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An IncP plasmid carrying the colistin resistance gene *mcr-1* in *Klebsiella pneumoniae* from hospital sewage

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Abstract

A *Klebsiella pneumoniae* strain of ST313 recovered from hospital sewage was found to carry the plasmid-borne colistin resistance gene *mcr-1*, which was bracketed by two copies of the insertion sequence IS*Apl1* on a 57-kb self-transmissible IncP type plasmid of a new IncP-1 clade. The carriage of *mcr-1* on a self-transmissible broad-host-range plasmid highlights that *mcr-1* has the potential to be spread beyond the *Enterobacteriaceae*.

Colistin is the last resort antimicrobial agents to treat the infections caused by many Gram-negative bacteria. Recently, a plasmid-borne colistin resistance gene, *mcr-1*, has been found in *Escherichia coli* and *Klebsiella pneumonia* from human and animals in China (1). A few follow-up studies have found *mcr-1*-carrying *E. coli* in many countries in Africa (2), Europe (3-8), Asia (1-3, 9, 10) and North America (9, 11). In addition, *mcr-1* has also been found in *Kluyvera ascorbata* (12) and several species of *Salmonella* (13-15). All of above findings suggest that *mcr-1* has been widely distributed and imposes an emerging threat for clinical management and public and animal health. During a screening study for the presence of colistin-resistant *Enterobacteriaceae* in hospital sewage, we found a *mcr-1*-carrying *K. pneumonia*, which is reported here.

K. pneumoniae strain WCHKP1511 was recovered from the influx mainstream of hospital sewage at West China Hospital, Chengdu, western China, in November 2015. Strain WCHKP1511 grew on CHROMAgar Orientation agar plates (CHROMAgar, Paris, France) containing 4 μg/ml colistin and 64 μg/ml linezolid. Species identification was established by partially sequencing the *gyrB* gene (16). Strain WCHKP1511 was resistant to colistin (MIC, 8 μg/ml), polymyxin B (MIC, 8 μg/ml), chloramphenicol (MIC, 128 μg/ml) and tetracycline (MIC, 64 μg/ml) but was susceptible to amikacin (MIC, 0.5 μg/ml), ceftazidime (MIC, ≤0.5 μg/ml), cefotaxime (MIC, 0.03 μg/ml), ciprofloxacin (MIC, 0.03 μg/ml)，imipenem (MIC, 0.125 μg/ml)

and tigecycline (MIC, 1 μg/ml) as determined using the microdilution broth method following recommendations of the Clinical Laboratory Standards Institute (CLSI) (17). WCHKP1511 was susceptible to fosfomycin (MIC, 32 μg/ml) as determined using the agar dilution method following recommendations of CLSI (17). In addition, strain WCHKP1511 was resistant to ampicillin and trimethoprim-sulphamethoxazole, intermediate to amoxicillin-clavuanate, gentamicin, tobramycin and nitrofurantoin, and susceptible to aztreonam, cefazolin, cefepime, cefoxitin, ceftriaxone, ertapenem, levofloxacin and piperacillin-tazobactam as determined using the Vitek II automated system (bioMerieux, Lyon, France). Breakpoints defined by FDA and by EUCAST were used for tigecycline and colistin, respectively; otherwise, those defined by CLSI were applied. Strain WCHKP1511 was found to carry *mcr-1* by PCR and sequencing (1, 12). Although *mcr-1* has been widely found in *E. coli*, *mcr-1*-carrying *K. pneumoniae* remains uncommon and had only been found in eastern China (Jiangsu and Zhejiang provinces) before (1, 18).

Strain WCHKP1511 was subjected to the 150-bp paired-end whole genome sequencing with a ca. 200× coverage using the HiSeq 2500 Sequencer (Illumina, San Diego, CA, USA). A total of 3,784,972 reads and 567,745,800 clean bases were generated, which were assembled into 275 contigs (230 contigs ≥ 1,000 bp) in length (N50, 67,217 bp) with a 57.46% GC content using the Spades program (19).

