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Prevalence and characterizaton of *Salmonella* in chicken consumed in military cantines

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Abstract - *Salmonella* is a major cause of foodborne infections world-wide. *Salmonella* outbreaks linked to consumption of poultry meat and eggs constitute a major public health problem in view of the increasing consumption of these relatively inexpensive products. The aim of this study was to determine the presence of *Salmonella* in chicken consumed in Tunisian military cantines. In addition, we sought to characterise the isolates with regard to their sensitivity to antibiotics as well as their virulence. To this end, a total of fifty rawchicken carcasses were assessed for the presence of *Salmonella*. As the number of isolates turned out to be limited, and to provide significant data, we analysed sixteen additional pre-existing chicken-derived isolates. In addition to classic methods for *Salmonella* isolation and serotyping, the isolate's antimicrobial susceptibility was tested using the agar diffusion assay. Finally, the presence of class 1 and 2 integrons as well as virulence genes were determined by the Polymerase Chain Reaction assay. we found that*Salmonella*contaminated 16% of the samples and that all the isolates were of the *Enteritidis* serotype. Furthermore, while all the isolates were devoid of extended-spectrum beta-lactamase-producing *Salmonella*, 29.16% were resistant to two antibiotics. Among these, 16.66% were resistant to nalidixic acid, whereas 12.5% displayed resistanceto tetracyclines. In addition, the isolates were free of integrons, however they expressed virulence genes, including *invE/A*, *ttrC*, *mgtC*, *sop*B, and *spv*C. Overall, we show a high prevalence of *Salmonella* in chicken meat intended for military cantines. The *Enteritidis* serotype is resistant to some antibiotics and is endowed with an alarming potential for pathogenicity owing to expression of virulence genes.

Key words: chicken, *Salmonella*, antibiotic resistance, integrons, virulence genes.

1. Introduction

In view of its high protein content combined with the fact that it is relatively inexpensive, poultry, especially chicken, is among the most consumed type of meat in Tunisia. However, poultry meat constitutes an ideal environment for microbial growth. Meat can be contaminated during handling and processing, and the sources of contamination can be the human hands, cutting instruments and contaminated areas used for meat processing. In addition, inadequate conditions during storage and distribution are known to be favorable to bacterial infestation and growth.

Foodborne illnesses and other infectious diseases caused by contaminated food are usually associated with lack of observance of hygienic rules not only by professional food handlers but also by consumers themselves. Despite control and public awareness measures, *Salmonella* infections engendered by contaminated food still constitute an immense and challenging public health problem. Indeed, *Salmonella spp* can trigger gastroenteritis (also known as salmonellosis), or typhoid, a systemic infection. Humans can also be asymptomatic carriers,thus constituting a potential route for bacterial spread. Salmonellosis is notorious for being amongst the most widely distributed enteric infectious disease worldwide, with an estimated 1.3 billion cases per year (Chimalizeni et al., 2010).

Usually, *Salmonella* infections are of good prognosis, manifesting as a mild, self-limiting gastroenteritis not requiring treatment. The severity of the infection may depend not only on the strain

of the bacteria, but also on the host. Unfortunately, severe infections are life-threatening in immunocompromised individuals, children and the elderly. Salmonellosis in adults is treated with fluoroquinolones, whereas children are mostly treated with third-generation cephalosporins. Other antibiotics, including ampicillin, amoxicillin and chloramphenicol as well as trimethoprime-sulfamethoxazole are occasionally used as substitutes. However, the past decade has been marked by an alarming emergence and wide-spread of drug-resistant *Salmonella* strains, constituting a serious public health risk.

Antibiotic resistance genes are generally found in mobile genetic elements, including integrons and plasmids. Identification of genes encoding virulence factors is of outmost importance as it provides important information that can be useful to control infections. Continuous control through rigorous programs monitoring the prevalence of *Salmonella* in food products combined with molecular characterization are of pivotal importance as they lay down the foundations for well-defined prophylactic measures and a plan of action to limit the spread of potentially threatening bacteria.

