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Genotypic Characterization of Nalidixic Acid Resistant *Salmonella enteritidis* Isolated from Human and Nonhuman Origin in Morocco

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Abstract

Objectives: *Salmonella* serotype Enteritidis remains the major causative agent of foodborne diseases and human infections worldwide. The purpose of this study was to investigate the molecular characteristics of nalidixic acid resistant *Salmonella enteritidis* in Morocco.

Methods: A total of 862 human and nonhuman *Salmonella* strains were collected and isolated from 2002 to 2018 in different cities in Morocco. Antimicrobial susceptibility was determined using the disc diffusion assay and sequencing the PCR amplified *gyrA* and *parC* genes.

Results: The overall percentage of *Salmonella enteritidis* was 12% (100/862). Drug susceptibility assay revealed that 80% of *Salmonella enteritidis* isolates were resistant to nalidixic acid. Our data further show that this resistance was due to mutations in *gyrA* (Aspartate (D) 87 Asparagine (N)) and in *parC* (Threonine (T) 57 Serine (S)) genes. PCR amplification and plasmid profiling analysis revealed that 80% of strains harbored the *spvC* virulence gene (669 bp) and a 54 kb plasmid.

Conclusion: Our study showed a high rate of nalidixic acid resistant *Salmonella enteritidis* in human and nonhuman isolates in Morocco. Taking this into account is essential in order to take rapid measures in livestock to ensure control of this emergence of multi-resistant bacteria.

Keywords: *Salmonella enteritidis*; Quinolone; sequencing; *spvC*

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Introduction

Salmonella enterica is recognized worldwide as one of the major agents of human gastrointestinal diseases. *Salmonella Enteritidis* (SE) is the most commonly detected serovar in cases of human non-typhoid salmonellosis in Europe [1]. Animals and their products, particularly chicken, meat and eggs, are considered as prominent sources of human infections caused by this pathogen [2]. *Salmonella enteritidis* has been the serotype most frequently associated with human salmonellosis in many African countries, such as Morocco [3]. Antibiotics have been successfully used in poultry for different purposes such as growth promotion, prophylaxis, or therapeutics. However, their indiscriminate use caused an increased bacterial resistance, mainly in *Salmonella* strains. In the past, antimicrobial resistance had been unusual in SE; Since 1990s, the occurrence of resistance to ampicillin and nalidixic acid increased, whereas the resistance to other antibiotics remained sporadic [1]. The aim of the present work is to study the antimicrobial susceptibilities and genetic of *Salmonella enteritidis* isolates from humans and non-human origin in Morocco during the period 2002 to 2018. The strains were received for diagnosis in the Laboratory of food microbiology-Institute Pasteur du Morocco.

Materials and Methods

Bacterial strains

A total of 862 *Salmonella enterica* were collected from human and nonhuman strains

Table 1: Antibiotic pattern of 54 *Salmonella enteritidis* isolated from different cities and origins in Morocco during 2002 to 2018.

