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

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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*, $bla_{\text{CTX-M-55/14}}$ and *floR*, was detected in *mcr-1* positive strains recovered from blowflies indicating the environmental spread of multi-drug resistant pathogens.

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

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

Materials and Methods

Bacterial isolates from blow flies

A total of 300 blow flies were trapped at three different locations in Northern Thailand: a local market in an urban community, a rural area and a suburb of the city Phitsanulok. These locations are approximately 10 kilometers apart. Blow flies were collected by the use of a sterile sweeping net. Individual fly was kept in a sterile plastic tube and sacrificed by placing on ice for 30 min. They were identified to species level by using the taxonomic keys as described by Kurahashi and Bunchu [17]. Only *Chrysomya megaceph*ala flies [18], the most abundant blow flies in Thailand were selected for further analysis. The flies were individually pulverized in enriched peptone water for 30 min and then aliquots of the resultant suspensions (100 µl) were plated on Eosin-Methylene-Blue (EMB)-agar plates supplemented with 2 mg/l colistin and incubated at 37^{o}C overnight. One to three representative colonies with different colors from each plates were purified and subsequently screened for *mcr-1* gene by PCR. The *mcr-1*-positive bacteria were sub-cultured in liquid nutrient broth for 18 h before DNA extraction for species identification and whole-genome sequence. Minimum inhibitory concentrations (MICs) of colistin for 48 *mcr-1*-bearing isolates was performed by using broth microdilution, in accordance with the guideline of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), reference strain *E. coli* ATCC25922 served as a quality control.

Conjugation experiments

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

Whole-genome sequencing and bioinformatics analysis

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

Bioinformatics analysis: The CGE platform (http://www.genomicepidemiology.org/) were used for analysis of multilocus sequence typing (MLST-1.8), acquired resistance genes (ResFinder 3.1, all antibiotic resistance databases were selected with a cut-off value of 95% identity and 80% minimum coverage) and incompatibility group of plasmids (PlasmidFinder-1.3 version, using Enterobacteriaceae database with parameters of minimum 95% identity and 85% query coverage). All contigs were searched for *mcr-1* using standalone BLAST analysis, the putative coding sequences containing *mcr-1* gene were obtained using ORF finder programs (Geneious 10.0.7). Draft genome sequences were aligned and then applied for phylogenetic analysis using Parsnp in the Harvest package, and phylogenetic trees was visualized by iTOL (https://itol.embl.de/). MCRPKP strain p38 recovered from healthy human feces in Thailand was served as a reference strain in the SNPs analysis of 17 MCRPKP strains in this study.

Primer walking was performed to fill the gap in *mcr-1*-carrying contigs from strain PN105 with primers (PN_IncX4_forward: CGACCTTTAAGTCGTATTTGCAAGT; PN_IncX4_reverse: ATTGCGCCCGTAGTTCGCTA, Tm 60ºC) and the complete plasmid sequence were constructed by *de novo* assembly using Geneious (10.0.7). The circular comparisons among *mcr-1*-related IncX4 plasmid backgrounds were performed using BLAST Ring Image Generator (BRIG v0.9555). Briefly, *mcr-1*-containing contigs were extracted from genomic data, and a fully sequenced *mcr-1*-linked IncX4 plasmid was served as a central reference sequence. In this study, plasmid IncX4 from strain PN105 and pMR0617mcr (GenBank No. CP024041), act as reference sequences. The similarity of between the central reference sequence and other *mcr-1*-positive contigs from studied MCRPE strains, shows as concentric rings with representative colors.

Virulence factors of *K. pneumoniae* isolates

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

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

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

Results

Details of isolates

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

Whole genome sequencing (WGS) analysis

A total of 48 MCRPE isolates were sequenced using Illumina Miseq platform. The distinct MCRPEC isolates belonged to 12 STs (**Table 1**): ST10(n=7), ST648(n=5), ST549(n=4), ST58(n=3), ST181(n=3), ST218(n=2), ST201(n=1), ST162(n=1), ST457(n=1), ST1244(n=1), ST2345(n=1), ST2705(n=1) and ST5487(n=1). Most interestingly, all 17 MCRPKP isolates belonged to ST43, thus we further determined the clonal relationship of 17 ST43 *K. pneumoniae* isolates by SNPs analysis using Parsnps software. Phylogenetic tree analysis for 17 strains based on their raw sequencing reads showed that their core genome differed only by a few SNPs (the numbers of differences in SNPs are up to 15, Table S1), suggesting the clonal 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*$_{\text{TEM-1b}}$, *bla*$_{\text{CTX-M-55}}$ and *bla*$_{\text{CTX-M-14}}$ 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*$_{\text{TEM-1b}}$, *tetA*, *bla*$_{\text{SHV-40}}$ encoding SHV ß-lactamase and *fosA* mediating fosmycin resistance.