Therefore, our study was conceived to: (1) assess the presence of *Salmonella* in chicken meat consumed in military cantines, *i.e*. in an environment of large-scale food preparation; and (2) provide a phenotypic and genotypic characterization of 24 *Salmonella* isolates by assessing their antimicrobial susceptibilty as well as determining the genetic basis for antibiotic resistance and virulence.

2. Material and Methods

2.1. Sample collection

Fifty chicken carcasses were randomly recovered from military units in the Tunis, Bizerte and Béja regions. Samples were taken out by military veterinarians and rapidly transported under cooling conditions (similar to those used during industrial meat transport) to the Laboratoire Militaire d'Analyses Alimentaires. The study was carried out from February to June 2013. At the recieving laboratory, undamaged and non-modified samples were recorded before being tested.

2.2. Sample processing

Samples were processed based on the ISO 6887-2-2003 standard. Briefly, sample preparation included skin cauterization followed by removal of muscle tissue situated deep in the pectoral muscles. After weighing, buffered peptone water was added and the mix was thereafter incubated at ≈ 37°C for 16-20 hours.

2.3. Methods

The protocol for *Salmonella* isolation was done in four steps according to the ISO-6579:2002 standard. The steps are as follows: (1) non-selective pre-enrichment using buffered peptone water (Biokar Diagnostics); (2) selective enrichments I and II in Muller-Kauffman broth and Rappaport-Vassilliadis soy peptone broth (Biokar); (3) spread and selection on XLD and Hektoen enteric agar plates (Biokar). *Salmonella* presence was further confirmed by biochemical analyses using Galerie API 20^{E} (Biomerieux). Serotyping was performed using the agglutination test at the Institut Pasteur de Tunis.Antibiotic susceptibility was determined by using the agar diffusion method (Mueller-Hinton agar, Biokar) according to le Comité d'Antibiogramme de la Société Française de Microbiologie (CA-SFM, 2013).

Extraction of DNA from *Salmonella* isolates was performed using the thermal lysis technique. Briefly, isolates were grown overnight on XLD agar plates at 37°C. After testing for purity of the cultures, streaks of colonies were picked up and suspended in 500 µl distilled water in sterile Eppendorf tubes. To induce bacterial lysis, the tubes were incubated in boiling water for 10 min. After a 10 min-centrifugation at 8000 rpm, supernatants containing DNA from each isolate were recovered, transferred into new Eppendorf tubes and stored at 4°C until use for Polymerase Chain Reaction (PCR) analyses. The PCR reaction was carried out in a 25 µl final volume. The presence of class 1 and 2 integrons in all isolates as well as in positive controls was determined by PCR using the primer sets (Mazel et al., 2000; Saenz et al., 2004) shown in Table 1. Furthermore, PCR was used to detect *Salmonella* pathogenicity islands (SPI-1 to SPI-5) as well as the *Salmonella* plasmid virulence gene *spvC*. The primer sets (Soto et al., 2006) used are shown in Table 2.

Two PCR multiplex assays were used to detect virulence genes, with the first multiplex containing the *spvC* et *sopB* genes and the second multiplex containing genes belonging to pathogenicity islands SPI-1 to SPI-4.

After amplification, PCR products were analysed by gel electrophoresis (40 min at 100 V). Agarose gels (2%) were prepared in 0.5 x TBE buffer containing 0.5 µg/ml ethidium bromide. Ten µl of PCR products mixed with 5 µl loading buffer were loaded into the gel's wells. For fragment size determination, 100 bp or 1 kb DNA ladders (Promega) were included in separate wells. Following electrophoretic separation, DNA bands were visualized and photographed under UV illumination.