Strains	Date of isolation	Origin	Regions	Antibiotic pattern
3/18	2018	Stool	HSK	Na
40/17	2017	Stool	UHC/M	Na
34/17	2017	Stool	UHC/M	Na
26/17	2017	Stool	CLP	Na
14/17	2017	Stool	HSK	Na
17/11	2017	Stool	HSK	Na
17/1	2017	Stool	HSK	Na, CAZ, CRO, Smx, Te, Amp, Sss, C, G, Tmp, Sxt
20/16	2016	Human	UHC/M	Te, Smx, S, Sss, G
10/16	2016	Human	UHC/M	Na
5/6	2016	Stool	UHC/M	Na
1/15	2015	Human	UHC/C	Na, Te, Smx, G
2/15	2015	Human	UHC/C	Na, Te, Smx, Tmp, Sss, G, Sxt
3/15	2015	Human	UHC/C	Na, Te, Smx, Cf, Tmp, Sss, G, Sxt
5/15	2015	Human	UHC/C	Na
2/14	2014	Droppings	RSZZ	Na
3/14	2014	Droppings	RSZZ	Na, G
4/14	2014	Poultry feed	RSZZ	Na
5/14	2014	Poultry feed	RSZZ	Na
17/14	2014	Droppings	SMD	Na
18/14	2014	Droppings	SMD	Na, G
19/14	2014	Droppings	SMD	Sensitive
20/14	2014	Dust	SMD	Na, Te, Smx, G
21/14	2014	Dust	SMD	Na
22/14	2014	Droppings	SMD	Na
23/14	2014	Droppings	SMD	Na
34/14	2014	Droppings	SMD	Na
35/14	2014	Droppings	SMD	Na, Caz, Smx, Te, Amp, Sss, C, G
36/14	2014	Dust	SMD	Na
51/14	2014	Dust	GC	Na
52/14	2014	Droppings	GC	Na, Te, G
53/14	2014	Droppings	GC	Na
54/14	2014	Droppings	GC	Na
55/14	2014	Droppings	GC	Na
57/14	2014	Droppings	GC	S, G
58/14	2014	Droppings	GC	Na, Cf, G
59/14	2014	Droppings	GC	Na
60/14	2014	Droppings	GC	Na, Caz, Amp, Smx
61/14	2014	Droppings	GC	Na, Caz, Amp
69/14	2014	Poultry feed	GC	Amp, Te, Smx, S, Sss, G
74/14	2014	Human	UHC/M	Na, Caz, Amp, C
78/14	2014	Human	UHC/M	Amp, Te, Cro, Smx, Cf, Tmp, Sss, G, C, Sxt
12/6	2012	Human	LCN	Na
12/7	2012	Human	LCN	Na, Te, G, Sxt
12/15	2012	Raw chopped meat	GC	Na
63/12	2012	Poultry feed	GC	Na
11/17	2011	Poultry feed	Tetouan	Na, Caz, Amp, Te, C

47/11	2011	Droppings	Tetouan	Na
48/11	2011	Droppings	Tetouan	Na
9/5	2009	Mayonnaise	TIAC	Na, Cf, G
9/6	2009	Stool	TIAC	Na
49/07	2007	Raw chicken	GC	Na
50/07	2007	Raw chicken	GC	Na, Caz, Amp, Te, Cro, Smx, Sss, G
67/02	2002	Abattoir	Rabat	sensitive
68/02	2002	Abattoir	Rabat	sensitive

HSK: Hospital Sheikh Khalifa; UHC/C: University Hospital Center/Casablanca; RSZZ: Rabat-Sale Zemmour-Zaer; GC: Grand Casablanca; SMD: Souss-Massa-Daraa; UHC/M: University Hospital Center/Marrakech; LCN: private laboratory; CLP: private laboratory. Na: Nalidixic Acid; Amp: Ampicillin; Caz: Ceftazidime; C: Chloramphenicol; Cro: Ceftriaxone; G: Gentamycin; Sxt: Trimethoprim/Sulphametoxazole; Tmp: Trimethoprim; S: Streptomycin; Smx: Sulphametoxazole; Te: Tetracyclin; Sss: Sulfonamide; Cf: Cephalothin

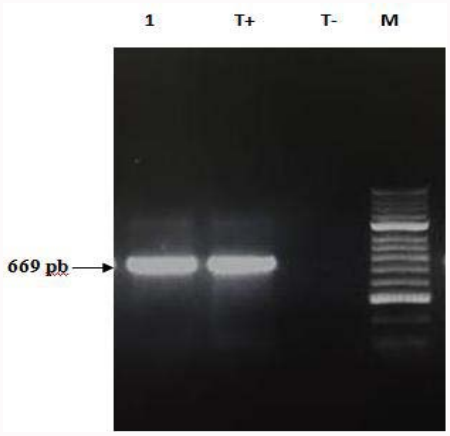

Figure 1: Amplification of the plasmid gene *spv C*.
1: *Salmonella* Enteritidis; T +: Positive control; T-: Negative control; M: Size marker

(environment, animals and food) in different cities in Morocco, from October 2002 to July 2018. Molecular confirmation of the *Salmonella* isolated was carried out using Polymerase Chain Reaction (PCR) technique in order to amplify a 1 kb fragment of DNA of the *InvA* gene of the chromosome specific to *Salmonella* genus (no. M 90846.1 of *Salmonella typhimurium*). All strains were serotype by agglutination tests with *Salmonella* specific anti-sera Bio- Rad (Marne-la-coquette-France). A total of 100 SE from 862 *Salmonella* enteric were identified. Indeed, our study was conducted on 45 *Salmonella enteritidis* from different origin [22 animal (laying hens, turkey, poultry), 12 human stool, 7 food (mayonnaise, ice cream) and 4 environment (dust and water from laying hens farms)].

