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Full Length Research Paper

Analysis of 16S rDNA sequence of isolates from Turkey meat to determine the antimicrobial susceptibility of Salmonella in the presence of virulence genes

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In this study, several *Salmonella* (n=25) isolated from turkey meat were characterized using conventional culture methods, biochemical, serological, antimicrobial testing and analysed for presence of virulence genes. For molecular analysis, the 16S rDNAs of all strains of *Salmonella* were sequenced and used for construction of a phylogenetic tree. Six different serotypes were identified of which *Salmonella Saintpaul* (n=10) was the most frequent, followed by *Salmonella Agona* (n=6), *Salmonella Typhimurium* (n=4), *Salmonella Heidelberg* (n=3), *Salmonella Infantis* (n=1) and *Salmonella Bredeney* (n=1). All strains were positive for nine virulence genes (*spi*A, *sif*A, *spa*N, *sop*B, *sip*B, *iro*N, *org*A, *sit*C and *prg*H), but none were positive for *spv*B genes. Salmonella isolates most frequently exhibiting resistance to 3-8 antibiotics were *Agona* (83%), *Typhimurium* (75%) and *Saintpaul* (60%), though one strain (*S. Agona*) is an Extended-spectrum beta-lactamase (ESBL) with a minimal inhibitory concentration (MIC) for ceftriaxone (16 µg/Ml). *Salmonella* strains were clustered into four phylogenetic groups and three sub clusters. However, *S. Infantis*, *S. Bredeney* and *S. Agona* AE136/2 betalactamase producing (ESBL) were placed separately on side branches separate from the remaining strains, while 100% (n=25) strains possessed a 16S rDNA sequence with ≥97% similarity to that of a genus *Salmonella*.

Key words: *Salmonella*, antibacterial resistance, 16S rDNA, phylogeny, virulence genes, Morocco.

INTRODUCTION

Food borne diseases are main problems, particularly in developing countries and they cause the majority of illnesses and death around the world. Food is the most important vehicle that transmits the microorganisms to humans (Varnam, 1999). Among these microorganisms, *Salmonellae* is still a major cause of food-borne human disease in most parts of the world (Soultose et al., 2003; Carraminana et al., 2004). In Morocco, *Salmonella, Staphylococcus aureus*, and *Clostridium perfringens* are reported to cause 42.8, 37 and 1.7% of food poisoning, respectively (Department of Epidemiology, 2005). Although the declaration and recording of 12% *Salmonella* cases remain underreported, *Salmonella* is the major cause of food poisoning in Morocco (Rouahi et al., 1998). Worldwide, epidemiological reports indicate that poultry meat is the food most incriminated in food poisoning (ICMSF, 1998). In addition, the prevalence of multidrug resistant *Salmonella enterica* in foods have been reported in many parts of the world from various host

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species including food animals as well as processed ready-to-eat meat products (Chen et al., 2004; Miko et al., 2005; Bouchrif et al., 2008; Guerra et al., 2010).

Reflecting a complex set of interactions with its host, *Salmonella* spp. require multiple genes for full virulence. Many of these genes are found in salmonella pathogenicity islands (SPI) in the chromosome. There are two species within the genus *Salmonella*: *Salmonella* enterica and *Salmonella* bongori, the former of which contains over 2,500 serotypes and is divided further into 6 subspecies known as *S.* enterica subsp. enterica, salamae, arizonae, diarizonae, houtenae, and indica (Grimont and Weill, 2007). In the United States, 99% of reported human isolates of *Salmonella* belong to S. enterica subsp. enterica (CDC, 2011).

Salmonella identification methods currently used were based on the analysis of morphological, physiological and biochemical data of the target bacteria. Isolates confirmed to be positive with these methods were then analyzed with Pulsed Field Gel Electrophoresis (PFGE) and submitted for serotype identification. This work can require 3-4 weeks (Rosalee et al., 2012). While these methods have proven to be effective in the identification and differentiation of *Salmonella*, they require a considerable amount of time and labor-factors which can impede the progress of foodborne outbreak investigations. Therefore, the use of rapid molecular methods has become indispensable. A comparison of the genomic sequences of bacterial species showed that the 16S ribosomal RNA (rRNA) gene is highly conserved within a species and among species of the same genus, and hence can be used as the new gold standard for the speciation of bacteria including *Salmonella* (Clarridge III, 2004; Woo et al., 2008). Using this new standard, phylogenetic trees based on base differences between species are constructed; bacteria are classified and re-classified into new genera (Olsen and Woese, 1993). This method utilizes universal primers to amplify and sequence either a partial region (~500 bp) of the 16S rRNA gene or the full gene (~1500 bp). In this method (rapid molecular), several genes were also used to detect *Salmonella* genus or serovars including: virulent chromosomal genes such as *inv*A (Zahraei et al., 2006), *iro*B (Soumet et al., 1999), *inv*E (Feder et al., 2001) and *sly*A (Del Cerro et al., 2003), fimbriae genes such as fimy (Yeh et al., 2002), *sef*A (Pan and Liu, 2002) and *sop*E (Mirmomeni et al., 2008), unique sequence such as *sdf* I (Agron et al., 2001), *S*T (Malkawi and Gharaibeh, 2004) and finally plasmid genes such as *spv* (Soumet et al., 1999).