Table 1. Primer sequences used for amplification of integrons

Target gene	Forward (F) /Reverse (R)	Primers (5'----3')	Amplified products
intI1	**IntI1-F**	GGGTCAAGGATCTGGATTTCG	483 bp
	IntI1-R	ACATGGGTGTAAATCATCGTC	
intI2	**IntI2-F**	CACGGATATGCGACAAAAAGGT	788 bp
	IntI2-R	GTAGCAAACGAGTGACGAAATG	

Table 2. Primer sequences for amplification of virulence genes

Localization of Target gene	Primers (5'----3')	Target gene/Amplified product
SPI1	CCTACAAGCATGAAATGG AAACTGGACCACGGTACAA	*InvE/A* (500 bp)
SPI2	GGGCGGTACAATATTTCTTTT TCACGAATAATAATCAGTAGC	*bpttrC* (920 bp)
SPI3	TGACTATCAATGCTCCAGTGAAT ATTTACTGGCCGCTATGCTGTTG	*mgtC* (655 bp)
SPI4	GAATAGAAGACAAAGCGATCATC GCTTTGTCCACGCCTTTCATC	*spi4D* (1231 bp)
SPI5	GATGTGATTAATGAAGAAATGCC GCAAACCATAAAAACTACACTCA	*sopB* (1170 bp)
Virulence plasmid (SPV)	ACTCCTTGCACAACCAAATGCGGA TGTCTTCTGCATTTCGCCACCATCA	*spvC* (424 bp)

3. Results and Discussion

3.1. The chicken samples are contaminated by *Salmonella Enteritidis*

We analysed 50 samples of chicken carcasses destined for consumption in military cantines. From these, we recovered eight*Salmonella* isolates of the*Enteritidis*serotype.The *S. Enteritidis* serotype emerged in poultry industry in all western countries during the period spanning 1965 and 1980. In fact, *S. Enteritidis* has become the most common serotype in poultry (Velge et al., 2005). Recent studies showed *that S. Enteritidis* is the first most common serovar in 37 countries with percentages varying between 19.2% (Cameroon) to 49% (Tunisia) in the African continent, and ranging from 5%, to 93.7% in Asia and Europe (Hendriksen et al., 2011).

3.2. Antibiograms revealisolates resistant to nalidixic acid and tetracyclines

Since the number of the above isolates was limited, we included an additional 16 pre-existing chicken-derived *Enteritidis* isolates (from the Laboratoire Militaire d'Analyses Alimentaires) in order to obtain significant data from the antibiotic sensitivity testings and molecular assays.

We thus studied the susceptibility of 24 isolates of *S. Enteritidis* to antibiotics. All isolates were sensitive to β-lactam antibiotics (Figure 1). This is in agreement with previous findings by others who found low prevalence of resistance to β-lactamins, ranging from 0 to 4.8% (Arlet et al., 2006). In general, as regards *enterobacteriaceae*, the emerging strains producing extended-spectrum beta-lactamases (ESBLs) constitute a major challenge. In *Salmonella* from poultry, strains producing ESBLs have been reported, however with low prevalence (Rino et al., 2006; Cloeckaert et al. 2007).

All our isolates were also sensitive to streptomycin, kanamycin, gentamicin, tobramycin, ciprofloxacin and trimethoprim/sulfamethoxazole (Bactrim). However, with quinolones, we found that 4 isolates (16.66 %) were resistant to nalidixic acid (Figure 1). Notably, similar percentages of resistance to quinolones were described previously (Martin et al., 2005). In fact the last decade had witnessed the emergence and spread of resistance to quinolones. This resistance is the result of mutations in the segment of the *gyrA* gene encoding the Quinolone Resistance-Determining Region (Hamidian et al., 2011). A second important mechanism found in *Salmonella* is provided by the active efflux system, specifically the AcrAB-TolC efflux system (Hur et al., 2011).

Finally, we found that 3 isolates (12.5%) were resistant to tetracyclines (Figure 1). Historically, tetracyclines are the most used antibiotics in poultry. Therefore, it is likely that this resistance can

manifest in different ways, including engagement of the efflux system, modification of the ribosomal target as well as enzymatic inactivation, since these mechanisms are the ones most deployed by Gram positive and Gram negative bacteria (Schwarz et al., 2001).

