp.	Switzerland	Neg
O33G3	*Aeromonas* spp.	Switzerland	Neg
O33G4	*Aeromonas* spp.	Switzerland	Neg
O33G5	*Aeromonas* spp.	Switzerland	Neg
O33G6	*Aeromonas* spp.	Switzerland	Neg
O33G7	*Aeromonas* spp.	Switzerland	Neg
O33G8	*Aeromonas* spp.	Switzerland	Neg
O33H1	*Aeromonas* spp.	Switzerland	Neg
O33H2	*A. media*	Switzerland	Neg
O33H3	*Aeromonas* spp.	Switzerland	Neg
O33H4	*Aeromonas* spp.	Switzerland	Neg
O33H5	*Aeromonas* spp.	Switzerland	Neg
O33H6	*Aeromonas* spp.	Switzerland	Neg
O33H7	*Aeromonas* spp.	Switzerland	Neg
O33H8	*Aeromonas* spp.	Switzerland	Neg
O34A1	*Aeromonas* spp.	Switzerland	Neg
O34A2	*Aeromonas* spp.	Switzerland	Neg
O34A3	*Aeromonas* spp.	Switzerland	Neg
O34A4	*Aeromonas* spp.	Switzerland	Neg

(*continued on next page*)

Table 2 (*continued*)

Name	Species	Isolation location	Multiplex PCR results
Bordeaux309	*A. media*	France (Bordeaux)	Neg
Bordeaux252	*A. hydrophila*	France (Bordeaux)	Neg
Bordeaux358	*A. caviae*	France (Bordeaux)	Neg
Bordeaux202	*A. caviae*	France (Bordeaux)	Neg
Bordeaux56	*A. caviae*	France (Bordeaux)	Neg
Bordeaux98	*A. media*	France (Bordeaux)	Neg
Bordeaux295	*A. caviae*	France (Bordeaux)	Neg
Bordeaux367	*A. media*	France (Bordeaux)	Neg
Bordeaux237	*A. eucrenophila*	France (Bordeaux)	Neg
Bordeaux250	*A. caviae*	France (Bordeaux)	Neg
Bordeaux96	*A. veronii*	France (Bordeaux)	Neg
Bordeaux281	*Aeromonas* spp.	France (Bordeaux)	Neg
Lyon276	*A. hydrophila*	France (Lyon)	Neg
Lyon309	*A. caviae*	France (Lyon)	Neg
Lyon60	*A. veronii*	France (Lyon)	Neg
Lyon24a	*A. caviae*	France (Lyon)	Neg
Lyon39	*A. bestiarum*	France (Lyon)	Neg
Lyon204	*A. hydrophila*	France (Lyon)	Neg
Lyon346	*A. hydrophila*	France (Lyon)	Neg
Lyon33c	*A. hydrophila*	France (Lyon)	Neg
Lyon243	*A. veronii*	France (Lyon)	Neg
Lyon36b	*A. hydrophila*	France (Lyon)	Neg
Lyon62	*A. hydrophila*	France (Lyon)	Neg

Neg, negative result.
[a] *S. xiamenensis* S12 has been published previously by Potron et al. [27].
[b] *Shewanella* spp. JAB-1 has been published previously by Jousset et al. [20].

