Antimicrobial Resistance, Virulence Genes, and Genetic Diversity of *Salmonella enterica* Isolated from Sausages

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Salmonella is a major cause of morbidity and mortality in humans worldwide, and the infection with multidrug-resistant strains can cause severe diseases. This study was designed to evaluate the antimicrobial resistance, to detect the virulence genes, and to study the genetic diversity of isolated *Salmonella* strains using 16S rRNA sequences. For this, 34 *Salmonella* strains isolated from sausages were identified using biochemical and serological methods. Molecular tools were used to evaluate the presence of virulence genes (*orgA*, *sitC*, *sipB*, *spiA*, *iroN*, and *sifA*) using simplex and multiplex polymerase chain reaction (PCR) and to sequence 16S rRNA genes for phylogenetic analysis. The susceptibility to 24 selected antibiotics was also studied. The results of this study showed that all isolated *Salmonella* were positive for targeted virulence genes and were resistant to at least one antibiotic. However, the multidrug resistance was observed in 44% of isolated strains. The phylogenetic analysis of 16S rRNA sequences highlighted that *Salmonella* isolates were divided into 3 clusters and 3 sub-clusters, with a ≥98% similarity to *Salmonella enterica* species. From this study, we conclude that sausages are considered as a potential source of *Salmonella*, which could be a major risk to public health.

Keywords: *Salmonella*, 16S rRNA gene, phylogenetic tree, virulence genes, sausages

Introduction

Salmonella species were discovered more than a century ago by Salmon, an American scientist. They are Gram-negative, motile, facultative anaerobic bacteria and classified within the Enterobacteriaceae family [1]. *Salmonella* are regarded as a major food-borne pathogen that causes an economic burden for health care systems worldwide [2]. The consumption of animal products contaminated with *Salmonella* is the leading cause of human Salmonellosis [3]. Globally, 94 million cases of gastroenteritis were caused by *Salmonella* with 155,000 deaths each year, knowing that 85% of them were related to food [4]. In the United States, a study estimated that nontyphoidal *Salmonella* were responsible for 1,027,000 cases of food-borne illness each year, with 19,586 hospitalizations and 378 deaths [5]. In Morocco, *Salmonella* are responsible for 42.8% of food-borne diseases [6].

The pathogenicity of *Salmonella* is influenced by a variety of virulence factors that play an important role in a wide range of pathogenic mechanisms, such as adhesion, invasion, intracellular survival, toxin production, and iron acquisition [7]. However, the presence of virulence genes in multidrug-resistant strains of *Salmonella* is associated with a severe infection and is a major public health concern [8].

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The identification of *Salmonella* is subjected to various constraints due to the greater diversity of its species and serovars. The *Salmonella* genus contains two species, *Salmonella bongori* and *Salmonella enterica*, which is divided into six subspecies with the presence of more than 2600 serotypes [9]. Nowadays, it is agreed that the identification of *Salmonella* by biochemical and serological techniques requires about a week [10]. Hence, molecular methods based on DNA sequences represent the best alternatives for accurate and rapid bacterial identifications.

In the last years, the use of DNA sequencing has increased in the determination of the evolutionary relationships of different bacteria [11, 12]. In relation to *Salmonella*, phylogenetic studies includes sequencing of 16S rRNA, 23S rRNA, housekeeping genes, virulence genes, and *inv-spa* invasion gene complex [12]. It has been shown that genetic analysis of 16S rRNA gene increases the resolution between genus and species of *Salmonella enterica* and has a major role in bacterial phylogeny and taxonomic studies [13].

In Africa, specifically in Morocco, we noticed a lack of information for *Salmonella* pathogenicity and molecular identification. Thus, in this context, the objectives of this study were (i) to analyze the 16S rRNA sequences of 34 *Salmonella* strains isolated from sausages and determine their phylogenetic relationship, (ii) to study the distribution of 6 virulence

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genes among the isolated strains, and (iii) to assess their antimicrobial resistance using 26 antibiotics.

