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Original Article

Antimicrobial susceptibility and MLVA analysis of *S.* Typhimurium strains isolated from human and poultry samples in Tunisia

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Abstract

Introduction: *Salmonella enterica* infections are a significant public health concern worldwide, being *Salmonella* Typhimurium one of the most prevalent serovars. Human salmonellosis is typically associated with the consumption of contaminated foods, such as poultry, eggs and processed meat. The extensive use of antimicrobials in humans and animals has led to an increase in multidrug resistance among *Salmonella* strains, becoming multidrug-resistant (MDR) strains a major public health concern.

Methodology: This study was designed to investigate the antimicrobial susceptibility and the genotypic diversity of *Salmonella* Typhimurium strains isolated in Tunisia from human and poultry sources from 2009 to 2015. Fortyfive strains were analyzed by disk-diffusion test to determine the antimicrobial susceptibility. The presence of antimicrobial resistance genes was tested by PCR, and genotyping was performed using multiple-locus variable-number tandem repeats analysis (MLVA).

Results: About 50% of the strains were resistant to at least 3 antibiotics (multidrug-resistant strains, MDR). The most frequent resistance profile in clinical strains was AMP-TIC-TET-MIN-SXT (n = 7) and TET-MIN in poultry origin strains (n = 7). The MLVA typing grouped the strains in 2 main clusters. Cluster I was mostly formed by human isolates, whereas in cluster II both human and poultry isolates were grouped. Simpson's diversity index was 0.870 and 0.989 for antimicrobial resistance profiles and MLVA, respectively.

Conclusions: Multiresistance is common in *Salmonella* Typhimurium isolated from human and poultry sources in Tunisia. The genotyping results suggest that some strains isolated from both sources may descend from a common subtype.

Key words: *Salmonella* Typhimurium; multidrug-resistance; antibiotic resistance genes; MLVA.

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Introduction

Salmonella enterica infections are a significant public health concern worldwide, with an estimated 93.8 million illnesses annually, of which 80.3 million are estimated to be food-borne [1]. Human salmonellosis is typically associated with the consumption of contaminated foods, such as poultry, eggs and processed meat [2,3]. The extensive use of antimicrobials in humans and animals has led to an increase in multidrug resistance among several bacterial strains. Multidrug-resistant (MDR) *Salmonella* strains have been among the major public health concerns worldwide [4]. Subsequent transmission of antimicrobial resistance to humans can be in the form of either resistant pathogens or commensal organisms carrying transferable resistance genes [5].

Over the last several decades, there have been significant changes in the predominant *Salmonella* serovars associated with poultry and human infections [6-8]. *Salmonella* serovar Typhimurium (*S.* Typhimurium) is one of the most important and frequent foodborne pathogens, being responsible of a high number of foodborne outbreaks [9]. In this sense, there is a need for highly discriminatory typing of isolates in order to be able to study the relatedness among strains, which can then aid in the contamination

sources tracking [10,11]. Bacterial genomes contain thousands of tandem repeats (TR) loci; some of them are polymorphic due to the variations in repeat copy number. Such loci, called variable-number tandem repeats (VNTRs), possess a wide range of mutability [12], making VNTRs useful for assessing genetic relatedness among strains [13]. In the last decade, many multilocus VNTR analysis methods have been developed to perform an accurate typing and phylogenetic analysis of bacterial pathogens [14]. Multiple-locus variable-number tandem repeats analysis (MLVA) is a PCR-based method that has recently become a widely used highly discriminatory molecular method for *Salmonella* typing based on amplification and analysis of the tamdem repeats copy number. This method was able to discriminate between sporadic cases of human clinical isolates in Tunis, meanwhile the pulsed-field gel electrophoresis (PFGE) technique was not [15].

Therefore, the aims of the present study were to assess the antimicrobial resistance, both phenotypically and genotypically, and to determine the usefulness of MLVA to characterize *S*. Typhimurium strains isolated from human and poultry sources in Tunisia.

Methodology

Bacterial strains

A total of 45 *Salmonella* Typhimurium isolates obtained from 2009 to 2015, including 27 human clinical isolates and 18 from poultry origin, were analyzed in this study. The human isolates were recovered from stool (n = 6), blood (n = 16), urine (n = 3) and pus (n = 2) at the Microbiology Laboratory of Charles Nicolle Hospital of Tunis. The poultry isolates, including sick poultry organs (n = 3), turkey meat (n = 2) and carcasses (n = 13) were obtained from the Microbiology Department of The National School of Veterinary Medicine of Tunis.