WCHKP1511 belonged to ST313, which was determined by using the genomic sequence to query the multi-locus sequence typing database of *K. pneumoniae* (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html). In the *K. pneumoniae* MLST database, only one ST313 strain, KML 2185, which was recovered from human blood in the Netherlands in 2007, has been deposited. *K. pneumoniae* strains carrying *mcr-1* in Jiangsu province belonged to ST25 (20), while the STs of those in Zhejiang province were unknown. ST313 was not closely related to ST25, as only 2 out of 7 alleles were identical between the two STs.

Antimicrobial resistance genes were predicted using ResFinder from the Center for Genomic Epidemiology (http://genomicepidemiology.org/). In addition to *mcr-1*, WCHKP1511 had multiple genes mediating resistance to aminoglycosides (*aac(3)-Iva*, *aadA2*, *aph(3')-Ia* and *aph(4)-I*), β-lactams ($bla_{\text{TEM-135}}$ and a new bla_{SHV} variant), chloramphenicol (*floR*), fosfomycin (*fosA*), quinolones (*oqxA* and *oqxB*), sulphonamides (*sul2*), trimethoprim (*dfrA12*) and tetracycline (*tet(A)*) as predicted by ResFinder. $bla_{\text{TEM-135}}$ encodes a broad-spectrum β-lactamase (21). Of note, although strain WCHKP1511 carried *fosA*, it was susceptible to fosfomycin, which warrants further investigation. bla_{SHV} in strain WCHKP1511 is a new variant, which encodes a SHV enzyme with an amino acid difference (Thr14Asn, the position is based on the ATG start condon) from SHV-111, the closest match. The new SHV has been assigned SHV-195 by the NCBI β-lactamase classification system

(www.ncbi.nlm.nih.gov/pathogens/submit_beta_lactamase/). As strain WCHKP1511 was susceptible to third generation cephalosporins, *bla*$_{SHV-195}$ is unlikely to encode an extended-spectrum β-lactamase (ESBL). The complete coding sequence of *bla*$_{SHV-195}$ was cloned onto the pBC SK vector (Agilent, Santa Clara, CA, USA), which was electroporated into *E. coli* DH5α. *E. coli* transformants containing *bla*$_{SHV-195}$ were resistant to ampicillin (MIC, >256 μg/mL) and cephalothin (32 μg/mL) but were susceptible to aztreonam, ceftazidime, cefotaxime, cefoxitin and imipenem determined using the broth microdilution method (17). This confirmed that *bla*$_{SHV-195}$ encodes a broad-spectrum rather than an ESBL.

In addition, there was a predicted bleomycin-resistance gene, designated ORFble here, which was not identified by Resfinder but was identified by the Prokka annotation tool (22) and was confirmed by Protein-BLAST in strain WCHKP1511. The complete coding sequence of ORFble was amplified with primers 1511_ble_BamHI_F (CGCGGATCCTTGGTTCACCATGAAGATG)/1511_ble_EcoRI_R (CCGGAATTCCGGCCGATTGCTGAACAGATTA), was cloned onto pBC SK and was electroporated into *E. coli* DH5α. ORFble-containing transformants were selected on LB agar plates containing 25 μg/mL chloramphenicol and the presence of ORFble in transformants was confirmed by PCR and sequencing. However, MIC (0.25 μg/mL) of zeocin (Thermo Fisher Scientific, Waltham, MA, USA), a bleomycin, against *E. coli* DH5α transformant containing ORFble was the same as that against DH5α as

