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Molecular typing of *Salmonella enterica* serovars Enteritidis, Corvallis, Anatum and Typhimurium from food and human stool samples in Tunisia, 2001–2004

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SUMMARY

During the period from 2001 to 2004, a total of 72 isolates of *Salmonella enterica* serovars: Anatum ($n=40$), Enteritidis ($n=18$), Corvallis ($n=8$), and Typhimurium ($n=6$), of various origins (mainly food and diarrhoeagenic stool samples), were collected and further characterized by antibiotic resistance, plasmid analysis, and pulsed-field gel electrophoresis (PFGE). Forty-five isolates presented multidrug resistance to antibiotics. Among which one *S. enterica* serovar Anatum isolate was resistant to 11 antibiotics, and one *S. enterica* serovar Typhimurium DT104 isolate was resistant to eight antibiotics. Plasmid profiling identified eight plasmid profiles (with 1–5 plasmids) among the isolates, of which one plasmid profile (P01) was predominant. *Xba*I PFGE analysis revealed the presence of a predominant clone of the four studied *Salmonella* serovars circulating in Tunisia throughout the years 2001–2004.

INTRODUCTION

The genus *Salmonella* includes more than 2400 different known serovars [1, 2]. *Salmonella* serovars are associated with considerable morbidity and mortality among livestock, thereby posing a significant threat to animal health and well-being and, as a result, to human health [2–4].

Non-typhoidal *Salmonella* serovars are increasing in importance as significant pathogens of both human and animals. According to the World Health Organization, there are about 17 million cases annually of acute gastroenteritis or diarrhoea due to non-typhoidal salmonellosis, with 3 million deaths [5, 6].

Phenotypic methods play an important role in identification to genus level. Serotyping, based on the Kauffmann–White scheme, remains the standard for classification of *Salmonella* isolates in outbreak investigations but now has been supplemented by a range of molecular genotyping methods [7–13].

In recent years, molecular-based techniques, such as plasmid profile analysis, ribotyping, random amplified polymorphic DNA analysis, and pulsed-field gel electrophoresis (PFGE) have been shown to be useful methods for discrimination among isolates of *Salmonella* spp. Among these techniques, PFGE is currently considered to be one of the most reliable typing procedures [14–17].

In Tunisia, the surveillance for *Salmonella enterica* is carried out by the National Centre of Enteropathogenic Bacteria (*Salmonella*, *Shigella*, and *Vibrio cholerae*). Annually, about 2000 *Salmonella* strains are reported from all over Tunisia to the National Centre for Enteropathogenic Bacteria. Any *Salmonella* strains isolated are analysed and serotyped.

Identification of isolates has been limited to the serovar level, without any additional characterization. Molecular typing data are useful for epidemiological studies; this information would facilitate

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the identification of the clonality of isolates and, in turn, would determine the epidemiological relationship and prevalence of different strains of *Salmonella* [18–23]. Molecular techniques were first introduced for *Salmonella* analysis in 2001. According to statistical data of an 11-year study period from 1994 to 2004 (R. Ben Aissa, unpublished observations), three *Salmonella* serovars were the most commonly isolated in Tunisia: *Salmonella enterica* serovars Enteritidis, Anatum, and Corvallis. Typhimurium is also of importance in Tunisia since it showed obvious peaks in different categories (food, human, animal and environment) over the 11-year study period and was therefore also included in our study.

The objectives of this study were to determine the extent of genetic variation and clonality among food and clinical strains of *S*. Enteritidis, *S*. Anatum, *S*. Corvallis, and *S*. Typhimurium in Tunisia. Specifically, this has been achieved by examination of susceptibility to common antibiotics, plasmid analysis, and PFGE patterns.

METHODS

Bacterial strains

A total of 72 *Salmonella* strains from different sources (mainly food and human stool samples) were isolated and serotyped during the period 2001–2004: Anatum (40), Enteriditis (18), Corvallis (8), and Typhimurium (6).

Serogrouping and serotyping

Salmonella strains were positively identified and serotyped according to the Kauffmann–White scheme with the use of antiserum (Bio-Rad, Marnes-la-Coquette, France). Serogrouping and serotyping were performed by slide agglutination to identify the somatic O antigen and flagellar H antigens.

