Rapid detection and quantification of plasmid-mediated colistin resistance genes (*mcr-1* to *mcr-5*) by real-time PCR in bacterial and environmental samples

Running headline: RT-PCR detection of *mcr* genes.

Roberta Tolosi[1], Ilias Apostolakos[1], Andrea Laconi[1], Lisa Carraro[1], Guido Grilli[2], Petra Cagnardi[2], Alessandra Piccirillo[1,*]

[a]Department of Comparative Biomedicine and Food Science, University of Padua, Viale dell'Università 16, 35020 Legnaro (Padua), Italy.

[b]Department of Veterinary Medicine, University of Milan, Via Celoria 10, 20133 Milano, Italy.

[*]Corresponding author

Address for correspondence: Prof. Alessandra Piccirillo, Dipartimento di Biomedicina Comparata e Alimentazione, Università degli Studi di Padova, Viale dell'Università 16, 35020 Legnaro (PD), Italy. Email: alessandra.piccirillo@unipd.it. Phone: +39 0498272793. Fax: +39 0498272973.

Abstract

Aim: the aim of the study was to validate a rapid method to detect and quantify colistin resistance genes (*mcr-1* to *mcr-5*) by real-time polymerase chain reaction (RT-PCR) in diverse matrices.

Methods and results: the detection limit of two newly designed SYBR Green real-time PCR assays for *mcr-4* and *mcr-5* and of previously published protocols for *mcr-1* to *mcr-3* was assessed using serial dilutions of reference strains. The assays could detect all five *mcr* genes with the lower limit of 10^2 copy numbers. *Escherichia coli* isolates (n=1,062) and environmental samples (n=93) were tested for the presence of *mcr* genes. The assays enabled the detection of colistin resistance genes both in bacterial isolates and in complex environmental samples.

Conclusions: this method represents a set of sensitive, rapid and effective assays for the screening of colistin resistance directly from the environment.

Significance and impact of the study: colistin is an antimicrobial commonly used in animals and has recently emerged as a last-resort treatment in humans. Plasmid-mediated *mcr* genes confer resistance to colistin and represent a major threat for public health since they can be easily disseminated through horizontal gene transfer. The rapid and sensitive detection of *mcr* genes is of utmost necessity.

Introduction

Colistin is a critically important antimicrobial and a last-resort treatment against human infections caused by multi-drug resistant Gram-negative bacteria (WHO CIA List, 2017). Currently, the clinical utility of colistin is facing a serious threat due to the emergence and dissemination of plasmid-mediated colistin resistance. The first report of a mobile colistin resistance gene was dated November 2015, and the discovered gene, identified in an *Escherichia coli* strain isolated from a pig, was named *mcr-1* (Liu *et al.*, 2016). Since then, novel variants of plasmid-mediated colistin resistance genes have been described, *mcr-2* (Xavier *et al.*, 2016), *mcr-3* (Yin *et al.*, 2017), *mcr-4* (Carattoli *et al.*, 2017), *mcr-5* (Borowiak *et al.*, 2017), *mcr-6* (AbuOun *et al.*, 2018), *mcr-7* (Yang *et*

al., 2018), *mcr-8* (Wang *et al.*, 2018), *mcr-9* (Carroll *et al.*, 2019) and *mcr-10* (Wang *et al.*, 2020). These additional *mcr* genes have been reported in different bacterial species isolated from multiple sources (i.e. human and livestock), raising concerns related to the emergence and spread of mobile colistin resistance. Moreover, *mcr* genes are harboured on conjugative plasmids belonging to various incompatibility groups, including IncI2 , IncX4, IncFIA, IncFII and IncHI2 (Xavier *et al.*, 2016; Yin *et al.*, 2017; Wang *et al.*, 2018; Yang *et al.*, 2018; Wang *et al.*, 2020). Although *mcr* genes have been discovered only recently, retrospective studies testing isolates dating back to 1980s identified bacteria harbouring *mcr* genes (Shen *et al.*, 2016). Of notice, most *mcr*-positives were detected in samples collected from 2009 onwards (Shen *et al.*, 2016). In the past few years, *mcr* genes have been identified in 47 different countries across six continents in human and animal associated bacteria (i.e. *Salmonella*, *E. coli*, *Pseudomonas*, etc.) (Nang *et al.*, 2019). Livestock is considered the main reservoir for *mcr* genes due to the use of colistin for prophylaxis, metaphylaxis and therapeutic purposes (Kempf *et al.*, 2016; Liu *et al.*, 2016). More worryingly, *mcr* genes have been also identified in water sources (Petrillo *et al.*, 2016; Zurfuh *et al.*, 2016) and soil (Xia *et al.*, 2019) suggesting that these resistance determinants might be transferred from the environment to humans via food of both vegetal and animal origin (Salisbury *et al.*, 2002; Hao *et al.*, 2014).