In this pilot study, (i) we analyzed the 16S rDNA sequences of 25 isolates of *S. enterica* isolated from retail turkey over a one year time period in Meknes, Morocco, (ii) we determined the antimicrobial susceptibility of *Salmonella* isolated, and (iii) we examined for presence of virulence genes the *spi*A, *sif*A, *spa*N, *sop*B, *sip*B, *iro*N, *org*A, *spv*B, *sit*C and *prg*H genes amplification by PCR.

MATERIALS AND METHODS

Sample collection

The samples of turkey meat (including breasts, legs, gizzards and livers) tested in this work were isolated between October 2011 and October 2012 from retail outlets in Meknès, Morocco. All strains were stored frozen at -70°C in 20% Glycerol and in conservation Agar cultures at room temperature in the Microbiology Laboratory at the College of Sciences, Moulay Ismail University, Meknès, Morocco.

Isolation and identification of *Salmonella*

All samples were analysed for isolation of *Salmonella* strains using the French AFNOR method V (AFNOR, 1944). Approximately 25 g of food were placed in 225 ml of Buffered Peptone Water (BPW) as pre-enrichment media, and incubated at 37°C for 18 h. After incubation, 0.1 ml of the BPW was added to Rappaport-Varsiliadis broth, an enrichment media, and incubated at 42°C for 18 h. A swab of the broth was inoculated onto Hektoen selective media. Suspected colonies for *Salmonella* were inoculated in Urea Indol at 37°C for 2 to 4 h, in Hajna Kligler at 37°C for 18 to 24 h, and with an ONPG disc for biochemical testing and presumptive identification. All isolates were biochemically identified by using the API20-E system (bioMérieux SA, Marcy– l'Étoile France). Serotyping of *Salmonella* isolates was performed by slide agglutination with commercial antisera following the Kauffmann-White serotyping scheme in collaboration with the Pasteur Institute of Morocco in Casa Blanca.

Susceptibility to antimicrobials

Antibiotic susceptibility testing was performed by a disc diffusion method on Mueller-Hinton agar and interpreted in accordance with the criteria of the National Committee for Clinical Laboratory Standards (Marjo et al., 2007). The strains were screened for their resistance to the following antibiotics (Sanofi Diagnostics Pasteur): amoxicillin, Amx 25 µg; colistin, Cs 50 µg; nalidixic acid, Na 30 µg; ciprofloxacin, Cip 5 µg; ceftazidime, Caz 30 µg; amoxicillin-clavulanic acid, Amc 20+10 µg; cefoxitin, Fox 30 µg; cefotaxime, Ctx 30 µg; bacitracin, B 130 µg; chloramphenicol, C 30 µg; streptomycin, S 10 µg; trimethoprim, Tmp 5 µg and ceftriaxone, Cro 5 µg. In this study, the Automated System (OSIRIS) was used for reading and interpretation of results (Bio-Rad). A screening test for the detection of ESBLs was carried out by the double disc diffusion test (using cefotaxime, ceftazidime and amoxicillin/clavulinic acid discs) according to the CLSI criteria (CLSI, 2007). *E. coli* ATCC 25922 was used as a quality control strain. The minimum

inhibitory concentration (MIC) of ceftriaxone for a single strain Agona producing ESBL were also determined by Etest strips (AB Biodisk) (Pfaller et al., 1998).

Extraction, PCR of 16S amplification and sequencing of bacterial DNA

The taxonomic identity of the strain isolated (Table 3) was confirmed by 16S rRNA gene sequencing. DNA was extracted using Power Soil DNA Isolation Kit (KAPA 2G Fast Hot Start de KAPA Biosystems) according to the manufacturer's instructions. PCR amplification of the 16S rDNA was performed at 25 µl reaction mixture containing 5 µl of template DNA, 0.1 PCR Taq polymerase buffer

(KAPA Biosystems, USA), 0.5 µM $MgCl_2$, 5 µl de tampon, 0.5 µM of each dNTPs (KAPA Biosystems, USA), 12.5

H_20 and 0.7 µM of each primer (Isogen) specific for the bacteria domain: 27f (AGAGTTTGATCMTGGCTCAG) and 1492r (TACGGYTACCTTGTTACGACTT) (Lane, 1991). PCR was carried out by Thermal Cycler (Applied Biosystems, USA) at the following conditions: initial denaturation of one cycle at 94°C for 10 min; 30 cycles at 94°C for 1 min; 52°C for 1 min; 72°C for 3 min and one final cycle at 72°C for 10 min, after which the temperature was maintained at 4°C following the final cycle. Electrophoresis in Tris-borate- EDTA buffer was performed at 100 V for 1.5 h. The gel was stained with ethidium bromide (0.5 µg/ml) for 15 min, rinsed and visualized using the «G Box» system (Applied Biosystems, USA). PCR amplicons were purified with a QIA quick PCR purification kit (ExoSAP-IT Affymetrix, USA) and eluted in Tris-HCl (10 mM, pH 8.5) prior to sequencing.