Antimicrobial susceptibility

Antimicrobial susceptibility testing was done using standard methods (disc diffusion method) using Mueller–Hinton agar, and interpreted according to the antibiogram guidelines of the French Committee of Microbiology (Société française de Microbiologie, 2002). Antimicrobials used for testing were ampicillin (AMP) 10 μg; cephalothin (CEF) 30 μg; ticarcillin (TIC) 75 μg; cefotaxime (CTX) 30 μg; chloramphenicol (CHL) 30 μg; amoxicillin (AMX) 25 μg; cefoxitin (FOX) 30 μg; amoxicillin + clavularic acid (AMC) 20/10 μg; trimethoprim–sulfamethoxazole (SXT) 1·25/23·7 μg; gentamicin (GEN) 10 μg; nalidixic acid (NAL) 30 μg; sulfonamide (SSS) 200 μg; tetracycline (TET) 30 μg; kanamycin (KAN) 30 IU; ciprofloxacin (CIP) 5 μg; streptomycin (STR) 10 IU; and ofloxacin (OFX) 5 μg. *S*. Choleraesuis strain ATCC14028 strain was used as a control. Characterization of strains as susceptible, intermediately resistant, or resistant was determined by Osiris software version 3.x (Bio-Rad).

Plasmid analysis

Plasmid DNA was isolated by the alkaline lysis method as described previously [24]. Plasmids were sized in comparison to *E. coli* V517 strain and compared by the use of Bionumerics software (Applied Maths, Kortrijk, Belgium). The molecular mass of the plasmids was calculated by comparison with plasmids in V517 and images normalized accordingly.

Genomic fingerprinting by PFGE

The *Xba*I PFGE patterns were determined for all 72 *Salmonella* strains using previously described PFGE methods with modifications, as described previously [25]. PFGE was performed on a 1% agarose gel (Bio-Rad) using CHEF DR III apparatus (Bio-Rad) in 0·5× TBE (Tris–borate–EDTA) buffer at 14 °C with 6 V/cm at a field angle of 120°: block 1, 8·5 h, with initial switching time of 7 s to final switching time of 12 s; block 2, 10·5 h, with initial switching time of 20 s to final switching time of 40 s. Gels were stained with ethidium bromide and photographed. A lambda DNA ladder with size range of 48·5 kb to 1 Mb (Amersham Biosciences, Buckinghamshire, UK) was used as a DNA size standard.

Numerical analysis of PFGE profiles

Together with visual analysis of the PFGE profile, a numerical analysis after conversion, normalization, and analysis of similarity in band pattern was performed using MVPS3.31 software (Media Cybernetics, GA, USA). Similarities between profiles were calculating using Dice coefficient, with a maximum position tolerance of 1%. PFGE patterns obtained were clustered by UPGMA. The capital letter A, E, C, and T were used to designate the different

Table 1. *Antibiotic resistance profile*

Salmonella serovar (no. of strains)	Antibiotic resistance profile	No. of strains	Total no.
S. Anatum (40)	STR	6	28
	TET	3	
	CHL	2	
	STR, TET	8	
	STR, CHL	2	
	STR, TET, NAL	1	
	STR, TET, CHL	2	
	TET, KAN, NAL	1	
	STR, TET, SXT, CHL	2	
	STR, TET, SXT, GEN, FOX, KAN, AMX, CEF, CAZ, CTX, AMC	1	
S. Enteritidis (18)	TET	7	9
	STR	1	
S. Corvallis (8)	TET, CHL	1	
	STR, TET	2	4
	STR, TET, NAL	1	
	STR, TET, KAN, NAL	1	
S. Typhimurium (6)	STR	2	4
	STR, TET	1	
	AMP, TET, CHL, STR, AMX, AMC, SSS, TIC	1	

AMC, Amoxicillin + clavularic acid; AMP, ampicillin; AMX, amoxicillin; CAZ, ceftazidime; CEF, cephalothin; CHL, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; OFX, ofloxacin; SSS, sulfonamide; STR, streptomycin; SXT, trimethoprim–sulfamethoxazole; TET, tetracycline; TIC, ticarcillin.

serovars: Anatum, Enteritidis, Corvallis, and Typhimurium. The numerical suffix between brackets was used to designate the different years of isolation.

RESULTS

Antimicrobial susceptibility

Of the 72 *Salmonella* isolates, 62·5% (27/72) were resistant to one or more antimicrobials (Table 1). Twelve (2 clinical and 10 food isolates) of the 40 *S.* Anatum isolates were sensitive to all antibiotics tested. One clinical strain was drug multiresistant with resistance to 11 different antibiotics (Table 1).

