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1 **Environmental dissemination of *mcr-1* positive Enterobacteriaceae by *Chrysomya* spp.**
2 **(common blowfly): an increasing public health risk**

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26 **Abstract**

27 Until recently, the role of insects, and particularly flies, in disseminating antimicrobial
28 resistance (AMR) has been poorly studied. In this study, we screened blowflies (*Chrysomya*
29 spp.) from different areas near the city of Phitsanulok, Northern Thailand, for the presence of
30 AMR genes and in particular, *mcr-1*, using whole genome sequencing (WGS). In total, 48 *mcr-*
31 *1*-positive isolates were recovered, consisting of 17 *mcr-1*-positive *Klebsiella pneumoniae*
32 (MCRPKP) and 31 *mcr-1*-positive *Escherichia coli* (MCRPEC) strains. The 17 MCRPKP were
33 shown to be clonal (ST43) with few single poly nucleomorphs (SNPs) by WGS analysis. In *in-*
34 *vitro* models, the MCRPKP were shown to be highly virulent. In contrast, 31 recovered
35 MCRPEC isolates are varied, belonging to 12 different sequence types shared with those
36 causing human infections. The majority of *mcr-1* gene are located on IncX4 plasmids (29/48,
37 60.42%), sharing an identical plasmid backbone. These findings highlight the contribution of
38 flies to the AMR contagion picture in low- and middle-income countries and the challenges of
39 tackling global AMR.

40

41 **Highlights**

- 42 • WGS-based analysis of *mcr-1*-carrying isolates from blowflies (*Chrysomya* spp.)
43 provides evidence that flies serve as an active vector for the environmental spread of
44 *mcr-1*-mediated colistin resistance pathogens.
- 45 • Hyper-virulent *mcr-1*-carrying *Klebsiella pneumoniae* isolates were identified from
46 blowflies, which post an acute public health risk.
- 47 • Diversity of antibiotic resistance genes including *qnrS1*, *fosA*, *bla*_{CTX-M-55/14} and *floR*,
48 was detected in *mcr-1* positive strains recovered from blowflies indicating the
49 environmental spread of multi-drug resistant pathogens.

50

51 **Keywords:** blow flies; *mcr-1* gene; *Klebsiella pneumoniae*; IncX4 plasmid; multidrug

52 resistance

53 **Running Title:** Environmental dissemination of *mcr-1* gene through blow flies

54 **Introduction**

55 There is growing public health concern for environmental dispersal of antibiotic resistance.
56 Historically, we have focused on resistomes in human and animal gut microbiomes, where
57 antimicrobial resistance (AMR) and pathogens can be widely spread via animal and human
58 wastes. However, there is increasing evidence indicating the important role of environmental
59 factors including wastewater[1,2], wildlife [3] and flies [4], in the dissemination of AMR in
60 different environments. In particular, flies have been recently recognized as potential reservoirs
61 exacerbating the spread of antibiotic resistance and pathogens among animals, environments
62 and human, as they can move freely, often unnoticed, among different public health sectors
63 including hospitals, human communities and animal farms [4-6]. It has been demonstrated that
64 houseflies are involved in the mechanical transmission of nosocomial infections with multidrug
65 resistance bacteria in hospital environments, such as *Shigella* spp.; *Escherichia coli*; *Klebsiella*
66 spp. and *Enterobacter* spp. [4,5]. According to a study analyzing the antibiotic resistome of
67 swine manure, the larvae (*Musca domestica*) gut microbiome was significantly affected the
68 resistant genotypes in manure-borne community [7].

69 Since the first discovery of a mobile colistin resistance mechanism (MCR-1) in November
70 2015 [8], *mcr*-mediated colistin resistance has been globally reported in Gram-negative
71 pathogens [9]. Additionally,, the co-resistance of colistin with other last-line antibiotics, has
72 revealed the emergence of extensively drug resistant (XDR)strains that are virtually untreatable
73 [10,11]. Recent studies reported housefly and blowfly are also responsible for the spread of
74 *mcr-1* gene conferring colistin resistance in a Chinese university hospital [12], as well as in pig
75 farms in Germany [13]. Among them, *mcr-1* was most commonly found in *E. coli* isolates,
76 although several other Enterobacteriaceae including *K. pneumoniae* have been detected in other
77 source such as animals [14] and human clinical isolates [15]. *K. pneumoniae* is known to be a
78 leading cause of hospital-acquired infections, such as pneumonia, post-surgical wound and

79 urinary tract infections [16]. It is especially problematic in hospitals when becoming resistant
80 to colistin, a last-resort antibiotic, leaves very limited therapeutic options. There is a marked
81 paucity of our understanding on the transmission of *mcr-1*-positive *Enterobacteriaceae*
82 (MCRPE), mainly in *E. coli* and *K. pneumoniae*, to negate the threat to human health posed by
83 MCRPE isolates. With this aim, we investigated the carriage of *mcr-1* positive isolates from
84 blowflies collected from different areas in the city of Phitsanulok in Thailand, using whole-
85 genome sequence to look for associations between MCRPE strains carrying *mcr-1*-linked
86 plasmids recovered from blowflies (*Chrysomya* spp.) and human clinical isolates.

