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frontiers

Plasmid-mediated colistin resistance among human clinical *Enterobacterales* isolates: National surveillance in the Czech Republic

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23 **Abstract**

24 The occurrence of colistin resistance has increased rapidly among *Enterobacterales* around the
25 world. We performed a national survey of plasmid-mediated colistin resistance in human
26 clinical isolates by a retrospective analysis of samples from 2009-2017 and a prospective
27 sampling in 2018-2020. The aim of this study was to identify and characterize isolates with *mcr*
28 genes from various regions of the Czech Republic using whole genome sequencing (WGS). Of
29 all 1932 colistin-resistant isolates analyzed, 73 (3.8%) were positive for *mcr* genes. Most
30 isolates carried *mcr-1* (48/73) and were identified as *Escherichia coli* (n=44)
31 and *Klebsiella pneumoniae* (n=4) of various sequence types (ST). Twenty-five isolates
32 including *Enterobacter* spp. (n=24) and *Citrobacter freundii* (n=1) carrying *mcr-9* gene were
33 detected, three of them (*Enterobacter kobei* ST54) co-harbored the *mcr-4* and *mcr-9* genes.
34 Multi-drug resistance phenotype was a common feature of *mcr* isolates and 14% (10/73)
35 isolates also co-harboured clinically important beta-lactamases including 2 isolates with
36 carbapenemases KPC-2 and OXA-48. Phylogenetic analysis of *E. coli* ST744, the dominant
37 genotype in this study, with the global collection, showed Czech isolates belonged to two major
38 clades, one containing isolates from Europe, while the second composed of isolates from
39 diverse geographical areas. The *mcr-1* gene was carried by IncX4 (34/73, 47%), IncHI2/ST4
40 (6/73, 8%) and IncI2 (8/73, 11%) plasmid groups. Small plasmids belonging to the ColE10
41 group were associated with *mcr-4* in three isolates while *mcr-9* was carried by IncHI2/ST1
42 plasmids (4/73, 5%) or the chromosome (18/73, 25%). We showed an overall low level of
43 occurrence of *mcr* genes in colistin-resistant bacteria from human clinical samples in the Czech
44 Republic.

45

46 **Keywords: antibiotic resistance; *Enterobacterales*; human; *mcr*; plasmids**

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47 **Introduction**

48 The excessive consumption of antimicrobial substances associated with faster spread of
49 antibiotic resistance represents a global concern. The dissemination of multi-drug resistant
50 (MDR) bacteria resulted in limited treatment options of infectious diseases in healthcare
51 systems. The interest in the administration of older antibiotics such as polymyxins has been
52 therefore renewed (Bitar et al., 2020). Colistin has been widely used in the past, but due to its
53 nephrotoxicity and neurotoxicity it has become a restricted antibiotic (Vińes et al., 2021).
54 Currently, colistin is administered for the treatment of life-threatening infections caused by
55 MDR Gram-negative pathogens as the last-resort antibiotic (Yilmaz et al., 2016; Hamel et al.,
56 2021; Vińes et al., 2021). In contrast, colistin has been widely used for prophylactic and
57 therapeutic purposes in veterinary medicine for decades (Quiroga et al., 2019; Vińes et al.,
58 2021). However, colistin overuse in livestock has led to the spread of colistin-resistant
59 pathogens worldwide and the development of different strategies used by bacteria to increase
60 resistance against colistin (El-Sayed Ahmed et al., 2020).
61 Resistance to colistin can be either associated with chromosomal mutations or with *mcr* genes
62 carried by plasmids that are facilitating horizontal transfer of colistin resistance between
63 bacteria (Zhu et al., 2019). Acquired colistin-resistance mechanisms have been recognized in
64 some members of Enterobacteriaceae family, such as *E. coli, Salmonella* spp., *Klebsiella* spp.,
65 and *Enterobacter* spp. These include genes and operons responsible for encoding enzymes that
66 have a direct role in LPS modification, such as the *pmrC* and *pmrE* genes and the *pmrHFIJKLM*
67 operon (Aghapour et al., 2019). Apart from the chromosomally-mediated mechanisms, 10
68 variants, *mcr-1* to *mcr-10*, carried by various plasmid families have been so far identified in
69 *Enterobacterales*, especially in *E. coli* and *Enterobacter* spp. (Bitar et al., 2020; Li et al., 2020;
70 Javed et al., 2020; Wang et al., 2020). The most common variant, *mcr-1,* is usually located on
71 plasmids of various incompatibility (Inc) groups, but predominantly on IncX4, IncI2 and
72 IncHI2 (Caratolli et al., 2014; Doumith et al., 2016; Zelendova et al., 2021). These plasmid
73 types carrying *mcr-1* were found in *Enterobacterales* isolates from humans as well as farm
74 animals around the globe (Dalmolin et al., 2018; Quoriga et al., 2019), highlighting their wide
75 distribution in various niches. Besides *mcr-1*, other genes for plasmid-mediated colistin
76 resistance, such as *mcr-4* and *mcr-9,* have been reported (Bitar et al., 2020; Li et al., 2020). The
77 *mcr-4* gene is usually located on small ColE10-type plasmids (Caratolli et al., 2017; Marchetti
78 et al., 2021) while *mcr-9* is mostly carried by large IncHI2 plasmids or is incorporated into the
79 chromosome (Li at al. 2020; Tyson et al., 2020).
80 The emergence of colistin resistance in MDR bacteria is a significant clinical concern. Isolates
81 encoding extended-spectrum beta-lactamase (ESBL) or carbapenemase on a single plasmid
82 along with *mcr* have been detected (Caratolli et al., 2014; Katip et al., 2021). As the co-
83 occurrence of more resistance genes within the bacteria represents a threat for current medicine,
84 The European Centre for Disease Prevention and Control (ECDC) published the expert protocol
85 that recommends to perform the surveillance of co-resistance to both colistin and carbapenems
86 in *Enterobacterales* (ECDC technical report).
87 From the Czech Republic, only sporadic reports describing the identification of *mcr*-carrying
88 isolates in clinical samples have been published so far (Bitar et al., 2019; Bitar et al., 2020;
89 Krutova et al., 2021), however, overview data on prevalence of *mcr* genes in Czech patients are
90 not available. To fill in this gap, we aim to identify *mcr* genes in colistin-resistant human clinical
91 isolates of Gram-negative bacteria from the Czech Republic between 2009 and 2020, and to
92 determine characteristics of the *mcr*-positive strains using whole genome sequencing (WGS),
93 plasmid typing and transferability experiments.
94

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95 **MATERIALS AND METHODS**

96 **Sampling and detection of *mcr* genes**

97 A total of 1932 colistin-resistant isolates of Gram-negative bacteria with minimum inhibitory
98 concentration (MIC) to colistin >2 mg/L collected from Czech patients between 2009 and 2020
99 were examined. The collection consisted of 682 retrospective isolates obtained from 2009 till
100 2017 during various surveillance programs at the National Institute of Public Health that were
101 not targeting colistin resistance. The retrospective collection included mainly *Klebsiella*
102 *pneumoniae* (n=429), *Enterobacter* spp. (n=108), *Pseudomonas aeruginosa* (n=49),
103 *Acinetobacter baumanii* (n=33), *Stenotrophomonas maltophilia* (n=20), *Escherichia coli*
104 (n=14) and 29 isolates of other 13 species. Prospective surveillance of colistin resistance in
105 Czech hospitals was carried out during 2,5-year period between January 2018 and June 2020.
106 It resulted in the collection of 1250 isolates of *Klebsiella pneumoniae* (n=491), *Enterobacter*
107 spp. (n=311), *Escherichia coli* (n=179), *Pseudomonas aeruginosa* (n=99), *Acinetobacter*
108 *baumanii* (n=43), *Hafnia alvei* (n=28), *Klebsiella variicola* (n=20), *Acinetobacter* spp. (n=15),
109 *Salmonella enterica* (n=15), *Klebsiella oxytoca* (n=14), *Klebsiella aerogenes* (n=10), and 25
110 isolates of other 11 species. Strain identification was performed by matrix-assisted laser
111 desorption ionization-time of flight mass spectrometer (MALDI-TOF) using MALDI Biotyper
112 software (Bruker Daltonics, Bremen, Germany). All isolates were subjected to multiplex
113 polymerase chain reaction (PCR) to detect the variant of *mcr* genes (*mcr-1* to *mcr-9*) (Rebelo
114 et al., 2018; Kieffer et al., 2019; Wang et al., 2018).
115

116 **Antimicrobial susceptibility testing**

117 Susceptibility profiles of *mcr*-positive isolates was determined by broth microdilution method
118 using the following 15 antimicrobial substances: amikacin, ampicillin, ampicillin/sulbactam,
119 cefepime, cefotaxime, cefoxitin, ceftazidime, ceftolozane/tazobactam, colistin, cotrimoxazole,
120 ciprofloxacin, gentamicin, meropenem, piperacillin/tazobactam and tobramycin. The
121 production of ESBL and AmpC type beta-lactamase was tested by double-disk synergy test
122 (EUCAST 2017). The production of carbapenemase was tested by combination disc test method
123 (EUCAST 2017) and biochemical tests (BioRad-Beta-Carba test) while carbapenem hydrolysis
124 was tested by MALDI-TOF (Papagiannitsis et al., 2015).
125

126 **Conjugative transfer of *mcr* genes**

127 Conjugation assays were performed to determine the transferability of *mcr* genes into plasmid-
128 free sodium azide-resistant *E. coli* J53 K12 recipient cells using filter-mating method
129 (Borowiak et al., 2019). The transconjugants (TCs) were selected on LB agar plates (LBA) with
130 sodium azide (100 mg/L) and colistin (0.5 mg/L). Successful transfer of the plasmid-mediated
131 colistin resistance via conjugation was confirmed by PCR targeting the *mcr* gene (Rebelo at al.
132 2018, Kieffer et al., 2019; Wang et al., 2018) and *E. coli* J53 K12 (Bauer et al., 2007). The size
133 and number of plasmids transferred were estimated by pulsed-field gel electrophoresis (PFGE)
134 using S1 nuclease (CDC 2004) and PCR-based replicon typing (PBRT; Carattoli et al., 2005).
135

