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Antimicrobial resistance genes, virulence markers and prophage sequences in *Salmonella enterica serovar* Enteritidis isolated in Tunisia using whole genome sequencing

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ABSTRACT

Salmonella Enteritidis causes a major public health problem in the world. Whole genome sequencing can give us a lot of information not only about the phylogenetic relatedness of these bacteria but also in antimicrobial resistance and virulence gene predictions. In this study, we analyzed the whole genome data of 45 *S.* Enteritidis isolates recovered in Tunisia from different origins, human, animal, and foodborne samples. Two major lineages (A and B) were detected based on 802 SNPs differences. Among these SNPs, 493 missense SNPs were identified. A total of 349 orthologue genes mutated by one or two missense SNPs were classified in 22 functional groups with the prevalence of carbohydrate transport and metabolism group. A good correlation between genotypic antibiotic resistance profiles and phenotypic analysis were observed. Only resistant isolates carried the respective molecular resistant determinants. The investigation of virulence markers showed the distribution of 11 *Salmonella* pathogenicity islands (SPI) out of 23 previously described. The SPI-1 and SPI-2 genes encoding type III secretion systems were highly conserved in all isolates except one. In addition, the virulence plasmid genes were present in all isolates except two. We showed the presence of two fimbrial operons *sef* and *ste* previously considered to be specific for typhoidal *Salmonella*. Our collection of *S.* Enteritidis reveal a diversity among prophage profiles. SNPs analysis showed that missense mutations identified in fimbriae and in SPI-1 and SPI-2 genes were mostly detected in lineage B.

In conclusion, WGS is a powerful application to study functional genomic determinants of *S.* Enteritidis such as antimicrobial resistance genes, virulence markers and prophage sequences. Further studies are needed to predict the impact of the missenses SNPs that can affect the protein functions associated with pathogenicity.

1. Introduction

During the last two decades, whole-genome sequencing (WGS) has become the affordable tool that has the capacity to revolutionize different domains including genetics, microbiology, epidemiology and public health surveillance. The evolution of the current WGS technologies allowed to rapidly increase the realization of bacterial genome sequencing projects (Hu et al., 2021; Punina et al., 2015) . Since the entire genome is readily available for analysis, WGS has the capacity to replace traditional methods for characterizations such as serotyping, virulotyping and antimicrobial resistance which can easily be predicted from the genome. This improves the capacity of surveillance systems to quickly provide information on the probable source, to identify the path of disease transmission within a population and to detect the virulence factors of the pathogen. Therefore, WGS-based analyses are becoming the primary subtyping tool of choice for pathogens particularly *Salmonella* species (Deng et al., 2015). To date, there are over 400,000 *Salmonella enterica* genomes published in public databases, that have been

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widely exploited to track and investigate outbreaks ("Home - Pathogen Detection - NCBI,"). *Salmonella enterica subsp enterica serovar* Enteritidis (*S.* Enteritidis) is among the most frequent *Salmonella* serovars isolated worldwide. It is a human and animal pathogen that causes a major public health problem. The genome of Enteritidis serovar has approximately 5 million bases and codes for over 4000 genes, from which more than 200 genes are actively dysfunctional. A global comparison study of *S.* Enteritidis isolates demonstrates the emergence of closely related isolates during a long period and underline the genetic homogeneity of this serovar. The virulence of *Salmonella enterica* depends on diverse assortment of genes which are required for adhesion, invasion, intra-cellular survival, and replication. These genes are located on various elements of the genome including *Salmonella* pathogenicity islands (SPIs) (Gupta et al., 2019; Zhao et al., 2020). Different strains of *S.* Enteritidis have mobile genetic elements such as plasmids, prophages and transposon that can carry virulence or antimicrobial resistance genes (Hu et al., 2021).

Recently, WGS has been successfully used in our previous studies to assess the genetic diversity of *S.* Enteritidis and *Salmonella* Typhimurium serovars (Ksibi et al., 2020; Ktari et al., 2020). The use of these technologies has greatly enlarged our view of the genetic diversity of this bacterium. Furthermore, it produces an opportunity to provide more genetic information to study all genetic determinants such as virulence markers, antimicrobial resistance genes, mobile elements, bacteriophages and to determine genomic changes associated with pathogenicity and antibiotic resistance.

In this study, we sought to investigate the whole genome of 45 *S.* Enteritidis strains isolated from different sources, human, animals and food in order to predict the antimicrobial resistance, the potential pathogenicity, the prophages sequences and the substitution of an amino acid on the protein sequence.

2. Material and methods

2.1. Bacterial strains

45 *S.* Enteritidis strains isolated from human, animal, and food samples were included in this study. A collection of 29 clinical *S.* Enteritidis isolates were recovered in the Laboratory of Microbiology CHU Habib Bourguiba Sfax-Tunisia between 2000 and 2015. In addition, seven *S.* Enteritidis isolates were collected in 2009, 2014, and 2015 from animal samples. We also included nine *S.* Enteritidis isolates obtained from two distinct foodborne outbreaks that occurred in 2007 (Table 1).

2.2. Antibiotic susceptibility test

Antibiotic susceptibilities of *S.* Enteritidis isolates were determined with the disk diffusion method, as recommended by European Committee on Antimicrobial Susceptibility Testing (EUCAST 2013). Antimicrobial susceptibility was performed for 16 antimicrobials, including ampicillin (10 μg), amikacin (30 μg), chloramphenicol (30 μg), azithromycin (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), streptomycin (10 μg), sulfamide (30 μg), tetracycline (30 μg), tigecycline (15 μg), streptomycin (10 μg), spectinomycin (100 μg), kanamycin (30 μg), netilmicin (10 μg), nalidixic acid (30 μg), pefloxacin (5 μg), and trimethoprim (30 μg). .

2.3. Whole genome sequencing, assembly and phylogeny

Genomic DNA was extracted using a MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Life Science). Sequencing libraries were prepared with a Nextera XT DNA sample preparation kit (Illumina, Inc., San Diego, CA) and sequenced on an illumina NextSeq 500 platform (Illumina, San Diego, CA, USA) with 100 to 150-bp paired-end protocol according to the manufacturer's instructions. A threshold of 30X minimum coverage was applied. Genome sequences were assembled using SPAdes software V.3.6.0 with default settings (Bankevich et al., 2012). Genome assemblies consisted of several contigs ranging from ~3.6 to 4.8 Mbp, with an average GC content of ~52%. The sequencing depth of coverage ranged between 81% to 94%. The genomes were annotated with the NCBI Prokaryotic Genomes Annotation Pipeline (PGAP V.4.1) (http://ncbi.nlm.nih.gov/genomes/static/Pipeline.html) (Tatusova et al., 2016). We identified a total number of genes ranged between 4313 and 5147, a number of protein coding regions ranging from 4228 to 5075, and a number of pseudogenes ranging from 150 to 665. The phylogenetic relationship based on SNPs was constructed by mapping all the paired-end reads to the *S.* Enteritidis reference genome (GenBank:AM933172) as previously described (Ksibi et al., 2020).

