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Antimicrobial drug resistance and genetic properties of *Salmonella enterica* serotype Enteritidis circulating in chicken farms in Tunisia

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ABSTRACT

This study focused on 77 isolates of *Salmonella enterica* serotype Enteritidis collected during 2009 to 2013 from healthy and sick chickens and environmental farm samples in Tunisia. Resistance to 14 antimicrobials and the encoding genes were analyzed. 66, 26, 6.5, 3.9 and 1.3% were pan-susceptible or showed resistance to nalidixic acid (Asp87 to Tyr and Asp87 to Asn substitutions in GyrA), ampicillin ($bla_{\text{TEM-1-like}}$ and bla_{SHV}), sulfonamides (*sul1* and *sul3*) and streptomycin (*strB*), respectively. A single isolate with intermediate susceptibility to ciprofloxacin was positive for *qnrB*, whereas *qnrA*, *qnrS* or *aac*(6′)-*Ib-cr* were not detected. The virulotype of the isolates was established by testing ten virulence genes. The *orgA, ssaQ, mgtC*, *siiD*, *sopB* genes, located on *Salmonella* pathogenicity islands, and *spvC* of the serotype-specific virulence plasmid, were common to all isolates. In contrast, the prophage-associated *sopE-1, sodC1* and *gipA* genes and the fimbrial *bcfC* gene were variably represented. All isolates except one contained the virulence plasmid, which appeared either alone or together with one or more additional plasmids. One isolate carried a single plasmid of ca. 90 Kb which may be derived from the virulence plasmid (60 Kb). Overall, seven resistotypes, six virulotypes and six plasmid profiles were identified. *Xba*I-PFGE revealed four related pulsotypes (X1–X4), with 80% of the isolates sharing the X1 pattern. The latter isolates exhibited different resistance, virulence and plasmid profiles, suggesting that mobile genetic elements, particularly prophages and plasmids, are of central importance for the evolution and adaptation of *S.* Enteritidis circulating in chicken farms in Tunisia.

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Introduction

Salmonella enterica is a major zoonotic food-borne pathogen causing outbreaks and sporadic cases of gastroenteritis in humans worldwide [1,2]. Among more than 2500 serotypes of *S. enterica*, serotype Enteritidis (*S.* Enteritidis), which is mainly transmitted through consumption of contaminated poultry meat and egg products [3], is the primary cause of human salmonellosis in many countries, including Tunisia [4–6]. The infection develops with diarrhea, nausea, vomiting and abdominal cramps, and is usually self-limiting. However, *S. enterica* can also cause severe invasive infections, particularly in immune-compromised hosts, the elderly and the very young [7,8]. Antimicrobial agents are not essential to control most *Salmonella* infections, but severe, life-threatening infections require treatment. In these instances, fluoroquinolones and broad spectrum cephalosporins are the drugs of choice [8]. Development of resistance to these key antimicrobials is hence a major problem for public health.

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Multiple factors determine the virulence of *S. enterica*, which are primarily encoded by chromosomal genes, unlinked or clustered in islets (composed by a few genes) and larger pathogenicity islands (SPIs). In *Salmonella* more than 20 SPIs have been detected so far, with SPI1 to SPI5 being linked to well-defined pathogenic processes [9]. SPI-1 and SPI-2 encode different type III secretion systems which deliver effector proteins into the cytosol of the host cell, leading to bacterial invasion of the intestinal epithelium as well as proliferation of *Salmonella* within eukaryotic cells. SPI-3 encodes a magnesium transporter involved in adaptation of *Salmonella* to the adverse intracellular environment. SPI-4 encodes a giant non-fimbrial adhesin (SiiE) and a type I secretion system responsible for its transport. SiiE mediates close interaction with microvilli at the apical side of epithelial cells. SPI-5 is connected with inflammation and chloride secretion during the enteric phase of the disease [9,10]. Other non SPI-associated genes also play an important role during infection, for example, prophage-encoded genes and fimbrial gene clusters [11,12]. In addition, some serotypes of *S. enterica*, including *S.* Enteritidis, harbor virulence-plasmids of variable size, which share the *spv* (*Salmonella* plasmid virulence) locus. The *spv* region seems to promote rapid growth and survival of *S. enterica* within the host cells and is thus believed to play an important role in systemic infections [13].

In Tunisia, a retrospective study on the occurrence of *S. enterica* during an 11-year period (1994–2004), revealed *S.* Enteritidis as the most common serotype recovered from human specimens (24.1%; 1640/6815) and animals (69%; 1551/2249), and the second serotype found in food products (15.8%; 877/5539), only preceded by *S.* Anatum. In addition, *S.* Enteritidis was the most common serotype in poultry (70.3%; 1378/1959) and poultry meat (27.7%; 415/1496) [4]. A more recent study also supported the predominance of *S.* Enteritidis in chicken products along the 2008–2011 years [6]. Despite this, information available on the resistance and genetic properties of isolates from chickens and environmental farm samples in Tunisia is rather limited. The aim of the present study was the characterization of a collection of *S.* Enteritidis isolates obtained from these sources with respect to their resistance properties, virulence gene content, plasmid patterns and *Xba*I pulsed-field gel electrophoresis (PFGE) profiles.

