environmental microbiology

Environmental Microbiology (2020) **22**(1), 413–432 doi:10.1111/1462-2920.14858

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Emergence of new variants of antibiotic resistance genomic islands among multidrug-resistant *Salmonella enterica* in poultry

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Summary

Non-typhoidal *Salmonella enterica* (NTS) are diverse and important bacterial pathogens consisting of more than 2600 different serovars, with varying host-specificity. Here, we characterized the poultry-associated serovars in Israel, analysed their resistome and illuminated the molecular mechanisms underlying common multidrug resistance (MDR) patterns. We show that at least four serovars including Infantis, Muenchen, Newport and Virchow present a strong epidemiological association between their temporal trends in poultry and humans. Worrisomely, 60% from all of the poultry isolates tested (*n* = 188) were multidrug resistant, mediated by chromosomal SNPs and different mobile genetics elements. A novel streptomycin-azithromycin resistance island and previously uncharacterized versions of the mobilized *Salmonella* genomic island 1 (SGI1) were identified and characterized in *S*. Blockley and *S*. Kentucky isolates respectively. Moreover, we demonstrate that the acquisition of SGI1 does not impose fitness cost during growth under nutrient-limited conditions or in the context of *Salmonella* infection in the mouse model. Overall, our data emphasize the role of the poultry production as a pool of specific epidemic MDR strains and autonomous genetic elements, which confer resistance to heavy metals and medically relevant antibiotics. These are likely to disseminate to humans via the food chain and fuel the increasing global antibiotic resistance crisis.

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Introduction

Salmonella enterica (*S. enterica*) is a Gram-negative, abundant human and animal pathogen and a leading cause of foodborne diseases in both developing and developed countries. This versatile bacterial species includes more than 2600 serovars presenting different adaptation to a wide spectrum of animal hosts. Non-typhoidal *Salmonella* (NTS) such as *S. enterica* serovar Typhimurium (*S*. Typhimurium) or *S*. Enteritidis have a broad host range and can infect many different animal species including reptiles, birds and mammals (Gal-Mor, 2019).

Infection by *S. enterica* occurs through the faecal-oral route, subsequent to consumption of food products contaminated with the bacteria. In healthy humans, infection with NTS serovars results in most cases in a localized self-limiting inflammation of the terminal ileum and colon, known as gastroenteritis. The annual estimated global burden of gastroenteritis due to *Salmonella* infections is 78.7 million cases, and each year 59 000 people lose their lives because of NTS infections, mainly in developing countries (Havelaar *et al*., 2015). In the United States alone, the Centres for Disease Control and Prevention estimates that *Salmonella* causes about 1.35 million illnesses, 26 500 hospitalizations, and 420 deaths (Scallan et al., 2011), associated with an economic burden of US $3.66 billion every year (USDA, 2017).

NTS can infect and colonize the intestinal and reproductive tract, or be part of the normal intestinal flora of many food-producing and wild animals, consisting the environmental reservoirs of these bacteria (Chousalkar and Gole, 2016; Gal-Mor, 2019). Among many food-producing animals, poultry are one of the most significant NTS carriers, which can persist in the bird's intestinal tract asymptomatically and commonly shed in their faces (Reed *et al*., 2003). Hence, *Salmonella*-contaminated

poultry products are often the source of NTS food-borne outbreaks and human salmonellosis, (Foley *et al*., 2011; Painter *et al*., 2013; Shah *et al*., 2017), demonstrating very clearly the "One Health" paradigm (Silva *et al*., 2014).

Since the discovery of penicillin by Alexander Fleming in 1928, antimicrobial compounds are extensively used in human medicine, but also as prophylaxis or growth promoters in the animal food industry (Castanon, 2007). The extensive usage of antibiotics in the clinical and the agricultural sectors and the selective pressure exerted by antimicrobials has led to the troublesome phenomenon of antibiotic resistance strains of both pathogens and commensal bacteria. The World Health Organization (WHO) announced the problem of antimicrobial resistance as one of the main threats to human health in the 21st century (Morehead and Scarbrough, 2018). *Salmonella* resistance to commonly used antimicrobials including ampicillin, chloramphenicol, and sulfonamides has become relatively frequent among clinical isolates, and multidrug resistant salmonellae are often associated with increased mortality and morbidity (Varma *et al*., 2005; Parisi *et al*., 2018). Moreover, recently, the WHO included fluoroquinolone-resistant *Salmonella* as one of 12 antibiotic-resistant "priority pathogens" that pose the greatest risk to human health (WHO, 2017).

The genetic plasticity of bacteria allows acquisition of mobile genetic elements encoding antimicrobial resistance genes (ARGs) by horizontal gene transfer (HGT). The main mechanisms by which bacteria can acquire ARGs by HGT are transduction, transformation and conjugation, facilitating the transfer of phages, transposons, integrons or plasmids (Kurland *et al*., 2003; Gal-Mor and Finlay, 2006). When several ARGs are integrated in one chromosomal locus, a multi-resistance genomic island can be generated like in the case of *Salmonella* genomic Island 1 (SGI1). This is an integrative mobilized element, which was originally characterized in the worldwide epidemic strain of *S*. Typhimurium, DT104 (Boyd *et al*., 2001). Since the initial identification of SGI1 in *S*. Typhimurium DT104, variants of SGI1, containing a conserved 27 kb backbone with different arrays of class 1 integrons and transposons have been described in a wide range of *S. enterica* serovars (Hall, 2010), avian pathogenic *Escherichia coli* (Cummins et al., 2019) and *Proteus mirabilis* (Schultz et al., 2015) isolates.

Importantly, with the exception of just a few well-studied *Salmonella* serovars like Enteritidis and Typhimurium, other NTS serovars have received much less attention and their ecology, epidemiology and resistome remained poorly understood. Here, we illuminated the ecology and resistance mechanisms of poultry-associated serovars and demonstrated high MDR prevalence in a subset of these serovars. Specifically, we identified a new streptomycin-azithromycin resistance island in *S*. Blockley and novel organization of mobilized SGI1 elements in *S*. Kentucky. Furthermore, we demonstrate that harbouring SGI1 does not pose an apparent metabolic burden on the bacteria, under nutrient-limited conditions, and in the context of *Salmonella* infection in the mouse model. Overall, our data emphasize the role of the poultry production industry as a key reservoir of specific NTS serovars and as a potential source of epidemic MDR strains and mobile genetic elements, conferring resistance to heavy metals and medically relevant antibiotics.

Results

Human and poultry-associated salmonellae *in Israel, 2010–2018*

Although poultry products are known to be a possible reservoir of *S. enterica* (Chousalkar and Gole, 2016), due to variation in host-specificity and other ecological factors, *Salmonella* serovars may demonstrate a different association with humans and poultry. To characterize differences in *Salmonella* epidemiology between the poultry and the clinical sectors, we chose to compare the relative prevalence of the 13 most common serovars in Israel (Infantis, Kentucky, Hadar, Muenchen, Newport, Java, Bredeney, Orion, Montevideo, Typhimurium, Enteritidis, Blockley, and Virchow) among 16438 poultry and 27489 clinical (human) isolates over the last 9 years (2010-2018). Interestingly, all the examined serovars besides *S*. Muenchen and *S*. Blockley showed significantly different occurrence, between human and poultry. While serovars Typhimurium and Enteritidis were much more dominant in humans than in poultry (by 3- and 13-fold respectively), the relative occurrence of serovars Kentucky, Hadar, Newport, Java, Bredeney and Orion was high by at least twofold in poultry samples than in clinical isolates throughout these 9 years (Fig. 1A). Similar results were also obtained when the relative prevalence of each serovar was compared in humans versus poultry for each year separately, demonstrating that the observed differences in prevalence between these sectors are kept along the years (Fig. 1B). For example, see the predominance of *S*. Enteritidis in clinical samples, but its low prevalence in poultry and vice versa for *S*. Java and *S*. Kentucky.

To further test possible linkage between temporal trends in *S. enterica* serovars prevalence in clinical versus the poultry samples, the Spearman's rank correlation coefficient was applied. This analysis demonstrated significant ($p < 0.05$) association for serovars Infantis, Newport, Muenchen, and Virchow (Fig. 2), suggesting that

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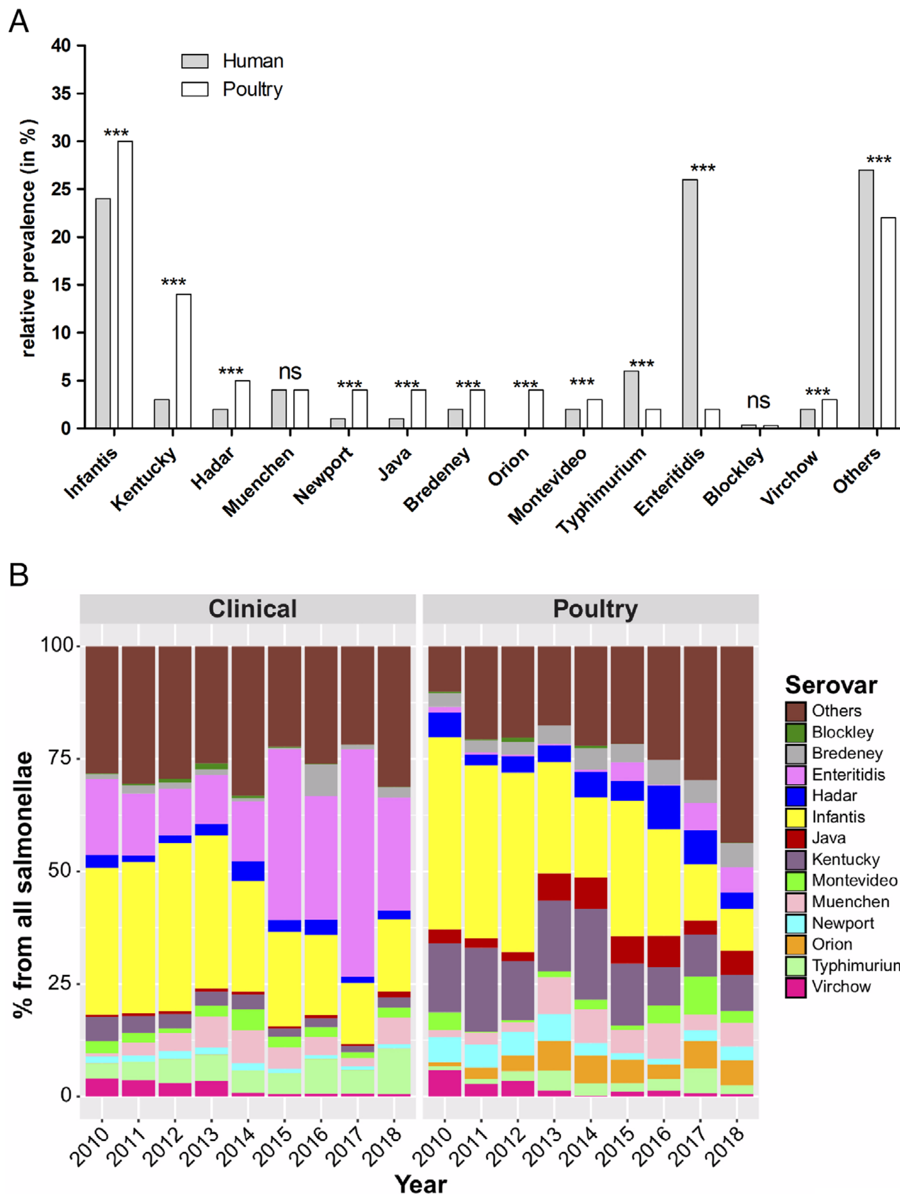