2.4. In silico analysis of virulence genes and prophage detection

Salmonella Pathogenicity Islands (SPIs) were detected using SPIFinder 1.0 database (https://cge.cbs.dtu.dk/services/SPIFinder/). Prophage sequences were detected using PHASTER web server (http://phaster.ca/): only "intact" prophages were considered for this analysis (Zhou et al., 2011).

2.5. In silico identification of resistance genes

Antimicrobial resistance genes were searched using the ResFinder database (https://cge.cbs.dtu.dk/services/ResFinder/) (Zankari et al., 2012) with the following thresholds: minimum length coverage of 90% and nucleotide sequence identity of 96%. PointFinder was used to detect chromosomal structural gene mutations, *gyrA, gyrB, parC*, and *parE* genes, which were analyzed for quinolone resistance-determining region (QRDR) mutations (Zankari et al., 2017).

2.6. SNPs annotation and Cog analysis

SNPs annotations were obtained using SnpEff v4.121 with Ensembl gene annotation database for *Salmonella* (Cingolani et al., 2012). Clusters of Orthologous Groups of proteins (COGs) database was used for the functional annotation of SNPs and determination the most variable COGs for *S.* Enteritidis (Tatusov, 2000). The amino acid sequences generated from the SnpEff were used as input for functional annotation based on orthologous group.

2.7. Nucleotide sequence accession numbers

Complete genome sequences of these *S.* Enteritidis isolates are available in GenBank under BioProject no. PRJNA579483 and the GenBank accession numbers listed in Table 1.

3. Results

3.1. Phylogeny and functional SNPs

A phylogenetic analysis based on 806 SNPs revealed the existence of two lineages named A and B with 345 to 470 SNPs difference. Examination of individual lineage revealed that lineage A grouped twenty-six isolates with two clades (C1 and C2) and five subclades (C1–1 to C1–5). Lineage B included eighteen *S.* Enteritidis subdivided into two clades (C3 to C4) and two subclades (C3–1 and C3–2) placed in lineage B (Fig. 1).

Variant annotations showed that 276 were silent SNPs (33.99%), 493 were missenses SNPs (61.16%) and 37 were nonsense mutations (4.59%). The distribution of 493 missense SNPs among serovar Enteritidis was examined using the COG database. This annotation reflected that the genes are scattered in an assorted range of 22 functional categories throughout the genome. The COG distribution showed that the five most prevalent groups were carbohydrate transport and metabolism

Table 1
List of 45 Samonella Enteritidis strains sequenced for comparison.

No.	Tree Label	biosample	Bioproject	genome	filename	Isolation date	Isolation type	Isolation source	Resistance patterns	Antimicrobial resistance genes	
										gyrA	blaTEM
1	C238	SAMN13108479	PRJNA579483	WICN00000000	C238.fasta	2000	Clinical	Stool	susceptible		
2	C5352	SAMN13110851	PRJNA579483	WICR00000000	C5352.fasta	2000	Clinical	unknown	susceptible		
3	X1500	SAMN13110876	PRJNA579483	WIDC00000000	X1500.fasta	2000	Clinical	unknown	susceptible		
4	B3015	SAMN13108482	PRJNA579483	WIBL00000000	B3015.fasta	2001	Clinical	Stool	susceptible		
5	D849	SAMN13108483	PRJNA579483	WICS00000000	D849.fasta	2002	Clinical	Stool	susceptible		
6	S1078	SAMN13108484	PRJNA579483	WIDB00000000	S1078.fasta	2003	Clinical	Stool	susceptible		
7	B3544	SAMN13108480	PRJNA579483	WIBM00000000	B3540.fasta	2004	Clinical	Stool	susceptible		
8	I42	SAMN13108481	PRJNA579483	WICY00000000	I42.fasta	2005	Clinical	Blood	susceptible		
9	B3790	SAMN13108485	PRJNA579483	WIBN00000000	B3790.fasta	2006	Clinical	Cerebro-Spinal Fluid	susceptible		
10	C4140	SAMN13108486	PRJNA579483	WICQ00000000	C4140.fasta	2007	Clinical	Stool	susceptible		
11	B1988	SAMN13108419	PRJNA579483	WIBK00000000	B1988.fasta	2007	Clinical	Stool	susceptible		
12	I1104	SAMN13110852	PRJNA579483	WICZ00000000	I1104.fasta	2007	Clinical	Stool	susceptible		
13	B5147	SAMN13108487	PRJNA579483	WICN00000000	B5147.fasta	2008	Clinical	Blood	susceptible		
14	G604	SAMN13110859	PRJNA579483	WICU00000000	G604.fasta	2008	Clinical	Stool	NAL	p.Asp87Asn	
15	C1248	SAMN13108488	PRJNA579483	WICO00000000	C1243.fasta	2009	Clinical	Blood	NAL Pef	p.Ser83Phe	
16	ED1075	SAMN13110877	PRJNA579483	WICT00000000	ED1075.fasta	2009	Clinical	Blood	NAL Pef	p.Asp87Asn	
17	H1349	SAMN13110860	PRJNA579483	WICX00000000	H1349.fasta	2009	Clinical	Blood	susceptible		
18	H175	SAMN13110878	PRJNA579483	WICV00000000	H175.fasta	2010	Clinical	Stool	NAL Pef	p.Ser83Phe	
19	H444	SAMN13110884	PRJNA579483	WICW00000000	H444.fasta	2010	Clinical	Stool	NAL	p.Asp87Asn	
20	C1458	SAMN13108489	PRJNA579483	WICP00000000	C1458.fasta	2010	Clinical	Stool	susceptible		
21	J316	SAMN13110853	PRJNA579483	WIDA00000000	J316.fasta	2010	Clinical	Blood	AMP NAL	p.Asp87Asn	blaTEM
22	C2644	SAMN13108490	PRJNA579483	WICC00000000	BK-C2644.fasta	2012	Clinical	Blood	susceptible		
23	B480	SAMN13108491	PRJNA579483	WICA00000000	BK-B480.fasta	2013	Clinical	Stool	NAL Pef	p.Asp87Asn	
24	NP294	SAMN13110854	PRJNA579483	WICF00000000	BK-NP294.fasta	2013	Clinical	Urine	NAL	p.Asp87Thr	
25	W355	SAMN13110881	PRJNA579483	WICM00000000	BK-W355.fasta	2013	Clinical	Blood	NAL Pef	p.Asp87Thr	
26	B2464	SAMN13110883	PRJNA579483	WICB00000000	BK-B2464.fasta	2013	Clinical	Stool	NAL Pef	p.Asp87Thr	
27	pl573	SAMN13108492	PRJNA579483	WICG00000000	BK-PL573.fasta	2014	Clinical	Stool	susceptible		
28	F74/14	SAMN13110858	PRJNA579483	WICE00000000	BK-F74.fasta	2014	Clinical	Blood	NAL Pef	p.Ser83Thr	
29	CR268	SAMN13108493	PRJNA579483	WICD00000000	BK-CR268.fasta	2015	Clinical	Blood	NAL Pef	p.Asp87Thr	
30	S2	SAMN13110855	PRJNA579483	WICH00000000	S2.fasta	2009	Animal	Chick	susceptible		
31	S10	SAMN13110856	PRJNA579483	WICI00000000	BK-S10.fasta	2014	Animal	Poultry	NAL	p.Asp87Thr	
32	S12	SAMN13110879	PRJNA579483	WICJ00000000	BK-S12.fasta	2014	Animal	Poultry	NAL Pef	p.Asp87Thr	
33	S19	SAMN13110880	PRJNA579483	WICK00000000	BK-S19.fasta	2009	Animal	Chick	susceptible		
34	S29	SAMN13110885	PRJNA579483	WICL00000000	BK-S29.fasta	2009	Animal	Chick	susceptible		
35	1922	SAMN13110889	PRJNA579483	WIBQ00000000		2014	Animal	Poultry	susceptible		