87

88

89 **Materials and Methods**

90 **Bacterial isolates from blow flies**

91 A total of 300 blow flies were trapped at three different locations in Northern Thailand: a local
92 market in an urban community, a rural area and a suburb of the city Phitsanulok. These
93 locations are approximately 10 kilometers apart. Blow flies were collected by the use of a
94 sterile sweeping net. Individual fly was kept in a sterile plastic tube and sacrificed by placing
95 on ice for 30 min. They were identified to species level by using the taxonomic keys as
96 described by Kurahashi and Bunchu [17]. Only *Chrysomya megacephala* flies [18], the most
97 abundant blow flies in Thailand were selected for further analysis. The flies were individually
98 pulverized in enriched peptone water for 30 min and then aliquots of the resultant suspensions
99 (100 μ l) were plated on Eosin-Methylene-Blue (EMB)-agar plates supplemented with 2 mg/l
100 colistin and incubated at 37°C overnight. One to three representative colonies with different
101 colors from each plates were purified and subsequently screened for *mcr-1* gene by PCR. The
102 *mcr-1*-positive bacteria were sub-cultured in liquid nutrient broth for 18 h before DNA
103 extraction for species identification and whole-genome sequence. Minimum inhibitory

104 concentrations (MICs) of colistin for 48 *mcr-1*-bearing isolates was performed by using broth
105 microdilution, in accordance with the guideline of the European Committee on Antimicrobial
106 Susceptibility Testing (EUCAST), reference strain *E. coli* ATCC25922 served as a quality
107 control.

108

109 **Conjugation experiments**

110 To investigate the transferability of *mcr-1*-carrying plasmids, we performed conjugation
111 assays with sodium-azide resistance *E. coli* J53 as the recipient strain. Briefly, overnight
112 cultures of 30 randomly selected *mcr-1*-producing donors (strains with transfer frequency
113 showed in Table 1) and the recipient *E. coli* J53 strain were 1:2 mixed and incubated in 37 °C
114 for 16-20 h. After incubation, we subsequently ten-fold serial diluted the mixed culture in
115 sterile saline and aliquoted 100 µl of diluted culture onto selective agar plates containing 2
116 mg/l colistin and 150 mg/l sodium azide. The *mcr-1*-positive transconjugants were confirmed
117 by PCR and transfer frequency was calculated by the number of transconjugants per recipient.
118 Plasmid analysis were done by whole genome sequence as described below.

119

120 **Whole-genome sequencing and bioinformatics analysis**

121 Total gDNA was extracted from an overnight culture (2 ml) on a QIAcube automated system
122 (Qiagen, Germany) with QIAamp DNA Microbiome kit (Qiagen, Germany), followed by
123 gDNA quantity measurement by fluorometric methods using a Qubit (ThermoFisher
124 Scientific). Genomic DNA libraries are constructed using the NexteraXT kit (Illumina),
125 according to manufacturer's instruction. Paired end sequencing was performed using the
126 Illumina MiSeq platform (MiSeq Reagent V3 Kit; 2 × 300 cycles). Raw sequence reads were
127 trimmed using Trim Galore and the genomes were *de novo*-assembled into contigs using
128 SPAdes (3.9.0) with pre-defined kmers set.

129

130 **Bioinformatics analysis:** The CGE platform (<http://www.genomicepidemiology.org/>) were
131 used for analysis of multilocus sequence typing (MLST-1.8), acquired resistance genes
132 (ResFinder 3.1, all antibiotic resistance databases were selected with a cut-off value of 95%
133 identity and 80% minimum coverage) and incompatibility group of plasmids (PlasmidFinder-
134 1.3 version, using Enterobacteriaceae database with parameters of minimum 95% identity and
135 85% query coverage). All contigs were searched for *mcr-I* using standalone BLAST analysis,
136 the putative coding sequences containing *mcr-I* gene were obtained using ORF finder
137 programs (Geneious 10.0.7). Draft genome sequences were aligned and then applied for
138 phylogenetic analysis using Parsnp in the Harvest package, and phylogenetic trees was
139 visualized by iTOL (<https://itol.embl.de/>). MCRPKP strain p38 recovered from healthy human
140 feces in Thailand was served as a reference strain in the SNPs analysis of 17 MCRPKP strains
141 in this study.

142 Primer walking was performed to fill the gap in *mcr-I*-carrying contigs from strain PN105
143 with primers (PN_IncX4_forward: CGACCTTTAAGTCGTATTTGCAAGT;
144 PN_IncX4_reverse: ATTGCGCCCGTAGTTCGCTA, Tm 60°C) and the complete plasmid
145 sequence were constructed by *de novo* assembly using Geneious (10.0.7). The circular
146 comparisons among *mcr-I*-related IncX4 plasmid backgrounds were performed using BLAST Ring
147 Image Generator (BRIG v0.9555). Briefly, *mcr-I*-containing contigs were extracted from genomic data,
148 and a fully sequenced *mcr-I*-linked IncX4 plasmid was served as a central reference sequence. In this
149 study, plasmid IncX4 from strain PN105 and pMR0617mcr (GenBank No. CP024041), act as reference
150 sequences. The similarity of between the central reference sequence and other *mcr-I*-positive contigs
151 from studied MCRPE strains, shows as concentric rings with representative colors.