All isolates were sequenced bi-directionally, and the sequencing reactions were performed using a Big Dye Terminator Kit version 3.1 (Applied Biosystems). Sequencing procedures were conducted using an Applied Biosystems 3130 XL Genetic Analyzer according to the manufacturer's instructions (Applied Biosystems, USA). Data were collected and analyzed using data collection software version 3.0 and sequencing analysis software version 5.3.1 (Applied Biosystems, USA).

Phylogenetic analysis

Nucleotide sequences were compared to sequences in the National Center for Biotechnology Information GenBank database using the BLASTn program (http://www. ncbi.nlm. nih.gov/BLAST). Moreover, databases and matrices of evolutionary distance were constructed using Clustal X (Jeanmougin et al., 1998), while the topology distance and probability of phylogenetic tree were determined with MrBayes program (Holder and Lewis, 2003). The phylogenetic trees were constructed from the evolutionary distances by Tree View software (Page, 1996). Nucleotide sequence accession numbers, that is, GenBank and EMBL accession number for reference 16S rDNA sequence used in this analysis, are listed in Table 3.

Criteria for identification

Identification at the species level was defined as a 16S rDNA sequence similarity of ≥ 99% with that of the prototype strain sequence in GenBank, while identification at the genus level was defined as a 16S rDNA sequence similarity of ≥ 97% with that of the prototype strain sequence in GenBank (Drancourt et al., 2000). The percentages of similarity are expressed in Bayesien posterior probability.

PCR detection of virulence genes

Salmonella isolates were screened for 10 virulence genes by simplex PCR method (Skyberg et al., 2006). Primers' sizes of virulence genes (Table 1) and PCR conditions are similar to those described by Skyberg et al. (2006). Briefly, total DNA was extracted from overnight cultures of the *Salmonella* isolates, using the Power Soil DNA Isolation Kit (KAPA 2G Fast Hot Start de KAPA Biosystems). The PCR reaction mixture, with a final volume of 25 µl, contained 150 ng of template DNA, 2 µl dNTP, 1 µl of forward and reverse primers, 0.4 µl of Taq

DNA polymerase (Taq recombinant d'Invitrogen), 2.5 µl de tampon and a sufficient amount of water with a volume of 25 µl. PCR cycle conditions were as follows: 5 min at 95°C, 30 cycles of 40 s at 94°C, 60 s at 66.5°C, and 90 s at 72°C, and a final elongation step of 10 min at 72°C. PCR products were analyzed by electrophoresis in 1×TAE buffer at 50 V for 85 min on 1.2% agarose gels. However, a wide-range molecular-weight DNA marker (100-bp DNA ladder, Promega) was used on each gel as a standard.

RESULTS

Serology

Among the 25 *Salmonella* isolates, 6 different serotypes were identified of which *S. Saintpaul* (n= 10) was the most frequent, followed by *S. Agona* (n= 6), *S.*

Typhimurium (n= 4), *S. Heidelberg* (n=3), *S. Infantis* (n= 1) and *S. Bredeney* (n=1) (Table 2).

Antibioresistance

Antibiotic resistance in *Salmonella* strains to 13 antimicrobial agents is shown in Table 5. Overall, the highest percentage of resistance was found in the following antimicrobial agents: bacitracin (96%), amoxicillin (68%), triméthoprim (44%), streptomycin (32%), colistin (20%) and chloramphenicol (12%), while the low resistance rates were returned (between 0 and 4%) for the remaining antibiotics. Multiple resistances (to

Table 1. Primers used for detecting virulence genes typing in *Salmonella* isolated [37].

