1   **Environmental dissemination of *mcr-1* positive Enterobacteriaceae by *Chrysomya* spp.**

2   **(common blowfly): an increasing public health risk**

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5   Qiu E Yang[1], Uttapoln Tansawai[2], Diego O. Andrey[1,3], Shaolin Wang[4], Yang Wang[4], Kirsty

6   Sands[1], Anong Kiddee[2], Kanit Assawatheptawee[2], Nophawan Bunchu[2], Brekhna Hassan[1],

7   Timothy Rutland Walsh[1], Pannika R. Niumsup[2]

8

9   **Address information:**

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11   1.   Department of Medical Microbiology and Infectious Disease, Division of Infection

12   and Immunity, Cardiff University, Cardiff CF14 4XN, UK

13   2.   Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan

14   University, Phitsanulok, 65000, Thailand.

15   3.   Service of Infectious Diseases, Geneva University Hospitals and Faculty of Medicine,

16   1211Geneva, Switzerland

17   4.   Beijing Advanced Innovation Centre for Food Nutrition and Human Health, College of

18   Veterinary Medicine, China Agricultural University, Beijing, 100193, China

19

20   *To whom correspondence should be addressed: Qiu-E Yang (YangQe@cardiff.ac.uk) or

21   Timothy R. Walsh (WalshTR@cardiff.ac.uk)

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Abstract

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

Highlights

- WGS-based analysis of *mcr-1*-carrying isolates from blowflies (*Chrysomya* spp.) provides evidence that flies serve as an active vector for the environmental spread of *mcr-1*-mediated colistin resistance pathogens.

- Hyper-virulent *mcr-1*-carrying *Klebsiella pneumoniae* isolates were identified from blowflies, which post an acute public health risk.

- Diversity of antibiotic resistance genes including *qnrS1*, *fosA*, *bla*CTX-M-55/14 and *floR*, was detected in *mcr-1* positive strains recovered from blowflies indicating the environmental spread of multi-drug resistant pathogens.

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

Introduction

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

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

5

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 ^{o}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-1* using standalone BLAST analysis,

136   the putative coding sequences containing *mcr-1* 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-1*-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-1*-related IncX4 plasmid backgrounds were performed using BLAST Ring

147   Image Generator (BRIG v0.9555). Briefly, *mcr-1*-containing contigs were extracted from genomic data,

148   and a fully sequenced *mcr-1*-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-1*-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 (*fimABCDEFGH* 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   µl 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].

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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.

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

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

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**).

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

K. pneumoniae is recognized as a serious threat to patients due to the emergence of MDR strains associated with hospital outbreaks and that they demonstrate a number of virulence factors associated with bacterial pathogenicity and poor patient outcome [36]. There are at least five groups of pathogenicity factors found in 17 MCRPKP isolates (**Table S1**), these include gene clusters associated with serum resistance (*traT*); adhesins (type I fimbrial operon *fimABCDEFGH*, and type III firmbrial operon *mrkBCDEF*); lipopolysaccharide (*wabGHN*); siderophore systems enterobactin-*entABCDEFS*, and aerobactin-*iucABCD-iutA*. Using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/), IncFIB-plasmid-related aerobactin was found in at least 14 isolates, suggesting potential aerobactin-mediated virulence transferability. Capsular synthesis loci matched type KL61 (wzi/wzc 412/61 typing) (Kaptive). Similarly, the iron acquisition operons *kfuABC*, *iroE*, urease-synthesis operon *ureABCDEFG* associated with gastric ulceration and urinary stone formation, and gene *ycfM* encoding surface protein were identified in all MCRPKP isolates. At least 10 different virulence factors were found in the 17 ST43 MCRPKP strains, which we further analysed by using a *G. mellonella* model [34]. The effect of different inoculum of 10 randomly selected ST43 MCRPKP strains was assessed in this model using K1 *rmpA*-positive *K. pneumomiae* A58300, as a hypervirulent strain reference strain [35]. As shown in **Fig. 4**, after 12-hour post-infection, with an inoculum of approx. $1x10^6$ CFU, 100% of mortality was observed with the K1 strain and all 10 ST43 MCRPKP strains. At an inoculum of $1x10^5$ CFU, the survival rate was 70% with K1 strain and 0% with 10 ST43 MCRPKP strains. With an inoculum of $1x10^4$ CFU, 100% survival was seen with the K1 strain but only 20% with ST43. The consistency between genotypic virulence factors and the reproducible results of the *G. mellonella* infection model suggest that the ST43 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. pneumomiae* strain ff101 and a KPC-positive *K. pneumomiae* strain p35,