Species identification

Strains were identified using conventional methods and API 20E (bioMérieux, Marcy l'Etoile, France) and confirmed by *Salmonella* genus specific PCR [16]. Serotyping was done using commercially available poly and monovalent antisera at the Microbiology Laboratory of Charles Nicolle Hospital, and confirmed at the National Center of Enteropathogenic Bacteria (Pasteur Institute of Tunis). Molecular serotyping was also performed by multiplex PCR at the Immunology, Microbiology and Parasitology department of the UPV/EHU [17].

Antimicrobial susceptibility testing and resistance genes detection by PCR

Antimicrobial susceptibility was performed by the disk diffusion method on Mueller-Hinton agar (Bio-Rad, Hercules, USA) and interpreted according to the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST) [18]. The following antibiotics were tested: ampicillin-AMP (10 µg), amoxicillin/clavulanic acid - AMC (20/10 µg), ticarcillin - TIC (75 µg), piperacillin – PIP (30 µg), cephalotin - CF (30 µg), cefoxitin - FOX (30 µg), cefuroxime - CXM (30 µg), cefotaxime - CTX (5 µg), ceftriaxone - CRO (30 µg), ceftazidime - CAZ (10 µg), cefepime - FEP (30 µg), aztreonam - ATM (30 µg), imipenem - IMP (10 µg), ertapenem - ETP (10 µg), gentamicin - GM (10 µg), tobramycin - TM (10 µg), netilmicin - NET (10 µg), amikacin - AKN (30 µg), nalidixic acid - NAL (30 µg), ofloxacin - OFX (5 µg), ciprofloxacin - CIP (5 µg), tetracycline - TET (30 µg), minocycline - MIN (30 µg), chloramphenicol - CHL (30 µg) and sulfamethoxazole/trimethoprim - SXT (1.25/23.75 µg).

Genes encoding resistance to beta-lactams (bla_{TEM-1}, bla_{SHV}, bla_{CTX-M}), tetracyclines (*tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*), and sulphonamides (*sul1*, *sul2*) were screened by simplex PCRs using previously described primers and conditions [19-21]. Amplification and sequencing of the quinolone-resistance-determining region of *gyrA* and *parC* genes were used to detect mutations encoding quinolone resistance [22]. Genes encoding plasmid-mediated quinolone resistance (PMQR) (*qnrA*, *qnrB*, *qnrS*, *aac (6')-Ib, oqxAB* and *qepA*) were also searched by PCR [23].

MLVA assay

Bacterial cells were cultured in tryptic soy broth (TSB) and incubated at 37 °C overnight. The cultured bacterial suspensions were boiled for 10 min. After centrifugation at 14,000 rpm for 10 min, the resulting supernatant was stored at -20 °C for future use as a DNA template. MLVA was performed analyzing five VNTR loci (ST7-ST6-ST5-STTR10-ST8) described in the PulseNet MLVA protocol of *Salmonella* Typhimurium (PulseNet Standard Operating Procedure for PulseNet MLVA of *Salmonella enterica* serotype Typhimurium – Applied Biosystems Genetic Analyzer 3500 Platform (2013) https://www.cdc.gov/pulsenet/pdf/se-abi-3500-508c.pdf) [24].

Forward and reverse primers were the same as described in the PulseNet protocol, but with a change in

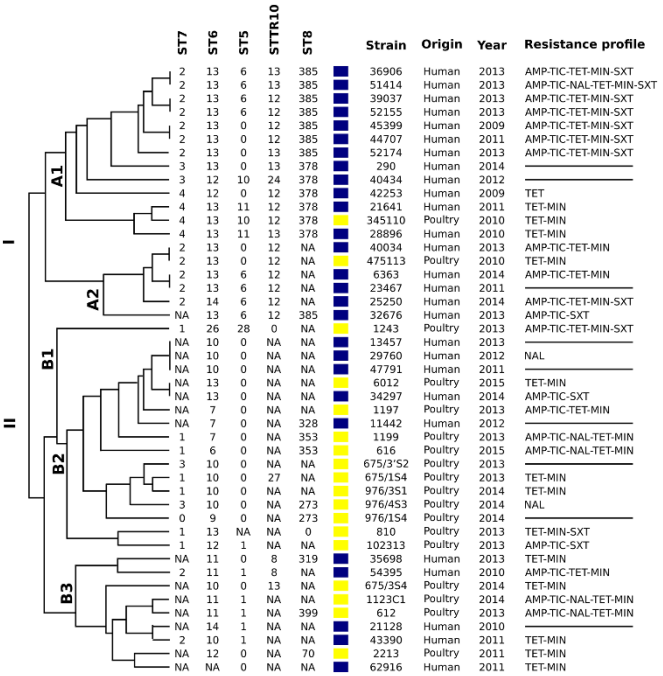
the forward primer labeling. The characteristics of the target locus and the primers used are shown in Table 1.

