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Mikhayel Myriam, Karine Praud, Sébastien Leclercq, Dolla Karam Sarkis, Benoît Doublet. Genomic insights into epidemic plasmids carrying bla CTX-M and mcr-1 genes in Escherichia coli from Lebanese broiler production. JAC-Antimicrobial Resistance, 2024, 6 (5), pp.dlae149. 10.1093/jacamr/dlae149 . hal-04703886

HAL Id: hal-04703886
https://hal.inrae.fr/hal-04703886v1

Submitted on 3 Oct 2024

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JAC Antimicrob Resist
https://doi.org/10.1093/jacamr/dlae149

JAC-Antimicrobial Resistance

Genomic insights into epidemic plasmids carrying *bla*$_{CTX-M}$ and *mcr-1* genes in *Escherichia coli* from Lebanese broiler production

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Received 16 April 2024; accepted 12 August 2024

Background: In a previous nationwide survey in the Lebanese broiler production, multidrug-resistant CTX-M-producing *E. coli* were found to carry the mobile colistin resistance gene *mcr-1*.

Objectives: To investigate the mobile genetic supports responsible for the spread of these resistance genes among *E. coli* in healthy broilers in Lebanon.

Methods: Thirty-three *bla*$_{CTX-M}$ and *mcr-1* positive *E. coli* of various sequence types from 17 broilers farms were subjected to conjugation assays. Long-read sequencing (Oxford Nanopore Technologies) and hybrid assembly were performed to determine complete plasmid sequences and their phylogenetic diversity.

Results: Twenty-nine conjugative IncFII plasmids harboured the extended-spectrum β-lactamase genes *bla*$_{CTX-M-3}$ ($n=25$) or *bla*$_{CTX-M-55}$ ($n=4$). Highly related IncF2:A-:B-/*bla*$_{CTX-M-3}$ plasmids differing only through IS-mediated genetic rearrangements in antibiotic resistance gene clusters were found in genetically diverse *E. coli* strains isolated from distant farms. The mobile colistin resistance genes *mcr-1.1* and *mcr-1.26* were carried by IncX4 and IncI2 plasmids. Worryingly, in one isolate, the IS*Ecp1*-*bla*$_{CTX-M-55}$ transposable unit was found integrated in a *mcr-1.26*-carrying IncX4 plasmid. Beside expanded cephalosporins and colistin resistances, all *E. coli* isolates were multidrug-resistant with different additional resistances against aminoglycosides, (fluoro) quinolones, fosfomycin, phenicols, sulphonamides, tetracycline and trimethoprim.

Conclusions: Closely related *bla*CTX-M-3/55-borne IncF2:A-:B- plasmids harbouring variable MDR regions and *mcr-1* carrying IncX4 plasmids are widely disseminated in the *E. coli* population of healthy broilers in Lebanon. Further surveillance programmes of antimicrobial resistance and interventions to reduce the abusive use of medically important antibiotics are necessary to limit the spread of resistances in food-producing animals in Lebanon.

Introduction

Among food-producing animals, the industrial broiler production has been incriminated for its high prevalence of ESBL and/or plasmidic AmpC Cephalosporinase (pAmpC) producing *Enterobacterales* as well as mobile colistin resistance (*mcr*) encoding genes in intensive farms.[1,2] The epidemiology of these critically important resistances is rather complex, since they are conferred by numerous resistance gene variants and families located on various mobile genetic elements.[3] Plasmids are recognized to play a crucial role in antimicrobial resistance spread among Gram-negative bacteria.[3] Plasmids of the IncC, IncF, IncH, IncI, IncN and IncX replicon types are the most frequently described carriers of ESBL/pAmpC, and *mcr* genes in isolates from food-producing animals.[3–5] Among ESBL genes, the *bla*$_{CTX-M}$ gene families predominate in food and food-producing animals.[6]

