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Official URL: https://doi.org/10.1016/j.jgar.2020.11.002

To cite this version:

Hage, Rima el and Losasso, Carmen and Longo, Alessandra and Petrin, Sara and Ricci, Antonia and Mathieu, Florence and Abi Khattar, Ziad and El Rayess, Youssef *Whole-genome characterisation of TEM-1 and CMY-2 β-lactamase-producing Salmonella Kentucky ST198 in Lebanese broiler chain.* (2020) Journal of Global Antimicrobial Resistance, 23. 408-416. ISSN 2213-7165

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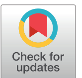
Journal of Global Antimicrobial Resistance 23 (2020) 408–416

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Whole-genome characterisation of TEM-1 and CMY-2 β-lactamase-producing *Salmonella* Kentucky ST198 in Lebanese broiler chain



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ARTICLE INFO

Article history:
Received 2 August 2019
Received in revised form 25 September 2020
Accepted 2 November 2020
Available online 16 November 2020

Keywords:
Lebanon
Poultry
Ciprofloxacin-resistant *Salmonella* Kentucky
ST198
bla$_{CMY-2}$
IS*10* transposition
IS*Ecp1*
Plasmids

ABSTRACT

Objectives: *Salmonella enterica* subsp. *enterica* serovar Kentucky has been associated with the worldwide ciprofloxacin-resistant (CIP^R) *Salmonella* Kentucky sequence type 198 (ST198) epidemic clone, mostly recovered from poultry farms and products. The aim of this study was to examine whether this expanding clone exists in the Lebanese broiler chain.
Methods: Eight CIP^R and extended-spectrum cephalosporin-resistant *Salmonella* Kentucky isolates previously recovered from Lebanese broilers were genetically characterised by whole-genome sequencing.
Results: Seven of the eight isolates belonged to ST198 and were phylogenetically closely related. They all harboured mutations in the chromosomal quinolone resistance genes*gyrA* and *parC* with double and single substitutions, respectively. The *bla*$_{TEM-1B}$ and *bla*$_{CMY-2}$ genes were both detected in six isolates. Insertion sequence IS*Ecp1* was located upstream of *bla*$_{CMY-2}$, harboured by IncI1 plasmids in four strains. An IS*10* transposition coupled to homologous recombination at transposition sites mediated CMY-2 plasmid integration into the chromosome of one strain. Resistance genes to aminoglycosides [*aadA7* and *aac(3)-Id*], tetracyclines [*tet(A)*] and sulfonamides (*sul1*) were detected in five strains, among which four were positive for the presence of *Salmonella* genomic island 1 (SGI1) variant SGI1-K. All studied isolates harboured a variety of *Salmonella* pathogenicity islands (SPIs) as well as common regulatory and virulence genes.
Conclusion: Here we report for the first time in Lebanon the detection and dissemination of the emerging highly drug-resistant*Salmonella* Kentucky ST198. Our findings shed new light on this clone as a potential public-health threat.
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1. Introduction

Uncommon in human salmonellosis, *Salmonella enterica* subsp. *enterica* serovar Kentucky is however widespread in poultry meat [1]. First recorded in Egypt in 2002, a new clone of ciprofloxacin-resistant (CIP^R) *Salmonella* Kentucky sequence type 198 (ST198) has spread worldwide [2,3], causing human infections linked to travellers returning from the Middle East, Southeast Asia and Africa [4]. Since the 1990s, this serovar acquired *Salmonella* genomic island 1 (SGI1) multidrug resistance determinants mainly to amoxicillin, gentamicin and sulfonamides as well as double mutations in topoisomerase-encoding *gyrA* and *parC* chromosomal genes conferring resistance to ciprofloxacin [5].

Mediterranean *Salmonella* Kentucky isolates have become producers of various carbapenemases (*bla*$_{VIM-2}$, *bla*$_{OXA-48}$, *bla*$_{OXA-204}$), extended-spectrum β-lactamases (ESBLs) (*bla*$_{CTX-M-1}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-25}$) and a mix of carbapenemases and ESBLs (*bla*$_{OXA-48}$ and *bla*$_{VEB-8}$), posing an imminent threat to public health [6].

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http://dx.doi.org/10.1016/j.jgar.2020.11.002
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Another class of β-lactamases, the AmpC β-lactamases, which is further divided into different families (CIT, CMY, FOX, etc.) and types or variants (CMY-2, DHA-1, FOX-1, etc.), has emerged worldwide as clinically relevant in *Enterobacteriaceae*. They confer resistance to aminopenicillins, cephalosporins (ceftriaxone, cefotaxime, ceftazidime), cephamycins (cefoxitin, cefotetan) and monobactams (aztreonam) [7]. According to the Ambler structural classification, AmpC β-lactamases belong to the molecular class C β-lactamases originally described as inducible chromosomal enzymes [8]. bla_{CMY-2} is the most prevalent AmpC β-lactamase gene, which has been reported in *S. enterica* and *Escherichia coli* worldwide owing to the spread of IncA/C and IncI1 plasmids from different sources including humans, animals and the environment [7]. The plasmid-borne bla_{CMY-2} gene most likely originated from the *Citrobacter freundii* chromosome by insertion sequence IS*Ecp1*-mediated transposition that also provides the promotor for its high-level expression [9]. AmpC β-lactamase production can be assessed using the cefoxitin disk screening test [8] as well as the cefoxitin/cloxacillin double-disk and AmpC induction tests that allow detection of AmpC β-lactamase production in *Enterobacteriaceae* naturally lacking chromosomal AmpC β-lactamases, such as *Salmonella* spp. Multiplex PCR assays have also been described for specific detection of six families of plasmid-acquired AmpC β-lactamase genes [10].