Materials and methods

Bacterial isolates

During the period 2009–2013, we have collected 142 *Salmonella* isolates, which were identified as *S.* Enteritidis (85; 60%), *S.* Eppendorf (22; 15.5%), *S.* Zanzibar (17; 12%), *S.* Typhimurium (7; 5%), *S.* Anatum (5; 3.5%), *S.* Kentucky (3; 2.1%), *S.* Seftenberg (2; 1.3%), *S.* Virchow (1; 0.7%) and *S.* Solt (1; 0.7%). Phenotypic and genotypic characterization of the *S.* Eppendorf isolates have previously been reported [14]. In the present study we focused on *S.* Enteritidis isolates owing to their high incidence. Only 77 out of the 85 originally detected isolates could be recovered for further analysis. They were obtained from samples (feces, organs and/or embryonic eggs) of healthy (38) and sick chickens (8; suffering from colibacillosis or pasteurellosis), and of the farm environment (31; waste of hatching, feathers, dust and water). The isolates were collected in 17 farms (located in central and north eastern regions of the country) or during official controls along the period of four years. Initial detection was done according to ISO method 6579/2002 and the isolates were confirmed as *Salmonella* by API 20E (Bio-Mérieux, Marcy l'Etoile, France). Serotype was determined by slide agglutination with the use of antisera (Bio-Rad, Marnes-la-Coquette, France) to identify somatic O antigens and flagellar H antigens, according to the Kauffmann–White–Le Minor scheme [15].

Antimicrobial susceptibility testing by disk diffusion

Antimicrobial susceptibilities for all *S.* Enteritidis isolates were tested by the disk diffusion method on Mueller–Hinton agar using commercial disks (Oxoid, Madrid, Spain), and results were interpreted according to the Clinical and Laboratory Standards Institute guidelines [16]. The following antimicrobials were used: ampicillin 10 μg (AMP), amoxicillin/clavulanic acid 30 μg (AMC), cefotaxime 30 μg (CTX), cefoxitin 30 μg (FOX), apramycin 15 μg (APR), chloramphenicol 30 μg (CHL), gentamycin 10 μg (GEN), ciprofloxacin 5 μg (CIP), nalidixic acid 30 μg (NAL), streptomycin 10 μg (STR), sulfonamides 30 μg (SUL), tetracycline 30 μg (TET), tobramycin 10 μg (TOB) and trimethoprim 5 μg (TMP). *S.* Enteritidis ATCC 13076 and *S.* Typhimurium ATCC 14028 were used as control strains.

Genes encoding antimicrobial resistance

Genes encoding resistance to ampicillin [bla_{OXA-1}, bla_{PSE-1}, bla_{SHV}, $bla_{TEM-1-like}$], streptomycin [*aadA1*-like, *aadA2*, *strA* and *strB*] and sulfonamides [*sul1*, *sul2*, *sul3*], selected according to the resistance phenotypes, were screened by PCR, using previously reported primers and conditions [17]. The genetic bases of nalidixic acid resistance were established by PCR amplification and sequencing of the quinolone resistance-determining region (QRDR) of the *gyrA* gene of nine isolates selected as representative of different resistance profiles [18]. Sequencing was conducted at Macrogen Europe (Amsterdam, Netherlands). For all *S.* Enteritidis isolates, the *qnrA*, *qnrB*, *qnrS* and *aac*(6′)-*Ib-cr* genes, coding for plasmid-mediated quinolone resistance (PMQR) were also screened by PCR [19]. To investigate the presence of class 1 integrons in *sul1*- and *sul3*-positive isolates, the presence of the *int1* gene was investigated [17].

Virulence genotyping

All *S.* Enteritidis isolates were tested for 10 virulence genes, selected for their established association with *Salmonella* pathogenicity [9–13]. For this, PCR amplifications were performed using previously published primers and conditions [20,21]. Five target genes [*orgA*, *ssaQ*, *mgtC*, *spi*_4D (*siiD*) and *sopB*] are located on SPI1 to SPI5, one (*spvC*) on the virulence plasmid, three (*gipA*, *sodC1* and *sopE1*) on prophages, and one (*bcfC*) on a fimbrial gene cluster.

Plasmid profiles

Plasmid DNA was extracted by the technique of Kado and Liu [22] and analyzed by electrophoresis on 0.6% agarose gels. Plasmids from *Escherichia coli* strains 39R861 and V517 were included as size controls.