(*continued on next page*)

Table 1 (*continued*)

No.	Tree Label	biosample	Bioproject	genome	filename	Isolation date	Isolation type	Isolation source	Resistance patterns	Antimicrobial resistance genes gyrA	blaTEM
					BK-1922.fasta						
36	153 (F)	SAMN13110886	PRJNA579483	WIBP00000000	BK-153.fasta	2014	Animal	Poultry	NAL Pef	p.Asp87Asn	
37	3174	SAMN13110933	PRJNA579483	WIBX00000000	BK-3174.fasta	2007	Foodborne		susceptible		
38	3156	SAMN13110887	PRJNA579483	WIBT00000000	BK-3156.fasta	2007	Foodborne		susceptible		
39	3157	SAMN13110890	PRJNA579483	WIBU00000000	BK-3157.fasta	2007	Foodborne		susceptible		
40	3220	SAMN13110940	PRJNA579483	WIBY00000000	BK-3220.fasta	2007	Foodborne		susceptible		
41	3222	SAMN13110941	PRJNA579483	WIBZ00000000	BK-3222.fasta	2007	Foodborne		susceptible		
42	3155	SAMN13110882	PRJNA579483	WIBS00000000	BK-3155.fasta	2007	Foodborne		susceptible		
43	3173	SAMN13110895	PRJNA579483	WIBW00000000	BK-3173.fasta	2007	Foodborne		susceptible		
44	3171	SAMN13110893	PRJNA579483	WIBV00000000	BK-3171.fasta	2007	Foodborne		susceptible		
45	3152	SAMN13110857	PRJNA579483	WIBR00000000	BK-3152.fasta	2007	Foodborne		susceptible		

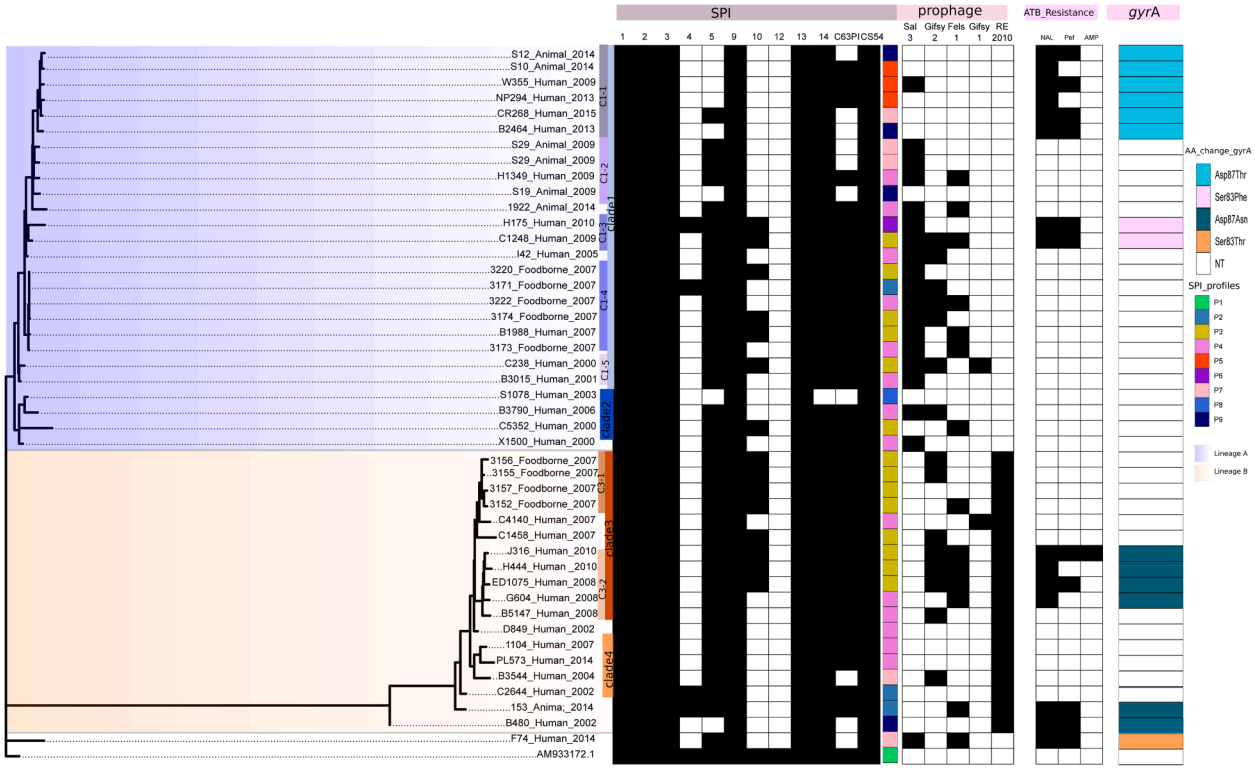

Fig. 1. Maximum likelihood tree of 45 *Salmonella* Enteritidis genomes with reference strain AM933172.1. Branch lables represent isolate number_Isolation type_-isolation date. Heatmaps showing the presence and absence of SPI, prophage and antimicrobial resistance with a white box indicates absence while a black box shows presence. Point mutations in *gyrA* and SPI profile are indicated by color as shown in the legend.