Fig. 1. The prevalence of NTS in poultry versus humans
The relative prevalence (as % from all salmonellae) of the 13 most common serovars in Israel (Infantis, Kentucky, Hadar, Muenchen, Newport, Java, Bredeney, Orion, Montevideo, Typhimurium, Enteritidis, Blockley, and Virchow) was determined over 9 years (2010–2018) among 16 438 poultry and 27 489 clinical (human) isolates that were submitted to and serotyped at the national *Salmonella* reference centre.
A. The overall differences in the relative prevalence of *Salmonella* serovars in human (grey bars) and poultry (white bars) are shown for the entire period. z score test for two population proportions with two-tailed hypothesis was used to determined statistical significance between the prevalence in the poultry versus the clinical sectors. ***$p < 0.0001$); ns, not significant.
B. The proportion (in %) of each serovar in the clinical (left) and the poultry (right) sectors from all NTS isolates obtained at each year is shown and color-coded by serovars.

poultry are likely a significant reservoir for human salmonellosis caused by these four *S. enterica* serovars.

Poultry is a major reservoir of MDR salmonellae

To gain insight into the occurrence of antibiotic resistance among *Salmonella* in poultry, we determined the antibiotic resistance profile of 188 animal (mostly poultry) isolates, representing the 11 leading serovars in the poultry sector. These serovars including Infantis, Kentucky, Hadar, Muenchen, Newport, Java, Bredeney, Orion, Montevideo, Blockley, and Virchow were tested against 13 antibiotic compounds [streptomycin, azithromycin, trimethoprim-sulfamethoxazole, tetracycline, sulfisoxazole, nalidixic acid, gentamycin, ciprofloxacin, chloramphenicol, ceftriaxone, cefoxitin, ampicillin, and amoclan (Amoxicillin-Clavulanate)]. Overall, phenotypic profiling was clustered in conjunction with the serovars classification, suggesting that many of the characterized serovars are represented by independent isolates with similar resistance profile. The most abundant tolerance was to nalidixic acid (65% of all isolates), tetracycline (65%) and streptomycin (48%), while no isolate was resistant to cefoxitin or to ceftriaxone (Fig. 3). Strikingly, 60% (113/188) of the poultry isolates were MDR, presenting resistance to three or more antibiotic compounds from different classes. The serovars that showed the

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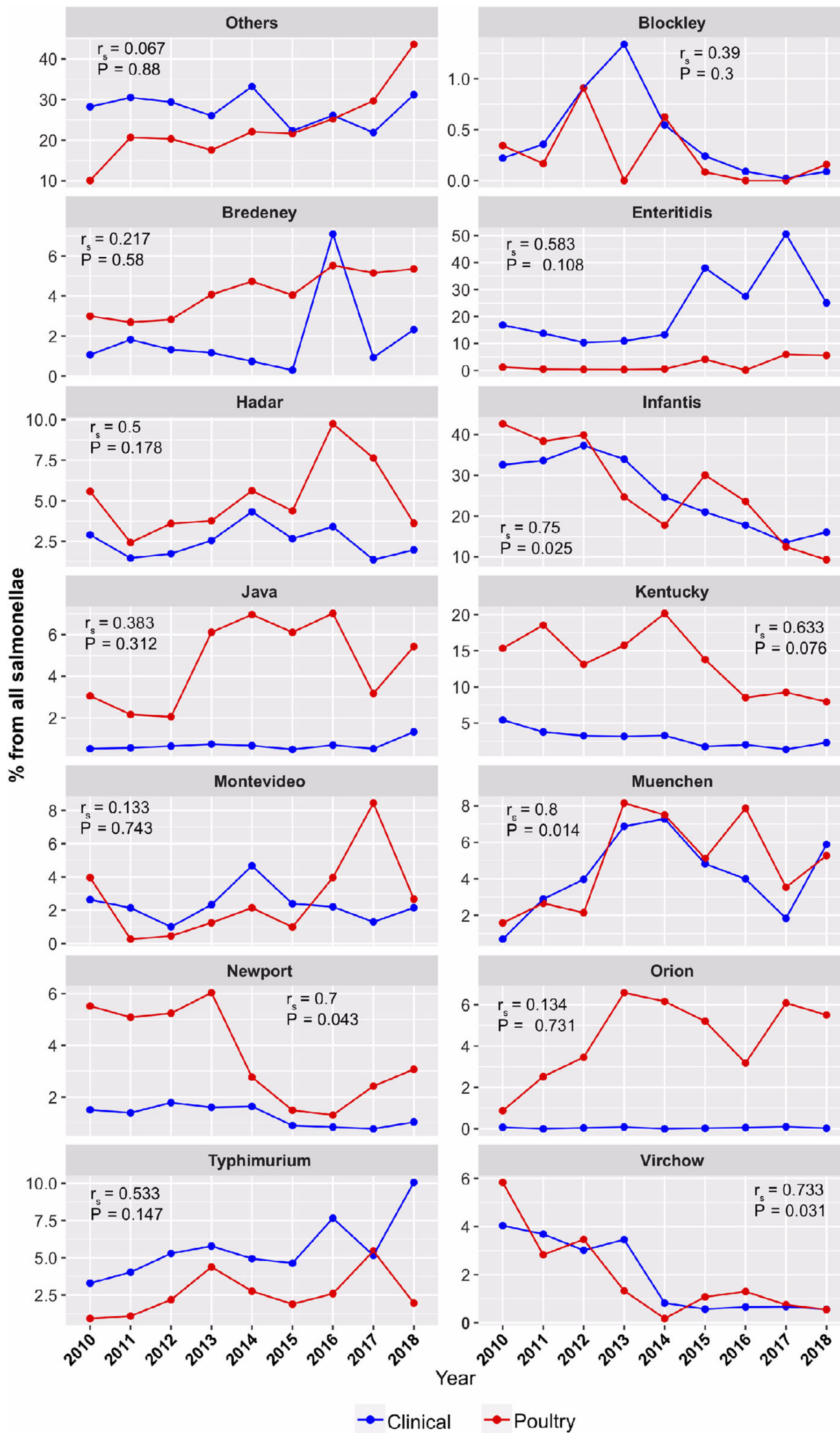

Fig. 2. Temporal trends in *Salmonella* prevalence in poultry and humans
The Spearman's rank-order correlation test and *p* values were calculated using the R software to study statistical dependence between the temporal prevalence changes in the poultry vs. the clinical sectors. The closer Spearman correlation (r_S) to 1, the strongest positive association exist between changes in prevalence in humans and poultry.

highest occurrence of MDR phenotype were Kentucky, as 38 out of 39 examined isolates (97%) were MDR followed by Hadar (80%), Java (75%) and Infantis (60%) (Fig. 4).

The resistome of MDR poultry isolates

A broad array of resistance genes and resistance-mediating point mutations (collectively known as the

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resistome) has been identified in NTS serovars (Michael and Schwarz, 2016). To better characterize the *Salmonella* resistome in zoonotic isolates, we sequenced and assembled the whole genome of 19 MDR animal (mainly poultry) isolates from seven different serovars. This analysis included representative isolates from the serovars that presented the highest MDR prevalence (Kentucky, Hadar, and Java) as well as poultry isolates from serovars Blockley, Muenchen, Orion and Newport. Since the resistome of *S*. Infantis was extensively characterized by us previously (Gal-Mor *et al*., 2010; Aviv *et al*., 2014), isolates from this serovar were not included in the current analyses. Genome assembly resulted in 28–66 contigs >500 bp from each isolate, covering each genome 127–150 times (Supporting Information Table S1). *In silico* resistance analysis using the ResFinder (Zankari *et al*., 2012) and RGI-CARD (Jia *et al*., 2017) tools successfully identified both SNPs, and horizontal gene acquisitions potentially contribute to antibiotic resistance. Gene acquisition of *bla*TEM-1B or *bla*TEM-57 encoding beta-lactamase enzymes was dominant in amoxicillin-clavulanate and ampicillin resistant isolates and were found in 85% (16/19) of all sequenced isolates. The genes encoding for aminoglycoside-3''-O-adenyltransferases, *aadA1* and *aadA7* that confer resistance to streptomycin and spectinomycin; the streptomycin phosphotransferase genes *strA* and *strB*; aminoglycoside-N-acetyltransferase genes like *aac*CA5 and *aac*(6')-ly, conferring gentamicin resistance; and genes for aminoglycoside 3'-phosphotransferases such as *aph*(3')-lc and *aphA1* were abundant among aminoglycosides-resistant strains. The genes *sul1*, *sul2* and *sul3* encoding dihydropteroate synthases and the genes *dfr*A1 encoding dihydrofolate reductase were associated with sulfonamides resistant isolates. Tetracyclines resistant strains harboured the *tetA* gene, codes for efflux pump; and erythromycin-resistance was associated with the presence of the *mphA* gene, encoding to the macrolide 2'-phosphotransferase I enzyme. A single isolate (159196) from serovar Java, which was identified to be resistant to chloramphenicol, possessed *floR* accounting for phenicol resistance in *Salmonella*. Variants of the plasmid-mediated quinolone resistance genes, *qnrB* (*qnrB*19) and *qnrS* (*qnrS*1), coding for DNA topoisomerase protecting proteins were

