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Short Communication

CTX-M-15/27-positive *Escherichia coli* and VIM-2-producing *Pseudomonas putida* in free-living pigeons (*Columba livia*) in Tunisia

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ABSTRACT

Objectives: Wild birds are vectors of antimicrobial resistance. Birds living in close contact with humans or other animals, like feral pigeons (*Columba livia*), might be especially prone to acquire resistance genes such as those encoding extended-spectrum beta-lactamases (ESBLs) and carbapenemases.

Methods: Cloacal samples ($n = 206$) of free-living feral pigeons (*C. livia*) were collected in Sousse and Monastir, Tunisia. Antimicrobial susceptibility profiles were determined by disc-diffusion, and resistant isolates were short- and long-read whole-genome sequenced. Sequence analysis was performed using tools of the Centre for Genomic Epidemiology, and Phylogenetic analysis was performed based on the core-genome MLST.

Results: Fourteen (14/206, 6.8%) pigeons harboured Enterobacterales resistant to last-generations cephalosporins, of which 10 were CTX-M-15- or CTX-M-27-producers, while two (1.0%) carried a VIM-2-producing *Pseudomonas putida*. Positive pigeons lived on four different livestock farms. Three STs (ST206, ST5584, ST8149) were identified among *E. coli*, of which ST5584 and ST8149 were found in two different farms. Genetic diversity was also observed in *Enterobacter cloacae* and *P. putida* isolates. The *bla*_{CTX-M-27} genes were chromosomally encoded, while the *bla*_{CTX-M-15} genes were carried on highly similar IncF/F-A-B53 plasmids. The *bla*_{VIM-2} gene was located on a class 1 integron co-harboring several resistance genes.

Conclusion: Pigeons living on livestock farms carried clinically important resistance genes encoding ESBLs and carbapenemases. Our results evidenced that both clonal (ST8149 and ST5584) and plasmidic (IncF/F-A-B53) transfers played a role in the spread of resistance genes among pigeons. Further studies are needed to identify factors favouring the transfer and persistence of resistance genes within the pigeon communities.

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1. Introduction

Antimicrobial resistance has become one of the most important global health issues in the 21st century, requiring multi-sectoral One Health approaches to understand the transmission dynamics of resistant bacteria and avoid the emergence of new pathogens. Wild birds have been identified as potential sentinels, reservoirs and vectors of antimicrobial resistance [1]. Synanthropic animals

such as urban pigeons (*Columba livia*) are of interest to public health because of their proximity to humans and their interactions with livestock and wildlife [2]. Feral pigeons are among the most common bird species in urban and suburban areas, found in all continents except Antarctica. Pigeons have been reported to be an important reservoir of many pathogens, including bacteria such as *Salmonella* spp. or Shiga-toxin producing *Escherichia coli*, which represents an important health hazard to humans [3,4]. In addition, clinically-relevant resistant bacteria such as Enterobacterales producing extended-spectrum beta-lactamases (ESBLs) [2,5,6], carbapenemases (*bla*_{VIM}, *bla*_{OXA-48}) and colistin resistance genes have also been reported in these birds [7,8]. *Acinetobacter* spp. resistant to carbapenems were also identified [9].

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Urban pigeons are opportunistic and omnivorous, feeding mainly from food waste, sewage-contaminated environments, landfills, or areas contaminated with animal faeces. This contributes to the possible horizontal transfer of resistant bacteria and resistance genes among pigeons, humans, animals, and their environment, making these birds potential sentinels of anthropogenic activities and indicators of the dissemination of antimicrobial resistance in the environment. Our main objective was to characterize Gram-negative bacteria resistant to extended-spectrum cephalosporins and/or carbapenems isolated from the faecal samples of feral pigeons collected from two Tunisian cities, in order to assess to which extent pigeons can act as potential indicators of environmental contamination.