[G] (11.7%), amino acid transport and metabolism [E] (9.46%), transcription [K] (9.17%), cell wall/membrane/envelope biogenese [M] (6.88%) and energy production and conversion [C] (6.59%) (Fig. 2).

3.2. *In vitro and in silico assessments of antimicrobial resistance*

Out of 45 isolates, 15 exhibited antimicrobial resistance (Table 1, Fig. 1). Among all these resistant isolates, only four were strictly resistant to nalidixic acid, ten showed resistance to nalidixic acid and intermediate resistance to pefloxacin, and one clinical isolate was resistant to nalidixic acid and ampicillin (Fig. 1). The antimicrobial resistance gene results showed that 15 resistant isolates harbored single nucleotide polymorphism in the gene *gyr*A: Asp87Thr ($n = 6$), Ser83Phe ($n = 2$), Ser83Tyr ($n = 1$) and Asp87Asn ($n = 6$) (Fig. 1). No other known quinolone resistance mutations were detected. Only one isolate contained a marker for β-lactamase $bla_{\text{TEM-1b}}$ gene conferring ampicillin resistance. No antimicrobial resistance genes have been identified in the genome of the susceptible isolates. These results agreed with those

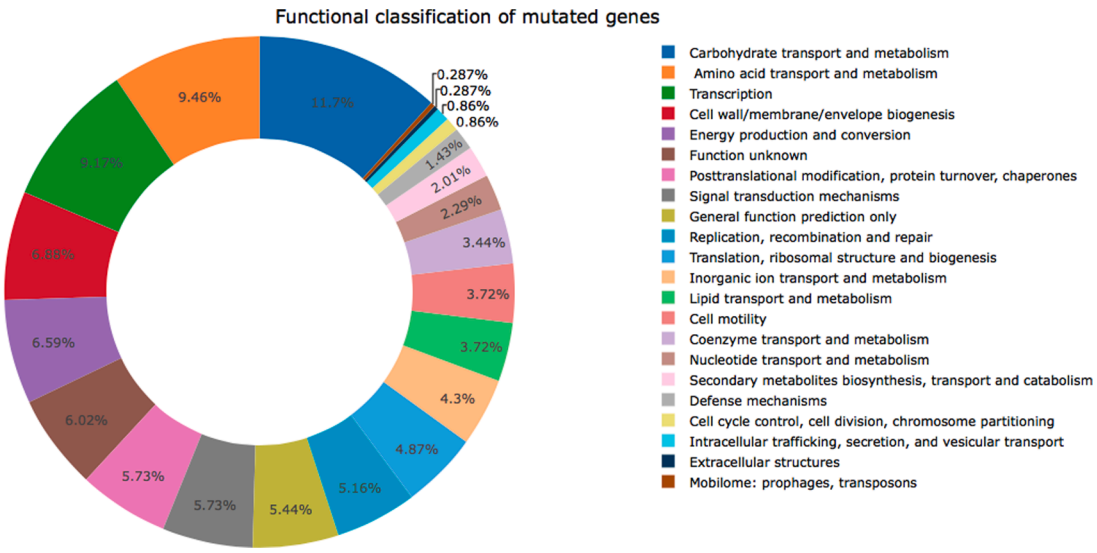

Fig. 2. Functional classification of mutated orthologues genes of 45 *Salmonella* Enteritidis genomes with Clusters of Orthologous Groups COGs. Each functional group is indicated by color as shown in the legend.

obtained from the phenotypic analysis.

Phylogenetic tree analysis demonstrated that resistant isolates were separated in two lineages. The six isolates carrying the amino acid change Asp87Thr were grouped in the clade C2–5. The amino acid change from serine to phenylalanine at the site 83 were detected in two isolates grouped in C2–3. At the same site, the singleton isolate showed