Due to the emergence of multiple mobile colistin resistance genes in human, animal and environmental samples (Osei Sekyere, 2019), an increased demand of rapid and reliable methods for their detection is warranted. Routine susceptibility testing is considered unreliable since colistin is a large cationic molecule, diffuses poorly into media and adheres to common plastic labware (Kempf *et al.*, 2016), making the agreement of results between replicates and between laboratories difficult (Poirel *et al.*, 2017). A previous study proposed a molecular method for detection of *mcr-1* to *mcr-5* (Rebelo *et al.*, 2018); however, this approach does not allow detection and quantification in complex environmental matrices and, as a conventional method, it does not offer results in real-time. Recently, a real-time polymerase chain reaction (RT-PCR) method for *mcr-1, mcr-2* and *mcr-3* detection has been proposed (Li *et al.*, 2017). In the present study we aimed to implement this

method towards the detection and quantification of *mcr-4* and *mcr-5* by using newly designed *mcr* specific RT-PCR primers. Further, we optimised the *mcr-1* to *mcr-5* detection protocols and validated their application in both bacterial isolates and complex environmental samples.

Materials and methods

Reference strains

The reference strains for *mcr-1* (*E. coli* 412016126), *mcr-2* (*E. coli* KP37), *mcr-3* (*E. coli* 2013-SQ352), *mcr-4* (*E. coli* DH5a harbouring the entire *mcr-4* gene in a pCR2 plasmid) and *mcr-5* (*Salmonella* Paratyphi B 13-SA01718) (Table 1) were kindly provided by the EU Reference Laboratory for Antimicrobial Resistance (DTU, Denmark) and were used for the optimization, the assessment of efficiency, sensitivity and specificity of the assays and as positive control when testing bacterial isolates and environmental samples. Briefly, reference strains were streaked onto nutrient agar (Microbiol, Uta, Italy) and incubated at 37±0.5 °C for 20±2h. Bacterial genomic DNA (gDNA) was extracted with the Invisorb Spin Tissue Mini Kit (Invitek Molecular, Berlin, Germany) according to the manufacturer's instructions. Quality and quantity of positive controls' DNA was assessed with NanoDrop spectrophotometer (ThermoScientific, Massachusetts, United States). Based on the assessment of the limit of detection of the assay, we used 0.4 ng/µl as cut-off value to select the sample to be tested.

mcr-4 and *mcr-5* RT-PCR primers design and confirmation by end-point PCR

Primer BLAST software (Jian *et al.*, 2012) was used to design the specific RT-PCR primers for *mcr-4* and *mcr-5* genes and the specificity was checked *in silico* against the NCBI database (Table 1) (Johnson *et al.*, 2008). Primer specificity was confirmed by end-point PCRs, using reference strains DH5a and 13-SA01718 harbouring *mcr-4* and *mcr-5*, respectively. To avoid false positive results, together with the *in silico* evaluation, the assays for the detection of *mcr-4* and *mcr-5*