2. Material and methods

2.1. Birds sampling and bacterial isolation

Free-living feral pigeons (*C. livia*) ($n = 206$) were collected between April 2021 and May 2023 in Sousse and Monastir (22 km apart), Tunisia. All sampled pigeons were wild birds only having occasional contacts with humans since they lived in proximity. Samples were obtained either using cloacal swabs when birds were captured ($n = 66$), or by collecting fresh droppings when birds could not be captured ($n = 140$) (Table S1). In Sousse, 178 pigeons were sampled, which were living either on four different livestock farms (farm 1–4; $n = 74$), or in the city near the faculty and the university hospital ($n = 104$) (Figure S1). In Monastir, 28 pigeons were collected from three livestock farms (farms 5–7). Feral pigeons living on farms were free to move in and out from the farms and were in close proximity to other animals.

Swabs were discharged into 5 mL Trypto-casein soy broth (TSB; Biokar), homogenized and incubated for 18–24 h at 37 °C. Overnight cultures were inoculated on MacConkey agar plates supplemented with cefotaxime or imipenem (3 mg/L), for the detection of Gram-negative bacteria resistant to extended-spectrum cephalosporins or carbapenems, respectively. One colony per morphology and per plate was picked up. Identification was performed using API20E and API20NE galleries (bioMérieux).

2.2. Antimicrobial susceptibility testing

Susceptibility testing was performed using the disc diffusion method on Mueller-Hinton agar, according to the guidelines and clinical breakpoints of the Antibiogram Committee of the French Society for Microbiology (CA-SFM; www.sfm-microbiologie.org). For Enterobacterales, 16 β -lactam (amoxicillin, piperacillin, ticarcillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, ticarcillin/clavulanic acid, cefalotin, cefuroxime, cefotaxime, ceftiofur, ceftazidime, cefoxitin, cefepime, cefquinome, aztreonam and ertapenem) and 14 non- β -lactam (tetracycline, kanamycin, tobramycin, gentamicin, amikacin, apramycin, netilmicin, streptomycin, florfenicol, chloramphenicol, sulfonamides, trimethoprim, nalidixic acid and enrofloxacin) antibiotics were tested, using *E. coli* ATCC 25,922 strain as quality control. Extended-spectrum beta-lactamase (ESBL) producing Enterobacterales were detected using the Double Disc Synergy Test (DDST). For *Pseudomonas* spp., the following antibiotics were tested using *Pseudomonas aeruginosa* ATCC 27,853 as quality control: piperacillin, ticarcillin, piperacillin/tazobactam, ticarcillin/clavulanic acid, cefepim, ceftazidime, meropenem, imipenem, aztreonam, tobramycin, gentamicin, amikacin, ciprofloxacin, fosfomicin, and tigecycline. MICs to colistin were determined by broth microdilution according to the EUCAST recommendations.

2.3. Illumina short-read sequencing and data analyses

DNA was extracted using the NucleoSpin Microbial DNA extraction kit (Macherey-Nagel) and sequencing was performed on a NovaSeq 6000 instrument (Illumina). Reads were quality trimmed and *de novo* assembled using Shovill v1.0.4. The quality of assemblies was assessed using QUAST v5.0.2 (Table S2).

Identification was performed using Kraken (<https://github.com/DerrickWood/kraken>); sequence types (STs), resistance genes and virulence factors as well as the replicon content and the plasmid sub-typing were determined using the online tools (<http://www.genomicpidemiology.org/>) of the centre for genomic epidemiology. Detection of the phylogenetic groups of each *E. coli* isolate was performed *in silico* using Clermontyping online tool (<http://clermontyping.iame-research.center/>). The genetic elements harbouring the *bla*_{VIM-2} gene were analysed by blastn and Easyfig v2.2.5 and compared to two similar elements found in the publicly NCBI database (CP104583.1 and CP016446.1).

The cgMLST phylogeny was determined through the pyMLST pipeline (<https://github.com/bvalot/pyMLST>) using the scheme available on the www.cgmlst.org/ncs, which comprised 2513 alleles for *E. coli* (for allelic matrix, see Table S3). Cut-off for highly related strains was <10 allelic differences. The resulting tree was visualized using iTol v6 (<http://itol.embl.de/itol.cgi>).