Pulsed-field gel electrophoresis analysis

Thirty six representative isolates with different resistance, virulence and plasmid combined profiles were typed by PFGE, using the PulseNet protocol with the *Xba*I (40 U; Takara Biomedical, Madrid, Spain) enzyme (http://www.pulsenetinternational.org/). Electrophoresis was performed in a CHEF-DR III (Bio-Rad Laboratories, Madrid, Spain) with the following settings: initial switch time 2 s, final switch time 63 s, a gradient of 6 V/cm, 120° angle and 21 h of electrophoresis. Migration of the DNA fragments was achieved in 1% agarose gels (w/v; Ultra-Pure DNA Grade Agarose, Bio-Rad) submerged in 0.5X TBE buffer. *Xba*I-digested DNA of *S.* Braenderup H9812 was included as size marker. The gels were visualized under

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312 nm UV light and the images were taken with Gel Doc TM XR (Bio-Rad).

Results

Amongst the 77 *S.* Enteritidis isolates, resistances to nalidixic acid, ampicillin, sulfonamides and streptomycin were observed in 20 (26%), five (6.5%), three (3.9%) and one (1.3%) isolates, respectively. In addition, a single isolate showed intermediate susceptibility to ciprofloxacin, but resistances to cefotaxime, cefoxitin, chloramphenicol, gentamicin, tobramycin and trimethoprim were not detected (Table 1). Overall, 51 isolates (66.2%) were susceptible to all antimicrobials tested while only 26 (33.8%) were resistant to one or more compounds. The genes responsible for the observed resistances are shown in Table 1. Sulfonamide resistance was encoded by *sul1* or *sul3*, detected in one and two isolates, respectively. The streptomycin-resistant isolate harbored the *strB* gene but was negative for *strA*. The $bla_{\text{TEM-1-like}}$ and bla_{SHV} genes were found in four and one of the ampicillin resistant isolates, respectively. The gene *intI1*, encoding the integrase of class 1 integrons, was not detected in the *sul1*- or *sul3*-positive *S.* Enteritidis isolates. Two mutations in the *gyrA* gene, changing Asp87 (GAC) to Tyr (TAC) and Asp87 (GAC) to Asn (AAC) were identified as responsible for nalidixic acid resistance in three and two of the isolates tested, respectively. In four other cases, mutations in the QRDR of *gyrA* were not detected; thus further research will be needed to establish the genetic basis of their resistance. With regard to PMQR genes, *qnrB* was present in the single isolate with intermediate susceptibility to ciprofloxacin (1.3%), while *qnrA*, *qnrS* and *aac*(6′)-*Ib-cr* genes were not observed. By combining resistant-phenotypes and genes involved, a total of seven profiles (R1 to R7) were identified (Table 1).

The presence or absence of ten virulence genes was investigated in the panel of 77 *S.* Enteritidis isolates by PCR amplification with specific primer pairs (Table 1). The *orgA, ssaQ, mgtC, siiD* (*spi4_D*) and *sopB*, selected as markers of SPI1 to SPI5, and the *spvC* used as indicator of the virulence plasmid of *S.* Enteritidis, were found in all isolates. The prophage-associated *sopE1*, *gipA* and *sodC* genes were detected in 75 (97.4%), 74 (96.1%) and 37 (48%) isolates, respectively. The *bcfC* fimbrial gene was present in 75 (97.4%) isolates. Overall, we observed six distinct virulence profiles (V). The V1 profile that included all tested genes was found in 34 (44.1%) isolates; the V2 profile lacking the *sodC1* gene was found in 36 (46.8%) isolates; the V3, V4 and V5 profiles lacking *bcfC*, *sopE* plus *sodC1* and *sodC1* plus *gipA*, respectively, were each shown by two isolates; and the V6 profile lacking *gipA* was represented by a single isolate.

Six plasmid patterns (P1–P6) were found in the present study (Fig. 1A; Table 1). All except one isolate harbored a plasmid of ca. 60 Kb, which is the size expected for the virulence plasmid of *S.* Enteritidis. This plasmid appeared either alone (67 isolates; 87%; P1 profile) or together with one or more additional plasmids ranging in size from ca. 2 Kb up to 80 Kb (profiles P2–P5, with two, five, one and one isolates, respectively). The remaining isolate carried a single plasmid of ca. 90 Kb (profile P6).

Altogether, 19 combinations of resistance, virulence and plasmid profiles were identified (Table 1). The most frequent combination (35.1%) corresponded to susceptible isolates (R0), carrying all virulence genes tested (V1) and containing the virulence plasmid alone (P1). Isolates positive for the virulence plasmid, lacking *sodC1* and being either susceptible (23.4%) or resistant to nalidixic acid (15.6%) were also very common. The genetic relatedness of 36 isolates covering different combinations of resistance, virulence and plasmid profiles, were investigated by PFGE. Four closely related pulsotypes, named X1–X4, were found (Fig. 1B; Table 1). The major pulsotype was X1 that encompassed 29 isolates, while X2, X3 and X4 included only one, two and four isolates, respectively. X1 was associated with isolates of different origin (healthy and sick chickens as well as environmental samples) and with diverse resistance, virulence and plasmid profiles.