Overall, it emerges that 70.83% (n=17) of our 24 isolates were sensitive to all antibiotics, whereas 29.16% (n=7) exhibited resistance to two antibiotics commonly used in treatments for *Salmonella* infections (Figure 1).

During the early years of the 1990s, isolates from several *Salmonella* serotypes were found to display resistance to multiple antimicrobial compounds, as a result of excessive use of antibiotics in human and veterinarian medicine (Foley and Lynne, 2008). The trends towards resistance to multiple antimicrobial agents has been described in numerous pathogenic bacteria, including *Salmonella* (Hur et al., 2011). Furthermore, strains harboring a conjugative or transmissible plasmid encoding resistance factors constitute a major source for geographic dissemination of antibiotic resistant bacteria.

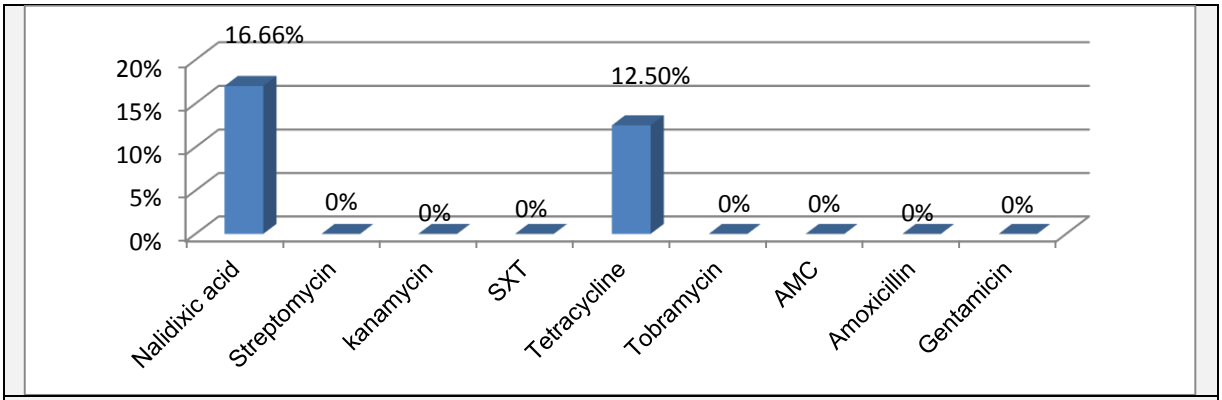

Figure 1. Prevalence of resistance to antibiotics in *S. Enteritidis*

3.3. Absence of class 1 and 2 integrons in the *S. Enteritidis* isolates

Factors for resistance to antibiotics are generally encoded by genes residing within integrons or transposons. These genetic units are located on chromosomes or on plasmids. In *Enterobacteriaceae*, integrons constitute an important basis for capture and expression of resistance genes. Integrons can harbour 1 to 8 resistance genes (Gillings et al., 2008). Drug-multi-resistance in enterobacteria is in part caused by the presence of integrons containing several cassettes of resistance genes.

Several PCR analyses indicated that our 24 isolates were devoid of class 1 and 2 integrons. This finding is in agreement with previous studies by others who found that *S. Enteritidis* usually exhibits weak resistance to antibiotics, likely as a result of lack of integrons (Su et al., 2004).

3.4. Presence of virulence genes in the the *S.Enteritidis* isolates

Non-typhoidal *Salmonella* serovars are the major cause of infections originating from contamined food in developed countries. *Salmonella* is armed with several virulence factors the majority of which are organized into the so-called *Salmonella* pathogenicity islands (SPIs). Other *Salmonella* virulence factors are encoded by genes localizing on prophages and plasmids, *i.e.* outside the SPIs.

It has been shown (Markus et al., 2000; Fierer and Guiney, 2001; Soto et al., 2006) that numerous serovars harbour virulence plasmids (*SPV*) the size of which depends on the serovar. All the V plasmids share in common a highly conserved region of 8 Kb carrying five genes known as *spvRABCD* (spv, *Salmonella* plasmid virulence). The *spv* region plays an important role for rapid growth and survival of *Salmonella* in the host cell, implying that it is of importance in systemic infections (van Asten and van Dijik, 2005).