Two multiplex PCRs combinations were performed for the MLVA analysis. Reaction 1 included primers for ST5, ST7 and STTR10 loci, and in reaction 2 primers for ST6 and ST8 were used. Both multiplex reactions were prepared in a total volume of 10 µL including 1X PCR buffer, 2.25mM $MgCl_2$, 0.2mM dNTPs, 1.5 µM of primers amplifying ST5, 0.4 µM of ST7, 0.03 µM of STTR10, 0.28 µM of ST6, 0.24 µM of ST8, and 1 µL of DNA template. Both multiplex PCR solutions were run with the same cycling conditions of 95 °C for 5 minutes, 35 cycles of 94 °C for 20 seconds, 63 °C for 20 seconds and 72 °C for 20 seconds, followed by a final elongation step of 72 °C for 5 minutes with a GeneAmp Thermal Cycler 2720 (Applied Biosystems, Foster City, California). PCR products were subjected to capillary electrophoresis on ABI-310 Genetic Analyser (Applied Biosystem, Foster City, USA) after which the size of the PCR products was determined with Peak Scanner v.1.0 (Applied Biosystem, Foster City, USA). GeneScan 600 LIZ was used as size standard.

MLVA Data analysis

Each peak was identified according to color and size and was binned into alleles so that each new multiple of the repeat was assigned to a distinct allele number. MLVA profiles were expressed as a string of five numbers (ST7-ST6-ST5-STTR10-ST8). For ST7, ST6, ST5 and STTR10 locus, each value represent the repeat copy number according to the nomenclature suggested by Larsson *et al.* [10], and for ST8 locus, the value was designated by the size of repeat array region as suggested by Chiou *et al*. [25]. To assign the copy number the value was rounded down to the nearest complete copy number [26]. An absent locus was named as "NA" and loci with 0 repeats with a "0" [10]. All null alleles (the ones that have no amplification = NA) were confirmed by singleplex PCR reactions, in order to rule out suboptimal multiplex conditions as a cause of amplification failure [26].

Figure 1. Dendrogram representing genetic relationships among 45 *Salmonella* Typhimurium strains based on MLVA profiles. The data were clustered by UPGMA method. Blue: strains isolated from humans; Yellow: strains isolated from poultry.



AMP: Ampicillin; TIC: Ticarcillin; NAL: Nalidixic acid; TET: Tetracycline; MIN: Minocycline; SXT: Sulfamethoxazole-trimethoprim.

A dendrogram of genetic relationships based on allelic profiles was generated using the Dice similarity coefficient, and the unweighted pair group method with arithmetic mean (UPGMA), by the software available at *in silico* platform [27] . Simpson's index of diversity (DI) with a 95% confidence intervals were calculated for each locus using the V-DICE software (Health Protection Agency, London, UK; http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl).

Table 1. Characteristics of the target locus and primers used in MLVA assay.

VNTR locus	Size of repeat unit (bp)	Dye	Primer sequences (5'-3')
ST7	9	NED	F-CGATTGACGATATCTATGACTT R-GTTTTTCACGTTTGCCTTTC
ST6	6	NED	F-TCGGGCATGCGTTGAAA R-CTGGTGGGGAGAATGACTGG
ST5	6	FAM	F-ATGGCGAGGCGAGCAGCAGT R-GGTCAGGCCGAATAGCAGGAT
STTR10	6	PET	F-CGGGCGCGGCTGGAGTATTTG R-GAAGGGGCCGGGCAGAGACAGC
ST8	27/33	VIC	F-GCAGGTGTGGCTATTGGCGTTGAAA R-GATGGTGACGCCGTTGCTGAAGG

F: forward primer; R: reverse primer.

