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Rapid detection and differentiation of *mobile colistin resistance*, *mcr-1* to *mcr-10,* genes by real time PCR and melt curve analysis

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Summary

Background: Emergence of multidrug resistant (MDR) microorganisms prompted new interest in older antibiotics like colistin that were previously abandoned due to limited efficacy or high toxicity. Over the years, several chromosomal-encoded colistin resistance mechanisms were described; more recently, ten plasmid-mediated mobile colistin resistance (*mcr*) genes have also been identified. Spread of these genes among MDR Gram-negative bacteria is a matter of serious concern, therefore reliable and timely *mcr* detection is paramount.

Aim: To design and validate a multiplex real-time PCR for detection and differentiation of *mcr* genes.

Methods: All available *mcr* alleles were downloaded from the NCBI Reference Gene Catalog, aligned with Clustal Omega and primers designed using Primer-BLAST. Real-time PCR monoplexes were optimized and validated using a panel of 120 characterised Gram-negative strains carrying a wide range of resistance genes, often in combination. Melt-curve analysis was used to confirm positive results.

Findings: *In silico* analysis allowed to design a "screening" assay for detection of mcr-1/2/6, mcr-3, mcr-4, mcr-5, mcr-7, mcr-8 and mcr-9/10 paired with an internal control assay to discount inhibition. A "supplementary" assay was then designed to differentiate mcr-1, mcr-2, mcr-6, mcr-9 and mcr-10. Expected results were obtained for all strains (100% sensitivity and specificity). Melt-curve analysis showed consistent Tm results. Inhibition was not observed.

Conclusions: The assay is rapid and easy to perform, enabling unequivocal *mcr* detection and differentiation even when more than one variant is simultaneously present. Adoption by clinical and veterinary microbiology laboratories would aid the surveillance of *mcr* genes amongst Gram-negative bacteria.

Introduction

The rise of multidrug resistant (MDR) microorganisms, especially Gram-negative bacteria resistant to carbapenems such as imipenem and meropenem, has resulted in some older antimicrobials being brought back into clinical practice and used as last resort. Colistin (also known as polymyxin E) is probably the most important amongst these agents due to its strong activity against serious pathogens like MDR *Pseudomonas aeruginosa*, MDR *Acinetobacter baumannii* and carbapenem-resistant *Enterobacterales* [1, 2]. Originally introduced into clinical practice during the 1950s, colistin is a polypeptide antibiotic of the polymyxin family that is able to bind the lipopolysaccharide (LPS) and then disrupt the outer membrane of Gram-negatives causing death of the bacterial cell. However, severe side effects, namely nephrotoxicity and neurotoxicity, progressively limited colistin usage over the years to infection prevention and treatment in livestock [3, 4].

The most common mechanism responsible for resistance to polymyxins is an increased presence of cationic groups, such as phosphoethanolamine or 4-amino-4-deoxy-L-arabinose, in the LPS, preventing the positively charged antibiotic from binding the target site. *Proteus mirabilis* and *Serratia marcescens* show intrinsic resistance, while acquired resistance due to mutations in enzymes involved in the LPS biosynthesis, such as PmrA, PmrB, PmrC, PhoP, PhoQ and MgrB, was identified in several other *Enterobacterales*, in *P. aeruginosa* and in *A. baumannii* [4]. In 2015, the first plasmid-mediated colistin resistance mechanism (i.e. *mobile colistin resistance 1*, *mcr-1*) was identified in a strain of *Escherichia coli* isolated from a pig farm in China [5]; presently, a total of ten different variants (i.e. *mcr-1* to *mcr-10*) have been described [6, 7]. These genes confer resistance by adding a phosphoethanolamine residue to the lipid A in the LPS and, more importantly, they are carried by mobile elements, thus they have the ability to easily spread amongst different Gram-negative bacteria by horizontal gene transfer [8]. Carbapenem resistant isolates acquiring *mcr* genes are the ultimate threat in clinical settings as they further limit the already scarce treatment options; they must be quickly identified and contained.