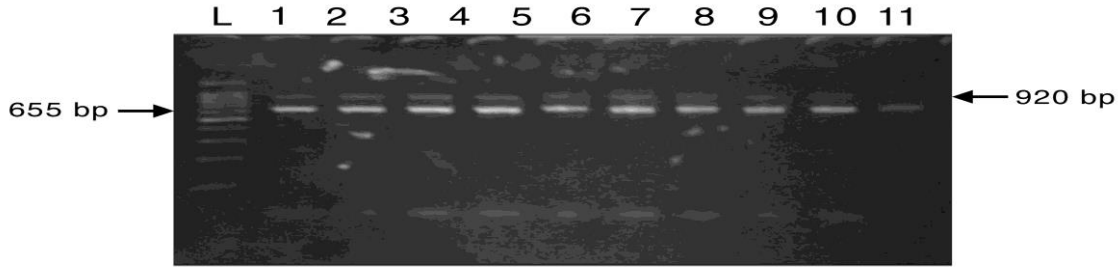

Figure 2. Representative image of PCR products showing bands corresponding to the *ttrC* (SPI-2) and *mgtC* (SPI-3) genes. L, 100 bp DNA ladder. Lanes 1-10 show isolates harbouring the two genes.

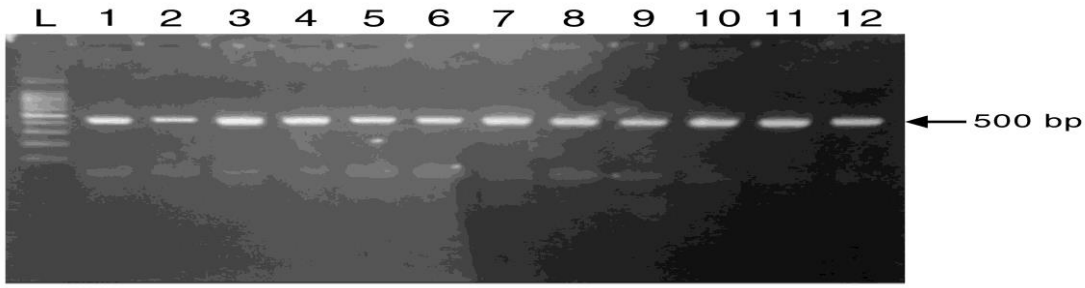

Figure 3. Representative image of PCR products showing bands corresponding to the *siiD* (SPI-4) and *invE/A* (SPI-1) genes. L, 100 bp DNA ladder. Isolates showing the presence of the *invE/A*gene (lanes 1-12).

We have screened the 24 isolates to determine the presence of 6 virulence genes, five of which belonging to the five SPIs (*mgtC, invE/A, ttrC, siiD/spi4D* and *sopB)*, and one is located on the V plasmid of *S. Enteritidis* (*spvC*). PCR amplification (Figs. 2 & 3) disclosed the *invE/A* (SPI-1), *ttrC* (SPI-2) and *mgtC* (SPI-3) genes in all isolates, whereas the *siiD* (SPI-4) gene was absent (data are summarized in Table 3). Furthermore, the *sopB* (SPI-5) gene was readily detectable in 12.5% of the isolates (Figure 4, Table 3). These data are similar to those reported previously (Huehn et al., 2010). In fact, genes belonging to SPI-1, -2 and -3 are known to play an important role in host invasion, while factors encoded by genes at SPI-4 and -5 are of importance for pathogenicity.

The *Salmonella* plasmid virulence gene *spvC* was amplified in11 *Enteritidis* isolates (45.8%; Figure 4; Table 3). This plasmid is generally specific to *S. Enteritidis*. However, while it has also been detected in *Typhimirium*, it was absent in *Infantis*, *Virchow* and *Hadar* (Huehn et al., 2010).

The presence of virulence genes in our isolates reveals their pathogenicity and underscores their potential threat amid an outbreak. This urgently calls for further studies that cover the entire military cantines in the country where food is prepared on a large scale.