developed in the present study were tested against DNA extracted from bacteria not harbouring the respective *mcr* target gene, including the positive reference strains for *mcr-1* (412016126), *mcr-2* (KP37) and *mcr-3* (B 13-SA01718) genes. Following this experimental setting, we also evaluated the absence of cross-reactivity among the five assays. Both end-point PCRs were performed in a final volume of 25 µL amplification mix containing 12.5 µL of DreamTaq PCR Master Mix 2X (ThermoScientific), 0.5 µL of 10 pmol mL^{-1} of each primer (Table 1), and 5 ng of gDNA as template. The thermal profile for the end-point PCR was initialised by holding 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 20 s, and 72 °C for 20 s, and a single final elongation at 72 °C for 7 min using an Applied Biosystems 2720 Thermal Cycler (ThermoScientific). The expected amplicon lengths (Table 1) were evaluated by agarose gel electrophoresis (2% agarose, 100 V, 60 min).

Optimization of RT-PCR conditions for *mcr-1* to *mcr-5* assays

The optimisation steps were conducted for both novel (*mcr-4*, *mcr-5*) and previously published (*mcr-1* to *mcr-3*) primers (Li *et al.*, 2017) in order to acquire a unique protocol for detection and quantification of genes. RT-PCR amplification was performed in a LightCycler®480 Roche (Basel, Switzerland), using a final volume of 10 µL. Two different reactions were carried out using either 2.5 µL of DNA template at different concentrations (2.0-0.2-0.02-0.002 ng μL^{-1}) or 2.5 µL of previous end-point PCR amplicons diluted at 0.0001 ng μL^{-1}, to assess respectively the optimal DNA concentration to analyse bacterial and environmental samples and the highest dilution to create a standard curve.

The PowerUp™ SYBR® Green Master Mix (ThermoScientific) was used together with different concentrations of each primer (*i.e.* 300/300 pmol mL^{-1}, 300/600 pmol mL^{-1}, 600/300 pmol mL^{-1}, 600/600 pmol mL^{-1}, 600/900 pmol mL^{-1}, 900/600 pmol mL^{-1} and 900/900 pmol mL^{-1} for forward and reverse primer, respectively). The cycling conditions were as follows: initial incubation at 50 °C for 2 min, followed by 2 min at 95 °C, and 45 cycles at 95 °C for 10 s and 60 °C for 40 sec. A

melting curve between 40 and 95 °C was determined by adding a dissociation step after the last amplification cycle at a temperature transition rate of 4.4 °C/s. RT-PCR data analysis was performed using LightCycler®480 software version 1.5 (Roche). For each sample, the crossing point (Cp) was used to determine the amount of target gene. Specificity of RT-PCR products was determined by the analysis of amplification profiles and melting curves. Furthermore, positive RT-PCR products were purified using the SPRIselect purification kit (Beckman Coulter, Pasadena, United States) and templates were sent to Macrogen Inc. (Madrid, Spain) for direct Sanger sequencing. The visualization, analysis and editing of chromatograms were performed with FINCHTV 1.4.0 software. Standard BLAST search against the CARD (Comprehensive Antibiotic Resistance Database) (Jia *et al.*, 2017) reference sequences was performed. All RT-PCR reactions were performed in duplicate.

Efficiency, sensitivity and specificity of *mcr-1* to *mcr-5* RT-PCR assays

To evaluate the efficiency and the dynamic range of each primer pair, serial dilutions (1:4 and 1:5) were prepared for each DNA template. Standard curves were constructed to evaluate the efficiency and the sensitivity of *mcr* primer sets using serial dilutions of gDNA. The assays' sensitivity was further tested by pooling five *E. coli* DNA samples with different concentrations and combinations of positive and negative samples (median concentration: 11.9 ng μL^{-1}, min: 0.4 ng μL^{-1}, max: 23.4 ng μL^{-1}). Melting profile analysis was used to assess the specificity of the amplification plots.