Materials and Methods

Isolation, Identification and Antimicrobial Susceptibility Testing. Thirty four *Salmonella enterica* belonging to 12 serotypes were isolated from sausages (beef sausages, turkey sausages, and artisanal sausages "merguez") sold in Meknes city in Morocco and confirmed by biochemical, serological, and molecular tests [10]. The disc diffusion method on Mueller–Hinton agar was used to study the antimicrobial susceptibility profiles of isolated *Salmonella* against 26 antibiotics (Table 1). Results were interpreted according to the recommendation of the Clinical and Laboratory Standards Institute [14]. *E. coli* ATCC 25922 was used as a positive control of this study, and the isolated *Salmonella* strains showing a decrease in susceptibility (intermediate) were considered as resistant.

Table 1. The antimicrobial agents used in this study

Antimicrobial agents	Code	Disk Content
Ampicillin	AMP	10 μg
Amoxicillin	AML	25 μg
Amoxicillin–clavulanic acid	AMC	20 μg/10 μg
Imipenem	IPM	10 μg
Ceftriaxone	CRO	30 μg
Cefuroxime sodium	CXM	30 μg
Ceftazidime	CAZ	30 μg
Cefamandole	MA	30 μg
Cefotaxime	CTX	30 μg
Cefoxitin	FOX	30 μg
Aztreonam	ATM	30 μg
Sulfamethoxazole	SMX	200 μg
Amikacin	AK	30 μg
Colistin sulfate	CT	50 μg
Kanamycin	K	30 μg
Ofloxacin	OFX	5 μg
Enrofloxacin	ENR	5 μg
Ciprofloxacin	CIP	5 μg
Nalidixic acid	NA	30 μg
Flumequine	UB	30 μg
Sulfonamide	SSS	200 μg
Gentamicin	CN	30 μg
Chloramphenicol	C	30 μg
Tetracycline	TE	30 μg
Streptomycin	S	10 μg
Trimethoprim–*sulfamethoxazole*	SXT	1.25 μg/23.75 μg

Table 2. Description of genes functions used in this study

Genes	Virulence-related function	References
orgA	Host recognition/invasion	[35]
sipB	Entry into nonphagocytic cells, killing of macrophages	[36]
sitC	Iron acquisition	[37]
spiA	Survival within macrophage	[30]
iroN	Iron acquisition	[27]
sifA	Filamentous structure formation	[29]

Amplification of the Virulence Genes Using Multiplex Polymerase Chain Reaction (PCR). Simplex and multiplex PCR methods were used to examine the presence of 6 virulence genes in the isolated *Salmonella* (Table 2), using their gene specific primers as described in Table 3. The simplex PCR method was used to amplify the genes *spiA*, *iroN*, and *sifA* as described previously by Mezal et al. [15]. Meanwhile, the multiplex PCR method was used to amplify the genes *orgA*, *sipB*, and *sitC* as described by Skyberg et al. [7]. The amplification was done in a Mastercycler gradient (Perkin-Elmer, Boston, MA) under the following conditions: 5 min at 95 °C, 30 cycles of 30 s at 94 °C, 30 s at 66.5°C, and 2 min at 72 °C, with a final cycle of 10 min at 72 °C. The isolated strains were considered positive for the tested virulence genes if they produce an amplicon with the expected size (Table 3). *Salmonella enterica* serovar typhimurium DT104 was used as positive control.

