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Rapid multiplex polymerase chain reaction for detection of *mcr-1* to *mcr-5* genes

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A rapid (total time <2 h) and reliable multiplex polymerase chain reaction for screening of *mcr-1* to *mcr-5* genes conferring resistance to colistin has been developed. This technique has been tested on a collection of isolates previously identified as bearing *mcr-1*, *mcr-2*, and *mcr*-like genes and had a sensitivity and a specificity of 100%. Using this method, we were also able to identify a single isolate possessing both *mcr-1* and *mcr-5* genes.

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Polymyxin resistance6
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Infections due to multidrug-resistant bacteria are increasing in health care facilities, which lead physicians to reintroduce the old antibiotic polymyxins (colistin, polymyxin B) as possible therapeutic options (Vincent et al., 2009; Wright et al., 2017). However, resistance to polymyxins in Gram-negative bacteria is now increasingly described and is mostly due in Enterobacteriaceae to chromosomal mutations in genes involved in modification of the lipopolysaccharide (LPS) (Poirel et al., 2017). Recently, the plasmid-mediated polymyxin resistance determinant MCR-1, responsible for acquired resistance to polymyxins, has been reported from *Escherichia coli* and *Klebsiella pneumoniae* isolates in China (Liu et al., 2016). Then, the *mcr-1* gene has b6een reported worldwide in various enterobacterial species including *Enterobacter*, *Salmonella*, and *Shigella* and from various animal and environmental origins (Poirel et al., 2017). The encoded MCR-1 protein is a phosphoethanolamine transferase that adds a phosphoethanolamine goup to the lipid A, a portion of the LPS, leading to a more cationic LPS structure and consequently to resistance to polymyxins (Liu et al., 2016). Epidemiologic surveys show that the livestock is the most important reservoir of MCR producers (Irrgang et al., 2016; Liu et al., 2016; Perrin-Guyomard et al., 2016). A recent study showed a very high prevalence (99%) of MCR-1–producing Enterobacteriaceae among pigs receiving colistin from 2 farms in Portugal (Kieffer et al., 2017). A recent large epidemiologic study in Chinese individuals showed a prevalence of *mcr-1*–positive Enterobacteriaceae isolates close to 1% in clinical samples, and comprised between 0.5% and 3% in carriage samples from volunteers and patients (Wang et al., 2017). In parallel, other mobile colistin resistance genes have been identified recently (*mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5*) mostly from single isolate (Borowiak et al., 2017; Carattoli et al., 2017; Xavier et al., 2016; Yin et al., 2017). Comparison of protein sequences of MCR-like proteins shows a low level of identity (ca. 30%–50%), except MCR-1 and MCR-2 that share ca. 80% amino acid identity (Table 1). Searching the *mcr* genes progenitors among *Moraxella* sp. strains led to the identification of the progenitor of *mcr-2* gene (Poirel et al., 2017) being *Moraxella pluranimalium*, and other *Moraxella* species (commensal of the tracheal flora) were considered as potential sources of other *mcr*-like genes (Kieffer et al., 2017). Multiple variants have been reported within the past 2 years, to date, 5 major groups, namely, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* each possessing 13, 2, 10, 2 and 2 variants, respectively (Table S1). Although *mcr-2* and *mcr-4* have been only reported in Europe, *mcr-1* and *mcr-3* are identified worldwide (Di Pilato et al., 2016; Liu et al., 2017; Lu et al., 2017; Teo et al., 2017; Zhao et al., 2017). Most of the bacteria carry a single *mcr* gene, but a single

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Table 1
Amino acid identity of MCR polymyxin resistance determinants.

MCR determinant	Amino acid identity level			
	MCR-1	MCR-2	MCR-3	MCR-4
MCR-2	80.7			
MCR-3	32.5	31.7		
MCR-4	34.0	35.0	49.0	
MCR-5	36.1	35.3	34.7	33.7

isolate possessing 2 *mcr* genes has been reported (Liu et al., 2017). More studies including the detection of *mcr* genes are needed to evaluate the emergence of these colistin resistance genes with high transferability potency.

The aim of this work was to design an easy-to-perform polymerase chain reaction (PCR) technique to detect all of *mcr-1* to *mcr-5* genes in a single mix. For this purpose, a PCR-based method was designed and has the advantage to give result in less than 2 h (amplification and electrophoresis).