Some studies have shown that multidrug-resistant (MDR) *Salmonella* and ESBL-producing isolates became more pathogenic by co-carrying several virulence genes on plasmids, prophages and *Salmonella* pathogenicity islands (SPIs) [11]. Some virulence genes were identified to confer pathogenicity more than others. *Salmonella* Kentucky is thought to be unharmful to humans owing to the lack of many virulence genes such as *grvA*, *sseI*, *sopE* and *sodC1* [12], or *sopD2*, *pipB2*, *sspH2* and *srfH* [13]. The relevant concern with *Salmonella* Kentucky is its accelerated dissemination in chickens attributed to a better acid response than other serovars [14]. Others attributed the differential regulation of core *Salmonella* genes via the stationary-phase sigma factor RpoS to the metabolic adaptation in the chicken caecum [12].

In Lebanon, *Salmonella* Kentucky is among the most predominant serovars in the broiler production chain (broiler breeder farms, broiler farms, slaughterhouses and retail) and layer flocks. The global prevalence of this serovar was 21.4% among the total identified ones (unpublished results), although it was not related to human ingestion [15]. It has been shown that all isolated strains were CIP^R, 65.4% were MDR and 6.8% were also extended-spectrum cephalosporin-resistant (ESC^R). The aim of this study was therefore to determine whether these CIP^R and ESC^R *Salmonella* Kentucky strains belonged to the expanding ST198-SGI1 clone. In line with this, a deep genomic characterisation was performed.

2. Materials and methods

2.1. Collection of Salmonella Kentucky strains

Eight *Salmonella* Kentucky isolates were chosen from a collection of strains isolated during the same year in our laboratory as a part of a previous study on *Salmonella* prevalence in Lebanese poultry production. Of a total 133 CIP^R *Salmonella* Kentucky isolates, only 8 were selected for whole-genome sequencing (WGS) according to their ESC^R phenotypic profile. The collected strains originated as follows: seven strains [17-70328(K12), 17-70460 (K24), 17-70462(K31), 17-70464(K32), 17-70468(K38), 17-70469 (K43) and 17-70472(K48)] from retail chicken cuts and one strain [17-70474(A66C)] from commercial slaughterhouse broiler caecum (unpublished results).

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed according to the Clinical and Laboratory Standards Institute (CLSI) [16]. The Kirby–Bauer disk diffusion method was first performed for a panel of 26 antimicrobials (Oxoid Ltd., Basingstoke, UK) of veterinary and human health importance, including ampicillin (10 μg), amoxicillin/clavulanic acid (30 μg), piperacillin/tazobactam (110 μg), cefalotin (30 μg), cefuroxime (30 μg), cefoxitin (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), ceftazidime (30 μg), ceftiofur (30 μg), cefepime (30 μg), imipenem (10 μg), aztreonam (30 μg), gentamicin (10 μg), tobramycin (10 μg), streptomycin (10 μg), amikacin (30 μg), netilmicin (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), enrofloxacin (5 μg), trimethoprim (5 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), tetracycline (30 μg) and chloramphenicol (30 μg). Minimum inhibitory concentrations (MICs) for resistant strains were determined by broth microdilution for the following antimicrobials (breakpoint values in parentheses): cefalotin (≥32 μg/mL); cefuroxime (≥32 μg/mL); cefoxitin (≥32 μg/mL); cefotaxime (≥4 μg/mL); ceftriaxone (≥4 μg/mL); ceftazidime (≥16 μg/mL); ceftiofur (≥8 μg/mL); gentamicin (≥16 μg/mL); nalidixic acid (≥32 μg/mL); ciprofloxacin (≥1 μg/mL); norfloxacin (≥16 μg/mL); and enrofloxacin (≥2 μg/mL). *Escherichia coli* ATCC 25922 was used as a quality control strain. Antimicrobial resistance to at least three classes of antibiotics was considered MDR.

2.3. Whole-genome sequencing analysis

Genomic DNA was extracted using a QIAamp® DNA Mini Kit (QIAGEN, Valencia, CA, USA) and was quantified with a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Libraries for sequencing were prepared using a Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA). High-throughput sequencing was performed on an Illumina MiSeq system (Illumina Inc.) with 2 × 250-bp paired-end reads. Raw sequence data were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under accession no. **PRJEB27597.** Raw reads were assembled in contigs using Assembler 1.2 (https://cge.cbs.dtu.dk/services/Assembler/) [17] or SPAdes 3.9 (https://cge.cbs.dtu.dk/services/SPAdes/) [18]. All isolates were then subjected to in silico serotyping using SeqSero 1.2 (www.denglab.info/SeqSero) [19] starting from assembled data to confirm in vitro serotyping. When concordance was not verified, analysis was repeated starting from raw reads. To verify the presence of acquired antimicrobial resistance genes, assembled genomes were analysed using ResFinder2.1 (https://cge.cbs.dtu.dk/services/ResFinder/) (selected threshold for %ID = 90%; selected minimum length = 60%), while ResFinder3.0 (https://cge.cbs.dtu.dk/services/ResFinder-3.0/) [20] was used to detect known chromosomal point mutations that can confer antimicrobial resistance. Multilocus sequence typing (MLST), plasmid identification and plasmid MLST (pMLST) were performed using MLST 1.8 (https://cge.cbs.dtu.dk/services/MLST/) [17], PlasmidFinder 1.3 (https://cge.cbs.dtu.dk/services/PlasmidFinder/) (selected threshold for %ID = 85%) and pMLST 1.4 (https://cge.cbs.dtu.dk/services/pMLST/) [21], respectively. MyDbFinder (https://cge.cbs.dtu.dk/services/MyDbFinder/) was used to investigate the presence of SGI1-K (GenBank accession no. **AY463797.8)** [22], which is frequently integrated into the *Salmonella* Kentucky genome. The reference used to find IS*Ecp1* was the deposited sequence of *Salmonella* Typhimurium strain 110516 [**KX377449.1:780**–1276].