In Wales, bacteria isolated from clinical settings showing certain resistant phenotypes are referred to the Specialist Antimicrobial Chemotherapy Unit (SACU) in Cardiff (UK) for phenotypic and molecular characterisation plus therapeutic guidance. Where the use of colistin is required, susceptibility testing is performed by microbroth dilution as recommended by EUCAST and according to ISO 20776-1 [9, 10] and colistin resistant strains are tested locally for *mcr-1* by block-based PCR or referred to another

reference laboratory testing *mcr-1* to *mcr-5*. So far only *mcr-1* has been detected in Wales from an *Escherichia coli* and a *Salmonella* Typhimurium strain [11], however an ongoing project for whole genome sequencing (WGS) of blood culture and carbapenem resistant isolates has identified Gram-negative *Enterobacterales* carrying *mcr-4.3*, *mcr-9.1* and *mcr-10.1* (unpublished data). This finding raised concerns regarding the undetected circulation of strains carrying *mcr* genes in Wales and the importance of their rapid identification to prevent spread was acknowledged.

A more reliable molecular approach, able to detect all *mcr* variants, was needed to rapidly test isolates referred to SACU showing colistin resistance by microbroth dilution. Real-time PCR is a well-established methodology for detecting genetic markers in bacteria. It can provide results much faster than block-based PCR and also does not normally require the use of high risk reagents like ethidium bromide (although safer alternatives are now available). A real-time PCR assay based on SYBR Green chemistry was designed *in silico* to detect the ten, so far described, *mcr* variants and thoroughly validated *in vitro* using a large panel of previously characterised Gram-negative isolates.

Methods

Assay design

All available allele sequences of the ten *mcr* variants were downloaded (as of October 2020) from the NCBI Reference Gene Catalog [12] and aligned using Clustal Omega [13]. A maximum likelihood phylogenetic tree of *mcr* sequences was generated from the alignment using RAxML v 8.2.8 [14]. BLASTn was also used to determine the nucleotide identity between pairs of variants [15]. Ten different primer sets per target were designed using Primer-BLAST [16] and then compared to the relevant alignment to identify the pair that at least *in silico* was able to amplify the highest number of alleles. Where necessary, degenerate bases (a maximum of two per primer) were inserted to cover non-conserved positions. An *in silico* PCR was used in case of cross-reaction to investigate primer specificity [17].

Bacterial strains

A total of 120 previously characterised Gram-negative isolates from 18 different species were selected from the SACU, Animal and Plant Health Agency (APHA), Cardiff University (CU), German Federal Institute for Risk Assessment (BfR), NCTC and ATCC collections (Supplementary data). Forty-two strains carried one *mcr* gene (i.e. thirteen *mcr-1.1*; one *mcr-2.2* and one *mcr-2.3;* two *mcr-3.1,* two *mcr-3.5* and one *mcr-3.21*; one *mcr-4.3*; one *mcr-5.1*; one *mcr-6.1*; three *mcr-8.1*; thirteen *mcr-9.1* and three *mcr-10.1*); eight strains carried *mcr-1.1* and *mcr-3.1*, *mcr-3.4*, *mcr-3.5* or *mcr-3.6* simultaneously, while two carried *mcr-1.1* and *mcr-8.1*. The remaining 68 strains were *mcr* negative however carried a wide range of different antimicrobial resistance genes. After overnight aerobic growth on blood agar at 35±1°C, bacterial growth approximately equivalent to a third of a loopful (10μL loop) was re-suspended in 250 μL of nuclease-free water and heat killed at 100°C for 10 min. Supernatant was separated by centrifugation at 12,000 *g* for 2 min and diluted 1:20 in nuclease-free water prior to testing. An extraction control (i.e. 250 μL of nuclease-free water) was always included to discount cross contamination. A genetically modified (GM) strain of *Escherichia coli* DH5α carrying a custom pEX-A128 plasmid (Eurofins, Germany) containing mcr-1.1, mcr-3.1, mcr-4.1, mcr-5.1, mcr-7.1, mcr-8.1 and mcr-9.1 PCR fragments was used as positive control. The GM *E. coli* DH5α strain was re-suspended in 1 mL of nuclease-free water, then after heat-treatment and centrifugation the supernatant was diluted 1:200 to compensate the extremely high copy number and obtain Ct values within the range produced by other strains. Additionally, *E. coli* NCTC 13846 (*mcr-1.1*), *E. coli* CU NP50 (*mcr-2.3*), *Moraxella pluranimalium* APHA MSG47-C17 (*mcr-6.1*), *Citrobacter freundii* SACU 33417 (*mcr-9.1*) and *Enterobacter cloacae* CU NKBR-1540 (*mcr-10.1*) were used as positive controls in the “supplementary” assay.