Amplification and Sequencing of the 16S rRNA Genes. DNA was extracted from a culture on liquid medium using DNA Isolation Kit (Gen Elute Bacterial Genomic DNA kit, Sigma) according to the manufacturer's instructions. The amplification of 16S rRNA gene was performed in a reaction mixture of 25 μL, with 12.5 μL of ddH_2O, 2.5 μL of 10*x* PCR buffer, 3.0 μL of 50 mM $MgCl_2$, 0.5 μL of 10 mM dNTPs (KAPA Biosystems, USA), 2 μL of template DNA (70 ng/μL), 0.2 μL of Taq DNA polymerase enzyme (5 U/μL; KAPA Biosystems, USA), and 1.25 μL of 10 μM from each primer: fD_1 (AGAGTTTGATCCTGGCTCAG) and rP_2 (ACGGCTACCTTGTTACGACTT) [16]. The amplification was performed in "Veriti" thermal cycler (Applied Biosystems, USA) under the following conditions: 10 min at 94 °C, 36 cycles of 1 min at 94 °C, 30 s at 52 °C, 2 min at 72 °C, and a final cycle of 10 min at 72 °C. Electrophoresis migration was done in 1% agarose (Sigma) for 1 h at 100 V. Then, gel was stained in ethidium bromide with a concentration of 0 .5 μg/mL for 20 min, rinsed, and visualized using «G Box» system (Applied Biosystems, USA).

The obtained PCR products were purified by QIAquick PCR purification kit (ExoSAP-IT Affymetrix, USA), and sequenced bi-directionally using Big Dye Terminator Kit version 3.1 (Applied Biosystems) to prepare sequencing reactions. The sequencing procedure was conducted by 3130 XL Genetic Analyzer according to the manufacturer's instructions (Applied Biosystems, USA). Then, the collected data were analyzed by data collection software version 3.0 and sequencing analysis software version 5.3.1 (Applied Biosystems, USA). The sequences were manually trimmed and analyzed with the Basic Local Alignment Search Tool (BLAST).

Phylogenetic Analysis. The sequences were aligned with ClustalW, the evolutionary history was inferred using the neighbor-joining method, and the evolutionary distances

Table 3. Primers sequences used to amplify the virulence genes of isolated *Salmonella*

Virulence genes	Primer sequence (5′-3′)[a]	Size (bp)	GenBank no.	References
orgA	F: TTTTTGGCAATGCATCAGGGA R: GGCGAAAGCGGGGACGGTATT	255	NC003197	[7]
sitC	F: CAGTATATGCTCAACGCGATGTGGGTCTCC R: CGGGGCGAAAATAAAGGCTGTGATGAAC	768	NC003197	[7]
sipB	F: GGACGCCGCCCGGGAAAAACTCTC R: ACACTCCCGTCGCCGCCTTCACAA	875	NC003197	[7]
spiA	F: CCAGGGGTCGTTAGTGTATTGCGTGAGATG R: CGCGTAACAAAGAACCCGTAGTGATGGATT	550	NC003197	[7]
iroN	F: ACTGGCACGGCTCGCTGTCGCTCTAT R: CGCTTTACCGCCGTTCTGCCACTGC	1205	NC003197	[7]
sifA	F: TTTGCCGAACGCGCCCCCACACG R: GTTGCCTTTTCTTGCGCTTTCCACCCATCT	449	NC003197	[7]

[a]F = forward; R = reverse.

Table 4. Virulence genes and antimicrobial resistance profile of *Salmonella* serovars isolated from sausages