2.4. Phylogenomics

Assembled genomes and a reference genome (*Salmonella* Kentucky CVM29188 [23]) were used to build a single nucleotide polymorphism (SNP)-based phylogenetic tree using CSI Phylogeny 1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny/) [24], with default parameters for SNP filtering and SNP pruning.

3. Results

3.1. MLST, plasmid detection and pMLST

All isolates submitted to WGS belonged to ST198, except for 17-70472(K48) for which it was not possible to assign a MLST sequence type. This was most likely due to a bad assembly compared with other isolates, since 17-70472(K48) showed a high number of contigs (3495 contigs) and a low N_{50} value (Table 1).

Using WGS data, all plasmids recovered from all isolates belonged to incompatibility group I1 (IncI1) and ColRNAI replicon types. Using pMLST based on WGS data, two IncI1-type plasmids were ST12 and two other plasmids were identified as belonging to ST2 and ST65. The four others were untypeable, but two of them closely matched ST12 and ST23 (Table 1).

3.2. Phenotypic and genotypic antimicrobial resistance

Antimicrobial susceptibility testing showed high MICs to ciprofloxacin (12.5 μg/mL to >32 μg/mL) for all strains, among which six were classified as MDR. Phenotypic antimicrobial resistance patterns are reported in Table 2.

WGS data revealed that all of the isolates had all genes correlating with the antimicrobial resistance phenotypes determined by disk diffusion and broth microdilution methods. Indeed, ESC^R strains were found to carry resistance genes to third-generation β-lactams, bla_{TEM-1B} (class A) and/or cephamycinase bla_{CMY-2} (class C), with six strains carrying both of them (Table 2). For strain 17-70468(K38), although initial disk diffusion test revealed a resistant phenotype to cefoxitin, the bla_{CMY-2} resistance gene was not detected. Broth microdilution demonstrated that this strain had reduced susceptibility to cefoxitin (MIC = 12.5 μg/mL) compared with the other cefoxitin-resistant strains (MIC ≥ 200 μg/mL).

Moreover, mutations in the quinolone resistance-determining regions (QRDRs) of the target *gyrA* and *parC* gene loci were detected in all strains. The strains harboured double amino acid substitutions in GyrA [serine to phenylalanine at codon 83 (S83F) and aspartic acid to asparagine at codon 87 (D87N)] and a single substitution in ParC [serine to isoleucine at codon 80 (S80I)]. These mutations were therefore responsible for high-level ciprofloxacin resistance (Table 2). Resistance genes to aminoglycosides [*aadA7* and *aac(3)-Id*], tetracyclines [*tet(A)*] and sulfonamides (*sul1*) were detected in five strains, among which four harboured the SGI1-K variant. Strain 17-70462(K31) was the only strain to have the *floR* gene conferring cross-resistance to chloramphenicol and florfenicol (Table 2).

3.3. CMY-2 plasmid analysis

Nucleotide sequence analysis revealed the presence of IS*Ecp1* in five strains at 117 bp upstream of the bla_{CMY-2} gene. The latter was co-localised on the same contig with both IncI1-type plasmid replicon and IS*Ecp1*. However, strain 17-70460(K24) lacked IS*Ecp1* and harboured bla_{CMY-2} and IncI1 on two different contigs. As shown in Table 3, the four IS*Ecp1*–bla_{CMY-2} containing contigs from strains 17-70462(K31), 17-70464(K32), 17-70469(K43) and 17-70474(A66C) displayed relatively short lengths. They also shared high identities (98.59–100%) with the previously described bla_{CMY-2} IncI1 plasmid pCVM29188_101 (GenBank accession no. **CP001121.1**) of *Salmonella* Kentucky from poultry [23] and its close variants (p12-4374_96, **CP012929.1** and pSA01AB09084001_92, **CP016533.1**) in *Salmonella* Heidelberg recently isolated from different sources in Canada [25]. These observations indicated that the IS*Ecp1*–bla_{CMY-2} transposition unit resided on IncI1 plasmids belonging to two close variants 1 and 2 (Fig. 1A), seemingly horizontally spread among *Salmonella* serovars worldwide. IS*Ecp1*–bla_{CMY-2} was followed by full-length *blc* (encoding an outer membrane lipoprotein) and *sugE* (encoding a quaternary ammonium compound resistance protein) genes along with a 551-bp fragment (Fig. 1A). This fragment showed null identity to *Salmonella* Kentucky sequences including the pCVM29188_101 plasmid, but was 100% identical to a region containing a 5′-truncated LuxR family transcriptional regulator (*ecnR*) gene belonging to IncA/C plasmids from other serovars.