136 **Whole genome sequencing and plasmid characterization**

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137 Genomic DNA of all *mcr*-positive isolates was extracted using NucleoSpin® Tissue kit
138 (Macherey-Nagel GmbH & Co, Duren, Germany). The libraries were prepared using Nextera
139 XT DNA Sample Preparation Kit and sequenced on MiSeq or NovaSeq 6000 platform
140 (Illumina, San Diego, CA, USA). Raw reads were quality- and adaptor-trimmed using
141 Trimmomatic v0.39 (Bolger at al., 2014) and assembly was performed by SPAdes v3.12.0
142 (Bankevich et al., 2012) and assembled data were analyzed using the CGE tools
143 (https://cge.cbs.dtu.dk/) that were used to identify antibiotic resistance genes (ResFinder 4.1)
144 (Zankari et al., 2012), multi-locus sequence types (MLST 2.0) (Larsen et al., 2012), plasmid
145 replicons (PlasmidFinder 2.1) and plasmid sequence types (STs) (pMLST 2.0) (Carattoli et al.,
146 2014). Chromosomal mutations for resistance to fluoroquinolones and colistin in *E. coli* and *K.*
147 *pneumoniae* isolates were determined by PointFinder (Zankari et al., 2017). Sequences of six
148 IncX4 plasmids carrying *mcr-1* were extracted from Illumina data and gaps were filled by PCR-
149 based strategy and Sanger sequencing.
150 Complete nucleotide sequence of 12 selected isolates was obtained using long-read sequencing
151 on MinION platform (Oxford Nanopore technologies, ONT, Oxford, UK). Genomic DNA was
152 extracted by Genfind V3 (Beckman Coulter, USA). Libraries were constructed using a SQK-
153 RBK004 rapid barcoding 1D kit according to the manufacturer's protocol. The barcoded library
154 mix was loaded onto a flow cell (FLO-MIN106 R9.4 SpotON) and sequenced for 48 h. The raw
155 electrical signals were basecalled using Guppy v4.2.2 (ONT) and raw reads in fastq format were
156 obtained. BBDuk (https://jgi.doe.gov/data-and-tools/software-tools/bbtools/bb-tools-user-
157 guide/) and Porechop v0.2.4 (ONT) were used for adaptor and quality trimming ($Q \leq 7$) and for
158 demultiplexing, respectively. Whole plasmid sequences were assembled using Unicycler v0.4.8
159 (Wick et al., 2017) and Flye v2.6 (Lin et al., 2016) and polished by Illumina reads using Pilon
160 v1.23 (Walker et al., 2014). For sequence analysis and annotation, BLAST
161 (www.ncbi.nlm.nih.gov/BLAST), the ISfinder database, and the open reading frame (ORF)
162 finder tool (www.bioinformatics.org/sms/) were used. Comparative genome alignment with
163 corresponding reference plasmids was performed using Mauve v.2.3.1 (Darling et al., 2010).
164 Figures were generated from sequence data using BRIG v.0.95 (Alikhan et al., 2011) and
165 clinker v0.0.23 (Gilchrist and Chooi, 2021).
166

167 Phylogenetic analysis

168 In total, four different datasets were subjected to phylogenetic analysis. Two of them were local
169 phylogenetic trees including only isolates from our collection: the first one comprised all
170 detected *mcr*-carrying *E. coli* isolates and the second one showed the phylogeny of
171 *Enterobacter* spp. genomes. The third tree was global and comprised genomes of 449 *E. coli*
172 ST744 isolates that were available at EnteroBase in April 2021
173 (http://enterobase.warwick.ac.uk/) along with ten ST744 isolates from our collection. These
174 three trees were generated based on a core-genome determined employing a Roary pipeline
175 v3.12.0 (Page et al., 2015) and aligned with MAFFT v7.313 (Katoch et al., 2013). Trees were
176 inferred under GTR+CAT model using FastTree v2.1.11 (Price et al., 2010) compiled with
177 double precision arithmetic.
178 Remaining detailed tree topology was constructed based on a pipeline described in previous
179 study (Forde et al., 2022) using Python scripts that are available on GitHub
180 (https://github.com/matejmedvecky/anthraxdiversityscripts). Based on *E. coli* ST744 global
181 tree, 38 Illumina SRA archives belonging to isolates that were closely related to ten ST744
182 isolates from our collection were gathered from the GenBank database in May 2021. Raw
183 sequencing reads of those 38 isolates along with another ten from our collection were subjected
184 to quality trimming via Trimmomatic tool v0.36 (Bolger et al., 2014) and consequently mapped
185 to *E. coli* str. K-12 substr. MG1655 reference genome (GenBank accession U00096.3) using

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186 Bowtie2 v2.3.4.2 (Langmead et al., 2012). Single nucleotide polymorphisms (SNPs) were
187 detected in individual isolates by VarScan v2.4.4 (Koboldt et al., 2012) using following
188 parameters: minimum read depth of 8; minimum base quality of 20; variant allele frequency ≥
189 0.80. Problematic sites were then removed based on the following rules: occurred in phage
190 regions as detected by PHASTER (Arndt et al., 2016); occurred in repetitive/homologous
191 genomic regions; more than 5 isolates at a particular site showed prevalent base frequency
192 below 80% or/and read depth below 8. Resulting alignment file was then subjected to
193 maximum-likelihood analysis using RAxML v8.2.11 (Stamatakis, 2014) under GTR+CAT
194 model of nucleotide substitution with 500 rapid bootstrap replicates using sample SRR9990292
195 as an outgroup. Tree topologies were visualised via iTOL v6.3 (Letunic and Bork 2021) and
196 edited using Inkscape v1.1 (https://inkscape.org/cs/).
197 Species level discrimination of *Enterobacter* spp. was performed using average nucleotide
198 identity (ANI) (Yoon et al., 2017) and digital DNA-DNA hybridization (Meier-Kolthoff et al.,
199 2013) of whole genome sequences. Eight type strains were used as reference species including
200 *Enterobacter asburiae* (ATCC35953^{T}), *Enterobacter bugandensis* (DSM 29888^{T}),
201 *Enterobacter cloacae* ATCC13047^{T}), *Enterobacter dykesii* (DSM111347^{T}), *Enterobacter*
202 *hormaechei* (ATCC49162^{T}), *Enterobacter kobei* (DSM13645^{T}), *Enterobacter vonholyi*
203 (DSM110788^{TT}), *Enterobacter roggenkampii* (DSM16690^{T}).
204

205 **Nucleotide sequence accession numbers**

206 Genome assemblies, SRA archives and annotated plasmid sequences (Table 1) were deposited
207 in NCBI under BioProject with accession number PRJNA772899.
208

209 **Results**

210 ***mcr*-positive *Enterobacterales* isolates**

211 From all 1932 examined colistin-resistant isolates, 73 (3.8%) were identified to carry *mcr* genes
212 (Supplementary Table S1). Most (65/73) isolates were detected during the prospective years
213 including eight isolates in 2018 (3%, n=274), 27 isolates in 2019 (4%, n=634) and 30 isolates
214 in 2020 (9%, n=342). From the retrospective analysis using a collection of isolates at National
215 reference laboratory for antibiotics, seven isolates (1%, n=682) carrying *mcr* genes were found
216 including seven *Enterobacter* spp. from 2010 (n=1), 2012 (n=3), 2013 (n=1), 2014 (n=2) and
217 one isolate of *K. pneumoniae* (n=1) from 2017. Isolates carrying *mcr-1* were identified as *E.*
218 *coli* (n=44) and *K. pneumoniae* (n=4), while the remaining 25 isolates carried the *mcr-9.1* allele.
219 Three of the *mcr-9.1*-carrying isolates were also positive for *mcr-4.2*/*mcr-4.3*. The isolates
220 carrying *mcr-9.1* were identified as *Citrobacter freundii* (n=1), *Enterobacter asburiae* (n=13),
221 *Enterobacter kobei* (n=6), *Enterobacter cloacae* (n=3). *Enterobacter roggenkampii* (n=1) and
222 *Enterobacter hormaechei* (n=1). Plasmid-mediated colistin resistance genes were the most
223 common among *E. coli* as 19% (44/231) colistin-resistant isolates carried *mcr-1* while the
224 occurrence in other species was rare (4.4% in *Enterobacter* spp., 0.4% in *K. pneumoniae*).
225 Colistin-resistant isolates carrying *mcr-1* (48/73, 66%) showed phenotypic resistance to beta-
226 lactam antibiotics including ampicillin (46/48, 96%), ampicillin/sulbactam (43/48, 90%),
227 cefoxitin (9/48, 19%), piperacillin/tazobactam (9/48, 19%), cefotaxime (5/48, 10%),
228 ceftazidime (5/48, 10%), cefepime (4/48, 8%) and ceftolozane/tazobactam (2/48, 4%).
229 Resistance to other antimicrobials including cotrimoxazole (34/48, 71%), ciprofloxacin (34/48,
230 71%), trimethoprim (34/48, 71%), gentamicin (11/48, 23%), tobramycin (10/48, 21%) and
231 amikacin (1/48, 2%) was found.

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232 The majority of colistin-resistant isolates carrying *mcr-9* were resistant to cefoxitin (25/25,
233 100%), ampicillin (23/25, 92%), ampicillin/sulbactam (23/25, 92%) and cefotaxime (12/25,
234 48%). Furthermore, they showed resistance to ceftazidime (9/25, 36%), cotrimoxazole (6/25,
235 24%), ciprofloxacin (5/25, 20%), tobramycin (5/25, 20%), trimethoprim (5/25, 20%),
236 piperacillin/tazobactam (7/25, 28%), gentamicin (4/25, 16%), cefepime (3/25, 12%), amikacin
237 (2/25, 8%), ceftolozane/tazobactam (2/25, 8%) and meropenem (2/25, 8%).
238 Nine isolates including *E. coli* (n=4), *Enterobacter* spp. (n=3), *Citrobacter freundii* (n=1) and
239 *K. pneumoniae* (n=1) with resistance to seven or more different antibiotics were simultaneously
240 positive for ESBL production. AmpC beta-lactamase was detected in six *Enterobacter* spp.
241 isolates and one *E. coli* isolate.
242