Validation of *mcr-1* to *mcr-5* RT-PCR assays

To validate the assays, DNA was extracted from *E. coli* isolates and environmental samples. Samples were stored at -80°C up to DNA isolation, while extracted DNAs were stored at -20°C up to processing. A total of 1,062 *E. coli* isolated from various stages of the broiler production pyramid (i.e. breeders, broilers and carcasses) and assessed for the presence of extended-spectrum β-lactamase (ESBL)- and plasmid-mediated AmpC β-lactamase (pAmpC) genes (n=587 positive)

(Apostolakos *et al.*, 2019) were tested. gDNA was extracted by suspending 1-2 bacterial colonies in a solution of 5% Chelex (BioRad, Hercules, United States) and boiling for 10 min. Debris in the Chelex suspension was removed by centrifugation at 12,000 ×g for 5 min. Bacterial isolates were analysed by pooling five DNA samples; when a pool tested positive, each sample was analysed individually to detect the positive sample(s) within the pool. No 16S amplification of *E. coli* isolates was performed because the aim of the study was to assess the diagnostic performance of the assays, i.e. their ability to identify rather than quantify *mcr* genes, and to optimize the assays with pooled isolates. DNA was extracted also from 93 environmental samples, namely agricultural soil (62 samples) and manure from dairy (11 samples), swine (10 samples) and chickens farms (10 samples), using the PowerSoil® DNA Isolation kit (Qiagen, Hilden, Germany). In each RT-PCR round, a positive (*mcr*+) and negative (*mcr*-) control for each gene was used. When testing the environmental samples, the 16S rRNA gene was used (Nadkarni *et al.*, 2002) as internal process control and to normalize the *mcr-1* to *mcr-5* gene copy numbers.

Results

Analytical performances of *mcr-1* to *mcr-5* RT-PCR assays

We established optimal amplification conditions for all *mcr* genes with 600 pmol mL^{-1} concentration of each primer. Assays' efficiencies ranged from 90.7% to 92.9%, and all R^2 values were >0.98 (Fig. 1). The limit of quantification copies was 13.7, 31.8, 82.4, 60.5 and 149.1 for *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* assays, respectively. No primer-dimers or non-specific peaks were observed in any of the assays. In detail, no increase in fluorescence associated with a sigmoidal amplification curve was observed for none of the template controls or when *mcr* primer pairs were used against non-target *mcr* genes. Pooling of bacterial DNA enabled fast screening of multiple samples in a single assay, since detection of positive controls was possible in all tested concentrations and combinations.

SYBR Green RT-PCR followed by melting curve analysis enables the detection of *mcr-1* to *mcr-5* from bacterial isolates and environmental samples

Two different matrices were screened in this study: bacterial (i.e. *E. coli* cultures) and complex environmental (i.e. manure and soil) samples. Starting from 1,062 pure cultures, seven samples were found positive for *mcr* genes. In particular, two samples were positive for *mcr-3* (one breeder, one broiler), one for *mcr-4* (breeder) and four for *mcr-5* (broilers). Three of the *mcr*-carrying isolates were also harbouring ESBL/pAmpC genes. Out of 93 environmental samples, 43 (46.2%) were positive for *mcr* genes; 20 (eight manure, twelve soil) for *mcr-1*, three (manure) for *mcr-2*, eleven (seven manure, four soil) for *mcr-3*, four (three manure, one soil) for *mcr-4* and five (two manure, three soil) for *mcr-5* (Fig. 2). The RT-PCR assays showed that copies number in the environmental samples for *mcr-1*, *mcr-3* and *mcr-5* ranged from below the limit of quantification to 21.0, 2072.68 and 30.6 copies, respectively. The copies number for *mcr-2* and *mcr-4* was below the limit of quantification in all positive samples.

Discussion

The aim of this study was to develop a fast, sensitive and reliable method for detection and quantification of colistin mobile resistance genes (*mcr-1* to *mcr-5*). We extended and optimized the method of Li *et al.* (2017) by constructing two novel assays for *mcr-4* and *mcr-5*. The method, based on a SYBR Green RT-PCR paired with melting curve analysis, enables the rapid and confident detection and quantification of *mcr-1* to *mcr-5* genes in bacterial isolates and environmental samples.