mcr-1-associated plasmid types and transferability of _mcr-1_ gene

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

Based on our previous study, the acquisition of *mcr-1*-carrying plasmid leads to virulence loss in *E. coli* strain [34]. In this study, three *mcr-1*-carrying plasmids were transferred into a clinical susceptible *K. pneumomiae* strain ff101 and a KPC-positive *K. pneumomiae* strain p35, followed by infection of *G. mellonella* larvae with an inoculum of ~1x10^5 CFU. As shown in **Fig. 5,** two *K. pneumomiae* strains ff101 and p35 caused more than 80% and 90% of mortality after 72h infection, respectively. After acquiring of IncX4-*mcr-1* plasmid, the survivals of larvae have been increased to 40% - 80% with strain ff101, and survivals are more than five times higher from ~10% to ~50% with strain IncX4-*mcr-1*-carrying p35 strain at 72h after infection, suggesting that IncX4-*mcr-1* plasmid are responsible to reduce bacterial virulence.

Discussion

The *mcr-1* gene was first discovered in *E. coli*, which has become the major host of *mcr-1* gene, and has subsequently been found in all continents crossing more than 50 countries [8,37]. From 'one health' perspective, environmental factors seem to be closely associated with the health of human and animals [38], for instance, the heavy use of antibiotic in livestock or human and their entry into sewage system, is considered as the major cause of resistance developing in zoonotic bacteria. MCRPE isolates have been mainly recovered from animal samples [39] and infrequently from human normal flora and clinical samples [40,41], there is a dearth of evidence on the link between these different populations. Here we present evidence that blowflies serve an environmental pathway for the transmission of MCR-positive bacteria including human pathogens. The importance of blowflies in the dissemination of MDR bacteria is only becoming recognized and represents an additional public health concern. Due to their habitation and their association with food animals and human, flies present a critical but undervalued 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 $5.93x10^{-3}$), when comparing to other *mcr-1*-linked Inc types, such as IncHI1A (mean $2.27x10^{-7}$, 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 *mcr-1* genes [47-49]. Interestingly, lacking *ISApl1* or other insert element were found in *mcr-1*-associated IncX4 genetic context in this study (Fig. 3), which lead to the hypothesis that *ISApl1* initially mediates the movement of *mcr-1* genes, and lose one or both copies of *ISApl1* during subsequent recombination [9,46,48].