243 **Analysis of WGS results**

244 The *mcr-1*-positive *E. coli* isolates belonged to 26 various STs of which the *E. coli* ST744 was
245 the most common (10/44). Fifteen *E. coli* isolates were assigned to ST88 (n=3), ST538 (n=3),
246 ST1011 (n=3), ST69 (n=2), ST162 (n=2), and ST453 (n=2), while the remaining nineteen
247 isolates belonged to unique STs (Table S1, Figure 1). Four *K. pneumoniae* isolates carrying
248 *mcr-1* were assigned to four different STs (ST147, ST231, ST290 and ST726) and one *C.*
249 *freundii* isolate with *mcr-9* belonged to ST18. *Enterobacter* spp. with *mcr-9* belonged
250 predominantly to *E. asburiae* of two different STs including ST484 (n=11) and ST358 (2)
251 (Figure 2). Six isolates of *E. kobei* belonged to ST32 (n=1), novel ST (n=1), ST591 (n=1) and
252 ST54 (n=3). All *E. kobei* ST54 isolates originated from patients in a single hospital and carried
253 *mcr-4* apart from *mcr-9*. Remaining two isolates were identified as *E. cloacae* ST1525 and were
254 obtained from one patient.
255 Most *mcr-1*-positive isolates carried genes (Supplementary Table S1) conferring resistance to
256 aminoglycosides (36/48), macrolides (42/48), sulphonamides (36/48), tetracycline (33/48) and
257 trimethoprim (31/48). Additionally, 44 *mcr-1*-positive isolates harboured genes for resistance
258 to narrow-spectrum beta-lactams including *bla*$_{\text{TEM-1B}}$ (n=27), *bla*$_{\text{TEM-135}}$ (n=4) and *bla*$_{\text{TEM-32}}$
259 (n=3). In two isolates, AmpC beta-lactamase genes *bla*$_{\text{CMY-2}}$ or *bla*$_{\text{DHA-1}}$ were detected while in
260 three isolates, ESBL genes *bla*$_{\text{CTX-M-1}}$ (n=1), *bla*$_{\text{CTX-M-27}}$ (n=2) were found. All four *mcr-1*-
261 positive *K. pneumoniae* isolates carried *fosA*, *oqxA*, *oqxB* and *bla*$_{\text{SHV}}$ genes.
262 On the other hand, the majority of *mcr-9*-positive isolates contained resistance genes to beta-
263 lactams (n=25) and fosfomycin (n=22). Specifically, the *E. asburiae* ST484 isolates carried the
264 *bla*$_{\text{ACT-6}}$ gene, encoding the intrinsic AmpC beta-lactamase, while the *E. asburiae* ST358
265 isolates carried *bla*$_{\text{ACT-9}}$ or *bla*$_{\text{ACT-10}}$ variants (Figure 2). The two *E. cloacae* ST1525 isolates
266 harboured only *bla*$_{\text{CMH-3}}$ gene, encoding the chromosomal AmpC. The ST54 *E. kobei* isolates
267 carried the *bla*$_{\text{ACT-9}}$ and *fosA* genes. Moreover, ESBL (*bla*$_{\text{CTX-M-9}}$, *bla*$_{\text{CTX-M-15}}$) and AmpC beta-
268 lactamase (*bla*$_{\text{DHA-1}}$, *bla*$_{\text{CMY-117}}$) were identified in three and two *mcr-9*-positive isolates,
269 respectively. One *E. hormaechei* ST91 isolate carried carbapenemase gene *bla*$_{\text{OXA-48}}$. The single
270 *Citrobacter freundii* isolate carried five beta-lactamase genes including carbapenemase-
271 encoding gene *bla*$_{\text{KPC-2}}$.
272 Chromosomal mutations to fluoroquinolones (*acrR*), cephalosporins (*ompK36*) and
273 carbapenems (*ompK37*) were identified in four *K. pneumoniae* isolates (Table S1). In contrast
274 with the susceptibility testing, three of these *K. pneumoniae* isolates were resistant to
275 fluoroquinolones and only one to cephalosporins. Twenty-five *E. coli* isolates carried at least
276 one of four mutation variants including *gyrA, gyrB, parC* and *parE* for resistance to quinolones
277 and thirteen *E. coli* isolates carried quinolone-resistance genes *qnrB* (n=5) or *qnrS* (n=8) (Table
278 S1) while MIC profiles showed quinolone resistance in thirty-one isolates. Nine different types
279 of mutations in *pmrA/pmrB* genes associated with colistin resistance were also found in eleven
280 *E. coli* isolates (Table S1).

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281

282 **Phylogenetic analysis of *E. coli* ST744 and *Enterobacter cloacae* ST484**

283 Phylogenetic analysis of ST744 isolates from our collection (n=10) along with other 38 closely
284 related genomes from public resources showed formation of 2 major clades; four isolates were
285 considered as outliers (Figure 3). First major clade (samples 60462 – 54343, green branch)
286 comprised 17 mostly European isolates from animals and humans, and was further divided into
287 two subclades. Second dominant clade (samples ERR3531597 – ERR1971525, violet branch)
288 was composed of 27 cosmopolitan isolates originating from various sources, and was divided
289 into several smaller subclades. Isolates belonging to different major clades showed a variable
290 number of pairwise SNP differences against each other, ranging from 600 up to 3000. In both
291 major clades, there were apparent clusters of isolates from humans and animals, exhibiting few
292 dozens of SNPs from each other (Supplementary Table S2). Isolates from our collection were
293 scattered across the tree, five of them belonged to the first clade, four to second clade, while
294 one sample was an outlier. Our clinical samples 48907, 52857, 55923 and 54343, belonging to
295 the first clade, showed 46-58 SNP differences from three clinical samples from Germany, and
296 39-49 SNP differences from the two Romanian (RO) isolates from poultry that carried $bla_{\text{CMY-}}$
297 $_{\text{2}}$. One of the RO isolates also carried the *mcr-1.1* gene that was borne by all isolates from our
298 Czech clinical collection. Those RO isolates were also closely related to three clinical isolates
299 from Germany (exhibited <20 SNP differences from each other). Isolate 60061 from the Czech
300 collection clustered with clinical isolate from Thailand (110 SNPs) and Chinese isolate from a
301 pig (128 SNPs). Notably, the Swiss isolate also carried *mcr-1.1*. Our isolates 45082 and 54444
302 were related to another clinical isolate from the United Kingdom (66 and 73 SNPs) and also to
303 an environmental isolate from a river in Japan (73, and 80 SNPs, respectively). Isolate 52637
304 from our collection showed the least SNP counts against three Australian isolates from gulls
305 (36-37 SNPs) and three clinical isolates, one coming from Switzerland (36 SNPs), and another
306 from Germany (39 SNPs) and Russia (42 SNPs).
307

308 **Structure of *mcr-1*-carrying plasmids**

309 The *mcr-1* gene was located predominantly on 33 kb IncX4 plasmids (34/48). Six complete
310 plasmids from *E. coli* and *K. pneumoniae* obtained by long-read sequencing (Table 1) showed
311 high level of nucleotide similarity (>99.9%) to each other as well as to plasmids from raw meat
312 from Czech retails (Zelendova et al., 2021) (Supplementary Figure S1). The *mcr-1* gene was
313 bordered by a hypothetical protein and a PAP2 transmembrane protein, which is the typical
314 genetic surrounding for *mcr-1* gene within IncX4 plasmids (Zelendova et al., 2021).
315 The *mcr-1* was also carried by ~60 kb IncI2 plasmids (n=8). Plasmid pMCR1-53288 originating
316 from *E. coli* ST538 from urine obtained by MinION sequencing shared high sequence similarity
317 (>98%) with several plasmids available in GenBank database including pMCR_1884_C3 and
318 pMCR_1138_A1 from *C. braakii* and *E. coli* ST162, respectively, isolated from imported meat
319 sold in Czech retails (Zelendova et al., 2021) (Figure S2). The *mcr-1* region was inserted
320 downstream the *nikB* gene, encoding a DNA topoisomerase III, as observed in other IncI2 *mcr-*
321 *1*-positive plasmids like pMCR_1884_C3. No other resistance genes were located on IncI2
322 plasmids.
323 From our collection, six *Enterobacterales* isolates were found to harbour IncHI2 plasmids with
324 *mcr-1* gene. The complete sequence of three *mcr-1* positive IncHI2 plasmids pMCR1-59496,
325 pMCR1-43934 and MCR1-51133 was determined using MinION technology. BlastN analysis
326 showed that all sequenced IncHI2 plasmids, ranging from ~225 kb to ~255 kb in size, belonged
327 to ST4 and were closely related (coverage 80-99%, identity 99%) to each other (Figure 4), as

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328 well as to other *mcr-1*-carrying IncHI2 plasmids, like pMCR_915_C1 and pMCR_1085_C1
329 from *E. coli* recovered from imported meat (Zelendova et al., 2021), and plasmid pKP121-1-
330 mcr (Ruan et al., 2019) of human clinical origin from China. All IncHI2 plasmids contained
331 regions for conjugative transfer (*htd*, *orf*, *tra* genes) and plasmid maintenance (*par* gene).
332 Additionally, IncHI2 plasmids carried tellurium resistance genes in two clusters including
333 *terZABCDEF* and *terXYW* (except p56099). In all InHI2 plasmids, characterized during this
334 study, *mcr-1* gene was inserted downstream the *terY2* gene, as observed in other IncHI2
335 plasmids like pMCR_1085_C1. Similar to pMCR_1085_C1, the *mcr-1* gene was bounded by
336 an IS*Apl1* element and PAP2 transmembrane protein (Zelendova et al., 2021). All IncHI2 *mcr-*
337 *1*-positive plasmids exhibited at least one MDR region, which ranged in size from 950 to 36097
338 bp (Figure 4).
339

340 **Structure of *mcr-4*-encoding plasmids**

341 *mcr-4* was located on ColE10 plasmids in three *E. kobei* ST54 isolates. Plasmid pMCR4-26153
342 of size 12,808 kb recovered from a rectal swab of a patient in the Czech Republic, was identical
343 (100% coverage, 100% identity) to pIB2020_ColE_MCR (Marchetti et al. 2021) from *E. kobei*
344 ST54 strain from a rectal swab of a 56 years old male patient hospitalized in 2019 in Italy
345 (Figure S3).
346

347 **Structure of *mcr-9.1*-carrying elements**

348 Out of the twenty-five *mcr-9.1*-positive isolates, eight were characterized by MinION
349 technology. Among the latter isolates, three carried the *mcr-9.1* allele on IncHI2 plasmids
350 (Table 1) while, in the five remaining isolates, the *mcr-9.1* was found on the chromosome.
351 Plasmid pMCR9-57185 originated from *C. freundii* ST18 recovered from rectal swab while
352 pMCR9-16539 was obtained from *E. kobei* ST591 from blood and pMCR9-17620 came from
353 *E. hormaechei* ST91 recovered from wound swab.
354 Following the IncHI2 pDLST scheme, plasmids pMCR9-57185 and pMCR9-16539 were typed
355 as ST1, while pMCR9-17620 that differed by a single nucleotide polymorphism in *smr0199*
356 locus was assigned to a novel ST. All plasmids exhibited closely related sequences (>89%
357 coverage, 99.99% identity) to other *mcr-9.1*-positive IncHI2 plasmids (Figure S4), like
358 p49790_MCR from an *E. hormaechei* isolate recovered previously from Czech hospitals (Bitar
359 et al., 2020). Similar to p49790_MCR, the *mcr-9.1* was inserted upstream the *pcoS* gene
360 (encoding a membrane protein for resistance to copper), in all IncHI2 plasmids like
361 p49790_MCR. Additionally, in plasmids pMCR9-57185 and pMCR9-16539, the *mcr-9.1* gene
362 was bounded by an IS element (upstream) and an ORF (downstream), encoding a cupin fold
363 metalloprotein, followed by IS*26*. However, in plasmid pMCR9-17620, an IS*1* was found
364 downstream of *mcr-9.1*. Furthermore, IncHI2 plasmids contained at least one MDR region
365 including genes for resistance to aminoglycosides, tetracyclines, trimethoprim,
366 chloramphenicol, sulfonamides, quinolones, and/or macrolides (Table 1). Moreover, IncHI2
367 plasmids carried tellurium resistance genes (*terZABCDEF*) commonly associated with this
368 plasmid family, and genes conferring arsenic resistance (*arsCBRH*).
369 The *mcr-9.1* gene was integrated into the chromosomes of four *E. cloacae* complex isolates
370 obtained by long-read assembly. The upstream genetic surroundings were identical in all
371 isolates consisting of *mcr-9.1*, IS*903B*, *pcoS*, *pcoE*, *rcnA* and *rcnR* genes while the downstream
372 sequences differed. In isolates 50607 and 59720, the *mcr-9.1* was followed (downstream) by
373 *wbuC*, IS*26* and IS*1A* forming a region *rcnR-rcnA-pcoE-pcoS*-IS*903B-mcr-9.1-wbuC*-IS*26*
374 identical to the respective region of the plasmid pMCR9-57185. On the other hand, the