No great variation of susceptibility among the 18 strains of *S.* Enteritidis was observed (Table 1). Seven strains from different food sources were resistant to tetracyclines, and one from turkey meat to streptomycin. Among the eight strains of *S.* Corvallis, only strains isolated from turkey meat were resistant. Of the six *S.* Typhimurium, only one isolate from white cheese was multi-resistant (eight antibiotics: AMP, STR, TET, CHL, AMX, AMC, TIC, and SSS). This strain was phage-typed at the French National Center for *Salmonella* (Institut Pasteur, Paris, France) by the method described by Anderson *et al.* [26] and was defined as multi-resistant serovar Typhimurium DT104.

Plasmid profiles

Seven different plasmid profiles with 1–5 plasmids were identified (Table 2). The most prevalent plasmid profile was P01, containing one plasmid of 53·7 kb; PFGE in 62 out of 72 isolates (86%) exhibited this.

PFGE

Four out of 72 isolates were untypable because of DNA degradation. The PFGE patterns of *Xba*I-digested chromosomal DNA of the remaining 68 isolates are summarized in Table 3.

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Table 2. *Number of plasmids and plasmid size of each plasmid profile in the 72* Salmonella *strains characterized in this study*

Salmonella serovar	Plasmid type	No. of plasmids	Plasmids (kb)	No. of isolates
S. Anatum	P01	1	53·7	38
	P02	3	53·7, 5·07, 3·03	1
	P03	3	53·7, 7·6, 4	1
S. Enteritidis	P01	1	53·7	16
	P04	2	53·7, 5·46	2
S. Corvallis	P01	1	53·7	2
	P05	5	53·7, 5·46, 5·07, 3·03, 2	2
	P06	2	53·7, 7·2	2
	P07	2	53·7, 4	2
S. Typhimurium	P01	1	53·7	6

Table 3. *The PFGE patterns and plasmid types of the 68* Salmonella *enterica serovars: Anatum, Enteriditis, Corvallis, and Typhimurium from patients, food and environmental samples in Tunisia*

Serovar	Total no. of strains	Origin	No. of strains	*Xba*I type	Plasmid type
Anatum	40	Food	5	X01	P01
			9	X02	P01
			2	X03	P01
			2	X04	P01
			1	X04	P03
			1	X05	P01
			3	X06	P01
			2	X07	P01
			1	X08	P01
			1	X09	P01
			1	X10	P01
			1	X11	P01
		Human stool*	5	X01	P01
			1	X05	P01
			1	X07	P01
			1	X07	P02
Typhimurium	6	Food	2	X0a	P01
			2	X0b	P01
			1	X0c	P01
		Tap water	1	X0a	P01
Enteritidis	18	Food	10	X0*I*	P01
			1	X0*I*	P04
			2	X0*II*	P01
			1	X0*III*	P01
		Human stool*	2	X0*II*	P01
			1	X0*I*	P04
Corvallis	8	Food	1	X0A	P01
			2	X0A	P05
			1	X0A	P06
			2	X0B	P07
			1	X0D	P01
		Human stool*	1	X0C	P01

* Diarrhoeagenic cases.