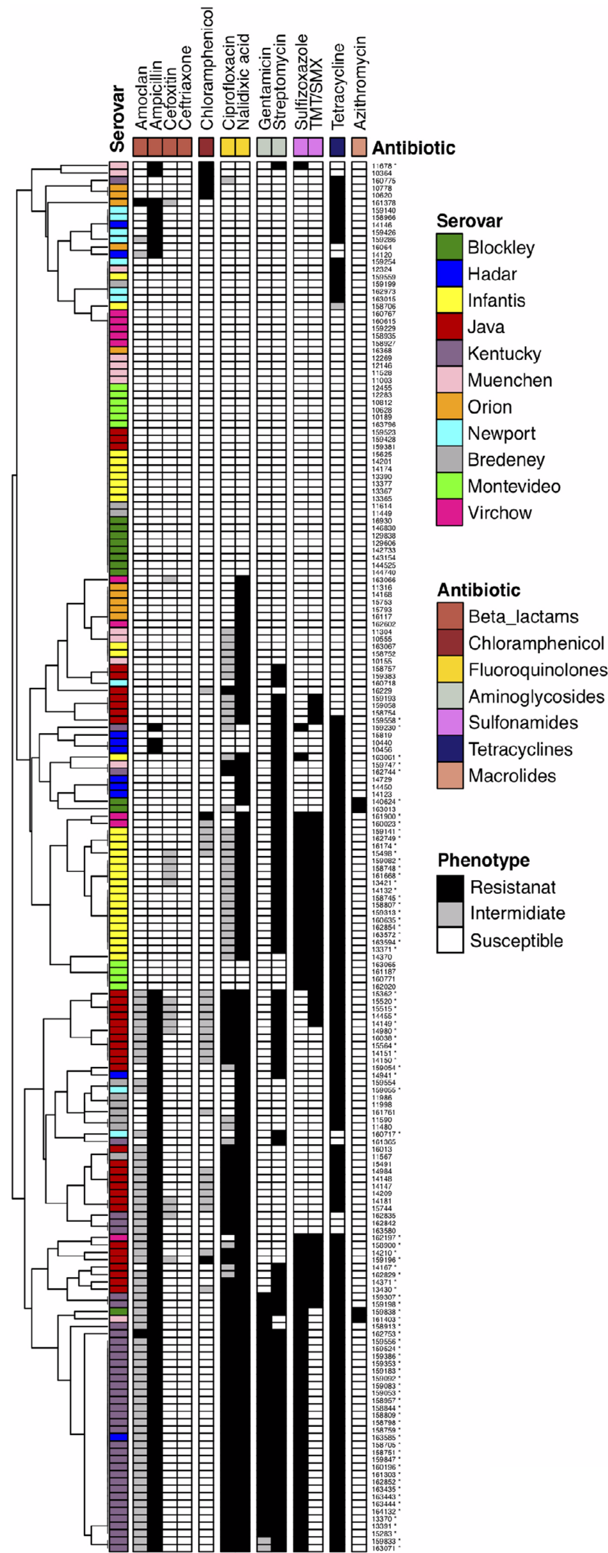

Fig. 3. Legend on next coloumn.

Fig. 3 Antibiotic resistance among *Salmonella* poultry isolates
The antibiotic resistance of 188 animal and food isolates from the 11 leading serovars in the poultry sector (Infantis, Kentucky, Hadar, Muenchen, Newport, Java, Bredeney, Orion, Montevideo, Blockley, and Virchow) to 13 antibiotic compounds [streptomycin, azithromycin, trimethoprim-sulfamethoxazole, tetracycline, sulfisoxazole, nalidixic acid, gentamycin, ciprofloxacin, chloramphenicol, ceftriaxone, cefoxitin, ampicillin, and amoclan (Amoxicillin-Clavulanate)] was determined. Resistance, intermediate resistance and susceptibility to each compound are indicated by black, grey and white colours respectively. MDR isolates are marked with an asterisk. Phenotypic clustering according to the resistance profile is shown by the dendrogram on the left.

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Fig. 4. Distribution of multidrug resistance among poultry isolates of *Salmonella*
The distribution of MDR was defined as resistance to ≥3 antibiotics from different classes. The MDR results for the 11 poultry-associated serovars are shown as percentage from all isolates tested from each serovar.

Fig. 5. Antibiotic resistance and the resistome of zoonotic *Salmonella* isolates
The genome of 19 representative MDR isolates of animal origin from serovars Blockley, Hadar, Java, Kentucky, Muenchen, Newport and Orion was sequenced and assembled. Resistome analysis using the ResFinder and RGI-CARD tools was used to identify the presence of ARGs and point mutations known to confer antibiotic resistance. The resistance phenotype to streptomycin, azithromycin, trimethoprim-sulfamethoxazole, tetracycline, sulfisoxazole, nalidixic acid, gentamycin, ciprofloxacin, chloramphenicol, ceftriaxone, cefoxitin, ampicillin, and amoclan was determined by using CMV4AGNF plates read by the VIZION digital MIC viewing system. The resistance phenotype to kanamycin was tested by direct plating on LB plates supplemented with 50 μg ml^{-1} kanamycin. Resistance, intermediate resistance and susceptibility are indicated in the left table (phenotype) by black, grey and white colours respectively. The presence of ARGs is indicated in the right table (genotype) by black squares.

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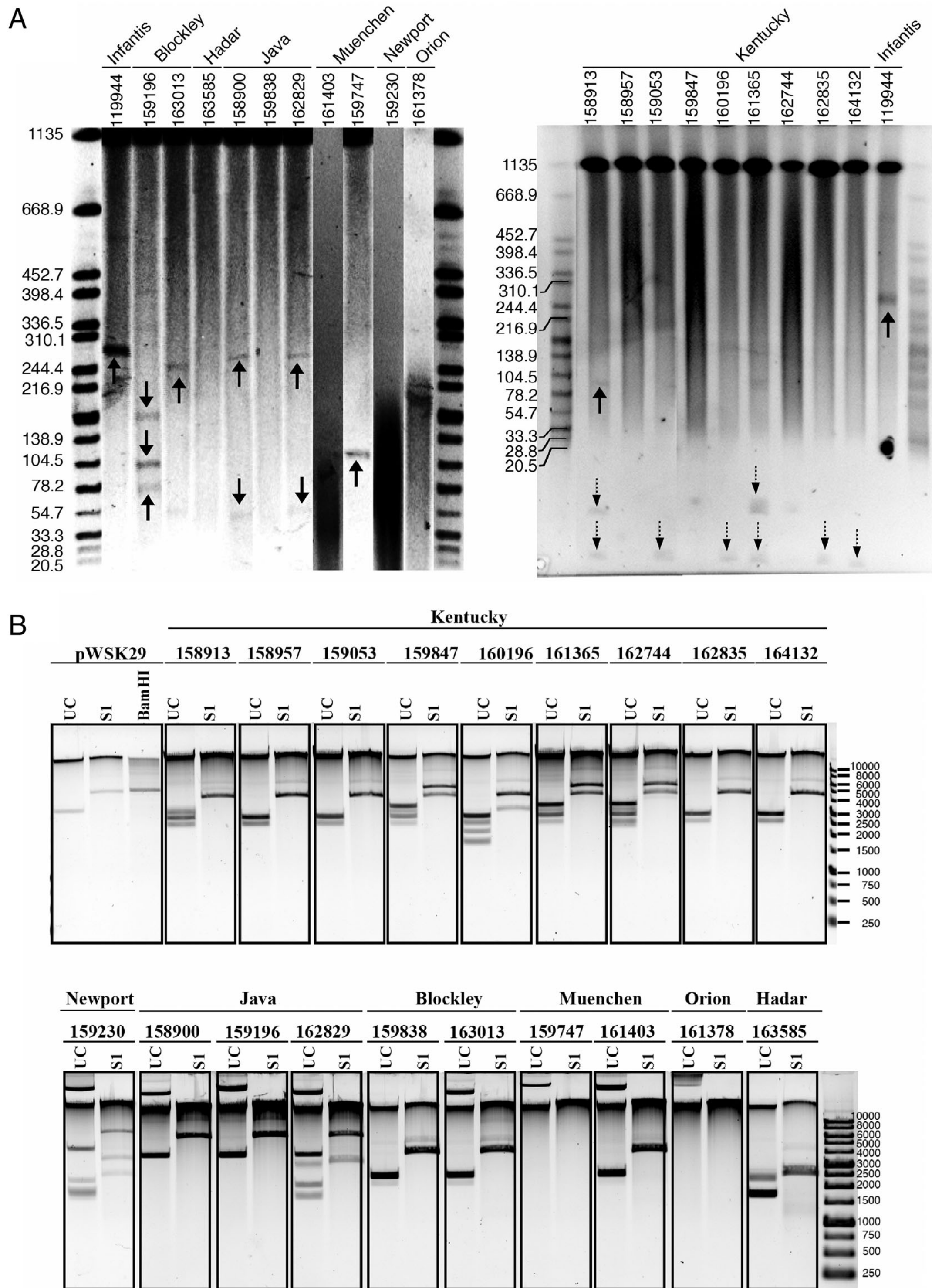

Fig. 6. Mega and small plasmid analysis of MDR *Salmonella* isolates
A. The genome of 19 NTS MDR animal and food isolates was subjected to S1-nuclease digest followed by PFGE. *S.* Infantis 119 944, which harbours the pESI plasmid (~300 kb) was included as a positive control. The presence of large plasmids is shown by black arrows and the presence of small plasmids (≤ 15 kb) is shown by broken arrows.
B. Small plasmids were extracted from the 19 sequenced NTS poultry isolates using mini preparation kit and were subjected to S1-nuclease treatment. Equal amounts (200 ng) of uncut (UC) and S1-digested (S1) plasmids were run side by side on a 1% agarose gel. Uncut pWSK29 (5434 bp), digested by S1-nuclease and cut by BamHI was included as controls.

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identified in isolates from serovars Hadar, Muenchen and Newport. Moreover, multiple SNPs in the *gyr*A and/or *par*C genes that are also associated with resistance to quinolones were found in 14 out of the 19 sequenced isolates. These results demonstrate multiple mechanisms contributing to quinolones and floroquinolones resistance and the high prevalence of such resistance among poultry-associated salmonellae. The phenotypic and genotypic analyses of antibiotic resistance of the 19 MDR poultry isolates are summarized in Figure 5.