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375 downstream environment of *mcr-9.1* in isolates 56674 and 57166 consisted of *wbuC*, *qseC*,
376 *qseB* and ATPase ORF similar to the corresponding region in the chromosome of a Japanese
377 human isolate *Enterobacter asburiae* A2563 (AP022628), visualized in Figure 5.
378

379 **Horizontal transfer of *mcr* gene**

380 Resistance to colistin associated with *mcr-1* was transferred to recipient *E. coli* laboratory
381 strains via conjugation in the majority of *E. coli* isolates (38/44, 86%) and in all isolates of *K.*
382 *pneumoniae* (4/4, 100%). The most frequently transferred plasmid harbouring *mcr-1* included
383 33 kb plasmid IncX4 (31/34, 91%), followed by 55 kb IncI2 (8/8, 100%) and 220 kb IncHI2
384 (4/6, 67%) plasmids. IncHI2 plasmid with *mcr-9* was transferred via conjugation in one *E.*
385 *asburiae* isolate. Also, ColE10 plasmid carrying *mcr-4* was not transferred via conjugation,
386 since this plasmid family does not contain any transfer region.
387

388 **Structure of carbapenemase-encoding plasmids**

389 The $bla_{\text{OXA-48}}$ was carried by a 63.7 kb IncL plasmid pOXA_17620 (OQ127401) of the *E.*
390 *hormaechei* ST91 isolate. The plasmid was identical with a previously described plasmid
391 pRIVM_C012525_20 (CP068332). The $bla_{\text{OXA-48}}$ was surrounded by IS*10A* and IS*1R* upstream
392 and by LysR family transcriptional regulator and IS*10A* downstream (Figure S5).
393 The $bla_{\text{KPC-2}}$ was carried by a 50.4 kb IncR plasmid pKPC_57185 (OQ127401) of the *C.*
394 *freundii* ST18 isolate. The gene was surrounded by Tn*4401* and IS*Kpn7* upstream, and DNA
395 resolvase and Tn*5403* downstream. The pKPC_57185 plasmid was similar (coverage 92%,
396 identity 100%) to a previously described IncR plasmid p46903_KPC (CP070521) (Figure S5).
397 However, the pKPC_57185 carried a MDR region identical with a larger IncN-IncR plasmid
398 (CP070576) from the same study, as visualised in Figure S5.
399

400 **Discussion**

401 Within this study, we performed a surveillance of *mcr*-encoding genes among colistin-resistant
402 *Enterobacterales* collected from Czech hospitals between 2009 and 2020. Our findings
403 indicated a low prevalence (3.8%) of *mcr* genes among colistin-resistant isolates with slightly
404 increasing prevalence during the study period (3% in 2018 while 9% in 2020). However, the
405 prevalence of *mcr*-positive isolates may be overestimated since our collection was composed
406 only of colistin-resistant isolates and did not include the bacterial population susceptible to
407 colistin. Moreover, another study limitation is the fact that isolates from retrospective sampling
408 from the period 2009-2017 were obtained during various surveillance programs at the National
409 Institute of Public Health. As these programs were focused mainly on *Klebsiella* sp, invasive
410 *E. coli* or they were targeting MDR strains, the data of *mcr* prevalence from this period needs
411 to be interpreted with caution.
412 Another Czech study (Tkadlec et al., 2021) published a low prevalence of the *mcr-1*
413 gene (4/1922, 0.21%) in *E. coli* from fecal samples from hospitalized patients between June
414 2018 and September 2019. A study from Switzerland reported that the fecal carriage rate of
415 colistin-resistant (MIC value >2 mg/l) *Enterobacterales* was 1.5% for healthy people and 3.8%
416 for primary care patients, while none of the isolates harboured the *mcr-1* or *mcr-2* genes
417 (Zurfluh et al. 2017). Additionally, in Finland, only one *mcr-1*-positive *E. coli* was
418 characterized from fecal samples collected from 176 healthy volunteers (Kirsi Gröndahl-Yli-
419 Hannuksela 2018), during 2016. Other studies from Europe have reported low prevalence of

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420 colistin-resistant isolates and of *mcr*-positive *Enterobacterales*. In Spain, the overall prevalence
421 of colistin resistance in clinical isolates of *Enterobacterales* was 0.67%. The rate was higher in
422 *E. cloacae* (4.2%) than *E. coli* (0.5%) and *K. pneumoniae* (0.4%) while *mcr-1* was detected
423 only in *E. coli* (0.15%) (Prim et al. 2017). Similar prevalence levels were observed for
424 Romagna, Northern Italy, where the prevalence of colistin-resistant isolates among human
425 *Enterobacterales* was 0.5% and the *mcr-1* gene was found in 0.14% *E. coli* isolates (Bianco
426 2018). On the other hand, higher percentages have been reported regarding the prevalence of
427 colistin-resistant isolates in different geographical areas. Giani et al. (2018) reported a high
428 proportion (38.8%) of *mcr-1* carriers among healthy children (129/337) from Bolivia.
429 Furthermore, in Chinese hospitals across 24 provincial capital cities and municipalities, human
430 carriage of *mcr-1*-positive *E coli* was identified in 644 (14.3%) of 4498 samples in 2016 (Wang
431 2020). However, different methodological approaches and study design (e.g., selective
432 cultivation on colistin-supplemented media, targeting isolates despite their susceptibility
433 profiles, PCR detection of *mcr* genes in either total enterobacterial microbiota or directly in a
434 clinical sample) significantly limit the comparison of prevalence data between the studies.
435 Moreover, the discrepancy in the prevalence of *mcr* carriers between studies and geographical
436 regions underlines the other factors, like antibiotic use and stewardship protocols, contributing
437 in the emergence and spread of colistin-resistant isolates.
438 In this study, we found *pmrA* or *pmrB* genes mutations associated with chromosomal colistin
439 resistance in *E. coli* isolates carrying *mcr* (11/44, 25%). However, our collection also contained
440 twenty-four *Enterobacter* spp. isolates and one *C. freundii* in which colistin resistance is often
441 associated with mutations in two-component regulator systems PmrAB and PhoPQ (Hong and
442 Ko, 2019; Wand and Sutton, 2020) and these mutations were not the target of the study.
443 Majority of those isolates carried *mcr-1* allele (n=48) while twenty-two isolates harboured the
444 *mcr-9.1* allele and the three remaining isolates co-carried the *mcr-4.2*/*mcr-4.3* and *mcr-9.1*
445 genes. These results are consistent with the current global epidemiology of *mcr* genes where
446 *mcr-1* and *mcr-9* are most widely disseminated (Ling et al., 2020). Most *mcr-1* carriers were *E.*
447 *coli* and as the gene was present in 23% of all resistant isolates of this species, which is in
448 agreement with findings of previous study (Zelendova et al., 2021). Additionally, a
449 retrospective screen of colistin-resistant *Enterobacterales* reported in the National Institute of
450 Public Health from Czech hospitals, during 2009-2017, revealed the presence of nine additional
451 isolates carrying *mcr* genes. Eight of the latter isolates produced MCR-9, whereas the *E. kobei*
452 ST54 strain also expressed the MCR-4 protein. The remaining isolate, a *K. pneumoniae* ST2590
453 isolated in 2017, produced MCR-1.1 protein. Most of *mcr-9.1*-carrying isolates belonged to
454 *Enterobacter* spp. Previous studies have shown that *mcr-9* gene is commonly associated with
455 isolates belonging to *Salmonella* and *Enterobacter* genus (Zhang 2022; Liao 2022; Bitar et al.,
456 2020). Interestingly, the low resistance levels to colistin of MCR-9-producing *Enterobacter*
457 isolates has been reported (Bitar et al., 2020). This observation may explain the unnoticed
458 spread of those isolates in Czech hospitals. Remarkably, 96% of isolates (70/73) carried
459 AmpC/ESBL or carbapenemases, raising the concern that the spread of *mcr*-carrying isolates
460 might also be related to the use of other antimicrobial agents including clinically important
461 beta-lactams. IncHI2 plasmids carrying *mcr-9.1* harboured also genes for resistance to
462 aminoglycosides, beta-lactams, trimethoprim, sulphonamides and/or tetracyclines (Table 1).
463 MLST revealed the presence of *mcr* genes in various STs of *E. coli*, *K. pneumoniae*, and
464 *Enterobacter* sp., highlighting the significant impact of horizontal gene transfer in the spread
465 of colistin resistance determinants via plasmids. Phylogenetic analysis of *E. coli* ST744 isolates,
466 the dominant *E. coli* genotype, showed formation of 2 major clades (Figure 3). Five isolates
467 from our collection belonged to the first clade, which comprised mostly European isolates from
468 animals and humans, were closely related with three clinical isolates from Germany and two
469 samples of poultry origin from Romania. The second dominant clade contained four isolates