Results

Antimicrobial susceptibility

Among the 45 *S*. Typhimurium strains tested for antimicrobial resistance, only 7 human and 2 poultry strains were fully susceptible to all antibiotics. Good correspondence between phenotypical and genotypical detection of antibiotic resistance was observed, although a combination of methods was needed to determine the antimicrobial resistance of an isolate. Antibiotic resistance rates of clinical and poultry strains were: 48.1% - 38.8% for penicillins, 7.4% - 27.7% for nalidixic acid, 62.9% - 77.7% for tetracycline, 59.2% - 77.7% for minocycline, and 37% - 16.6% for sulfamethoxazole/trimethoprim, respectively. Thirteen strains (28.8%) were multi-drug resistant, which were defined as resistant to three or more antibiotics of different classes. Greater incidence of multiresistant phenotype was observed during 2013-2015 (Figure 1). The most frequent resistance profile in clinical strains was AMP-TIC-TET-MIN-SXT (n = 7); it was also encountered in one poultry strain (strain number 1243). In poultry strains, TET-MIN was the most frequent one (n = 7), but also in 6 of the 18 strains resistance to AMP-TIC-TET-MIN was observed (Table 2). Over the 20 strains resistant to penicillins, 14 harbored the *bla*$_{TEM-1}$ gene and only in one strain (poultry strain number 616)

Table 2. Antimicrobial resistance profile and resistance genes of *Salmonella* Typhimurium strains isolated from humans and poultry.

Clinical strains (n = 27)			Poultry strains (n = 18)		
Strain number	**Resistance profile**	**Resistance genes**	**Strain number**	**Resistance profile**	**Resistance genes**
42253	TET	*tetA,*	**345110**	TET-MIN	*tetA*
45399	AMP-TIC-TET-MIN-SXT	*tetA, sul1,* bla$_{TEM-1}$	**475113**	TET-MIN	*tetA*
54395	AMP-TIC-TET-MIN	*tetA,* bla$_{TEM-1}$	**2213**	TET-MIN	*tetA*
21128	----------	------	**102313**	AMP-TIC-SXT	-----
28896	TET-MIN	*tetA*	**612**	AMP-TIC-NAL-TET-MIN	*tetA, blaTEM-1*
47791	----------	------	**1243**	AMP-TIC-TET-MIN-SXT	*tetA, sul1, blaTEM-1*
21641	TET-MIN	*tetA*	**1197**	AMP-TIC-TET-MIN	*tetA, blaTEM-1*
23467	----------	------	**616**	AMP-TIC-NAL-TET-MIN	*tetA, gyrA* (Ser83-Phe), *parC* (Ser80-Ile), *blaSHV*
44707	AMP-TIC-TET-MIN-SXT	bla$_{TEM-1}$	**1199**	AMP-TIC-NAL-TET-MIN	*tetA, blaTEM-1*
43390	TET-MIN	*tetA*	**810**	TET-MIN-SXT	*tetA*
62916	TET-MIN	*tetA*	**675/1S4**	TET-MIN	*tetA*
11442	----------	------	**675/3S4**	TET-MIN	*tetA*
40434	----------	------	**1123C1**	AMP-TIC-NAL-TET-MIN	*tetA, gyrA* (Ser83-Phe), *blaTEM-1*
29760	NAL	------	**976/4S3**	NAL	-------
13457	----------	------	**675/3'S2**	----------	-------
32676	AMP-TIC-SXT	------	**675/3S1**	TET-MIN	*tetA*
35698	TET-MIN	------	**6012**	TET-MIN	*tetA*
36906	AMP-TIC-TET-MIN-SXT	*tetA, sul1,* bla$_{TEM-1}$	**976/1S4**	----------	-------
39037	AMP-TIC-TET-MIN-SXT	*tetA,* bla$_{TEM-1}$			
40034	AMP-TIC-TET-MIN	------			
51414	AMP-TIC-NAL-TET-MIN-SXT	*tetA, gyrA* (Asp87-Asn), bla$_{TEM-1}$			
52155	AMP-TIC-TET-MIN-SXT	bla$_{TEM-1}$			
52174	AMP-TIC-TET-MIN-SXT	*tetA,sul1*			
290	----------	------			
6363	AMP-TIC-TET-MIN	*tetB,* bla$_{TEM-1}$			
25250	AMP-TIC-TET-MIN-SXT	*tetA,* bla$_{TEM-1}$			
34297	AMP-TIC-SXT	-------			

AMP: Ampicillin; TIC: Ticarcillin; NAL: Nalidixic acid; TET: Tetracycline; MIN: Minocycline; SXT: Sulfamethoxazole/trimethoprim.