Virulence-related gene	Primer 5'- 3'	Size (pb)	Function of gene
Spv B	F- CTATCAGCCCCGCACGGAGAGCAGTTTTTA R- GGAGGAGGCGGTGGCGGTGGCATCATA	717	Growth within host
Spi A	F- CCAGGGGTCGTTAGTGTATTGCGTGAGATG R- CGCGTAACAAAGAACCCGTAGTGATGGATT	550	Survival within macrophage
Sip B	F- GGACGCCGCCCGGGAAAAACTCTC R- ACACTCCCGTCGCCGCCTTCACAA	875	Entry into nonphagocytic cells, killing of macrophages
Iron N	F- ACTGGCACGGCTCGCTGTCGCTCTAT R- CGCTTTACCGCCGTTCTGCCACTGC	1205	Iron acquisition
Sif A	F- TTTGCCGAACGCGCCCCCACACG R- GTTGCCTTTTCTTGCGCTTTCCACCCATCT	449	Filamentous structure formation
Spa N	F- AAAAGCCGTGGAATCCGTTAGTGAAGT R- CAGCGCTGGGGATTACCGTTTTG	504	Entry into nonphagocytic cells, killing of macrophages
prgH	F- GCCCGAGCAGCCTGAGAAGTTAGAAA R- TGAAATGAGCGCCCCTTGAGCCAGTC	756	Host recognition/invasion
Sit C	F- CAGTATATGCTCAACGCGATGTGGGTCTCC R- CGGGGCGAAAATAAAGGCTGTGATGAAC	768	Iron acquisition
Sop B	F.CGGACCGGCCAGCAACAAAACAAGAAGAAG R- TAGTGATGCCCGTTATGCGTGAGTGTATT	220	Host recognition/invasion
orgA	F- TTTTTGGCAATGCATCAGGGAACA R- GGCGAAAGCGGGGACGGTATT	255	Host recognition/invasion

Table 2. Antigenic Formula of *Salmonella* strains used in this study.

Strains	Antigenic formulae				Serotype
	O		H1	H2	
AE136/2, , AE6, AE10, AE28/1, AE156, AE24/1	B	4,12	fgs	-	*Agona*
AE28/1, AE10/21, AE88/1, AE81/2, AE111, AE113/1, AE121/1, AE91/2, AE168/1, AE10/21	B	1,4,5,12	eh	1,2	*Saintpaul*
AE70, AE10/25, AE159, AE31/1	B	1,4,5,12	i	1,2	*Typhimurium*
AE92/1	C1	6,7	r	1,5	*Infantis*
AE40/5	B	1,**4**,12,27	l,v	1,7	*Bredeney*
AE111/3, AE75/1, AE93/3	B	1,**4,5,12**	r	1,2	*Heidelberg*

Antigenic formulae were determined by conventional serotyping at the reference laboratories of Salmonellae and shigellae of the Pasteur Institute of Morocco in Casablanca.

two or more) was observed in 19 strains (76%) (Table 4). Therefore, a high prevalence of multiresistance among foodborne *Salmonella* strains was observed. *S. Agona* showed the highest percentages of resistance to the tested drugs. When analyzed by serovar, *Salmonella* isolates most frequently exhibiting resistance to 3-8 antimicrobials were *Agona* (83%), *Typhimurium* (75%) and *Saintpaul* (60%). While the results showed only one

Table 3. Distribution and molecular identification of *salmonella* strains isolated of turkey meat employing 16S rRNA gene and sequencing techniques.

Sequences	Representative sequence in GenBank	The closest sequence	Similarity (%)	Previous reported association and characteristics	Classification with this 16S type
AE121/1	KF509916	*Salmonella* sp. 4063 **FJ405336.1**	98	_	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; *Salmonella*
AE88/1	KF509910	Saintpaul 382 JQ694568.1	98	From food (Rosalee et al., 2012).	
AE31/1	KF509907	Typhimurium str. 08-1736 CP006602.1	99	Gram -negative pathogen isolated from human (Jarvik et al., 2012)	
AE168/1	KF509919	Saintpaul 382 JQ694568.1	98	From food (Rosalee et al., 2012).	
AE75/1	KF509909	Heidelberg str. B182 CP003416.1	98	Isolated from bovine feces in France (Le Bars and al., 2012).	
AE111/3	KF509914	Heidelberg str. SL476 CP001120.1	98	Isolated from ground turkey (Fricke et al., 2012).	
AE93/3	KF509912	Heidelberg strain ATCC 13311 KC768785.1	98	_	
AE24/1	KF509904	*Salmonella* sp. D194-2 FJ463825.1	97	_	
AE126/1	KF509894	Saintpaul strain 382 JQ694568.1	98	_	
AE136/2	KC960690	*S.* Agona SA5 JQ228522.1	99	_	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; *Salmonella*.
AE159	KF509903	Typhimurium AF227869.1	98	_	
AE6	KF509901	*S.* Agona str. SL483 CP001138.1	98	From human isolates mildly pathogenic (Fricke et al., 2011)	
AE10	KF509902	*Salmonella* sp. 4063 FJ405336.1	97	_	
AE113/1	KF509915	Saintpaul strain 382JQ694568.1	98	Isolated from food (Rosalee et al., 2012).	
AE28/1	KF509895	*S.* Agona strain 312 JQ694192.1	97	Isolated from food (Rosalee et al., 2012).	
AE156	KF509897	Agona str. SL483 CP001138.1	98	Isolated from human isolates mildly pathogenic (Fricke et al., 2011).	
AE10/21	KF509913	*S.* Saintpaul strain384n JQ694569.1	99	Isolated from food (Rosalee et al., 2012).	
AE91/2	KF509911	Saintpaul 382 JQ694568.1	98	Isolated from food (Rosalee et al., 2012).	
AE81/2	KF509918	*Salmonella* sp. 4066 FJ405339.1	99	_	
AE111	KF509908	*Salmonella* sp. 9KF188417.1	98	_	
AE10/25	KF509913	Typhimurium UK1 (ATCC 68169). CP002614.1	98	Exhibits the highest invasion and virulence attributes among the most frequently studied strains. Has been used as the foundation for developing recombinant vaccines (Luo et al., 2011).	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; *Salmonella*
AE70	KF509906	Typhimurium strain 85 JQ694621.1	99	Isolated from food (Rosalee et al., 2012)	
AE92/1	KF509899	Infantis strain 343 JQ694375.1	99	Isolated from food (Rosalee et al., 2012).	
AE40/5	KF509898	*Salmonella* sp. APK1 KF574808.1	98	_	
AE26/1	KF509900	*Salmonella* sp. XJ-ZG1	99	_	

