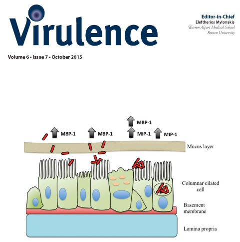

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ISSN: 2150-5594 (Print) 2150-5608 (Online) Journal homepage: http://www.tandfonline.com/loi/kvir20

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To cite this article: Jiali Lv, Mashkoor Mohsin, Sheng Lei, Swaminath Srinivas, Raja Talish Wiqar, Jingxia Lin & Youjun Feng (2018): Discovery of a mcr-1-bearing plasmid in commensal colistin-resistant Escherichia coli from healthy broilers in Faisalabad, Pakistan, Virulence, DOI: 10.1080/21505594.2018.1462060

To link to this article: https://doi.org/10.1080/21505594.2018.1462060

Publisher: Taylor & Francis

Journal: *Virulence*

DOI: http://dx.doi.org/10.1080/21505594.2018.1462060

Discovery of a *mcr-1*-bearing plasmid in commensal colistin-resistant *Escherichia coli* from healthy broilers in Faisalabad, Pakistan

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Keywords: Lipid A; Gut microbiome; Enteric bacteria; Colistin resistance; *mcr-1*; IncI2; Poultry; Pakistan

Running title: *mcr-1* in healthy broilers in Pakistan

Letter to the Editor

Polymyxins, like colistin (polymyxin E), are a group of cationic antimicrobial cyclic polypeptide, which have been extensively-used as prophylactic feed additives in animal production since the 1960s (1). A strong association has especially been drawn between antimicrobial use and resistance in poultry and pig farms (2-5). The advent and rise of multi-drug resistant bacteria has now prompted the re-introduction of colistin as a last-resort treatment option in human medicine (6, 7). However, the recent emergence and diversity of plasmid-borne mobile colistin resistance determinants (*mcr-1* to *mcr-5*) in *Enterobacteriaceae* has severely challenged its use in a clinical setting (8-12). In the past two years, *mcr-1* has been detected in over 40 countries across 5 of 7 continents worldwide (13-15). Except rare cases of chromosomally-integrated *mcr-1* (16, 17), its prevalent transmission relies on the transfer by diversified plasmids of different replication incompatibilities (18, 19).

Mechanistically, the *mcr-1* gene that encodes phosphoethanolamine transferase mediates the modification of the lipopolysaccharide layer (LPS) of the outer membrane of Gram-negative bacteria through the addition of phosphoethanolamine (PEA) to the 1 (or 4′)-phosphate position of lipid A moieties (20, 21). This reduces the affinity of polymyxin antibiotics to their primary target, the

LPS layer. Since its discovery during routine surveillance in China (8), increasingly-accumulated evidence suggested the presence of *mcr-1*-bearing bacteria in food producing animals and humans across the world. To the best of our knowledge, the leading two types of *mcr-1*-harboring plasmids referred to IncI2 (8, 18, 22, 23) and IncX4 (24-26), have greatly facilitated global dissemination of *mcr-1* colistin resistance (14, 27). In Pakistan, the *mcr-1* gene has been identified in *Escherichia coli* (*E. coli*) isolates from human (28), wildlife (29) and a broiler suffering from colibacillosis (30). However, little is known about the prevalence of *mcr-1* and its genetic environment in commensal *E. coli* isolates from poultry in Pakistan.

From December 2016 to January 2017, cloacal swabs from a total of 100 healthy broiler chicken were obtained from four commercial farms (n=25 each) in the Faisalabad region of Pakistan. To screen the colistin resistant *E. coli*, all the samples were seeded directly onto MacConkey agar supplemented with 4 µg/ml of colistin and were incubated at 37°C for 24 hours. Of 100 birds, colistin resistant *E. coli* were found in only 8 (8%) samples. A single colony of *E. coli* was selected per sample and identified using API 20E biochemical strips (bioMérieux, Marcy l'Etoile, France). The presence of *mcr-1* gene was confirmed among all 8 *E. coli* isolates by conventional PCR as we recently conducted (19, 31). Subsequently, the minimum inhibitory concentration (MIC) of colistin among these strains was tested by micro-dilution according to the guidelines of Clinical and Laboratory Standards Institute (32, 33). The *mcr-1*-positive *E. coli* gave MIC of colistin between 2-8 µg/ml (**Table 1**). Plasmids were extracted from *mcr-1*-positive *E. coli* using alkaline lysis method. To elucidate the genetic context of *mcr-1* on these plasmids, the conventional multiplex PCR with 7 primer sets was performed (**Table S1**) as we recently described (3, 19). The plasmids isolated from the different strains had unexpectedly similar PCR profiles with the exception of pPK112 which lacks the *tnpA* loci (**Fig. 1B**). Genetic