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470 from our collection, which were closely related with isolates from different geographical areas
471 and various sources (Figure 3). Additionally, phylogenetic analysis uncovered the association
472 of *mcr* genes with specific clones, like *E. kobei* ST54, which has been previously reported to
473 produce MCR-4.3 from clinical samples recovered in Italy (Marchetti et al., 2021). Of note,
474 these observations underline the important role of travelling across the borders, that has
475 contributed to the spread of MDR bacteria.
476 Finally, analysis of *mcr*-carrying plasmid sequences showed the presence of *mcr-1*, mainly on
477 IncX4 replicons, but also on IncI2 and IncHI2 plasmids. On the other hand, the *mcr-9* allele
478 was found on IncHI2 plasmids (n=3) or it was integrated into the chromosome of *Enterobacter*
479 isolates (n=5). The *mcr-4* gene was located on ColE10 plasmids. These findings are in
480 agreement with the previously published data, showing the emergence of *mcr* genes on the
481 specific Inc groups of plasmids that were characterized from *Enterobacterales* recovered from
482 different sources including animals, food and humans (El Garch et al., 2018; Xavier et al., 2016;
483 Zurfluh et al., 2017; Bitar et al., 2020; Li et al., 2020; Zelendova et al., 2021; Marchetti et al.,
484 2021). Furthermore, our experiments demonstrated a high efficiency of conjugative transfer of
485 *mcr-1*-carrying IncX4 plasmids. Also, the conjugative transfer of IncHI2 plasmids carrying
486 *mcr-1* or *mcr-9* was confirmed. Thus, the horizontal transfer of plasmid-mediated *mcr* genes
487 represents an important risk factor for public health since colistin is considered as one of the
488 last-resort antibiotics for the treatment of serious infections in human medicine. Therefore,
489 studying the spread of MDR pathogens is vital for analysis of transmission pathways and risk
490 factors for public health.
491 The prospective epidemiological survey performed in this study brought the first information
492 on the plasmid-mediated dissemination in the Czech Republic and showed that a surveillance
493 system is essential to monitor the diffusion of plasmid mediated colistin resistance.
494

Table 1. The characteristics of sequenced *mcr*-encoding plasmids

Plasmid name	Organism (ST)[1]	Plasmid-carrying *mcr*[2]	*mcr*	Other ARGs in *mcr* plasmid[3]	WGS platform	Accession no.
pMCR1-40331	*K. pneumoniae* (ST290)	IncX4 (33,303)	*mcr-1.1*	-	Illumina	OP428973
pMCR1-42913	*E. coli* (ST448)	IncX4 (33,304)	*mcr-1.1*	-	Illumina	OP428974
pMCR1-44158	*E. coli* (ST156)	IncX4 (33,303)	*mcr-1.2*	-	Illumina	OP428975
pMCR1-44653	*E. coli* (ST1196)	IncX4 (33,303)	*mcr-1.1*	-	Illumina	OP428976
pMCR1-45082	*E. coli* (ST744)	IncX4 (34,068)	*mcr-1.1*	-	Illumina	OP428977
pMCR1-46049	*K. pneumoniae* (ST147)	IncX4 (33,303)	*mcr-1.1*	-	Illumina	OP428978
pMCR1-53288	*E. coli* (ST538)	IncI2 (60,733)	*mcr-1.1*	-	MinION	OP434482
pMCR1-43934	*E. coli* (ST8186)	IncHI2/ST4 (225,732)	*mcr-1.1*	*aph(6)-Id, aph(3")-Ib, catA1, tet*(A)	MinION	OP950834
pMCR1-51133	*E. coli* (ST117)	IncHI2/ST4 (237,743)	*mcr-1.1*	*aadA1, aadA2b,* $bla_{\text{TEM-1B}}$, *catA1, cmlA1, qacE, sul1, tet*(A)	MinION	OP950835
pMCR1-59496	*K. pneumoniae* (ST726)	IncHI2/ST3 (254,909)	*mcr-1.1*	*aac(3)-IV, aadA1, aadA2b aph(3')-Ia, aph(4)-Ia,* $bla_{\text{CTX-M-14}}$, *cmlA1, floR, fosA3, mph*(A), *sul2, sul3*	MinION	OP950836
pMCR9-16539	*E. kobei* (ST591)	IncHI2/ST1 (285,283)	*mcr-9.1*	*aadA2b, aph(3")-Ib, aph(6)-Id,* $bla_{\text{TEM-1B}}$, $bla_{\text{SHV-12}}$, *catA2, dfrA19, qacE, qnrA1, sul1, tet*(D)	MinION	OP950838
pMCR9-17620	*E. hormaechei* (ST91)	IncHI2/ST1 (276,870)	*mcr-9.1*	*aadA2b, ant(2")-Ia,* $bla_{\text{CTX-M-9}}$, *catA1, dfrA16, qacE, qnrA1, sul1*	MinION	OP950833
pMCR9-57185	*C. freundii* (ST18)	IncHI2/ST1 (330,692)	*mcr-9.1*	*aac(6')-IIc,* bla_{TEM-1B}, $bla_{\text{DHA-1}}$, *catA2, ere*(A), *qacE, qnrB4, sul1, tet*(D)	MinION	OP950837
pMCR4-26153	*E. kobei* (ST54)	ColE10 (12,808)	*mcr-4.2*	-	MinION	OP428979

[1]ST, sequence type.
[2]Plasmid carrying *mcr* include the information on plasmid incompatibility group (Inc), ST (if available) and plasmid size in bp.
[3]ARGs, antibiotic resistance genes

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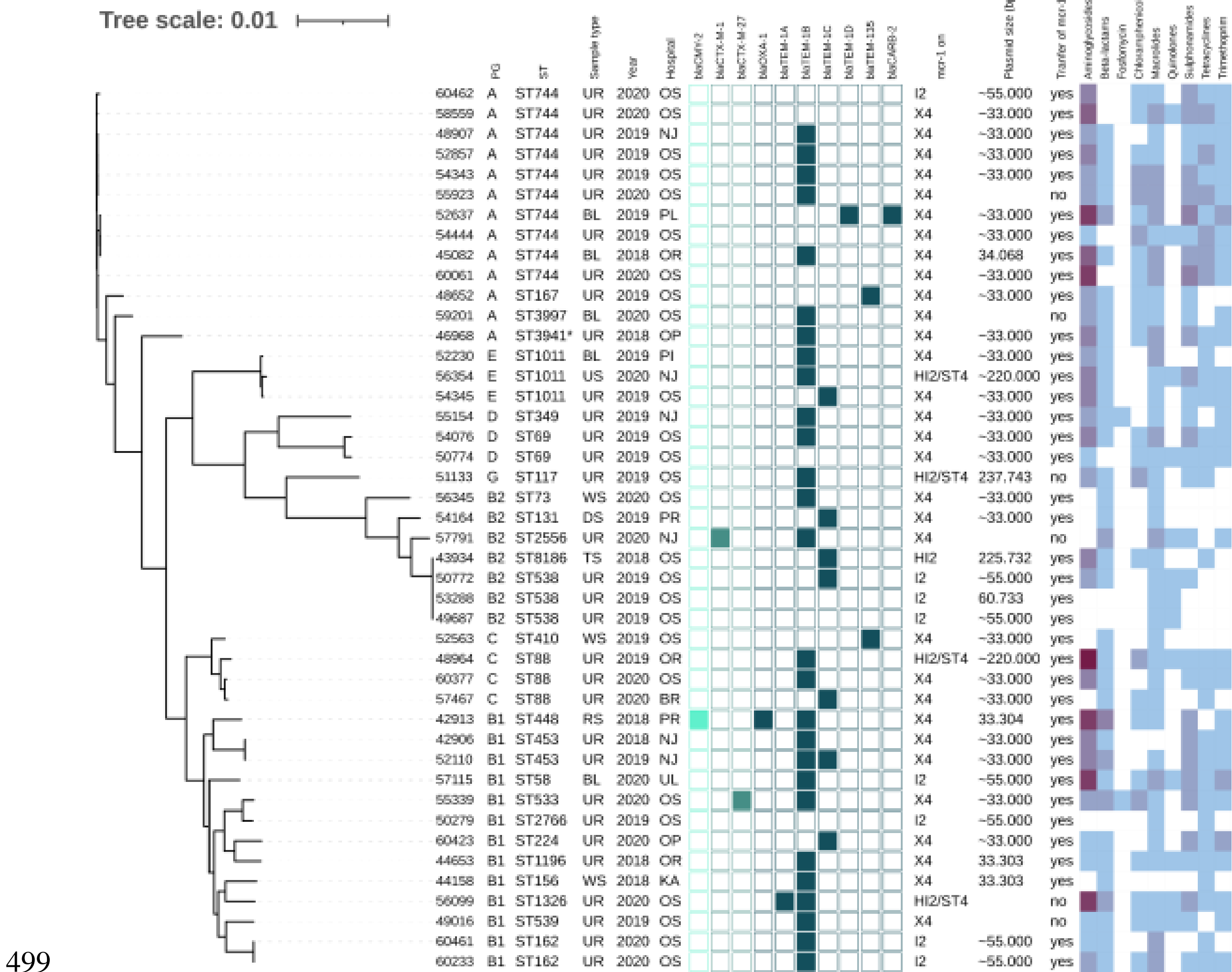

499

500

501 **Figure 1.** Phylogenetic tree of *E. coli* isolates with *mcr-1* of Czech clinical origin. The
502 metadata in columns represents phylogenetic group (PG); sequence type (ST); type of sample
503 (Sample type): urine (UR), blood (BL), rectal swab (RS), tonsil swab (TS), wound swab
504 (WS), decubitus swab (DS), urethra swab (US); year of isolation (Year) and city where is the
505 hospital related to the isolate recovery (Hospital): Novy Jicin (NJ), Prague (PR), Ostrava
506 (OS), Karvina (KA), Ostrava-Poruba (OR), Opava (OP), Pribram (PI), Plzen (PL), Usti nad
507 Labem (UL), Brno (BR). The turquoise squares represent presence (full square) or absence
508 (empty square) or respective beta-lactamase encoding genes divided as AmpC (bright
509 turquoise), ESBL (medium) and narrow-spectrum beta-lactamases (dark).The next section
510 (mcr-1 on) reveals which plasmid carried *mcr-1* gene; the size of the plasmid (Plasmid size) in
511 bp while approx. sizes (~) are estimated based by S1-PFGE while the more precise values
512 come from plasmid sequencing; the success of conjugative transfer is indicated (Transfer of
513 mcr-1). The heat map in the last section indicated the amount of antibiotic resistance genes
514 carried by the respective isolate in specified category of antibiotics from zero (white) to
515 maximum of six (dark purple).

