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• LETTER TO THE EDITOR •

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Diversified variants of the *mcr-1*-carrying plasmid reservoir in the swine lung microbiota

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Dear Editor,

Emergence of bacteria with multiple drug resistance is a major problem in global public health. Polymyxin comprising polymyxin E (colistin) and polymyxin B is generally recognized as the last-resort antibiotics with broad-spectrum activity against the lethal infections by the multi-drug resistant gram-negative bacteria (Ye et al., 2016). Very recently, Yi-Yun Liu and colleagues reported, for the first time, that the plasmid-encoded mobilized colistin resistance gene (*mcr-1*) confers the transmissible colistin resistance in Enterobacteriaceae (Liu et al., 2015), which attracted much attention /debate from scientific society and triggered extensive public worrisome. Given the fact that the pHNSHP45 (isolated from swine *Escherichia coli*) is only one *mcr-1*-harbouring plasmid with full genome reported, after screening thousands of samples from five provinces of China, from 2011 to 2014 (Liu et al., 2015), we speculated that diversified plasmids and/or pHNSHP45 variants carrying the *mcr-1* colistin resistance gene might be present in the microbiota of swine populations where the *mcr-1*-harbouring mobile elements are transmissible by bacterial conjugation-aided gene horizontal transfer. Similar scenarios were found in the human gut microbiota (Zhang et al., 2016; Ye et al., 2016).

Driven by this hypothesis, we re-analyzed our bacterial samples collected from six provinces of China, from 2011 to 2013 (Table S1). Apart from the three provinces (Guangdong, Hunan, and Zhejiang) investigated by Liu and coworkers (Liu et al., 2015), we sampled three more provinces (Jiangsu, Henan, and Hubei, in Figure 1A). Among hundreds of bacteria isolated from the different tissues (like lung, liver, and throat trachea) of pigs, totally 16 bacterial strains were determined to be of colistin resistance where the minimum inhibitory concentrations (MIC) of polymyxin B varied from 4 mg L^{-1} to 32 mg L^{-1} (Table S1). The routine biochemical assays combined with 16S rDNA sequencing (Figure 1C) validated that all these colistin-resistant bacteria belong to *E. coli*. Following the PCR screening, six of the above 16 isolates (namely WH03, WH07, WH09, WH12, WH13 and WH15) were totally revealed to be positive for *mcr-1* (Figure 1B, 1C and S1). The six strains consistently exhibited the colistin resistance at appreciable level up to 32 mg mL^{-1} (Figure S2). In contrast, the colistin resistance in the *mcr-1*-negative *E. coli* isolates might indicate a different/unknown mechanism. Direct DNA sequencing showed that all the six *mcr-1* genes we screened can match 100% to the counterpart from the paradigm *mcr-1*-harbouring plasmid pHNSHP45 (Figure 1B). We also confirmed the function of the single *mcr-1* gene *in vivo* using an arabinose-inducible pBAD24 expression system in *E. coli* (Figure S2).

To further address the genetic context of the *mcr-1* gene from our bacterial isolates, we carried out PCR-based molecular dissection through the pHNSHP45 plasmid-guided