Circulation of megaplasmids among MDR poultry Salmonella *isolates*

Often, ARGs are encoded on mobile genetic elements such as plasmids (Madec and Haenni, 2018). Previously, we showed that the MDR phenotype of the emerging strain of *S*. Infantis is largely due to the presence of a large virulence-resistance plasmid, called pESI (Gal-Mor *et al*., 2010; Aviv *et al*., 2014; Aviv *et al*., 2016). To investigate similar contribution of megaplasmids to the MDR of other poultry *Salmonella* serovars, the genomes of the 19 MDR isolates were subjected to plasmid profiling using S1-endonuclease, followed by pulsed-field gel electrophoresis (PFGE). As a positive control, we included the *S*. Infantis 119 944 isolate that harbour ~300 kb pESI plasmid. As demonstrated in Fig. 6A and Table 1, nine isolates from serovars Blockley, Java, Muenchen, Newport, Orion and Kentucky were found to harbour large plasmids ranging from 40 to 245 kb including plasmids from the incompatibility groups IncI1 and IncX1. Noteworthy, at least four out of the megaplasmids identified in serovars Muenchen Java, and Orion were found to be conjugative and their antibiotic resistance phenotype was able to be mobilized into a recipient *E. coli* strains at frequency of 1.02×10^{-2} to 1.72×10^{-4} transconjugants per donor cell (Table 1).

Moreover, the above analysis indicated that in addition to the large plasmids, some isolates harbour smaller (<15 kb) plasmids, indicated by broken arrows in Fig. 6A. Thus, to further characterize the distribution of small episomes, minipreparation of plasmid DNA followed by S1-nuclease digest and DNA separation by gel electrophoresis was conducted. As shown in Fig. 6B and summarized in Table 1, this analysis successfully profiled small plasmids with estimated size of 2.2–6 kb in 17/19 isolates, with replicon types of ColRNAI, Col440I, Col8282, and Col156. Interestingly, several of these isolates contained two to three small plasmids in addition to at least one megaplasmid (Table 1). Collectively, these results

Table 1. Plasmid analysis of MDR strains. Integrated plasmid characterization that was based on the S1-nuclease and bioinformatics analyses shows the distribution of small and megaplasmids found in the 19 sequenced MDR strains. The estimated plasmids size (in kb) is shown and the presence of known conjugation systems genes and measured conjugation frequency are indicated as well. Identification of plasmid replicons was done by the PlasmidFinder tool and in cases where we were able to link the plasmid replicon with a specific plasmid sequence, the size of the plasmid is indicated in brackets next to the replicon type.

Strain	Serovar	Small plasmids (estimated size in kb)	Mega plasmids (estimated size in kb)	Presence of conjugation genes	Conjugation frequency	Replicon type (plasmid size)
159838	Blockley	4			ND	
163013	Blockley	4	245	VirB11; Mobilazation protein	ND	IncX1 (245)
163585	Hadar	2.6		MobA/MobL family	-	Col440I (2.6)
162829	Java	6; 3.5	245:50	Pilx; MbeB	-	IncX1
158900	Java	6	245; 50	Pilx2 and others (T4SS VirB2 component)	-	IncX1
159196	Java	6	185;104;78	Type IV secretory pathway, VirD2 components	1.72E-04	IncI1,IncX1
159847	Kentucky	5; 4.6		mbeB,MobA/MobL family	ND	ColRNAI (4.6)
162744	Kentucky	5; 4.6		MbeB;MobA	-	Col8282 (5), ColRNAI (4.6)
162835	Kentucky	4.6		MbeB	ND	Col440I (4.6)
164132	Kentucky	4.6		MobA/MobL family	-	ColRNAI (4.6)
158913	Kentucky	4.6	90	MobA/MobL family	-	ColRNAI;Col156 (4.6)
158957	Kentucky	4.6		MobA/MobL family	ND	ColRNAI (4.6)
159053	Kentucky	4.6		MobA/MobL family	ND	ColRNAI (4.6)
160196	Kentucky	4.6 ;3.2		MbeB	-	ColRNAI (4.6)
161365	Kentucky	5; 4.6		MbeB	-	ColRNAI (4.6)
159747	Muenchen		111	Tra system	1.02E-02	IncI1/IncQ1 (111)
161403	Muenchen	4	111	TrbC	7.84E-03	IncI1 (111)
159230	Newport	6; 3.2; 2.2	44.6	MobC; MbeA; VirB11	ND	IncX1 (44.6); Col440I (2.2)
161378	Orion		111	TrbC	1.50E-02	IncI1 (111)

-, conjugation was tested, but not detected; ND, conjugation was not determined.

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showed diverse and abundant distribution of both small and megaplasmids among MDR isolates from the poultry sector and demonstrated the ability of some of these resistance plasmids to conjugate to other bacterial species, further disseminating the resistance phenotype.

Identification of a new streptomycin and azithromycin resistance island

In additions to ARGs encoded on plasmids, sequence analysis of isolates 163013 and 159838 belonging to serovar Blockley identified chromosomally encoded macrolide inactivation gene cluster containing the genes *mphA*, *mrx* and *mphr*(A). In agreement with the whole-genome sequencing (WGS) data, isolates 163013 and 159838 were resistant to 200–400 μg ml^{-1} of erythromycin in comparison to erythromycin-sensitive *S*. Blockley strain (isolate 147117; Fig. 7A).

Recently, Nair and colleagues reported the identification of a new island designated *Salmonella* azithromycin resistance genomic island (SARGI), which harbours these three genes (Nair *et al*., 2016). To confirm the genetic organization of the corresponding region in *S*. Blockley 159838, its genome was resequenced using MinION technology and the obtained long reads (mean reads length 6.1 kb; maximum read length 100 kb) were combined with the short Illumina MiSeq reads for complete assembly of the *S*. Blockley 159838 genome. Surprisingly, pairwise sequence comparison with the originally described SARGI in *S*. Blockley H123780513 revealed an insertion of 12 884 bp harbouring additional ARGs, into this island immediately upstream to *penDE*. This new region contains the ARGs *aph*(3')-I (encoding aminoglycoside phosphotransferase) and *strB*, conferring resistance to streptomycin and another, possibly non-functional copy of *aph*(3'')-Ib (Fig. 7B).

To further confirm this insertion and clustering of additional ARGs in this locus, a PCR using the primers aph (3'')-Ib-F (P1) and penDE-R (P2) was used to amplify a 2986 bp fragment spanning the insertion point of the macrolide and streptomycin-resistance cluster in the *S*. Blockley genome. As shown in Fig. 7C, a single and specific PCR product of the expected size was amplified from the genomes of the erythromycin resistant *S*. Blockley isolates 159838 and 163013, but not from the erythromycin-sensitive *S*. Blockley isolate 147117 or from *S*. Typhimurium SL1344 strain (that was included as a negative control). These results provided additional direct molecular evidence for the unique genetic organization of these ARGs in the *S*. Blockley MDR isolates.

This new genomic island was possibly created by an insertion of a 12 884 bp region flanked by two IS26 elements through homologous recombination at the IS26 site, located upstream to *penDE* in the ancestral strain. The origin of this insertion is most likely from a plasmid, as one of the genes encoded on this accessory fragment is a plasmid replication initiator protein, RepE (accession number AKM38258).

To the best of our knowledge, this is the first report demonstrating a new composition of SARGI, which harbours resistance gene clusters to both macrolides and aminoglycosides in a single genomic island and we therefore propose to designate this novel locus '*Salmonella* streptomycin and azithromycin resistance island' or SASARI for short.

Identification of new variants of Salmonella *genomic island 1 (SGI1)*

Additional unusual clusters of resistance genes were identified in nine *S*. Kentucky isolates, carrying new variants of SGI1. The canonical organization of SGI1, previously identified in *S*. Kentucky isolates and known as variant SGI1-K comprises a mosaic resistance segment made of transposons Tn*3*-like, Tn*5393*, Tn*1721*, Tn*21* and an In4-type integron, conferring resistance to ampicillin, streptomycin, gentamicin, sulfamethoxazole and tetracycline (Levings *et al*., 2007; Hawkey *et al*., 2019). Interestingly, our WGS data indicated that nine MDR *S*. Kentucky isolates harboured the backbone of SGI1 (that was integrated into the known locus in the chromosome between *yidY* and *trmE* genes; Le Hello *et al*., 2012 and Fig. 8A), but presented a distinct composition and location of its associated transposons and the integron, carrying the ARGs. To verify the different organization of SGI1-K, isolates 161365 and 162835 were subjected to a second whole genome sequencing by MinION platform and the resulted long reads were combined with the short MiSeq reads for complete assembly and closing their genome sequences.

Pairwise comparison with the canonical SGI1-K sequence of *S*. Kentucky strain SRC73 (accession number AY463797) identified two new compositions of SGI1-K-like islands (Fig. 8A). In *S*. Kentucky 162835, we identified a single insertion event of Tn3 harbouring the *bla*-TEM-1B gene into the SGI1 backbone gene S044 that was mediated by an IS26 element. Nevertheless, this version of SGI1-K did not contain Tn*5393*, Tn*1721*, Tn*21* or the In4-type integron and in agreement with this partial content, isolate 162835 was resistant to ampicillin but sensitive to gentamycin, tetracycline, and sulfamethoxazole (Fig. 5).

Similar non-canonical organization of SGI1-K was identified in isolate 161365; however, this isolate harbours additional insertion of resistance complex outside of the SGI1 backbone. A 15 924 bp mosaic resistance element made of IS26, the transposons Tn1721 and Tn21 and the In4-type integron was integrated into the