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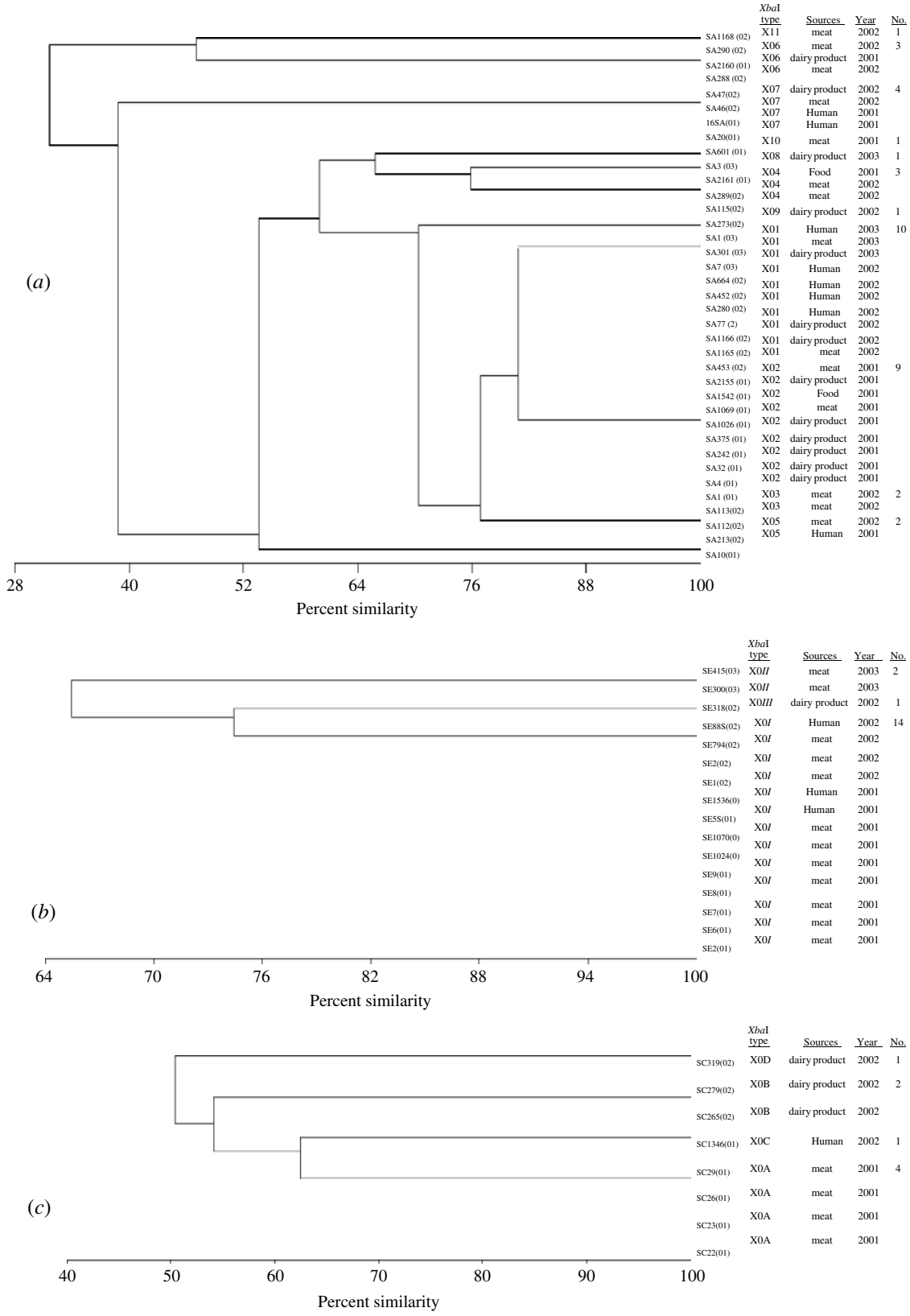

Fig. 1. Dendogram showing percent similarity calculated by the Dice similarity index of PFGE restriction endonuclease digestion profiles among the 66 *Salmonella* isolates: (*a*), *S.* Anatum; (*b*), *S.* Enteritidis; (*c*), *S.* Corvallis. The different patterns, sources, year of isolation and number of strains are indicated.

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(i) *S*. Anatum. A total of 11 PFGE patterns were identified (Fig. 1*a*). A summary of these patterns is shown in Table 3. Most of these strains belonged to clusters X01 (10/40 isolates) and X02 (9/40 isolates). Cluster X01 was detected in strains isolated from food and human stool samples during the years 2002 and 2003 in the same seasons. The PFGE patterns of strains belonged to cluster X01 were 100% genetically similar (identical profiles). Cluster X02 was detected in food strains during different seasons of the year 2001. The genetic similarity among X01 and X02 was 80%. The remaining strains clustered in one of nine different PFGE patterns. Seven of these clusters (X03, X04, X06, X08, X09, X10, X11) were unique for food isolates.

(ii) *S*. Enteriditis. PFGE permitted the resolution of *Xba*I macrorestriction fragments of the 18 *S*. Enteritidis isolates into three distinct clusters (Table 3). The largest cluster was X01 (14/18 isolates). All 14 isolates were produced between 2001 and 2002 throughout different seasons. The genetic similarity among these three PFGE clusters was 64% (Fig. 1*b*).

(iii) *S*. Corvallis. The eight strains of *S*. Corvallis were assigned to four different clusters (Table 3). All food strains of 2001 clustered in pattern X0A, while human stool strains of 2001 clustered alone in pattern X0C (unique profile) with 62% similarity with pattern X0A (Fig. 1*c*). The remaining PFGE patterns are summarized in Table 3.