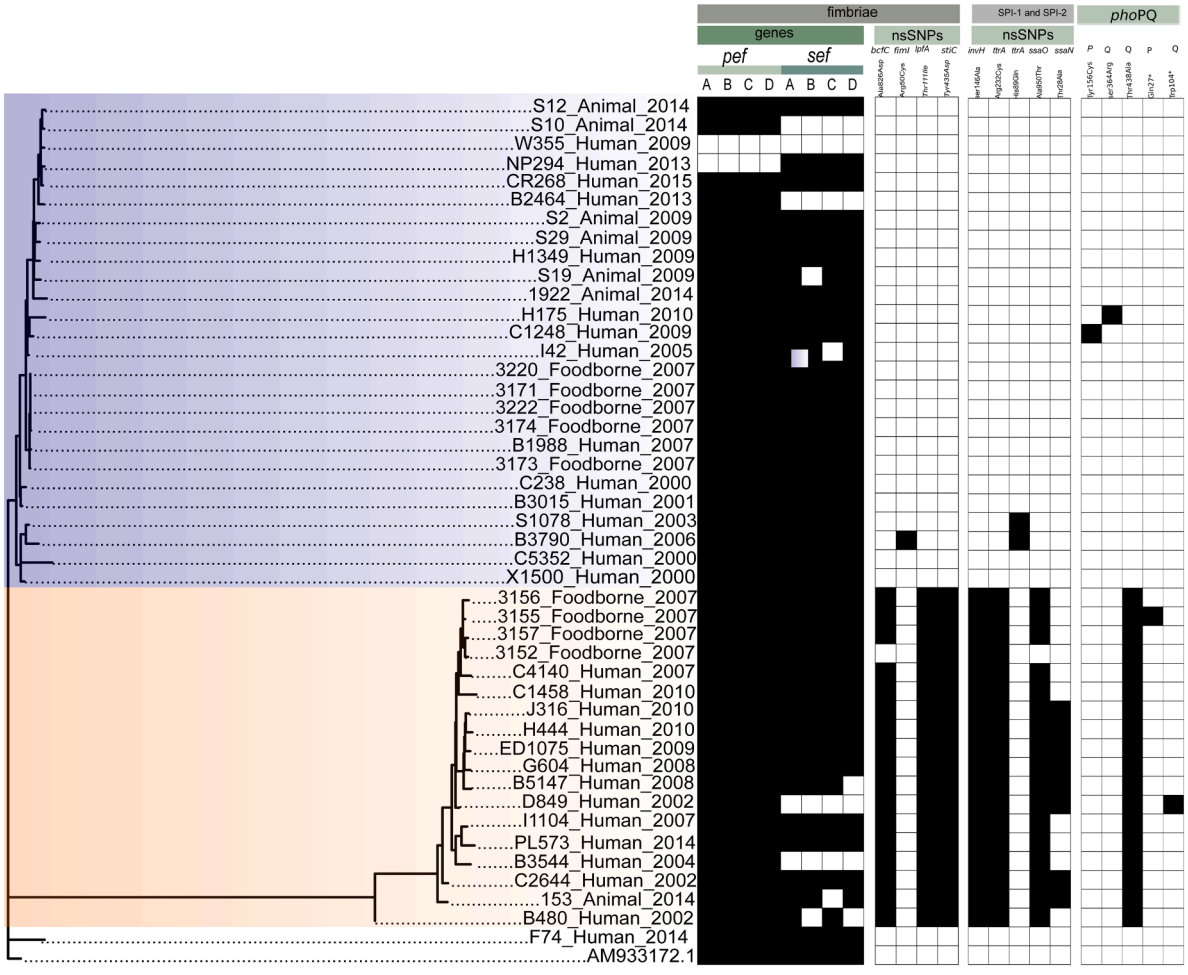

Fig. 3. Maximum likelihood tree of 45 *Salmonella* Enteritidis genomes with reference strain AM933172.1. Branch lables represent isolate number_Isolation type_-isolation date. First heatmap showing the presence and absence of *pef* and *sef* genes. Second and third heatmap showing the presence and absence of missense mutations in fimbriae, SPI-1, SPI-2 and *pho*PQ genes with a white box indicates absence and a black box shows presence.

the variation from serine to tyrosine. Finally, six isolates having the mutation at the site 87 from aspartic acid to asparagine were assembled in lineage B (Fig. 2).

3.3. In silico assessments of virulence genes

To elucidate genomic features of virulence, we used the SPIFinder database. This analysis identified 11 out of 23 previously described SPIs (Fig. 1). All 45 *S.* Enteritidis carried the same six SPIs, including SPI-1, SPI-2, SPI-3, SPI-9, SPI-13 and CS54. The SPI-14 was detected in all isolates except one. Eight profiles of SPIs, arbitrarily designated as P1 to P8, were identified with two major profiles P3 and P4 (14 isolates for each) which differ at the level of SPI-10. These profiles shared SPI-1_SPI-2_SPI-3_SPI-5_SPI-9_SPI-13_SPI-14_C63PI_CS54 (Fig. 1).

WGS data was used to screen SPI-1 and SPI-2 genes from 45 *S.* Enteritidis. SPI-1 and SPI-2 genes were common to all isolates except one clinical strain isolated in 2000 and lacking the *spi*C and *sse*A genes. In addition, the *pho*P and *pho*Q genes, encoded the *pho*P/Q system, were detected in all our isolates.

All *S.* Enteritidis isolates harbored 12 fimbrial operons *agf, bcf, fim, lpf, peg, saf, stb, std, ste, stf, sth* and *sti.* Conversely, *sta, stc, stg, stj, stk* and *tcf* genes were absent in all isolates. It is noticeable that fimbrial adherence operon *sef* was detected in all isolates except five. Moreover, four isolates showed the absence of one or two *sef* operon genes (Fig. 3).

Finally, the virulence plasmid genes (*spv*ABCDR) involved in intra-macrophage survival, plasmid-encoded fimbriae (*pef*ABCD), transfer gene (*tra*AVKELY) and resistance to complement killing (*rck*) were present in all our isolates except two (Fig. 4). None of these plasmids contained genes associated with antimicrobial resistance. The expression of several genes on the virulence plasmid that contribute to efficient systemic infections was regulated by alternative sigma factor *RpoS*. This factor across all isolates.

3.4. Mutations of the target virulence genes

We identified 15 synonymous, 20 missense and 7 nonsens mutations among the selected target genes of SPI-1and SPI-2 genes, fimbrial adherence genes and virulence plasmid genes. The most commonly mutations were changes between G and A (15 times), C and T (15 times) and C and A (9 times); the less frequent changes were those between T and A (2 times) and T and G (1 time). Regarding the mutations in each gene, we identified four missenses SNPs in fimbrial adherence genes (Fig. 3). For one isolate of lineage A, the *fim*I gene was found to have one missense mutation resulted in an acid amine change of Arginine to cysteine at the site 50. The missense mutations in *lpf*A and *sti*C genes, at the site 111 (from threonine to isoleucine) and site 435 (from tyrosine to aspartic acid), respectively, were detected across all the isolates of lineage B (Fig. 3; Table 2).

Among SPI-1 and SPI-2 genes, we found five genes mutated with missense SNPs. The phylogenetic analysis demonstrated that two clinical isolates of lineage A carried a missense mutation in the *ttr*A gene (from alanine to Aspartate) at the site 950 (Fig. 3; Table 2). For the lineage B isolates, four missense mutations have been identified in *inv*H (Ser146Ala), *ttr*A (Arg232Cys), *ssa*Q (Thr28Ala) and *ssa*N (His89Gln)

