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Brief Report

Multidrug Resistance Dissemination in *Escherichia coli* Isolated from Wild Animals: Bacterial Clones and Plasmid Complicity

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Abstract: Objectives. Epidemiological data concerning third-generation cephalosporin (3GC) resistance in wild fauna are scarce. The aim of this study was to characterize the resistance genes, their genetic context, and clonal relatedness in 17 *Escherichia coli* resistant to 3GC isolated from wild animals. **Methods.** The isolates were characterized by short-read whole genome sequencing, and long-read sequencing was used for the hybrid assembly of plasmid sequences. **Results.** The 3GC resistance gene most identified in the isolates was the extended-spectrum β-lactamases (ESBL)-encoding gene $bla_{\text{CTX-M-1}}$ (82.3%), followed by $bla_{\text{CTX-M-32}}$ (5.9%), $bla_{\text{CTX-M-14}}$ (5.9%), and $bla_{\text{SHV-12}}$ (5.9%). *E. coli* isolates mainly belonged to the sequence types (STs) rarely reported from humans. The single nucleotide polymorphism (SNP)-based typing showed that most *E. coli* genomes from wild animals (wild boars, birds of prey, and buzzards) formed clonal clusters (<5 SNPs), showing a clonal dissemination crossing species boundaries. $bla_{\text{CTX-M-1}}$-harboring IncI1-ST3 plasmid was the predominant ESBL-encoding plasmid (76.4%) in wild animal isolates. Plasmid comparison revealed a 110-kb self-transferable plasmid consisting of a conserved backbone and two variable regions involved in antimicrobial resistance and in interaction with recipient cells during conjugation. **Conclusion.** Our results highlighted the unexpected clonal dissemination of $bla_{\text{CTX-M-1}}$-encoding clones and the complicity of IncI1-ST3 plasmid in the spread of $bla_{\text{CTX-M-1}}$ within wild fauna.

Keywords: antibiotic resistance; ESBL; CTX-M-1; *Escherichia coli*; enterobacteriaceae; environment; animals; wild fauna; plasmid

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Citation: Sabença, C.; Igrejas, G.; Poeta, P.; Robin, F.; Bonnet, R.; Beyrouthy, R. Multidrug Resistance Dissemination in *Escherichia coli* Isolated from Wild Animals: Bacterial Clones and Plasmid Complicity. *Microbiol. Res.* **2021**, *12*, 123–137. https://doi.org/10.3390/microbiolres12010009

Academic Editor: Luca Grispoldi

Received: 17 December 2020
Accepted: 23 February 2021
Published: 2 March 2021

1. Introduction

Antibiotics overuse is a key factor in the emergence of antimicrobial resistance (AMR) mechanisms, especially extended-spectrum β-lactamases (ESBL) [1]. ESBLs are enzymes that confer resistance to penicillins and cephalosporins, including third-generation cephalosporins (3GC), classified by the World Health Organization (WHO) as critically important antimicrobial agents in human medicine [2].

Antimicrobial resistance is also a complex and multifaceted threat to humans, animals, and the environment. Advanced knowledge on AMR has demonstrated that it not only affects the human sector but is also widely distributed across animals and the environment [3]. A major cause of the AMR burden is the capability of AMR to transmit within and

between individuals, including across sectors. There is a great concern that contacts with animals may enhance the risk of ESC-E/CP-E acquisition by humans [3]. An increase in ESBL-producing bacteria carriage was demonstrated for farmers and employees in contact with broilers [4].

The occurrence of antibiotic-resistant bacteria in wild fauna has also been reported, with the first description of chloramphenicol-resistant *Escherichia coli* isolates in Japanese wild birds in 1977 [5,6]. The ESBL-producing *E. coli* isolates from wild animals were first reported in Portugal in 2006 [7]. Since then, the occurrence of ESBL-producing bacteria within diverse animal species, including urban and wild birds with no evident exposure to antimicrobial drugs, has been recently reported across different geographical areas [6,8,9]. On the other hand, wild animals, especially migratory birds, have been identified as potential reservoirs for ESBL, AmpC β-lactamase, carbapenemase, and colistin resistance genes in *Enterobacteriaceae* [6,10]. Because of their frequent presence in close proximity to human-influenced environments, such as urban areas, landfills, wastewater, and livestock facilities, they can be colonized by resistant bacteria and then disseminate them over long distances, thus constituting a possible transmission factor of resistant bacteria that can contaminate animal food and natural water reservoirs [9,11]. However, few data were collected on the spread of resistant bacteria in wildlife.

CTX-M-type are the most common plasmid-mediated ESBL among *Enterobacteriaceae* isolates of human and veterinary origin worldwide [12–16]. The CTX-M-1-encoding gene $bla_{\text{CTX-M-1}}$ has been frequently associated with IncI1 and IncN plasmids in *Enterobacteriaceae* of human and veterinary origin [17–19], and it has been mainly associated with IncI1 plasmids in wildlife in Europe [20,21]. In addition, IncI1 plasmids can also carry other ESBL-encoding genes such as $bla_{\text{TEM-52}}$ and $bla_{\text{SHV-12}}$ in wild animals [6,10]. However, genomic data for ESBL-encoding plasmids in wild animals are scarce [22,23].

This study aimed to characterize a collection of 17 ESBL-producing *E. coli* isolates collected in distinct regions of Portugal from wild animals with different eating habits to analyze the clonal diversity of isolates and the ESBL genetic context. The results showed both an unexpected clonal dissemination of ESBL-producing *E. coli* and the epidemic spread of ESBL-encoding plasmids in wild fauna.