281 followed by infection of *G. mellonella* larvae with an inoculum of ~1x10^5 CFU. As shown in

282 **Fig. 5,** two *K. pneumomiae* 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 ~10% to ~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-

valued link between the environment and human communities. It has been previously identified that flies can carry the same ESBL-producing *E. coli* clone as found in chicken manure in the Netherlands [42], and identical antibiotic resistance genes were characterized from both flies and swine feces [43]. In a modelling study, eight calves were exposed to flies, which were inoculated with *E. coli* O157:H7, after 24 hours, fecal samples from all calves and drinking water were positive for *E.coli* O157:H7 [44], suggesting that flies act an effective vector for the spread of bacteria between animals and the synanthropic environment through feeding and defecation. In our study, in the region Northern Thailand, 16% (48/300) of studied blowflies possessed *mcr-1* gene, predominantly located on IncX4 plasmids (29/48, 60.42%) with higher frequency (up to 5.93×10^{-3}), when comparing to other *mcr-1*-linked Inc types, such as IncHI1A (mean 2.27×10^{-7}, Table 1). This high transferability of *mcr-1*-bearing plasmids from insect-borne bacteria indicates that these bacteria can act as environmental reservoirs of MCR-positive bacteria,which can potentially become human pathogens. So far, no less than 14 different *mcr-1* bearing plasmids incompatibility types have been identified with approximately 35.2% of published *mcr-1*-carrying plasmids belonging to IncX4 plasmid, which has been circulating in human, animal and environmental sectors [45]. More importantly, the identical nucleotide sequences of 26 *mcr-1*-carrying IncX4 plasmid in our study share an identical plasmid backbone to that obtained from human and animal samples in Thailand (**Fig. 3B**), with typical IncX4-plasmid housekeeping functions and an accessory *-mcr-1-pap2-*cassette[34], further implying that IncX4-type plasmids serve as a *mcr-1* gene pool in Thailand. The conjugative function of these *mcr-1*-linked plasmids allow *mcr-1* gene to spread horizontally in and/or cross the species. Many insert elements have been recognized as a 'copy-out-paste-in' mechanism, which can facilitate the acquisition and mobilization of antibiotic resistance genes between bacterial pathogens [46]. For example, two copies or one copy of *ISApl1* flanking in *mcr-1-papA* segment, is actively involved in capture and dissemination of

327   *mcr-1* genes [47-49]. Interestingly, lacking *ISApl1* 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   *ISApl1* initially mediates the movement of *mcr-1* genes, and lose one or both copies of *ISApl1*

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. penumoniae*

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 ß-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*

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377 detected from blowflies is located on transferable plasmids increasing the possibility of

378 horizontal transfer of *mcr-1* gene between bacteria as part of the blowflies microbiota, thereby

379 increasing the environmental gene pool and posing a greater public health risk.

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399 **All authors have no conflict of interest to declare in the article**.