152

153 **Virulence factors of *K. pneumoniae* isolates**

154 Based on whole-genome sequence data, we have developed a database with publicly
155 available genomes [NTUH-K2044 (Genbank accession No. AP006725.1, AB117611.1),
156 pK2044 (Genbank accession No. NC_006625.1), pLVPK (Genbank accession No.
157 NC_005249.1), allantoin metabolism (Genbank accession No. AB115590.1), SB3193
158 (Genbank accession No. LK022716.1), *uge* CDS (Genbank accession No. AY294624.1),
159 Kp52.145 (Genbank accession No. FO834906.1), SB4536_2858 (Genbank accession No.
160 HG518478.1), pO26-Vir (Genbank accession No. NC_012487.1), IHE3034 (Genbank
161 accession No. AM229678.1), *kvg* operon (Genbank accession No. AJ250891.2)] to determine
162 the key virulence factors in *K. pneumoniae* strains. Our database includes a set of virulence
163 genes: capsular biosynthesis genes (*wzy/magA*, *K2A*) [20,21]; mucoid factor regulator (*rmpA*,
164 *rmpA2*) [22]; allantoin metabolism operons (*allABCDRS*, *gcl* and *glxRK*) [23]; an iron-uptake
165 system (*kfuABC*) [24]; two-component operon (*kvgAS*) [25]; gene clusters for siderophores
166 dependent iron acquisition (aerobactin *iucBCD-iutA*, yersiniabactin *ybtAEPQSTUX-irp1-irp2-*
167 *fyuA*, colibactin *clbBCDEFGHHIJKLMNOPQR*, salmochellin *iroBCDN*, enterobactin
168 *entABCDEF*) [26]; gene clusters of type I and type III fimbriae (*fimABCDEF* and
169 *mrkABCDFHIJ*, respectively) [27,28]; outer membrane lipoprotein (*ycfM*); serum resistance
170 factor (*traT*) [29]; hemolysin transport protein (*hlyABCD*) [30]; urease operon (*ureABCDEFG*)
171 [31] and type IV secretory system gene cluster (*virB1* to *B11*) [32]. Annotation of genes with
172 75% identity to reference sequences was performed by Geneious (10.2; Biomatters
173 Ltd.). Capsular (KL) loci were evaluated using Kaptive platform
174 (<http://kaptive.holtlab.net/jobs>) [33].

175 A *Galleria mellonella* model has been used for virulence test for MCRPKP isolates. Log-
176 phase cultures of *K. pneumoniae* strains were washed with sterile saline twice, followed by
177 standardization of bacterial concentrations to approximately 1×10^9 - 1×10^5 CFU/mL and 10
178 μ l were injected into each *G. mellonella* larvae at a final inoculum ranging from 1×10^6 - 1×10^4

179 cfu/ml, as described previously [34]. The ST23 *Klebsiella pneumoniae* A58300 strain,
180 harboring the K1 capsule serotype (hyperviscosity phenotype widely associated with
181 hypervirulent strains) served as virulent control strain in the *G. mellonella* model [35].

182

183

184 **Results**

185 **Details of isolates**

186 Overall, we recovered 48 MCRPE isolates from 300 collected blowflies (16.0% flies
187 positivity for *mcr-1*), consisting of 31 *mcr-1*-positive *E. coli* (MCRPEC) and 17 *mcr-1*-positive
188 *K. pneumoniae* (MCRPKP). Bacterial species were determined by whole genome sequencing
189 data. Among them, 4 MCRPE strains were recovered from local market in urban community,
190 16 from rural area and 28 from suburb area. MICs of colistin for all MCR-1-producing isolates
191 are ranging from 4-16 mg/l (**Table 1**).

192

193 **Whole genome sequencing (WGS) analysis**

194 A total of 48 MCRPE isolates were sequenced using Illumina Miseq platform. The distinct
195 MCRPEC isolates belonged to 12 STs (**Table 1**): ST10(n=7), ST648(n=5), ST549(n=4),
196 ST58(n=3), ST181(n=3), ST218(n=2), ST201(n=1), ST162(n=1), ST457(n=1), ST1244(n=1),
197 ST2345(n=1), ST2705(n=1) and ST5487(n=1). Most interestingly, all 17 MCRPKP isolates
198 belonged to ST43, thus we further determined the clonal relationship of 17 ST43 *K.*
199 *pneumoniae* isolates by SNPs analysis using Parsnps software. Phylogenetic tree analysis for
200 17 strains based on their raw sequencing reads showed that their core genome differed only by
201 a few SNPs (the numbers of differences in SNPs are up to 15, Table S1), suggesting the clonal
202 dissemination of ST43 *K. pneumoniae* isolates in *Chrysomya* spp. from Thailand.