2. Materials and Methods

2.1. Bacterial Isolates

A total of 17 ESBL-producing *E. coli* isolates from different species of wild animals with different eating habits living in natural parks and reserves in distinct regions of Portugal were included in this study, ranging from carnivores such as birds of prey (n = 4), buzzards (n = 4), lynx (n = 2), and wolves (n = 2) to omnivores such as wild boar (n = 3) and seagulls (n = 1) and herbivores such as a bat (n = 1) (Table 1) [24–30].

Table 1. Metadata and key features of *Escherichia coli* isolates collected from the stool of different wild animals.

Strain ID	Source	Country/Year	Phylogroup/ST	ESBL Gene/Replicon [a]	Plasmid-Mediated Resistance Genes [b]	Chromosome-Mediated Resistance Genes [b]
TB63	Bat	Portugal/2014	B1/ST155	$bla_{\text{CTX-M-1}}$/IncI1-ST3	*aadA5*, *dfrA17*, *sul2*	*gyrA-S83L*
1-102	Bird of prey	Portugal/2008	C/ST1998	$bla_{\text{CTX-M-1}}$/IncI1-ST3	bla_{TEM-1}, *aadA5*, *dfrA17*, *sul2*, *tet(A)*, *tet(R)*,	*gyrA-S83L-D87N*, *parC-S80I*
13-103	Bird of prey	Portugal/2008	B1/ST1800	$bla_{\text{CTX-M-1}}$/IncI1-ST3	bla_{TEM-1}, *aadA5*, *dfrA17*, *sul2*, *tet(A)*, *tet(R)*	*gyrA-S83L-D87N*, *parC-S80I*
2-101	Bird of prey	Portugal/2008	C/ST1998	$bla_{\text{CTX-M-1}}$/IncI1-ST3	bla_{TEM-1}, *aadA5*, *dfrA17*, *sul2*, *tet(A)*, *tet(R)*,	*gyrA-S83L-D87N*, *parC-S80I*
21-101	Bird of prey	Portugal/2008	C/ST1998	$bla_{\text{CTX-M-1}}$/IncI1-ST3	bla_{TEM-1}, *aadA5*, *dfrA17*, *sul2*, *tet(A)*, *tet(R)*	*gyrA-S83L-D87N*, *parC-S80I*
BU10A	Buzzard	Portugal/2007	A/ST48	$bla_{\text{CTX-M-1}}$/IncI1-ST3	bla_{TEM-1}, *aadA5*, *dfrA17*, *sul2*, *tet(A)*, *tet(R)*, *qnrS1*, *strAB*, *aadA1*, *dfrA1*, *sul1*	
BU10B	Buzzard	Portugal/2007	A/ST48	$bla_{\text{CTX-M-1}}$/IncI1-ST3	bla_{TEM-1}, *aadA5*, *dfrA17*, *sul2*, *tet(A)*, *tet(R)*, *qnrS1*, *strAB*, *aadA1*, *dfrA1*, *sul1*	
BU22A	Buzzard	Portugal/2007	B1/ST1800	$bla_{\text{CTX-M-1}}$/IncI1-ST3	bla_{TEM-1}, *aadA5*, *dfrA17*, *sul2*, *tet(A)*, *tet(R)*	*gyrA-S83L-D87N*, *parC-S80I*
BU41A	Buzzard	Portugal/2007	A/ST48	$bla_{\text{CTX-M-1}}$/IncI1-ST3	bla_{TEM-1}, *aadA5*, *dfrA17*, *sul2*, *tet(A)*, *tet(R)*, *qnrS1*, *strAB*, *aadA1*, *dfrA1*, *sul1*	
L16	Lynx	Spain/2010	C/ST23	$bla_{\text{CTX-M-14}}$/IncK	*aadA1*, *aac(3)-VIa*, *tet(A)*, *tet(R)*, *sul1*	
L98	Lynx	Spain/2010	F/ST117	$bla_{\text{SHV-12}}$/IncI1-ST3	*aadA1*, *aadA2*, *cmlA1*, *sul3*, *strA*, *dfrA5*	*gyrA-S83L*
GV23	Seagull	Portugal/2007	A/ST710	$bla_{\text{CTX-M-1}}$/IncN-ST1		bla_{OXA-1}, *aadA1*, *catA1*, *sul1*, *tet(B)*, *tet(R)*
J31	Wild boar	Portugal/2006	A/ST48	$bla_{\text{CTX-M-1}}$/IncI1-ST3	bla_{TEM-1}, *aadA5*, *dfrA17*, *sul2*, *tet(A)*, *tet(R)*, *qnrS1*, *strAB*, *aadA1*, *dfrA1*, *sul1*	
J64	Wild boar	Portugal/2006	C/ST1998	$bla_{\text{CTX-M-1}}$/IncI1-ST3	bla_{TEM-1}, *aadA5*, *dfrA17*, *sul2*, *tet(A)*, *tet(R)*	*gyrA-S83L-D87N*, *parC-S80I*
J69	Wild boar	Portugal/2006	A/ST10430	$bla_{\text{CTX-M-1}}$/IncI1-ST3	*aadA5*, *dfrA17*, *sul2*	*gyrA-S83L*, *parC-S80R*, *tet(B)*, *tet(R)*
W151	Wolf	Portugal/2008	A/ST361	$bla_{\text{CTX-M-32}}$/IncN-ST1		
W4	Wolf	Portugal/2008	B1/ST155	$bla_{\text{CTX-M-1}}$/IncI1-ST3	*tet(A)*, *tet(R)*	*gyrA-S83L*

[a] The association of ESBL genes with replicons was determined from Southern blot experiments and in silico analysis of WGS; [b] deduced from in silico NGS data analysis.

