A novel plasmid mediated polymyxin resistance determinant (*mcr-1.8*) in *Escherichia coli* recovered from broiler chickens in Brunei Darussalam

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Running Title: Novel plasmid mediated polymyxin resistance determinant *mcr-1.8.*

Sir,

Multi-drug resistant (MDR) Gram-negative bacteria are identified as critical pathogens and their effective treatment increasingly relies on the polymyxins (polymyxin B, colistin), either alone or, as part of unorthodox combination therapies.[1] The rapid emergence of polymyxin resistance due to mutations / insertions in genes involved in LPS modifications (*lpxCAD, pmrA/B, mgrB, phoP/Q, ccrAB*) has been reported among individuals exposed to or treated with polymyxins.[1] Of greater concern are increasing reports of resistance due to the acquisition of phosphoenthanolamine (PEtN) transferases, enzymes that catalyze the addition of phosphoethanolamine to lipid A resulting in lower binding affinity of polymyxins.[1] Since the first identification in China,[1] multiple gene variants have been reported in diverse bacterial genera recovered from a range of human, retail food, food producing animal and environmental sources.[2] Here, we report a novel variant of PEtN transferase, designated MCR-1.8, and its genetic context in an MDR *E. coli* isolate recovered from poultry in Brunei Darussalam.

Isolate C22 was isolated pre-slaughter from a healthy bird during a surveillance study (2017) and identified as *E. coli* by MALDI-TOF MS (Bruker, Coventry, UK). Susceptibility testing according to EUCAST / CLSI methods confirmed resistance to colistin (MIC=8 mg/L), polymyxin B (MIC=12 mg/L) and an MDR phenotype (Table S1). Screening of *E.coli* C22 for *mcr-like* genes by PCR[3] and Sanger sequencing, revealed a variant with a single non-synonymous nucleotide difference (A8G) from *mcr-1.1* (Table S2) designated *mcr-1.8* by NCBI (Accession number KY683842.1) and resulting in a glutamine to arginine substitution (Q3R) in the transmembrane portion of the protein, physically distant from the extracellular catalytic domain. The previously identified MCR-1.2 and MCR-1.12 also contain respectively the amino acid substitutions Q3L and Q3H with no attributable loss of catalytic function.[4] The purified *mcr-1.8* amplicon was cloned into pCR-Blunt II TOPO vector

(Invitrogen, Paisley, UK) and expressed in *E. coli* TOP10 under its native promoter, conferring a 4- to 16-fold increase in the MIC of colistin and polymyxin B (Table S1).

Whole-genome sequencing of *E. coli* C22 with the Illumina HiSeq platform (Illumina, Inc., San Diego, CA) followed by *de novo* assembly and annotation identified the *mcr-1.8* gene as the only antimicrobial resistance gene, located between the *topB* (encoding a DNA topoisomerase III) and *nikB* (relaxase) genes, on a 63,056 bp IncI2 plasmid (pEC-MCR1.8) (Figure 1). Illumina reads was aligned using the nucleotide sequence of pMRY16-002_4 as an IncI2 reference plasmid (Ref) .

Analysis of pEC-MCR1.8 (KY792081.1) confirmed a similar position of *mcr-1.8* and absence of the IS*Apl1* locus as in other IncI2 plasmids carrying *mcr-1*-like genes (Fig. 1). Apart from the *mcr-1.8* gene, pEC-MCR1.8 is predicted to encode 85 open reading frames in total, including genes for replication, maintenance, partitioning and stability, as well as conjugal transfer/ formation of type IV pilus. Immediately downstream of *mcr-1.8* was the *pap2* gene predicted to encode a membrane-associated phosphatase enzyme able to catalyze the removal of terminal phosphate groups from lipid carriers essential in the transport of hydrophilic small molecules across the outer membrane.[5] The role of PAP2-like phosphatases is unclear, although PAP2 does not seem to influence susceptibility to polymyxins.[6]

Transfer of plasmid-mediated resistance genes by conjugation was investigated using *E. coli* J53 (recipient) with transconjugants (ECJ53/C22) selected by plating onto MH II agar supplemented with colistin (4 mg/L) and sodium azide (150 mg/L). Polymyxin resistance was transferable with a frequency of 2.6 x 10^{-4} transconjugants per donor cell, suggesting pEC-MCR1.8 has the potential to spread into key human pathogens.