2.4. MinION long-read sequencing

MinION long-read sequencing libraries were prepared according to the Oxford Nanopore Technologies using the native barcoding expansion kit (EXP-NBD104) and the ligation sequencing kit (SQK-LSK109). Sequencing was performed on a MinION sequencer using a SpotON Mk 1 R9 version flow cell (FLO-MIN106D). Assembly of both Illumina and Nanopore reads was performed using Unicycler. The assembled contigs were annotated using Bakta (Web version (Software: 1.8.1| DB: 5.0.0) [10]).

2.5. Accession number

The project was deposited in DDBJ/EMBL/GenBank under the BioProject accession number PRJNA1019937.

3. Results and discussion

3.1. Prevalence of AmpC/ESBL-producing isolates

Among the 206 pigeon samples tested, 14 (14/206, 6.8%) presented growth on plates containing cefotaxime, and two (2/206, 1.0%) on plates containing imipenem. All positive birds were living on four different farms (Figure S1). None of the birds sampled in the city and near a hospital presented resistant isolates, suggesting that contamination in these areas was not sufficient for resistant bacteria to be transmitted to the pigeons. In farm environments, faeces of livestock may contain antibiotic-resistant bacteria and resistance genes [11], and both soil contamination and close contacts between livestock and pigeons may offer favourable conditions for the transmission of resistance to these birds.

One isolate was selected per bird, except for two samples presenting two colony morphologies, resulting in a final collection of 18 resistant isolates. The 16 cefotaxime-resistant isolates were Enterobacterales, including 11 *E. coli* and one *Klebsiella pneumoniae* with an ESBL phenotype (12/206, 5.8%), and four *Enterobacter hormaechei* hyperproducing their intrinsic AmpC gene. The proportion of ESBL-producing *E. coli* observed in Tunisian pigeons was comparable to the one reported in international studies. Indeed, slightly lower rates were reported in France (1/71; 1.4%), Brazil (3/107; 2.8%) and Bangladesh (7/150; 4.7%) [2,5,12], while slightly higher

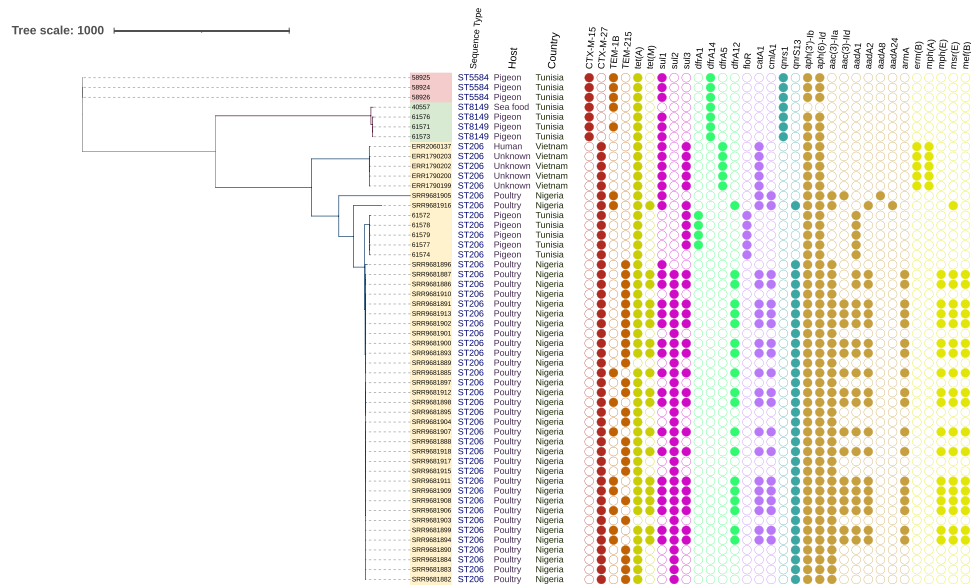


Fig. 1. cgMLST-based phylogeny of *E. coli* isolates using iTOL. ST206 and ST1849 found in the publicly available NCBI database were included for comparison.

rates were reported in Algeria (18/276; 6.5%) and Portugal (9/100; 9%) [6,7]. These differences can be attributed to multiple factors, including protocols and methods, geographical variations, urbanization levels, and contamination of the environments.