2.2. Antibiotic Susceptibility Testing

Antimicrobial susceptibility testing was assessed with the disc (Bio-Rad, Marnes-la-Coquette, France) diffusion method according to the European Committee on Antimicrobial Susceptibility Testing guidelines (http://www.eucast.org/ (accessed on 1 December 2020)). The molecules tested were ampicillin (10 µg), amoxicillin-clavulanate combination (20–10 µg), ticarcillin (75 µg), piperacillin (30 µg), piperacillin-tazobactam combination (30–6 µg), cephalexin (30 µg), cefuroxime (30 µg), cefixime (5 µg), cefoxitin (30 µg), cefotaxime (5 µg), ceftazidime (10 µg), cefepime (30 µg), aztreonam (30 µg), ertapenem (10 µg), imipenem (10 µg), mecillinam (10 µg), tobramycin (10 µg), amikacin (30 µg), gentamicin (10 µg), netilmicin (10 µg), nalidixic acid (30 µg), norfloxacin (10 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), trimethoprim (5 µg), trimethoprim-sulfamethoxazole combination (1.25–23.75 µg), temocillin (30 µg), and fosfomycin (200 µg). ESBL production was detected by the double-disc synergy test using cefotaxime, an amoxicillin-clavulanate combination, and ceftazidime (http://www.eucast.org/ (accessed on 1 December 2020)). *E. coli* ATCC 25,922 was used as a reference strain.

2.3. Whole-Genome Sequencing

Whole-genome sequencing (WGS) was performed using the next-generation sequencing platform of the teaching hospital of Clermont-Ferrand, France. DNA was extracted with a DNeasy UltraClean Microbial kit (Qiagen). The libraries were prepared with a Nextera XT Kit (Illumina, San Diego, CA, USA), and sequenced by the Illumina MiSeq system generating 2 × 301-base pair (bp) paired-end reads. The Fastp v0.19.10 [31] was used for quality filtering of Illumina reads and SPAdes [32] for short reads assembly. The average sequencing depth was $\geq$75×; the number of assembled contigs ranged between 69 and 154, and genome sizes between 4,567,913 and 5,210,322 nucleotides. The raw reads have been deposited in the European Nucleotide Archive (ENA) under the project accession number PRJEB36175.

Genomic Data Analysis

E. coli phylogroups and multi-locus sequence typing (MLST) were determined in silico according to ClermonTyping method [33] and Achtman's MLST scheme [34]. The molecular typing of isolates was performed by core genome SNP-based typing (cgSNP) and core genome MLST (cgMLST). BactSNP was used to perform cgSNP using the *E. coli* EC958 genome [35] as reference (genome size: 5,162,892 bases) as previously described [36,37]. After the filtration of recombination zones detected by Gubbins [38], a phylogenetic tree was inferred from the resulting alignment by maximum likelihood using RAxML [39]. The cgMLST was performed according to the Enterobase cgMLST scheme (https://enterobase.warwick.ac.uk (accessed on 4 February 2021)) and allele calling with package chewBBACA [40]. The antibiotic resistant genes were identified by alignment against a database including the online databases CARD [41], Resfinder [42], and the NCBI National Database of Antibiotic Resistant Organisms (https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/ (accessed on 1 December 2020)) using a 95% minimum threshold for the breadth of coverage and identity percentage, as previously described [43].

2.4. Plasmid Analysis

The plasmid content of bacteria and the size of plasmids were determined using plasmid DNA extracted using the method of Kado and Liu [44]. The PCR-based replicon typing (PBRT) scheme (Diatheva, Fano, Italy) and Southern blots using probes specific for the IncI1, IncN, and IncK replicons and CTX-M-1, CTX-M-14, and SHV-12-encoding genes were used to localize the ESBL-encoding genes in plasmids, as previously described [45]. The PCR-generated probes derived from $bla_{\text{CTX-M}}$-harboring sequenced strains and PBRT kit controls (Diatheva, Fano, Italy). The experimental results were confirmed in silico from WGS using plasmidFinder and the plasmid MLST typing, as previously described [46]. Long reads were obtained from *E. coli* isolates 2-101 (birds of prey) and J31 (wild boar) using

the ligation sequencing kit 1D SQK-LSK109 with the barcoding extension kit EXP-NBD 104 according to the Oxford Nanopore Technologies protocols (Oxford, United Kingdom). The same DNA extract was used for sequencing across Illumina and Nanopore platforms to minimize biological variability introduced in bacterial culture. A flowcell FLO-MIN106 connected to the MinION sequencing device was used to sequence the library during 48 h. Real-time base calling of MinION reads was performed with the MinIT and integrated Guppy software to produce fastQ files. The reads size from Nanopore varied between 120 and 69,617 bases for the J31 dataset (39,647 reads with a median of average quality score per read of Q15—base call accuracy: 97%—and an average size of 11,691 bases corresponding to a total of 463,529,496 bases) and between 107 and 87,164 bases for 2-101 dataset (85,034 reads with a median of average quality score per read of Q15—base call accuracy: 97%—and an average size of 10,090 bases corresponding to a total of 857,958,899 bases). These long reads were de novo assembled with the Illumina short reads using Unicycler v. 0.4.7 [47]. The average sequencing depth of the hybrid assembly was 573× (minimum 139×), and 169× (minimum 30×) for J31 isolate and 370× (minimuFm 89×) and 110× (minimum 20×) for 2-101 isolate from Nanopore and Illumina reads, respectively. Plasmids annotation was performed by the National Center for Biotechnology Information prokaryotic genome annotation pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/ (accessed on 4 May 2020), project accession number PRJEB36175).