In recent years, end-point PCR, real-time PCR, loop-mediated isothermal amplification and microrray-based methods have been developed to detect colistin resistance genes. Although these methods proved to be sensitive and effective, they present some limitations, such as detection of only one to three *mcr*-genes (Chabou *et al.*, 2016; Irrgang *et al.*, 2016; Xavier *et al.*, 2016; Bernasconi *et al.*, 2017; Donà *et al.*, 2017; Li *et al.*, 2017) and/or requirement of specific and

expensive equipment (Bernasconi *et al.*, 2017; Zhong *et al.*, 2019). A multiplex PCR for the detection of *mcr-1* to *mcr-5* has been also recently developed (Rebelo *et al.*, 2018); however, this method is a standard end-point PCR, hence it is time consuming as it has to rely on gel electrophoresis to visualize the results of the amplification and more prone to contamination. By comparison, SYBR Green based assays are faster and less expensive, making our set of assays an ideal tool for *mcr-1 to mcr-5* genes detection, in particular when testing large number of samples including complex matrices such as those from the environment. The set of assays described here supports samples pooling, allowing the screening of five individual samples in a single reaction tube, further decreasing time and cost of the analyses. Due to its cost-effectiveness, our method might also have wide employment in developing countries, where financial limitations represent a huge issue toward antimicrobial resistance gene surveillance studies. The method proposed here is not itself deprived of limitations, as it cannot detect all five *mcr* genes in a single reaction, unlike the multiplex PCR developed by Rebelo et al. (Rebelo *et al.*, 2018), nor emerging *mcr* genes. However, SYBR Green RT-PCRs are relatively easy to implement, and the detection of emerging *mcr* genes might be easily achieved via designing new specific primer pairs.

The detection and quantification of colistin resistance genes are crucial because they are considered an emerging risk for public health (Poirel *et al.*, 2017), since colistin is employed as a last-resort antimicrobial for the treatment of multidrug-resistance Gram-negative bacterial infection in humans. Non-prudent use of colistin in human medicine but also in farming settings contributed to the spread and persistence of resistance genes in the natural environment. The simultaneous presence of *mcr* and ESBL/pAmpC genes in three *E. coli* isolates is notable given the importance of 3rd generation cephalosporins and colistin in human health.

The novel set of RT-PCR primers and the optimised *mcr-1* to *mcr-5* assays developed in the present study represent a useful method for future surveillance studies for determination of *mcr* genes prevalence in human, animal and environmental samples in a fast, sensitive and reproducible way.

Funding

This work was supported by the Italian Ministry of Research and University (PRIN Project 2015, prot. 2015KA3XFH).

Acknowledgements

The authors wish to thank Arianna Scalabrin, Sara Petratti, Sabrine Ben Hamed and Ikbel Hadj Hassine for their contribution to the realization of the work. The Authors wish also to thank the EU Reference Laboratory for Antimicrobial Resistance, National Food Institute, Technical University of Denmark for kindly providing the positive controls.

Conflict of interest

None to declare.

References

AbuOun, M., Stubberfield, E.J., Duggett, N.A., Kirchner, M., Dormer, L., Nunez-Garcia, J., Randall, L.P., Lemma, F., Crook, D.W., Teale, C., Smith, R.P. and Anjum, M.F. (2018) Erratum in: *mcr-1* and *mcr-2* (*mcr-6.1*) variant genes identified in *Moraxella* species isolated from pigs in Great Britain from 2014 to 2015 (*J Antimicrob Chemother* (2017) 72 (2745-2749). *J Antimicrob Chemother.* **73**, 2904.

Apostolakos, I., Mughini-Gras, L., Fasolato, L. and Piccirillo, A. (2019) Assessing the occurrence and transfer dynamics of ESBL/pAmpC-producing *Escherichia coli* across the broiler production pyramid. *PLoS One*. **14**, 1-13.

Bernasconi, O.J., Principe, L., Tinguely, R., Karczmarek, A., Perreten, V., Luzzaro, F. and Endimiani, A. (2017) Evaluation of a new commercial microarray platform for the simultaneous detection of β-lactamase and *mcr-1* and *mcr-2* genes in Enterobacteriaceae. *J Clin Microbiol*. **55**, 3138-3141.