We recently described a worrying prevalence of ESC-resistant *E. coli* in healthy broilers in Lebanon.[7] The most prevalent ESBL gene was *bla*$_{CTX-M-3}$, distributed in numerous broiler farms in different poultry-associated *E. coli* STs (sequence types). Moreover, a

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significant part of these ESBL-producing *E. coli* isolates also harboured the colistin resistance gene *mcr-1*. The important diversity of *E. coli* genetic backgrounds found in this previous study suggested that the bla_{CTX-M} and *mcr-1* genes are largely spreading among *E. coli* in the Lebanese broiler production through horizontal transfer.[7]

Therefore, the aim of the present study was to investigate the mobile genetic supports responsible of *mcr-1* and ESBL genes dissemination among *Escherichia coli* isolated from healthy broilers in Lebanon.

Materials and methods

Bacterial collection, culture and antimicrobial susceptibility testing

Thirty *E. coli* strains carrying ESBL/pAmpC and *mcr-1* genes and three additional strains harbouring only ESBL/pAmpC genes were selected from a national survey previously published (Table S1, available as Supplementary data at *JAC-AMR* Online).[7] Antibiotic Susceptibility and the production of ESBLs were determined by the discs diffusion method on Mueller–Hinton agar and using the double-disc synergy test, respectively, as recommended by EUCAST (http://www.eucast.org/) using 31 discs (BioRad, Marne-la-Coquette, France), and the *E. coli* control strain ATCC 25922, as previously described.[8] Colistin resistance of *mcr-1* positive isolates was confirmed by MIC using a broth microdilution method.

Conjugation experiments

Conjugative mating experiments were carried out in liquid cultures using sodium azide resistant *E. coli* K-12 J5-3 (F- *proB22 metF63*) as recipient strain and field strains as donors, as previously described.[8] Transconjugants were selected on BHI solid media containing either cefotaxime at 2 mg/L or colistin at 4 mg/L and sodium azide at 250 mg/L. Transconjugants were screened by PCR for bla_{CTX-M}, *mcr-1* and Inc replicons, using primers previously described.[2,8,9]

Plasmid sequencing and bioinformatic analysis

Plasmidic DNA extraction and Oxford Nanopore sequencing

AMR plasmids were extracted from selected transconjugants using Macherey–Nagel NucleoBond Xtra Midi plasmid purification kit (Hoerdt, France) following the manufacturer's recommendations for high molecular weight plasmids. Conjugative AMR plasmids were sequenced using the Oxford Nanopore Technologies. Briefly, plasmidic DNAs (40–80 ng) were barcoded and multiplexed using the rapid-barcoding kit SQK-RBK110.96 following the manufacturer's recommendations. Long-read sequencing was performed using the MinION sequencing device with FLO-FLG001 flongle flow cells (R9.4.1) for 16 h. Two sequencing runs were realized producing 62 486 and 47 376 raw reads that were base-called and demultiplexed using the super-accurate algorithm (Guppy v.6.0.1), with read lengths N50 of 20 048 and 25 245 bp, respectively.

Plasmid hybrid assembly and comparative analysis

Oxford Nanopore raw reads were quality-filtered at >Q10 and assembled using the FullForce Plasmid assembler (https://github.com/NorwegianVeterinaryInstitute/FullForcePlasmidAssembler) followed by polishing with short Illumina reads using polypolish.[10] The Illumina pair-end, 150-bp-long raw reads were generated from genomic DNAs of the corresponding field donor isolates in the previous study.[7] Complete plasmid sequences have been deposited in the European Nucleotide Archive under BioProject accession number PRJEB74339. All 40 complete AMR plasmids were analysed using various tools with default threshold parameters (ResFinder v.4.1, PlasmidFinder v.2.1, pMLST and COPLA) to determine their resistance genes content and genetic characteristics (incompatibility group, FAB formula, pTUs, relaxase, conjugative systems).[4,5,11] Genetic maps and comparative genomic analysis of plasmids were performed using GenoFig v.1.1.0 with blastN homologies (https://forgemia.inra.fr/public-pgba/genofig). In addition, a SNP phylogenetic tree of IncF plasmids was produced using the parSNP tool.[12,13] The phylogenetic tree was visualized and annotated with the iTOL web interface tool v.6.[14]