Antimicrobial susceptibility testing

Antimicrobial susceptibility of the isolates to a panel of antibiotics was determined by the disc diffusion method on Mueller-Hinton agar according to the recommendation of the CA-SFM/EUCAST. Commercials antibiotics discs (n=16) (Bio-Rad, Marne's-la- Coquette-France) were used (µg): ampicillin (10), amoxicillin + clavulanic acid (20/10), Ceftazidime (30), Cefotaxime (30), chloramphenicol (30), ceftriaxone (30), gentamicin (500), Trimethoprim/sulphametoxazole (1,25/23,75), Trimethoprim (5), streptomycin (10), Sulfamethoxazole (300), tetracycline (30), ciprofloxacin (5), sulfonamide (200), cephalothin (30) and nalidixic acid (30). The *Salmonella enteritidis* ATCC 13076 was used as a control to validate stereotyping, antimicrobial susceptibility and PCR methods.

Plasmid analysis

The presence of plasmids in all the *Salmonella Enteritidis* strains was investigated using the modified rapid alkaline method of Kado and Liu [4]. *Escherichia coli* VA517 strain was used as a control.

Detection of *spvC* genes in *Salmonella enteritidis*

All *Salmonella enteritidis* isolates were analyzed for detection of *spvC* gene using specific primers as previously described [5].

Detection of mutations in the Quinolone Resistance-Determining Region (QRDR)

All nalidixic-acid resistant *Salmonella enteritidis* were investigated for the presence of point mutations in the Quinolone Resistance-Determining Region (QRDR) of Topoisomerase genes by sequencing the PCR amplified *gyrA* and *parC* genes as previously described [5]. The genomic DNA was extracted using the rapid "boiling-prep" method. The sequences obtained were compared with those available in Gen Bank (http://www.ncbi.nlm.nih.gov/genbank/index.html).

Results

Antimicrobial susceptibility testing

The results of antimicrobial resistance showed that 80% of *Salmonella enteritidis* were resistant to nalidixic acid, 24% to gentamycin, 15% to tetracycline, 13% to ampicillin, 11% to Sulfamethoxazole, 7% to sulfonamide, cephalothin, 4% to Sulfamethoxazole/Trimethoprim, chloramphenicol, streptomycin and 2% to Trimethoprim (Table 1). It is also important to note, that none of the *Salmonella enteritidis* isolates was resistant to ciprofloxacin or produced beta-lactamases (extended-spectrum beta-lactamases). The resistance to nalidixic acid was variously distributed among isolates of different origin [100% the from environment (dust and water from lying hens farms), 82% in animals (laying hens, turkey, poultry), 75% in human stool and 71% from food (mayonnaise, ice cream)].

Detection of mutations in the Quinolone Resistance-Determining Region (QRDR)

Table 1 showed that nalidixic acid resistant *Salmonella enteritidis* carried one mutation in *gyrA* and one in *parC* genes, which caused the substitution aspartate (D) to asparagines (N) at codon 87 in the *gyrA* gene and the substitution Threonine (T) to serine (S) at codon 57 of the *parC* gene.

Plasmids analysis and *spvC* virulence gene

The amplification of the *spvC* virulence gene using specific primers as previously described generated products of the expected size (669 bp) (Figure 1) [5]. Table 1 showed that *spvC* gene was

detected in 80% (36/45) of the *Salmonella enteritidis*. The plasmid analysis showed that all positive strains for the *spvC* virulence gene harbored a plasmid size 54 kb.