Code	Serotypes	Accession Number	Resistant profiles	Virulence genes					
				orgA	*sipB*	*sitC*	*spiA*	*iroN*	*sifA*
8D	Agona	KX355299	AMP	+	+	+	+	+	+
63 D	Muenster	KX355305	AMP	+	+	+	+	+	+
55 MA	Livingstone	KX355303	AMP	+	+	+	+	+	+
57 B	Anatum	KX355304	AMP, S, CT	+	+	+	+	+	+
3MA	Give	KX355298	AMP, S	+	+	+	+	+	+
42MA	Give	MG869141	AMP, S	+	+	+	+	+	+
58MA	Give	MG869142	AMP, S	+	+	+	+	+	+
29MA	Give	MG869140	AMP, S	+	+	+	+	+	+
2B	Bovismorbificans	KX355297	AMP, S	+	+	+	+	+	+
12B	Bovismorbificans	MG869128	AMP, S	+	+	+	+	+	+
51B	Mbandaka	MG869131	AMP	+	+	+	+	+	+
31B	Mbandaka	MG869129	AMP	+	+	+	+	+	+
80B	Mbandaka	KX355306	AMP, S, CT	+	+	+	+	+	+
9MA	Mbandaka	MG869136	AMP, S, CT	+	+	+	+	+	+
17D	Montevideo	KX355301	AMP, S, CT	+	+	+	+	+	+
108D	Montevideo	MG869146	AMP, S, CT	+	+	+	+	+	+
72D	Montevideo	MG869143	AMP, S, CT	+	+	+	+	+	+
100MA	Corvallis	MG869133	AMP, S	+	+	+	+	+	+
97MA	Corvallis	KX355307	AMP, S	+	+	+	+	+	+
25B	Corvallis	MG869139	AMP, S	+	+	+	+	+	+
95D	Corvallis	MG869134	AMP, S	+	+	+	+	+	+
94MA	Corvallis	MG869144	AMP, CT	+	+	+	+	+	+
18B	Corvallis	MG869137	AMP, CT	+	+	+	+	+	+
19MA	Corvallis	MG869138	AMP, AML, SMX, SSS	+	+	+	+	+	+
113MA	Corvallis	KX355309	AMP, S, AML, CXM,	+	+	+	+	+	+
130 D	Saintpaul	KX355311	AMP, S, CT, SMX, SSS, SXT	+	+	+	+	+	+
104D	Kentucky	MG869145	AMP, S	+	+	+	+	+	+
143D	Kentucky	MG869135	AMP, S	+	+	+	+	+	+
30D	Kentucky	KX355302	AMP, S, TE, ENR, OFX, CIP, NA, UB	+	+	+	+	+	+
116B	Kentucky	KX355310	AMP, AML, MA, SMX, NA, ENR, UB, OFX, CIP, TE, S, SSS	+	+	+	+	+	+
1D	Kentucky	MG869130	AMP, AML, AMC, SMX, NA, ENR, UB, OFX, CIP, TE, S, SSS	+	+	+	+	+	+
14D	Kentucky	KX355300	AMP, AML, AMC, MA, SMX, NA, ENR, UB, OFX, CIP, TE, S, SSS	+	+	+	+	+	+
102D	Typhimurium	MG869132	AMP, AML, AMC, CRO, MA, CXM, CTX, SXT, SMX, K, AK, CT, TE, S, C, SSS	+	+	+	+	+	+
105D	Typhimurium	KX355308	AMP, AML, AMC, CRO, MA, CXM, CTX, SXT, SMX, NA, UB, K, AK, CT, TE, S, C, SSS	+	+	+	+	+	+

were computed using the maximum composite likelihood method (Mega7 software) with 1000 bootstrap replications. *Salmonella enterica* str. 08-00436, *Salmonella* Mbandaka str. SA20026234, *Salmonella* Agona str. 460004 2-1, and *Salmonella* Typhi str. CT18 were used as in-group strains, and *Escherichia coli* ATCC 8739 was used as an out-group strain to root the phylogenetic tree.

Ethics. Ethical approval was not required in this study since no live animals or humans samples were used in the experiments.

Results

Isolation, Identification and Antimicrobial Susceptibility Testing. Among the 34 isolated *Salmonella*, 12 serotypes were identified (Table 4). In addition, the antimicrobial resistance analysis has shown a high rate of resistance to several antibiotics, with presence of 13 different phenotypic profiles (Table 4). Moreover, multidrug resistance (more than two antibiotics) was detected in 15 strains (44%). From them, one *Salmonella* typhimurium strain was considered as the highest by exerting resistance against 18 different antimicrobial compounds (Table 4).