Results and discussion

We previously identified 14 out of 102 ESC-resistant *E. coli* isolates also carrying the mobile colistin resistance gene *mcr-1*.[7] The screening of 111 additional sequenced ESC-resistant *E. coli* isolates from the same sampling campaign increased the bacterial collection to 30 isolates co-harbouring *mcr-1* and bla_{CTX-M} genes (Table S1). Three bla_{CTX-M}-positive only isolates were also included in the present study for comparison purpose. These isolates belong to 15 different STs and were distributed in 17 farms across the whole Lebanese territory. The main ESBL genes were $bla_{CTX-M-3}$ (25/33) and $bla_{CTX-M-55}$ (5/33). Two distinct variants of the *mcr-1* gene were present in these isolates: *mcr-1.1* and *mcr-1.26* in 19 and 11 isolates, respectively. The 33 MDR field *E. coli* isolates contained a large diversity of replicon types, with up to 14 replicon families per isolate (Table S1).

Conjugative mating and complete plasmid sequencing

All field isolates except those carrying $bla_{CTX-M-14b}$ (S34CTX and S35CTX) and $bla_{CTX-M-27}$ (S25CTX) transferred their ESC-resistance phenotype by conjugative mating experiments using cefotaxime selection. On the other hand, conjugative transfer of the colistin resistance phenotype was successful for fewer than half (14/30) (Table S1). Interestingly, eight field isolates (A17FEP, B30FEP, B31CTX, B34FEP, B40FEP, N11CTX, N12CTX, N15CTX) could co-transfer both genes, i.e. bla_{CTX-M} and *mcr-1*, in repeated conjugative mating experiments regardless of the antibiotic selection (cefotaxime or colistin) (Table S2). In all other matings, tranconjugants were positive for bla_{CTX-M} or *mcr-1* gene only according to the respective antibiotic selection. PCR-based replicon typing indicated that all but two bla_{CTX-M} positive transconjugants were found IncFII-positive and those also positive for *mcr-1* were all positive for IncX4 (Table S2). The two transconjugants from A17FEP (TC-CTX and TC-COL) were only positive for IncX4 suggesting that $bla_{CTX-M-55}$ and *mcr-1.26* genes were probably located on the same IncX4 plasmid (Table S2).

Complete circular sequences from 40 conjugative plasmids of 34 transconjugants were obtained after hybrid assembly (Tables S2 and S3). These assemblies confirmed (i) that A17FEP-TC-CTX harboured a single 36 546-bp plasmid and (ii) that all other bla_{CTX-M}/*mcr-1*/IncFII/IncX4-positive transconjugants contained two distinct plasmids of ~33 kb and 71–98 kb, respectively (Table S3). All other bla_{CTX-M} or *mcr-1* only positive transconjugants harboured a single plasmid (Tables S2 and S3).

$bla_{CTX-M-3/55}$-carrying IncFII plasmids

IncFII plasmids carrying $bla_{CTX-M-3/55}$ genes range in size from 61 959 to 100 908 bp, and show the same pMLST formula F2:A-:B-,