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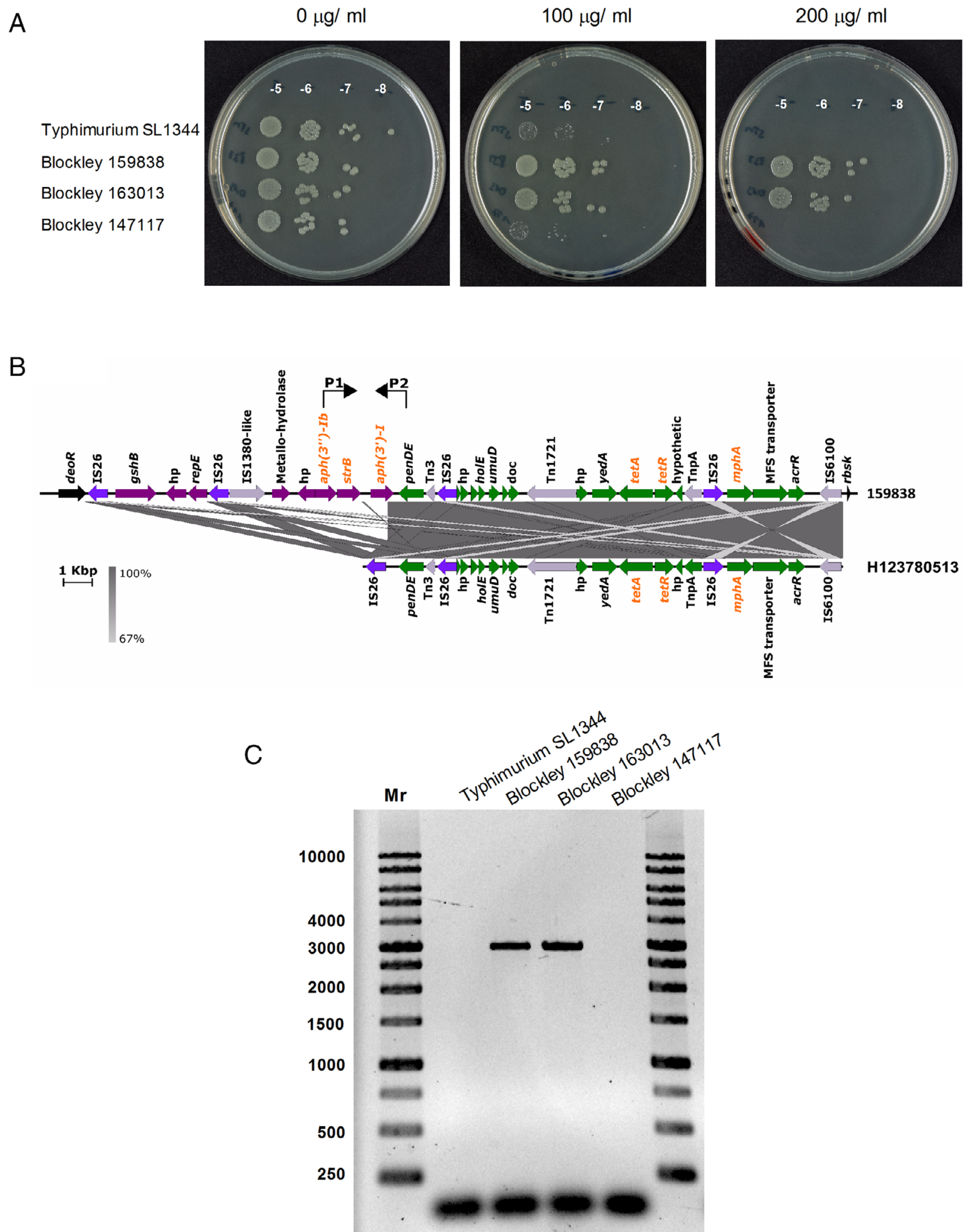

Fig. 7. Identification of a new streptomycin-azithromycin genomic island (SASARI) in *S.* Blockley

A. Serial dilutions of *S.* Typhimurium SL1344, and *S.* Blockley isolates 159838, 16013 and 147117 that were grown in LB broth were plated on LB agar plates supplemented with 0, 100, or 200 µg ml^{-1} of erythromycin. Plates were imaged after 16 h of incubation at 37°C.

B. Pairwise alignment of the *Salmonella* azithromycin resistance genomic island (SARGI) defined in *S.* Blockley strain H123780513 (accession number KX237654) with *S.* Blockley isolate 159838 (position 4 326 383–4 352 831). ARGs are indicated in orange and transposase genes are marked in purple. The degree of sequence similarity is shown by shades of grey.

C. The genome of *S.* Blockley isolates 159838 and 16013 was used as a template to amplify a 2986 bp PCR product, spanning the insertion point of the macrolide and streptomycin-resistance cluster in the *S.* Blockley genome using the primers aph(3'')-Ib-F (P1) and penDE-R (P2). *S.* Typhimurium SL1344 and *S.* Blockley isolate 147117 that do not carry the insertion of the streptomycin-azithromycin genomic island were used as negative controls.

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rbsK ribokinase gene located about 50 kb downstream from the original SGI1 backbone integration locus, creating a "split" organization of SGI1-K. As a result, this strain was resistant to ampicillin, gentamicin, sulfamethoxazole and tetracycline (Fig. 5). Similar organization of such resistance island was found in the *S.* Kentucky poultry

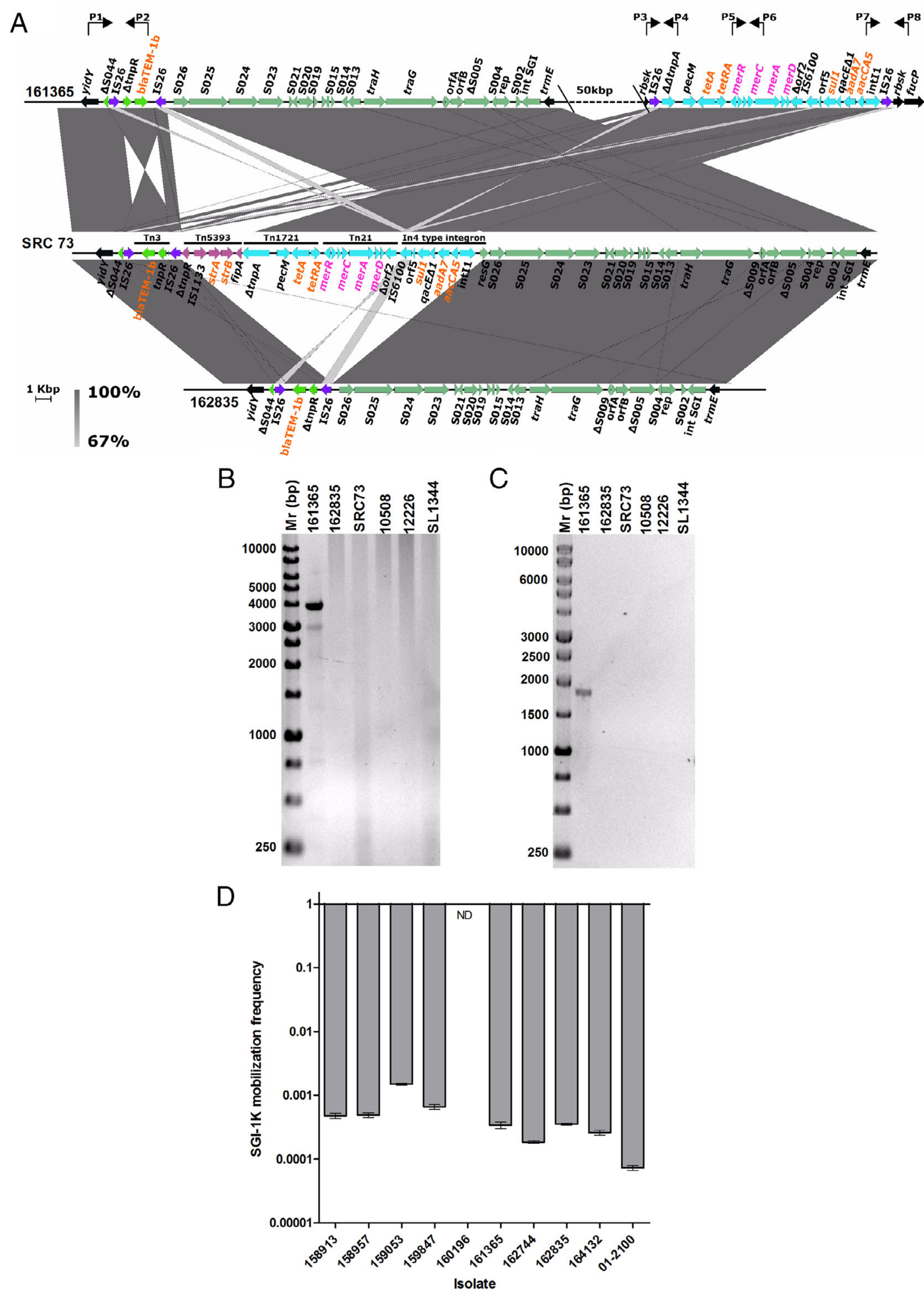

Fig. 8. Legend on next page.

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isolates 158957, 159847, 162744, 164132, and 158913; turkey isolates 159053 and 162835; and the horse isolate 160196, that all of them seem to be clonal (Supporting Information Fig. S1). To the best of our knowledge, such unique organization of SGI1-K was not reported before. To further endorse this unusual organization of SGI1-K, a series of confirmatory PCRs were applied. Using the primers P1 and P2, a resulted 3.9 kb amplicon confirmed the insertion of Tn3 downstream to the core gene *yidY* within the S044 SGI1 backbone gene in isolate 161365. This amplicon was not generated in *S*. Kentucky SARC 73 harbouring the canonical SGI1-K nor in isolate 162835 that harbours the transposon Tn3 in an opposite orientation. Negative control templates consisting of *S*. Kentucky SGI1-K-negative strains (10508 and 12226) and *S*. Typhimurium SL1344, did not amplify the 3.9 kb fragment as well (Fig. 8B).

Likewise, the primers P3 and P4 resulted in a 1864 bp PCR product, which confirmed the insertion of Tn1721 downstream to core *rbsK* gene. Additional analyses using the primer pairs P5 and P6; and P7 and P8, produced PCR products of 1.6 kb and 2.9 kb, respectively, which verified the integration of Tn21 and In4 integron upstream to the core gene *fucP* (data not shown). Altogether, hybrid genome assembly and molecular confirmation using PCR demonstrated previously unknown organizational structures of SGI1 among MDR *S*. Kentucky zoonotic isolates of animal origin.

To estimate the distribution of these novel SGI1-K-like islands among poultry isolates on the national level, 39 poultry isolates of *S*. Kentucky that were collected between 2014 and 2018 were tested for the presence of SGI1-K and its derivatives by PCR using the primers P5 and P6. Among the 39 *S*. Kentucky isolates tested, 31 isolates (80%) were found to carry some version of SGI1-K. Moreover, using the primers P3 and P4, we showed that in 23 out of 31 (74%) *S*. Kentucky SGI1-K-positive strains, this resistance island was split and presented a similar organization to the one found in isolate 161365 (data not shown). These results suggested abundant distribution of the split SGI1-K-like structure among the local population of *S*. Kentucky. Therefore, we named this genomic island SGI1-KIV standing for *Salmonella* genomic island 1 Kentucky Israeli version.

