Journal of Infection and Public Health xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Infection and Public Health

journal homepage: http://www.elsevier.com/locate/jiph

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

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ARTICLE INFO

Article history:
Received 5 August 2016
Received in revised form
21 November 2016
Accepted 28 January 2017

Keywords:
Poultry
Antimicrobial drug resistance
qnrB
Virulence genes
PFGE

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.

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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http://dx.doi.org/10.1016/j.jiph.2017.01.012

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

Please cite this article in press as: Ben Salem R, et al. Antimicrobial drug resistance and genetic properties of *Salmonella enterica* serotype Enteritidis circulating in chicken farms in Tunisia. J Infect Public Health (2017), http://dx.doi.org/10.1016/j.jiph.2017.01.012