Discussion

In this study, 77 *S.* Enteritidis isolates recovered in Tunisia from chicken facilities (healthy and sick animals as well as the farm environment) during a period of five years were examined for antimicrobial susceptibility and the genetic basis of the observed resistances. More than half of the isolates were susceptible to all antimicrobials tested, which is consistent with other studies indicating that antimicrobial drug resistance is uncommon in *S.* Enteritidis [23–25]. In the present study, a low incidence of streptomycin, ampicillin and sulfonamides resistance (1.3%, 3.9% and 6.5%, respectively), and a relatively high level of nalidixic acid resistance (26%) were observed. The spread of sulfonamides resistance in *Enterobacteriaceae* including *S. enterica* is largely due to the frequent location of *sul1* and *sul3* genes in class 1 integrons, which often contain gene cassettes encoding resistance also to other antimicrobials. Although these genes were identified as responsible for sulfonamides resistance in the *S.* Enteritidis isolates from Tunisia, neither *intI1* nor any gene cassette characteristically associated with class 1 integrons, were detected [26]. However, the presence of defective class 1 integrons cannot be ruled out.

Resistance to quinolones has increasingly been reported in *Enterobacteriaceae* from animal and human origin worldwide. Particularly, poultry-derived *S.* Enteritidis isolates have been shown to be highly resistant to nalidixic acid, probably due to the widespread use of quinolones in poultry production systems [27]. Antimicrobial agents used to be widely administered in animal husbandry for growth promotion, a practice now banned in many countries, but still common in others. In Tunisia it was banned since 1999, although the law is not enforced. Unfortunately, official documents about the use of antimicrobials in Tunisian avian husbandry are not available. However, according to veterinarians and farmers, florfenicol (fenicol), ofloxacin (second generation fluoroquinolone) and doxycycline (tetracycline) are commonly administered in avian farms to treat some infections, specifically chronic respiratory diseases, colibacillosis or necrotic enteritis. These antimicrobials are sometimes excessively applied with a concurrent lack of bacteriological analysis. Thus, a better control of antimicrobial usage in chicken production systems in Tunisia is required.

Quinolone resistance is usually associated with alterations in the *gyrA* gene which encodes the A subunit of the target DNA gyrase. In fact, Asp87 to Tyr or Asp87 to Asn mutations were found in the *gyrA* genes of five isolates tested, these being the most common mutations associated with nalidixic acid resistance in *S.* Enteritidis [23,28,29]. A single isolate carried the *qnrB* gene, but none of the isolates was positive for *qnrA*, *qnrS* and *aac*(6′)-*Ib-cr* genes. This is consistent with a low-level rate of dissemination of PMQR among chicken-associated isolates of *S.* Enteritidis in Tunisia. In fact, PMQR genes (*qnrA*, *qnrB*, *qnrS* and *aac(6′)-Ib-cr*) have been previously reported in only five out of 113 nalidixic acid resistant isolates of this serotype (4.4%) recovered from different food sources, but mainly from chicken, during the 2008–2011 period in Tunisia [6]. The frequency of nalidixic acid resistance reported in the latter study (31.4%) was close to the value obtained for the 2009–2013 period reported herein (26%).

The 77 *S.* Enteritidis isolates were further characterized according to virulotype and plasmid content, which resulted in six profiles each. Nearly half of the isolates (44%) shared the same virulotype (V1), which included all virulence genes tested. These genes were selected as markers of regions known to be highly conserved (SPIs

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Table 1
Resistance properties, virulotypes, plasmid profiles and *Xba*I-PFGE profiles of *Salmonella enterica* serotype Enteritidis from chicken and farm environmental samples.