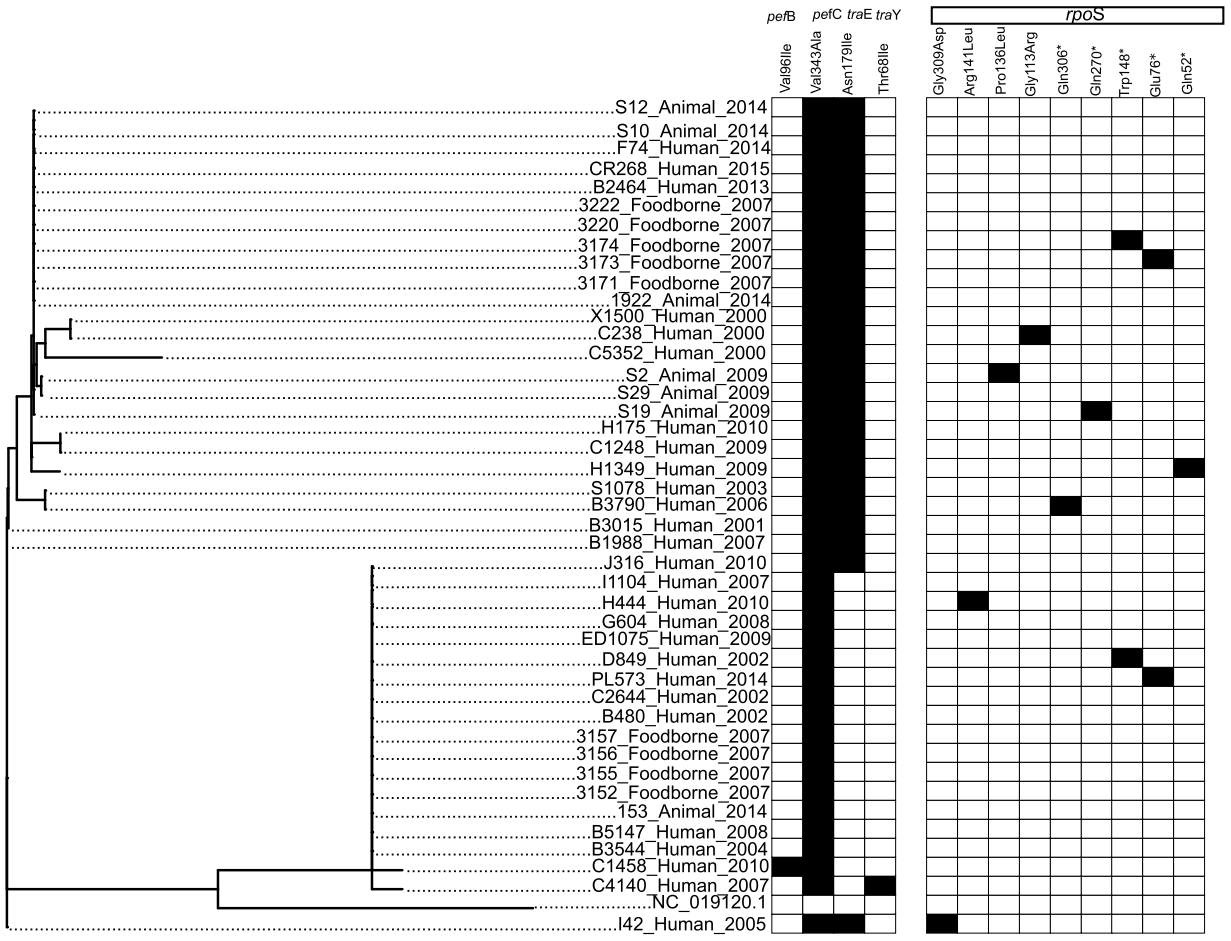

Fig. 4. Maximum likelihood tree of 43 plasmid sequences of *Salmonella* Enteritidis with reference strain NC_019120.1. Branch lables represent isolate number_-Isolation type_isolation date. Heatmap showing the presence and absence of missense mutations in plasmid virulence genes and *rpoS* gene with a white box indicates absence and a black box shows presence.

Table. 2
: Non-synonymous mutations determined in target virulence genes in 45 *Salmonella* Enteritidis isolates.

		Genes	Nucleotide position	Nucleotide change	Protein position	Amino acid change	Associated isolates
chr_genome	fimbriea	*bcf*C	28,045	C->A	826	Ala -> Asp	17 isolates (13 Human, 1 Animal, 3 Foodborne)
		*fim*I	584,376	C->T	50	Arg -> Cys	1 Foodborne isolate
		*lpf*A	3,708,664	G->*A*	111	Thr->Ile	18 isolates (13 Human, 1 Animal, 4 Foodborne)
		*sti*C	208,100	A->C	435	Tyr->Asp	18 isolates (13 Human, 1 Animal, 4 Foodborne)
	SPi-1&2	*inv*H	2,929,483	T->*G*	475	Ser->Leu	18 isolates (13 Human, 1 Animal, 4 Foodborne)
		*ttr*A	1,760,475	C->T	232	Arg->Cys	18 isolates (13 Human, 1 Animal, 4 Foodborne)
		*ttr*A	1,762,630	C->A	950	Ala->Thr	2 Human isolates
		*ssa*O	1,732,159	T->C	28	Thr->Ala	17 isolates (13 Human, 1 Animal, 3 Foodborne)
		*ssa*N	1,733,278	A->C	89	His->Gln	8 isolates (7 Human, 1 Animal)
	phoPQ-regulated genes	*Pho*P	1,919,806	A->*G*	156	Tyr->Cys	1 Human isolate
		*Pho*Q	1,921,103	A->C	364	Ser->Arg	1 Human isolate
		*Pho*Q	1,921,325	A->*G*	438	Thr->Ala	18 isolates (13 Human, 1 Animal, 4 Foodborne)
		*Pho*P	1,919,418	C->T	27	Gln->stop codon	1 Human isolate
		*pho*Q	1,920,324	G->*A*	104	Trp->stop codon	1 Human isolate
	Alternative sigma factor	*rpo*S	2,950,320	C->T	309	Gly->Asp	1 Human isolate
			2,950,824	C->A	141	Arg->Leu	1 Human isolate
			2,950,839	G->*A*	136	Pro->Leu	1 Animal isolate
			2,950,909	C->T	113	Gly->Arg	1 Human isolate
			2,950,330	G->*A*	306	Gln->stop codon	1 Human isolate
			2,950,438	G->*A*	270	Gln->stop codon	1 Animal isolate
			2,950,803	C->T	148	Trp->stop codon	1 Foodborne isolate
			2,951,020	C->A	76	Glu->stop codon	1 Foodborne isolate
			2,951,092	G->*A*	52	Gln->stop codon	1 Human isolate
Virulence plasmid genes	Plasmid-encoded fimbriae	*pef*B	17,597	G->*A*	96	Val->Ile	1 Human isolate
		*pef*C	19,667	T->C	343	Val->Ala	All isolates (43 isolates)
	Transfer gene	*tra*E	35,699	T->*A*	179	Asn->Ile	24 isolates (16 Human,3 Animal, 5 Foodborne)
		*tra*Y	37,011	G->*A*	68	Thr->Ile	1 Human isolate

(Fig. 3; Table 2).