111 determined using the broth microdilution method (17). This suggests that ORFble did
112 not mediate resistance to bleomycin and its function remains undetermined.
113
114 Conjugation experiments were carried out in broth using azide-resistant *E. coli* strain
115 J53 as the recipient and 2 μg/ml colistin plus 150 μg/ml sodium azide was used for
116 selecting transconjugants. The presence of *mcr-1* in transconjugants was confirmed
117 using PCR. In strain WCHKP1511, *mcr-1* could be transferred to *E. coli* J53 at a
118 frequency of 10^{-2} cells per donor cell by mating, suggesting that *mcr-1* was carried by
119 a self-transmissible plasmid, which was assigned pMCR_1511. The sequence of
120 pMCR_1511 was completely circularised with gaps between contigs closed by Sanger
121 sequencing of amplicons from PCRs using primers designed based on available contig
122 sequences. pMCR_KP1511 was 57,278 bp in size and had no known antimicrobial
123 resistance genes other than *mcr-1*. Unlikely the previously-described *mcr-1*-carrying
124 IncI2 plasmid pHNSHP45 (GenBank accession number KP347127) (1),
125 pMCR_KP1511 belonged to the IncP type, a broad-host-range incompatibility group.
126 Plasmid pKH-457-3-BE carrying *mcr-1* in *E. coli* from Belgium was found to have an
127 IncP backbone (3). However, the sequence of pKH-457-3-BE was not available for
128 further analysis. Nonetheless, it has been suggested that pKH-457-3-BE was 99%
129 similarity and 73% coverage with the IncHI2 plasmid pHXY0908 (GenBank
130 accession number KM877269) in *Salmonella enterica* serotype Typhimurium (8).
131 pMCR_KP1511 had only a 6% coverage with pHXY0908, indicating that

pMCR_1511 was very different from pKH-457-3-BE and pKH-457-3-BE may not be a true IncP plasmid but is likely of IncHI2.

pMCR_1511 has the typical IncP-1 plasmid backbone (23) containing the *trfA* encoding the replication initiation protein, two *par* modules for plasmid partitioning, two conjugative regions *tra* (17.6 kb) and *trb* (12.7 kb), the host-lethal protein-encoding *kil* genes and their regulator *kor* (stands for *kil*-override) genes, and a toxin-antitoxin *higA-B* system (Figure 1). The backbone of pMCR_1511 was highly similar (99% identity) with that on plasmid pHNFP671 (GenBank accession number KP324830), which was an IncP plasmid in *E. coli* isolate FP671 from Guangzhou, China but did not carry *mcr-1*. IncP-1 plasmid has six assigned clades, i.e., α, β, γ, δ, ε and ζ, among which β clade has β1 and β2 two subclades (24). To determine of which clade pMCR_1511 was, the sequence of 30 genes belonging to the IncP-1 backbone was retrieved from pMCR_1511, concatenated and then aligned to the counterparts of one representative plasmid of each clade including β1 and β2 subclades as described previously (25). Phylogenetic analysis of the IncP-1 plasmid backbone revealed that pMCR_1511 and pHNFP671 belonged to a new IncP-1 clade (Figure S1 in the supplementary file).

When compared the sequence of pHNFP671, pMCR_1511 has two unique regions (Figure 1). One of the unique region harbored *mcr-1* and the other contained ORFble.

The sequence comparison of pMCR_1511 and pHNFP671 allowed us to analyze the genetic context of *mcr-1* in detail. Like most genetic contexts available in the GenBank, *mcr-1* was located downstream of the insertion sequence IS*Apl1* on pMCR_1511. However, there was another IS*Apl1*, which was interrupted (see below), downstream and therefore *mcr-1* was bracketed by two copies of IS*Apl1* on pMCR_1511. It has been known that IS*Apl1* is able to generate 2-bp direct target repeats (DR) upon insertion (26) (https://www-is.biotoul.fr/index.html?is_special_name=ISApl1). The 2-bp flanking sequences of the region bracketed by the two copies of IS*Apl1* were identical (AC, Figure 2). When the region formed by two copies of IS*Apl1* and one of the 2-bp flanking sequences were subtracted artificially, the joined sequence perfectly matched that of an open reading frame (orf) with unknown function on plasmid pHNFP671. It therefore proved that the 2-bp sequence was truly DR generated by IS*Apl1* rather than coincidence and the two IS*Apl1* formed a composite transposon to mobilize *mcr-1* gene. A very recent analysis revealed that the IS*Apl1*-formed composite transposon carrying *mcr-1* has also been seen on either the chromosome or a plasmid (IncH or unknown Inc groups) of seven *E. coli* strains (27). The IS*Apl1*-formed composite transposon carrying *mcr-1* is all located at different locations, which are also different from the location on pMCR_1511, in the seven *E. coli* strains and is flanked by 2-bp DR in five strains (27). The previous analysis (27) and the findings in the present

study suggest that the IS*Apl1*-formed composite transposon is a common vehicle for mediating the spread of *mcr-1*.