203 Analysis of genomic accessory modules including acquired resistance genes, virulence
204 factors and metal resistance genes, showed significant variations in resistance gene content.
205 Apart from *mcr-1* gene, multiple antibiotic resistance genes were identified in most of the
206 isolates, with the average number 9.35 and 7 in MCRPEC strains and MCRPKP strains,
207 respectively. In the 31 MCRPEC collection, 25 difference resistance genes were identified by
208 ResFinder 3.1 (<https://cge.cbs.dtu.dk/services/ResFinder/>, updated on 2018-09-10), conferring
209 resistance to nearly all currently available antibiotics, such as β -lactams, aminoglycoside,
210 chloramphenicol, fluoroquinolones and sulfonamide (**Fig.1**). The most prevalence resistance
211 genes are *mdfA* resistant to macrolide (n=28, 90.32%), followed by gene *aadA2* conferring
212 streptomycin resistance (n=26, 83.87%). Besides *aadA2* gene, several genes resistant to
213 aminoglycoside were detected: *aadA1* (n=16), *aadA17* (n=2), *aac(3')-IId* (n=8), *aph(3')-Ib*
214 (n=11), *aph(6')-Id* (n=11) and *aph(3')-Ia* (n=1). Furthermore, the plasmid-mediated
215 fluoroquinolone resistance gene, *qnrS1*, was found in 18 MCRPEC isolates, and three β -
216 lactamase-producing genes, *bla_{TEM-1b}*, *bla_{CTX-M-55}* and *bla_{CTX-M-14}* are detected in 19 (61.29%),
217 6 (19.35%) and 1 (3.23%) isolates, respectively. Additionally, resistant genes responsible to
218 other groups of antibiotics were observed in Fig. 1, including tetracycline resistance (*tetA*, *tetB*
219 and *tetM*), phenicol resistance (*cmlA*, *floR* and *catA*), and sulphonamine resistance (*sul2* and
220 *sul3*).

221 In contrast, identical resistant genotypes among MCRPKP strains were observed, and seven
222 acquired antibiotic resistance genes were detected in 17 MCRPKP strains, namely, *mcr-1*,
223 *mcr-8*, *qnrS1*, *bla_{TEM-1b}*, *tetA*, *bla_{SHV-40}* encoding SHV β -lactamase and *fosA* mediating
224 fosmycin resistance.

225

226 ***mcr-1*-associated plasmid types and transferability of *mcr-1* gene**

227 *De novo* bacterial genome assembly was performed and the *mcr-1*-carrying contigs were
228 analyzed. Replication origin are located in the *mcr-1* contigs, allowing to analyze
229 incompatibility groups of these plasmids by using PlasmidFinder
230 (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>). In 35 out of 48 isolates, replicon sequence
231 type of *mcr-1*-harbouring plasmids could be identified: IncX4 (n=29, 12 *E. coli* and all 17 *K.*
232 *pneumoniae* isolates), IncHI1A (n=2), IncHI1B (n=3) and IncHI1A-IncHI1B (n=1).
233 Representative 10 *mcr-1*-bearing IncX4 plasmids obtained from *K. pneumoniae* isolates were
234 probed for *mcr-1* gene using S1-PFGE. As shown in **Fig.2**, the 10 *mcr-1* genes were all located
235 on a ~32-kb IncX4 plasmid. PCR was performed to fill the gap in *mcr-1*-carrying contigs, as a
236 result, complete sequencings of 26 IncX4-*mcr-1*-carrying plasmids were achieved (**Fig. 3**).
237 Alignment of 26 *mcr-1*-carrying IncX4 plasmids visualized by software BRIG v0.9555 showed
238 that all *mcr-1*-carrying plasmid share the identical plasmid backbone, including the typical
239 region encoding ~11kb T4ss conjugation system and a toxin-antitoxin system *hicAB*. More
240 importantly, in **Fig. 3B**, the backbone of *mcr-1*-linked IncX4 plasmids from blowflies, are
241 highly similar to those recovered from other sources including companion animals, human
242 feces and poultry, further suggesting that this type of IncX4 plasmid facilitate the transmission
243 of *mcr-1* gene. Furthermore, the transferability of *mcr-1*-bearing plasmids were performed by
244 conjugation with *E.coli* J53 as a recipient. We randomly selected 31 *mcr-1*-positive isolates as
245 donors, containing four different *mcr-1*-linked Inc-type plasmids: IncX4 plasmids (n=8, three
246 *mcr-1*- positive *Klebsiella* isolates and five *mcr-1*- positive *E. coli*), IncHI1B plasmids (n=3),
247 IncHI1A (n=2) and IncHI1A_HI1B (n=1) (**Table 1**). 12 out of 14 *mcr-1*-bearing plasmids were
248 successfully transferred to *E. coli* J53, IncX4-*mcr-1* plasmids are able to transferred into the
249 recipient at a higher frequency (mean 1.46×10^{-3} in *E. coli* and mean 2.11×10^{-5} in *K.*
250 *pneumoniae*), compared to other *mcr-1*-related IncHI1 plasmid types (2.77×10^{-7}), IncHI1A
251 (mean 2.27×10^{-7}), IncHI1B (1.85×10^{-7}) and IncHI1A-IncHI1B (8.7×10^{-8}) (**Table 1**).