The two imipenem-resistant isolates were *Pseudomonas putida*, which is a bacterial species rarely reported in pigeons.

3.2. Genetic diversity of resistant isolates and genes conferring resistance to extended-spectrum cephalosporins and carbapenems

Three STs were identified among ESBL-*E. coli* isolates. ST5584 ($n = 3$) presenting the *bla*_{CTX-M-15} gene was the unique ST identified in farm 1 in Monastir and was absent in all other farms, while ST1849 ($n = 3$) carrying the *bla*_{CTX-M-15} gene and ST206 ($n = 5$) carrying the *bla*_{CTX-M-27} gene were both found in farm 2 in Sousse and in farm 6 in Monastir (Fig. 1, Table S4). ST5584 isolates differed by 1–2 alleles, proving clonal expansion of these STs in each farm and highlighting that sharing a same habitat favours the spread of successful resistant clones. Whether the use of antibiotics to treat animals might play a role in this spread of resistance in pigeons remains to be studied. The presence of ST1849 and ST206 in pigeons collected from farms located 18 km apart was rather unexpected since these birds usually live in a small perimeter, but flights over longer distances were also observed especially for foraging in fields [13]. Another hypothesis might be that these resistant bacteria are carried by determinants shared between the two farms, such as care-takers, animals or food. The STs identified here were not pandemic but all have already been reported. ST206 has mainly been associated with animal hosts so far; in particular, one isolate carrying a *bla*_{CTX-M-15} gene has been identified in urban pigeons in Portugal [6], and isolates displaying the *bla*_{CTX-M-27} gene were identified in numerous poultry flocks in Nigeria as well as in a human isolate in Vietnam [14]. Since genomic data from the two last studies were available, a cgMLST comparison was conducted (Fig. 1, Table S4), which showed that isolates from Tunisian pigeons were genetically similar but not identical (28–37 allelic differences) to 33/35 poultry isolates from Nigeria, while they shared no genetic link with isolates from Vietnam (>600 allelic differences). Surprisingly, *bla*_{CTX-M-15}-carrying ST1849 isolates have already been reported in two different studies in Tunisia, once from a fish and once from a cockroach [15,16]. The fish isolate was similar but not

identical to the pigeon isolates (21–22 allelic differences). ST5584 was more infrequent, sporadically reported as *bla*_{CTX-M-15}-carrier in seawater in Ireland and in a Chinese patient [17,18].

In farm 6, one *K. pneumoniae* ST29 carrying the *bla*_{CTX-M-15} gene was also isolated. ST29 has been recurrently reported from animal, water and human isolates carrying diverse resistance genes such as *bla*_{CTX-M}, *bla*_{NDM} or *mcr-1*.