Internal process control

An assay detecting a 76 bp fragment of the *green fluorescent protein* (*gfp*) from *Aequorea victoria* was included to discount PCR inhibition [18]. A custom-made pEX-A128 plasmid (Eurofins, Germany) containing the entire *gfp* sequence (Genbank Accession: M62653) was diluted to a working concentration of 1 pmol/μL before being added to the GFP reaction mix.

Real Time PCR

Monoplex PCR assays were optimized for the QuantStudio 6-Flex (Applied Biosystems) using the PowerUp SYBR Green MasterMix (Life Technologies) and Microamp Fast Optical 96-Well Reaction

Plate 0.1 mL (Thermo-Fisher Scientific). Briefly, reactions were performed in a final volume of 10 μL containing 5 μL of MasterMix, 2.5 μL of template, 2 μL of PCR grade water and 0.5 μL of relevant primer mix. The final concentration of each primer detailed in Table I. In the GFP reaction mix, 0.5 μL of pGFP [1 pmol/μL] were added therefore only 1.5 μL of PCR grade water was added to obtain the 10 μL final volume. After an initial uracil-DNA-glycosylase step at 50°C for 2 min, the Dual-Lock™ DNA polymerase was activated at 95°C for 2 min followed by 35 cycles of denaturation at 95 °C for 1 sec and annealing/extension at 60°C for 20 sec. Amplification results were analysed with the threshold set at 0.5 ΔRn for all targets and the baseline set between 5 and 15 cycles only for the GFP target. Melting curve analysis was performed as follows: 95°C for 15 sec (ramp rate = 1.6°C/sec), 60°C for 1 min (ramp rate = 1.6°C/sec) and 95°C for 15 sec (ramp rate = 0.15°C/sec) with fluorescence fluctuation analysed during the latter.

Colistin susceptibility testing

Colistin susceptibility of the 52 strains carrying *mcr* genes was performed by micro-broth dilution using the ComASP™ Colistin Kit (Liofilchem, Italy) according to manufacturer's instructions. Two-fold dilutions ranging from 0.25 mg/L to 16 mg/L were tested; *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* NCTC 25922 were used as susceptible controls, while *E. coli* NCTC 13846 (*mcr-1.1*) was used as a resistant control. Results were interpreted according to EUCAST version 10 breakpoints where available [9].

Results

In silico analysis

A maximum likelihood tree constructed using a Clustal Omega alignment of all the available *mcr* sequences showed the levels of relatedness among variants (Figure I). Many of the variants aligned poorly, and this is reflected by low levels of relatedness in the phylogenetic tree. However, the tree demonstrated higher levels of similarity between mcr-1, mcr-2 and mcr-6 (78.8% to 86.0% nucleotide identity over their length), and between mcr-9 and mcr-10 variants (79.7% nucleotide identity across

their length). This finding allowed to design primers able to detect more than one *mcr* variant and so maximise the number of strains that could be tested in a 96-well plate. A "screening" assay targeting mcr-1/2/6, mcr-3, mcr-4, mcr-5, mcr-7, mcr-8 and mcr-9/10 plus the gfp assay used as internal control was designed to initially test isolates; then a "supplementary" assay was designed to differentiate (where necessary) mcr-1, mcr-2 mcr-6, mcr-9 and mcr-10. Primer sequences, final concentrations and amplicon sizes are detailed in Table I. Sequence alignments and primer binding sites are detailed in the Supplementary Data.

***In vitro* analysis**

The eight "screening" monoplex PCR assays (i.e. mcr-1/2/6, mcr-3, mcr-4, mcr-5, mcr-7, mcr-8, mcr-9/10 and GFP) were performed in 96-well plates with PCR mixes added in rows; DNA extracts were tested in column 1 to 10, while columns 11 and 12 were used to test the extraction control and the GM positive control respectively. The time necessary to complete PCR amplification and melt curve analysis was approx. 40min. Testing of undiluted DNA extracts resulted in strong PCR inhibition (as revealed by absence of, or delayed, GFP amplification), consequently 1:20 dilutions were prepared prior to testing.