the bla_{SHV} gene was detected. Resistance to tetracycline was observed in 17 clinical strains, from which 12 harbored *tetA* and one *tetB*. However, *tetA* was found in all tetracycline resistant poultry strains. *sul1* gene was detected in 3 clinical and 1 poultry strains. Concerning the mechanisms of resistance to quinolones, a point mutation in the *gyrA* gene (GAC into AAC) at codon 87 (Asp87-Asn) was detected in only one of the 2 clinical strains resistant to nalidixic acid (strain 51414). Among the 5 poultry resistant strains, one mutation (TCC into TTC) in *gyrA* (Ser83-Phe) in strain 1123C1, and doble mutations in *gyrA* (Ser83-Phe) and *parC* (Ser80-Ile) in strain 616 were identified. The plasmid-mediated quinolone resistance genes were not detected in any isolate. In some cases, the resistance to an antibiotic *in vitro* was not asociated with none of the genetic determinants analysed (Table 2).

MLVA data results

Among the 45 *S.* Typhimurium strains typed with MLVA, 37 distinct MLVA profiles were detected, from which 7 were repeated (Figure 1). The most frequently encountered profiles were: 2-13-6-13-385 (n = 2); 2-13-6-12-385 (n = 2); 2-13-0-12-385 (n = 2); 2-13-0-12-NA (n = 2); 2-13-6-12-NA (n = 2); NA-10-0-NA-NA (n = 3) and NA-13-0-NA-NA (n = 2), and they included 33.3% of the *S.* Typhimurium isolates. Isolates from same and different sources with an identical antibiotic resistance and MLVA profiles were identified. The diversity index obtained for MLVA (0.989) was higher that the one obtained for antimicrobial susceptibility testing (0.870). The loci with highest discriminatory power were ST6 (DI = 0.805), followed by ST7 (DI = 0.774). Whereas a lower diversity was observed for ST5 and STTR10 (Table 3). For all loci, there were some strains for which no amplicon was obtained, nearly the half of the strains lacked STTR10 or ST8 locus, and in most of these cases no amplification of both loci was observed. The unique MLVA profiles, defined as profiles that were exclusively displayed in one strain, comprised 31.1% of the human origin isolates and 35.5% of the poultry ones. Little genetic variation was observed among strains from different sources isolated in different years. For instance, a clinical strain (23467) from 2011 shared identical MLVA profile with another clinical strain from 2014 (6363); a poultry strain (475113) from 2010 shared identical MLVA profile with a clinical strain (40034) from 2013 (Figure 1).

The clustering of the MLVA profiles revealed the presence of two major clusters designated as I and II. The cluster I was subdivided into A1 and A2 and the cluster II into B1, B2 and B3. The A1 subcluster comprised 13 strains (28.8%) with a similarity above 28.5% between them. Twelve strains isolated from humans and one from poultry between 2009 and 2014 were grouped in this subcluster. The A2 subcluster comprised 5 strains isolated from humans and one from poultry. Most of the poultry origin strains were grouped in cluster II, except for two strains that were in cluster I. The B1 subcluster comprised only one poultry strain, in B2 subcluster 16 strains were grouped (5 isolated from humans and 11 from poultry) with a similarity above 31.2%, and B3 subcluster contains 9 strains (5 from humans and 4 from poultry) with a similarity above 40% (Figure 1). MLVA based clusters showed also similarities with respect to antimicrobial resistance. The most common antimicrobial resistance pattern in cluster I was AMP-TIC-TET-MIN-SXT (36.8%), and TET-MIN pattern (30.7%) in cluster II.

Discussion

Human infections caused by *Salmonella enterica* serovar Typhimurium are commonly considered to be associated with foods of animal origins, mainly poultry meat, eggs and pork [28], and previous reports indicate possible transmission of antibiotic resistant serovar Typhimurium isolates from animals to humans [29,30]. A combination of phenotypical and genotypical methods for the detection of antibiotic resistance is still needed for a better characterization and to assess their genetic relatedness.

In our study, 50% of the *S.* Typhimurium isolates showed a multiresistant phenotype, with a greater incidence during 2013-2015 as previously described [31-33] suggesting an increase in the use of antibiotics during this period. Hence, a better control to prevent *S.*

Table 3. Diversity index results obtained for each locus included in the multiple-locus variable-number of tandem repeats analysis (MLVA).