SGI1-K variants are frequently mobilized into naïve bacterial hosts

SGI1 was previously characterized as a site-specific integrative mobilizable element (Cloeckaert *et al*., 2007) that can excise from the chromosome and mobilize in the presence of broad-host-range IncA/C helper plasmids (Douard *et al*., 2010; Carraro *et al*., 2014). To determine the capability of the new SGI1-like variants to disseminate to a new host, the pVCR94ΔX4 IncC plasmid (Carraro *et al*., 2014) was introduced into the 10 *S*. Kentucky strains carrying the SGI1-K-like islands and their mobilization frequency into a donor *E. coli* strain (ORN 172) was examined, by measuring the transfer of the *bla*TEM-1b, ampicillin resistance gene. With the exception of one isolate (160196) that was unable to mobilize the resistance island; all the other strains tested showed conjugative transfer frequency ranging from 1.4×10^{-3} to 7×10^{-5} transconjugants per donor, dependent on the donor strain (Fig. 8D), demonstrating significant dissemination of this mosaic element into other bacterial species hosts. Moreover, these results showed similar transfer efficiency of SGI1-K for both 161365 and 162835 that harbour a different composition of transposons in the SGI1-K, indicating that the number and the composition of the different antibiotic resistance cassettes do not necessarily affect the transfer efficiency of SGI1-K. Noteworthy, as opposed to the SGI1-K in *S*. Kentucky 01-2100, antibiotic resistance to tetracycline and sulfamethoxazole encoded on Tn1721 and In4 type integron, respectively, separated from the SGI1 backbone in isolate 161365, were not conjugated together with the *bla*TEM-1b resistance gene into the recipient strain. These results further support the conclusion that Tn1721 and In4 type integron are not integral components of the SGI1-KIV island.

Fig. 8. Identification of new types of *Salmonella* genomic island 1 in *S*. Kentucky
A. Pairwise alignment of the SGI1-K that was integrated into the *S*. Kentucky chromosome between *yidY* and *trmE* genes characterized in SRC 73 (accession number AY463797) with the *S*. Kentucky isolates 161365 (position 297 955–397 892) and 162835 (position 4 814 961–4 847 944). ARGs are indicated in orange and transposase genes are marked in purple. The SGI1-K backbone genes are shown as green arrows. The degree of sequence similarity is shown by shades of grey.
B. S. Kentucky SGI1-K-positive strains 161365, 162835, and SRC73 as well as the SGI1-negative strains 10508 and 12226 and *S*. Typhimurium SL1344 were subjected to PCR using the primers P1 and P2. The resulted 3.9 Kb amplicon indicates the insertion of Tn3 in a specific orientation downstream to the core gene *yidY*.
C. The same strains were subjected to PCR using the primers P3 and P4, resulting in a 1864 bp specific amplicon, indicating the insertion of Tn1721 transposon downstream to *rbsK*.
D. The IncC helper plasmid pVCR94ΔX4 (Cm-resistant) was introduced by conjugation to 10 *S*. Kentucky strains harbouring different versions of SGI1-K. The mobilization frequency of SGI1-K and its derivatives into a recipient *E. coli* ORN 172 (Kn-resistant) was tested by plating the transconjugants onto LB agar plates supplemented with ampicillin (to select for SGI1-positive clones) and kanamycin (to select for the recipient). Mobilization frequency was calculated as the number of transconjugants obtained after 16 h of conjugation at 37°C per number of donor CFUs. No transconjugant were obtained when isolate 160196 was used as the SGI1-K donor. ND, not detected.

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SGI1 variants provide tolerance to toxic mercury

Elemental mercury and its compounds are toxic to all free-living organisms and circulated in various environments. Many of the mercury-resistant bacteria, which belong to a broad range of Gram-negative and Gram-positive bacterial species, harbour the *mer* operon. This cluster of linked genes encodes proteins involved in regulation (*merR* and *merD*), transport (*merT merP merC* and *merE*), and enzymatic detoxification (*merA*) of inorganic mercury compounds by reduction of Hg^{2+} to its metallic form (Hg^0) (Matsui and Endo, 2018).

The presence of the *mer* operon (*merRTPCADE* genes) as part of aTn21-like transposon integrated in SGI1-K derivatives, suggested that these strains might be resistant to toxic mercury. To test the resistance of the *S*. Kentucky SGI1-K-like-positive strains to mercury, the growth of nine *S*. Kentucky SGI1-K-like-positive isolates was studied in the presence and absence of 25 μM of $HgCl_2$. As a negative control, we included two *S*. Kentucky SGI1-K-negative strains (10508 and 12226) and as a positive controls we tested the 01-2100 and SRC 73 strains previously characterized as carrying the complete SGI1-K (Doublet *et al*., 2008). In LB broth, all the examined *S*. Kentucky strains presented similar growth rate (Fig. 9A). Nevertheless, in the presence of 25 μM of HgCl2, SGI-K1-negative strains were unable to grow, while the strains that carried the intact SGI-K1 or its Tn21-positive derivatives were fully resistant to mercury. As expected, strain 162835, which lacks the Tn21 transposon (carrying the *mer* operon) was unable to grow in the presence of mercury as well (Fig. 9B). These results indicate that the *mer* genes are functional and provide protection against toxic concentrations of mercury, illuminating the role of SGI1-K-like elements in bacterial resistance not only to antibiotics but also to environmental stressors such as heavy metals.

Carrying the SGI1-K does not confer metabolic burden or compromise Salmonella *virulence* in vivo

Horizontal acquisition of large DNA elements is thought to impose metabolic burden on the bacterial host and to reduce bacterial fitness (Baltrus, 2013). Thus, we were interested in testing whether the integration of SGI1-K into the *S*. Kentucky genome affects the fitness of its host in the context of infection and under nutrient-limited conditions. We constructed in-frame deletion of the SGI1-KIV lacking 28.8 kb and used competitive growth approach to compare the fitness of *S*. Kentucky 161365 harbouring the SGI1-KIV deletion (ΔSGI1-KIV; carrying a kanamycin resistance cassette) against its isogenic wild-type strain (WT) possessing the full SGI1-KIV locus (resistant to ampicillin). Equal volumes of stationary phase cultures that were normalized to the same optical density diluted 1:100 into M9 minimal medium, mixed and grown in the same flask at 37°C for 120 h. At different time points during 5 days, aliquots from the mixed culture were taken and platted on selective plates to determine the ratio between the WT and the ΔSGI1-KIV populations. Interestingly, during 5 days of culturing, similar numbers of

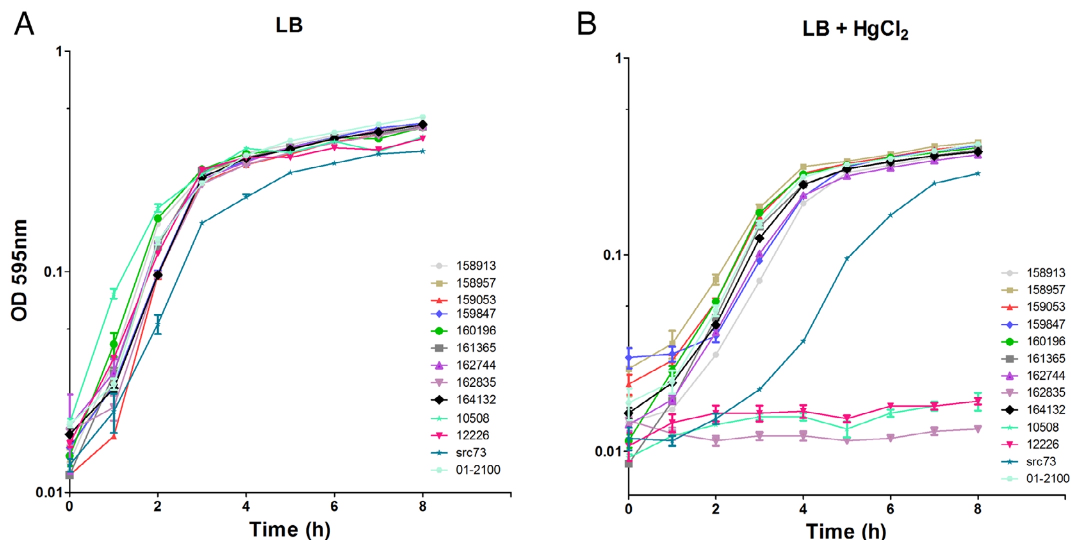

Fig. 9. SGI1-KIV provides tolerance to toxic mercury in *S*. Kentucky
Ten *S*. Kentucky Israeli isolates, harbouring SGI1-K-like islands (158913, 158957, 159053, 159847, 160196, 161365, 162744, 164132, 10508, 12226); two reference SGI1-positive *S*. Kentucky strains (SRC 73 and 01-2100); an Israeli isolates that harbours the SGI1-K backbone but lacks the *mer* operon (162835); and two Israeli *S*. Kentucky strains that lacks the entire SGI1-K locus (10508 and 12226) were grown in LB broth (A) or in LB supplemented with 25 μM of $HgCl_2$ (B) for 8 h at 37°C. Optical density at 595 nm is shown. Each point shows the average of triplicates with SEM indicated by the error bars.

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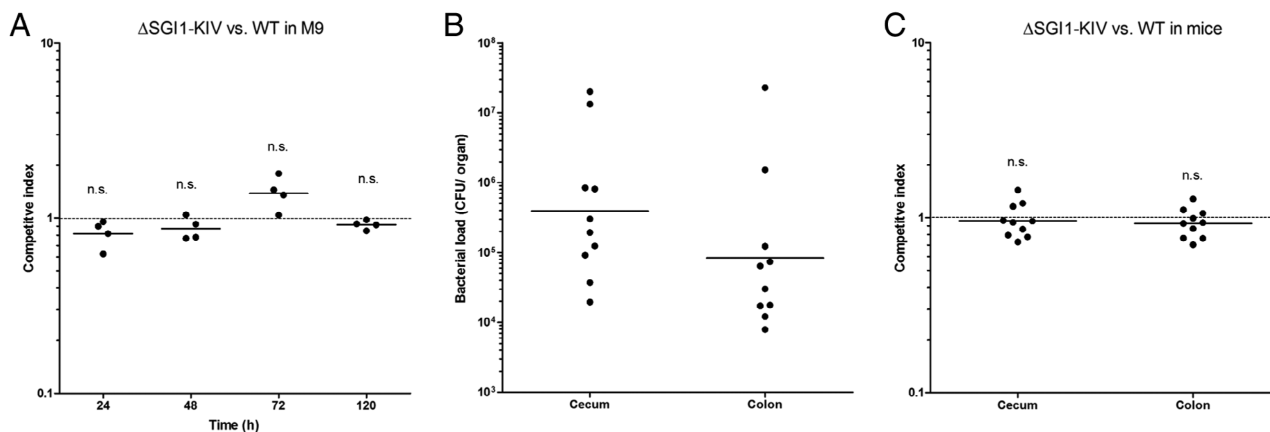

Fig. 10. SGI1-KIV does not confer fitness cost *in vitro* or *in vivo*
A. *S*. Kentucky isolate 161365 (WT; Amp-resistance) and its isogenic strain that lacks SGI-KIV (ΔSGI1-KIV) carrying kanamycin resistance cassette were grown in LB broth for 16 h. Both cultures were normalized to same optical density and diluted 1:100 at equal volumes. The mixed culture was grown for five days in two independent flasks and at 24, 48, 72 and 120 h, four independent samples (two from each culture) were plated onto LB plates supplemented with ampicillin (for WT counting) and kanamycin (for ΔSGI1-KIV counting). The ratio between the counted CFUs of the ΔSGI1-KIV versus the wild type is expressed as the competitive index at each time point. One sample t-test against a theoretical mean of one was used to determine if the obtained competitive index is different than one (C.I. of one indicates that both strains have a similar fitness under the experimental conditions). n.s., not significant.
B. Ten female C57BL/6 mice at age of 8 weeks were infected with equal CFUs (6×10^6) of WT and ΔSGI1-KIV strains by oral gavage. At day 4 post infection, mice were euthanized and tissues were harvested, homogenized aseptically and plated on selective media for bacterial enumeration. Bacterial load in the cecum and colon is shown as total CFU per organ.
C. Homogenized tissues were serial diluted and plated onto LB plates supplemented with ampicillin (for WT counting) and kanamycin (for ΔSGI1-KIV counting). The ratio between the counted CFUs of the WT vs. the ΔSGI1-KIV is expressed as the competitive index that was obtained in the cecum and the colon of each mice. Each dot represents the competitive value in one mouse as the geometrical mean is shown by a horizontal bar. A competitive value of one indicates no significant difference in the fitness between both strains.