Cross-reaction was observed between the mcr-3 assay and 15 strains known to be *mcr* negative. Ct values were > 20 and melt temperatures were not consistent with that of the positive control (Supplementary data). Using genome sequences from five of these strains (i.e. *Klebsiella pneumoniae* ATCC 700603, *E. cloacae* NCTC 13406, *K. pneumoniae* NCTC 13443, *Klebsiella aerogenes* SACU 27329 and *E. cloacae* complex SACU 32799) and the mcr-3 primer sequences (i.e. mcr-3_F and mcr-3_R), an *in silico* PCR identified a marker with high homology to *pmrC*, one of the chromosomal genes able to confer colistin resistance by addition of phosphoethanolamine residues, as the likely cause of the cross-reaction; furthermore, Clustal Omega showed that the amplified fragments were 139bp long in both the *pmrC-like* genes and *mcr-3* (Supplementary Data). When new mcr-3 primers (i.e. F3 and R2) were designed taking into account the *pmrC-like* sequences, cross-reaction was no longer observed (Table II).

Amplification results produced Ct values ranging between 11.01 and 18.33 with Standard Deviation (SD) values between 0.32 and 1.53 (Table II). Melt-curve analysis was used to confirm positive results by comparing dissociation curves to that of the relevant positive control. Overall, minor Tm variations were observed (SD $\leq$ 0.60), while greater differences were noted with the mcr-1/2/6 monoplex.

The expected result was initially obtained for 119 of the 120 previously characterised isolates. *Enterobacter cloacae* complex SACU 31819, previously shown to carry *mcr-9.1,* produced the expected results when tested using the mcr-9/10 screening assay (Ct = 16.32; Tm = 77.46°C); however when tested with the supplementary primers, it produced unequivocal mcr-10 amplification (Ct = 14.47; Tm = 81.25°C) while the mcr-9 assay was negative. Further investigation showed that the WGS analysis of this strain was initially performed prior to the publication of the *mcr-10.1* sequence, therefore only a partial match with *mcr-9.1* (79.7 % identity) was identified. When the analysis was recently repeated, *mcr-10.1* was correctly identified. Indeed, the newly designed assay provided the expected result for all 120 tested isolates (100% sensitivity and specificity) including those carrying two *mcr* genes simultaneously. Cross-reaction with other resistance markers present in the isolates used in this study was not observed.

Colistin susceptibility testing was performed by microbroth dilution on the 52 strains carrying *mcr* genes included in this study (supplementary data). Fourteen strains were susceptible to colistin: one carried *mcr-4.3*, twelve carried *mcr-9.1* and one carried *mcr-10.1*. Simultaneous presence of *mcr-4.3 or mcr-9.1* and a carbapenemase gene (i.e. $bla_{\text{IMP-4}}$, $bla_{\text{NDM-1}}$ or $bla_{\text{OXA-48}}$) was noted in eleven strains; among these strains only *E. cloacae* complex SACU 31955 carrying *mcr-9.1* and $bla_{\text{IMP-4}}$ was resistant to colistin (MIC = 16 mg/L or >16 mg/L).

Discussion

We describe here the design and validation of an array of monoplex real-time PCRs optimised for a 96-well assay format to rapidly detect and differentiate the ten *mcr* variants so far described. A rational approach that took into account all the relevant allele sequences available (as of October 2020) from

the NCBI Reference Gene Catalog [12] was applied *in silico* to design specific primers and to then predict whether they could also detect those alleles that were not available to the authors for *in vitro* testing. A wide collection of previously characterised Gram-negative isolates was then tested *in vitro* to fully validate the newly designed monoplexes. An assay targeting the *gfp* was tested in parallel as an internal control to discount inhibition [18].

The consideration that *mcr* genes were described relatively recently and that new alleles are frequently added to the NCBI Reference Gene Catalog, prompted the choice of SYBR Green chemistry combined with melting curve analysis in monoplex PCR reactions over multiplexing using primers and probes. Newly described SNPs in primers and/or probe binding sites would potentially result in assays having to be re-designed and then re-validated. This is time consuming and costly. The absence of probes in this assay reduces the number of binding sites from three to two and consequently the likelihood that a newly described SNP falls within them. Should a new SNP be identified in either of the two primer binding sites causing the assay to be re-designed, the use of separate monoplexes limits any revalidation to just the affected monoplex rather than the whole assay. Furthermore, the use of separate monoplexes provides greater flexibility to combine testing of different target panels if the need arises: one or more of the monoplexes described here could be quickly replaced with others validated separately to detect relevant resistance genes (e.g. *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{GES}$, *bla*$_{CTX-M}$, etc…) in an outbreak scenario without the need to perform any further validation on the entire assay panel.