252

253 **Virulence factors in MCRPKP and virulence loss in *G. mellonella* model**

254 *K. pneumoniae* is recognized as a serious threat to patients due to the emergence of MDR
255 strains associated with hospital outbreaks and that they demonstrate a number of virulence
256 factors associated with bacterial pathogenicity and poor patient outcome [36]. There are at
257 least five groups of pathogenicity factors found in 17 MCRPKP isolates (**Table S1**), these
258 include gene clusters associated with serum resistance (*traT*); adhesins (type I fimbrial operon
259 *fimABCDEFGH*, and type III fimbrial operon *mrkBCDEF*); lipopolysaccharide (*wabGHN*);
260 siderophore systems enterobactin-*entABCDEF*S, and aerobactin-*iucABCD-iutA*. Using
261 PlasmidFinder (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>), IncFIB-plasmid-related
262 aerobactin was found in at least 14 isolates, suggesting potential aerobactin-mediated virulence
263 transferability. Capsular synthesis loci matched type KL61 (*wzi/wzc* 412/61 typing) (Kaptive).
264 Similarly, the iron acquisition operons *kfuABC*, *iroE*, urease-synthesis operon *ureABCDEFG*
265 associated with gastric ulceration and urinary stone formation, and gene *ycfM* encoding surface
266 protein were identified in all MCRPKP isolates. At least 10 different virulence factors were
267 found in the 17 ST43 MCRPKP strains, which we further analysed by using a *G. mellonella*
268 model [34]. The effect of different inoculum of 10 randomly selected ST43 MCRPKP strains
269 was assessed in this model using K1 *rmpA*-positive *K. pneumoniae* A58300, as a hypervirulent
270 strain reference strain [35]. As shown in **Fig. 4**, after 12-hour post-infection, with an inoculum
271 of approx. 1×10^6 CFU, 100% of mortality was observed with the K1 strain and all 10 ST43
272 MCRPKP strains. At an inoculum of 1×10^5 CFU, the survival rate was 70% with K1 strain
273 and 0% with 10 ST43 MCRPKP strains. With an inoculum of 1×10^4 CFU, 100% survival was
274 seen with the K1 strain but only 20% with ST43. The consistency between genotypic virulence
275 factors and the reproducible results of the *G. mellonella* infection model suggest that the ST43
276 MCRPKP strains recovered from blowflies are highly virulent clones.

277

278 Based on our previous study, the acquisition of *mcr-1*-carrying plasmid leads to virulence
279 loss in *E. coli* strain [34]. In this study, three *mcr-1*-carrying plasmids were transferred into a
280 clinical susceptible *K. pneumoniae* strain ff101 and a KPC-positive *K. pneumoniae* strain p35,
281 followed by infection of *G. mellonella* larvae with an inoculum of $\sim 1 \times 10^5$ CFU. As shown in
282 **Fig. 5**, two *K. pneumoniae* strains ff101 and p35 caused more than 80% and 90% of mortality
283 after 72h infection, respectively. After acquiring of IncX4-*mcr-1* plasmid, the survivals of
284 larvae have been increased to 40% - 80% with strain ff101, and survivals are more than five
285 times higher from $\sim 10\%$ to $\sim 50\%$ with strain IncX4-*mcr-1*-carrying p35 strain at 72h after
286 infection, suggesting that IncX4-*mcr-1* plasmid are responsible to reduce bacterial virulence.

287

288

289 **Discussion**

290 The *mcr-1* gene was first discovered in *E. coli*, which has become the major host of *mcr-1*
291 gene, and has subsequently been found in all continents crossing more than 50 countries [8,37].
292 From 'one health' perspective, environmental factors seem to be closely associated with the
293 health of human and animals [38], for instance, the heavy use of antibiotic in livestock or
294 human and their entry into sewage system, is considered as the major cause of resistance
295 developing in zoonotic bacteria. MCRPE isolates have been mainly recovered from animal
296 samples [39] and infrequently from human normal flora and clinical samples [40,41], there is
297 a dearth of evidence on the link between these different populations. Here we present evidence
298 that blowflies serve an environmental pathway for the transmission of MCR-positive bacteria
299 including human pathogens. The importance of blowflies in the dissemination of MDR bacteria
300 is only becoming recognized and represents an additional public health concern. Due to their
301 habitation and their association with food animals and human, flies present a critical but under-