R[a] (N[b])	Phenotype[c]	Genotype (N[b])	V[d] (N[b])	Detected virulence genes	RV[e]	P[f] (N[b])	RVP[g] (N[b])	RI[h] (O[i])	X[j]
R0 (51)			V1 (28)	*orgA-ssaQ-mgtC-siiD-sopB-sopE1-sodC1-gipA-bcfC-spvC*	R0V1	P1 (27)	R0V1P1 (27)	432I (HCk)	X1
						P4	R0V1P4	49I (HCk)	X1
			V2 (20)	*orgA-ssaQ-mgtC-siiD-sopB-sopE1-gipA-bcfC-spvC*	R0V2	P1 (18)	R0V2P1 (18)	816/09lit (F_{env})	X1
						P2	R0V2P2	1990Dv (F_{env})	X3
						P6	R0V2P6	198/09Dv3 (F_{env})	X3
			V3	*orgA-ssaQ-mgtC-siiD-sopB-sopE1-sodC1-gipA-spvC*	R0V3	P1	R0V3P1	510/09 (F_{env})	X1
			V5 (2)	*orgA-ssaQ-mgtC-siiD-sopB-sopE1-bcfC-spvC*	R0V5	P1 (2)	R0V5P1 (2)	728/09Dch (F_{env})	X1
R1 (17)	NAL	GAC/AAC (Asp87Asn)	V1 (3)	*orgA-ssaQ-mgtC-siiD-sopB-sopE1-sodC1-gipA-bcfC-spvC*	R1V1	P1 (2)	R1V1P1 (2)	227Dch (F_{env})	X4
		ni				P2	R1V1P2	Ssp1 (HCk)	X2
		GAC/TAC (Asp87Tyr) GAC/AAC (Asp87Asn)	V2 (12)	*orgA-ssaQ-mgtC-siiD-sopB-sopE1-gipA-bcfC-spvC*	R1V2	P1 (12)	R1V2P1 (12)	ST2 (HCk)	X1
								312I (HCk)	X1
		GAC/TAC (Asp87Tyr)	V3	*orgA-ssaQ-mgtC-siiD-sopB-sopE1-sodC1-gipA-spvC*	R1V3	P1	R1V3P1	685/09lit (F_{env})	X1
		ni	V6	*orgA-ssaQ-mgtC-siiD-sopB-sopE1-sodC1-bcfC-spvC*	R1V6	P1	R1V6P1	560-S2 (HCk)	X1
R2 (3)	AMP	$bla_{TEM-1-like}$ (2) bla_{SHV}	V2 (3)	*orgA-ssaQ-mgtC-siiD-sopB-sopE1-gipA-bcfC-spvC*	R2V2	P3 (3)	R2V2P3 (3)	11/013 (SCk)	X1
R3	STR	*strB*	V1	*orgA-ssaQ-mgtC-siiD-sopB-sopE1-sodC1-gipA-bcfC-spvC*	R3V1	P1	R3V1P1	58/013 (SCk)	X1
R4 (2)	SUL	*sul3*	V1	*orgA-ssaQ-mgtC-siiD-sopB-sopE1-sodC1-gipA-bcfC-spvC*	R4V1	P1	R4V1P1	238S (HCk)	X1
			V2	*orgA-ssaQ-mgtC-siiD-sopB-sopE1-gipA-bcfC-spvC*	R4V2	P1	R4V2P1	539W (HCk)	X1
R5	AMP-NAL	$bla_{TEM-1-like}$-GAC/AAC (Asp87Asn)	V4	*orgA-ssaQ-mgtC-siiD-sopB-gipA-bcfC-spvC*	R5V4	P3	R5V4P3	1024E (F_{env})	X4
R6	SUL-NAL	*sul1*-ni	V1	*orgA-ssaQ-mgtC-siiD-sopB-sopE1-sodC1-gipA-bcfC-spvC*	R6V1	P3	R6V1P3	595/09Dch (F_{env})	X1
R7	AMP-NAL-CIP[I]	$bla_{TEM-1-like}$-ni-*qnrB*	V4	*orgA-ssaQ-mgtC-siiD-sopB-gipA-bcfC-spvC*	R7V4	P5	R7V4P5	1025E (F_{env})	X4

+, positive and −, negative for the indicated gene; ni, not identified.
[a] Resistance profile.
[b] Number of isolates when more than one.
[c] AMP, ampicillin; CIP, ciprofloxacin; I, intermediate susceptibility; NAL, nalidixic acid; STR, streptomycin; SUL, sulfonamides.
[d] V, Virulence profile.
[e] RV, combined Resistance and Virulence profile.
[f] P, Plasmid profile.
[g] RVP, combined Resistance, Virulence and Plasmid profile.
[h] RI, Representative Isolate.
[i] O, Origin; HCk, Healthy Chicken; SCk, Sick Chicken; F_{env}, Farm environment.
[j] X, *Xba*I-pulsed-field gel electrophoresis profile.