3. Results

3.1. Resistance Phenotype and Resistome

The *E. coli* isolates exhibited a positive synergy test and resistance to penicillin and cephalosporins, including the oxyimino-cephalosporins cefotaxime or ceftazidime, in agreement with ESBL production. A total of 41% of the isolates were resistant to all quinolones, 76.4% to trimethoprim, and 82.3% to sulphonamides. All the tested isolates were susceptible to amikacin, and only one isolate was resistant to gentamicin. The antimicrobial susceptibility profiles are listed in Supplementary Table S1.

WGS analysis detected ESBL-encoding genes in all isolates (Table 1). The predominant ESBL gene was $bla_{\text{CTX-M-1}}$ (82.3%), followed by the point variants $bla_{\text{CTX-M-32}}$ (5.9%), $bla_{\text{CTX-M-14}}$ (5.9%), and $bla_{\text{SHV-12}}$ (5.9%). Analysis of *gyrA* and *parC* mutations responsible for quinolone resistance showed the predominance of the double mutations *gyrA* (S83L and D87N) associated with *parC* mutation (S80I; 35.3%). The gene *qnrS1* that decreases quinolone susceptibility was identified in 23.5% of the isolates. The most prevalent aminoglycoside resistance genes were the genes *aadA* (*aadA1*, *aadA2*, and *aadA5*), *strA*, and *strB* that affect susceptibility to streptomycin. Only one gene known to affect the susceptibility to gentamicin was detected (*aac(3)-VIa*; 5.9%), and none affecting amikacin were detected. Overall, the content in resistance genes agreed with the resistance phenotype.

3.2. Whole-Genome Typing of E. coli Isolates

Most *E. coli* isolates belonged to phylogroup A (41.2%); the others belonged to phylogroups C (29.4%), B1 (23.5%), and F (5.9%). Nine STs were identified among the isolates, and 70% of the wild animal isolates were clustered in four STs: ST48 (n = 4), ST1998 (n = 4), ST1800 (n = 2) and ST155 (n = 2) (Table 1). None of these isolates belonged to the predominant STs that carry ESBLs in humans [48–64], suggesting that the dissemination of ESBLs in wild animals is not due to human strains.

The core genome alignment consisted of 3,499,887 bp, with the isolates differing from each other by 0 to 77,550 SNPs. Three clonal clusters comprising 2 to 4 isolates differed from each other by less than 5 SNPs, while the others diverged by 1831 to 77,550 SNPs (Figure 1a). To provide an unbiased phylogeny, recombination zones were removed. The resulting alignment consisted of 568,547 nucleotides with 0 or 1 SNP for the clonal isolates ($\leq$5 SNPs) and of 211 to 32,758 SNPs for the other isolates. The clonality relationships were also inferred by a cgMLST scheme including 2513 conserved loci (Figure 1b). In agreement with the cgSNP approach, the isolates differed from each other by 0 or 3 alleles for the clonal

isolates (≤5 alleles) and by 258 to 2375 alleles for the other isolates (Figure 1b). The clonal clusters belonged to ST48, ST1800, and ST1198, and corresponding isolates were isolated from carnivore birds and a wild boar. These results reveal the clonal dissemination of ESBL-producing *E. coli* strains for 10 out of 17 isolates in wild fauna.

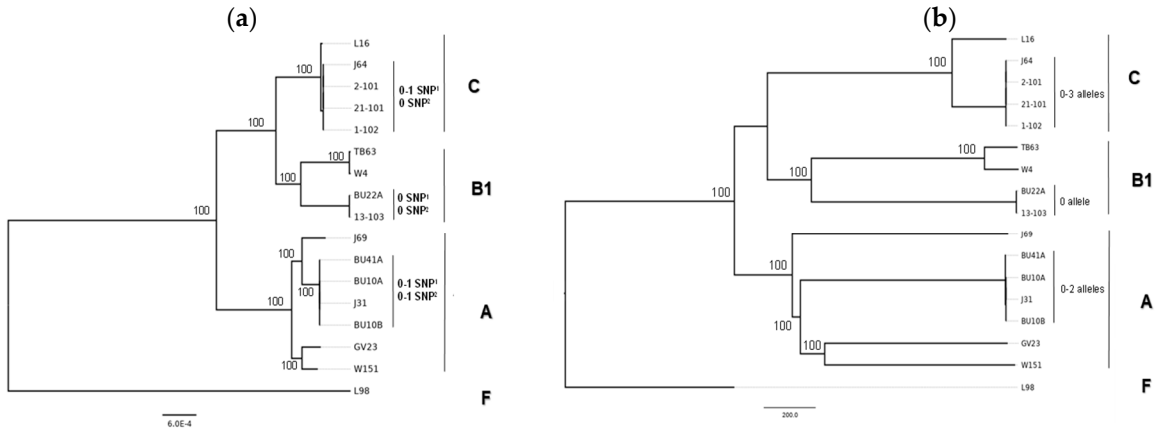

Figure 1. The core genome of extended-spectrum β-lactamases (ESBL)-encoding *E. coli* isolates analyzed by the cgSNP (**a**) and cgMLST (**b**) approaches. The dendrogram from cgSNP was inferred from recombination-free core genome SNPs by the maximum likelihood method (scale: Number of mutations per site). The dendrogram from cgMLST was inferred by the neighbor-joining method (scale: Number of differences in allele). The bootstrap values indicated for the main nodes were calculated from 100 replicates. The clusters corresponding to *E. coli* phylogroups A, B1, B2, and D are indicated. The number of differences in allele or SNP are indicated for the clonal isolates according to the cgMLST and cgSNP approaches ([1] before recombination filtration, and [2] after recombination filtration). The clonal relatedness of the strains was determined using less than five variant sites as clonality criteria.