CFUs from the WT and the ΔSGI1-KIV strains were counted, indicating similar fitness of both strains under these conditions (Fig. 10A).

To examine their relative fitness in the context of infection, we have implemented the colitis mouse model for *Salmonella* infection. Ten BALB/c female mice were orally gavaged with equal CFUs of *S*. Kentucky WT and the ΔSGI1-KIV strains that established a significant intestinal colonization (Fig. 10B). Four days post infection, mice were euthanized and tissues were harvested and homogenized aseptically and plated for bacterial enumeration. As illustrated at Figure 10C, very similar numbers of CFUs from both strains were recovered from the cecum and ilium of the infected mice. The obtained competitive index (CI) value (the ratio between the CFUs of ΔSGI1-KIV and the WT background) of ~1 indicated no effect of SGI1-KIV on *S*. Kentucky fitness during infection, under non-selective conditions.

Collectively, these results demonstrated that the acquisition of the SGI1-KIV into the chromosome of *S*. Kentucky does not lead to an apparent decrease in bacterial fitness, under the tested conditions *in vitro* and *in vivo*.

Discussion

Despite increasing awareness and control programs implemented by governments and global health organizations aimed at reducing the *Salmonella* contamination in the food chain, *Salmonella* infections continue to pose a significant health threat and economic burden in both developed and developing countries (Scallan *et al*., 2011; Painter *et al*., 2013; Founou *et al*., 2016).

Although more than 2600 NTS serovars have been identified thus far (Gal-Mor, 2019), many of which differ in their host specificity and ecology and only a subset of them are commonly associated with poultry (Shah *et al*., 2017). Traditionally, NTS serotypes such as Enteritidis and Typhimurium were known to be widely prevalent in the poultry production. However, in recent years, due to the *Salmonella* control measures that were implemented in the poultry production chain, a shift in poultry-associated salmonellae has been reported, resulting in the spread of certain poultry-adapted and antibiotic resistant clones (Antunes *et al*., 2016). These trends are now apparent in Israel too and in recent years, the leading NTS serovars in poultry were Infantis, Kentucky and Hadar, characterized by high prevalence of antibiotic resistant clones. These results are in agreement with other recent reports showing that *S*. Kentucky has emerged to become the most predominant serotype associated with poultry in the United States (Shah *et al*., 2017) and with the emergence of *S*. Infantis in European poultry (EFSA, 2018). This shift in population dynamics of poultry-associated salmonellae should not be

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overlooked as it may present direct consequences on the safety and economy of the global food chain.

Noticeably, while *S*. Typhimurium and *S*. Enteritidis are prevalent among clinical isolates, their frequency among poultry samples is as low as 2%, due to mandatory surveillance, biosafety and vaccination measures, targeted specifically against these serovars in flocks of broilers in Israel. Concurring, the correlation coefficient shows disparity between the temporal epidemiological trends of these serovars in the poultry and the clinical sectors, suggesting that the main reservoir of human salmonellosis caused by serovars Typhimurium and Enteritidis, is most likely not the broiler production in Israel. On the other hand, the poultry-associated serovars Infantis, Muenchen, Virchow and Newport display a statistically significant correlation between temporal occurrence in poultry and humans, which may imply that broiler is one of the main sources for human infection caused by these serovars.

Since the late 1990s, several MDR *Salmonella* strains have been identified and since then, their occurrence in humans, domestic and wild animals has been increasing globally (Jajere, 2019). More recently, the prevalence of *Salmonella* resistance to first-line treatment agents such as fluoroquinolones and third-generation cephalosporins has been spreading worldwide (Angelo *et al*., 2016; Michael and Schwarz, 2016; Iwamoto *et al*., 2017). Our results point out a worrisome escalation as 60% from all NTS isolates from poultry in Israel were found to be resistant to three or more classes of antibiotics. These results are moderately higher than the overall rate of MDR NTS isolates in broiler meat in the EU that was recently reported to be 50.3% (EFSA, 2018). High prevalence of antimicrobial resistance exceeding 60% in poultry isolates is an emerging problem and was reported as a significant concern in the poultry production in the United States, as well (Shah *et al*., 2017).

It is now well accepted that extensive usage or misuse of antimicrobial agents for treating human and animal infections as well as growth promoters in livestock has facilitated the emergence and dissemination of MDR strains (Antunes *et al*., 2016; Founou *et al*., 2016; Helke *et al*., 2017). In agreement with this notion, when considering the ARGs repertoire found in the current cohort, it becomes apparent that the majority of these genes confer resistance to antimicrobial compounds that are normally used for extended period of time in both clinical and veterinary practice, including ampicillin, tetracyclines, streptomycin, and sulfonamides. Furthermore, many of these genes are coded on mobile genetic elements that can be circulated between different bacterial species not limited to *Salmonella enterica*. Indeed, in the context of the One Health paradigm, it is important to mention that emergence of resistant bacterial strains and their transmission from food producing animals to humans is not specific to *Salmonella* and have been also reported for *Campylobacter jejuni*, *Escherichia coli*, *Staphylococcus aureus* and other bacteria (Lammie and Hughes, 2016).

Among the 11 poultry-associated serovars analysed, *S*. Kentucky was the most conspicuous as 97% of all its isolates were MDR and 92% of all *S*. Kentucky isolates were ciprofloxacin resistant. Despite the high prevalence of *S*. Kentucky in chickens, this serovar appeared to cause relatively low rate (3%) of human salmonellosis. Similar results were also reported when the *S*. Kentucky prevalence was compared between humans and poultry in the United States (Shah *et al*., 2017), suggesting that this serovar is a prolific colonizer of the chicken gut and possibly more adapted to the poultry host than to humans. Interestingly, Cheng and colleagues suggested that *S*. Kentucky has a reduced virulence and that it is metabolically adapted to the chicken cecum (Cheng *et al*., 2015).

An MDR clone of *S*. Kentucky has been emerging globally since the 1990s, and it is frequently isolated from chicken and turkey farms (Le Hello *et al*., 2011; Westrell *et al*., 2014). This epidemic of ciprofloxacin-resistant *S*. Kentucky clone belongs to sequence type (ST) 198 and was shown to acquire a certain type of SGI1-K into its chromosome around 1989, probably in Egypt (Le Hello *et al*., 2012).

Recently, Hawkey *et al*. showed high diversity of SGI1-K derivatives among *S*. Kentucky ST198 isolates that were shaped by IS26-mediated insertions and deletions events. Some of these isolates harbour large deletions of the SGI backbone but retained their multidrug-resistance region between the *trmE* and *yidY* genes (Hawkey *et al*., 2019). Our results demonstrate new types of SGI1-K organization. One type that was found in isolate 161365, harbours an intact SGI1 backbone, but possesses the mobile elements Tn1721, Tn21 and In4 in a separated region, located about 50 kb downstream from *trmE*, which normally define the 3' border of SGI1-K. The discontinuation of the ARGs cluster containing Tn1721, Tn21 and In4 from the SGI backbone was likely mediated by the mobilized activity of IS26, which flanks this region. The other SGI1-K type that was identified in isolate 162835 harbours the SGI1 backbone and Tn3 in the known locus, between *yidY* and *trmE*, but lacks the other normally associated elements (Tn5393, Tn1721, Tn21 and In4). Therefore, this strain may represents the ancestral form of SGI1-K, that later collected the rest of the mobile elements comprising the full SGI1-K. Hence, our results demonstrate the high modularity and the genetic plasticity of the SGI1-K element, which is largely shaped by the dynamic transposable nature of IS26.

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Importantly, as site-specific integrative mobilized elements, these types of SGI1-KIV were able to move into recipient *E. coli* strains in the presence of helper IncC plasmid at relatively high frequencies. These results exhibit the public health risk associated with such strains and the dissemination potential of multiple antibiotic resistance cassettes into pathogenic and environmental new bacterial species. The fact that this SGI1-KIV also confer resistance to toxic mercury is likely to contribute to the stability and maintenance of these integrative mobilized elements under antibiotic-free conditions. Several studies have reported the presence of mercury in poultry and their feeds (Van Overmeire *et al*., 2006; Shah *et al*., 2010; Yin *et al*., 2017), which may enhance the selective forces acting to maintain SGI1 in the *S*. Kentucky chromosome.

The genetic flexibility and modularity of resistance islands is not specific to SGI1 and was further demonstrated in the case of the new azithromycin-resistant island in *S*. Blockley. In this case, we found that the known azithromycin-resistant island was genetically linked with the chromosomal aminoglycoside 3'-phosphotransferases *aph* (3'')-Ib and the streptomycin phosphotransferase *strB* genes, creating a new type of combined streptomycin and azithromycin resistance island, we called SASARI. A chromosomal *mphA-mrx-mphR* gene cluster has been recently described in *S*. Blockley from the United Kingdom (Nair *et al*., 2016) as well as in *Proteus mirabilis* from swine farms in China, in which the macrolide inactivation gene cluster was inserted within a SGI1-B element (Lei *et al*., 2015). Antimicrobial resistance against macrolides, specifically erythromycin, may be selected in poultry-associated bacteria, due to the usage of this antibiotic to treat and prevent chicken coccidiosis and respiratory diseases, as growth promoter, to improve feed efficiency and to increase pigmentation. As appose to the SGI1-KIV, SASARI was unable to mobilize into recipient *E. coli* strains in the presence of a helper plasmid; however, it is likely that this region could still disseminate between bacteria by transduction mediated by bacteriophages as an alternative vehicle of HGT.