516

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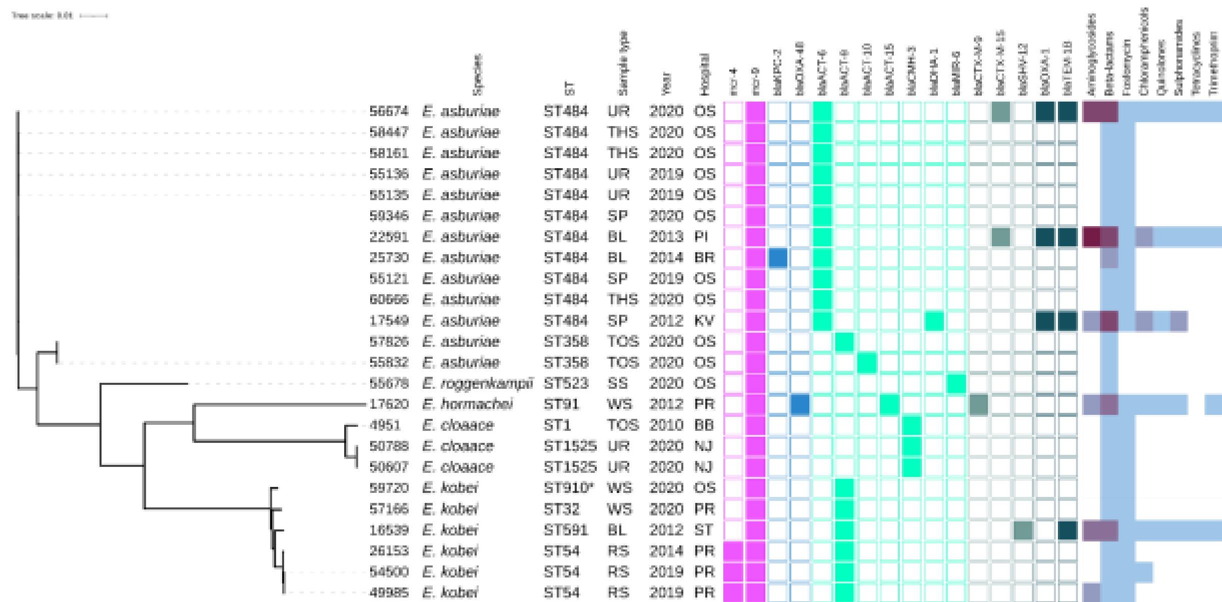

517

518

519 **Figure 2.** Phylogenetic tree of *Enterobacter* spp. isolates of Czech clinical origin. The
520 metadata specify the species (Species); sequence type (ST), type of sample (Sample type):
521 urine (UR), throat swab (TSH), sputum (SP), blood (BL), tonque swab (TOS), skin swab
522 (SS), wound swab (WS), rectal swab (RS); year of isolation (Year) and city where is the
523 hospital related to the isolate recovery (Hospital): Ostrava (OS), Pribram (PI), Brno (BR),
524 Karlovy Vary (KV), Prague (PR), Brno-Bohunice (BB), Novy Jicin (NJ), Strakonice (ST).
525 The colour squares represent presence (full square) or absence (empty square) or respective
526 antibiotic resistance genes divided to genes encoding resistance to colistin (pink),
527 carbapenemases (blue) and other beta-lactamases (turquoise, see legend Figure 1). The heat
528 map in the last section indicated the amount of antibiotic resistance genes carried by the
529 respective isolate in specified category of antibiotics from zero (white) to maximum of five
530 (dark purple).

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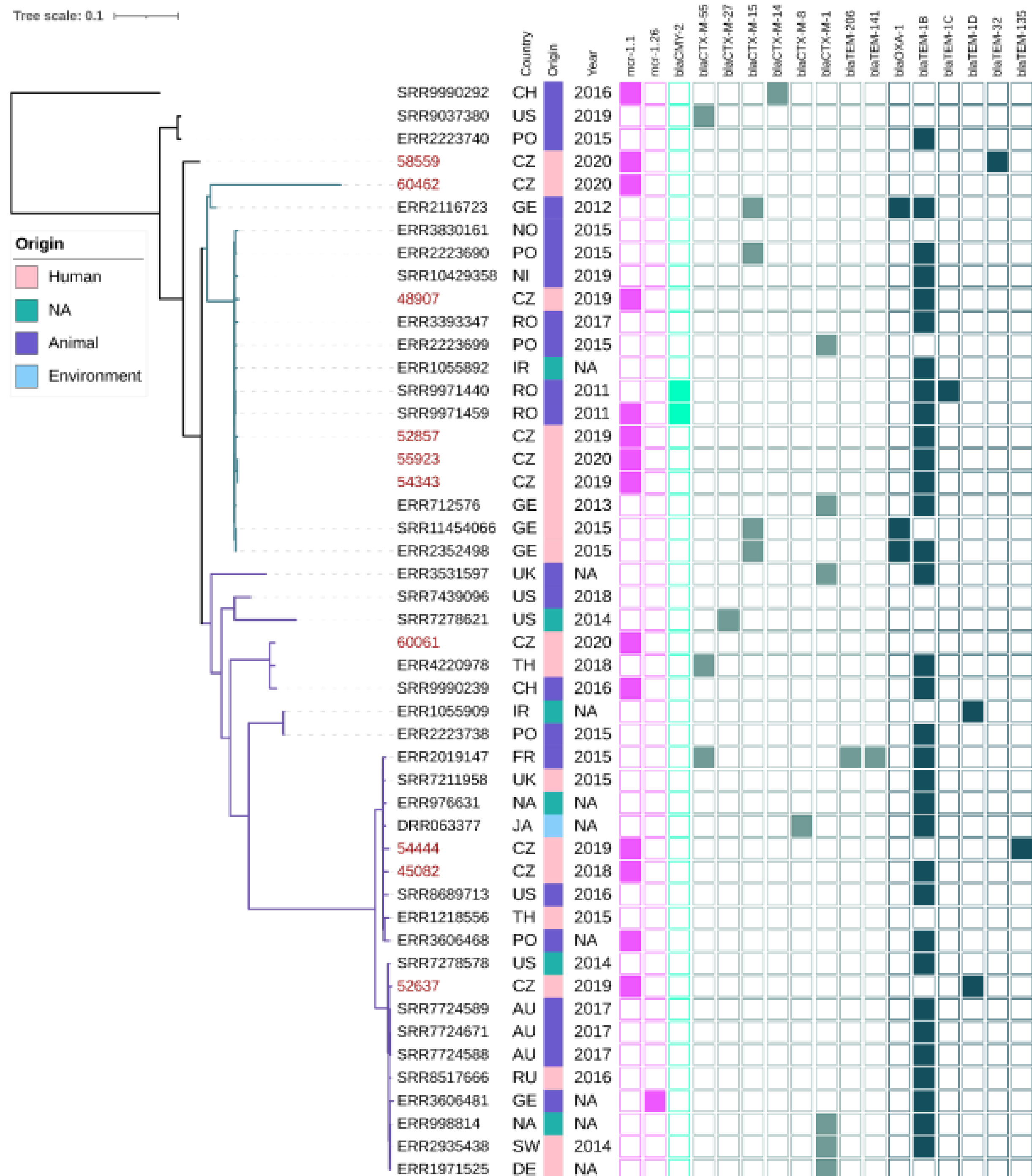

Figure 3. Phylogenetic tree of Czech clinical *E. coli* ST744 isolates with selected sequences from global collection. The red labels indicate isolates coming from this study. The metadata specifies country of origin (Country): China (CH), The United States (US), Poland (PO), Czech Republic (CZ), Germany (GE), Norway (NO), Nigeria (NI), Romania (RO), United Kingdom (UK), Thailand (TH), Ireland (IR), France (FR), Ukraine (UK), Australia (AU), Russia (RU), Switzerland (SW), not available (NA), the source of origin (Source) and the year of isolation (Year). The colour squares represent presence (full square) or absence (empty square) or respective antibiotic resistance genes divided to genes encoding resistance to colistin (pink), carbapenemases (blue) and other beta-lactamases (turquoise, see legend Figure 1).

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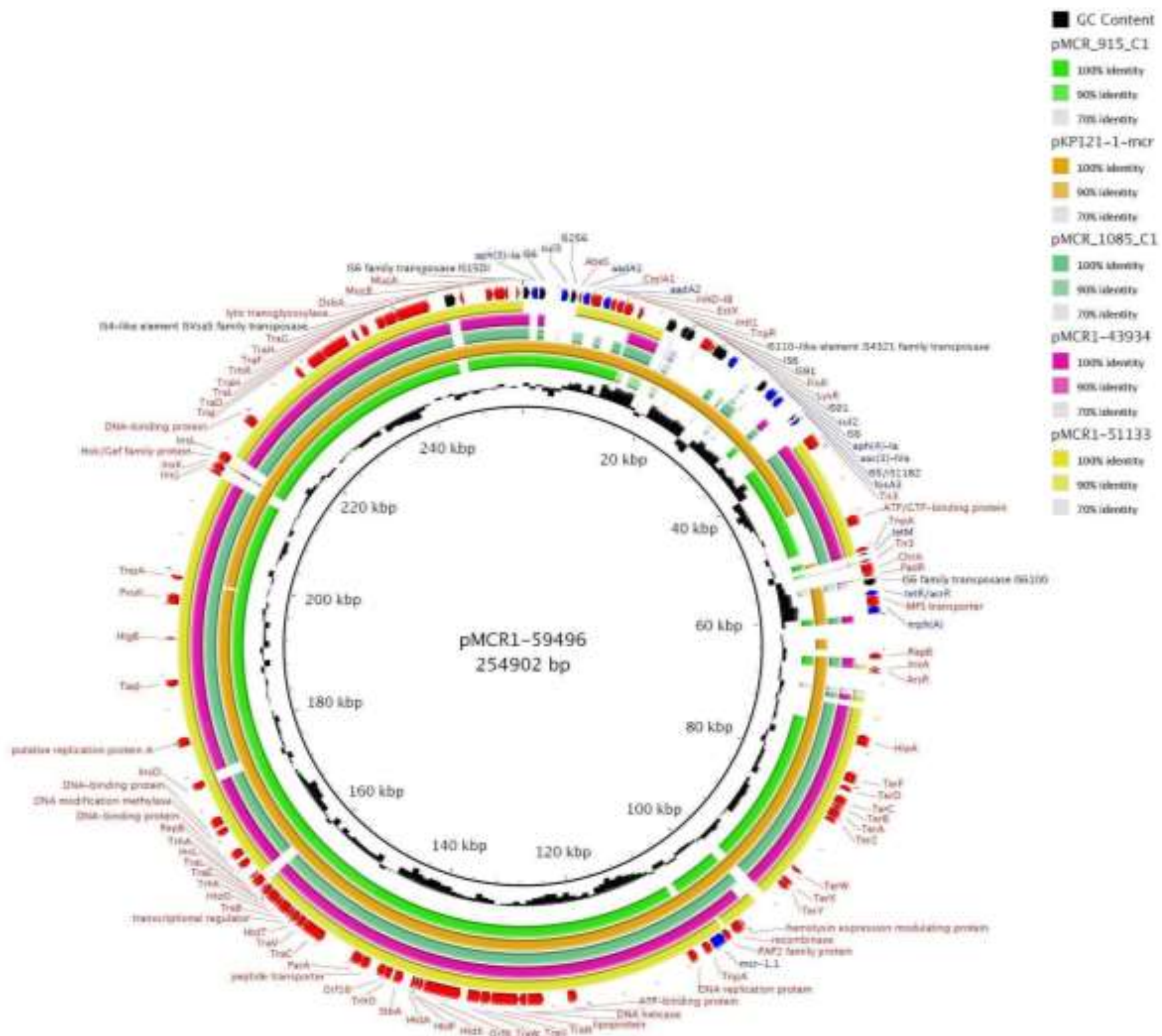

543

544 **Figure 4.** BRIG comparison of *mcr-1.1*-encoding IncHI2/ST4 plasmids. The plasmid
545 pMCR1-59496 from *K. pneumoniae* ST726 identified in our study from urine sample
546 (OP950836) was used as a reference. Two other plasmids originated from our collection
547 including pMCR1-43934 from *E. coli* ST8186 from tonsil swab sample and pMCR1-51133
548 from *E. coli* ST117 from a urine sample. The sequence alignment includes pMCR_915_C1
549 (MT929284.1) and pMCR_1085_C1 (MT929286.1) from *E. coli* recovered from raw turkey
550 meat imported to the Czech Republic from Poland and one of plasmid pKP121-1-mcr
551 (CP031850.1) from *K. pneumoniae* ST2570 from human blood in China.