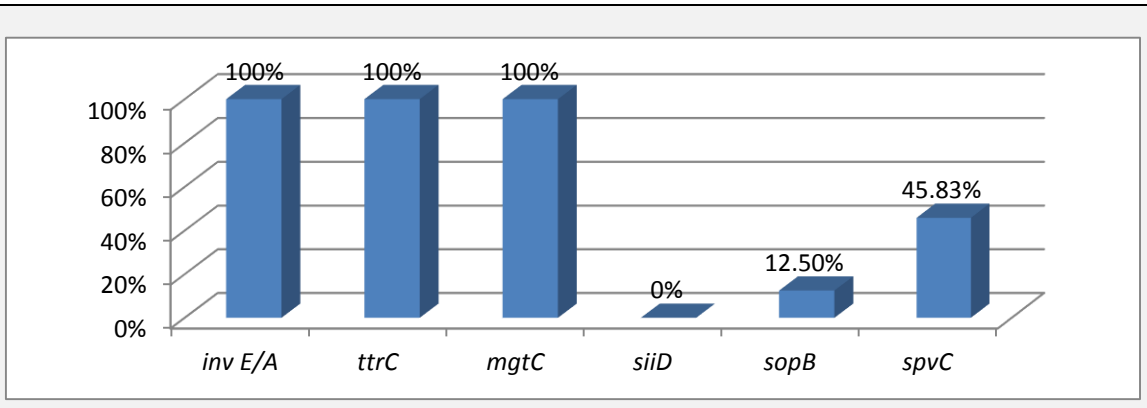

Figure 4 . Prevalence of virulence genes in *S. Enteritidis*

Table 3. Summary of PCR data showing the presence (+) or absence (-) of virulence genes in the different *S. enteritidis* isolates

Number of isolate	SPI-1 *(invE/A)*	SPI-2 *(ttrC)*	SPI-3 *(mgtC)*	SPI-4 *(siiD)*	SPI-5 *(sopB)*	Virulence plasmid *spvC*
1	+	+	+	–	–	+
2	+	+	+	–	–	+
3	+	+	+	–	–	+
4	+	+	+	–	–	+
5	+	+	+	–	+	–
6	+	+	+	–	–	+
7	+	+	+	–	–	+
8	+	+	+	–	–	+
9	+	+	+	–	–	+
10	+	+	+	–	–	–
11	+	+	+	–	+	–
12	+	+	+	–	–	–
13	+	+	+	–	+	–
14	+	+	+	–	–	–
15	+	+	+	–	–	–
16	+	+	+	–	–	–
17	+	+	+	–	–	+
18	+	+	+	–	–	+
19	+	+	+	–	–	–
20	+	+	+	–	–	–
21	+	+	+	–	–	–
22	+	+	+	–	–	–
23	+	+	+	–	–	–
24	+	+	+	–	–	+

4. Conclusion

In view of its ubiquity and its prevalence in food products, combined with its virulence and treacherous adaptability, *Salmonella* has a major impact on public health and global economy. *Salmonella* is among the major causes of bacterial foodpoisoning outbreaks. Our data show a high prevalence of *Salmonella* in chicken carcasses (16%). Furthermore, 29.16% of the isolates were resistant to two antibiotics (the 16 isolates from our pre-existing collection were all sensitive to all antibiotics). Nevertheless, the absence of ESBL-producing *Salmonella* among our isolates is remarkable. Noteworthy also is the fact that our isolates did not harbour integrons, hence, they are devoid of a crucial genetic basis for capture and expression of antibiotic resistance genes. However, the presence of virulence genes in the isolates strongly suggests a potential for pathogenicity, entailing considerable public health risks.

Accurate determination of the prevalence of *Salmonella* in meat from poultry as well as understanding the molecular mechanisms of pathogenicity and antibiotic resistance of this bacteria are essential objectives of food-related bacteriology, that are crucial for implementing well-functioning prophylactic and therapeutic strategies.

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