302 valued link between the environment and human communities. It has been previously identified
303 that flies can carry the same ESBL-producing *E. coli* clone as found in chicken manure in the
304 Netherlands [42], and identical antibiotic resistance genes were characterized from both flies
305 and swine feces [43]. In a modelling study, eight calves were exposed to flies, which were
306 inoculated with *E. coli* O157:H7, after 24 hours, fecal samples from all calves and drinking
307 water were positive for *E. coli* O157:H7 [44], suggesting that flies act an effective vector for
308 the spread of bacteria between animals and the synanthropic environment through feeding and
309 defecation. In our study, in the region Northern Thailand, 16% (48/300) of studied blowflies
310 possessed *mcr-I* gene, predominantly located on IncX4 plasmids (29/48, 60.42%) with higher
311 frequency (up to 5.93×10^{-3}), when comparing to other *mcr-I*-linked Inc types, such as IncHI1A
312 (mean 2.27×10^{-7} , Table 1). This high transferability of *mcr-I*-bearing plasmids from insect-
313 borne bacteria indicates that these bacteria can act as environmental reservoirs of MCR-
314 positive bacteria, which can potentially become human pathogens. So far, no less than 14
315 different *mcr-I* bearing plasmids incompatibility types have been identified with
316 approximately 35.2% of published *mcr-I*-carrying plasmids belonging to IncX4 plasmid,
317 which has been circulating in human, animal and environmental sectors [45]. More importantly,
318 the identical nucleotide sequences of 26 *mcr-I*-carrying IncX4 plasmid in our study share an
319 identical plasmid backbone to that obtained from human and animal samples in Thailand (**Fig.**
320 **3B**), with typical IncX4-plasmid housekeeping functions and an accessory *-mcr-I-pap2-*
321 cassette [34], further implying that IncX4-type plasmids serve as a *mcr-I* gene pool in Thailand.
322 The conjugative function of these *mcr-I*-linked plasmids allow *mcr-I* gene to spread
323 horizontally in and/or cross the species. Many insert elements have been recognized as a ‘copy-
324 out-paste-in’ mechanism, which can facilitate the acquisition and mobilization of antibiotic
325 resistance genes between bacterial pathogens [46]. For example, two copies or one copy of
326 *ISAp11* flanking in *mcr-I-papA* segment, is actively involved in capture and dissemination of

327 *mcr-1* genes [47-49]. Interestingly, lacking *ISAp11* or other insert element were found in *mcr-*
328 *1*-associated IncX4 genetic context in this study (Fig. 3), which lead to the hypothesis that
329 *ISAp11* initially mediates the movement of *mcr-1* genes, and lose one or both copies of *ISAp11*
330 during subsequent recombination [9,46,48].

331

332 WGS analysis provided comprehensive information for the *mcr-1*-carrying bacteria and their
333 phylogenetic relationship. Twelve different STs were identified in 31 MCRPEC strains, which
334 is consistent with other studies that MCRPEC isolates are highly diverse [9,50,51], but ST10-
335 like *E. coli* seems to represented the higher proportion in MCRPEC isolates (7/31, 22.58%).
336 *E. coli* ST10 frequently recovered from meat products [52], food-borne animals [53] and
337 human clinical samples [54], has been strongly associated with human infections and ESBL-
338 production [55]. Interestingly, in a surveillance study, ESBL-producing *E.coli* ST10 is the most
339 predominant lineage obtained from a military medical center in America [56]. *E. coli* ST10 is
340 common among MCRPEC isolates [45], recovered from human [57], animals [58] and
341 environmental sectors [59]. Apart from *mcr-1* gene, a variety of acquired resistance genes were
342 detected in all MCRPEC isolates (Fig.1), including plasmid-mediated quinolone resistance
343 gene (*qnrS1*) and ESBL-dependent *bla*_{CTX-M-14} and *bla*_{CTX-M-55} genes. Interestingly, a higher
344 number of acquired resistance genes has been found in seven *E. coli* ST10 isolates (mean 11.71,
345 ranging from 10 to 14), compared to other STs groups with average 9.67 ranging from 2 to
346 11(**Fig.1**), further supporting the previously findings that ST10-like *E. coli* strains are linked
347 to *mcr-1* gene [50].

348

349 Compared with the prevalence of MCRPEC, the reported incidence of MCRPKP is
350 comparatively rare. In a recent study, *mcr-1*-positive *E.coli*, *Providencia* spp and *Enterobacter*
351 *cloacae* strains were recovered from blowflies in China, but no *mcr-1*-carrying *K. pneumoniae*