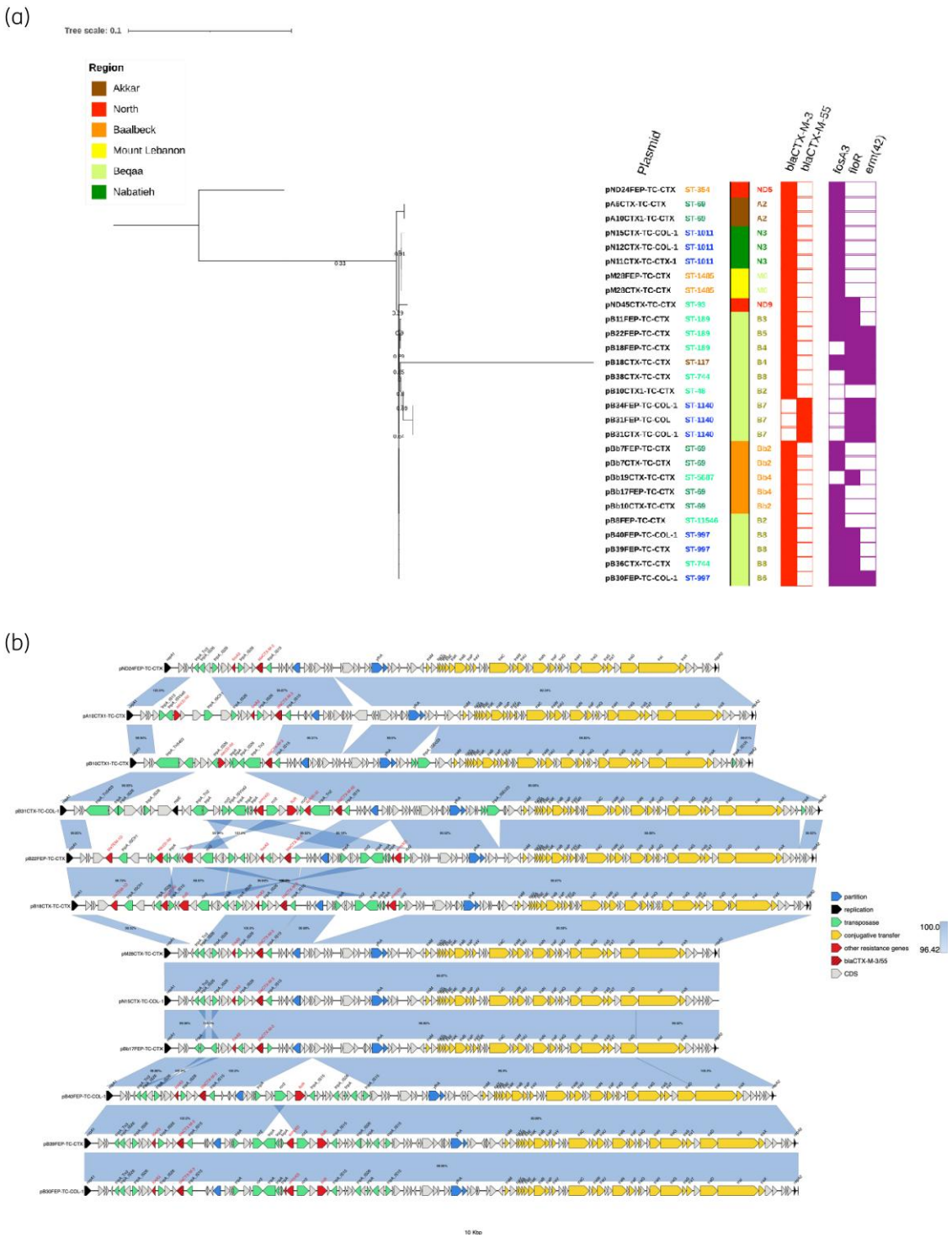

Figure 1. (a) Phylogenetic relationship of 28 IncF2:A-:B-/$bla_{CTX-M-3/55}$ plasmids. The tree was built with a maximum likelihood algorithm from 1458 bp SNPs present in the core plasmid alignment using IncFII plasmid pB38CTX-TC-CTX as internal reference for SNP calling. The tree was rooted using the IncF33:A-:B- plasmid pB6CTX-TC-CTX as outgroup, subsequently removed to improve branch length visibility (see also Figure S3). Local branching support values are based on the Shimodaira–Hasegawa test. Values of 1, indicating maximum support, are not displayed. Columns 1 to 4 on the right side of the tree correspond to plasmid name, sequence type of the *E. coli* donor strain, region and farm of origin, respectively. Sequence types are colour-coded according to the *E. coli* phylotype: cyan, A; green, D; blue, E; orange, F and brown, G. Presence of resistance genes [$bla_{CTX-M-3}$ or $_{-55}$, *fosA3*, *floR*, *erm*(42)] are indicated with a filled box. (b) Schematic map and genomic comparisons of 12 representative IncF2:A-:B-/$bla_{CTX-M-3/55}$ plasmids of different clonal groups generated using Genofig v.1.1.0 (see also Figure S2 showing all IncFII plasmids). Nucleotide identities are displayed for homologous regions >1500 bp. ORF functions are colour-coded according to the panel legend.