The IS*Apl1* downstream of *mcr-1* on pMCR_1511 was interrupted by the insertion of Tn*3* with the characteristic 5-bp DR (Figure 2). The Tn*3* was also disrupted by IS*26* and most of the Tn*3* was absent, which may be due to the action of IS*26*. It is well known that the insertion of IS*26* can lead to the deletion of the adjacent sequence of the insertion site (28). Alternative explanation for the absence of most part of Tn*3* is that the insertion of the second IS*26* and the recombination between the two IS*26* could lead to the loss of the intervening region. Although IS*Apl1* downstream of *mcr-1* was interrupted by Tn*3*, the right-end inverted repeat (IRR) of the IS*Apl1* remained intact (Figure 2). The transposase encoded by the IS*Apl1* upstream of *mcr-1* had the potential to recognize the IRR of the IS*Apl1* downstream of *mcr-1* and then could realize the mobilization of the region bracketed by the two copies of the IS*Apl1*.

In conclusion, the plasmid-borne colistin resistance gene *mcr-1* was found in a *K. pneumoniae* of an infrequently encountered ST from hospital sewage. *mcr-1* was carried by a self-transmissible IncP plasmid, which is a broad-host-range type of plasmids and has the potential to mediate the dissemination of *mcr-1* from the *Enterobacteriaceae* to other Gram-negative bacteria such as *Pseudomonas aeruginosa*. *mcr-1* was bracketed by two copies of IS*Apl1*, which were able to form a composite

transposon and represented a common mechanism for mediating the mobilization of *mcr-1*.

Nucleotide sequence accession number. Reads and the Whole Genome Shotgun Sequencing project of *K. pneumoniae* strain WCHKP1511 have been deposited into DDBJ/EMBL/GenBank under accession number SRR3170679 and LSMF00000000, respectively. The sequence of pMCR_1511 has been deposited into DDBJ/EMBL/GenBank under accession number KX377410.

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305

306

Figure legends

Figure 1. Genetic structure of IncP plasmid pMCR_1511 carrying *mcr-1* and the comparison with IncP plasmid pHNFP671. Regions and genes that are indicated are ORFble (a predicted [but actually not] bleomycin resistance gene), *higA-B* (encoding a toxin/antitoxin system), *mcr-1*, *kor-par-kli* (for plasmid maintenance), *tra* and *trb* (the two conjugation-encoding regions), and *trfA* (encoding the plasmid replication initiation protein). Backbones of the two IncP plasmids are almost identical. Compared to pHNFP671, pMCR_1511 carried two additional regions that contained either *mcr-1* or ORFble.

Figure 2. Genetic context of *mcr-1* on pMCR_1511. Genetic context of *mcr-1* on the IncI2 plasmid pHNSHP45 (GenBank accession number KP347127) and the corresponding region on the IncP plasmid pHNFP671 (GenBank accession number KP324830) are shown for comparison. orfs that encode hypothetical proteins with unknown function are indicated in white except that the one disrupted by the IS*Apl1*-formed composite transposon on pMCR_1511 is shown in black (Δ1 and Δ2). Other genes shown are *nikB* (encoding relaxase of the plasmid), *ydgA* (DNA topoisomerase III), *ydfA* (transcriptional regulator), *parA* (resolvase), bla_{TEM} (shown as a white arrow in Tn*3*) and *traB* (conjugative protein). The 2-bp direct repeat (GA) abutting the IS*Apl1-mcr-1-pho* region on pHNSHP45 and the 2-bp direct repeat (AC) abutting the IS*Apl1*-formed composite transposon on pMCR_1511 are shown. On

pMCR_1511, the IS*Apl1* downstream of *mcr-1* was interrupted by Tn*3*, which was interrupted by IS*26*.