Analysis of the entire genome sequence of *E. coli* C22 was performed as previously described[8] and confirmed the presence of multiple resistance genes to aminoglycosides

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Elucidation of quantitative structural diversity of remarkable rearrangement regions, shufflons, in IncI2 plasmids.
Sekizuka T[1], Kawanishi M[2], Ohnishi M[3], Shima A[4], Kato K[5], Yamashita A[5], Matsui M[4], Suzuki S[4], Kuroda M[5].

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(*aph(4)-Ia*, *aadA1*, *aac(3)-IVa*, *aph(3')-Ic*), β-lactams (*bla*$_{TEM-1B}$, *bla*$_{CTX-M-65}$), fluoroquinolones (*qnrS1*), fosfomycin (*fosA4*), phenicols (*cmlA1*, *floR*), trimethoprim (*dfrA15*, *dfrA14*), sulphonamides (*sul3*) and tetracyclines (*tetA*, *tetM*).

No mutations in the *lpxCAD* or *mgrB* genes associated with polymyxin resistance were identified but polymorphisms were present in *phoP/Q* and *pmrA/B* predicted to encode the amino acid changes I44L (*phoP)*, I165F (*phoQ)*, S29G (*pmrA)*, D282G and Y358N (*pmrB*) relative to the K12 sequence. These substitutions have not previously been linked with reduced susceptibility to polymyxins in *E. coli* but D282G and Y358N are predicted to occur in the ATP binding domain of *pmrB* in *Salmonella* spp[9] and could contribute to polymyxin resistance.

Apart from pEC-MCR1.8, *in silico* analysis confirmed the presence of multiple plasmid replicons belonging to FIA, FIB, FIC, FII, HI1A, HI1B, I1, X1 and Y types, several of which were associated with genes encoding antibiotic resistance (Table S3). Of note β-lactam- (*bla*$_{CTX-M-65}$), tetracycline- (*tetA*), phenicol- (*floR*) and aminoglycoside-resistance (*aph(4)-Ia*, *aac(3)-IVa*,) were associated with an I1 plasmid whereas fosfomycin- (*fosA4*) and sulfonamide-resistance (*sul3*) were localised to a HI mulitreplicon (HIA, HIB and FIA) plasmid.

Analysis of the WGS sequencing data with SerotypeFinder 1.1 predicted an O88:H31 serotype. Multi-locus sequence typing analysis designated *E. coli* C22 to a globally disseminated sequence type (ST) 101, associated with polymyxin resistance across South East Asia South America and Europe.[10] Of more concern, are reports that highlight its potential to act as a reservoir for additional resistances including to carbapenems, suggesting that ST101 could represent a 'high-risk' clone able to promote global dissemination of polymyxin and multi-drug resistance in *E. coli.*

Although only recently identified, 15 functional variants of the MCR-1 enzyme have now been described, including the *mcr1.8* allele encoded by the pEC-MCR1.8 plasmid that was characterized here. This highlights the need for continual and enhanced surveillance for plasmids, host strains and bacterial species able to support the success and dissemination of these resistance determinants. Given the existing knowledge of the global epidemiology of MCR-producing strains this may be particularly important for countries in South East Asia.

Funding

This study forms part of M. H. F. Abdul Momin Ph. D. project funded through the Brunei Darussalam Government in-service training scheme.

Genome sequencing was provided by MicrobesNG (http://www.microbesng.uk), which is supported by the BBSRC (grant number BB/L024209/1).

Transparency declarations

None to declare.

Supplementary data

Tables S1-S3 are available as Supplementary data at JAC Online.