Amplification of Virulence Genes Using Multiplex PCR. In order to evaluate the pathogenicity of *Salmonella* strains and to have an idea about its capacity to survive in host environments, simplex and multiplex PCR methods were used to detect 6 virulence genes, namely, *orgA*, *sipB*, *sitC*, *spiA*, *iroN*, and *sifA* (Figures 1 and 2), involved in its virulence system. The results showed that all isolated strains contain the 6 virulence genes (Table 4).

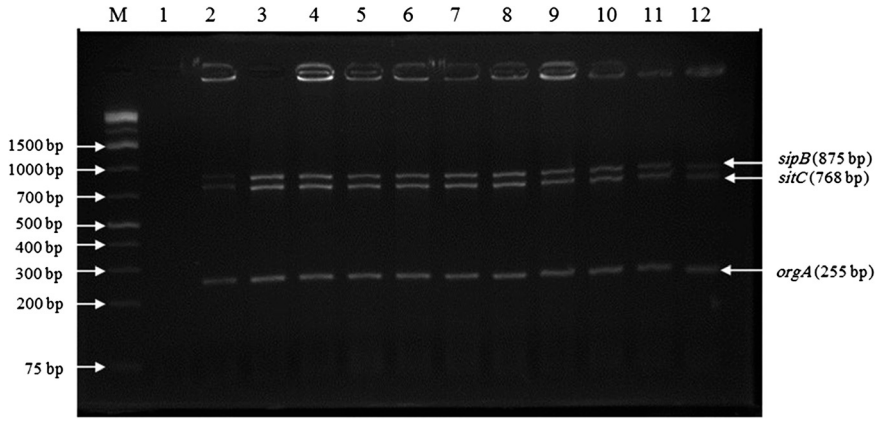

Figure 1. PCR products amplified with the universal virulence gene primers (*orgA*, *sipB*, and *sitC*). Lanes: (M) DNA ladder (GeneRuler 1Kb Plus), (1) negative control, (2) positive control, and (3–12) *Salmonella* tested.