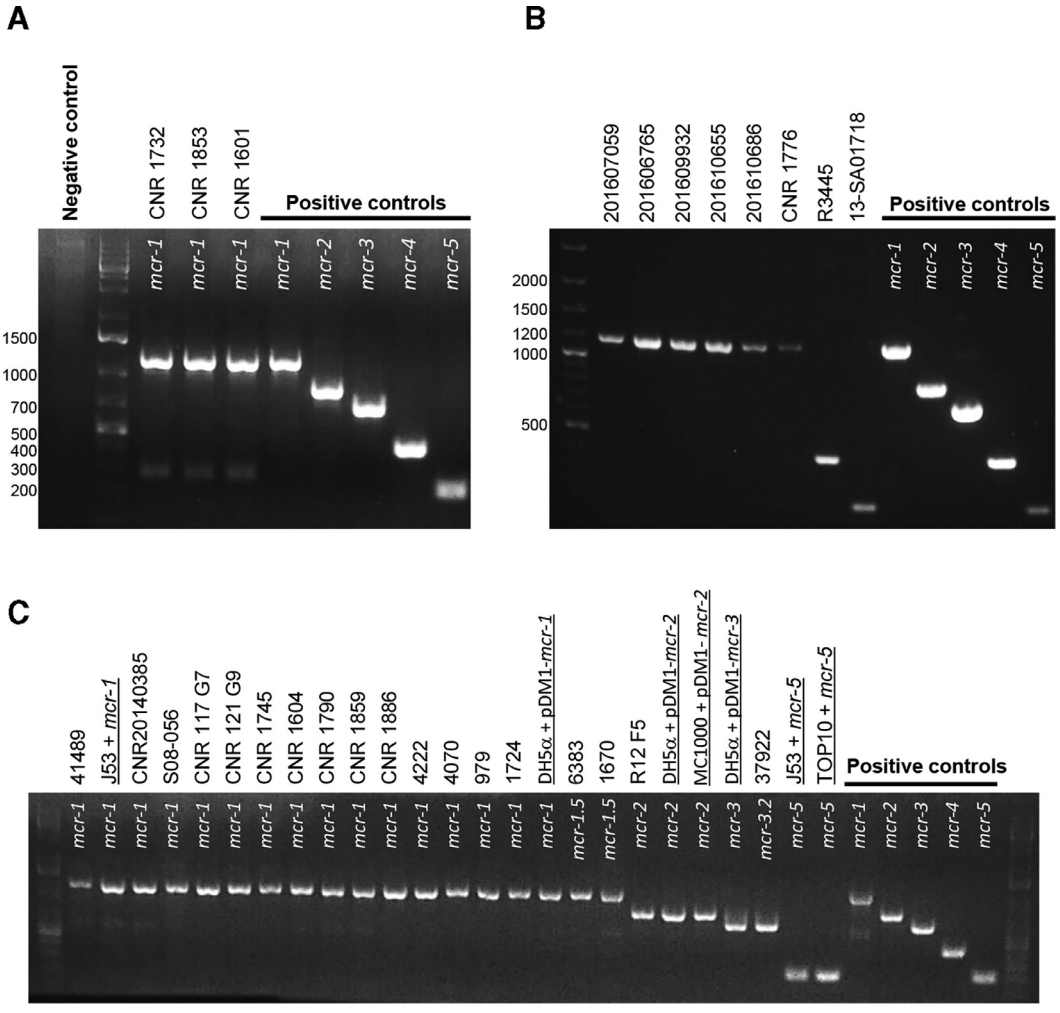

Fig. 1. Representative results of the multiplex polymerase chain reaction for *Klebsiella pneumoniae* (A), *Salmonella enterica* (B) and *Escherichia coli* (C).

nia alvei, were identified, for which colistin resistance corresponds to a wild-type phenotype [4,21]. Among the remaining 19 isolates, 11 *Enterobacter cloacae* complex, four *K. pneumoniae*, one *Klebsiella oxytoca*, one *Citrobacter freundii* and two *E. coli* were identified. As described by Guerin et al. [22], all *E. cloacae* complex isolates belong to subspecies known to be naturally resistant to polymyxins. For the 14 *K. pneumoniae*, the *K. oxytoca* and the *C. freundii* isolates, with colistin MICs ranging from 16 to 32 mg/L, no putative phosphoethanolamine transferase encoding gene was found, suggesting chromosome-encoded acquired resistance to colistin. Concerning the two colistin-resistant *E. coli* isolates, the *mcr* multiplex PCR gave a positive result for *mcr-1*-like gene for one isolate with a colistin MIC of 4 mg/L. This result was confirmed by the detection of *mcr-1* in the genome of this strain. Of note, this MCR-1-producing *E. coli*. possessed decreased susceptibility to carbapenems due to CTX-M-3 ESBL production associated with decreased outer membrane permeability. No putative phosphoethanolamine transferase encoding gene was identified for the last colistin-resistant *E. coli* isolates that harboured colistin MIC of 32 mg/L. Overall, during this prospective evaluation, the performance of the PCR multiplex assay was 100% (95% CI 54.6–100%) sensitivity, 100% (95% CI 99.1–100%) specificity, 100% (95% CI 54.6–100%) positive predictive value and 100% (95% CI 99.1–100%) negative predictive value.

Of note, only 3.2% (11/346) of the carbapenemase producers were resistant to colistin, including seven isolates with natural colistin resistance (four *E. cloacae* complex, one *M. morganii* and two *P. mirabilis*) and four isolates with acquired resistance (one KPC-3-, one NDM-1- and one OXA-48-producing *K. pneumoniae,* and one OXA-48-producing *C. freundii*). No *mcr*-like-encoding plasmid was identified among the carbapenemase producers.