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552

553 **Figure 5.** Genetic surroundings of *mcr-9.1* gene located on chromosomes in *Enterobacter*.
554 The linearized coding sequences of MCR-9.1 region of four isolates were compared using
555 clinker with identity threshold 90%. The MCR-9.1 (pink) surrounded by mobile genetic
556 elements (turquoise) and other coding sequences (grey) in isolates 50607 and 59720 formed a
557 region corresponding to the *mcr-9* region observed in IncHI2/ST1 plasmids. On the other
558 hand, 57166 and 56674 isolates contained downstream sequence similar to the previously
559 described *mcr-9* environment in *E. asburiae* (AP022628). White numbers in links correspond
560 to the similarity of coding sequences.

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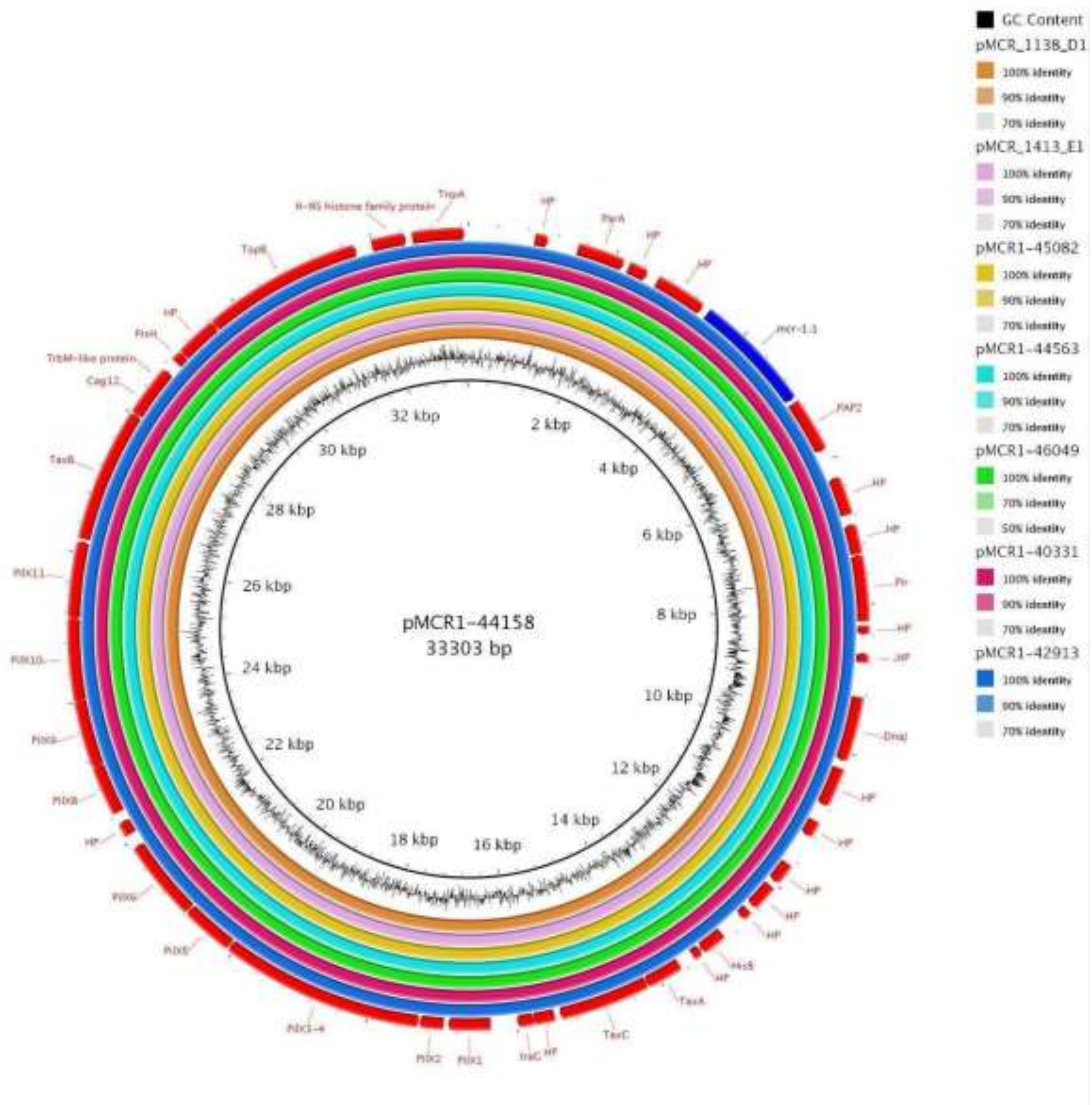

561

562 **Figure S1.** BRIG comparison of *mcr-1*-encoding IncX4 plasmids. Six representative plasmids
563 from our study subjected to MinION sequencing were used in the alignment. The plasmid
564 pMCR1-44158 carrying *mcr-1.2* from *E. coli* ST156 recovered from wound swab
565 (OP428975) was used as a reference for the comparison. Other plasmids originated from *E.*
566 *coli* of different STs including pMCR1-42913 (from *E. coli* ST448, rectal swab), pMCR1-
567 44563 (from *E. coli* ST1196, urine) and pMCR1-45082 (from *E. coli* ST744, blood). Other
568 two plasmids from our study came from *K. pneumoniae* including pMCR1-40331 (*K.*
569 *pneumoniae* ST290, urine) and pMCR1-46049 (*K. pneumoniae* ST147, pus). The sequence
570 alignment contains plasmid sequences from other sources including pMCR_1413_E1
571 (MT929275) from *E. coli* ST354 from Czech raw turkey meat and pMCR_1138_D1
572 (MT929276) from *E. coli* ST744 from raw turkey meat imported to the Czech Republic from
573 Germany.

574

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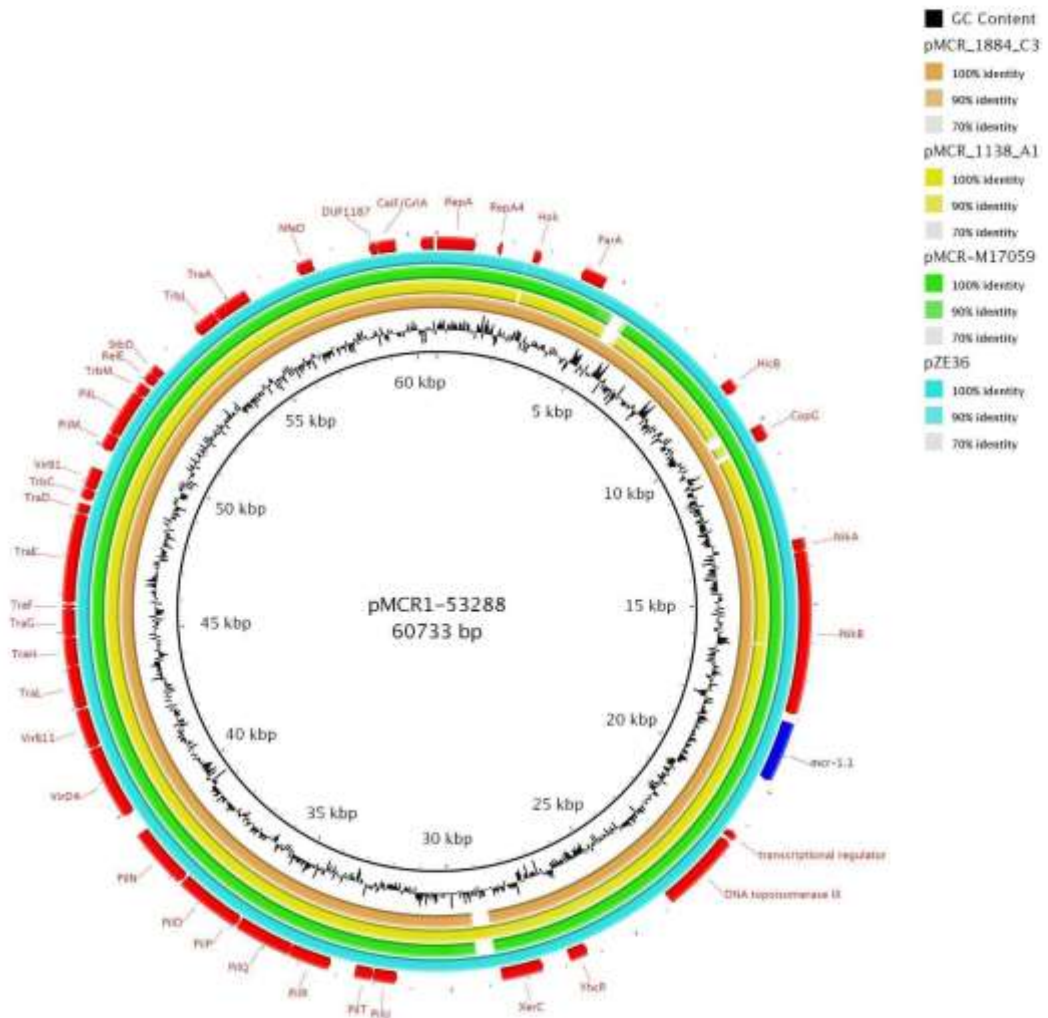

575

576 **Figure S2.** BRIG comparison of *mcr-1.1*-encoding IncI2 plasmids. Plasmid pMCR1-53288
577 originating from *E. coli* ST538 recovered from a urine sample (OP434482) from our study
578 was used as a reference. Sequence alignment contains pMCR_1884_C3 (MT929290)
579 identified in *C. braakii* from raw rabbit meat imported to the Czech Republic from China,
580 while pMCR_1138_A1 (MT929289) originated from *E. coli* ST162 from raw turkey meat
581 imported to the Czech Republic from Germany. The plasmids pMCR-M17059 (KY471310)
582 and pZE36 (KY802014), both of clinical origin, were obtained from *E. coli* ST1488 from
583 Argentina and from *E. coli* ST156 from China.