257 Borowiak, M., Fischer, J., Hammerl, J.A., Hendriksen, R.S., Szabo, I. and Malorny, B. (2017)
258 Identification of a novel transposon-associated phosphoethanolamine transferase gene, *mcr-5*,
259 conferring colistin resistance in d-tartrate fermenting *Salmonella enterica* subsp. *enterica*
260 serovar Paratyphi B. *J Antimicrob Chemother*. **72**, 3317-3324.
261 Carattoli, A., Villa, L., Feudi, C., Curcio, L., Orsini, S., Luppi, A., Pezzotti, G. and Magistrali, C.F.
262 (2017) Novel plasmid-mediated colistin resistance *mcr-4* gene in *Salmonella* and *Escherichia*
263 *coli*, Italy 2013, Spain and Belgium, 2015 to 2016. *Eurosurveillance*. **22**, 1-5.
264 Carroll, L.M., Gaballa, A., Guldimann, C., Sullivan, G., O Henderson, L. and Wiedmann, M.
265 (2019) Identification of novel mobilized colistin resistance gene *mcr-9* in a multidrug-resistant,
266 colistin-susceptible *Salmonellaenterica* Serotype Typhimurium isolate. *mBio*. **10**, 1-6.
267 Chabou, S., Leangapichart, T., Okdah, L., Le Page, S., Hadjadj, L. and Rolain, J.M. (2016) Real-
268 time quantitative PCR assay with Taqman®probe for rapid detection of MCR-1 plasmid-
269 mediated colistin resistance. *New Microbes New Infect*. **13**, 71-74.
270 Donà, V., Bernasconi, O.J., Kasraian, S., Tinguely, R. and Endimiani, A. (2017) A SYBR ® Green-
271 based real-time PCR method for improved detection of *mcr-1*-mediated colistin resistance in
272 human stool samples. *J Glob Antimicrob Resist*. **9**, 57-60.
273 Hao, H., Cheng, G., Iqbal, Z., Ai, X., Hussain, H.I., Huang, L., Dai, M., Wang, Y., Liu, Z. and
274 Yuan, Z. (2014) Benefits and risks of antimicrobial use in food-producing animals. *Front*
275 *Microbiol*. **5**, 1-11.
276 Irrgang, A., Roschanski, N., Tenhagen, B.A., Grobbel, M., Skladnikiewicz-Ziemer, T., Thomas, K.,
277 Roesler, U. and Käsbohrer, A. (2016) Prevalence of *mcr-1* in *E. coli* from livestock and food in
278 Germany, 2010-2015. *PLoS One*. **11**, 2010-2015.
279 Jia, B., Raphenya, A.R., Alcock, B., Waglechner, N., Guo, P., Tsang, K.K., Lago, B.A., Dave,
280 B.M., Pereira, S., Sharma, A.N., Doshi, S., Courtot, M., Lo, R., Williams, L.E., Frye, J.G.,
281 Elsayegh, T., Sardar, D., Westman, E.L., Pawlowski, A.C., Johnson, T.A., Brinkman, F.S.L.,
282 Wright, G.D. and McArthur, A.G. (2017) CARD 2017: Expansion and model-centric curation