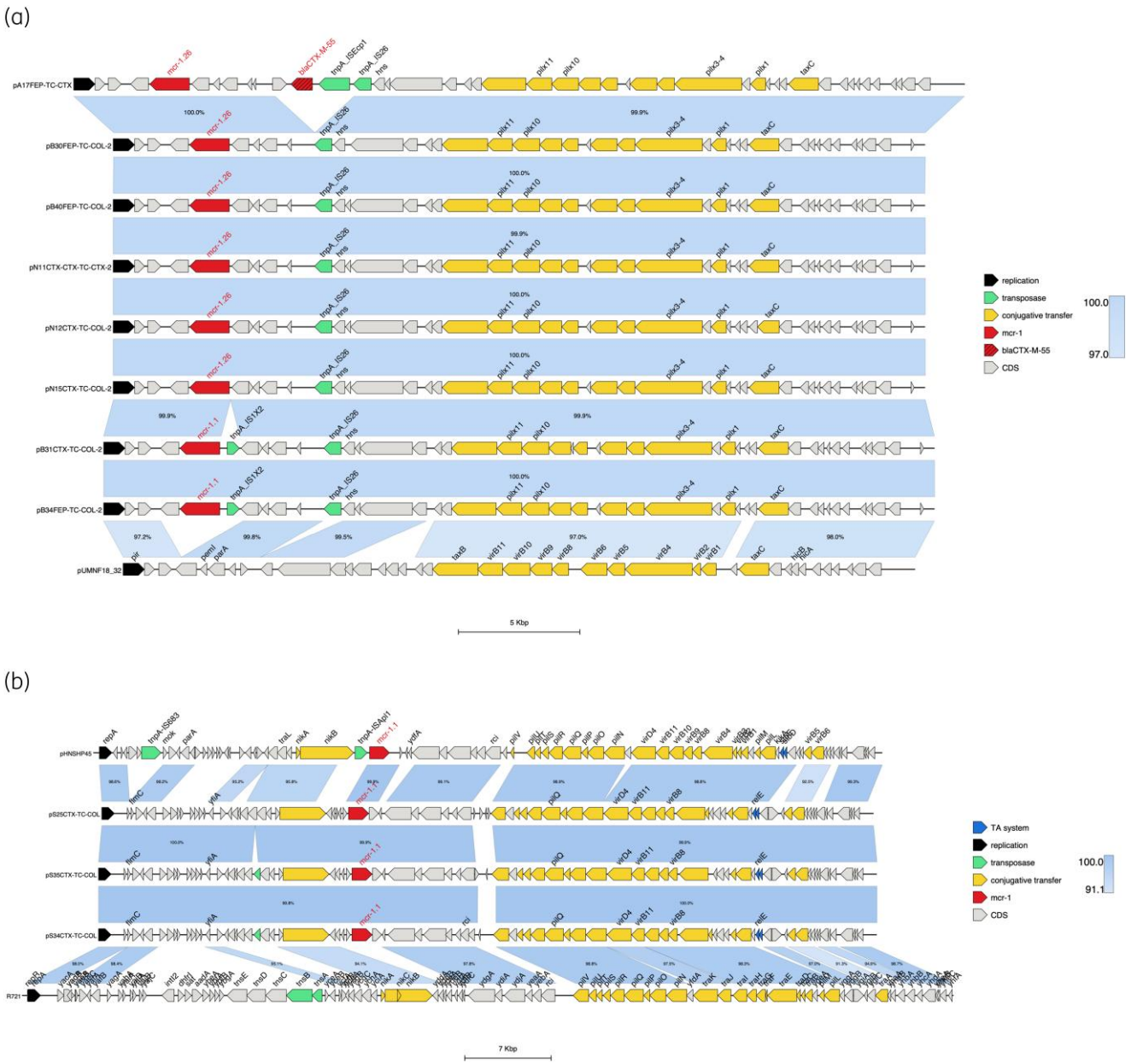

Figure 2. (a) Genomic comparison of the eight IncX4 plasmids carrying *mcr-1*-like genes generated using Genofig v.1.1.0. Plasmid pUMNF18-32 represents a reference IncX4 plasmid without antibiotic resistance gene. Nucleotide identities are displayed for homologous regions >1000 bp. ORF functions are colour-labelled according to the panel legend. (b) Schematic map and genomic comparisons of the three IncI2 plasmids carrying the *mcr-1.1* gene generated using Genofig v.1.1.0. Plasmids pHNSHP45 and R721 represent reference IncI2 plasmids carrying or not the *mcr-1.1* gene, respectively. Nucleotide identities are displayed for homologous regions >1000 bp. ORF functions are colour-coded according to the panel legend.

except for the F33:A-:B- plasmid pB6CTX-TC-CTX (100 879 bp) (Figures S1 and S2, Table S3). They belong to the plasmid taxonomic unit pTU-FE and harbour a MOB-F type relaxase as well as a mating pair formation system of type F.[11] A phylogenetic tree was inferred from the core 1458 SNP positions shared between all IncFII plasmids (Table S4). The F33:A-:B- plasmid pB6CTX-TC-CTX carrying $bla_{CTX\text{-}M\text{-}55}$ clearly branches outside of a large cluster containing all F2:A-:B- plasmids (Figure S3). To gain into resolution, plasmid pB6CTX-TC-CTX was removed from the phylogenetic tree (Figure 1a). Apart from two distantly related F2:A-:B- plasmids (pND24FEP-TC-CTX and pB18CTX-TC-CTX), all other plasmids share between 0 and 23 SNPs (Figure 1a, Table S4). Among them, several clonal plasmid groups showing no SNP difference are found in *E. coli* strains of different STs and phylogroups carrying from 2 to 10 replicons and isolated in distinct farms from distant regions in Lebanon (Figure 1a, Table S4).