HGT is a powerful genetic force allowing bacteria evolutionary quantum leaps by acquisition of new virulence and resistance traits (Gogarten and Townsend, 2005). Nonetheless, despite the potential benefit, these events often confer fitness cost (Baltrus, 2013). For example, it was shown that acquisition of antibiotic resistance could result in reduced competitive ability in antibiotic-free environments (Gibreel *et al*., 2005; Vogwill and MacLean, 2015). In contrast, here, we showed that the lateral acquisition of SGI1-KIV into the *S*. Kentucky chromosome does not affect the ability of the carrier strain to grow under nutrient-limited conditions or to cause disease in the mouse, even under non-selective conditions. These results may provide further explanation for the wide dissemination of SGI1-K-like elements among *S*. Kentucky strains in Israel. This possibility is also supported by the PCR screen that included nearly 40 randomly selected *S*. Kentucky poultry isolates, which indicated the presence of SGI1 in 80% of them.

Overall, the data presented here, emphasize the role of the poultry production industry as a reservoir of epidemic MDR strains and mobile genetic elements conferring resistance to medically relevant antibiotics. Poultry emerging MDR clones and new mobile genetic elements harbouring clusters of antibiotic resistance cassettes are likely to disseminate to humans via the food chain. Moreover, decreasing in the prevalence of historically dominant serovars such as Typhimurium and Enteritidis in poultry resulted in the emergence of other previously uncommon serovars and new adapted MDR clones. Integrated measures of inspection, surveillance, next generation sequencing and advanced bioinformatics tools, while implementing the One-Health approach are required to better control antibiotic usage in animal production and to reduce contamination and transmission of *Salmonella* along the food chain.

Experimental Procedures

Epidemiology

Since salmonellosis is a reportable disease in Israel by law, all the microbiology laboratories nationwide are required to submit isolated salmonellae from all sources to the National *Salmonella* Reference Center (NSRC), where serological identification according to the Kauffmann–White-Le-Minor scheme (Issenhuth-Jeanjean *et al*., 2014) is performed. In this study, 16 438 poultry and 27 489 clinical (human) isolates, reported to the NSRC during 2010 and 2018 were included in analyses.

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in the Supporting Information Table S2. Bacterial cultures were routinely grown in Lennox Luria-Bertani (LB; BD Difco) medium at 37°C.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was carried out by the broth microdilution method following the CLSI guidelines (M02-A12, 2017). Azithromycin breakpoints for NTS was calculated according to the CLSI breakpoints for *S*. Typhi, and streptomycin breakpoints were set based on (Garcia-Migura *et al*., 2012).

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In this study 188 NTS isolates were tested for susceptibility to 13 antibiotics (Amoxicillin/ Clavulanic acid, Ampicillin, Azithromycin, Cefoxitin, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Gentamicin, Nalidixic Acid, Streptomycin, Sulfisoxazole, Tetracycline and Trimethoprim/Sulfamethoxazole) by the CMV4AGNF AST Plate (Sensititre, TREK diagnostic systems, Thermo scientific), according to the requirements of the National Antimicrobial Resistance Monitoring System (NARMS). Minimal inhibitory concentration (MIC) was determined by reading the CMV4AGNF AST plates with the VIZION digital MIC viewing system (Thermo scientific).

Whole genome sequencing

Nineteen *S. enterica* MDR isolates from six different serovars were subjected to WGS with Illumina MiSeq platform using 2 × 300 bp paired end reads at the Technoin Genome Center, Haifa, Israel. Low-quality bases and adapter sequences were trimmed using Trimmomatic (Bolger *et al*., 2014) and the average reads size of 276 bp yielded a mean of 137-fold coverage per genome. Three genomes (isolates 161365 and 162835 of *S*. Kentucky and isolate 159838 of *S*. Blockley) were also subjected to Oxford Nanopore MinION sequencing yielded a mean reads size of 6 kb (the maximum read size obtained was 101 kb). All the 19 assembled genomes have been deposited at the NCBI nucleotide database, according to the accession numbers shown in the Supporting Information Table S1.

de novo *assembly, annotation and genome analysis*

The short reads from the Illumina MiSeq sequencing were assembled using Unicycler (Wick *et al*., 2017a) and SPAdes-optimizer (Bankevich *et al*., 2012). Hybrid assemblies (Wick *et al*., 2017b) were generated from the MinION long reads of the three serovars combined with the Illumina short reads using Unicycler. The resulted assemblies were uploaded to the RAST server (Aziz *et al*., 2008) for genome annotating. AMR gene alleles were determined by ResFinder (Zankari *et al*., 2012) and by the RGI-CARD software (Jia *et al*., 2017). Episomal DNA encoding ARGs was visualized with Bandage (Wick *et al*., 2015) and the ARG-ANNOT resistance database (Gupta *et al*., 2014). Plasmid replicons were identified by PlasmidFinder 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/). SGI-K and SASARGI regions were compared to the published reference sequences using Easyfig tool (http://mjsull.github.io/Easyfig/).

S1 nuclease digestion and PFGE analysis

Plasmids size was determined by S1 nuclease digestion followed by PFGE. Briefly, *Salmonella* isolates were grown on LB agar plates for overnight and suspended in cell suspension buffer (100 mM Tris, 100 mM EDTA [pH 8.0]) to an OD_{600} of 1.3–1.4 used to cast DNA extraction agarose plugs. DNA plugs were incubated with 1.7 U of S1 nuclease (New England Biolabs) in a restriction buffer (0.2 M Nacl, 2 mM ZnSO4, 60 mM acetic acid [pH 4.6]) for 1 h at 37°C. Digestion was stopped by incubation of the plugs with 200 μl of ES buffer (1% sodium lauroylsarcosine, 0.5 M EDTA) on ice. Digested DNA was then separated by PFGE according to the PulseNet protocol (Ribot *et al*., 2006). Small plasmids (<15 kb) were purified using AccuPrep Plasmid Mini Extraction Kit (Bioneer) according to the manufacturer instructions. The purified plasmids were linearized by the S1 nuclease as above. The known vector pWSK29 was digested with S1 nuclease and BamHI (New England Biolabs) as a control for plasmid linearization.

Mating experiments

ARGs transfer by conjugation was performed between the MDR isolates as donors and the recipient strains *E. coli* ORN172 (kanamycin resistant) or J5-3 (rifampin resistant) on LB agar plates for 16 h at 37°C. Both strains were grown in LB broth for 16 h with aeration and 1 ml from each strain was harvested by centrifugation and resuspended in 100 μl of fresh LB medium. Equal amounts (10 μl; ~2×10^9 CFU) from each strain were mixed and placed onto LB agar plates for 6 h at 37°C. The conjugation mixture was scraped from the plate and resuspended in 0.5 ml of LB broth. Serial dilutions were plated on LB agar plates supplemented with ampicillin or tetracycline (to select for SGI1-K), or chloramphenicol (to select for isolate 159196 or pVCR94ΔX4 plasmid), or kanamycin (to select for isolates 161378 or 161403) together with kanamycin or rifampin to select for the recipient strain ORN172 and J5-3 respectively.

Molecular biology and cloning

All primers used in this study are listed in the Supporting Information Table S3. Oligonucleotides were purchased from IDT and PCR was carried out using Phusion Hot Start Flex DNA Polymerase (New England Biolabs) or Red load taq master (LAROVA GmbH). Null mutants were constructed using the λ-red-recombination system and a three-step PCR method to produce an amplimer containing the antibiotic resistance gene, as previously described (*Serra-Moreno et al*., 2006). Resistant cassette

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was then eliminated from the genome by using a helper plasmid encoding the FLP recombinase.

Murine CI infections

All mice experiments were conducted according to the ethical requirements of the Animal Care Committee of the Sheba Medical Centre (approval numbers 933/14 and 1182/18) and in line with the guidelines of the National Council for Animal Experimentation. Eight week old female C57BL/6 mice (Envigo, Israel) were pretreated with streptomycin (20 mg per mouse in 100 μl HEPES buffer) 24 h prior to infection. Mice were infected with 1.25×10^7 CFU of a mixed (1:1) inoculum containing the wild-type *S*. Kentucky isolate 161365 (Amp^R) and its isogenic strain lacking SGI1-KIV harbouring kanamycin resistance cassette. *S*. Kentucky strains were grown aerobically with the appropriate antibiotics for 16 h in LB at 37°C. Four days p.i. mice were euthanized and the GI organs were collected on ice and homogenized in 0.7 ml saline. Serial dilutions of the homogenates were plated on XLD agar plates supplemented with ampicillin or kanamycin. CFUs were counted and the competitive index was calculated as $[\Delta\text{SGI1-KIV/wild-type}]_{output}/[\Delta\text{SGI1-KIV/wild-type}]_{input}$.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5 software package (GraphPad Software). A student t-test against a theoretical mean of 1.0 was used to determine statistical significance of the CI values. The Z score test for two population proportions with a two-tailed hypothesis was used to compare the prevalence of the most common serovars in the clinical or environmental sectors. Spearman ranked correlation and *p* values were calculated using *R*'s function cor.test(). *P*-value smaller than 0.05 was considered statistically significant and was indicated in the figures as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant. Error bars show the standard error of the mean.

Acknowledgements

The authors are grateful to Dr. Vincent Burrus from Université de Sherbrooke, Canada for sharing the pVCR94ΔX4 plasmid; to Dr. Benoît Doublet from INRA Val de Loire, France for the *S*. Kentucky 01-2100 strain; and to Dr. Ruth Hall from University of Sydney, Australia for the *S*. Kentucky SRC73 strain. We also thank Mr. Liron Mendel for a valuable help with statistical analyses. The work in the Gal-Mor lab was supported by grant numbers: 20-14-0032 from the Ministry of Agriculture and Rural Development of Israel; I-41-416.6-2017 from the German-Israeli Foundation for Scientific Research and Development (GIF); 3-12435 from Infect-Era /Chief Scientist Ministry of Health; and 2616/18 from the joint ISF-Broad Institute program. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Phylogenetic tree of the sequenced *S*. Kentucky MDR isolates A phylogenetic tree of nine *S*. Kentucky MDR strains was created using Enterobase (Alikhan *et al*., 2018). *S*. Typhimurium LT2 and *S. bongori* SARC 11 were used as outgroup. The bar represents the number of substitutions per site.

Table S1. Genome sequencing and assembly data of the MDR isolates

Table S2. Bacterial strains and plasmids used in this study

Table S3. Primers used in this study

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