3.3. CTX-M-1-Encoding Plasmids

Hybridization experiments revealed that ~110-kb IncI1 plasmids were mainly associated with $bla_{\text{CTX-M-1}}$ (n = 13) and, to a lesser extent, with $bla_{\text{SHV-12}}$ (n = 1). Furthermore, $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-32}}$ were also localized on ~50-kb IncN plasmid in isolates GV23 and W151, respectively. The probe specific for the $bla_{\text{CTX-M-14}}$ gene hybridized with a ~90-kb IncK plasmid in L16 isolate. The in silico analysis of WGS performed with plasmidFinder identified seven replicons among the isolates, with IncF and IncI1-ST3 replicons among the predominant ones (88.2%). The experimental and in silico plasmid analysis results are listed in the Supplementary Table S2.

The genetic context of the predominant ESBL-encoding gene $bla_{\text{CTX-M-1}}$ was further investigated. Based on short-read mapping and WGS assembly, $bla_{\text{CTX-M-1}}$ was localized in large contigs (size ranged between 38,271–88,238 bp), including the replicon origin IncI1 (60%) or at least a fragment of the region previously called shufflon that is specific to IncI1 plasmids [65,66]. These data agreed with the hybridization experiments and the detection of plasmid IncI1-ST3 in the 13 $bla_{\text{CTX-M-1}}$ isolates. The mapping of corresponding reads against IncI1 reference plasmid R64 confirmed the results and showed a highly similar structure of IncI1-ST3 plasmids except in two variable regions, including the shufflon.

While the ESBL-producing plasmid IncI1-ST3 has already been sequenced in strains isolated from human and human-influenced habitats [67,68], data that have been collected from wild fauna are scarce [23]. The isolates J31 (wild boar) and 2-101 (bird of prey) were, therefore, sequenced to closure by Nanopore sequencing. The hybrid assembly of the J31 dataset generated five circular contigs (sizes: 4871 kb, 50 kb, 108 kb, 126 kb, and 160 kb), including the complete and circular IncI1-ST3 plasmid pJ31 harboring $bla_{\text{CTX-M-1}}$ (108,661 bases, accession number: CP053788). The hybrid assembly of the 2-101 dataset generated a linear contig (size: 150 kb) and two circular contigs (sizes: 4759 kb and

108 kb), including complete and circular IncI1-ST3 plasmid p2-101 harboring $bla_{\text{CTX-M-1}}$ (108,661 bases, accession number: CP053786).

These data confirmed the presence of two variable regions. The first variable region was located downstream of the gene *repZ* and flanked by an IS*Sbo1* and *ISVsa3*, two IS*91*-like elements in plasmids p2-101 and pJ31. This region (12.725 kb) harbored a class 1 integrase gene *int1*, two resistance gene cassettes (*dfrA17* and *aadA5*), IS*26*, and truncated IS*5075* insertion sequences and *sul2* (Figure 2a,b), as previously reported IncI1-ST3 plasmids (e.g., KJ484638, MH846978, and LT985235), but was absent in plasmid of wolf's strain W4 (Table 1).

The second variable region, the shufflon, was located between the conserved genes *rci* and *pilVA* and usually consisted of four segments A to D randomly rearranged by the activity of the *rci*-encoded recombinase [69]. In contrast to IncI1 reference plasmid R64, shufflon segment D was absent in the InI1-ST3 plasmids, and the remaining segments exhibited different organizations in the circularized plasmids p2-101 and pJ31 (Figure 2b). An additional level of rearrangement was provided by the presence of insertion sequence IS*Ecp1* associated with $bla_{\text{CTX-M-1}}$. The element IS*Ecp1*-$bla_{\text{CTX-M-1}}$, which is well-known to be mobile, was flanked by direct repeats (TTTTTA) and was inserted in the same site within the shufflon segment B. However, this block IS*Ecp1*-$bla_{\text{CTX-M-1}}$-B exhibited two different orientations in plasmids p2-101 and pJ31 (Figure 2b). Since the shufflon segments are involved in the synthesis of PilV adhesin of the type IV conjugative pilus, the insertion of IS*Ecp1*-$bla_{\text{CTX-M-1}}$ associated with the shuffling of segments could provide diversity in PilV adhesin and, therefore, modify the recognition and the binding of recipient cell during IncI1-ST3 conjugation [65]. The resistance element IS*Ecp1*-$bla_{\text{CTX-M-1}}$ could thereby modulate the transfer efficiency of IncI1-ST3 $bla_{\text{CTX-M-1}}$ plasmids and may be, therefore, involved their successful spread in a broad diversity of *E. coli* lineages.