For the remaining strain 17-70328(K12), the bla_{CMY-2} gene resided on the largest contig (1758.535 kb) corresponding to the

Table 1
Results of genomic assembly, SeqSero, multilocus sequence typing (MLST), PlasmidFinder and plasmid MLST (pMLST) and accession number of eight Lebanese *Salmonella* Kentucky isolates.

ID IZSVe (Ref. Lebanon)	Source	Genome size (bp)	No. of contigs	N_{50}[a]	Serovar	MLST	Plasmids	pMLST[b]	Accession no.
17-70328(K12)	Chicken cuts/retail	4 922 807	93	534 536	Kentucky	ST198	IncI1, ColRNAI	IncI1 [ST65]	**ERR2681948**
17-70460(K24)	Chicken cuts/retail	5 002 563	251	238 030	Kentucky	ST198	IncI1, ColRNAI	IncI1 [ST12]	**ERR2681949**
17-70462(K31)	Chicken cuts/retail	4 967 065	105	534 536	Kentucky	ST198	IncI1, ColRNAI	IncI1 [ST12]	**ERR2681950**
17-70464(K32)	Chicken cuts/retail	4 916 713	80	450 673	Kentucky	ST198	IncI1, ColRNAI	IncI1 [unknown, closest match ST23]	**ERR2681951**
17-70468(K38)	Chicken cuts/retail	4 980 697	458	26 113	Kentucky	ST198	IncI1, ColRNAI	IncI1 [unknown, closest match ST12]	**ERR2681952**
17-70469(K43)	Chicken cuts/retail	4 896 047	93	293 715	Kentucky	ST198	IncI1, ColRNAI	IncI1 [unknown]	**ERR2681953**
17-70472(K48)	Chicken cuts/retail	4 417 617	3495	1670	Kentucky	Unknown	IncI1	IncI1 [unknown]	**ERR2681954**
17-70474(A66C)	Chicken caecum/ slaughterhouse	4 942 747	149	120 678	Kentucky	ST198	IncI1, ColRNAI	IncI1 [ST2]	**ERR2681955**

[a] The N_{50} statistic defines assembly quality. Given a set of contigs ordered from the shortest to the longer, N_{50} is defined as the shortest sequence length among contigs that covers at least one-half of the genome size.
[b] pMLST is defined only for schemed plasmids (i.e. IncI1): plasmid replicon and identified alleles in square brackets are given.

Table 2
Phenotypic and genotypic antimicrobial resistance (AMR) results of the eight Lebanese ciprofloxacin-resistant *Salmonella* Kentucky isolates using ResFinder 2.1, ResFinder 3.0 and MyDbFinder.

ID IZSVe (Ref. Lebanon)	AMR phenotype	AMR genotype	QRDR point mutations		SGI1-K
			gyrA	*parC*	
17-70328(K12)	AMP-AMC-CEF-CXM-FOX-CTX-CRO-CAZ-TIO-NAL-CIP-NOR-ENR	bla_{CMY-2}, bla_{TEM-1B}	S83F, D87N	S80I	Absence
17-70460(K24)	AMP-AMC-CEF-CXM-FOX-CTX-CRO-CAZ-TIO-GEN-STR-NAL-CIP-NOR-ATM-TET-ENR	*aadA7*, *aac(3)-Id*, bla_{CMY-2}, bla_{TEM-1B}, *sul1*, *tet(A)*	S83F, D87N	S80I	Absence
17-70462(K31)	AMP-AMC-CEF-CXM-FOX-CTX-CRO-CAZ-TIO-NAL-CIP-NOR-CHL-ENR	bla_{CMY-2}, bla_{TEM-1B}, *floR*	S83F, D87N	S80I	Absence
17-70464(K32)	AMP-AMC-CXM-FOX-CRO-CAZ-TIO-GEN-STR-NAL-CIP-NOR-ATM-TET-ENR	*aadA7*, *aac(3)-Id*, bla_{CMY-2}, bla_{TEM-1B}, *sul1*, *tet(A)*	S83F, D87N	S80I	Presence
17-70468(K38)[a]	AMP-AMC-CEF-CXM-FOX-CTX-CRO-CAZ-TIO-GEN-STR-NAL-CIP-NOR-TET-ENR	*aadA7*, *aac(3)-Id*, bla_{TEM-1B}, *sul1*, *tet(A)*	S83F, D87N	S80I	Presence
17-70469(K43)	AMP-AMC-CEF-CXM-FOX-CTX-CRO-CAZ-TIO-GEN-STR-NAL-CIP-NOR-ATM-TET-ENR	*aadA7*, *aac(3)-Id*, bla_{CMY-2}, bla_{TEM-1B}, *sul1*, *tet(A)*	S83F, D87N	S80I	Presence
17-70472(K48)	AMP-AMC-CEF-CXM-FOX-CTX-CRO-CAZ-TIO-NAL-CIP-NOR-ENR	bla_{CMY-2}	S83F, D87N	S80I	Absence
17-70474(A66C)	AMP-AMC-TZP-CEF-CXM-FOX-CTX- CRO-CAZ-TIO-GEN-STR-NAL-CIP-NOR-ATM-TET-ENR	*aadA7*, *aac(3)-Id*, bla_{CMY-2}, bla_{TEM-1B}, *sul1*, *tet(A)*	S83F, D87N	S80I	Presence