In silico analysis showed that this assay should detect all the described *mcr* allele variants (as of October 2020). Sequence homology among *mcr-1*, *mcr-2* and *mcr-6*, and between *mcr-9* and *mcr-10* allowed to design a “screening” assay for the rapid detection of all ten variants in a single 96-well assay format that also included an internal control to discount inhibition. A “supplementary” set of primers was also designed to differentiate (where necessary) among the above five *mcr* variants.

In vitro experiments proved the assay to be 100% sensitive and specific on a large panel of previously characterised isolates available to the authors. A strain carrying *mcr-7.1* was not available, however

successful amplification of the mcr-7 target from the GM *E. coli* DH5α strain used as positive control, proved that all *mcr* variants can be detected at least in principle.

As stated by Lund *et al.* [19], assays are often validated using a limited selection of target variants that are available locally; furthermore, testing high numbers of unsequenced isolates might simply result in redundant analysis of a small number of variants which then provides little indication about the actual sensitivity and specificity of a particular assay. These issues were addressed in this study by applying a robust *in silico* approach to confidently predict amplification of those variants that were not available for *in vitro* testing and by then including isolates where the *mcr* sequence had previously been characterised.

Interpretation of melting profiles can be problematic when dissociation curves do not precisely overlap with that of the relevant positive control. This was observed in particular when analysing results of the mcr-1/2/6 assay as a likely consequence of the sequence variability of mcr-2 and mcr-6 compared to the *mcr-1.1* PCR fragment used as positive control. Interestingly, this observation proved helpful when preliminarily differentiating between mcr-1 and mcr-2 or mcr-6: an average Tm of 79.80 was observed for mcr-1 PCR products compared to 76.87 and 77.42 for mcr-2 and mcr-6 respectively. The same was not clear for the mcr-9/10 assay as the average Tms were 77.43 and 77.67 for mcr-9 and mcr-10 respectively, therefore a definitive differentiation could only be obtained by running the supplementary mcr-9 and mcr-10 assays. It is noteworthy that variations in Tm results were observed also for the GFP PCR product that is identical in every PCR assay, therefore it seems that the actual Tm value cannot be reproducibly obtained by the PCR platform between different PCR runs. A Tm SD of 0.29 was calculated for the GFP amplified fragment, this value could be considered as an indication of the Tm uncertainty of measurement intrinsic to this procedure.

If at all necessary, our results highlight the importance of updating databases of resistance genes used by WGS pipelines. *Enterobacter cloacae* SACU 31819, previously found to carry *mcr-9.1* by WGS analysis, instead gave a positive mcr-10 amplification. This result triggered an investigation showing that, at the time of the WGS analysis, the antimicrobial resistance database used did not contain the *mcr-10.1* sequence and thus it identified *mcr-9.1* as the closest match.

A positive result obtained by this assay is a reliable indication of *mcr* presence, however it does not necessarily mean resistance to colistin. Indeed, despite carrying *mcr* genes, several isolates analysed in this study tested susceptible to colistin; this finding had already been observed in several isolates carrying *mcr-9* [20, 21, 22] and more recently in an isolate of *Aeromonas veronii* carrying *mcr-3.30* [23]. We report here an *E. coli* isolate (SACU 24218) carrying *mcr-4.3* and an *E. cloacae* complex isolate (SACU 31819) carrying *mcr-10.1* that are not resistant to colistin (MIC = 0.5 mg/L for both strains). The association between certain *mcr* genes and colistin resistance is certainly worth further investigation, however this topic does not fall within the scope of this study. Similarly, a negative result cannot completely exclude the presence of a new *mcr* allele or a totally new variant that are not amplified by the primers described in this study. Colistin resistant strains that are negative for the amplification of *mcr* genes need further investigation (e.g. by WGS analysis) before discounting presence of a mobile element as the cause of the resistant phenotype.

Simultaneous presence of *mcr* and a carbapenemase gene was identified in 11 strains (supplementary data). Among these strains only *E. cloacae* complex SACU 31955 was resistant to colistin (MIC $\geq$ 16 mg/L), nevertheless this finding is particularly concerning as strains carrying the above genes have the potential to cause severe damage in hospital settings where vulnerable patients are present. These strains must be rapidly and reliably identified to contain their spread.