(iv) *S*. Typhimurium. PFGE patterns of *Xba*I-digested chromosomal DNA of six *S*. Typhimurium isolates are shown in Fig. 2. Three patterns were observed: X0A (three strains), X0B (2 strains) and X0C (one strain) (Table 3).

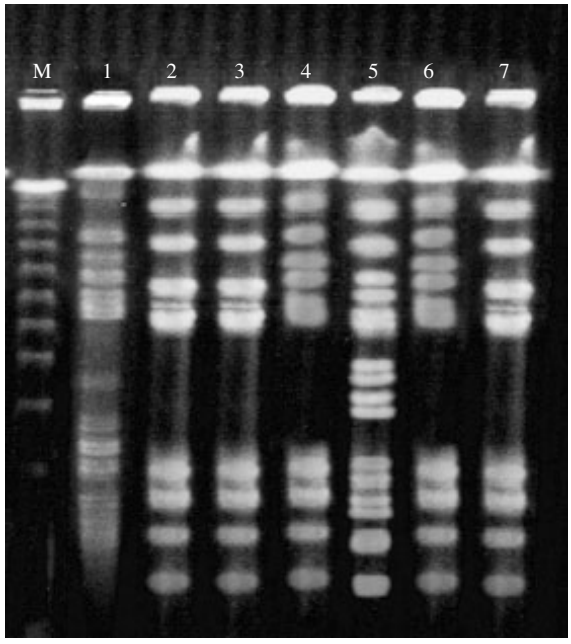

Fig. 2. PFGE patterns of *Xba*I digests of chromosomal DNA of *S*. Typhimurium. The numerical prefix was used to designate the serovar of the strain. The alphabetical suffix was to designate the code of the strain in our laboratory. *Salmonella enterica* serovar *cholereasuis* ATCC14028 strain was used as a control. M, lambda DNA ladder; lane 1, *S*. Choleraesuis ATCC14028; lane 2, ST21(01); lane 3, ST1031(01); lane 4, ST2157(01); lane 5, ST1161(02); lane 6, ST179(03); lane 7, ST299(03).

DISCUSSION

This study has utilized a combination of phenotypic and genotypic typing methods to define relationships between 72 strains of *S*. Anatum, *S*. Enteritidis, *S*. Corvallis, and *S*. Typhimurium from human stool, food, animal, and environmental sources in Tunis. In this investigation 62·5% of the 72 isolates of the four serovars were resistant to at least one antibiotic (tetracycline), and most were multiresistant (to tetracyclines, streptomycin, and chloramphenicol). Both resistance and multiresistance were more common in *S*. Anatum, *S*. Corvallis and *S*. Typhimurium. One *S*. Anatum strain, from a human stool diarrhoeagenic case, was resistant to 11 antibiotics. In contrast, isolates of *S*. Enteritidis were predominantly drug-sensitive.

S. Typhimurium DT104 with multidrug resistance is an important international human pathogen, and it is widespread in Western and Eastern Europe, North America, and the Middle East [2, 27]. In our study, one *S*. Typhimurium strain, from a dairy product (cheese), was multidrug-resistant type DT104 (according to the phage typing). This strain was resistant to eight antibiotics.

When studied by PFGE, within *S*. Anatum the majority of strains fell into two major PFGE patterns (X01, X02). Strains with PFGE patterns of X01 were detected exclusively in the winter months in Tunis in 2002 and 2003, and were isolated from different sources. Isolates of the X02 pattern were detected throughout 2001 from different food sources (Fig. 1*a*). Other strains belonged to four different minor patterns (X04, X05, X06, and X07); each of these patterns exhibited only two or three genetically related strains.

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The present study showed that the PFGE profiles of most of the *S*. Anatum and *S*. Enteritidis isolates from food and human stool sources belonged to two clones: X01 and X02. These clones were stable and persisted over a considerable period of time in Tunis. This supports the notion that infected animals and humans are important sources of contamination of the environment and the food chain.

Our findings suggest that certain clones of *S*. Enteritidis, *S*. Anatum, *S*. Corvallis and *S*. Typhimurium are in circulation in Tunis, suggesting an endemic status for these organisms in Tunisia. PFGE can be regarded as the method most suitable for epidemiological studies. Greater numbers of isolates are needed to evaluate their clonal origins, and the epidemiology of *Salmonella* in Tunis.

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DECLARATION OF INTEREST

None.

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