283 of the comprehensive antibiotic resistance database. *Nucleic Acids Res*. **45**, D566-D573.
284 Jian, Y., George, C., Irena, Z., Ioana, C., Steve, R. and Madden Thomas, L. (2012) Primer- BLAST:
285 A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*.
286 **13**, 134.
287 Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S. and Madden, T.L. (2008)
288 NCBI BLAST: a better web interface. *Nucleic Acids Res*. **36**, 5-9.
289 Kempf, I., Jouy, E. and Chauvin, C. (2016) Colistin use and colistin resistance in bacteria from
290 animals. *Int J Antimicrob Agents*. **48**, 598-606.
291 Li, J., Shi, X., Yin, W., Wang, Y., Shen, Z., Ding, S. and Wang, S. (2017) A multiplex SYBR green
292 real-time PCR assay for the detection of three colistin resistance genes from cultured bacteria,
293 feces, and environment samples. *Front Microbiol*. **8**, 1-5.
294 Liu, Y.Y., Wang, Y., Walsh, T.R., Yi, L.X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B.,
295 Huang, X., Yu, L.F., Gu, D., Ren, H., Chen, X., Lv, L., He, D., Zhou, H., Liang, Z., Liu, J.H.
296 and Shen, J. (2016) Emergence of plasmid-mediated colistin resistance mechanism *mcr-1* in
297 animals and human beings in China: A microbiological and molecular biological study. *Lancet*
298 *Infect Dis*. **16**, 161-168.
299 Nadkarni, M.A., Martin, F.E., Jacques, N.A. and Hunter, N. (2002) Determination of bacterial load
300 by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology*. **148**,
301 257-266.
302 Nang, S.C., Li, J. and Velkov, T. (2019) The rise and spread of *mcr* plasmid-mediated polymyxin
303 resistance. *Crit Rev Microbiol*. **45**, 131-161.
304 Osei Sekyere, J. (2019) *Mcr* colistin resistance gene: a systematic review of current diagnostics and
305 detection methods. *Microbiologyopen*. **8**, 1-21.
306 Petrillo, M., Angers-Loustau, A. and Kreysa, J. (2016) Possible genetic events producing colistin
307 resistance gene *mcr-1*. *Lancet Infect Dis*. **16**, 280.
308 Poirel, L., Jayol, A. and Nordmanna, P. (2017) Polymyxins: antibacterial activity, susceptibility

309 testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev*.
310 **30**, 557-596.
311 Rebelo, A.R., Bortolaia, V., Kjeldgaard, J.S., Pedersen, S.K., Leekitcharoenphon, P., Hansen, I.M.,
312 Guerra, B., Malorny, B., Borowiak, M., Hammerl, J.A., Battisti, A., Franco, A., Alba, P.,
313 Perrin-Guyomard, A., Granier, S.A., de Frutos, C., Escobar, Malhotra-Kumar, S., Villa, L.,
314 Carattoli, A. and Hendriksen, R.S. (2018) Multiplex PCR for detection of plasmid-mediated
315 colistin resistance determinants, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* for surveillance
316 purposes. *Eurosurveillance*. **23**, 1-11.
317 Salisbury, J.G., Nicholls, T.J., Lammerding, A.M., Turnidge, J. and Nunn, M.J. (2002) A risk
318 analysis framework for the long-term management of antibiotic resistance in food-producing
319 animals. *Int J Antimicrob Agents*. **20**, 153-164.
320 Shen, Z., Wang, Y., Shen, Y., Shen, J. and Wu, C. (2016) Early emergence of *mcr-1* in *Escherichia*
321 *coli* from food-producing animals. *Lancet Infect Dis*. **16**, 293.
322 Wang, X., Wang, Y., Zhou, Y., Li, J., Yin, W., Wang, S., Zhang, S., Shen, J., Shen, Z. and Wang,
323 Y. (2018) Emergence of a novel mobile colistin resistance gene, *mcr-8*, in NDM-producing
324 *Klebsiella pneumoniae*. *Emerg Microbes Infect*. **7**, 1-9.
325 Wang, C., Feng, Y., Liu, L., Wei, L., Kang, M. and Zong, Z. (2020) Identification of novel mobile
326 colistin resistance gene *mcr-10*. *Emerg Microbes Infect*. **9**, 508-516.
327 WHO CIA List (2017) *Critically Important Antimicrobials for Human Medicine*. *World Heal*
328 *Organization*.
329 Xavier, B.B., Lammens, C., Ruhal, R., Malhotra-Kumar, S., Butaye, P., Goossens, H. and
330 Malhotra-Kumar, S. (2016) Identification of a novel plasmid-mediated colistin resistance gene,
331 *mcr-2*, in *Escherichia coli*, Belgium, june 2016. *Eurosurveillance*. **21**, 6-11.
332 Xia, X., Wang, Z., Fu, Y., Du, X. dang, Gao, B., Zhou, Y., He, J., Wang, Y., Shen, J., Jiang, H. and
333 Wu, Y. (2019) Association of colistin residues and manure treatment with the abundance of
334 *mcr-1* gene in swine feedlots. *Environ Int*. 361-370.