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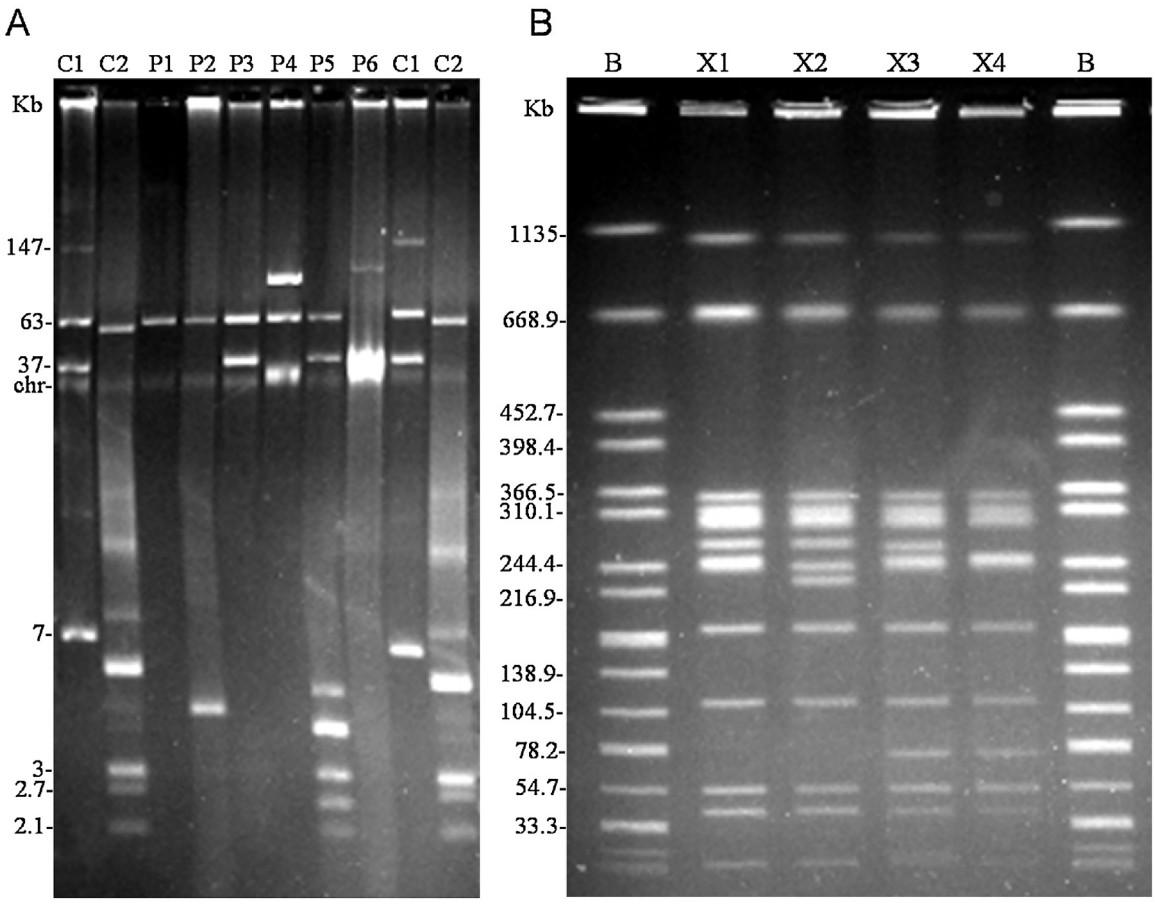

Fig. 1. Plasmid (A) and *Xba*I-pulsed-field gel electrophoresis (B) profiles of *Salmonella enterica* serotype Enteritidis isolates recovered from chicken samples in Tunisia (2009–2013). (A) Lanes C1 and C2, plasmids obtained from *Escherichia coli* 39R861 (NCTC 50192) and V517 (NCTC 50193) used as size standards for undigested DNA; lanes P1 to P6, plasmid profiles; Chr, chromosomal DNA. (B) lane B, *Xba*I-digested DNA of *Salmonella enterica* serovar Braenderup H9812 used as size standard; lanes X1 to X4, *Xba*I-PFGE profiles.

and virulence plasmid) or variable (prophages and fimbrial operons) in *S.* Enteritidis. It is well known that prophages play a key role in the evolution of pathogenic bacteria such as *S. enterica* [11], leading to genotypic and phenotypic diversity even between isolates of the same serotype. Fimbriae also contribute to bacterial diversity and are considered to be important for host adaptation. Accordingly, all isolates tested here were positive for targets located on SPIs and for *spvC*, while the observed variations were related to the *gipA*, *sodC1* and *sopE1* genes, carried by prophages Gifsy-1, Gifsy-2 and a cryptic λ-like phage in *S.* Enteritidis, as well as to the *bcfC* fimbrial gene [11,12,30]. The least frequent virulence target was *sodC1*, found in less than half of the isolates, while the frequency of all other variable genes was ≥95%. The gene *sodC1* encodes a periplasmic Cu–Zn superoxide dismutase that promotes survival of *S.* Typhimurium in macrophages [31]. In contrast to the present study, high and low frequencies were respectively reported for *sodC1* and *gipA* in *S.* Enteritidis isolates from different sources (human, poultry and food) in nine European countries [21,32]. This raises the interesting possibility of geographical variations between Europe and North Africa, but similar variations have not been observed within Europe [21].

As mentioned above, the *spvC* gene was detected in all isolates analyzed in the present study, although *S.* Enteritidis isolates lacking the virulence plasmid have occasionally been reported [20,21,23,33]. It should be noted that all except one of the isolates carried a 60 Kb plasmid, which is the size expected for the virulence plasmid. The remaining isolate contained a ca. 90 Kb plasmid which may have originated from the smaller version, as the isolate was positive for *spvC*. In fact, larger derivatives of the virulence plasmid, most carrying multiple resistance genes, have previously been reported in clinical isolates of *S.* Enteritidis [20,34,35]. However, the Tunisian isolate harboring the 90 Kb plasmid was pan-susceptible, so the role of the DNA which was possibly acquired remains unknown. In *S.* Enteritidis, plasmids of different sizes (ca. 7, 40, 90 and 100 Kb) have been implicated in the spread of the bla_{TEM-1} gene for ampicillin resistance [23,36]. In this study, all ampicillin resistant isolates carried a 40 Kb plasmid where the bla_{TEM-1} gene could be located. Similarly, PMQR genes have been found on plasmids of variable size and incompatibility groups, indicating that multiple plasmids are responsible for the worldwide spread of these genes [37]. Together with 60 Kb and 40 Kb plasmids, several small plasmids, ranging in size from 2 to 6 Kb, were detected in the single *qnrB*-positive isolate identified in this study, which was additionally resistant to ampicillin.