context of *mcr-1* shows that all the plasmids lack the insertion element IS*Apl*1 (**Fig. 1A**), which has been responsible for insertion of *mcr-1* in previous studies (34). Also, these plasmids (**Fig. 1A**) are identical to the *mcr-1*-carrying plasmid pE15017 isolated in China (19, 22). These bacteria were subjected to multi-locus sequence typing analyses with the Warwick method (http://mlst.warwick.ac.uk/mlst/). Diversified sequence types were detected, namely, ST10, ST2847, ST155, ST361 and ST6395. Evidently, all strains belonged to different STs with the exception of two strains of ST361 (**Table 1**). Despite that none of these STs have been reported from Pakistan in the past, ST10 and ST155 have been reported in *mcr-1*-harboring *E. coli* isolated from chicken in China (17, 35).

Subsequently, a representative *mcr-1*-carrying plasmid pPK105 (**Table 1**) was subjected to whole genome sequencing using the method of Illumina HiSeq X-ten. The plasmid sequences were annotated by RAST, and the genome maps were drawn with the Circos program. As a result, the genome size of pPK105 was determined to be 60.499 kb (Acc. no.: MG808035, **Fig. 2A**), encoding hundreds of open reading frames with a GC content of 42.3%. Unlike the prevalent IncX4 type plasmid reported in Pakistan, Plasmid Finder at the web server (https://cge.cbs.dtu.dk/services/PlasmidFinder/) indicated that pPK105 is a member of IncI2-type plasmid family (**Fig. 2B**). Notably, *mcr-1* is the only resistance gene detected in pPK105 (**Fig. 2B**). It is quite different from the scenarios observed in the other *mcr-1*-containing Pakistan isolate coharboring ESBL (extended-spectrum β-lactamase gene) and heavy-metal resistance. Although that IS*Apl1* sequence is missing, pPK105 retains the two conjugative genes *vir* and *pil* (**Fig. 2**). Intriguingly, we noticed that the *mcr-1* gene in pPK105 is next to a *sdrI* gene, a serine-aspartate repeat surface protein known to bind collagen (**Fig. 2B**). However, its relevance

remains unclear. Nevertheless, it has been shown earlier that IS*Apl1* transposon element is highly unstable in IncI2 plasmid (36).

For further investigation, the chemical modification of lipid A by the *mcr-1* protein product MCR-1, the bacterial LPS was extracted as we conducted earlier (20, 37) and then subjected to matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (38). In comparison to *E. coli* MG1655 (m/z 1796.29, **Fig. 3A**), a shift in the predominant lipid A species (m/z 1920.136, **Fig. 3A**) was observed in the *mcr-1*-expressing PK105 strain (Δ_{mass} is close to 123) corresponding to the addition of a PEA moiety (**Fig. 3B**). This new peak corresponds to a single modification that may occur at either the 1 or the 4′ position (**Fig. 3B**). This highlighted that surface remodeling by the *mcr-1*-encoding phosphoethanolamine transferase contributes to the resultant colistin resistance (14).

The discovery of the *mcr-1* gene, prompted a shift in focus from chromosomal mutations causing colistin-resistance to a transmissible plasmid-borne colistin resistance determinant. In addition to clinical isolates in humans, antimicrobial surveillance programs in Europe have identified *mcr-1* in commensal bacterial populations from broilers, pigs and turkeys (39). This study shows a similar threatening scenario in the Faisalabad region of Pakistan where high rates of *mcr-1* positive *E. coli* were identified in healthy broilers. Retrospectively, the discovery of diverse clonal backgrounds of *E. coli* harboring the plasmid-borne *mcr-1* is similar to scenarios observed earlier in Chinese poultry.