In *pho*P and *pho*Q genes, we found two and three non-synonyms mutations, respectively. The *pho*P gene exhibited one missense mutation (Tyr156Cys) in one clinical of the lineage A and one nosense mutation (Gln27*) in one clinical isolate of the lineage B. For the *pho*Q gene, we identified missense mutation (from theronine to alanine) at the site 438 in all isolates of lineage B. In addition, one clinical isolate of the lineage A carried mutation in the *pho*Q (from Serine to arginine) at the site 364. Another mutation at the site 104 in the gene *pho*Q, resulting a change from tryptophane to codon stop was detected in one clinical isolate of lineage B (Fig. 3; Table 2)

For the virulence plasmid genes, one clinical isolate of lineage B carried missense mutation at the site 197 (form valine to alanine) (Fig. 4). The 43 *S.* Enteritidis carried virulence plasmid genes carried a missense mutation in *pef*C gene (Val343Ala). Other missense mutations have been identified in genes that code for plasmid transfer *tra*E (Asn179Ile) and *tar*Y (Thr68Ile). Two mutation types were detected in alternative sigma factor *RpoS*, including four missense and five nonsense (Fig. 4). No missense mutations were observed for the other target virulence genes among the isolates studied.

3.5. Prophages in S. enteritidis

A total of 21 prophage regions were detected using the PHASTER prophage analysis web server. Five out of these different phages were intact lysogenic phages (Salmon_118,970_Sal3; Gifsy_2; Salmon_Fels_1; Gifsy_1 and salmon_re_2010) (Fig. 1). 11 prophage profiles were identified, the most prevalent profiles were Salmon_118,970_sal3 ($n = 7$); Salmon_RE_2010 ($n = 6$); Salmon_118,970_sal3-Salmon_Fels_1 ($n = 6$) and Gifsy_2-Salmon_RE_2010 ($n = 5$). No intact prophage sequence was detected in six isolates. We identified only one phage sequence in nine isolates. The genomes of the other isolates carried two to three prophage sequences. The most prevalent intact prophages detected in our collection of *S.* Enteritidis were Salmon_118,970_sal3 and Fels-2 which were present in 20 and 18 genomes, respectively. The prophages Gifs _2 and Salmon_Fels_1 were defined in 15 isolates, while Gifsy_1 was present in only two isolates. Phylogenetic analysis based on the SNPs of all isolates showed that Salmon_118,970_sal3 and Salmon_RE_2010 were specific for the lineage A and lineage B isolates, respectively while three prophages (Gifsy_2, Salmon_Fels_1 and Gifsy_1) were common among the two lineages (Fig. 1).

4. Discussion

In the present study, a collection of 45 *S.* Enteritidis isolates were studied through WGS and subsequent *in silico* to determine the distribution of virulence genes, antimicrobial resistance genes, prophage sequences and the sequence variation of target virulence genes.

The use of WGS became the most powerful tool for determining genomic variation especially in homogeneous bacterial genome. Several

studies have demonstrated the homogeneity of S. Enteritidis (Allard et al., 2012; Graham et al., 2018). In the current report, we have identified the existence of two lineages of S. Enteritidis circulating in Tunisia based on 802 SNPs whole-genome. Among these SNPs, 493 missense SNPs were detected. The COG distribution predicted that 349 orthologue genes mutated by one or two missense SNPs were classified into 22 functional groups. The major group was carbohydrate transport and metabolism [G] followed by amino acid transport and metabolism [E] and transcription [K]. Previous studies indicated that the orthologue genes of the majority of *Salmonella* serovars including Enteritidis are mainly classified in the transport and metabolism of glucid [G], transport and metabolism of amino acids [E], and transcription [k]. Zhou et al. (2018), reported that 1622 missenses SNPs distributed in 928 ORFs were detected in Rissen and Typhi serovar genomes. The distribution of the mutated orthologue genes showed that the majority were classified in the defense mechanisms [V], intracellular passage, secretion and vesicular transport [U] (Zhou et al., 2018). This classification differs from one serovar to another. The most abundant COG functional group was the carbohydrate transport and metabolism [G] for Typhimurium serovar however for the Worthington and Ohio serovars, the orthologous genes were mainly classified in the groups of transcription [K] amino acids transport and metabolism [E] (Gupta et al., 2019). The orthologous genes of *Salmonella* Napoli were mainly classified in the functional group of translation, ribosome structure and biogenesis [J] (Mastrorilli et al., 2020).

WGS has been previously applied for the prediction of antibiotic resistance genes in several microbes including the genus *Salmonella* (Ellington et al., 2017; Pornsukarom et al., 2018). It has been reported that WGS had more than 96% sensitivity and 97 to 100% specificity in predicting resistance phenotypes and as well it is concluded that WGS can be used as an alternative method to conventional phenotypic antimicrobial susceptibility testing methods. In accordance, our results show a correlation between genotypic antibiotic resistance profiles and phenotypic analysis. The nalidixic acid-resistant isolates showed a single point mutation in the *gyrA* gene at the codon Asp87 or Ser83. These mutations were previously detected in *S.* Enteritidis recovered from different sources resistant to nalidixic acid (Aldrich et al., 2019; Ben Salem et al., 2017; Wasyl et al., 2014). In addition, to single point mutation in the *gyrA* gene, one isolate carried the $bla_{\text{TEM-1B}}$ gene which is associated with resistance to ampicillin. Previous studies showed that $bla_{\text{TEM-1B}}$ gene is the most common β-lactamase in *Salmonella* . In our study, we note that the ampicillin-resistant isolate carried the plasmid *Inc*X-1 additional to plasmid virulence genes *Inc*F. This result suggests that IncX plasmid can carry $bla_{\text{TEM-1B}}$ gene. The association of *Inc*X-1 conjugative plasmids with bla_{TEM} genes was previously described by Matsumoto et al., al.(2014) which showed that the bla_{TEM} gene has been found to be located on a 38-kb *Inc*X-1 plasmid (Matsumoto et al., 2014). Similarly in a study conducted by Tran-Dien et al. (2018), the authors revealed that WGS identified $bla_{\text{TEM-1B}}$ gene on two different plasmids *Inc*X-1 and *Inc*F in three *S.* Typhimurium isolates collected in France and Tunisia between 1959 and 1960 (Tran-Dien et al., 2018).