For optimization of the multiplex PCR, 5 control strains harboring the *mcr-1* to *mcr-5* genes were used as positive controls, being all enterobacterial isolates. We obtained the positive *mcr*-5 isolate by eletroporating in the *E. coli* strain TOP10 a plasmid in which the *mcr*-5 gene was cloned by the manufacturer RD-Biotech (http://www.rd-biotech.com). An easy interpretation (avoiding similar-in-size amplicons for 1 given PCR tube) of the PCR results was obtained. We also tested mixes of all couples of DNA of the 5 positive controls (mixes of DNA of isolates containing *mcr-1* and *mcr-2*, *mcr-1* and *mcr-3*, *mcr-1* and *mcr-4*, *mcr-1* and *mcr-5*, *mcr-2* and *mcr-3*, *mcr-2* and *mcr-4*, *mcr-2* and *mcr-5*, *mcr-3* and *mcr-4*, *mcr-3* and *mcr-5*, *mcr-4* and *mcr-5* genes, separately). We also tested isolates containing other *mcr*-like genes (a *M. pluranimalium* isolate harboring the *mcr-2.2* gene, an isolate of *Moraxella porci*, and 2 recombinant strains of *E. coli* bearing *mcr*-like genes from *Moraxella osloensis* and *Moraxella lincolnii*; Table S1) (Kieffer et al., 2017).

Different primers were designed for each gene by using the Primer Blast software from the NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to amplify fragments of *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* genes. We used an internal control detecting fragment of 16S DNA gene previously designed (Arunasri et al., 2013). We used for the development of the PCR method the 5 control isolates, the mix of DNA of the different positive controls, and the isolates containing *mcr*-like genes. This PCR method amplifies fragments of 502 bp for *mcr-1* (primers mcr1–mtpF [5′-ATGCCAGTTTCTTTCGCGTG-3′] and mcr1–mtpR [5′-TCGGCAAATTGCGCTTTTGGC-3′]), 379 bp for *mcr-2* (primers mcr2–mtpF [5′-GATGGCGGTCTATCCTGTAT-3′] and mcr2–mtpR [5′-AAGGCTGACACCCCATGTCAT-3′], 296 bp for *mcr-3* (primers mcr3–mtpF [5′-ACCAGTAAATCTGGTGGCGT-3′] and mcr3–mtpR [5′-AGGACAACCTCGTCATAGCA-3′], 207 bp for *mcr-4* (primers mcr4–mtpF [5′-TTGCAGACGCCCATGGAATA-3′] and mcr4–mtpR [5′-GCCGCATGAGCTAGTATCGT-3′], 608 bp for *mcr-5* (primers mcr5–mtpF [5′-GGACGCGACTCCCTAACTTC-3′] and mcr4–mtpR [5′-ACAACCAGTACGAGAGCACG-3′], and finally 93 bp for the 16S rDNA as control (primers 16SF [5′-GTGCAATATTCCCCACTGCT-3′] and 16SR [5′-CGATCCCTAGCTGGTCTGAG-3′]). DNA template of each isolate was extracted by using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, USA), and 200 ng (2 μL) of total DNA was subjected to the multiplex PCR in a 20-μL reaction mixture containing 2 μL of 10× buffer (supplied with *Taq* polymerase); 20 pmol of each primer detecting *mcr-1*, *mcr-2*, and *mcr-3* genes; 10 pmol of each primer detecting *mcr-4* and *mcr-5* genes; and 5 pmol of each primer detecting the 16S rDNA gene, each deoxynucleoside triphosphate at a concentration of 2 μM, 2 U of *Taq* polymerase (GenScript, USA). We used the cycling conditions of the rapid PCR method previously developed (Clermont et al., 2000) to determine the phylogroups of *E. coli* and which last 48 min. The conditions were as follows: denaturation at 94 °C for 4 min; followed by 30 cycles of 94 °C for 5 s, 59 °C for 20 s, and a single, final, elongation step at 72 °C for 5 min. We avoided the elongation step because all PCR products have sizes under 600 bp. The final elongation step is sufficient to allow for the elongation of PCR products. DNA fragments were analyzed by electrophoresis in a 2.5% agarose gel (Fig. 1) for 50 min. The DNA from the 5 positive controls (used separately and in mix of 2 DNA) (Fig. 1) and the progenitor of *mcr-2* gene, *M. pluranimalium,* presented the expected bands of *mcr-1* to *mcr-5* gene fragments. The *M. porci* isolate and the 2 *E. coli* recombinant strains harboring the *mcr*-like genes from the *M. osloensis* and *M. lincolnii* isolates did not give a positive result and confirmed the specificity of the PCR primers used in this multiplex approach.

