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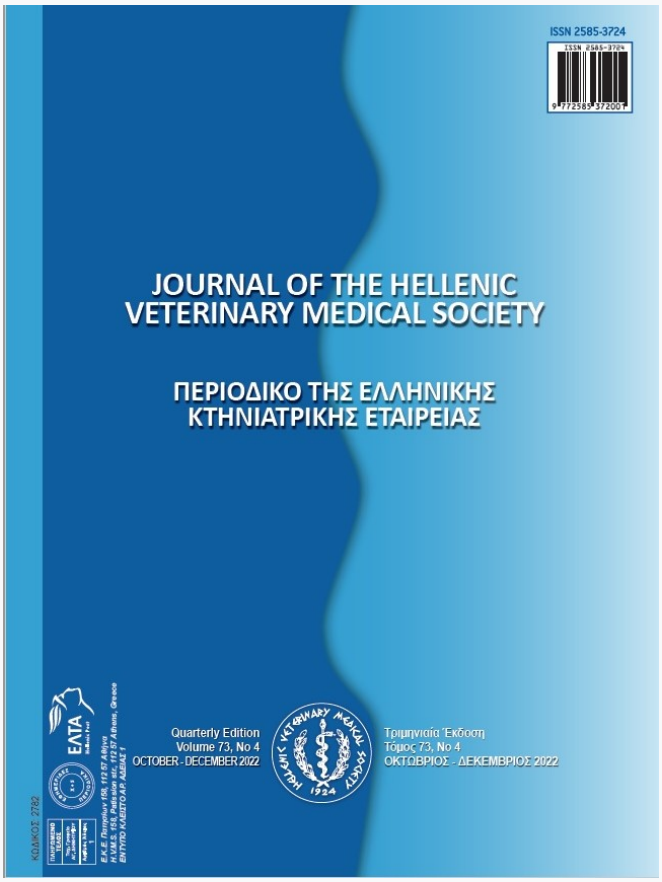

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Research article
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Distribution of *Salmonella* virulence factors originated from sheep and cattle in Algerian slaughterhouses

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ABSTRACT: The present study was carried out to determine the virulence gene profiles associated with *Salmonella* subsp. *enterica* strains recovered from cattle and sheep samples, derived from carcasses and feces, by polymerase chain reaction (PCR). A total of 84 *Salmonella* isolates belonging to 10 serovars were screened for presence of eight different virulence genes (*inv*A, *pef*A, *sef*A, *pip*B, *sse*C, *ssa*P, *spv*C and *iro*B) using their specific primers. Different distribution patterns of these genes were noticed amongst the isolates. While the *iro*B gene was the most identified (65/84;77.4%), the *sef*A gene was not detected in all tested strains. *pef*A and *spv*C genes were detected in *S*. Typhimurium (3/84;3.6%). The four other genes were found to be present in 61.9% (52/84) each. Occurrence of virulence genes did not vary significantly ($P > 0.05$) by serovar or animal species, neither by type of sample. These results show that transmissible pathogenic *Salmonella* strains are circulating in slaughterhouses in Algeria.

Key words: *Salmonella*; red meat; virulence genes; polymerase chain reaction

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INTRODUCTION

Salmonella is still a major cause of human foodborne outbreaks and diseases all around the world with attendant public health problem resulted in 95.1 million cases and 50,771 deaths in 2017 (Lee et al., 2021). Foods of animal origin such as beef, poultry meat, eggs, and dairy products have been well recognized as vehicles for transmitting salmonellosis to humans (Bertelloni et al., 2017; Thung et al., 2018).

Salmonella's capacity to cause disease can be due to a variety of virulence genes sited in the chromosome or in large virulence related plasmids (Fazl et al., 2013; Rowlands et al., 2014; Ilyas et al., 2017). These genes encode products that help *Salmonella* to interact with host organism at different stages including colonization, invasion, intracellular replication and damage of tissues (Parvathi et al., 2011; Das et al., 2012; Mthembu et al., 2019).

The aim of this study is to characterize *Salmonella* strains isolated from carcasses and feces of sheep and cattle slaughtered at Algiers by virulence gene profiling, focusing on eight virulence determinants that have been shown to be relevant for the success of *Salmonella* as an intracellular pathogen.