Antibiotic resistance genes are gathered in complex genetic clusters including different insertion sequences (IS*15*, IS*26*, IS*CR2*, ...)

and/or partial transposons of the Tn*3* family (Tn*5403*, Tn*3*, Tn*2*). Genomic comparisons highlighted various genetic rearrangements between and within clonal groups that probably occurred by transposition, homologous recombination or co-integrate formation (Figure 1b). Several examples of nearly identical plasmids strongly suggested that these plasmids are spreading horizontally by conjugation between different *E. coli* STs in broilers at the country level (Figure 1). It is worth noting that most (22 out of 29) of these $bla_{CTX-M-3/55}$-carrying IncFII plasmids also harboured the fosfomycin resistance gene *fosA3* representing an additional critically important resistance gene for human medicine (Figures 1, Figures S2 and S3). Since 2012, such plasmids have been sporadically described in clinical isolates of *Salmonella* and *E. coli* of worldwide origins.[15,16] A search for $bla_{CTX-M-3/55}$-carrying F2:A-:B- plasmids in the PLSDB database (59 895 entries, 18 March 2024) retrieved only 26 complete plasmids. Nine of them, mainly from *E. coli* of various origins (humans and animals from Asia, Russia and Switzerland), strongly clustered with the poultry-associated $bla_{CTX-M-3/55}$--carrying F2:A-:B-plasmids described here (data not shown).