Table 4. Resistance of *Salmonella* serotypes isolated from turkey carcasses and giblets to antimicrobial agents.

Serotypes	Sample examined	Antibiotype
S. Agona	Legs	Amx, Amc, C, S, B
S. Agona	Breast	Amx, C, Tmp, B
S. Agona	Breast	Amx, Tmp, B
S. Agona	Gizzard	Amx, Tmp, B
S. Agona	Breast	Amx, C, S, B, Ctx, Caz, Cro, Cs
S. Agona	Liver	Tmp, B
S. Saintpaul	Legs	Amx, Tmp, B, S
S. Saintpaul	Breast	Amx, S, Tmp, B, Cs
S. Saintpaul	Breast	Amx, Tmp, B, Cs, S
S. Saintpaul	Gizzard	Amx, S, Tmp, B
S. Saintpaul	Breast	Amx, B
S. Saintpaul	Liver	B
S. Saintpaul	Liver	Amx, Tmp, B
S. Saintpaul	Gizzard	Amx, B
S. Saintpaul	Brest	Amx, B, CS
S. Saintpaul	Liver	B
S. Heidelberg	Breast	B
S. Heidelberg	Breast	B
S. Heidelberg	Gizzard	Tmp, B
S. Bredeney	Gizzard	B
S. Infantis	Breast	B
S. Typhimurium	Breast	Amx, B
S. Typhimurium	Breast	Amx, S, B
S. Typhimurium	Gizzard	Amx, S, Tmp, B, Cs
S. Typhimurium	Legs	Amx, B, S

S: streptomycin, Tmp: triméthoprime, B: bacitracin, Cs: colistine, Cro: céftriaxone Amc: amoxicillin+clavulinic acid, Amx: amoxicillin, C: chloramphenicol, Caz: ceftazidime, Cip: ciprofloxacin, Ctx: cefotaxime, Fox: cefoxitine.

Table 5. Resistance (%) to antibiotics of *Salmonella* strains isolated from sampled at retail outlets in Meknès, Morocco.

	% of strains resistant to antibiotics						
Serotypes	**A**	**S**	**T**	**H**	**I**	**B**	**Total**
Number of strains	**6**	**10**	**4**	**3**	**1**	**1**	25
B	6	9	4	3	1	1	96
Amx	5	8	4	0	0	0	68
C	3	0	0	0	0	0	12
Amc	1	0	0	0	0	0	4
Caz	1	0	0	0	0	0	4
S	2	3	3	0	0	0	32
Tmp	4	5	1	1	0	0	44
Na	0	0	0	0	0	0	0
Cip	0	0	0	0	0	0	0
Fox	0	0	0	0	0	0	0
Cs	1	3	1	0	0	0	20
Cro	1	0	0	0	0	0	4
Ctx	1	0	0	0	0	0	4

S: S. Saintpaul, H: S. Heidelberg, A: S. Agona, T: S. Typhimurium, I: S. Infantis, B: S. Bredeney.