*Xba*I-PFGE analysis conducted for representative isolates identified four closely or possibly related pulsotypes, according to the criteria of Tenover et al. [38], with a clear predominance of one of them (X1). This pulsotype was previously associated with human isolates recovered from diarrheagenic stool samples in Tunisia, as well as from feces and other clinical samples in different countries [5,20,39]. Interestingly, X1 isolates exhibited different resistance, virulence and plasmid patterns, suggesting that mobile genetic elements, particularly prophages and plasmids, are playing an active role in the evolution of *S.* Enteritidis circulating in chicken farms in Tunisia.

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Competing interest

Not declared.

Ethical approval

Not required.

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References

[1] EFSA (European Food Safety Authority). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012. EFSA J 2014;12:3547.
[2] Huang JY, Henao OL, Griffin PM, Vugia DJ, Cronquist AB, Hurd S, et al. Infection with pathogens transmitted commonly through food and the effect of increasing use of culture-independent diagnostic tests on surveillance—foodborne diseases active surveillance network, 10 U.S. Sites, 2012-2015. MMWR Morb Mortal Wkly Rep 2016;65:368–71.
[3] Foley SL, Nayak R, Hanning IB, Johnson TJ, Han J, Ricke SC. Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. Appl Environ Microbiol 2011;77:4273–9.
[4] Ben Aissa R, Al-Gallas N, Troudi H, Belhadj N, Belhadj A. Trends in *Salmonella enterica* serotypes isolated from human, food, animal, and environment in Tunisia, 1994-2004. J Infect 2007;55:324–39.
[5] Abbassi-Ghozzi I, Jaouani A, Aissa RB, Martinez-Urtaza J, Boudabous A, Gtari M. Antimicrobial resistance and molecular analysis of non-typhoidal *Salmonella* isolates from human in Tunisia. Pathol Biol 2011;59:207–12.
[6] Al-Gallas N, Abbassi MS, Gharbi B, Manai M, Ben Fayala MN, Bichihi R, et al. Occurrence of plasmid-mediated quinolone resistance determinants and *rmtB* gene in *Salmonella enterica* serovar Enteritidis and Typhimurium isolated from food-animal products in Tunisia. Foodborne Pathog Dis 2013;10:813–9.
[7] Parry CM. Antimicrobial drug resistance in *Salmonella enterica*. Curr Opin Infect Dis 2003;16:467–72.
[8] Butaye P, Michael GB, Schwarz S, Barrett TJ, Brisabois A, White DG. The clonal spread of multidrug-resistant non-typhi *Salmonella* serotypes. Microbes Infect 2006;8:1891–7.
[9] Schmidt H, Hensel M. Pathogenicity islands in bacterial pathogenesis. Clin Microbiol Rev 2004;17:14–56.
[10] Gerlach RG, Jackel D, Stecher B, Wagner C, Lupas A, Hardt WD, et al. *Salmonella* pathogenicity island 4 encodes a giant non-fimbrial adhesin and the cognate type 1 secretion system. Cell Microbiol 2007;9:1834–50.
[11] Ehrbar K, Hardt WD. Bacteriophage-encoded type III effectors in *Salmonella enterica* subspecies 1 serovar Typhimurium. Infect Genet Evol 2005;5:1–9.
[12] van Asten AJ, van Dijk JE. Distribution of "classic" virulence factors among *Salmonella* spp. FEMS Immunol Med Microbiol 2005;44:251–9.
[13] Guiney DG, Fierer J. The role of the *spv* genes in *Salmonella* pathogenesis. Front Microbiol 2011;2:129.
[14] Ben Salem R, Abbassi MS, García V, García-Fierro R, Njoud C, Messadi L, et al. Detection and molecular characterization of *Salmonella enterica* serovar Eppendorf circulating in chicken farms in Tunisia. Zoonoses Public Health 2016;63:320–7.
[15] Issenhuth-Jeanjean S, Roggentin P, Mikoleit M, Guibourdenche M, de Pinna E, Nair S, et al. Supplement 2008-2010 (no. 48) to the White-Kauffmann-Le Minor scheme. Res Microbiol 2014;165:526–30.
[16] Clinical and Laboratory Standards Institute (C.L.S.I.). Performance standards for antimicrobial susceptibility testing: twenty-fifth informational supplement M100-S25; 2015.
[17] García P, Guerra B, Bances M, Mendoza MC, Rodicio MR. IncA/C plasmids mediate antimicrobial resistance linked to virulence genes in the Spanish clone of the emerging *Salmonella enterica* serotype 4,[5],12:i. J Antimicrob Chemother 2011;66:543–9.