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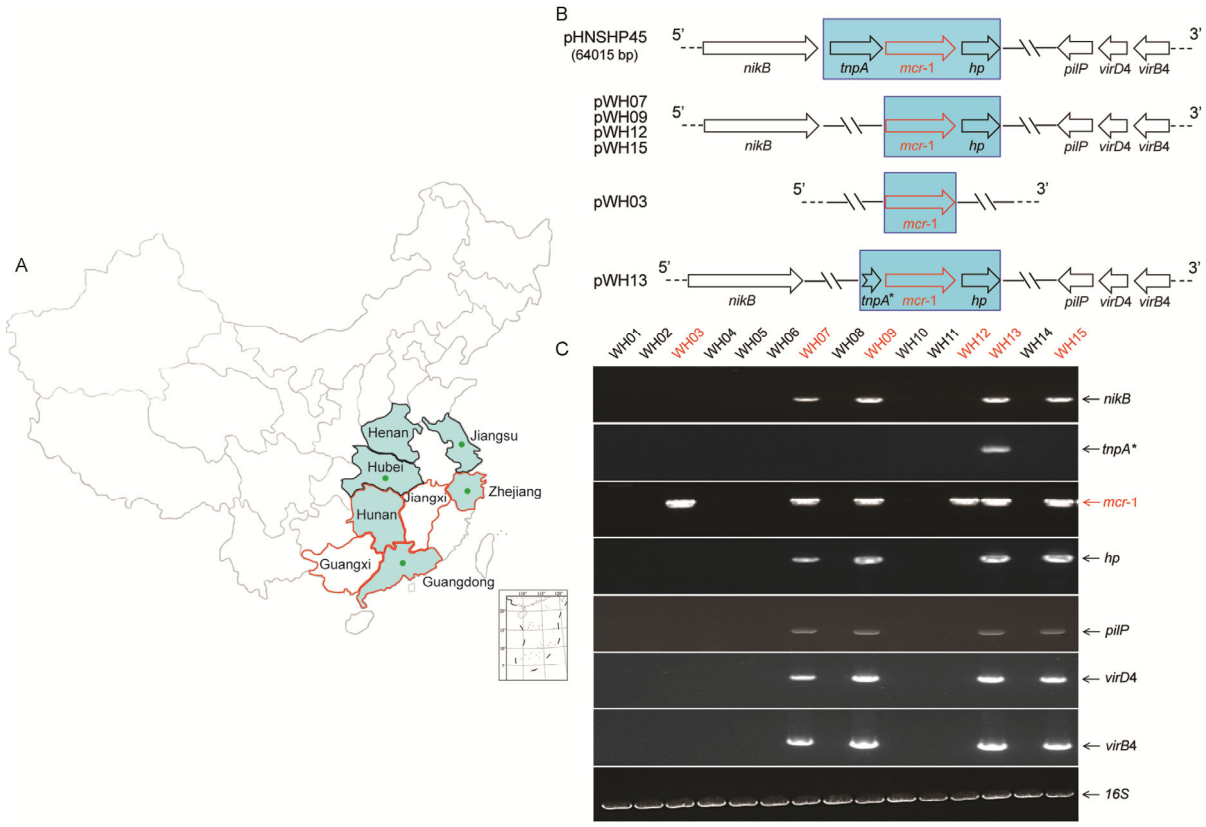

Figure 1 Dissemination of the *mcr-1* colistin resistance gene in China and occurrence of *mcr-1*-carring plasmid pHNSHP45 variants in swine lung microbiota. A, Dissemination of the *mcr-1* colistin resistance gene in China. This map of China is used to illustrate the positions with *mcr-1*/colistin resistance recorded. The provinces investigated by Liu and coauthors are labelled with red boundaries. In our epidemiological study, the provinces are highlighted in blue upon the bacterial colistin resistance was found, some of which are further indicated with green dots in that the *mcr-1* colistin resistance gene is detected. B, Cartoon illustration for the paradigm *mcr-1*-harbouring plasmid pHNSHP45 and its three types of variants.pHNSHP45 refers to a swine isolate with known genome sequence(Liu et al., 2015), the other six plasmids (namely pWH03, pWH07, pWH09, pWH12, pWH13, and pWH15) correspond to the bellowed six *E. coli* strains isolated from the tissue of swine lungs (WH03, WH07, WH09, WH12, WH13, and WH15) sampled in China from 2011 to 2013 (Figure S1). The genes are represented with arrows. The colistin resistance gene *mcr-1*is highlighted in red. With an exception of pWH13, all the other five *mcr-1* gene are 100% identical to that of the counterpart of *E. coli* SHP45 strain plasmid, pHNSHP45 (Liu et al., 2015). The broken arrow denotes the partial sequence of *tnpA* gene (*tnpA**). In light of the genomic information of the pHNSHP45 plasmid, we designed a series of primers to perform the molecular dissection for our *mcr-1*-positive bacterial isolates. Apart from the strain WH03, all the other five strains (WH07, WH09, WH12, WH13 and WH15) are PCR-positive for the four genes (*nikB, pilP*, *virD*4 and *virB*4), as well as a hypothetical protein (*hp*), an adjacent locus of *mcr-1* in pHNSHP45. Unlike the pHNSHP45 plasmid harbouring an intact *tnpA* adjacent to the *mcr-1* at 5′-end, the five isolates (WH03, WH07, WH09, WH12, andWH15) are PCR-negative for *tnpA* gene (note: a truncated version of *tnpA*, *tnpA** is present in WH13). C, PCR screen for the *mcr-1* gene in the colistin-resistant *E. coli* strains and molecular mapping of relevant plasmid-encoded loci. The *mcr-1*-positive *E. coli* isolates (WH03, WH07, WH09, WH12, WH13, and WH15) were highlighted in red letters, and the *mcr-1* amplicon with expected size of 1.6 kb was indicated with a red arrow. As we recently reported (Ye et al., 2016), PCR assays were conducted using eight pairs of specific primers targeting the following genes (Ye et al., 2016): the *mcr-1* colistin resistance gene plus the two neighboring loci (the transposase-encoding gene *tnpA* and the hypothetical protein (*hp*)-encoding gene), *nikB*, *pilP*, a type IV secretion system-encoding genes *virD4-virB4*, and a house-keeping gene, *16 S rDNA*.