QRDR, quinolone resistance-determining region; SGI1-K, *Salmonella* genomic island 1 variant; AMC, amoxicillin/clavulanic acid; AMP, ampicillin; ATM, aztreonam; CAZ, ceftazidime; CEF, cefalotin; CHL, chloramphenicol; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; ENR, enrofloxacin; FOX, cefoxitin; GEN, gentamicin; NAL, nalidixic acid; NOR, norfloxacin; STR, streptomycin; TET, tetracycline; TIO, ceftiofur; TZP, piperacillin/tazobactam.
[a] Categorised as resistant according to disk diffusion method and of reduced susceptibility according to broth microdilution.

chromosome, suggesting that this plasmid might have integrated into the chromosome. An alignment of this contig against sequences from various online databases identified a 93.8-kb large fragment sharing the highest significant identity (91% coverage and 98.59% identity) with pCVM29188_101 plasmid (Fig. 1B). The plasmid insertion site was flanked by two IS*10* with the same orientation [left (IS*10*L) and right (IS*10*R)] accompanied by typical target site 9-bp duplication (DR1). The resulting genetic structure IS*10*L-93.8 kb fragment-IS*10*R mimicked a composite transposon that has been inserted into the chromosomal hemin uptake protein-encoding *hemP* gene. Similarly, two other 9-bp DR2 were detected next to inverted repeats IR_R and IR_L of IS*10*L and IS*10*R, respectively. Genetic mapping analysis revealed that such a genomic organisation could have resulted from homologous recombination at the IS*10* site itself between the IncI1 plasmid and the chromosome.

Sequence analysis of strain 17-70328(K12) also showed that the IS*Ecp1*–bla_{CMY-2} transposition unit was inserted into the SeKA_C0024 gene at ~21.4 kb upstream of IS*10*R, followed by the *blc* gene and a truncated form of *sugE* (Fig. 1B). All typical 14-bp IRs and 5-bp AT-rich DRs (DR3) of IS*Ecp1* were identified, among which the IR_L detected at 20-bp upstream of the 3′ end of the *yagA* gene and the alternative IR_{alt} barely recognised directly upstream of the SeKA_C0024 3′-part. This explained why the terminal 34-bp of *yegA* belonged to the IS*Ecp1* transposition unit that was extended beyond the IR_R causing a larger region to be captured and inserted into the SeKA_C0024 locus. The 5′-part of *sugE* was left in tandem with the remaining part of *yagA*. Many genes indicative of motility, including transposases and recombinases, were observed along with deletions and insertions of sequences identical to the IncFIB/IncFIIA pCVM29188_146 (**CP001122.1**) and IncI1 pCS0010A (**CP0020901**) plasmids previously found in *Salmonella* Kentucky. The presence of such diverse plasmid sequences suggests that plasmids of different Inc groups could have coexisted in ancient *Salmonella* Kentucky hosts where their genes were subject to intermolecular and intramolecular rearrangements. In line with this, we observed the presence of a 'TGGGT' DR (typical of IncI1 pCVM29188_101) on the *sugE* 5′-end at the original deletion site of IS*Ecp1*. This could indicate that recombinations in this region have occurred before horizontal acquisition of the bla_{CMY-2} plasmid and its integration into the 17-70328(K12) chromosome. Finally, several genes were not integrated into the chromosome, including *yacABC* and *ccdAB* encoding toxin–antitoxin plasmid stability systems. Therefore, the loss of these systems along with continuous exposure to increased cephalosporin concentrations could have promoted the CMY-2 plasmid integration into the chromosome.

3.4. Virulence gene analysis

Screening of SPIs, virulence genes, RpoS-regulated core genes as well as other genes related to pathogenicity and survival of *Salmonella* Kentucky was performed (Fig. 2). All sequenced strains were shown to have conserved genes implicated in fimbriae-mediated adhesion, curli formation, iron acquisition, galactose transport, propionate catabolism, nitrate respiration, type III secretion system (T3SS) and regulation of stress factors. However, SPI-1 and SPI-2 were detected in four and three strains, respectively, whilst only two strains harboured them both. The SPI-1 *sopE2* gene was absent from two strains, while all strains lacked the SPI-2 *sspH2* gene. The *ssek2* gene was identified in six strains but was missing in all *sopE2*-lacking strains. Finally, it is noteworthy to state that strain 17-70472(K48) lacked many virulence genes, namely *lpfD*, *stjB*, *siiE*, *sopE2*, *ssek2*, *pipA*, *pipD*, *mgl*, *prp* and *nar*, which could impact its fitness in chicken colonisation and human infections.

3.5. Phylogenetic SNP analysis

SNPs identified from whole-genome comparisons were used to determine the relationships between the eight CIP^R *Salmonella* Kentucky strains with *Salmonella* Kentucky CVM29188 strain selected as a reference genome. As shown by the phylogenetic tree in Fig. 3, a close relatedness between all strains was observed, with the exception of 17-70472(K48). Once again, this was most probably due to the bad assembly achieved for this particular strain. SNP difference among strains varied between 12 and 7491 nucleotides.

Table 3
Results related to the presence/absence of insertion sequence IS*Ecp1* in the genomes of the Lebanese ciprofloxacin-resistant *Salmonella* Kentucky strains and the co-localised antimicrobial resistance genes (ARGs) in the same contig.