352 strain was identified [12]. Sporadic *mcr-1*-positive isolates of *K. pneumoniae* have been
353 identified from patients [60-62], animal samples [63] and environmental sector (sewage water)
354 [64]. However, a recent outbreak of *bla*_{KPC} positive MCRPKP in Portugal as further raised the
355 seriousness of MCRPKP [65]. In this study 17 MCRPKP isolates were recovered from 300
356 blowflies (5.67%, 17/300) and all belonged to ST43 which has been reported globally in
357 clinical bacteria associated with abdominal infections [66,67], bacteremia [68] and intensive
358 care unit infections [69,70]. In addition, ST43 *K. pneumoniae* strains can carry clinically
359 relevant β -lactamases including NDM-1, CTX-M-15, VIM-5, and OXA-181 [66,70]
360 (supplementary Table S2). Furthermore, the pairwise analysis of SNPs data (no more than 15
361 SNPs, Table S2) further suggest ST43 MCRPKP clonality. This scenario is worrying, as
362 blowflies can act as an efficient and “unseen” environmental vectors of virulent bacteria, and
363 are associated with outbreaks of enteric pathogens in rural areas in low- and middle income
364 countries where sanitation and hygiene infrastructure is poor [6,71]. Additionally, the ST43
365 MCRPKP isolates described in our study also contain at least four major virulence
366 determinants responsible for disease progression: capsular synthesis loci KL61;
367 lipopolysaccharide; siderophores enterobactin and (mobilizable) aerobactin, iron acquisition
368 *kfuABC* that are responsible for binding ferric iron in the host cell; and adherence factors
369 (fimbria type I and III) that allow bacteria to attach to the host cell surface [24,36,72]. The
370 virulence potential of these isolates performed in a *G. mellonella* model (**Fig. 4**), suggest that
371 ST43 MCRPKP are virulent clones circulating with blowflies in Thailand. Thus, MCRPE
372 strains obtained from blowflies present a global public health problem owing to: i) common
373 blowflies inhabit the environment and global communities, and act as bacterial environmental
374 reservoirs via animals/humans and waste; ii) most of MCRPE strains in these study are
375 multidrug resistant, especially the virulent MCRPKP strains that can be transmitted and cause
376 infections in humans via contact with blowflies e.g post-surgical wounds, and iii) *mcr-1*

377 detected from blowflies is located on transferable plasmids increasing the possibility of
378 horizontal transfer of *mcr-I* gene between bacteria as part of the blowflies microbiota, thereby
379 increasing the environmental gene pool and posing a greater public health risk.

380

381 **Data availability:** Whole genomic sequences of 48 studied MCRPE strains have been
382 deposited in the NCBI database (BioProject accession No. PRJNA503337 and BioSample
383 accession No. SAMN10358806 to SAMN10358853). Genomic data of additional 17 MCRPEC
384 strains showed in Fig.3B were also submitted to the NCBI database (BioProject accession No.
385 PRJNA504530 and BioSample accession No. SAMN10394864 to SAMN10394880).

386

387

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624 **Table 1** characteristics of 48 MCRPE strains recovered from blowflies in Northern Thailand

Strain code	Species	Isolated area	colistin MIC(mg/L)	MLST	mcr-1-bearing Inc plasmid type	Conjugation frequency
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PN100	<i>K. pneumoniae</i>	Suburb area	4	43	IncX4	2.1x10 ⁻⁵
PN104	<i>K. pneumoniae</i>	Rural area	16	43	IncX4	1.5x10 ⁻⁶
PN105	<i>K. pneumoniae</i>	Suburb area	8	43	IncX4	1.5x10 ⁻⁵
PN106	<i>K. pneumoniae</i>	Suburb area	8	43	IncX4	2.1x10 ⁻⁵
PN107	<i>K. pneumoniae</i>	Rural area	16	43	IncX4	9.45x10 ⁻⁷
PN110	<i>K. pneumoniae</i>	Rural area	16	43	IncX4	2.08x10 ⁻⁶
PN114	<i>K. pneumoniae</i>	Suburb area	8	43	IncX4	7.63x10 ⁻⁶
PN118	<i>K. pneumoniae</i>	Suburb area	8	43	IncX4	2.44x10 ⁻⁵
PN120	<i>K. pneumoniae</i>	Rural area	4	43	IncX4	2.0x10 ⁻⁵
PN77	<i>K. pneumoniae</i>	Suburb area	4	43	IncX4	1.5x10 ⁻⁶
PN79	<i>K. pneumoniae</i>	Rural area	4	43	IncX4	3.13x10 ⁻⁵
PN81	<i>K. pneumoniae</i>	Suburb area	4	43	IncX4	2.86x10 ⁻⁵
PN84	<i>K. pneumoniae</i>	Suburb area	4	43	IncX4	3.0x10 ⁻⁵
PN95	<i>K. pneumoniae</i>	Suburb area	8	43	IncX4	1.0x10 ⁻⁵
PN96	<i>K. pneumoniae</i>	Suburb area	4	43	IncX4	7.11x10 ⁻⁷
PN97	<i>K. pneumoniae</i>	Suburb area	4	43	IncX4	2.31x10 ⁻⁶
PN98	<i>K. pneumoniae</i>	Suburb area	4	43	IncX4	8.03x10 ⁻⁷
PN123	<i>E.coli</i>	Local market in urban community	4	2345	IncHI1A	1.44X10 ⁻⁷
PN33	<i>E.coli</i>	Local market in urban community	4	10	NA	1.33x10 ⁻³
PN93	<i>E.coli</i>	Local market in urban community	4	162	IncX4	NA
PN103	<i>E.coli</i>	Rural area	8	10	IncHI1A	3.1x10 ⁻⁷
PN109	<i>E.coli</i>	Rural area	16	1244	IncX4	7.6x10 ⁻⁴
PN111	<i>E.coli</i>	Rural area	8	457	NA	NA
PN116	<i>E.coli</i>	Rural area	4	648	NA	NA
PN119	<i>E.coli</i>	Rural area	8	549	NA	NA
PN74	<i>E.coli</i>	Rural area	16	10	IncX4	NA
PN75	<i>E.coli</i>	Rural area	4	58	IncHI1B	NA
PN87	<i>E.coli</i>	Rural area	4	549	NA	NA
PN88	<i>E.coli</i>	Rural area	4	10	IncX4	NA
PN91	<i>E.coli</i>	Rural area	4	181	IncX4	6.67x10 ⁻⁵
PN101	<i>E.coli</i>	Suburb area	4	58	NA	NA
PN102	<i>E.coli</i>	Suburb area	4	648	NA	NA
PN108	<i>E.coli</i>	Suburb area	8	549	IncX4	NA
PN112	<i>E.coli</i>	Suburb area	8	181	IncX4	NA
PN117	<i>E.coli</i>	Suburb area	8	201	IncX4	5.94x10 ⁻³
PN121	<i>E.coli</i>	Suburb area	8	5487	NA	NA
PN122	<i>E.coli</i>	Suburb area	8	549	NA	NA
PN124	<i>E.coli</i>	Suburb area	8	181	IncX4	5.33x10 ⁻⁴
PN126	<i>E.coli</i>	Suburb area	4	648	IncHI1B	N.S
PN127	<i>E.coli</i>	Suburb area	4	648	IncHI1B	1.85x10 ⁻⁷
PN73	<i>E.coli</i>	Suburb area	4	648	IncHI1B	N.S
PN76	<i>E.coli</i>	Suburb area	4	58	IncHI1A_IncHI1B	8.70x10 ⁻⁸
PN78	<i>E.coli</i>	Suburb area	4	10	IncX4	NA
PN80	<i>E.coli</i>	Suburb area	8	218	NA	1.14x10 ⁻⁴
PN83	<i>E.coli</i>	Suburb area	8	10	IncX4	6.27x10 ⁻⁷
PN85	<i>E.coli</i>	Suburb area	4	2705	NA	N.S
PN86	<i>E.coli</i>	Suburb area	8	218	IncX4	NA
PN92	<i>E.coli</i>	Suburb area	4	10	NA	NA