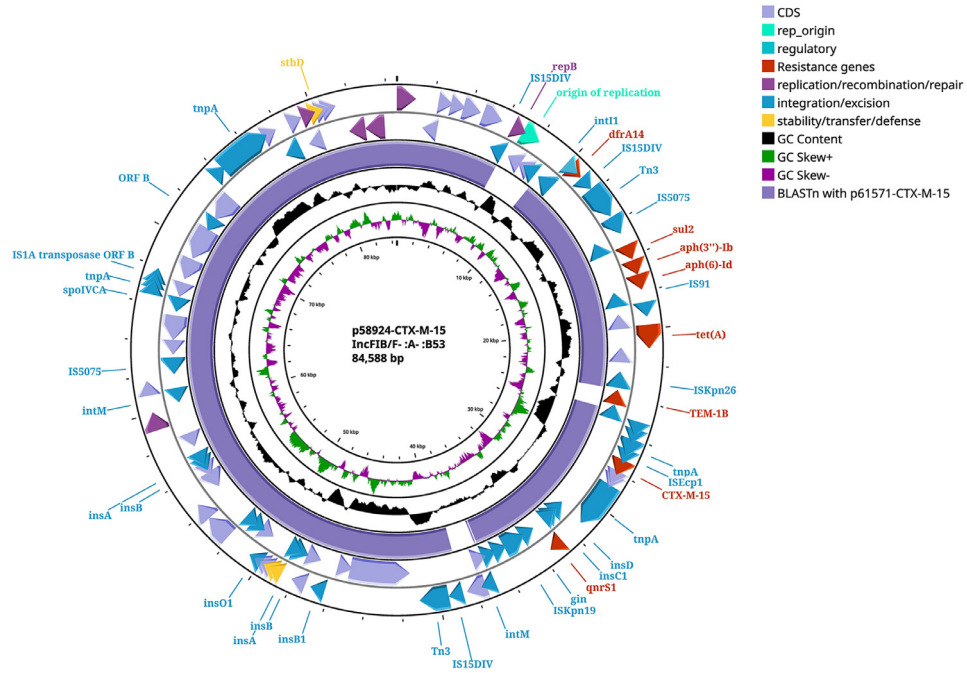
Finally, in farm 4, four *E. hormaechei* hyperproducing their intrinsic AmpC gene were identified, as indicated by resistance to ticarcillin, resistance or decreased susceptibility to ceftazidime, and the absence of synergy with amoxicillin + clavulanic acid on the antibiogram by disk diffusion, as well by the absence of identified resistance genes by WGS despite the non-susceptible phenotype. One isolate belonged to ST102 while the three other ones to two new STs assigned to ST2819 and ST2824 (Table S4). In the same farm, two different carbapenem-resistant *P. putida* presenting the *bla*_{VIM-2} genes were identified, which presented the new clearly diverging ST275 and ST276. A *bla*_{VIM} gene has already been described in pigeons, but carried by an *E. coli* isolate and the gene variant was not determined [8].

3.3. Genetic determinants carrying resistance genes in CTX-M-positive *E. coli*

One representative of each ST was long-read sequenced to characterize the genetic elements carrying the resistance genes. The ST206 *E. coli* #61,579 isolate presented an IncX1/IncFIBk small (48 kbp) hybrid plasmid carrying numerous resistance genes (*tet(A)*, *floR*, *sul3*, *dfrA1*, *aadA1*, *aph(6)-Id* and *aph(3'')-Ib*) which conferred resistances to tetracyclines, chloramphenicol/florfenicol, sulfonamides-trimethoprim and streptomycin. Conversely, the *bla*_{CTX-M-27} was located on the chromosome in an atypical genetic environment since it was not preceded by an IS26 but only followed by an IS903B family transposase. The chromosomal localization of *bla*_{CTX-M} genes has been frequently described, including in the Nigerian *bla*_{CTX-M-27}-producing isolates collected from poultry [14], and is supposed to help stabilising the resistance genes even in the absence of selective pressure.

The *bla*_{CTX-M-15} gene was carried by an IncF/F-: A-: B53 plasmid (Fig. 2A) in the *E. coli* ST5584 (#58,954) and ST1849 (#61,571) isolates that were collected from two different farms in Sousse and Monastir. Plasmids co-harboured the *dfrA14*, *qnrS1*, *tet(A)* and

A.



B.