The real time PCR assay described here was implemented to investigate presence of *mcr* genes in colistin resistant Gram-negative isolates in Wales. Testing colistin susceptible isolates, which are the great majority, would not be practical in busy microbiology laboratories and would not have an immediate benefit for the treatment of patients; instead, surveillance studies performed on a suitable selection of isolates should be organised. The assay allows to rapidly screen colistin resistant isolates for the ten *mcr* variants so far described; then the supplementary assay allows to differentiate between mcr-1, mcr-2, mcr-6, mcr-9 and mcr-10. Ten isolates (plus the extraction control and positive control) can be tested simultaneously using 96-well plates. Given the short PCR running time (ca. 40 min), DNA could be extracted simultaneously from a greater number of isolates to allow a quick second PCR experiment to be prepared while the first is running; up to 20 isolates could be then tested in less than

2 hours. Alternatively, 384-well plates may be used for high throughput, allowing a greater number of strains to be tested simultaneously. The assay was deemed easy to perform by members of staff with limited experience with real-time PCR and melt-curve analysis. Importantly, the real time PCR assay allowed unequivocal result interpretation even when more than one *mcr* gene was present.

To the authors' knowledge, this is the first real time PCR targeting all the ten *mcr* variants so far described. The assay was easily implemented, allowing reliable detection and unequivocal differentiation of the different genes. Adoption by both clinical and veterinary microbiology laboratories would facilitate procedures aimed at controlling the spread of colistin resistant Gram-negative strains. Further studies will be necessary to elucidate the lack of colistin resistance in certain strains carrying *mcr* genes.

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Conflict of interest statement.

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References

[1] Bonomo RA, Burd EM, Conly J, Limbago BM, Poirel L, Segre JA, *et al*. Carbapenemase-Producing Organisms: A Global Scourge. Clin Infect Dis. 2018 Apr 3;66(8):1290-1297. https://doi.org/10.1093/cid/cix893.

[2] Li J, Nation RL, Turnidge JD, Milne RW, Coulthard K, Rayner CR, *et al*. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. Lancet Infect Dis. 2006 Sep;6(9):589-601. https://doi.org/10.1016/S1473-3099(06)70580-1.

[3] European Medicines Agency. Updated advice on the use of colistin products in animals within the European Union: development of resistance and possible impact on human and animal health (EMA/231573/2016), https://www.ema.europa.eu/en/documents/scientific-guideline/updated-advice-use-colistin-products-animals-within-european-union-development-resistance-possible_en.pdf; 26 May 2016 [accessed 14 December 2020].

[4] Poirel L, Jayol A, Nordmann P. Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes. Clin Microbiol Rev. 2017 Apr;30(2):557-596. https://doi.org/10.1128/CMR.00064-16.

[5] 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 Feb;16(2):161-8. https://doi.org/10.1016/S1473-3099(15)00424-7.

[6] Ling Z, Yin W, Shen Z, Wang Y, Shen J, Walsh TR. Epidemiology of mobile colistin resistance genes mcr-1 to mcr-9. J Antimicrob Chemother. 2020 Nov 1;75(11):3087-3095. https://doi.org/10.1093/jac/dkaa205.

[7] Wang C, Feng Y, Liu L, Wei L, Kang M, Zong Z. Identification of novel mobile colistin resistance gene mcr-10. Emerg Microbes Infect. 2020 Mar 2;9(1):508-516. https://doi.org/10.1080/22221751.2020.1732231.

[8] Caselli E, D'Accolti M, Soffritti I, Piffanelli M, Mazzacane S. Spread of *mcr-1*-Driven Colistin Resistance on Hospital Surfaces, Italy. Emerg Infect Dis. 2018;24(9):1752-1753. https://doi.org/10.3201/eid2409.171386.

[9] The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Clinical breakpoints - bacteria (v 10.0), https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_10.0_Breakpoint_Tables.pdf; 2020 [accessed 14 December 2020].

[10] International Standardisation Organisation. ISO 20776-1:2019: Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices — Part 1: Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases https://www.iso.org/standard/70464.html [accessed 14 December 2020].