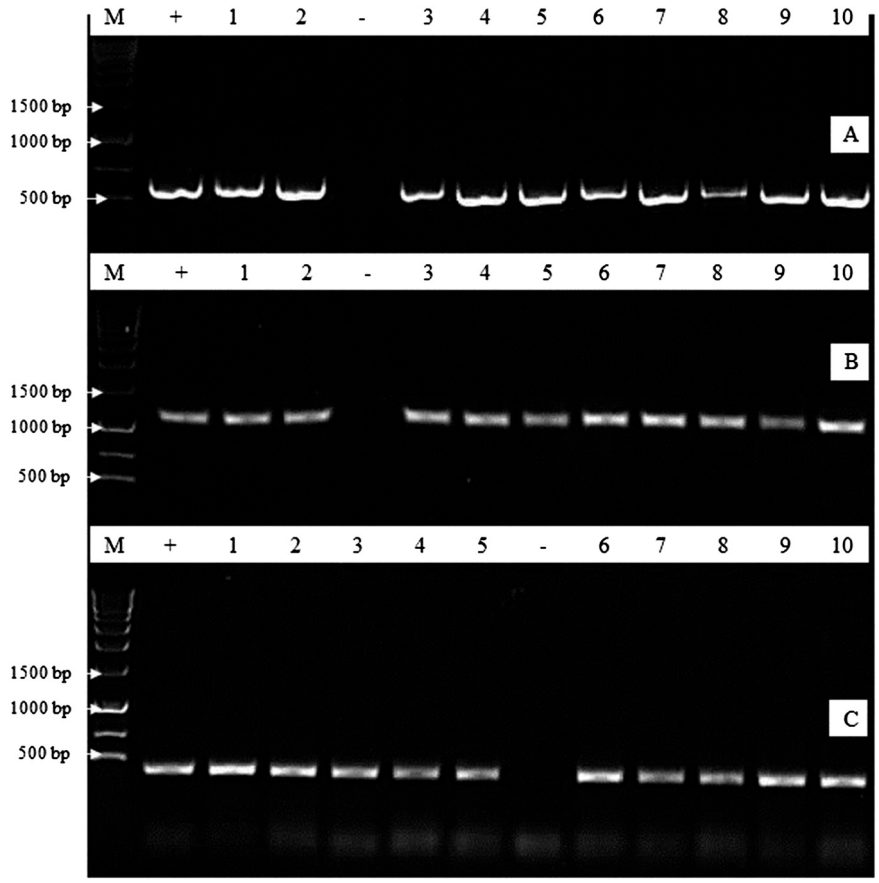

Figure 2. PCR products in agarose gel electrophoresis 1% with the expected molecular size of 550 bp for *SpiA* gene (A), 1205 bp for *IroN* gene (B), and 449 bp for *SifA* gene (C). Lanes: (1–10) *Salmonella* tested, (M) DNA ladder (Hyper Ladder 500 bp), (−) negative control, and (+) positive control

Amplification and Sequencing of the 16S rRNA Genes. Amplification of 16S rRNA genes was performed on extracted genomic DNA of isolated *Salmonella* strains. The results showed that all examined strains generate an amplicon of 1500 bp (Figure 3). Then, PCR products were sequenced and analyzed by the online software "BLAST" (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), and the obtained sequences were deposited in GenBank under the corresponding accession numbers (Table 4).

Phylogenetic Analysis and Identification Criteria. The phylogenetic analysis of 16S rRNA genes organized all the strains into 3 clusters (I, II, and III), and cluster I is divided into 3 sub-clusters (Ia, Ib, and Ic) (Figure 4). The analysis of 16S rRNA genes groups the serotypes Corvallis, Kentucky, Typhimurium, Anatum, Mbandaka, Livingstone, and Saintpau in cluster I, and cluster II is constituted by S. Montevideo, S. Bovismorbificans, S. Give, and S. Agona. However, cluster III is constituted by 2 strains, namely, S. Muenster and S. Kentucky, which are placed separately on side branches from the strains. Moreover, 16S rRNA based analysis showed that all 16S rRNA sequences possessed a ≥98% similarity to those of *Salmonella enterica* species.

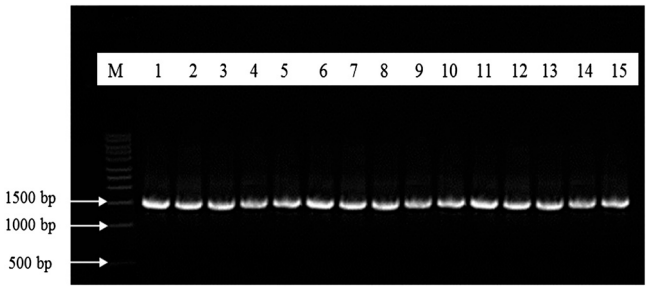

Figure 3. The amplification results of 16S rRNA genes using universal primers. Lanes: (1) S. Typhimirium, (2) S. Mbandaka, (3) S. Livingstone, (4), S. Muenster, (5) S. Give, (6) S. Bovismorbificans, (7–9) S. Kentucky, (10–11) *S. Corvallis*, (12) S. Montevideo, (13) S. Agona, (14) S. Anatum, (15) S. Saintpaul, and (M) 1Kb DNA ladder

Discussion

Worldwide, *Salmonella* are recognized as a major gastrointestinal pathogen for humans and animals, whereas, *Salmonella* typhimurium and *Salmonella* enteritidis were considered as the major serovars that can be infecting human after eating food of animal origin, but this data may be different over the years and countries.

In this study, the antimicrobial resistance analysis revealed the presence of *Salmonella* Kentucky resistant to quinolones and *Salmonella* Typhimurium resistant to multiple antibiotics including 3rd generation cephalosporins. Other studies showed that the resistance to quinolones, fluoroquinolones, and 3rd generation cephalosporins in *Salmonella* has recently increased [17–20]. This resistance may be due to the intensive use of antibiotics in the veterinary and human fields by contributing to the acquisition of resistance genes and exerting a selective pressure for the development of resistant bacteria [21] and may change depending on serotype, time, source of microorganism, and geographic region of isolate [22].