primer designing (Figure 1B and 1C). The two loci upstream of the *mcr-1* genes assayed here refers to *nikB* and *tnpA* that encodes relaxases and transposases, respectively (Figure 1B), whereas the four genes at 3′-end of the *mcr-1* gene we concerned, encode a hypothetical protein, PilP (a Type IV pilus biogenesis protein), and two components (VirD4 and VirB4) of a type IV secretion system, respectively (Figure 1B). To our surprise, it seems likely that the *mcr-1*-containing plasmid pWH13 (Strain WH13) is far different from the paradigm *mcr-1*-harbouring plasmid pHNSHP45 (Figure 1B) in that none of the six genetic loci for the plasmid pHNSHP45-derived backbone can be positive in PCR assays (Figure 1C). In contrast, PCR screen suggested that all the remaining five strains (WH07, WH09, WH12, WH13 and WH15) carry the other four genes (*nikB, pilP*, *virD*4 and *virB*4), plus *hp*, an adjacent locus of *mcr-1* in pHNSHP45. The identity of the DNA fragments with origin of pHNSHP45 plasmid was verified by subsequent DNA sequencing. Intriguingly, the *tnpA* locus adjacent to the *mcr-1* of the pHNSHP45 plasmid is consistently absent in the five *mcr-1*-positive isolates (WH03, WH07, WH09, WH12, and WH15), whereas only a truncated version of *tnpA*, *tnpA** appears in the strain WH13 (Figure 1C and D).

Obviously, our results established that variants of the

mcr-1-barbouring plasmids occur at least in the swine microbiota (Figure S1). Fortunately, the whole genome sequencing of the *mcr-1*-positive plasmid has verified this speculation (Gao et al., 2016). Also, the similar scenario was observed in our recent investigation of the human gut microbiome (Ye et al., 2016). Given the fact that all the six *mcr-1*-positive *E. coli* are consistently traced to swine lung samples from four different provinces (Table S1), it raised a potential food-chain dissemination pathway for *mcr-1* colistin resistance (Gao et al., 2016b) and reinforced the risk for co-existence of *mcr-1* together with other antibiotic resistance genes, like β-lactamase on a hybrid plasmid.

Compliance and ethics *The author(s) declare that no conflict of interest is present, and financial funders had no roles in design and process of this project.*

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SUPPORTING INFORMATION

Table S1 Epidemiological description for the polymyxin-resistant *E. coli* isolates sampled from pig lungs, in China, from 2011 to 2013

Figure S1 Isolation and identification of *mcr-1*-harbouring plasmid variants from the swine lung microbiota. A, 0.7% agarose gel analyses of different plasmids isolated from the swine lung microbiota.The plasmids are namely pWH03, pWH07, pWH09, pWH12, pWH13, and pWH15. B, PCR-based determination of presence of *mcr-1* in the isolated plasmids Abbreviation: M, DNA marker; kb, kilo-base pair.

Figure S2 Performance of clinical *E. coli* strains grown on LBA plated supplemented with variety levels of colistin.The strain of MG1655 with/without the pBAD24 vector is negative control, whereas the MG1655 with the arabinose-inducible expression of the *mcr-1* gene refers to the positive control. All the clinical *mcr-1*-positive strains are highlighted in red. To determine the colistinminimal inhibitory concentration (MIC), the mig-log phase cultures (OD600=0.8) in serial dilution were spotted on LBA plates supplemented with colistin at a series of level (0, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0 and 64.0 mg/L) and 0.2% arabinose. The LBA plates were kept overnight at 37°C.

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