(a)

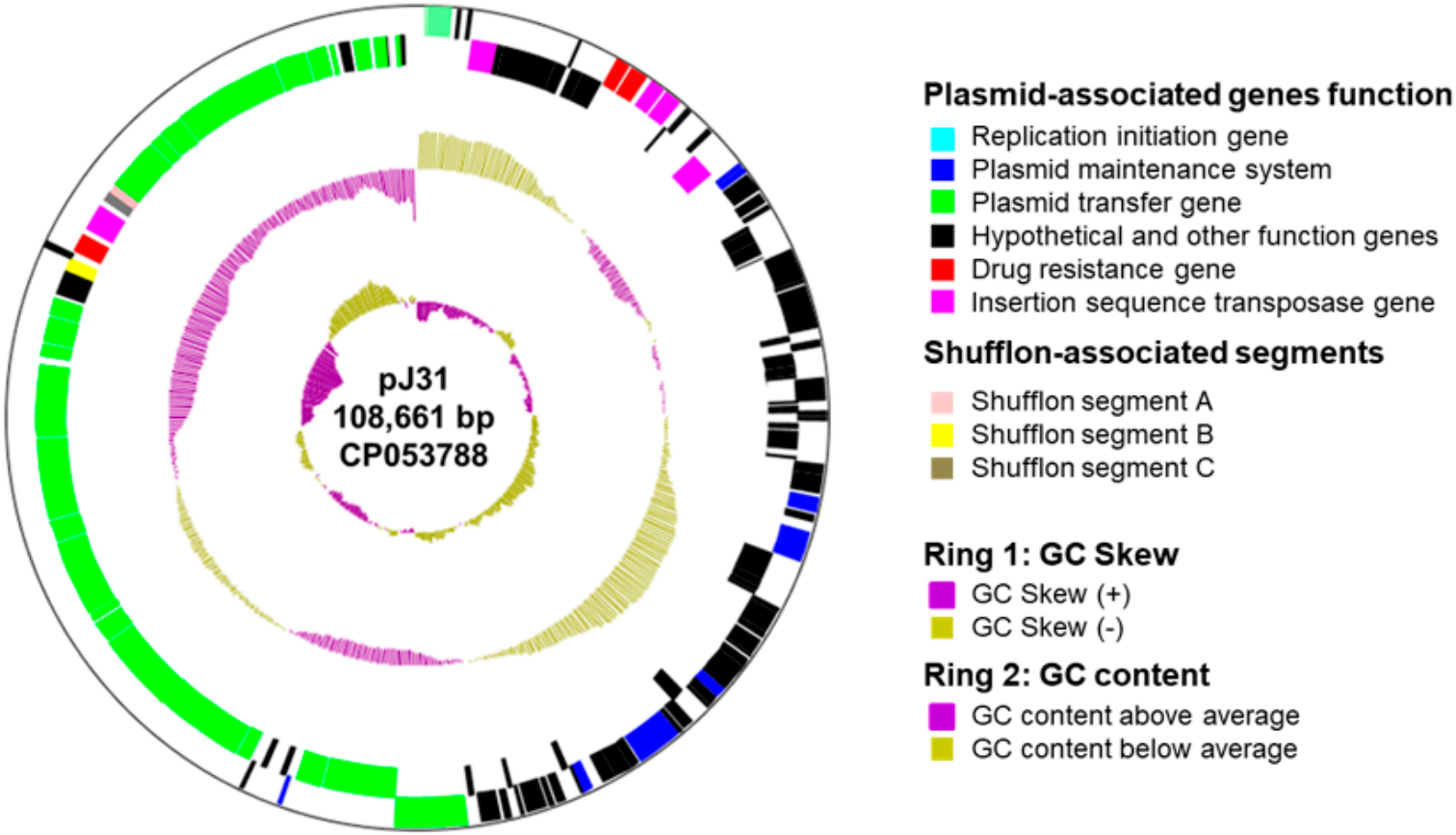

Figure 2. *Cont.*

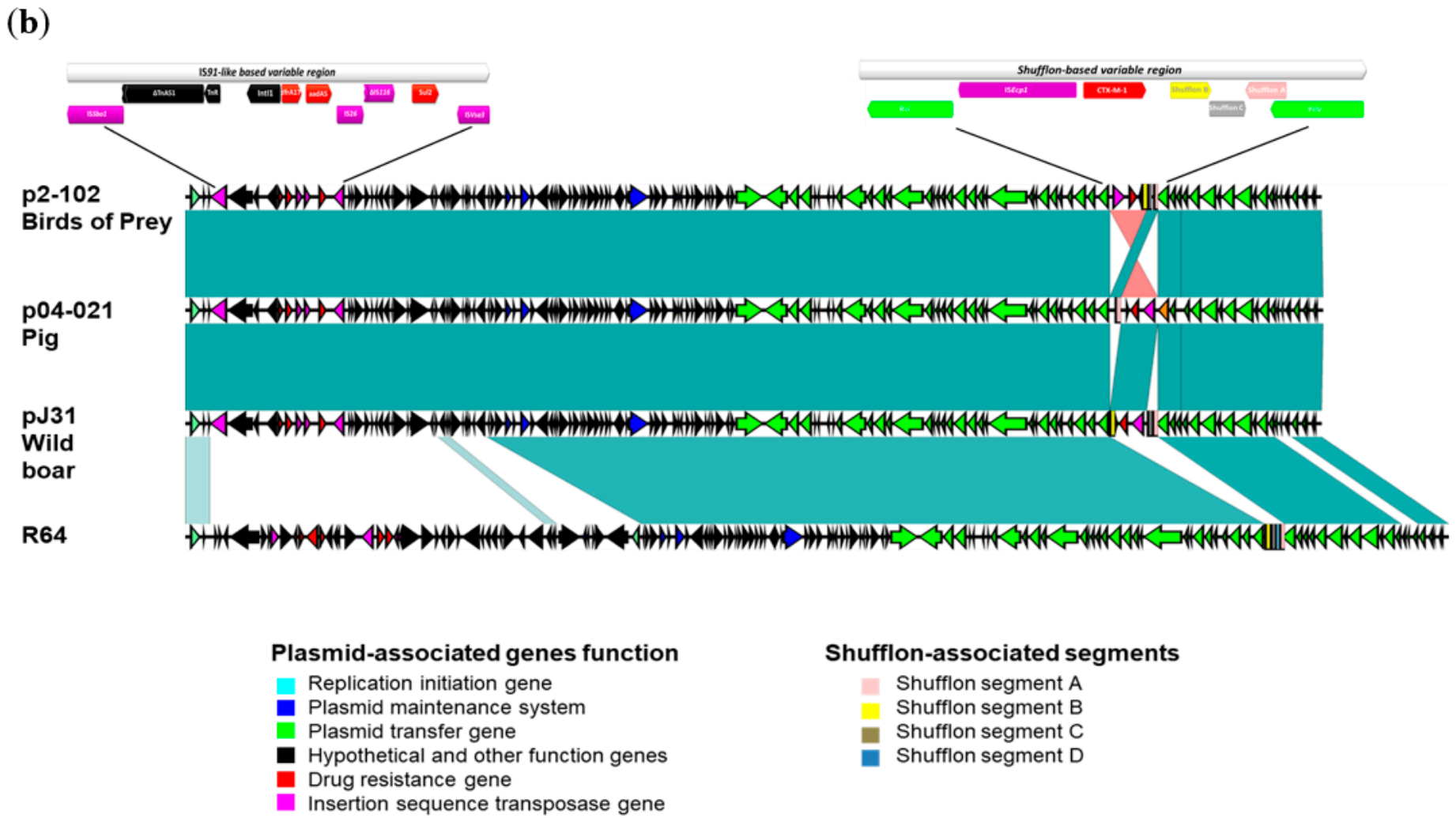

Figure 2. A CTX-M-1-encoding IncI1-ST3 plasmid recovered from *E. coli* isolated in wild animals. (**a**) Schematic representation of the complete plasmid pJ31 (CP053788) from a wild boar. The two outer rings represent the forward and reverse open reading frames, respectively. (**b**) Comparative sequence analysis of p2-101 (CP053786), and pJ31 (CP053788) with the typical IncI1-ST3 $bla_{\text{CTX-M-1}}$ plasmid p04-021 (MH847038) and the reference IncI1 plasmid R64 (AP005147). Vertical blocks between sequences indicate regions of shared similarity shaded according to BLASTn sequence identity (>95% nucleotide similarity).

4. Discussion

This work aimed to analyze at the genomic level ESBL-producing *E. coli* collected from wild animals with different eating habits and living in natural parks and reserves or other rural conservation areas in distinct regions of Portugal [24–30]. All ESBL-producing *E. coli* strains were resistant to penicillin, cephalosporin 1st generation (cephalexin), and cefuroxime and 94% were resistant to cefotaxime. The isolates also exhibited associated resistances to quinolones, fluoroquinolones, sulfonamides, and trimethoprim, showing that multidrug-resistant bacteria have emerged in wild birds, as previously reported [6], and in a broad range of other wildlife species.

The $bla_{CTX\text{-}M\text{-}1}$ gene was the predominant ESBL-encoding gene (82.3%), followed by $bla_{CTX\text{-}M\text{-}14}$ (5.9%), and $bla_{CTX\text{-}M\text{-}32}$ (5.9%). In addition to $bla_{CTX\text{-}M}$ genes, we detected the $bla_{SHV\text{-}12}$ gene, previously reported in isolates from humans, livestock, companion animals, and wild birds [6,70–73]. We also detected genes $bla_{OXA\text{-}1}$ and $bla_{TEM\text{-}1}$, which have been reported in waterbirds on the Baltic sea coast of Poland [20] and from wild boars and Barbary macaques [74], respectively. Except for the absence of $bla_{CTX\text{-}M\text{-}15}$, these results were also consistent with those previously reported from wild birds [6], in which the $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}15}$ genes were the most widespread ESBL genes, followed by $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}32}$. The $bla_{CTX\text{-}M\text{-}15}$ gene is the predominant ESBL in humans [6]. The absence of $bla_{CTX\text{-}M\text{-}15}$ in our study may reflect a weak anthropization of the wild animals included in this study. Previous reports identified the impact of the human-influenced habitat in the diffusion of the antimicrobial resistance determinant in the wildlife, especially in urban areas where wild birds can live and feed [6,10,75]. Our isolates were collected from natural parks and reserves or other rural conservation areas [24–30], which are less influenced by human activity than urban areas.

E. coli ST131 and ST405 are the major STs associated with the rapid worldwide rise in $bla_{CTX\text{-}M}$ genes, including $bla_{CTX\text{-}M\text{-}15}$ [76] and other CTX-M genes, such as $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}1}$ [77]. None of our $bla_{CTX\text{-}M}$-positive isolates belonged to ST131 or ST405, showing that the dissemination of $bla_{CTX\text{-}M}$ genes in our isolates is not related to well-known clonal strains, in contrast to previous reports about wildlife [6,10]. Furthermore, *E. coli* isolates from this study essentially belonged to novel STs (ST1998, ST1800, ST10430), STs rarely reported in human and human-influenced habitats (ST361, ST155, ST117, ST710) and/or STs reported in wild animals (ST117 and ST155) [10]. A striking finding from this study is the clonal dissemination of ESBL-producing *E. coli* in wild fauna. The isolates belonged to ST48, ST1800, and ST1198 formed clusters that diverged by less than 5 SNPs, suggesting direct and/or indirect transmissions between wild animals. This contrasts with data obtained from human isolates, in which ESBLs are often observed in distantly related *E. coli* isolates [48–64]. Overall, the genetic background of isolates suggests that ESBL spread in the wild animals of this study is not directly linked to contacts with humans or human-influenced habitats and is marked by direct or indirect transmission within wild fauna despite a probable low antibiotic selection pressure.