ID IZSVe (Ref. Lebanon)	IS*Ecp1*	ARGs in same contig	Contig length (kb)
17-70328(K12)	NODE_1; Position: 771647..772143	bla_{CMY-2}: NODE_1; Position: 772260..773405	1758.535
17-70460(K24)		None	
17-70462(K31)	NODE_14; Position: 71183..71679	bla_{CMY-2}: NODE_14; Position: 71796..72941	88.499
17-70464(K32)	NODE_74; Position: 20105..20476	bla_{CMY-2}: NODE_74; Position: 20593..21738	29.323
17-70468(K38)	NODE_315; Position: 579..1075	None	
17-70469(K43)	NODE_70; Position: 4743..5118	bla_{CMY-2}: NODE_70; Position: 5235..6380	20.277
17-70472(K48)	NODE_1431; Position: 400..895	None	
17-70474(A66C)	NODE_54; Position: 9143..9518	bla_{CMY-2}: NODE_54; Position: 9635..10780	18.357

Presence (100% coverage; 100% identity)
Presence (75.65% coverage; 100% identity)
Presence (74.84%coverage; 100% identity)
Presence (100% coverage; 98.79% identity)
Absence

4. Discussion

In this study, for the first time in Lebanon, MLST analysis performed on eight *Salmonella* Kentucky isolates from poultry showed that seven isolates belonged to the international emerging CIP^R *Salmonella* Kentucky ST198 clone, of which six were MDR. Double substitutions in GyrA (Ser83 and Asp87) and a single substitution in ParC (Ser80) are frequently identified in CIP^R strains [4], which was also the case for all our strains highly resistant to fluoroquinolones.

The bla_{TEM-1B} and/or bla_{CMY-2} genes were detected in our isolates, confirming the current hypothesis that the Mediterranean Basin is the ecological niche of β-lactam-resistant *Salmonella* Kentucky ST198. Liakopoulos et al. showed that the emergence of ESC^R *Salmonella* in the Netherlands was due to the presence of bla_{CMY} on IncI1 plasmids [26]. Similarly, such an IncI1 plasmid replicon was found in all isolates investigated. Moreover, plasmid sequences were diverse within these isolates, among which two were identified as the IncI1/ST12. The latter has been disseminated worldwide, being related to the spread of bla_{CMY}-type plasmid-mediated AmpC genes among *Enterobacteriaceae* [27]. IS*Ecp1* is often inserted by transposition at the 5′ end of β-lactamase genes providing promoter sequences for expression, thereby enabling an increase in MICs of ESCs such as cefotaxime, ceftiofur and ceftazidime [28]. We showed here that the highly cefoxitin-resistant phenotype was associated with the IncI1 plasmid-encoded CMY-2 located downstream of IS*Ecp1*. Indeed, the only known β-lactam resistance gene detected in strain 17-70468(K38) with reduced cefoxitin susceptibility was bla_{TEM-1B}. Nonetheless, cefoxitin is known to be stable against TEM-1 activity [29]. Thus, it is quite likely that the reduced cefoxitin susceptibility of this particular isolate could have resulted from other mechanisms related to outer membrane permeability not investigated in this study [30].