Prophages sequences constitute the accessory genome of bacteria and appear to be a major source of genomic variability in *Salmonella*. These sequences can carry virulence genes, toxins and antimicrobial resistance genes (Mottawea et al., 2018; Wahl et al., 2019). Previous studies reported that *Salmonella enterica* are characterized by high degree of variability of prophage profiles (Gymoese et al., 2019; Hu et al., 2021). This is consistent with our results that prophage profiles are variable among *S.* Enteritidis. No prophage sequences were identified in six isolates. The number of prophage sequences detected ranged from 1 to 3 intact region per genome. We also noticed that prophage sequences Salmon_118,970_sal3 and Salmon_RE_2010 are highly prevalent among *S.* Enteritidis. In addition, the phylogenetic analysis informs that the Salmon118970_sal3 phage was detected only in the lineage A, while the Salmon RE_2010 phage was specific for the lineage B isolates. Many studies concluded that prophage sequences can differentiate between epidemiological subtypes of *S.* Enteritidis. Allard et al., 2012 exhibit the capacity of prophage RE_2010 to separate *S.* Enteritidis isolates with the same PFGE pattern. Furthermore, Ogunremi et al. (2014) proposed that the prophage composition could differentiate between *S.* Enteritidis subtypes during foodborne outbreaks (Ogunremi et al., 2014).

Many virulence genes of *Salmonella enterica* are organized on SPIs. 23 SPIs are identified in genus *Salmonella*, playing a fundamental role in pathogenesis and host specificity (Zhao et al., 2020). Suez et al. (2013) showed that *S.* Enteritidis reference strain P125109 contains 14 SPI (SPI-1 to 5, 9, 11–14, 16,17, 19 and cs54) (Suez et al., 2013). In our study, the investigation of 45 *S.* Enteritidis isolates showed that six SPIs, including SPI-1, SPI-2, SPI-3, SPI-9, SPI-13 and CS54 are conserved in all isolates while SPI-11 and SPI-12 were absent. Many studies suggest the universal presence of SPI-1 to SPI 5, SPI-13 and SPI-14, the absence of SPIs 7, 8 and 15 in all non-typhoidal *Salmonella* isolates, and the mosaic presence of SPIs 6, 10–12 and 16–19 across the serovars (Suez et al., 2013). The characterization of several genes carried by SPI-1 and SPI-2, encoding type III secretion systems which is important for the infection of the host cell and facilitate intracellular survival and replication, were highly conserved in all isolates except one. Previous studies reported that these genes are probably part of the core genes with an essential function for *Salmonella* serovars and the absence of certain genes could be explained by the possibility of losing the gene during their evolution (Ben Hassena et al., 2021; Suez et al., 2013). In our study, all tested isolates were positive for *pho*P and *pho*Q genes which are essential for the regulation of type III secretion systems. *Pho*Q is the transmembrane sensor histidine kinase of the bacterial and *pho*P is cytoplasmic regulatory protein (Hu et al., 2021).

Among virulence factors, fimbriae have a major role in pathogenesis and a source of diversity for *Salmonella* serovars. This factor is one of the most common adhesion systems and are differentially expressed and found in a specific pattern among each serovar (Dufresne and Daigle, 2017). In our analysis, the fimbrial operons *agf, bcf, fim, lpf, peg, saf, stb, std, ste, stf, sth* and *sti* were present in all *S.* Enteritidis genomes. Suez et al. (2013) showed the same results and indicated that five fimbrial clusters (bcf, agf, stb, sth and sti) were detected to be part of core genome for invasion and systemic disease in humans (Suez et al., 2013). In addition, it has been reported that the five Chaperone-Usher Pathway (CUP) fimbriae *sta, stg, ste, tcf* and *sef* were considered to be specific for typhoidal *Salmonella* (Dufresne et al., 2018; Forest et al., 2007; Suez et al., 2013). However, our study showed that *sef* and *ste* are not specific for typhoidal *Salmonella* as we detected the presence of *ste* operon in all isolates and the *sef* operon in 40 isolates. These findings agree with the study of Ben Hassena et al. (2021) that reported the presence and specificity of the *sef* operon for the Enteritidis serovar (Ben Hassena et al., 2021).

Virulence-associated plasmids are important genetic elements in *Salmonella*. They are required for bacterial multiplication in the reticuloendothelial system, which confers to *Salmonella* the ability to produce systemic disease. In our previous study, the plasmid characterization showed that among 45 isolates 43 carried the replicons *inc*FIB and *inc*FII belonging to FAB type [S1:A-:B22]. This FAB type was previously found to be associated with the *S.* Enteritidis virulence plasmid (Silva et al., 2017; Villa et al., 2010). This largely consistent with our finding that all *S.* Enteritidis isolates caring the replicons *inc*F contain *pef*ABCD, *spvABCDR, tra*AVKELY and *rck*.

Among the 45 *S.* Enteritidis isolates investigated in this study, both synonym and non-synonym mutations in the selected target virulence genes were detected. In plasmid genes, we note missense mutations in *pef*C gene (Val343Ala) and in transfer genes as *tra*E (Asn179Ile) and *tar*Y (Thr68Ile). No missenses mutations were detected in the *spv* and *rck* genes. The *spv* genes were reported to be highly conserved in *Salmonella* (Hu et al., 2021). In our study, we observed missense and nonsense variability in the regulated *rpo*S and *pho*PQ genes. Hu et al. (2021) observed that cytoplasmic regulatory protein *pho*P gene has been frequently used for *Salmonella* detection as it has a conserved sequence

in different serovars. Of note, the phylogenetic analysis highlights that missense SNPs detected in virulence genes is also able to discriminate isolates by lineages. Two missense SNPs were detected in *fim*H (Arg50Cys) and *ttr*A (Ala950Asp) genes in lineage A. However, six missense SNPs were found in *lpfA* (Thr111Ile), *sti*C (Tyr435Asp) *inv*H (Ser146Ala), *ttr*A (Arg232Cys), *ssa*Q (Thr28Ala) and *ssa*N (His89Gln) genes in lineage B. These results suggest that, in addition to their phylogenetic differences, each lineage may have differential phenotypic traits.

5. Conclusions

In conclusion, the WGS appears to be undisputed not only in typing of bacterial pathogens, epidemiological surveillance and outbreak investigation but also in functional genomics studies. This is the first study of the application of WGS to predict antimicrobial resistance genes, virulence markers, prophage sequences and the sequence variation among *S.* Enteritidis strains isolated from different sources in Tunisia. An excellent correlation between antimicrobial profiles obtained by phenotypic and genotypic analysis were observed. Our comparative genomics approach informs that our collection of *S.* Enteritidis is characterized by variability of prophage profiles. The prophage sequences can also be exploited for lineage tracking and epidemiological studies. The Salmon118970_sal3 phage was detected only in the lineage A, while the Salmon RE_2010 phage was specific for the lineage B isolates. We also observed that lineage B isolates acquired the majority of missenses SNPs detected in fimbriae, SPI-1 and SPI-2 genes. Further experiments will be needed to elucidate the impact of these mutations in the pathogenicity of *Salmonella*.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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