4. Discussion

To date, five *mcr* gene families have been reported in Enterobacteriaceae. Of note, *mcr-1* is more prevalent than the other *mcr* families in enterobacterial isolates recovered from human samples. The other *mcr* families (*mcr-2* to *mcr-5*) seem to be more widespread than *mcr-1* in animals. Although several commercialized molecular diagnostic tools have been developed for the detection of *mcr* genes, they cannot detect the latest reported families – *mcr-3, mcr-4* and *mcr-5*. This study developed a multiplex PCR scheme and evaluated its performance using a collection of colistin-resistant isolates, including *mcr-1* to *mcr-5* producers. Moreover, this multiplex assay was validated prospectively with isolates from the French NRC for Antimicrobial Resistance. All the *mcr* variants were detected correctly by this multiplex PCR, and no false-negative results were obtained. Recently, another multiplex assay using different primers was developed for identification of the same five *mcr* families [23]. Using this test, a multi-centre study led to the identification of 14 MCR-producing Enterobacteriaceae among 49 *E. coli* and *Salmonella* spp. isolates from animals and food from Spain, Germany, France and Italy. These two studies pave the way for the direct detection of the five *mcr* family genes on clinical samples using multiplex PCR. However, the multicentre study [23] did not evaluate the possibility of false-positive results if it is performed on samples containing potential progenitors of *mcr* genes such as *Shewanella* spp. or *Aeromonas* spp. [7,14]. In addition, this study obtained an amplicon for two *Shewanella* spp. Whole-genome sequencing revealed the presence of two putative phosphoethanolamine transferases sharing homology with *mcr-4*. Cloning and expression of *mcr-4*-like genes from *S. bicestrii* JAB-1 showed the functionality of the gene. Other shewanella and aeromonas isolates remained negative. These results highlight the risk of false detection of MCR-producing Enterobacteriaceae when molecular methods are applied directly to clinical samples (e.g. stool). Indeed, it was reported that the intestinal carriage of *Shewanella* spp., as a progenitor of $bla_{\text{OXA-48-like}}$ genes, might result in false detection of OXA-48-like-producing Enterobacteriaceae [24]. In the same way, it is likely that it falsely simulates the carriage of MCR-4-producing Enterobacteriaceae.

Despite the fact that the multiplex PCR does not allow identification of the variant inside the five *mcr* families, it allows rapid and accurate identification of all MCR-producing Enterobacteriaceae. Accordingly, this type of multiplex assay should be used on all Enterobacteriaceae isolates with diminished susceptibility to colistin to detect the presence of plasmid-mediated resistance as soon as possible. Thus, it might be helpful to rapidly assess the presence of plasmid-encoded resistance to: (i) implement infection control measures which aim to limit the spread of such resistance, as it is already proposed for the containment of CPE; and (ii) select the appropriate therapy in cases of infection.

5. Conclusions

In conclusion, a multiplex PCR was developed and validated to detect all *mcr* gene families described in Enterobacteriaceae to date. This type of test is critical for the epidemiological surveillance of plasmid-encoded resistance. This is also the case for laboratories that have access to whole-genome sequencing, as only *mcr-1, mcr-1.2* and *mcr-2* genes are included in the ResFinder database (http://www.genomicepidemiology.org/), one of the most commonly used websites for the identification of acquired resistance genes from whole-genome sequencing data.

Funding

This work was partially funded by the University Paris-Sud, France. ABJ, SB, TN, RAB and LD are members of the Laboratory of Excellence in Research on Medication and Innovative Therapeutics, supported by a grant from the French National Research Agency (ANR-10-LABX-33).

Competing interests

None declared.

Ethical approval

Not required.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.10.022.

References

[1] Falagas ME, Kasiakou SK. Colistin: the revival of polymyxins for the management of multidrug-resistant Gram-negative bacterial infections. Clin Infect Dis 2005;40:1333–41.
[2] Kontopidou F, Giamarellou H, Katerelos P, Maragos A, Kioumis I, Trikka–Graphakos E, et al. Infections caused by carbapenem-resistant *Klebsiella pneumoniae* among patients in intensive care units in Greece: a multi-centre study on clinical outcome and therapeutic options. Clin Microbiol Infect 2014;20:O117–23.
[3] Venditti C, Nisii C, D'Arezzo S, Vulcano A, Di Caro A. Surveillance of *mcr-1* and *mcr-2* genes in carbapenem-resistant *Klebsiella pneumoniae* strains from an Italian hospital. EuroSurveill 2017;22.
[4] Jeannot K, Bolard A, Plesiat P. Resistance to polymyxins in Gram-negative organisms. Int J Antimicrob Agents 2017;49:526–35.
[5] Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol 2014;5:643.