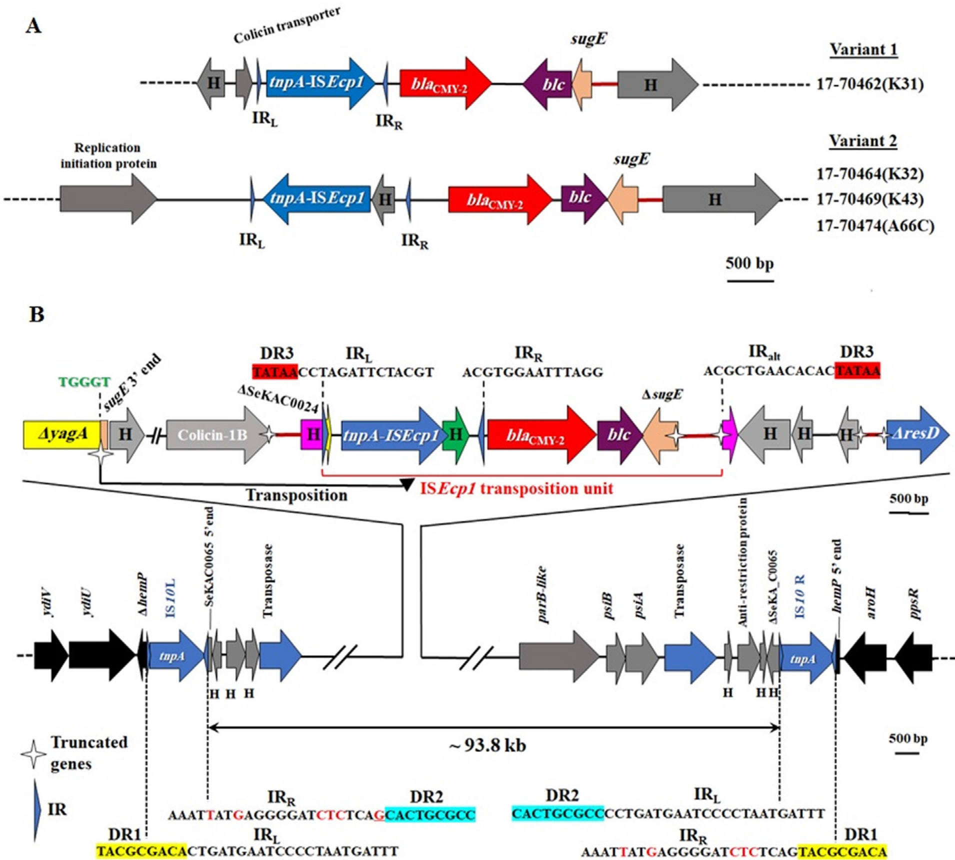

Fig. 1. Schematic diagrams showing the genomic organisation of the IS*Ecp1*–bla_{CMY-2} transposition units and flanking genes carried on (A) two extrachromosomal IncI1 plasmid variants from four *Salmonella* Kentucky strains and (B) a plasmid inserted into the chromosome of *Salmonella* Kentucky strain 17-70328(K12). The chromosomal insertion site was identified by comparison with the close IncI1 bla_{CMY-2} *Salmonella* Kentucky pCVM29188_101 plasmid previously characterised. The orientation of each gene and IS element is indicated by arrows. Black arrows represent chromosomal open-reading frames (ORFs) and grey arrows represent plasmid ORFs. The IS*Ecp1*–bla_{CMY-2} transposition unit was defined after transposition from its original site, and genes captured are represented by multicoloured arrows. Intergenic red lines represent regions with no identity to IncI1 plasmids used as a scaffold. Inverted repeats (IRs) flanking the transposase *tnpA* genes are represented by triangles as follows: IR_L, left inverted repeat; IR_R, right inverted repeat, IR_{alt}, alternative right inverted repeat. Nucleotide sequences of IRs are shown with point mutations written in red characters and G deletion underlined. IS*10*L direct repeat (DR1) nucleotide sequences are highlighted in yellow, while IS*10*R direct repeat (DR2) nucleotide sequences are highlighted in cyan blue. IS*Ecp1*-associated 5-bp AT-rich direct repeats (DR3) are highlighted in red. The TGGGT DR sequence of IS*Ecp1* was detected at the *sugE* 5′. The DR1 sequence overlaps with 9 bp of the *hemP* ORF extending from +2 to +10 nucleotide positions with respect to the translation initiation site. The DR2 sequence overlaps with 9 bp of the SeKA_C0065 ORF extending from +16 to +24 nucleotide positions with respect to the translation initiation site. Four-point stars indicate truncated genes following deletion events. H, hypothetical protein. Scales of 500 bp are shown.

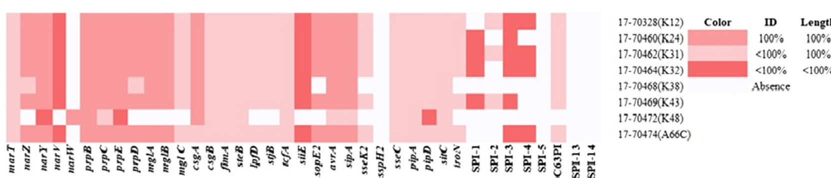

Fig. 2. Virulence determinants of the eight Lebanese *Salmonella* Kentucky isolates based on the protein sequences of the *Salmonella* spp. database.

In this study, a 93.8-kb CMY-2 plasmid region was found to have inserted into the chromosome of *Salmonella* Kentucky 17-70328 (K12) strain. The chromosomally-integrated IncI1 plasmid showed an unusual IncA/C genetic environment of bla_{CMY-2}, consisting of IS*Ecp1* upstream and *blc*, Δ*sugE* and Δ*ecnR* downstream of bla_{CMY-2}. This configuration seemed to be derived via recombination between IncA/C and IncI1 plasmids that have coexisted in ancient *Salmonella* Kentucky hosts. Previous reports on *Salmonella* and *E. coli* have assigned such plasmid insertions to an IS*Ecp1*-mediated transposition [25,31]. Interestingly, we showed that the integrated plasmid resided between two IS*10*, the insertions of which, as far as we know, took place at novel target DNA sites [32]. Full-length or partially inverted forms of IS*Ecp1* have already been described to co-localise with IS*10* [33]. An SXT/A391 integrative conjugative element (ICE) from *Proteus mirabilis* harboured a similar 14.2-kb Tn10-like composite transposon containing a truncated IS*Ecp1* upstream of bla_{CMY-2}, *blc*, *sugE* and *ecnR* [34]. However, in our case, no features indicative of ICE were identified. Taken together, all data provided full evidence that integration of the 93.8-kb CMY-2 fragment occurred by a two-step mechanism of transposition of IS*10* coupled to homologous recombination at the transposition site, and resulting in a cointegrate formation that contains a duplication of IS*10* at each chromosome–plasmid junction (Fig. 4). Such cointegrate production mechanisms by interplasmid or

Fig. 3. Single nucleotide polymorphisms (SNP)-based phylogenetic tree of the eight Lebanese ciprofloxacin-resistant (CIP^R) *Salmonella* Kentucky isolates with *Salmonella* Kentucky CVM29188 as reference genome.