[18] Martínez N, Mendoza MC, Rodríguez I, Soto S, Bances M, Rodicio MR. Detailed structure of integrons and transposons carried by large conjugative plasmids responsible for multidrug resistance in diverse genomic types of *Salmonella enterica* serovar Brandenburg. J Antimicrob Chemother 2007;60:1227–34.
[19] Minarini LA, Poirel L, Cattoir V, Darini AL, Nordmann P. Plasmid-mediated quinolone resistance determinants among enterobacterial isolates from outpatients in Brazil. J Antimicrob Chemother 2008;62:474–8.
[20] Soto SM, Rodríguez I, Rodicio MR, Vila J, Mendoza MC. Detection of virulence determinants in clinical strains of *Salmonella enterica* serovar Enteritidis and mapping on macrorestriction profiles. J Med Microbiol 2006;55:365–73.
[21] Huehn S, La Ragione RM, Anjum M, Saunders M, Woodward MJ, Bunge C, et al. Virulotyping and antimicrobial resistance typing of *Salmonella enterica* serovars relevant to human health in Europe. Foodborne Pathog Dis 2010;7:523–35.
[22] Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 1981;145:1365–73.
[23] Soto SM, González-Hevia MA, Mendoza MC. Antimicrobial resistance in clinical isolates of *Salmonella enterica* serotype Enteritidis: relationships between mutations conferring quinolone resistance, integrons, plasmids and genetic types. J Antimicrob Chemother 2003;51:1287–91.
[24] Guedda I, Abbassi MS, Soufi L, Debya R, Hassen AB, Hamami S. Phenotypic and genotypic typing of *Salmonella enterica* serovar Enteritidis isolates from poultry farms environments in Tunisia. Ann Microbiol 2009;59:1–5.
[25] Zou M, Keelara S, Thakur S. Molecular characterization of *Salmonella enterica* serotype Enteritidis isolates from humans by antimicrobial resistance, virulence genes, and pulsed-field gel electrophoresis. Foodborne Pathog Dis 2012;9:232–8.
[26] Hall RM, Collis CM. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. Mol Microbiol 1995;15:593–600.
[27] Hopkins KL, Davies RH, Threlfall EJ. Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: recent developments. Int J Antimicrob Agents 2005;25:358–73.
[28] Kilmartin D, Morris D, O'Hare C, Corbett-Feeney G, Cormican M. Clonal expansion may account for high levels of quinolone resistance in *Salmonella enterica* serovar Enteritidis. Appl Environ Microbiol 2005;71:2587–91.
[29] Kim KY, Park JH, Kwak HS, Woo GJ. Characterization of the quinolone resistance mechanism in foodborne *Salmonella* isolates with high nalidixic acid resistance. Int J Food Microbiol 2011;146:52–6.
[30] Mirold S, Rabsch W, Tschäpe H, Hardt WD. Transfer of the *Salmonella* type III effector *sopE* between unrelated phage families. J Mol Biol 2001;312:7–16.
[31] Craig M, Slauch JM. Phagocytic superoxide specifically damages an extracytoplasmic target to inhibit or kill *Salmonella*. PloS One 2009;4:e4975.
[32] Capuano F, Mancusi A, Capparelli R, Esposito S, Proroga YT. Characterization of drug resistance and virulotypes of *Salmonella* strains isolated from food and humans. Foodborne Pathog Dis 2013;10:963–8.
[33] Hur J, Kim JH, Park JH, Lee YJ, Lee JH. Molecular and virulence characteristics of multi-drug resistant *Salmonella* Enteritidis strains isolated from poultry. Vet J 2011;189:306–11.
[34] Rodríguez I, Guerra B, Mendoza MC, Rodicio MR. pUO-SeVR1 is an emergent virulence-resistance complex plasmid of *Salmonella enterica* serovar Enteritidis. J Antimicrob Chemother 2011;66:218–20.
[35] Rodríguez I, Rodicio MR, Guerra B, Hopkins KL. Potential international spread of multidrug-resistant invasive *Salmonella enterica* serovar Enteritidis. Emerg Infect Dis 2012;18:1173–6.
[36] Tassios PT, Markogiannakis A, Vatopoulos AC, Katsanikou E, Velonakis EN, Kourea-Kremastinou J, et al. Molecular epidemiology of antibiotic resistance of *Salmonella* Enteritidis during a 7-year period in Greece. J Clin Microbiol 1997;35:1316–21.
[37] Jacoby GA, Strahilevitz J, Hooper DC. Plasmid-mediated quinolone resistance. Microbiol Spectr 2014;2.
[38] Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995;33:2233–9.
[39] Pang J-C, Chiu T-H, Chiou C-S, Schroeter A, Guerra B, Helmuth R, Tsen H-Y. Pulsed-field gel electrophoresis, plasmid profiles and phage types for the human isolates of *Salmonella enterica* serovar Enteritidis obtained over 13 years in Taiwan. J Appl Microbiol 2005;99:1472–83.

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