VNTR Locus	No of variants	No of isolates where locus is not present	Simpson's diversity index
ST6	10	1	0.805
ST7	7	16	0.774
ST8	11	22	0.742
STTR10	8	21	0.692
ST5	8	2	0.682
TOTAL MLVA	37	0	0.989

Typhimurium food contamination is needed, but severe control is also needed to ensure a better usage of antimicrobials in animals and humans [34]. The most common resistance profile in human origin strains was AMP-TIC-TET-MIN-SXT (26%), and for poultry strains it was TET-MIN (38.8%) and AMP-TIC-TET-MIN (33.3%), suggesting that a possible gene transference of *S*. Typhimurium strains between these two sources might have happened. The *S*. Typhimurium exhibited high rates of resistance to traditional antibiotics such as ampicillin, nalidixic acid, tetracycline and sulfamethoxazole-trimethoprim; this is in accordance with the findings of other authors [35,36]. The detection of antimicrobial genes and a strict policy for the rational use of antibiotics for treating animals and humans is needed, if we do not want to have to limit the therapeutical choices for treating salmonellosis [5]. The majority of the human isolates were resistant to ampicillin and sulfamethoxazole-trimethoprim. However, the resistance to nalidixic acid was higher among poultry isolates, this can be explained by the wide utility of quinolones in veterinary practice [33,37]. It is known that the increase of nalidixic acid resistance, in some way, may reflect the emergence of fluoroquinolone resistance [38]. Like other members of the *Enterobacteriaceae*, resistance to fluoroquinolones in *Salmonella* is mostly due to mutations in the quinolone resistance determining region (QRDR) of the DNA gyrase genes [39]. In the present study, we found that one human isolate and another from poultry origin had a single mutation (Asp87/Asn) in *gyrA*. A double mutation *gyrA* Ser83-Phe/*parC* Ser80-Ile was found in another poultry origin strain. These mutations have been previously reported in the literature [40-43].

Potential spread of *Salmonella* from poultry to humans could be traced by molecular subtyping, such as PFGE and MLVA. It is reported that MLVA typing of serovar Typhimurium may perform better for subtyping than PFGE with enhanced resolution and good reproducibility. Epidemiological concordance has been also observed [8,15,33]. In this study, we have demonstrated the use of MLVA in characterizing *S*. Typhimurium to establish the genetic diversity among isolates from human and poultry origin in Tunisia. Only 5 of the 7 loci proposed by Pulsenet protocol were used because in one of them (ST3 locus) no amplification was observed in most of the strains analysed. So a 5-loci MLVA scheme was used, as it is mostly used in Europe (STTR9-STTR5-STTR6-STTR10-STTR3) [44]. The nomenclature of the loci is different in the MLVA protocols, but they are overlaping VNTR loci [24]. Moreover, it has been proved that the discrimination power obtained by a 4-loci or 5-loci MLVA scheme is realy close, and that adding more loci to the scheme does not increase the resolution power of the technique [25,33].

In this work, some identical MLVA types were found in both human and poultry isolates, indicating a possible route of transmission [37]. The study of the most frequent alleles obtained from the isolates of the different origins and the higher discriminatory index (DI), showed that ST6 was the loci with highest discriminatory index, and it may be of potential use for source attribution. Previous studies supported our results [9,36]. On the other hand, STTR10 and ST8 locus were absent in many strains (46.6% and 48.8%, respectively). Our results were in concordance with results obtained by other authors, as it has been described how STTR10 is the locus with the highest percentage of absence, but also ST8 has been shown to be absent in many strains [33,37]. In our study, 33.3% of *S.* Typhimurium isolates were dominated by 7 frequent MLVA profiles. The two MLVA clusters defined differ regarding frequency of MDR isolates. Most of the MLVA profiles in the two groups have already been described in other European countries [11]. The Simpson's indices found in our study, suggest that MLVA has a higher discriminatory power than antimicrobial resistance testing (0.989 to 0.960), as was concluded from earlier studies [11,45,46] and which would result in an improved surveillance and detection of possible outbreaks. Thus, two major MLVA types (2-13-0-12-NA; NA-13-0-NA-NA) present in both human and poultry isolates were revealed, suggesting that chickens may be a major source of human salmonellosis in Tunisia. Therefore, the data support that there is potentially substantial risk of transmission of serovar Typhimurium between humans and chickens, and hence, the dissemination of the prevalent clones in both humans and poultry.

Conclusion

A high number of *Salmonella* serovar Typhimurium MDR strains were detected at both groups of human and poultry strains in Tunis. Furthermore, the genotyping results using MLVA suggest that some strains isolated from humans and poultry may descend from a common subtype. The MDR strains isolated from poultry may indicate also the possibility of MDR strains transmission to humans. It is imperative to continue the survey of *Salmonella enterica* multiresistant lineages in Tunisia using discriminatory typing methods to characterize isolates from human and animal origins.

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