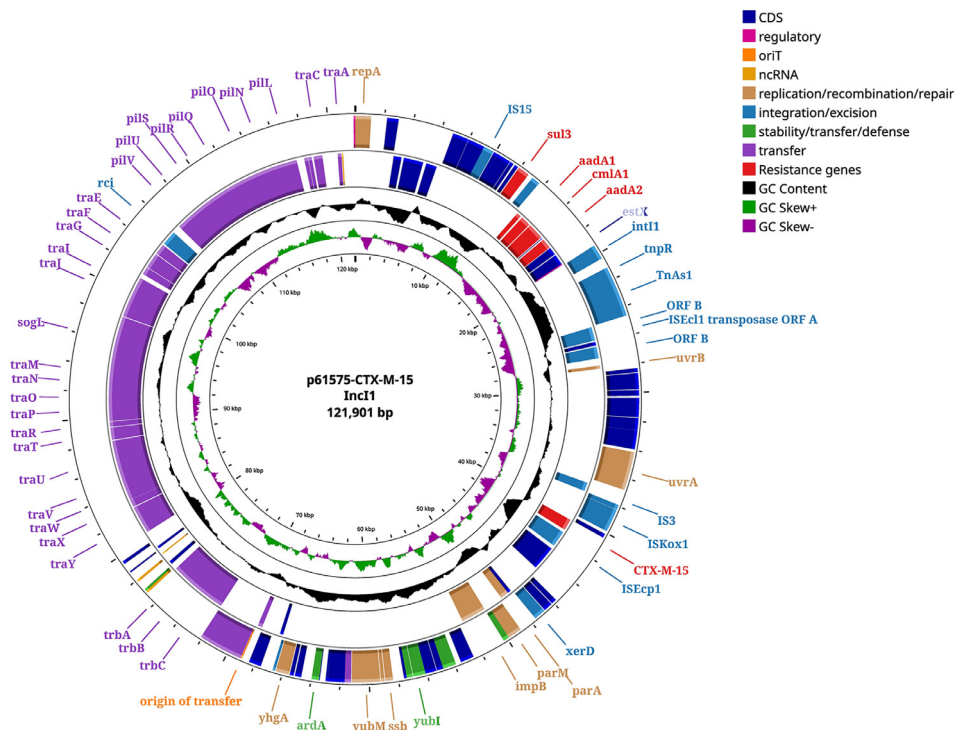


Fig. 2. PROKSEE visualization of the long-read sequenced plasmids carrying the *bla*_{CTX-M-15} genes: the two IncF/F-:A-:B53 plasmid carried by the *E. coli* #58 924 and #61 571 strains (A), and the IncI1 carried by the *K. pneumoniae* #61 575 strain (B).

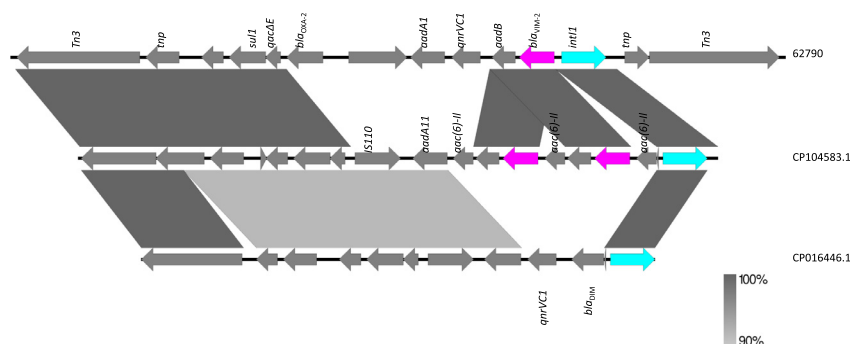


Fig. 3. Easyfig v.2.2.5 representation of the blastn alignment of the *bla*_{VIM-2} element and similar elements found in *Pseudomonas* spp. CP104583.1 and *Pseudomonas putida* CP016446.1.

[*sul2-aph(3'')-lb-aph(6)-ld*] genes, and p58954-CTX-M-15 (ST5584) additionally displayed the *bla*_{TEM-1b}. The two plasmids slightly differed by their size, p58954-CTX-M-15 being larger (84,588 bp) than p61571-CTX-M-15 (77,407 bp), but shared high similarity (99% nucleotide identity, 99% coverage). Such a similarity might suggest horizontal transfer from one genetic background to another, followed by differential evolution. This IncF/F-: A-: B53 plasmid might also be particularly successful since it has been reported in Tunisia in a fish isolate belonging to ST8149 [16], in humans, chicken and the environment in Madagascar [19] as well as in wild migratory birds in Pakistan [20]. Blastn search in the NCBI publicly available database further revealed high similarity (99% identity, 99% coverage) with three *E. coli* plasmids originating from a soldier in West Mali in 2014 (p121_MLI106-1; CP116930.1), from raw milk cheese in Egypt in 2014 (pCFSAN061768; CP042974.1) and from wastewater effluent in Tokyo in 2017 (pWP7-S17-ESBL-04_1 DNA; AP022178.1).