584

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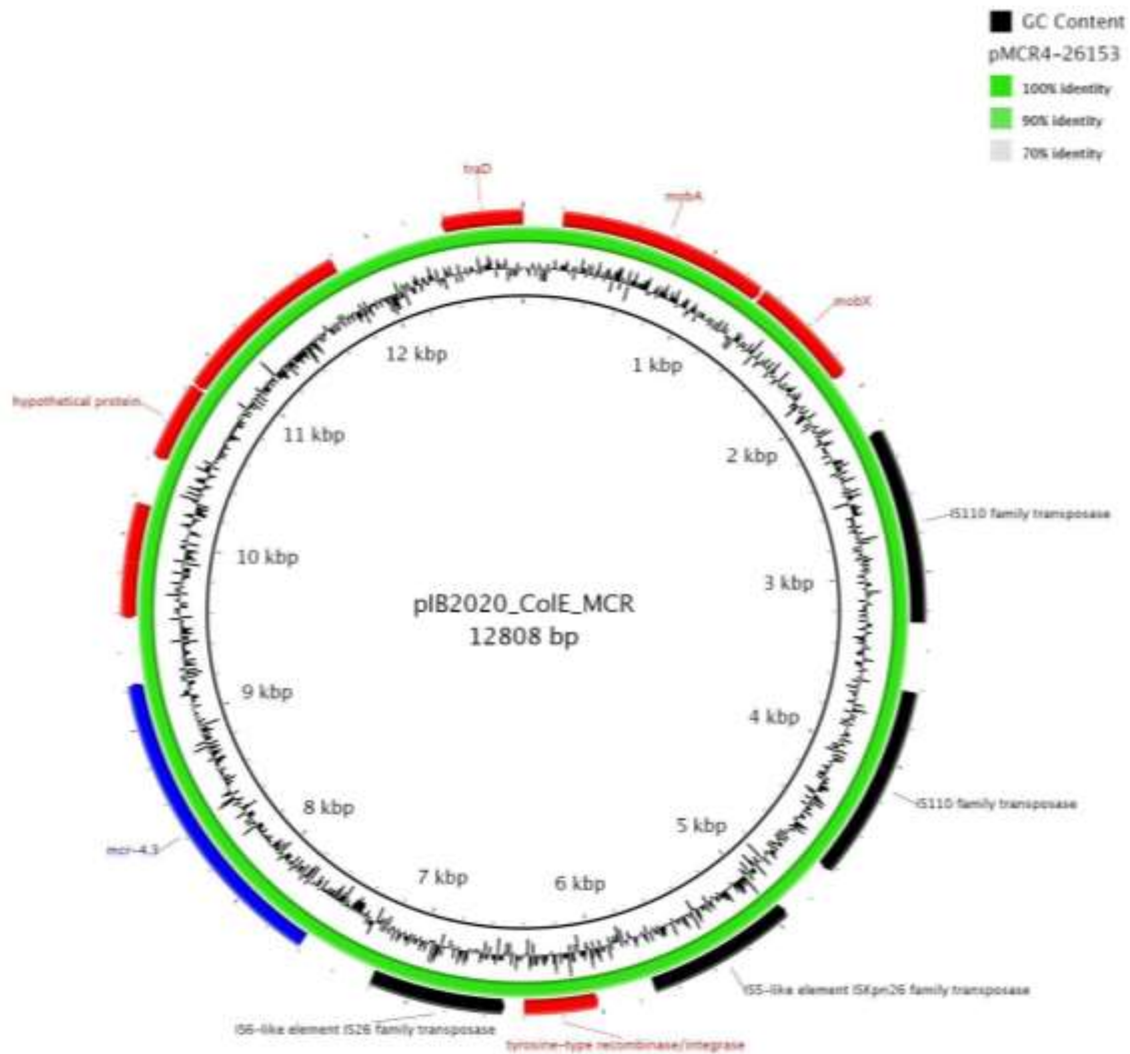

585

586

587 **Figure S3.** BRIG comparison of ColE10 plasmids with *mcr-4*. pMCR4-26153 originates from
588 *E. kobei* ST54 recovered from a rectal swab of a patient in the Czech Republic. Plasmid
589 pIB2020_ColE_MCR (CP059482) from *E. kobei* ST54 from a patient in Italy was used as a
590 reference.

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591

592 **Figure S4.** BRIG comparison of *mcr-9.1*-encoding IncHI2/ST1 plasmids. The plasmid
593 pMCR9-16539 from *E. kobei* ST591 recovered from a blood sample (OP950838) from our
594 collection was used as a reference. Other two plasmids from our study included pMCR9-
595 57185 (*C. freundii* ST18, rectal swab) and pMCR9-17620 (*E. hormaechei* ST91, wound
596 swab). Plasmids from human clinical isolates previously characterized from the Czech
597 Republic including *C. freundii* (p51929_MCR_VIM; CP059429) and *E. hormaechei*
598 (p48212_MCR; CP059413) were used for the comparison.

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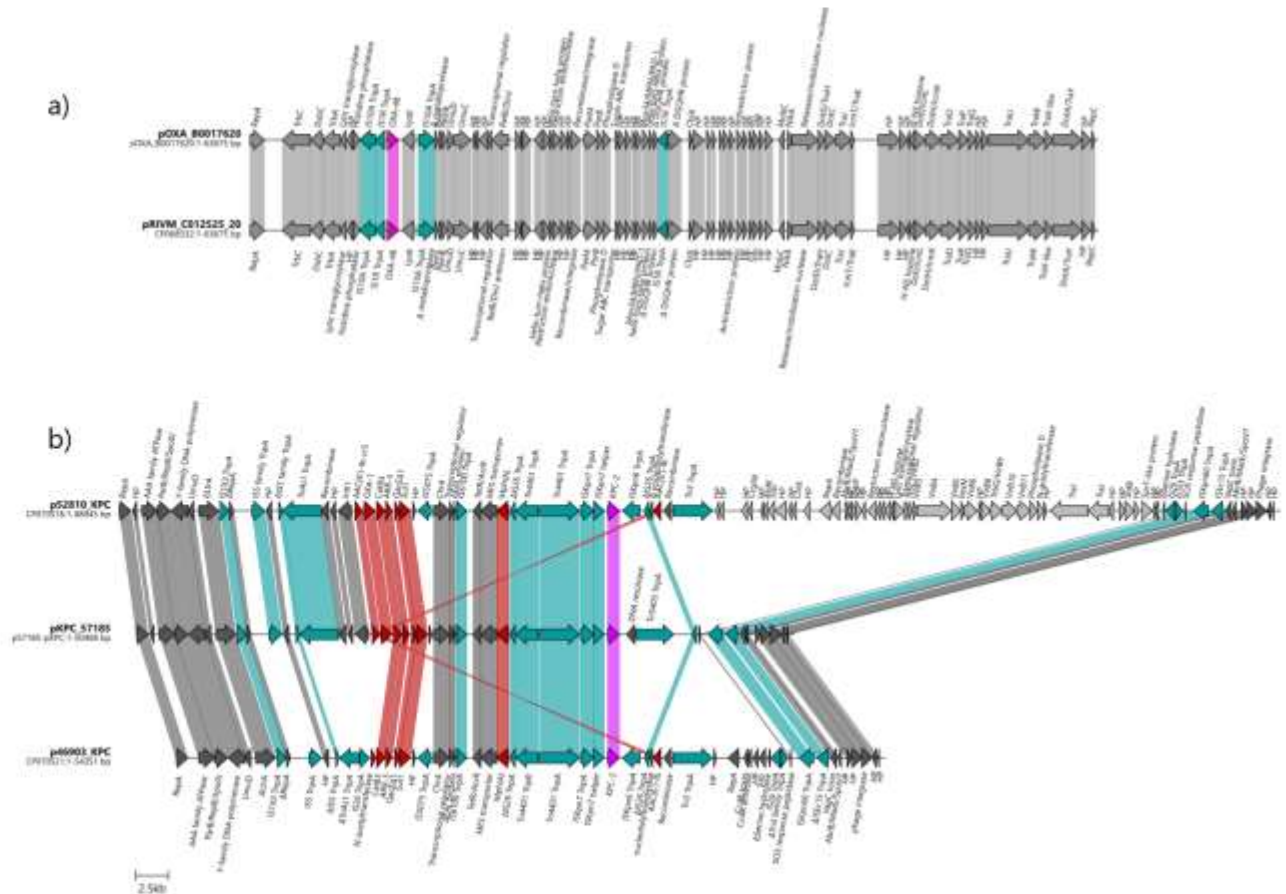

599

600 **Figure S5.** Genetic comparison of the carbapenemase-encoding plasmids. The linearized
601 coding sequences of the carbapenemase-encoding plasmids were compared with reference
602 plasmids using clinker with identity threshold 90%. The coding sequences of OXA-48 and
603 KPC-2 are visualised in pink and other antibiotic resistance CDS in dark red. Turquoise
604 shading indicates mobile genetic elements (MGEs) and grey suggests other coding sequences.
605 HP stands for hypothetical protein. a) The coding sequences of the pOXA_17620 were
606 identical with a reference plasmid pRIVM_C012525_20 (CP068332). b) The linearized
607 coding sequences of the pKPC_57185 were compared with reference plasmids p52810_KPC
608 (CP070576) and p46903_KPC (CP070521). The pKPC_57185 carried multiple MGEs and
609 antimicrobial resistance genes, similarly as the reference plasmid p52810_KPC. The
610 downstream sequence of the KPC-2 differs in the studied plasmid. The KPC-2 is followed by
611 DNA resolvase and Tn*5403*, unlike the reference plasmids carrying IS*Kpn6*, part of IS*26*,
612 AAC(6')-Ib and recombinase followed by Tn*3*. Other IncR and IncN coding sequences are
613 visualised in dark grey and light grey, respectively.

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614 **Conflict of Interest**

615 No conflict of interest declared.

616 **Author Contributions**

617 MZ performed laboratory work, data analysis and prepared the manuscript. CCP performed
618 data analysis and prepared the manuscript. PS performed laboratory work and helped with the
619 manuscript preparation. MM performed bioinformatic analyses of whole-genome sequencing
620 data. JP conducted MinION sequencing and helped with plasmid analysis and KN contributed
621 on figure preparation. VJ, KP and HZ provided the samples and IJ performed whole-genome
622 sequencing. MD supervised the project, performed data analysis and revised the manuscript.
623 All authors discussed the results. Members of the surveillance network provided the clinical
624 isolates and metadata obtained during the standard microbiological testing in their laboratories.

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