Similarly, data on global population structure of *mcr-1*-positive *E. coli* showed large diversity in STs but limited plasmid types, particularly with regional spread of IncHI2 plasmids in Europe and IncI2 in Asia (14). This indicated the possible spread of a single *mcr-1* colistin resistance gene across large geographical distances. It seems likely that farm animals act as a reservoir for the genetic diversity of *mcr-1* (40).

Funding

This work was supported by National Key R&D Program of China (2017YFD0500202, YF), National Key Basic Research Program of China (2016YFC1200100, YF), and National Natural Science Foundation of China (31570027 & 81772142, YF). Dr. Feng is a recipient of the “Young 1000 Talents” Award.

Author Contributions

YF and MM designed and supervised this project; YF, MM, JL, SL, SS, RW, and J-X L performed experiments; YF, JL, MM, and SL analyzed the data and prepared figures; YF, MM, SS and SL drafted this manuscript.

Disclosure of Potential Conflicts of Interest

We declare that no conflict of interest is present.

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Table 1 Characteristics of *mcr-1*-positive *E. coli* isolates from healthy broilers in Faisalabad, Pakistan

Strains	Source	Date	MIC (µg/ml)	MLST	Farm no.
PK102	cloacal	27/12/2016	≥ 8	ST10	1
PK103	cloacal	27/12/2016	≥ 4	ST2847	1
PK105	cloacal	27/12/2016	≥ **8**	**ST155**	1
PK107	cloacal	27/12/2016	≥ 8	New ST	1
PK109	cloacal	27/12/2016	≥ 4	ST361	1
PK110	cloacal	30/01/2017	≥ 4	ST6395	3
PK111	cloacal	30/01/2017	≥ 2	ST361	3
PK112	cloacal	30/01/2017	≥ 8	New ST	3