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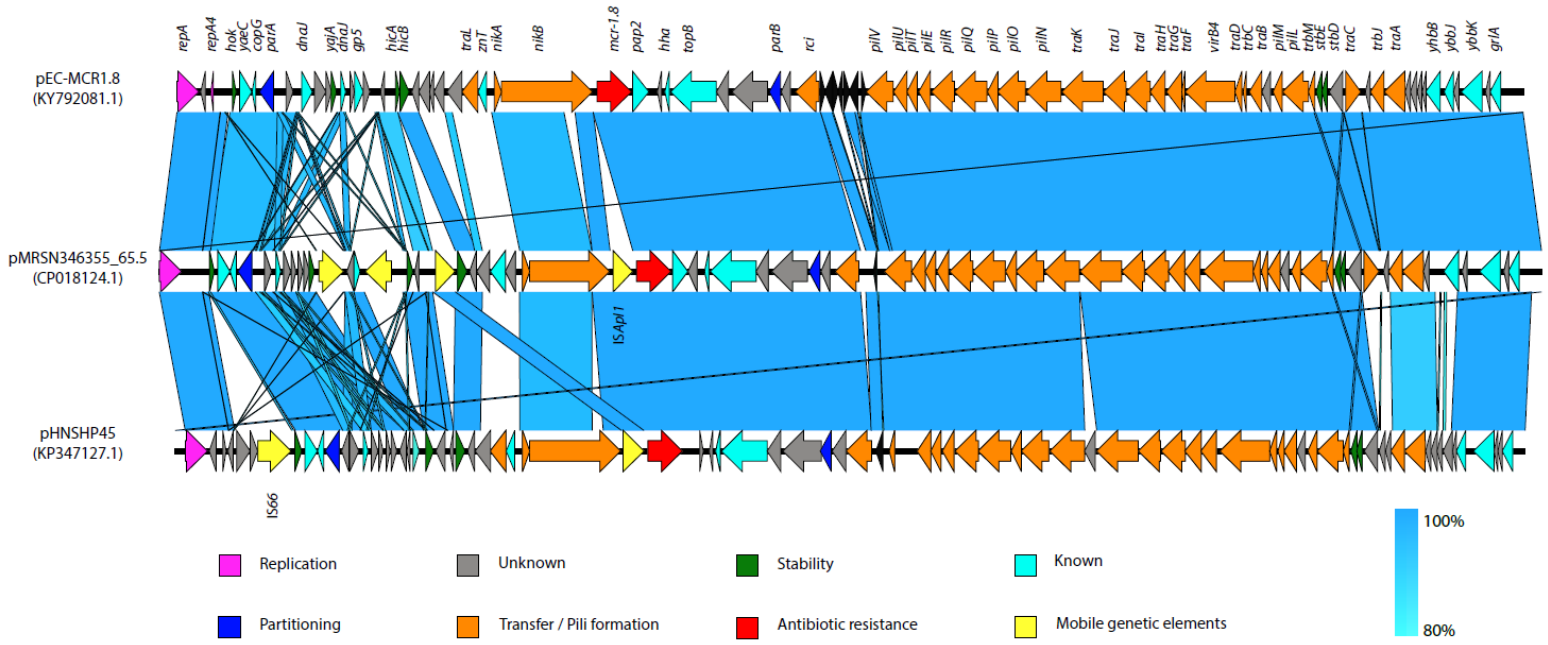
Figure 1: Comparison of pEC-MCR1.8 with selected IncI2 plasmids carrying *mcr-1.1*



Open reading frames are represented with arrows and the direction of transcription by arrowheads. Open reading frames encoding proteins involved in replication, partitioning, stability, transfer/ type IV pilus formation, antibiotic resistance and other known or unknown functions are colour-coded. The shufflon region is indicated with black rectangles. Areas shaded in blue indicate nucleotide identity. This figure is drawn to scale, appears in colour in the online version of JAC and in black and white in the printed version of the journal.