MATERIALS AND METHODS

Bacterial isolates

The 84 selected *Salmonella* strains are listed in the supplementary file. They were isolated from samples collected from El-Harrach and Hussein- Dey slaughterhouses in Algiers, Algeria, between 2013 and 2014. These strains were originally isolated from cattle carcasses (n= 39), sheep carcasses (n= 32), cattle feces (n= 11) and sheep feces (n=2). All *Salmonella* strains were isolated according to the International Organization of Standardization method (ISO 6579) and serotyped by slide agglutination as specified by the White- Kauffmann-Le Minor scheme (Grimont and Weill, 2007). The isolates belonged to 10 serovars among which *Salmonella enterica* ser. Muenster was the most prevalent (39%). The antibiotic resistance patterns of all the strains were reported in our previous study (Nouichi et al., 2018).

Detection of virulence genes of *Salmonella* isolates by PCR

Extraction of template DNA

Cultures were streaked onto the appropriate agar medium, and incubated for 24 hours at 37ºC prior to PCR experiments. Then, boiling methodwas used as previously described (Nouichi et al., 2018).

DNA primers

Eight sets of primers were selected. There sequences and corresponding genes are described in Table 1.

PCR analyses

Four reactions (singleplex and multiplex PCR) were used to amplify the eight genes. The genes *pef*A

Table 1. Oligonucleotide primer sequences used for virulence genotyping of *Salmonella* strains

Target gene	Location	Virulence- related function	Primer sequence (5'-3')	Size (bp)	Reference
*inv*A	SPI-1	Host recognition/ invasion	GTTGTACCGTGGCATGTCTG GCCATGGTATGGATTTGTCC	930	Fazl et al., 2013
*ssa*P	SPI-2	Survival within host cell	ATGCGTATTACCAAAGTTGA TCATTCGCTATTCTTAACAT	375	Fazl et al., 2013
*sse*C	SPI-2	Survival within host cell	TATGGTAGGTGCAGGGGAAG CTCATTCGCCATAGCCATTT	121	Fazl et al., 2013
*pip*B	SPI-5	Survival within host cell	AATATCGGATGGGGGAAAAG AACCTGACTCACGCAGACCT	230	Fazl et al., 2013
*sef*A	SPI-10	*Salmonella* Enteritidis fimbriaeis	GCAGCGGTTACTATTGCAGC TGTGACAGGGACATTTAGCG	330	Rowlands et al., 2014
*spv*C	Plasmid	Intracellular survival and replication	CGGAAATACCATCTACAAATA CCCAAACCCATACTTACTCTG	669	Rowlands et al., 2014
*pef*A	Plasmid	Invasion of host cells	TTCCATTATTGCACTGGGTG AAGCCACTGCGAAAGATGCC	497	Rowlands et al., 2014
*iro*B	Plasmid	Ferric uptake regulation	TGCGTATTCTGTTTGTCGGTCC TACGTTCCCACCATTCTTCCC	606	Baumler et al., 1997

and *iro*B were screened using two simplex reactions. While amplification was observed for *pef*A gene with the combinations of 2.5 µL 10X PCR reaction buffer, 3 µL MgCl2, 0.5 µL dNTP, 0.2 µL each primer, 3 µL DNA template, 0.3 µL Taq polymerase, and 15.3 µL water, the other simplex was carried out with a total reaction volume of 50 µL. The reaction mixture contained 5 µL 10X PCR reaction buffer, 6 µL MgCl2, 1µL dNTP, 0.2 µL each primer, 3 µL extracted DNA template, 0.3 µL Taq polymerase, and 35 µL molecular biology water.

Duplex PCR was performed for the screening of *sef*A and *spv*C as described by Rowlands et al. (2014). Primers (0.3 µL each) were used in a 50 µL reaction containing 5 µL10XPCR reaction buffer, 6 µL MgCl2, 0.5 µL dNTP, 3 µL DNA template, 0.3 µL Taq polymerase, and 29 µL molecular biology water.

The virulence genes *inv*A, *ssa*P, *sse*C, and *pip*B were investigated by tetraplex PCR as described by Fazl et al. (2013) with some modifications. The reaction mixture contained 5 µL 10X PCR reaction buffer, 3 µL MgCl2, 1 µL dNTP, 0.2 µL each primer, 3 µL extracted DNA template, 0.3 µL Taq polymerase, 36.1 µL of molecular biology water for completing a final volume of 50 µL.

The cycling conditions are summarized in Table 2. Electrophoresis of amplified products was carried out using 1.5% agarose gel containing 3 µL/g ethidium bromide. The amplified DNA fragments were visualized under ultraviolet light.

Statistical analysis

Statistical analysis was conducted using XLSTAT version 2014. Pearson's Chi-square (χ^2) and Fisher exact tests were applied for comparison of gene frequencies in different categories.