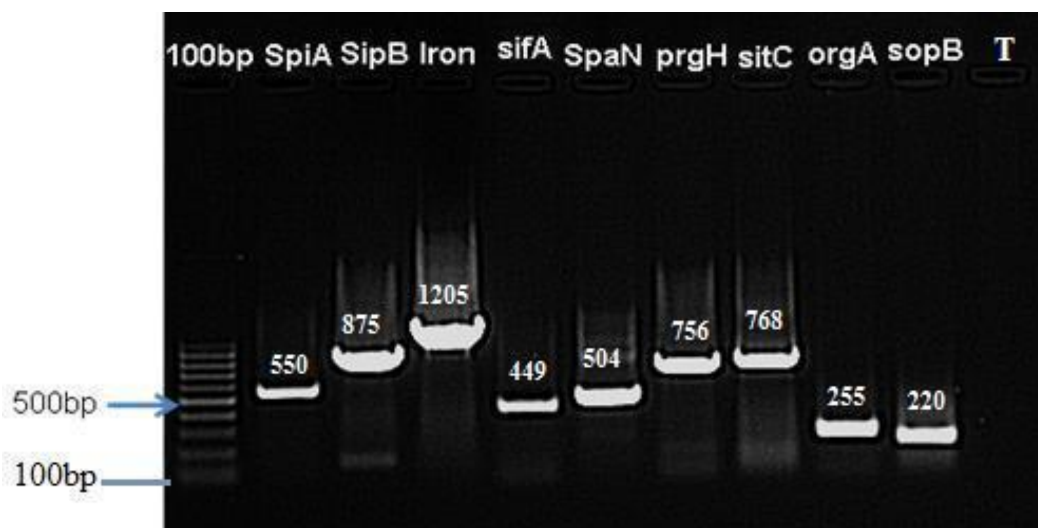

Figure 1. Agarose gel electrophoresis of PCR products after amplification of *spi*A, *sif*A, *spa*N, *sop*B, *sip*B, *iro*N, *org*A, *spv*B, *sit*C and *prg*H genes using specific primers (table 1). Lanes: 100 bp: molecular weight marker; *spi*A : *spi* A gene products; *sip* B: *sip* B gene products; *iro* N: *iro* N gene products; *sif* A: *sif* A gene products; *spa* N: *spa* N gene products; *prg* H: *prg* H gene products; *sit* C: *sit* C gene products; *org* A: *org* A gene products, T: Negatif control .

strain (*S. Agona*), the resistance profile (Amx, C, S, B, Ctx, Caz, Cro, Cs) is an ESBL with a MIC for ceftriaxone 16 µg/Ml (Table 4).

PCR detection of virulence genes

The presence of genes which play a role in invasion and survival of *Salmonella* spp. in host environments, *spi*A, *sif*A, *spa*N, *sop*B, *sip*B, *iro*N, *org*A, *sit*C and *prg*H was determined using simplex PCR (Skyberg et al., 2006). All of the 25 isolates were positive for these virulence genes (Figure 1) but none were positive for *spv*B genes.

16S rDNA sequence analysis and bacterial identification

Resulting sequences were confirmed from chromatogram analysis and sequences were compared with those stored in the GenBank using the BLASTn alignment software (http://www.blast.genome.ad.jp/). PCR amplifications on high quality genomic DNA preparations of these isolates generated amplicons of expected size for all isolates examined (Figure 2). All sequences were then deposited in GenBank with their corresponding accession numbers, as detailed in Table 3. 16S rDNA-based analysis resulted in the classification of the isolates into two categories: a total of 6 of 25 isolates (24%) possessed a 16S rDNA sequence with ≥99% similarity to that of a previously characterized bacterial species, while 100% (n=25) strains possessed a 16S rDNA sequence with ≥97% similarity to that of a genus *Salmonella* (Table 3).

Phylogenetic structure on the basis of 16S rRNA gene sequences

A total of 25 strains of *Salmonella* enterica were initially compared based on differences in 16S rDNA sequence. The 16S rDNA phylogenetic analysis organized the strains into four clusters (I, II, III, and IV) and three sub clusters (IIa, IIb and IIIa) (Figure 3). However, *S. Infantis, S. Bredeney* and *S. Agona* AE136/2 betalactamase producing (ESBL) were placed separately on side branches separate from the other strains. Overall, genetic distances between the sequences within the same serovars ranged from 0.006 to 0.04, and the degree of similarity within each group ranged from 95-99%. The intraspecies 16S rDNA sequence similarity levels generally exceeded 95%, but with one exception, which is the similarity between the *S. Agona* 16S rDNA sequences ranged from 75 to 95% (Figure 3).

Analysis of the 16S rDNA gene shows the grouping of the same serotype (three *S. Agona* and four *S. Saintpaul*) into cluster IV and cluster I respectively. The remaining clusters (II and III) were populated by a variety of different serotypes. Cluster II is populated by four *S. Saintpaul* in which three are grouped into sub cluster (IIb), whereas one *S. Typhimurium* and three *S. Heidelberg* came together to form sub cluster II (IIa). Cluster III was dominated by *S. Typhimurium* strains together (sub cluster IIIa), but also contained two *S. Agona* and two *S. Saintpaul* strains.