The pathogenicity of *Salmonella* is influenced by various factors encoded by different virulence genes that play an important role in different steps of pathogenicity, such as adhesion, invasion, intracellular survival, systemic infection, toxin production, and iron acquisition [7]. In this study, the amplification of virulence genes by PCR showed that all isolated strains contain the 6 tested virulence genes (*orgA*, *sitC*, *sipB*, *spiA*, *iroN*, and *sifA*).

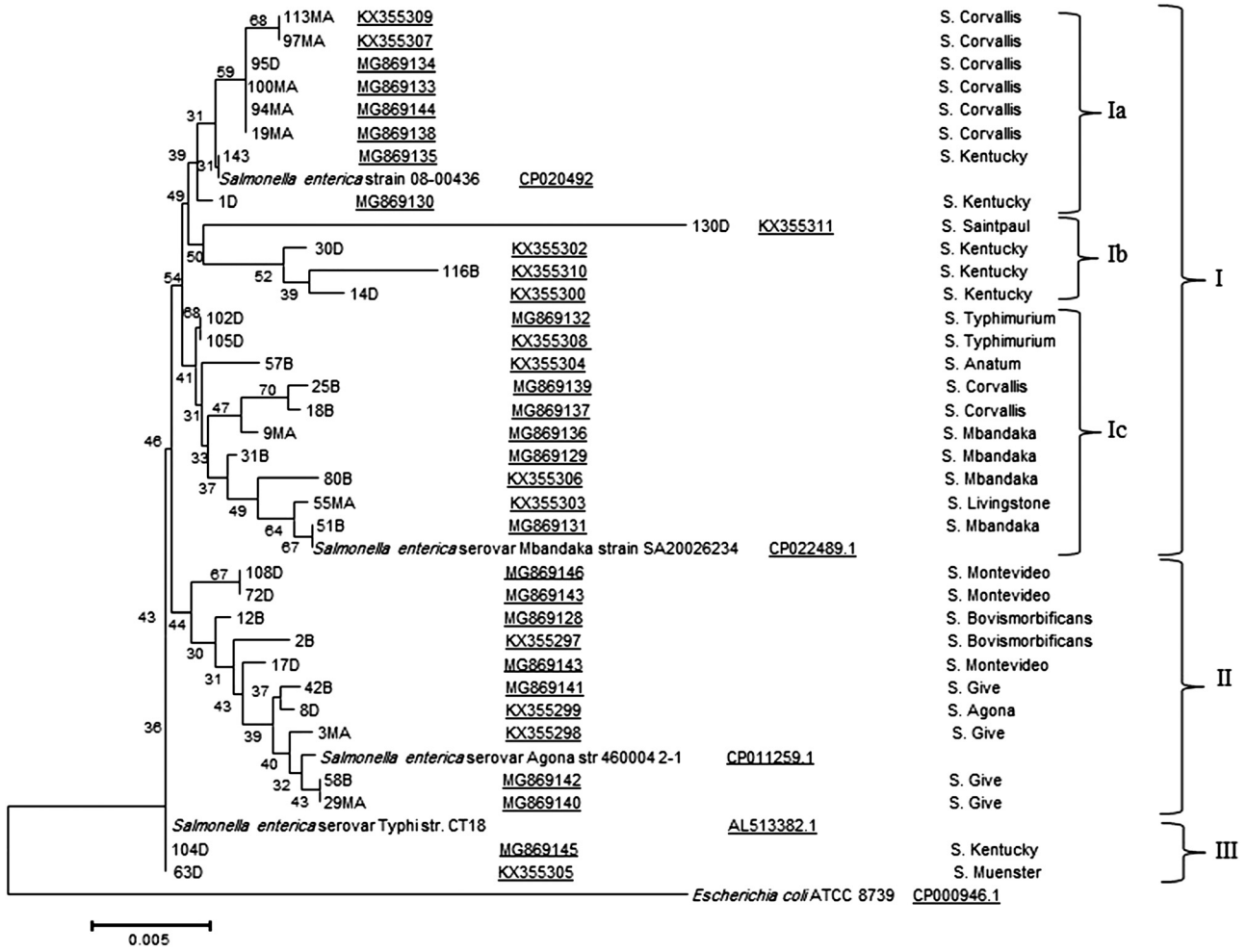

Figure 4. Phylogenetic tree of isolated *Salmonella enterica* strains based on 16S rRNA sequences analysis using MEGA7

The host cell invasion and intracellular survival of *Salmonella* depend on several genes that are clustered on pathogenicity islands (PIs), and these effector proteins are transferred by 2 type III secretion systems, namely, T3SS1 and T3SS2 [23]. Moreover, *Salmonella* strains expresses several invasion genes located on the *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2), which promote bacterial uptake and invasion of non-phagocytic cells to cross the epithelial barrier [24]. Among these genes, *orgA* is required for invasion and secretion system [25]. Thus, the *sipB* gene is responsible for the entry into non-phagocytic cells and the killing of macrophages. It has been shown that the targeting of *sipB* by the inv-Spa type III secretion apparatus is necessary and sufficient for the induction of macrophage apoptosis [26].

The *iroN* gene is implicated in iron acquisition [27]. However, the *sitC* gene encodes an important transporter of iron. Also, a study carried out by Boyer et al. on *Salmonella* Typhimurium showed that *sitABCD* is required for replication of *Salmonella* inside macrophages and for the creation of virulence in susceptible animals [28]. On the other hand, the *sifA* gene is required for the formation of filamentous structures in the lysosome vacuoles of infected epithelial cells [29].

A previous study conducted on *Salmonella* typhimurium showed that *spiA* gene codes for an outer membrane component that is essential for virulence in host cells [30]. Another study carried out by Dong et al. revealed that *spiA* gene is associated with biofilm formation mechanism [31]. It has been demonstrated that biofilm formation improves the ability of microorganisms to withstand stresses, such as desiccation, temperature extremes, antibiotics, and antiseptics [32], which allowed these bacteria to survive longer in animal farms and to contaminate meats and eggs, which remain the main vehicles of *Salmonella* transmission to humans.