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

Compared with the prevalence of MCRPEC, the reported incidence of MCRPKP is comparatively rare. In a recent study, *mcr-1*-positive *E.coli*, *Providencia* spp and *Enterobacter cloacae* strains were recovered from blowflies in China, but no *mcr-1*-carrying *K. penumoniae* strain was identified [12]. Sporadic *mcr-1*-positive isolates of *K. pneumoniae* have been identified from patients [60-62], animal samples [63] and environmental sector (sewage water) [64]. However, a recent outbreak of *bla*$_{\mathrm{KPC}}$ positive MCRPKP in Portugal as further raised the seriousness of MCRPKP [65]. In this study 17 MCRPKP isolates were recovered from 300 blowflies (5.67%, 17/300) and all belonged to ST43 which has been reported globally in clinical bacteria associated with abdominal infections [66,67], bacteremia [68] and intensive care unit infections [69,70]. In addition, ST43 *K. pneumoniae* strains can carry clinically relevant ß-lactamases including NDM-1, CTX-M-15, VIM-5, and OXA-181 [66,70] (supplementary Table S2). Furthermore, the pairwise analysis of SNPs data (no more than 15 SNPs, Table S2) further suggest ST43 MCRPKP clonality. This scenario is worrying, as blowflies can act as an efficient and “unseen” environmental vectors of virulent bacteria, and are associated with outbreaks of enteric pathogens in rural areas in low- and middle income countries where sanitation and hygiene infrastructure is poor [6,71]. Additionally, the ST43 MCRPKP isolates described in our study also contain at least four major virulence determinants responsible for disease progression: capsular synthesis loci KL61; lipopolysaccharide; siderophores enterobactin and (mobilizable) aerobactin, iron acquisition *kfuABC* that are responsible for binding ferric iron in the host cell; and adherence factors (fimbria type I and III) that allow bacteria to attach to the host cell surface [24,36,72]. The virulence potential of these isolates performed in a *G. mellonella* model (**Fig. 4**), suggest that ST43 MCRPKP are virulent clones circulating with blowflies in Thailand. Thus, MCRPE strains obtained from blowflies present a global public health problem owing to: i) common blowflies inhabit the environment and global communities, and act as bacterial environmental reservoirs via animals/humans and waste; ii) most of MCRPE strains in these study are multidrug resistant, especially the virulent MCRPKP strains that can be transmitted and cause infections in humans via contact with blowflies e.g post-surgical wounds, and iii) *mcr-1* detected from blowflies is located on transferable plasmids increasing the possibility of horizontal transfer of *mcr-1* gene between bacteria as part of the blowflies microbiota, thereby increasing the environmental gene pool and posing a greater public health risk.

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

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

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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	2.1x10^{-5}
PN104	*K. pneumoniae*	Rural area	16	43	IncX4	1.5x10^{-6}
PN105	*K. pneumoniae*	Suburb area	8	43	IncX4	1.5x10^{-5}
PN106	*K. pneumoniae*	Suburb area	8	43	IncX4	2.1x10^{-5}
PN107	*K. pneumoniae*	Rural area	16	43	IncX4	9.45x10^{-7}
PN110	*K. pneumoniae*	Rural area	16	43	IncX4	2.08x10^{-6}
PN114	*K. pneumoniae*	Suburb area	8	43	IncX4	7.63x10^{-6}
PN118	*K. pneumoniae*	Suburb area	8	43	IncX4	2.44x10^{-5}
PN120	*K. pneumoniae*	Rural area	4	43	IncX4	2.0x10^{-5}
PN77	*K. pneumoniae*	Suburb area	4	43	IncX4	1.5x10^{-6}
PN79	*K. pneumoniae*	Rural area	4	43	IncX4	3.13x10^{-5}
PN81	*K. pneumoniae*	Suburb area	4	43	IncX4	2.86x10^{-5}
PN84	*K. pneumoniae*	Suburb area	4	43	IncX4	3.0x10^{-5}
PN95	*K. pneumoniae*	Suburb area	8	43	IncX4	1.0x10^{-5}
PN96	*K. pneumoniae*	Suburb area	4	43	IncX4	7.11x10^{-7}
PN97	*K. pneumoniae*	Suburb area	4	43	IncX4	2.31x10^{-6}
PN98	*K. pneumoniae*	Suburb area	4	43	IncX4	8.03x10^{-7}
PN123	*E.coli*	Local market in urban community	4	2345	IncHI1A	1.44X10^{-7}
PN33	*E.coli*	Local market in urban community	4	10	NA	1.33x10^{-3}
PN93	*E.coli*	Local market in urban community	4	162	IncX4	NA
PN103	*E.coli*	Rural area	8	10	IncHI1A	3.1x10^{-7}
PN109	*E.coli*	Rural area	16	1244	IncX4	7.6x10^{-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	6.67x10^{-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	5.94x10^{-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	5.33x10^{-4}
PN126	*E.coli*	Suburb area	4	648	IncHI1B	N.S
PN127	*E.coli*	Suburb area	4	648	IncHI1B	1.85x10^{-7}
PN73	*E.coli*	Suburb area	4	648	IncHI1B	N.S
PN76	*E.coli*	Suburb area	4	58	IncHI1A_IncHI1B	8.70x10^{-8}
PN78	*E.coli*	Suburb area	4	10	IncX4	NA
PN80	*E.coli*	Suburb area	8	218	NA	1.14x10^{-4}
PN83	*E.coli*	Suburb area	8	10	IncX4	6.27x10^{-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

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

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

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

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

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

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

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