3.4. Genetic determinants carrying resistance genes in the *K. pneumoniae* isolate

The ST29 *K. pneumoniae* isolate carried the *bla*_{CTX-M-15} gene preceded by the *ISEcp1* element on a 121,901 bp IncI1 plasmid whose pMLST profile was unknown (4/6*/1*/2/21*, where * indicates a novel allele) (Fig. 2B). The plasmid additionally harboured the *aadA1*, *aadA2b*, *sul3* and *cmlA1* resistance genes; conversely, the *floR* gene was located on an additional IncX1 plasmid while the remaining intrinsic genes *bla*_{SHV-187}, *fosA* and *oqxAB* were located on the chromosome. Comparison with publicly available data showed that it only shared low similarity (99.99% identity, 72% coverage) with p90CM2-103k (CP071822.1), a *K. pneumoniae* plasmid found in healthy human's faeces in Thailand in 2018 and sharing a similar pMLST type (4/6*/1*/2/9*).

3.5. Genetic environment of the *bla*_{VIM-2} gene in *P. putida*

Two multidrug-resistant *P. putida* isolates carrying both the ESBL *bla*_{OXA-2} and carbapenemase *bla*_{VIM-2} genes (Fig. 3) were identified on a farm in Sousse. This is the second occurrence of a *bla*_{VIM} gene in pigeons to date, after its description in an *E. coli* isolate in Brazil [8], and one of the few studies reporting carbapenemases in pigeons. In Marseille (France) and Annaba (Algeria), carbapenemase-encoding genes were detected in 16 out of 73 *Acinetobacter* spp. isolates, 13 being positive for the *bla*_{OXA-58} gene and eight for the *bla*_{OXA-23} genes [9]. Also in Algeria, in the city of Batna, 11 *E. coli* strains harbouring the *bla*_{OXA-48} gene were found among the 276 droppings analysed [7]. The presence of OXA-48-producing *E. coli* has been recurrently reported in Batna, in hospital settings but also in humans in the community, in wastewater as well as in migratory storks [7]. This context probably cre-

ated favourable conditions for the acquisition of this gene by pigeons contributing to OXA-48 endemicity, and its further spread in a given bacterial population is then assured by its localization on the highly successful IncI plasmids.

In our study, the *bla*_{VIM-2} gene was located on a 13 kbp class 1 integron also carrying the *sul1*, *qacΔE*, *bla*_{OXA-2}, *qnrVC1*, *aadB* resistance genes bracketed by genes encoding transposases of the Tn3 family (Fig. 3). The very same integron was identified in two *P. putida* from different STs, suggesting its transfer between birds and subsequently from one *P. putida* isolate to a different one. Such cassette arrays have already been described in diverse *Pseudomonas* species, carrying carbapenemase genes (*bla*_{VIM}, *bla*_{IMP}, *bla*_{DIM}) as well as a high diversity of other resistance genes [21]. Cassette arrays presenting partial homologies with the integron described here were found in the publicly available databases (Fig. 3), but the overall genetic variability is very high. Such a genetic environment was described in a *Pseudomonas aeruginosa* from burn patients in Tunis in 2008 [22], but both the weak homology with the genetic structure of our isolates and the temporality rule out any possibility of genetic links between these isolates.

4. Conclusion

We report the presence of CTX-M-15 and CTX-M-27-producing Enterobacterales, mainly *E. coli*, and VIM-2-producing *P. putida* in pigeon droppings in Tunisia. Our study highlighted that both clonal (ST8149 and ST5584) and plasmidic (IncF/F-:A-:B53) transfers contributed to the spread of resistance genes among pigeons. Our results also corroborated that pigeons can be vectors of antimicrobial resistance, but specific conditions must be fulfilled to allow the colonization of acquired resistance. Indeed, no resistance genes were found in pigeons living in the city centre, where human-animal contacts and the proximity of hospitals seemed favourable conditions. Further studies are needed to identify the factors influencing the dissemination and persistence of resistant genes within these avian communities and to explore their potential impact on public health and ecosystems.

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Competing interest: The authors declare that the research was conducted in the absence of any commercial or financial interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2023.12.013.

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