RESULTS

Analysis of the presence of the eight virulence-associated genes in the tested isolates is shown in Table 3. Seven of the eight studied genes have been found in varying degrees from the four sample sources analyzed.

No statistical differences were observed in the distribution of the eight virulence genes among investigated serovars. Four different virulence profiles were identified. None of the strains possessed all investigated genes, the stains belonging to Typhimurium serovar were found to have seven different genes, and 39 strains were positive for five different genes. There was no statistically significant difference between frequencies of all the genes among carcasses [88.7% (63/71)] and feces [92.3% (12/13)], as well as difference between frequencies of genes between cattle and sheep species which were [94% (47/50)] and [82.4% (28/34)] respectively (Table 4).

DISCUSSION

In this study, the presence of eight virulence genes was studied to investigate the pathogenic potentials of the selected strains. Seven of these virulence determinants, namely: *inv*A, *ssa*P, *sse*C, *pip*B, *pef*A, *sef*A and *spv*C are associated with invasion and survival within cells and the production of adhesins or pili while *iro*B, is important for iron acquisition (Mthembu et al., 2019). Previous studies from several countries exhibited the prevalence of virulence genes in veterinary clinical *Salmonella* isolates, which varies in different regions globally, In Algeria, there are no data about virulence genotyping of *Salmonella* strains isolated from animals.

Seven of the eight screened genes were present in the studied strains. The heterogeneity of virulence profiles suggests multiple acquisition events of different virulence genes.

Table 2. Primers and PCR cycling conditions

Gene	Initial denaturation	Number of cycles	Cycling conditions			Final extension
			Denaturation	Annealing	Extension	
*pef*A	94 ºC, 2 m	30	94ºC, 30 s	50ºC, 45 s	72ºC, 1m	72ºC, 7 m
*iro*B	94ºC, 3 m	30	94ºC, 40 s	55ºC, 40 s	72ºC, 40 s	72ºC, 5 m
*sef*A *spv*C	93ºC, 3 m	30	94ºC, 30 s	55ºC, 45 s	72ºC, 1 m	72ºC, 5 m
*inv*A *ssa*P *sse*C *pip*B	94ºC, 3 m	30	94ºC, 1 m	50ºC, 1 m	72ºC, 1 m	72ºC, 5 m

Table 3. Distribution of virulence genes by serovar

Serovar	n	Virulence genes N (%)							
		*iro*B	*sef*A	*pef*A	*spv*C	*inv*A	*sse*C	*ssa*P	*pip*B
S. Muenster	**33**	21 (63.6)	0 (0)	0 (0)	0 (0)	21 (63.6)	21 (63.6)	21 (63.6)	21 (63.6)
S. Kentucky	**13**	10 (76.9)	0 (0)	0 (0)	0 (0)	5 (38.7)	5 (38.7)	5 (38.7)	5 (38.7)
S. Infantis	**12**	11 (91.7)	0 (0)	0 (0)	0 (0)	8 (66.7)	8 (66.7)	8 (66.7)	8 (66.7)
S. Anatum	**11**	9 (81.8)	0 (0)	0 (0)	0 (0)	8 (72.7)	8 (72.7)	8 (72.7)	8 (72.7)
S. Richmond	**4**	4 (100)	0 (0)	0 (0)	0 (0)	4 (100)	4 (100)	4 (100)	4 (100)
S. Havana	**3**	2 (66.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
S. Typhimurium	**3**	3 (100)	0 (0)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
S. Montevideo	**3**	3 (100)	0 (0)	0 (0)	0 (0)	2 (66.7)	2 (66.7)	2 (66.7)	2 (66.7)
S. Virginia	**1**	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
S. Braenderup	**1**	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)
Total	**84**	65 (77.4)	0 (0)	3 (3.6)	3 (3.6)	52 (61.9)	52 (61.9)	52 (61.9)	52 (61.9)