DISCUSSION

The widespread use of antibiotics as supplements for

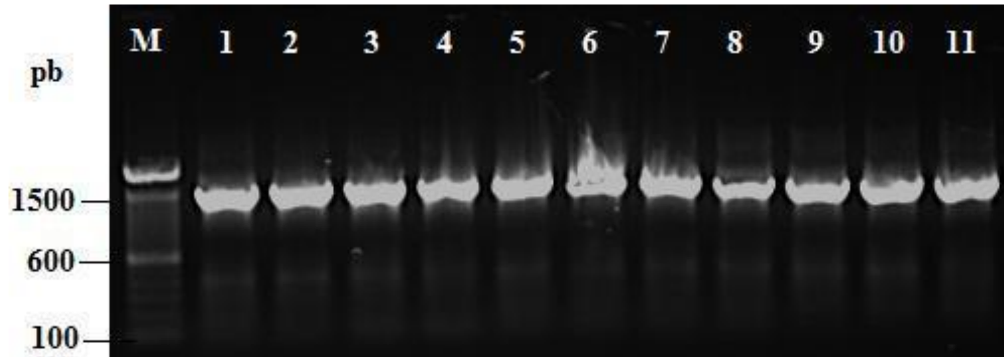

Figure 2. PCR products amplified with the universal 16S rDNA PCR primers. PCR products amplified from different strains, i.e., lanes 1-3, S. Saintpaul ; 4-5, S. Agona; 6-7, S. Typhimurium ; 8-9, S. Heidelberg; 10, S. Infantis; 11, S. Bredeney are shown. Lane M represents the 100-bp DNA ladder. Molecular weights of these PCR products were equal to 1546 bp.

used on poultry farms for years, cross-resistance or coresistance mechanisms could be the cause of the resistance observed to both drugs in the present study and other research (Yildirim et al., 2011). Almost all strains (96%) were resistant against bacitracin. Sangeeta et al. (2010) also observed complete resistance for bacitracin among the isolates from poultry farm and marketing channels. For *S. Agona*, nevertheless the cefotaxime results (Table 4) suggested the presence of an ESBL, and analysis for the presence of an ESBL was positive using the ceftazidime-ceftazidime/clavulinic acid Etest ESBL strip. It comes to one strain (*S. Agona*) isolated from Breast turkey collected at supermarket. Thus resistance of high serotype *Agona* isolated from meat with a MIC for ceftriaxone 16 µg /Ml was noted for the first time in Morocco.

Seven of these genes, namely: *org*A, *prg*H, *sif*A, *sip*B, *spa*N, *spi*A and *spv*B are associated with invasion and survival within cells, and the production of adhesin or pili, though the remaining genes are important for iron acquisition (*sit*C, *iro*N) (Nde and Logue, 2008). Each of the *Salmonella* isolates identified in this study harbored virulence genes including *spi*A, *sip*B, and *sop* that reside on SPI1, SPI5, as well as other virulence factors such as *pag*C. These genes encode *Salmonella* virulence factors which enable it to invade host cells, induce enteropathogenicity (diarrhea), and promote survival in macrophage cells (Abouzeed et al., 2000; Sameshima et al., 2000). The *spi*A gene within *S. Typhimurium*, which encodes an outer-membrane component of the SPI-2 type III secretion system, is essential for virulence in host cells (Ochman et al., 1996). It has also been demonstrated by transposon mutagenesis that the *spi*A gene may be associated with biofilm formation (Dong et al., 2001). Indeed, the formation of biofilms may improve the ability of these organisms to resist stresses such as desiccation, extreme temperatures, antibiotics and antiseptics (Marin et al., 2009; Scher et al., 2005). Biofilm formation allows *Salmonella* to survive long term in the poultry farm environment and to contaminate poultry meat and eggs, which remain the leading vehicles of food-borne salmonellosis outbreaks (Joseph et al., 2001). The *spv* operon is located on a large virulence plasmid in many subspecies I serovars of *S. enterica* (Fierer and Guiney, 2001). It has been proposed that the *spv* operon is mainly responsible for the pathogenicity of non-typhoidal human pathogenic strains, as a majority of invasive clinical strains harbor this virulence plasmid (Fierer and Guiney, 2001; Montenegro et al., 1999). Previous studies have demonstrated that *sif*A is an important virulence factor of *S. Typhimurium*, required for lethal infection of mice (Stein et al., 1996). This study demonstrates that the function of *sif*A is relevant to survival/replication in murine macrophages, and the host niche is exploited by *S. Typhimurium* during systemic phases of disease in these animals (Richter-Dahlfors et al., 1997). In this study, the presence of nine virulence genes in all *Salmonella* isolated from turkey meat was confirmed by other studies in *Salmonella* serovars from clinical, food and environmental samples (Tatsuya et al., 2011; Ezat et al., 2013). These similarities are consistent with the observation that these food isolates (turkey meat) may be capable of causing human infection (salmonellosis) (Blostein, 1991; Elward et al., 2006; Toth et al., 2002). The high prevalence of virulence markers that were investigated highlights the pathogenic potential of these isolated Salmonellas to cause disease in humans and contaminate food. Similar results are obtained for high prevalence of some virulence genes in strains isolated from North America and Africa (Dione et al., 2011; Shah et al., 2011; Zou et al., 2012). The results from this study support those of previous studies suggesting that these virulence genes are widely distributed among *Salmonella* (Skyberg et al., 2006).