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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

PN100	*K. pneumoniae*	Suburb area	4	43	IncX4	2.1×10^{-5}
PN104	*K. pneumoniae*	Rural area	16	43	IncX4	1.5×10^{-6}
PN105	*K. pneumoniae*	Suburb area	8	43	IncX4	1.5×10^{-5}
PN106	*K. pneumoniae*	Suburb area	8	43	IncX4	2.1×10^{-5}
PN107	*K. pneumoniae*	Rural area	16	43	IncX4	9.45×10^{-7}
PN110	*K. pneumoniae*	Rural area	16	43	IncX4	2.08×10^{-6}
PN114	*K. pneumoniae*	Suburb area	8	43	IncX4	7.63×10^{-6}
PN118	*K. pneumoniae*	Suburb area	8	43	IncX4	2.44×10^{-5}
PN120	*K. pneumoniae*	Rural area	4	43	IncX4	2.0×10^{-5}
PN77	*K. pneumoniae*	Suburb area	4	43	IncX4	1.5×10^{-6}
PN79	*K. pneumoniae*	Rural area	4	43	IncX4	3.13×10^{-5}
PN81	*K. pneumoniae*	Suburb area	4	43	IncX4	2.86×10^{-5}
PN84	*K. pneumoniae*	Suburb area	4	43	IncX4	3.0×10^{-5}
PN95	*K. pneumoniae*	Suburb area	8	43	IncX4	1.0×10^{-5}
PN96	*K. pneumoniae*	Suburb area	4	43	IncX4	7.11×10^{-7}
PN97	*K. pneumoniae*	Suburb area	4	43	IncX4	2.31×10^{-6}
PN98	*K. pneumoniae*	Suburb area	4	43	IncX4	8.03×10^{-7}
PN123	*E.coli*	Local market in urban community	4	2345	IncHI1A	1.44×10^{-7}
PN33	*E.coli*	Local market in urban community	4	10	NA	1.33×10^{-3}
PN93	*E.coli*	Local market in urban community	4	162	IncX4	NA
PN103	*E.coli*	Rural area	8	10	IncHI1A	3.1×10^{-7}
PN109	*E.coli*	Rural area	16	1244	IncX4	7.6×10^{-4}
PN111	*E.coli*	Rural area	8	457	NA	NA
PN116	*E.coli*	Rural area	4	648	NA	NA
PN119	*E.coli*	Rural area	8	549	NA	NA
PN74	*E.coli*	Rural area	16	10	IncX4	NA
PN75	*E.coli*	Rural area	4	58	IncHI1B	NA
PN87	*E.coli*	Rural area	4	549	NA	NA
PN88	*E.coli*	Rural area	4	10	IncX4	NA
PN91	*E.coli*	Rural area	4	181	IncX4	6.67×10^{-5}
PN101	*E.coli*	Suburb area	4	58	NA	NA
PN102	*E.coli*	Suburb area	4	648	NA	NA
PN108	*E.coli*	Suburb area	8	549	IncX4	NA
PN112	*E.coli*	Suburb area	8	181	IncX4	NA
PN117	*E.coli*	Suburb area	8	201	IncX4	5.94×10^{-3}
PN121	*E.coli*	Suburb area	8	5487	NA	NA
PN122	*E.coli*	Suburb area	8	549	NA	NA
PN124	*E.coli*	Suburb area	8	181	IncX4	5.33×10^{-4}
PN126	*E.coli*	Suburb area	4	648	IncHI1B	N.S
PN127	*E.coli*	Suburb area	4	648	IncHI1B	1.85×10^{-7}
PN73	*E.coli*	Suburb area	4	648	IncHI1B	N.S
PN76	*E.coli*	Suburb area	4	58	IncHI1A_IncHI1B	8.70×10^{-8}
PN78	*E.coli*	Suburb area	4	10	IncX4	NA
PN80	*E.coli*	Suburb area	8	218	NA	1.14×10^{-4}
PN83	*E.coli*	Suburb area	8	10	IncX4	6.27×10^{-7}
PN85	*E.coli*	Suburb area	4	2705	NA	N.S
PN86	*E.coli*	Suburb area	8	218	IncX4	NA
PN92	*E.coli*	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.

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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.

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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).

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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).

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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).