n= number of studied strains / N= number of strains harboring virulence genes

Table 4. Distribution of virulence genes by animal species and type of sample

Genes	Animal species			Type of sample		
	Cattle n/N (%)	Sheep n/N (%)	*P*	Carcass n/N (%)	Feces n/N (%)	*P*
*iro*B	38/50 (76%)	27/34 (79.41%)	0.713[a]	55/71 (77.46%)	10/13 (76.9%)	0.965[a]
*spv*C	0/50 (0%)	3/34 (8.82%)	0.062[b]	3/71 (4.22%)	0/13 (0%)	0.450[a]
*pef*A	0/50 (0%)	3/34 (8.82%)	0.062[b]	3/71 (4.22%)	0/13 (0%)	0.450[a]
*inv*A	28/50 (56%)	24/34 (70.58%)	0.176[a]	46/71 (64.78%)	6/13 (46.15%)	0.203[a]
*sse*C	28/50 (56%)	24/34 (70.58%)	0.176[a]	46/71 (64.78%)	6/13 (46.15%)	0.203[a]
*ssa*P	28/50 (56%)	24/34 (70.58%)	0.176[a]	46/71 (64.78%)	6/13 (46.15%)	0.203[a]
*pip*B	28/50 (56%)	24/34 (70.58%)	0.176[a]	46/71 (64.78%)	6/13 (46.15%)	0.203[a]
*sef*A	0/50 (0%)	0/34 (0%)	1.000[b]	0/71 (0%)	0/13 (0%)	1.000[b]

N: number of studied strains
n: number of strains harboring genes
[a] : χ^2 test.
[b] : Fisher's exact test.

Out of the 84 examined *Salmonella* strains, 52 (61.9%) harbored the *inv*A gene which is located on the SPI-1 pathogenicity islandand is involved in intestinal epithelial cell invasion(Pal et al., 2017). This finding was not expected since previous studies showed that *inv*A gene was detected in 94% to 100% of *Salmonella* isolates from different animal and food sources (red meat, chicken and pigs) (Karatuğ et al., 2018; Lan et al., 2018; Elkenany et al., 2019; Webber et al., 2019). This could be explained by many reasons, such as the choice of primers. In the current study, we used a primer set with 930 bp size, while the primer set 139-141 (284 pb) was chosen in an international research project to validate and normalize PCR to detect five major foodborne pathogens, including *Salmonella* as the most selective primer targeting the *inv*A gene (Pal et al., 2017). Besides, Barletta et al. (2013) reported that despite the fact that the sequences utilized as targets in a multiplex PCR are derived from highly conserved regions of the virulence genes, new variants of these genes may not be amplified by the described primers, which is a weakness of this assay or any multiplex assay.

In this study, *iro*B gene was the most frequently detected occurring in more than 77% (65/84) of isolated strains. Our results are slightly higher than those reported by Zishiri et al. (2016), who noted the presence of the *iro*B gene in *Salmonella* isolates from broilers in South Africa (68.6%) and Brazil (70.8%). In another study (Mthembu et al., 2019), a low prevalence (30.2%) of *iro*B gene was noticed in *Salmonella* spp. isolated from livestock production systems in two South African provinces. On the other hand, our results are lower than those of Parvathi et al. (2011) and Guedda et al. (2014), who detected *iro*B gene in 100% of the tested strains. The virulence factor *iroB* which plays an important role in *Salmonella* virulence, is not located on *Salmonella* pathogenicity islands (SPIs) but on plasmids (Parvathi et al., 2011). Due to its specificity, this ferric uptake regulator gene, could also be used to detect *Salmonella* in samples from clinical and food origin (Baumler et al., 1997).

Our results revealed that only 3.6% of the isolates were positive for *spv*C gene. In accordance, Karatuğ et al. (2018), Lan et al. (2018) and Nguyen Thi et al.(2020) have also reported very low prevalence of this gene in *Salmonella* isolates. The *spv*C gene determinant was present only in *Salmonella enterica* ser Typhimurium strains, which is an expected result as the *spvRABCD* operon is located in a high size plasmid (pSEV) restricted to specific serovars (i.e. Enteritidis, Typhimurium, Dublin, Gallinarum, Pullorum and Abortus-ovis) (Rowlands et al., 2014). This is consistent with previous virulotyping studies on *Salmonella* strains, where *spv*C was found only in serotype Typhimurium and Enteritidis among many serotypes isolated from several food sources in Italy (Bertelloni et al., 2017), Vietnam (Lan et al., 2018), Brazil (Borges et al., 2019) and China (Li et al., 2019; Chen et al., 2020). In contrast, Thung et al. (2018) did not detect the *spv*C gene in any of the serotypes isolated from beef and pork, including Enteritidis and Typhimurium. Mutations in *spv* genes have been shown to cause attenuation related to systemic salmonellosis (Pal et al., 2017).