Consequently, anthropization is probably not the main spreading factor of $bla_{CTX\text{-}M\text{-}1}$, which was observed in five different species (bird of prey, bat, wolf, buzzard, wild boar) out of the seven wild animal species included in this study. 92.8% of the gene $bla_{CTX\text{-}M\text{-}1}$ was encoded by the IncI1-ST3 plasmid. IncI1 is one of the most common plasmid families in Enterobacteriaceae, in both animals and humans [6,10,78], and one of the major plasmids contributing to the dissemination of the $bla_{CTX\text{-}M\text{-}1}$ gene in animals, the environment, and humans [79]. On the Baltic coast of Poland, ESBL-producing bacteria isolated from water-birds predominantly had the $bla_{CTX\text{-}M\text{-}1}$ gene located on plasmid IncI1 [20]. Similarly, in the Netherlands, the $bla_{CTX\text{-}M\text{-}1}$ gene was carried by IncI1 plasmids among *E. coli* isolates from several wild birds [21]. This association was also more recently detected in ESBL-producing *E. coli* from various wild animals isolated in Guadeloupe [23]. The identification of the epidemic plasmids IncI1-ST3 encoding $bla_{CTX\text{-}M\text{-}1}$ in samples of different sources, including humans, animals, and the environment, further underlined the role of horizontal transfer in the dissemination of resistance genes [80]. Since the IncI1 shufflon segments are involved

in the synthesis of PilV adhesin of the type IV conjugative pilus, the insertion of IS*Ecp1*-$bla_{\text{CTX-M-1}}$ associated with the shuffling of segments could modify the recognition and the binding of recipient cells during IncI1-ST3 conjugation [65], modulate the transfer efficiency of IncI1-ST3 $bla_{\text{CTX-M-1}}$ plasmids, and may be, therefore, involved in their successful spread in a broad diversity of *E. coli* lineages even in an antibiotic-free ecosystem.

5. Conclusions

It emerges from this study that the clonal dissemination of ESBL-producing *E. coli* strains in wild fauna crosses the species barriers. It is worth noting the moderate diversity of ESBLs detected in our *E. coli* isolates. The $bla_{\text{CTX-M-1}}$ gene, associated with tetracyclines, fluoroquinolones, aminoglycosides, sulphonamides, and trimethoprim-encoding resistance genes, was the predominant resistance mechanism observed in our isolates, which belonged to unusual *E. coli* lineages collected from wild animals. Our results also underline the key role of the epidemic plasmid IncI1-ST3 in the diffusion of CTX-M-1 in areas with low or no antibiotic exposure and weak anthropization. The present study is a step forward to understanding the dissemination of antibiotic resistance in the wild ecosystem. More surveillance programs focusing on the spread of antibiotic resistance mechanisms at the interface of ecosystems, including the wildlife, are needed to better assess the emergence of antibiotic resistance in the context of the "one health" approach.

Supplementary Materials: The following are available online at https://www.mdpi.com/2036-7481/12/1/9/s1, Table S1: Antimicrobial susceptibility profiles of *Escherichia coli* isolates collected from different wild animals., Table S2: Plasmid analysis of *Escherichia coli isolates* based on experimental and whole genome sequencing results.

Author Contributions: Conceptualization, R.B. (Racha Beyrouthy), G.I., and R.B. (Richard Bonnet); methodology, R.B. (Racha Beyrouthy), R.B. (Richard Bonnet); software, R.B. (Racha Beyrouthy).; validation, R.B. (Richard Bonnet), G.I., F.R., P.P., R.B. (Racha Beyrouthy); formal analysis, R.B. (Richard Bonnet), G.I., F.R., P.P., R.B. (Racha Beyrouthy); investigation, C.S., R.B. (Racha Beyrouthy); resources, R.B. (Richard Bonnet), G.I., data curation, R.B. (Racha Beyrouthy); writing—original draft preparation R.B. (Racha Beyrouthy), C.S., R.B. (Richard Bonnet), G.I., writing—review and editing, R.B. (Richard Bonnet), G.I., F.R., R.B. (Racha Beyrouthy); supervision, R.B. (Racha Beyrouthy), R.B. (Richard Bonnet), G.I.; project administration, R.B. (Racha Beyrouthy)., R.B. (Richard Bonnet), G.I.; funding acquisition, R.B. (Racha Beyrouthy), R.B. (Richard Bonnet), G.I., P.P. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Ministère de la Recherche et de la Technologie, Inserm (UMR 1071), INRAe (USC-2018), Santé Publique France; the Centre Hospitalier Regional Universitaire de Clermont-Ferrand; the French government's IDEX-ISITE initiative 16-IDEX-0001 (CAP 20-25); and the JPIAMR program TransComp-ESC-R of the European Commission. We also thank the 'Espectrometria de massa e sequenciação aplicadas à microbiologia clínica moderna: pesquisa e identificação de biomarcadores em espécies bacterianas multiresistentes' (Programa de Acções Universitárias Integradas Luso-Francesas, Ação n°: TC-14/2017) and the Associate Laboratory for Green Chemistry—LAQV, which is financed by national funds from FCT/MCTES (UID/QUI/50006/2019).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The Illumina raw sequence reads have been deposited in the European Nucleotide Archive under the project accession number PRJEB36175. The pJ31 and p2-101 complete plasmid sequence were deposited in GenBank under the accession number CP053788 and CP053786, respectively.

Acknowledgments: We thank Alexis Pontvianne and Lucie Pourpuech for their technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

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