625 MLST is analysed by seven allele sequence using MLST2.0 (See methods). NA, not available; N.S, not successful

626

627 **Fig.1** Phylogenetic trees of 31 MCRPEC isolates recovered from blowflies, were analysed by Parsnp
628 in the Harvest package, and visualized by iTOL (<https://itol.embl.de/>). The pink circles indicate
629 the presence of *mcr-1* gene. The presence or lack of AMR genes is colored in red or light yellow,
630 respectively.

631

632

633 **Fig.2** PFGE analysis of MCR-1-producing strains digested with S1 nuclease (right) and hybridization
634 with *mcr-1* gene probe (right). White arrows showed the location and size of *mcr-1*-carrying plasmids.

635

636

637 **Fig.3** (A). Alignment of 26 *mcr-1*-complete plasmids and visualized using BLAST Ring Image
638 Generator (BRIG v0.9555). First inner ring is the plasmid obtained from PN105, used as reference for
639 the alignment. (B) Alignment of 29 *mcr-1*-complete plasmids and visualized using BRIG
640 v0.9555. First inner ring is the plasmid pMCR0617mcr used as a reference for the alignment,
641 GenBank accession number and size of the reference plasmid indicated in the middle of rings.
642 These *mcr-1*-carrying plasmids are recovered from different sources, namely, 10 representative
643 *mcr-1*-linked plasmids from blowflies (PN78, PN83, PN86, PN88, PN91, PN93, PN112,
644 PN117, PN109 and PN120), 2 from companion animals (PN10 and PN11), 4 from poultry
645 (PN23, 24, 25 and 29), 11 from human feces (PN45, 42, 57, 41, 46, 51, 58, 60, 47, 49 and
646 71). Besides those strains from blowflies fully described in Table 1, whole genomic sequence
647 of all these MCRPE strains have been deposited in the NCBI database (see data availability).

648

649

650 **Fig.4** A. The image of *G. mellonella* over 12 h post-infection with ST43 MCRPKP strains and a clinical
651 reference strain K1. B and C, Kaplan-Meier plots showing the percent survival of *G. mellonella* over

652 24 h post-infection with the 10^4 CFU/ml (B) and 10^5 CFU/ml (C) inoculum of MCRPKP and strain K1.
653 Survival curves were plotted using the Kaplan-Meier method (GraphPad Software).

654

655 **Fig.5** A and B, Kaplan-Meier plots showing the percent survival of *G. mellonella* over 72 h post-
656 infection with the 10^5 CFU/ml inoculum of clinical susceptible *K. pneumoniae* ff101 and clinical KPC-
657 positive *K. pneumoniae* p35, with or without *mcr-1*-carrying plasmid. Survival curves were plotted
658 using the Kaplan-Meier method (GraphPad Software).