In Morocco, it is a challenge for laboratories to systematically identify *Salmonella* isolates on the basis of biochemical and serological identification using Kaufmann White scheme because of the cost of reagents and the time needed to get the results. In this study, we conducted a rapid and cost-effective method to identify the isolated *Salmonella* strains using DNA sequencing tools. Furthermore, the sequence analysis of the 16S rRNA genes is being regularly used to identify bacterial species and to perform the taxonomic studies in clinical and scientific investigations [33]. However, the phylogenetic analyses can be used to understand the emergence of pathogenic bacteria and to study the relationship between isolated strains and other reference strains, implicated in animal or human diseases [12, 19, 34].

In this study, the analysis of phylogenetic tree showed the presence of different 16S rRNA profiles among the isolated *Salmonella* serovars, which are grouped into 3 clusters and 3 sub-clusters, suggesting the presence of different contamination sources due to the diversity of raw materials and manufacturing processes used for each type of sausages.

Conclusion

The results obtained in this study showed that the sequencing of 16S rRNA genes was a suitable tool for identification of *Salmonella* strains at the genus and species level. In addition, the phylogenic analysis showed a high diversity in all clusters of this bacterium. Furthermore, the antimicrobial

resistance and the virulence gene analysis suggest that sausages are contaminated with *Salmonella* strains and present a major risk to public health.

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Authors' Contributions

This work was carried out in collaboration between all authors. FRF, BB and MM designed the experimental procedures. AED, BO and AE conducted the experimental analysis. SK and SO conducted the bio-informatics analysis. AED and FRF analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

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