[6] 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.
[7] Sun J, Zhang H, Liu YH, Feng Y. Towards understanding MCR-like colistin resistance. Trends Microbiol 2018;26:794–808.
[8] 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.
[9] Chabou S, Leangapichart T, Okdah L, Le Page S, Hadjadj L, Rolain JM. Real-time quantitative PCR assay with Taqman® probe for rapid detection of MCR-1 plasmid-mediated colistin resistance. New Microb New Infect 2016;13:71–4.
[10] Dona V, Bernasconi OJ, Kasraian S, Tinguely R, Endimiani A. A SYBR® Green-based real-time PCR method for improved detection of *mcr*-1-mediated colistin resistance in human stool samples. J Glob Antimicrob Resist 2017;9:57–60.
[11] Imirzalioglu C, Falgenhauer L, Schmiedel J, Waezsada SE, Gwozdzinski K, Roschanski N, et al. Evaluation of a loop-mediated isothermal amplification-based assay for the rapid detection of plasmid-encoded colistin resistance gene *mcr-1* in Enterobacteriaceae isolates. Antimicrob Agents Chemother 2017;61.
[12] Bernasconi OJ, Principe L, Tinguely R, Karczmarek A, Perreten V, Luzzaro F, et al. Evaluation of a new commercial microarray platform for the simultaneous detection of beta-lactamase and *mcr-1/-2* genes in Enterobacteriaceae. J Clin Microbiol 2017;55:3138–41.
[13] Borowiak M, Fischer J, Hammerl JA, Hendriksen RS, Szabo I, Malorny B. Identification of a novel transposon-associated phosphoethanolamine transferase gene, *mcr-5*, conferring colistin resistance in d-tartrate fermenting *Salmonella enterica* subsp. *enterica* serovar Paratyphi B. J Antimicrob Chemother 2017;72:3317–24.
[14] Carattoli A, Villa L, Feudi C, Curcio L, Orsini S, Luppi A, et al. Novel plasmid–mediated colistin resistance *mcr-4* gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. EuroSurveill 2017;22.
[15] Roer L, Hansen F, Stegger M, Sonksen UW, Hasman H, Hammerum AM. Novel *mcr-3* variant, encoding mobile colistin resistance, in an ST131 *Escherichia coli* isolate from bloodstream infection, Denmark, 2014. EuroSurveill 2017;22.
[16] Xavier BB, Lammens C, Ruhal R, Kumar-Singh S, Butaye P, Goossens H, et al. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. EuroSurveill 2016;21.
[17] Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing joint subcommittee, Recommendations for MIC determination of colistin (polymyxin E) as recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group. EUCAST, 2016. Available at: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf2017.
[18] Dortet L, Bonnin RA, Pennisi I, Gauthier L, Jousset AB, Dabos L, et al. Rapid detection and discrimination of chromosome- and MCR-plasmid-mediated resistance to polymyxins by MALDI-TOF MS in *Escherichia coli*: the MALDIxin test. J Antimicrob Chemother 2018;73:3359–67.
[19] Dortet L, Cuzon G, Ponties V, Nordmann P. Trends in carbapenemase-producing Enterobacteriaceae, France, 2012 to 2014. EuroSurveill 2017;22.
[20] Jousset AB, Dabos L, Bonnin RA, Girlich D, Potron A, Cabanel N, et al. CTX-M-15-producing *Shewanella* species clinical isolate expressing OXA-535, a chromosome-encoded OXA-48 variant, putative progenitor of the plasmid-encoded OXA-436. Antimicrob Agents Chemother 2018;62 pii:e01879–17.
[21] Jayol A, Saly M, Nordmann P, Menard A, Poirel L, Dubois V. *Hafnia*, an enterobacterial genus naturally resistant to colistin revealed by three susceptibility testing methods. J Antimicrob Chemother 2017;72:2507–11.
[22] Guerin F, Isnard C, Sinel C, Morand P, Dhalluin A, Cattoir V, et al. Cluster-dependent colistin hetero-resistance in *Enterobacter cloacae* complex. J Antimicrob Chemother 2016;71:3058–61.
[23] Rebelo AR, Bortolaia V, Kjeldgaard JS, Pedersen SK, Leekitcharoenphon P, Hansen IM, et al. Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* for surveillance purposes. EuroSurveill 2018;23.
[24] Antonelli A, Di Palo DM, Galano A, Becciani S, Montagnani C, Pecile P, et al. Intestinal carriage of *Shewanella xiamenensis* simulating carriage of OXA-48-producing Enterobacteriaceae. Diagn Microbiol Infect Dis 2015;82:1–3.
[25] Haenni M, Beyrouthy R, Lupo A, Chatre P, Madec JY, Bonnet R. Epidemic spread of *Escherichia coli* ST744 isolates carrying *mcr-3* and $bla_{CTX\text{-}M\text{-}55}$ in cattle in France. J Antimicrob Chemother 2018;73:533–6.
[26] Chang AC, Cohen SN. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J Bacteriol 1978;134:1141–56.
[27] Potron A, Poirel L, Nordmann P. Origin of OXA-181, an emerging carbapenem-hydrolyzing oxacillinase, as a chromosomal gene in *Shewanella xiamenensis*. Antimicrob Agents Chemother 2011;55:4405–7.