Discussion

The widespread use of antibiotics as supplements for prophylaxis and growth promotion may result in the emergence of antimicrobial-resistant *Salmonella* strains in poultry farms [6]. The results of the present study show a high level (80%) of nalidixic acid resistant *Salmonella enteritidis* strains isolated in Morocco. This high level of nalidixic acid resistance of *Salmonella enteritidis* was previously reported in other studies in Morocco (100% in poultry 37% from laying hens) and the comparable figure for Spain was 61% in food [7-9]. Our data further show that the resistance of *Salmonella enteritidis* isolates to nalidixic acid was observed regardless of its origin (human and nonhuman). In a previous study conducted in Senegal, 2% (1/50) of *Salmonella enteritidis* isolated from human and poultry were found to be resistant to nalidixic acid [10]. Whereas in Algeria, all *Salmonella enteritidis* isolates were reported to be resistant only to sulfonamide, while none of the *Salmonella enteritidis* isolates was resistant to nalidixic acid [11]. In South Korea, 30% of *Salmonella enteritidis* isolates were resistant to nalidixic acid with more than 70% of them being multi resistant [12]. The high rate of nalidixic acid resistance of *Salmonella enteritidis* isolates found in our study could be explained by a Tran's boundary dissemination of these resistant strains due to the geographical proximity of Morocco to Europe or by international trade with Europe. The overuse and misuse of antibiotics as supplements for prophylaxis and treatment of infections, is another potential source of the selection of these resistant organisms. Our data further show that two point mutations in the Topoisomerase genes were found to be associated with nalidixic acid resistance of *Salmonella enteritidis* isolated strains, with one mutation occurring in the *gyrA* gene and the other in *parC* gene. These findings are in agreement with the results of a previous study conducted in North Africa, showing that a mutation in the D87 codon resulted in an asparagines residue [13]. The same substitution was also reported to be associated with nalidixic acid resistance of *Salmonella* Hadar and in ciprofloxacin resistant *Salmonella* Kentucky isolated in Morocco that carried three mutations, two in *gyrA* gene and one in *parC* gene [14,15]. It is also noteworthy that all the *Salmonella enteritidis* strains isolated in the present study since 2007 were resistant to nalidixic acid. Interestingly, the first ciprofloxacin resistant *Salmonella* Kentucky was also isolated from turkeys during the same year. These data clearly suggest that the appearance of an additional mutation could engender a fluoro quinolone resistant *Salmonella enteritidis* population; resulting in a reduced with a diameter of 26 mm susceptibility to ciprofloxacin. The high level of resistance to nalidixic acid could limit or reduce the efficacy of fluoroquinolone used for the treatment of severe salmonellosis [14]. Based on plasmid analysis, our data showing that the *spvC* virulence gene was probably located on a 54 kb plasmid are in accordance with our previous finding and with the study of Madajczak and Binek indicating that the *spvC* virulence gene was located on a 59 kb plasmid [16,17]. Furthermore, Chu et al., [18] demonstrated that among the clinical *Salmonella enteritidis* isolates studied, 50% carried a 50 kb virulence plasmid and 44% carried a plasmid of more than 125 kb. Although, Murgia et al., [14] observed that plasmids of *Salmonella enteritidis* (63 kb) were virulence plasmids .The difference in these virulence plasmids sizes might result from a combination of plasmids to form a larger *pSCVs* (125 kb) by involving two mechanisms, namely co integration, or transposition. In fact, all large *pSCVs* were reported to carry a large portion of both the 50 kb *spvC* and a Non-*pSCV* and were found to be the product of a recombination. However, Chu et al., [18] demonstrated that Enteritidis serovar showed a regular size of virulence plasmids of 60 kb without deviation [18]. This high prevalence of the *spvC* virulence gene detected in *Salmonella enteritidis* strains was also reported in other studies [12]. This is in contrast to another study conducted by Swamy who reported that only 15% of *Salmonella enteritidis* isolates contained the *spvC* gene [19]. The assessed virulence gene is involved in the pathogen city of *Salmonella*, and its presence can cause salmonellosis. It enables this serovar to play a role in extra- intestinal infections, and make it more virulent towards humans [20]. The high level of nalidixic acid resistant *Salmonella enteritidis* in human and nonhuman isolates in Morocco, the risk of reduced with susceptibility to ciprofloxacin and the presence of virulence genes highlight the health risks for consumers. Our study further provided a better understanding on the risks related to the spread of virulent and highly quinolone resistant *Salmonella enteritidis*, which could generate fluoroquinolone resistant population and be directly associated with therapeutic failure. In light of these findings, the antibiotic resistance of *Salmonella* strains appears to be increasing which is of concern. We recommend that a program of health education be given to food preparers and handlers in which they are taught proper means of food handling, hygiene and storage as well as preventive control measures [21-24]. We also recommend more restrictions on the irrational use of antibiotics, and public awareness activities should be undertaken to alert the public to the risks of the unnecessary use of antibiotics.

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