Generally, a total of two clusters (I and IV), consisting solely of *S. Saintpaul* and *S. Agona* strains respectively and three sub clusters (IIa, IIb and IIIa) populated respectively by three serovars *Heidelberg*, three

Escherichia coli B50 GU594305
AE40/5 KF509898 B
AE92/1 KF509899 I
AE136/2 KC960690 A
1.00 AE126/1 KF509894 S
AE113/1 KF509915 S
0.75
AE88/1 KF509910 S
0.97
AE10 KF509913 S
I
0.99
AE81/2 KF509918 S
AE111/3 KF509914 H
1.00
AE75/1 KF509909 H
0.99
AE93/3 KF509912 H
a
0.57
AE168 KF509919 S
II
0.93
AE26/1 KF509900 S
b
0. 95
AE91/2 KF509911 S
0.77
AE31/1 KF509907 T
AE6 KF509901 A
AE10 KF509902 A
0.89
AE70 KF509906 T
0 98
AE159 KF509903 T
a III
0.95
0.54
AE10/25 KF509913 T
AE111 KF509908 S
1.00
AE121/1 KF509916 S
AE24/1 KF509904 A
0.95
AE28/1 KF509895 A
IV
0.75
AE156 KF509897 A
0.05

Figure 3. Dendrogram of Morocco *Salmonella* strains based on 16S rDNA analysis. Phylogenetic tree resulting from comparison of 16S rRNA gene sequences of strains (n=25), using MrBayes program [34]. The numbers at the nodes represent Bayesian posterior probability, the GeneBank accession numbers of the sequences are given next to the species name. The distance between any two strains can be determined by measuring the lengths of the connecting horizontal lines. The sequence fr om an Escherichia coli B50 strain (accession number GU594305) was used as an outgroup. Strains designated with the prefixes A, B, H,I,S and *T indicate S. Agona , S. Bredeney, S. Heidelberg, S. Infantis and S. Saintpaul* serovars respectively.

Typhimurium and three *Agona* strains were used in this study. However, *S. Infantis*, *S. Bredeney* and *S. Agona* AE136/2 betalactamase producing (ESBL) were placed separately on side branches separate from the remaining strains. Similar studies showed that *Salmonella* genetic distances between the sequences within the same serovars ranged from 0.001 to 0.003 and the degree of similarity within each group ranged from 99.6 to 100% (Tajbakhsh et al., 2011). BLASTN analysis of 16S rRNA gene of *Agona* strain AE6 when compared to known

sequences in NCBI showed close relation to *S. Agona* CP001138.1 (98% of similitude) which is mildly pathogenic isolated from humans (Fricke et al., 2011). On the other hand, *Typhimurium* strain AE31/1 exhibited 99% of similitude to *Typhimurium* str. 08-1736 (CP006602.1) which is Gram-negative pathogen isolated also from humans (Jarvik et al., 2012). Another strain Typhimurium AE10/25 was 98% similar to that of *Typhimurium* UK1ATCC68169 (accession number CP002614.1), which exhibits the highest invasion and virulence attributes among the most frequently studied strains. This serovar was also used as the foundation for developing recombinant vaccines (Luo et al., 2011) (Table 3).

Based on analysis of different bacterial groups, it has been argued that for 16S rDNA, similarity values above 97.0 ± 97.5% 16S rDNA sequencing alone cannot be used to determine species identities or relationships, and therefore DNA-DNA hybridisations have to be performed (Stackebrandt and Goebel, 1994). However, extensive phylogenetic analysis of several members of the Enterobacteriaceae has demonstrated that for these genera, sequencing of 16S rDNA has the same phylogenetic discriminative power as DNA-DNA reassociation (Spróer et al., 1999).

In this study, the overall performance of 16S rDNA sequence analysis was excellent, in that it separates more or less the different serotypes in clusters or sub clusters. However, in order to improve this performance, efforts should be made to complete 16S rDNA databases with high-quality sequences and develop electronic tools for sequence comparison and interpretation.

Conclusion

In conclusion, epidemiological survey, identification of *Salmonella*, and screening of virulence gene through PCR based procedures can have major benefit in public health specifically for rapid diagnosis, epidemiological investigations, ideal vaccine, development of treatment, and prophylactic strategies for salmonellosis in Morocco.

The presence of virulence genes represents a serious threat to public health. Therefore, prudent use of antimicrobials in animal husbandry and human therapy as well as good food hygiene practices is mandatory. More studies to track the evolution of virulence factors among *Salmonella* must be encouraged.

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