Then, a total of 43 isolates of enterobacterial species (38 *E. coli* and 2 *K. pneumoniae* isolates and 2 *Salmonella enterica*) bearing the *mcr-1* gene and a single *mcr-4* positive *S. enterica* isolate from our collections were tested (Table S2). We detected that one isolate, the *S. enterica* named FR-290, previously identified as *mcr*-1 gene–positive isolate, was also positive for *mcr*-5 gene. All other isolates yielded the expected band for *mcr-1* or *mcr-4* amplicons and no amplification of the other *mcr* genes as attempted (sensitivity and specificity of 100%).

PCR, real-time PCR, or loop-mediated isothermal amplification–based methods have previously been developed to assess the detection of colistin resistance genes. However, for most of these techniques, only a single *mcr* gene is detected (*mcr-1* gene) (Bontron et al., 2016; Donà et al., 2017; Liu et al., 2016; Xavier et al., 2016; Zou et al., 2017). In a single test, both the *mcr-1* and *mcr-2* genes could be detected (Mavrici

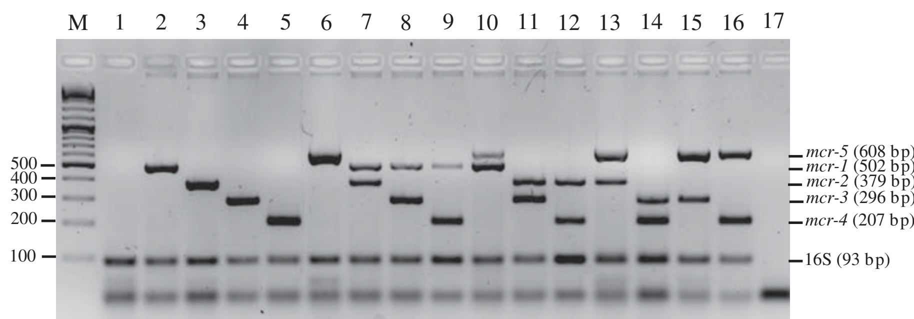

Fig. 1. Agarose gel electrophoresis (2.5%) used for the separation of multiplex PCR products. Lanes: 1, negative control (susceptible *E. coli* isolate); 2, *mcr-1*–positive isolate; 3, *mcr-2*–positive isolate; 4, *mcr-3*–positive isolate; 5, *mcr*-4–positive isolate; 6, *mcr-5*–positive isolate; 7, mix of DNA of *mcr-1*– and *mcr-2*–positive isolates; 8, mix of DNA of *mcr-1* and *mcr-3*–positive isolates; 9, mix of DNA of *mcr-1*- and *mcr-4*–positive isolates; 10, mix of DNA of *mcr-1* and *mcr-5* isolates; 11, mix of DNA of *mcr-2*– and *mcr-3*–positive isolates; 12, mix of DNA of *mcr-2*– and *mcr-4*–positive isolates; 13, mix of DNA of *mcr-2*– and *mcr-5*–positive isolates; 14, mix of DNA of *mcr-3*– and *mcr-4*–positive isolates; 15, mix of DNA of *mcr-3*– and *mcr-5*–positive isolates; 16, mix of DNA of *mcr-4*– and *mcr-5*–positive isolates; and 17, negative control (water). M = molecular size marker (GeneRuler™, 100-bp DNA Ladder Plus; Thermo Fisher Scientific, USA). The size of each PCR product is indicated in base pairs.

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et al., 2017), and in these studies, several *mcr* genes were separately detected (El Garch et al., 2018; Mavrici et al., 2017). A microarray detection method of several genes including *mcr-1* and *mcr-2* genes was also developed (Bernasconi et al., 2017). Also, a multiplex SYBR green real-time PCR detecting *mcr-1*, *mcr-2*, and *mcr-3* genes was developed (Li et al., 2017). However, although these methods are sensitive, they cannot be applied in numerous laboratories because they need specific and expensive equipment. A multiplex method detecting the 5 *mcr* genes together was recently developed (Rebelo et al., 2018); however, this method uses a traditional PCR program, consequently last in time 3 h (amplification and gel electrophoresis) and does not include an internal control.

Here we developed a rapid (<2 h; amplification and gel electrophoresis) and reliable multiplex PCR for a rapid screening of the *mcr-1* to *mcr-5* genes with an internal control. This technique can be adapted easily to any laboratory that possesses a PCR amplification machine. It could allow for the determination of the prevalence of these colistin resistance genes in other clinical collections such as human, animal, and environmental collections.

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Conflict of interest

All authors declare that they have no conflict of interest.

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