Figure Legends

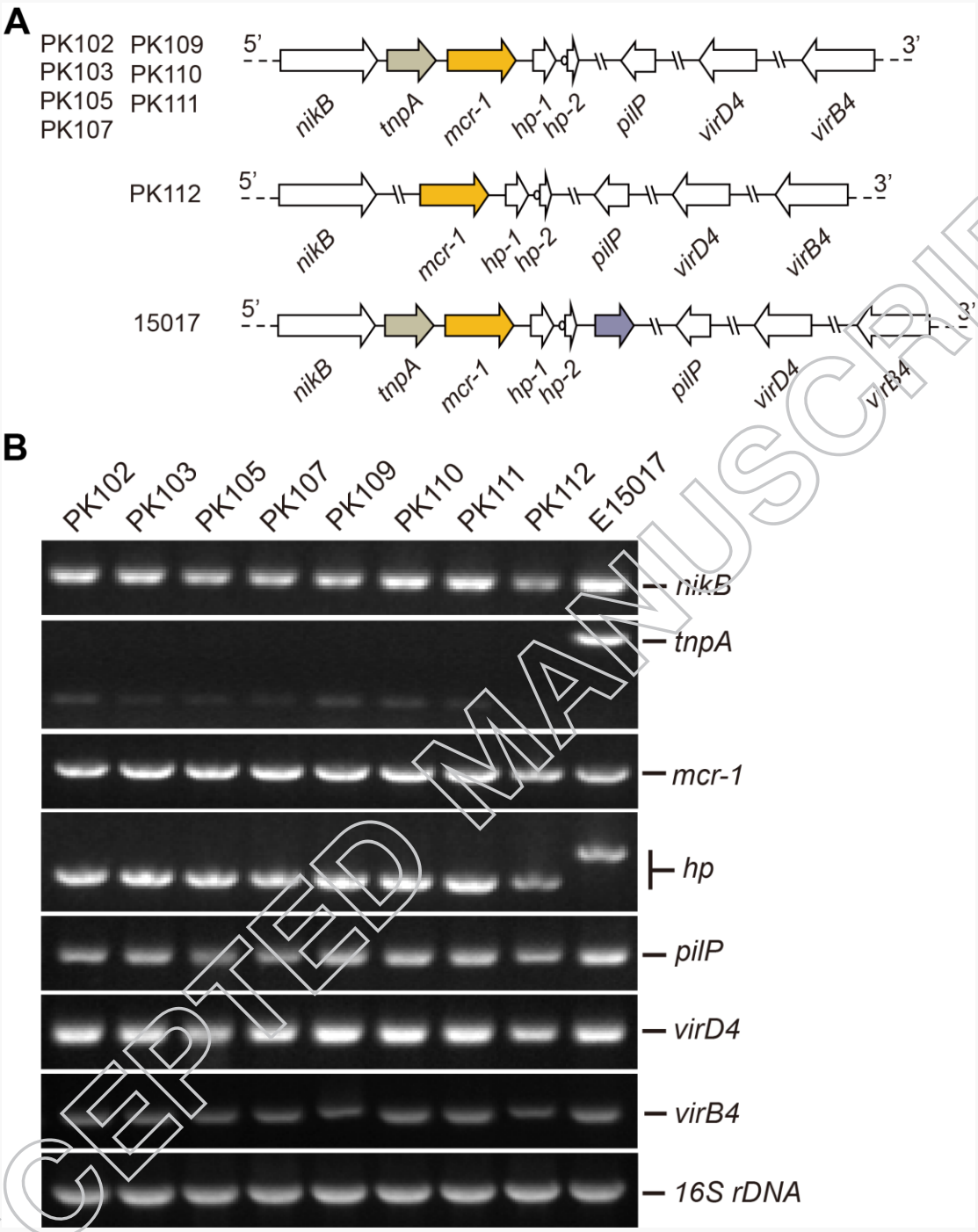

Figure 1 Genetic analyses of *mcr-1*-harboring plasmids in this study

A. Scheme of different *mcr-1*-bearing plasmids

B. PCR assays of *mcr-1* and neighboring loci in plasmids

16S rDNA is specific to the *E. coli* specie.