[11] Doumith M, Godbole G, Ashton P, Larkin L, Dallman T, Day M, *et al*. Detection of the plasmid-mediated mcr-1 gene conferring colistin resistance in human and food isolates of Salmonella enterica and Escherichia coli in England and Wales. J Antimicrob Chemother. 2016 Aug;71(8):2300-5. https://doi.org/10.1093/jac/dkw093.

[12] NCBI Reference Gene Catalog. https://www.ncbi.nlm.nih.gov/pathogens/refgene [accessed 14 December 2020].

[13] Chojnacki S, Cowley A, Lee J, Foix A, Lopez R. Programmatic access to bioinformatics tools from EMBL-EBI update: 2017. Nucleic Acids Res. 2017 Jul 3;45(W1):W550-W553. https://doi.org/10.1093/nar/gkx273.

[14] Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014 May 1;30(9):1312-3. https://doi.org/10.1093/bioinformatics/btu033.

[15] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990 Oct 5;215(3):403-10. https://doi.org/10.1016/S0022-2836(05)80360-2.

[16] Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 2012 Jun 18;13:134. https://doi.org/10.1186/1471-2105-13-134.

[17] *In silico* PCR. https://github.com/simonrharris/in_silico_pcr [accessed 14 December 2020].

[18] Murphy NM, McLauchlin J, Ohai C, Grant KA. Construction and evaluation of a microbiological positive process internal control for PCR-based examination of food samples for Listeria

monocytogenes and Salmonella enterica. Int J Food Microbiol. 2007 Nov 30;120(1-2):110-9. https://doi.org/10.1016/j.ijfoodmicro.2007.06.006.

[19] Lund M, Petersen MB, Jørgensen AL, Paulmann D, Wang M. Rapid real-time PCR for the detection of IMP, NDM, VIM, KPC and OXA-48 carbapenemase genes in isolates and spiked stool samples. Diagn Microbiol Infect Dis. 2018 Sep;92(1):8-12. https://doi.org/10.1016/j.diagmicrobio.2018.04.002.

[20] Börjesson S, Greko C, Myrenås M, Landén A, Nilsson O, Pedersen K. A link between the newly described colistin resistance gene mcr-9 and clinical Enterobacteriaceae isolates carrying blaSHV-12 from horses in Sweden. J Glob Antimicrob Resist. 2020 Mar;20:285-289. https://doi.org/10.1016/j.jgar.2019.08.007.

[21] Kananizadeh P, Oshiro S, Watanabe S, Iwata S, Kuwahara-Arai K, Shimojima M, *et al*. Emergence of carbapenem-resistant and colistin-susceptible *Enterobacter cloacae* complex co-harboring blaIMP-1 and mcr-9 in Japan. BMC Infect Dis. 2020 Apr 16;20(1):282. https://doi.org/10.1186/s12879-020-05021-7.

[22] Tyson GH, Li C, Hsu CH, Ayers S, Borenstein S, Mukherjee S, *et al*. The mcr-9 Gene of Salmonella and Escherichia coli Is Not Associated with Colistin Resistance in the United States. Antimicrob Agents Chemother. 2020 Jul 22;64(8):e00573-20. https://doi.org/10.1128/AAC.00573-20.

[23] Ragupathi NKD, Sethuvel DPM, Anandan S, Murugan D, Asokan K, Neethi Mohan RG, *et al*. First hybrid complete genome of Aeromonas veronii reveals chromosome-mediated novel structural variant mcr-3.30 from a human clinical sample. Access Microbiol. 2020 Feb 17;2(4):acmi000103. https://doi.org/10.1099/acmi.0.000103.

Table I. Summary of primer sets used in this study (F = Forward Primer; R = Reverse Primer; degenerate positions are underlined) including final concentrations and amplicon sizes.