335 Yang, Y.Q., Li, Y.X., Lei, C.W., Zhang, A.Y. and Wang, H.N. (2018) Novel plasmid-mediated
336 colistin resistance gene *mcr-7.1* in *Klebsiella pneumoniae*. *J Antimicrob Chemother.* **73**, 1791-
337 1795.
338 Yin, W., Li, H., Shen, Y., Liu, Z., Wang, S., Shen, Z., Zhang, R., Walsh, T.R., Shen, J. and Wang,
339 Y. (2017) Novel plasmid-mediated colistin resistance gene *mcr-3* in *Escherichia coli*. *MBio*. **8**,
340 e00543-17.
341 Zhong, L.L., Zhou, Q., Tan, C.Y., Roberts, A.P., El-Sayed Ahmed, M.A.E.G., Chen, G., Dai, M.,
342 Yang, F., Xia, Y., Liao, K., Liang, Y., Yang, Y., Feng, S., Zheng, X. and Tian, G.B. (2019)
343 Multiplex loop-mediated isothermal amplification (Multi-LAMP) assay for rapid detection of
344 *mcr-1* to *mcr-5* in colistin-resistant bacteria. *Infect Drug Resist*. **12**, 1877-1887.
345 Zurfuh, K., Poirel, L., Nordmann, P., Nüesch-inderbinen, M., Hächler, H., Yy, L., Wang, Y., Tr,
346 W., Lx, Y., Zhang, R., Spencer, J., Doi, Y. and Tian, G. (2016) Occurrence of the plasmid-
347 borne *mcr-1* colistin resistance gene in river water and imported vegetable samples in
348 Switzerland. *Antimicrob Agents Chemother*. **60**, 2594-2595.
349

350 **Table 1.** Primers and positive controls used for the detection of *mcr-1* to *mcr-5* and 16S genes in RT-PCR assays.

Primer name	Target gene	Sequence (5′ - 3 ′)	Product length (bp)	Reference	Positive control strains	
					Strain [a]	Origin [b]
mcr1-qf mcr1-qr	*mcr-1*	AAAGACGCGGTACAAGCAAC GCTGAACATACACGGCACAG	213	(Li *et al.*, 2017)	*E. coli* 412016126	Poultry
mcr2-qf mcr2-qr	*mcr-2*	CGACCAAGCCGAGTCTAAGG CAACTGCGACCAACACACTT	92	(Li *et al.*, 2017)	*E. coli* KP37	Swine faeces
mcr3-qf mcr3-qr	*mcr-3*	ACCTCCAGCGTGAGATTGTTCCA GCGGTTTCACCAACGACCAGAA	169	(Li *et al.*, 2017)	*E. coli* 2013-SQ352	Sewage
mcr4-qf mcr4-qr	*mcr-4*	AGAATGCCAGTCGTAACCCG GCGAGGATCATAGTCTGCCC	230	This study	*E. coli* DH5a (harbouring the entire *mcr-4* gene in a pCR2 plasmid)	Swine faeces
mcr 5-qf mcr 5-qr	*mcr-5*	CTGTGGCCAGTCATGGATGT CGAATGCCCGAGATGACGTA	98	This study	*Salmonella* Paratyphi B 13-SA01718	Poultry
16S-qf 16S-qr	16S	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTAATCCTGTT	467	(Nadkarni *et al.*, 2002)		

351 [a] Reference strains were kindly provided by the EU Reference Laboratory for Antimicrobial Resistance (DTU, Denmark).
352 [b] Origin of the initially isolated strains carrying the respective *mcr* genes.

353

Figure 1. Real-time PCR amplification curves, standard curves and melting curves. (A-E) Show real-time PCR amplification and standard curves for *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4 and *mcr*-5. For each standard curve R^2, efficiency and slope are reported (F). Shows melting curves for each *mcr* gene.

Figure 2. Detection of *mcr* genes in soil and manure samples.