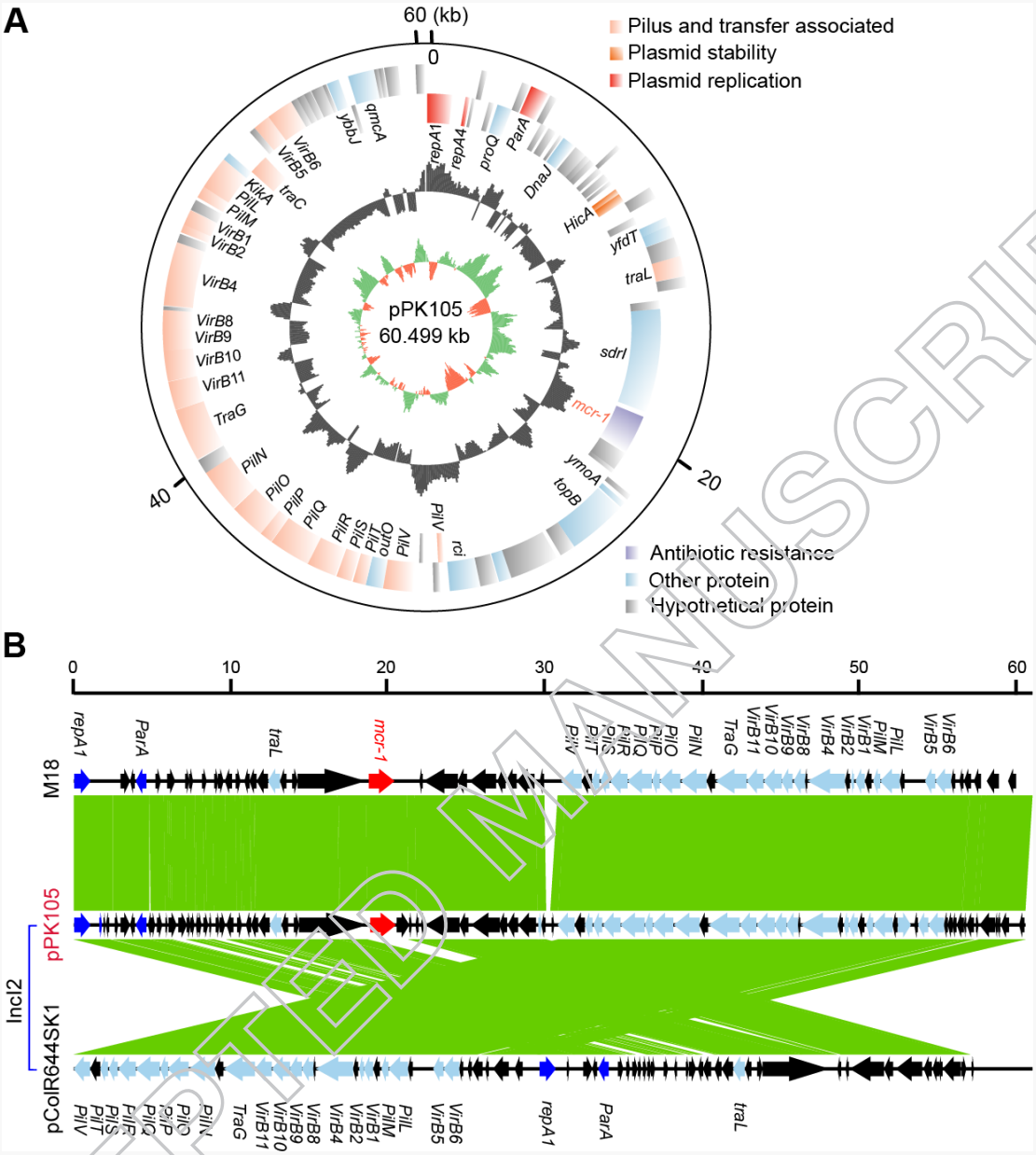

Figure 2 The representative *mcr-1*-bearing plasmid in a colistin resistant *E. coli* isolate PK105

A. Diagram for the *mcr-1*-positive plasmid (pPK105) that exists in the E. coli isolate PK105

B. Colinear comparison of the IncI2-type plasmid pPK105 with two closely-related plasmids *mcr-1*-postive M18 and *mcr-1*-negative pColR644SK1

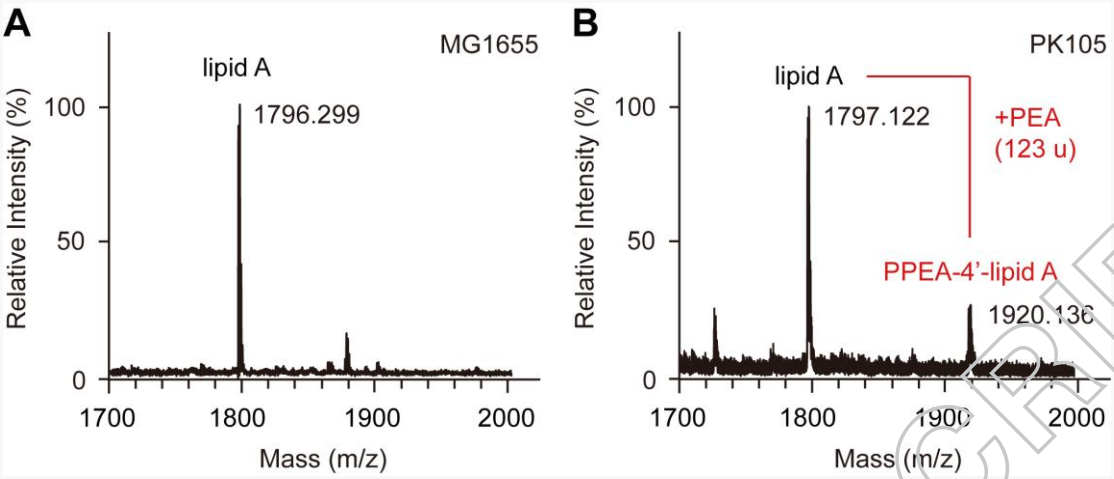

Figure 3 MALDI-TOF MS analyses of lipid A pools of *E. coli* strains with (or without) *mcr-1*

A. MS profile of the LPS-lipid A in the negative control strain *E. coli* MG1655

B. MS spectrum of the LPS-lipid A in PK105, a representative strain of *E. coli* carrying *mcr-1.* A single modification may occur at the 1 (or 4') position. The position indicated here is suggestive (20).