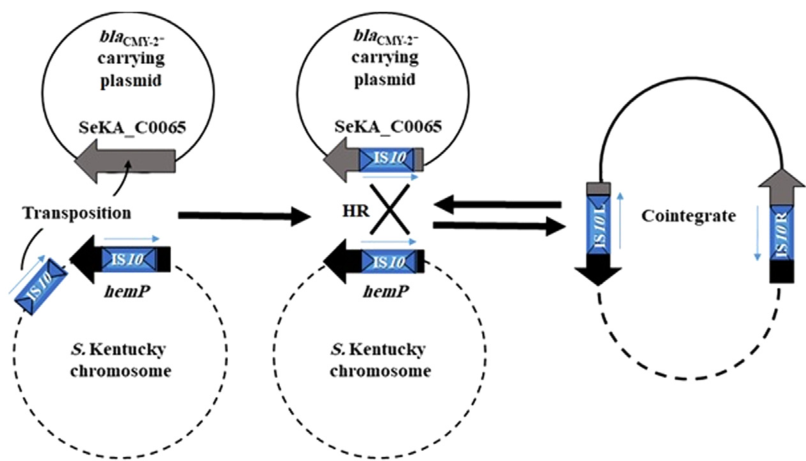

Fig. 4. Proposed model of a two-step mechanism of transposition followed by intermolecular homologous recombination for integration of the bla_{CMY-2}-carrying IncI1 plasmid into the chromosome of *Salmonella* Kentucky 17-70328(K12) strain. A chromosomal IS*10* is excised from the chromosome (donor) and inserted into the IncI1 plasmid-borne SeKA_C0065 target gene, causing its disruption. Recombination is assumed to have occurred via the IS*10* sequence itself since the sequences flanking IS*10* in the chromosome had no homology to the IncI1 plasmid as revealed by whole-genome sequencing (WGS). An additional copy of the IS*10* sequence must always exist in close proximity to the transposition complex in the chromosome [35], here inserted at the chromosomal *hemP* locus thereby serving as a donor for homologous recombination. Blue arrows indicate the orientation of IS*10* elements. Other probable sources of IS*10* at the SeKA_C0065 locus could be the pCVM29188_146 plasmid (already known to harbour IS*10*), assumed to have coexisted with the bla_{CMY-2}-carrying IncI1 plasmid in a ancient bacterial host, or even the chromosome of this ancient host.

chromosome-to-plasmid transpositions of IS*10* have been described previously [35]. It is worth mentioning that both IR_R of IS*10* were perfectly duplicated but were not 100% reverse complementary to their respective IR_L owing to some mutations (Fig. 1B), which could account for their autonomy and stability within the chromosome [32].

Gene transfer under antibiotic selective pressure facilitates the spread of drug resistance [36]. This could explain the dissemination of the highly MDR *Salmonella* Kentucky ST198 clone following the excessive therapeutic use of fluoroquinolones (enrofloxacin), third-generation cephalosporins (ceftiofur) and trimethoprim in the Lebanese poultry industry. These findings are in accordance with other reports in Africa and some parts of Asia [6]. In line with this, *Salmonella* Kentucky is well known for its genomic plasticity mediated by horizontal acquisition of plasmids or genomic islands [37]. These include the SGI1-K initially detected in *Salmonella* Kentucky strains isolated in Australia. It comprises a MDR region to aminoglycosides [*aadA7* and *aac(3)-Id*], tetracyclines [*tet(A)*] and

sulfonamides (*sul1*) as well as a mercury resistance module, all of which were located in a class 1 integron [22,38]. Similarly, we showed that five of eight strains carried this MDR region along with the SGI1-K, except for strain 17-70460(K24). Moreover, other non-negligiblecontributors could trigger this multidrug resistance, such as free trade and travel as well as the use of contaminated feeds of aquaculture origin in poultry farms [5]. Fishmeal is the most common source of mercury for farmed animals [39]. Consequently, selective pressure by mercury in contaminated feeds and/or excessive antibiotic usage in farms directly or indirectly favour the maintenance of such resistance genes among *Salmonella* isolates.

Virulotyping results revealed little gene variability among seven *Salmonella* Kentucky strains. The number of SPIs varied from one to five per isolate, with C63P1 being the most predominant, as described previously [40]. Most of T3SS-associated genes were detected in all our strains except for the T3SS-2 *sspH2* gene, as systematically reported for *Salmonella* Kentucky [41]. The reduced virulence of *Salmonella* Kentucky was then partially attributed to the absence of *sspH2* [13]. The putative iron transporters SitABCD and IroN are essential for *Salmonella* virulence and were shown to be always present in *Salmonella* Kentucky in comparison with many *Salmonella* serotypes being studied [13]. All our isolates carried *sitC*, but six isolates harboured the *iroN* gene. Therefore, the presence of these genes in *Salmonella* Kentucky isolates deserves attention in promoting the emergence of pathogenic *Salmonella* Kentucky isolates associated with human infection worldwide. Another explanation for *Salmonella* Kentucky emergence as a predominant coloniser of the chicken caecum might be the high expression levels of RpoS-regulated genes compared with *Salmonella* Typhimurium [12]. Indeed, this study highlighted the high conservation of RpoS-regulated genes involved in galactose catabolism and curli production for colonisation of *Salmonella* Kentucky in the caecum.

In conclusion, AmpC β-lactamase-producing *Salmonella* Kentucky ST198 strains reported here are the first evidence in Lebanon, thereby highlighting their high dissemination in the Mediterranean Basin. Also, the arsenal of resistance and virulence determinants identified in association with many mobile genetic elements could strongly promote the emergence of *Salmonella* Kentucky infection in humans. Further efforts are needed from health, food and agricultural authorities to control this emergence. In terms of food safety, our findings represent the first national database for future legislative amendments that will also serve the agricultural development policy of the Lebanese Agricultural Research Institute (LARI) and the Ministry of Agriculture. Inclusion of *Salmonella* Kentucky ST198 as a target strain in any national reduction plan of *Salmonella* in poultry is therefore worth being fully implemented.

Funding

None.

Competing interests

None declared.

Ethical approval

Not required.

Acknowledgments

The authors thank the Istituto Zooprofilattico Sperimentale delle Venezie (Italy) for performing the WGS. The authors also acknowledge Dr Michel Afram (General Director of LARI) for his great support.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2020.11.002.

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