mcr-1-*carrying IncX4 and IncI2 plasmids*

Colistin resistance gene variants *mcr-1.1* and *mcr-1.26* were carried by IncX4 plasmids ($n=8$) and IncI2 plasmids ($n=3$) (Table S3). Five nearly identical 33 304-bp IncX4 plasmids (0–1 SNP) carried the *mcr-1.26* gene variant linked to the *pap2* gene but not flanked by insertion sequences (Figure 2a). The *mcr-1.26* gene is located ∼3.4 kb upstream of a single IS*26* element, showing 8-bp target site duplication (CTGTGTGA) suggesting a distinct acquisition event (Figure 2a). Interestingly, the IncX4 plasmid pA17FEP-TC-CTX co-harbours the *mcr-1.26* gene and the ESBL gene $bla_{CTX-M-55}$ (Figure 2). The IS*Ecp1*-$bla_{CTX-M-55}$ transposable unit is inserted in the 3′ end of the IS*26* and is flanked by 5-bp target site duplication (TCAAA), consistent with the known mobilization of bla_{CTX-M} by IS*Ecp1* (Figure 2a). The last two IncX4 plasmids carried the *mcr-1.1* gene and a novel IS*1* family element, 96% identical to IS*1X2*, is found inserted at the 3′ end of the *mcr-1.1-pap2* mobilizable unit (Figure 2a). Finally, all IncI2 plasmids are highly related to each other and carry the *mcr-1.1* gene variant (Figure 2b). Comparisons of the insertion site of *mcr-1.1* with those of reference IncI2 plasmids pHNSHP45 and R721 carrying or not *mcr-1.1*, respectively, revealed a distinct acquisition in Lebanese IncX4 plasmids (Figures 2b and Figure S4). How this acquisition happened is still unclear since no mobile element could be detected in the vicinity of *mcr1.1*.

Numerous studies have described the occurrence of *mcr-1* positive Enterobacterales in various settings in Lebanon (human clinical isolates, carriage in healthy community, food-producing animals and the environment).[17–20] These studies also found the *mcr-1.1/1.26*-carrying IncX4 plasmids followed by IncI2 plasmids as main drivers of the colistin resistance dissemination.[21,22] A recent report described a *mcr-1.26*-carrying IncX4 plasmid in *E. coli* from fresh chicken wings collected from a retail meat market in Beirut,[23] highlighting the exposure risk of consumers.

In conclusion, the massive spread of $bla_{CTX-M-3/55}$-carrying IncF2:A-:B- plasmids and *mcr-1*-carrying IncX4 and IncI2 plasmids occurred in the *E. coli* population of healthy broilers at the entire Lebanese territory level. Identical plasmids differing only by IS-mediated genetic rearrangements in antibiotic resistance gene clusters confirmed their horizontal transfer in genetically diverse *E. coli* strains from distant farms. The routine use of colistin (9 out 17 farms in the present study) and other medically important antibiotics for prophylactic and therapeutic purposes probably participates in the persistence of these MDR plasmids in poultry farm.[10]

Acknowledgements

We are grateful to the Ministry of Agriculture of Lebanon for the help in contact with the different broiler farms. Finally, we warmly thank all involved veterinarians and broiler-farm owners who participated in this study. The present article is dedicated to the memory of our dear colleague Professor Dolla Karam Sarkis, who passed away while this paper was in preparation. She will be greatly missed, but her invaluable contribution to microbiology in Lebanon will continue to inspire and guide scientific research in the field.

Funding

This work was supported by the Saint Joseph University (USJ) of Beirut in Lebanon, by funding from the European Union's Horizon 2020 Research and Innovation program under grant agreement number 773830: One Health European Joint Program, joint research project FULLFORCE and by public funds from the National Research Institute for Agriculture, Food and Environment (INRAE) in France. M.M. was funded by an USJ PhD studentship (grant numbers FP72/73), by the National Center for Scientific Research in Lebanon (CNRS-L) and by the Francophone University Agency (AUF) in Lebanon during her stay in France.

Transparency declarations

None to declare.

Supplementary data

Figures S1 to S4 and Tables S1 to S4 are available as Supplementary data at *JAC-AMR* Online.

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