Assay	Primer set	Target	Primer name	Sequence (5′ → 3′)	Final Conc. [nM]	Amplicon size (bp)	Reference
SCREENING	A	MCR-1 MCR-2 MCR-6	MCR-1/2/6_F	GCGTAYTCTGTGCCGTGTATG	500	70	This study
			MCR-1/2/6_R	GGTATTTGGCGGTATCGACATCA	500		
	B	MCR-3	MCR-3_F3	GGTGATGATGCAAAACGGGATA	500	176	This study
			MCR-3_R2	GTCCCACACGAACGAACATCA	500		
	C	MCR-4	MCR-4_F	AGGCGTTACATTGTCCCTACCT	500	120	This study
			MCR-4_R	ACGACTGGCATTCTTCGCATCT	500		
	D	MCR-5	MCR-5_F	GCCATGCTGCGGAATCTGAT	500	73	This study
			MCR-5_R	AGGGCAGCATTCTCCATTGC	500		
	E	MCR-7	MCR-7_F	ATGCCAAAGTCGTCGCCAAA	500	57	This study
			MCR-7_R	CCCCCACCACCAGAAACATC	500		
	F	MCR-8	MCR-8_F	GGATGCGTGACGTTGCTATGA	500	59	This study
			MCR-8_R	GCTTTCCCCCAGCGATTCTC	500		
	G	MCR-9 MCR-10	MCR-9/10_F	GCAGCCATGGACCGACYTAT	500	46	This study
			MCR-9/10_R	CGATGCTCWGCCGGATAACG	500		
	H	GFP	GFP_F	CCTGTCCTTTTACCAGACAACCA	300	76	[18]
			GFP_R	GGTCTCTCTTTTCGTTGGGATCT	300		
SUPPLEMENTARY	1	MCR-1	MCR-1_F	ATCCCATCGCGGACAATCTC	500	177	This study
			MCR-1_R	AGACCGTGCCATAAGTGTCA	500		
	2	MCR-2	MCR-2_F	GTGTCAGCCTTGTGYTGTTG	500	112	This study
			MCR-2_R	ATCGGCGTAATCGGRTTRAT	1000		
	6	MCR-6	MCR-6_F	CCGTCCGGTCAATCCCTATC	500	157	This study
			MCR-6_R	CATCGCCCCAAATAGCACAAG	500		
	9	MCR-9	MCR-9_F	TCCTTCCTGCCATCCTCCTT	500	120	This study
			MCR-9_R	CGGCAACACCTGCAATCAAA	500		
	10	MCR-10	MCR-10_F	GCAATAACCCGACGCTGAAC	500	133	This study
			MCR-10_R	GTAACGCGCCTTGCATCATC	500		

433 **Table II.** Summary of Cycle threshold (Ct) and melting temperature (Tm) results for the primer sets
434 used in this study (* only one strain available for testing, consequently Min, Max and SD were not
435 calculated).
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Assay	Primer set	Target	Ct				Tm (°C)			
			Min	Mean	Max	SD	Min	Mean	Max	SD
SCREENING	A	*mcr-1*	13.42	**15.77**	18.33	1.53	79.47	**79.80**	80.15	0.17
		mcr-2	16.07	**16.08**	16.10	0.02	76.44	**76.87**	77.29	0.60
		mcr-6 *	-	**15.81**	-	-	-	**77.42**	-	-
	B	*mcr-3*	13.05	**14.80**	16.95	1.47	80.03	**80.27**	80.43	0.16
	C	*mcr-4* *	-	**16.65**	-	-	-	**79.76**	-	-
	D	*mcr-5* *	-	**11.01**	-	-	-	**83.00**	-	-
	E	*mcr-7* *	-	**12.75**	-	-	-	**79.80**	-	-
	F	*mcr-8*	16.04	**16.78**	17.19	0.54	75.57	**75.57**	75.58	0.04
	G	*mcr-9*	15.96	**17.44**	18.30	0.80	77.12	**77.43**	77.74	0.17
		mcr-10	15.69	**15.99**	16.32	0.32	77.46	**77.67**	77.96	0.26
	H	*gfp*	21.15	**23.02**	24.32	0.56	77.28	**77.87**	78.86	0.29
SUPPLEMENTARY	1	*mcr-1*	12.79	**14.83**	17.97	1.47	83.86	**84.08**	84.14	0.11
	2	*mcr-2*	15.78	**16.52**	17.26	1.04	81.01	**81.01**	81.01	0.00
	6	*mcr-6* *	-	**15.24**	-	-	-	**80.93**	-	-
	9	*mcr-9*	15.54	**16.45**	17.68	0.76	79.80	**79.80**	79.80	0.00
	10	*mcr-10*	14.34	**14.64**	15.11	0.41	80.95	**81.35**	81.84	0.45

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Figure I. Midpoint-rooted tree of the *mcr* allele variants available from the NCBI Reference Gene Catalog. The scale shows the number of SNPs per site in the alignment.