The *pef*A gene carried by large virulence plasmids is implicated in recognition and invasion of host cells (Parvathi et al., 2011; Webber et al., 2019). Detected by the presence of a 497 bp product, it was found to be present in only 3 of the 84 isolates (3.6%) screened during this study, all of them belonged to *S*. Typhimurium serovar. This result corroborates those of Thung et al. (2018), Borges et al. (2019) and Webber et al. (2019) who found *pef*A gene only in Enteritidis and Typhimurium serovars. In contrast, Ajayi et al. (2019) did not note any serovar-specific presence or absence of the *pef*A gene in *Salmonella* strains. Different studies have also reported low prevalence of the plasmid associated *pef*A gene in *Salmonella* isolates (Ahmed et al., 2016; Lan et al., 2018). Elkenany et al. (2019) noticed the absence of *pef*A gene in *Salmonella* isolates recovered from broiler chickens and chicken carcasses in Egypt. Additionally, in a study conducted in India, Parvathi et al. (2011) were unable to detect *spv*C and *pef*A genes in 60 *S*. Newport strains isolated from different food sources and other *S*. Typhi strains originated from human samples. These authors showed that these serovars lack large virulence plasmids and consequently the *pef*A gene. In this study, *spv*C and *pef*A genes were found to be originated from only sheep samples. This finding is consistent with those of Ajayi et al. (2019) who noticed that 94% of the *Salmonella* isolates from sheep harbored the *spv*C genes. Das et al. (2012) suggest that the spread of *Salmonella* virulence plasmids may be restricted to isolates from some specific origin of sources.

As regards *ssa*P and *sse*C genes encoded by SPI-2, they are required for systemic infection and survival within macrophages (Fazl et al., 2013). Bhowmick et

al. (2011) showed that deletion of this gene in mutants resulted in loss of ability to survive intracellular in Hela cells. We detected these two genes in 61.9% of the studied *Salmonella* strains. Our results are lower to those reported by Bhowmick et al. (2011) and Fazl et al. (2013) who recorded rates of 96% and 100% respectively for the *sse*C gene, and 100% for *ssa*P. This latter gene was detected in 75% of the isolates studied by El Bayomi et al. (2016)

As concerns the *pip*B gene encoded in SPI-5, it is activated when *Salmonella* enters host cells to facilitate intra-macrophage survival (Bertelloni et al., 2017; Ilyas et al., 2017). The present survey found the gene *pip*B in 52 out of 84 strains (61.9%). This result is lower than previously reported by Bhowmick et al. (2011), Fazl et al. (2013) and El Bayomi et al. (2016) who detected this gene in all of the *Salmonella* strains analyzed in their studies. On the other hand, Nguyen Thi et al. (2020) recovered *pip*B in 48.28% of *Salmonella* isolates from swine.

None of the studied isolates were positive for *sef*A virulence factor. This gene coding for *Salmonella* Enteritidis fimbriae is located on the SPI-10 (Webber et al., 2019). Our findings were expected since the genes of the *sef* operon (*sef*ABCD) are limited to the serovars of serogroup O: 9, which include among them *S.* Enteritidis, *S.* Typhi, *S.* Dublin, *S.* Berta, *S.* Gallinarum (Rowlands et al., 2014, Borges et al., 2019). It is worth noting that the *sef*gene has moreover been utilized for molecular detection of *Salmonella* Enteritidis (Webber et al., 2019).

The difference in virulence rates among *Salmonella* strains between studies could also be due to several factors such as the amplified nucleotide sequences, used technique and origin of the strains. Several environmental factors, such as oxygen levels, pH, contact with fatty acids and carbohydrates, have been shown to influence gene expression by activating or repressing their virulent effects (López-Garrido et al., 2015).

The detection of these virulence genes in 92.3% (12/13) of fecal isolates indicates that apparently healthy slaughtered animals could be carriers of highly pathogenic *Salmonella* which increases risks of direct or indirect spread of these germs to meat and meat products.

None of the isolates resulted positive for all 8 targeted genes, however all the isolates of *S.* Typhimurium were positive for 7 genes. It is worth mentioning that these strains were multidrug resistant (MDR) as previously reported (Nouichi et al., 2018) with a particular antibiotic resistance profile (ACSSuT, i.e., ampicillin, chloramphenicol streptomycin, sulphamethoxazole and tetracycline resistance) typically associated to more pathogenic strains (Nguyen Thi et al., 2020). The association between the presence of this pentaresistance pattern and each gene was significant. These findings would explain why *S.* Typhimurium strains are related to the majority of clinical infections and hospital outbreaks, and suggest the importance of controlling *Salmonella* especially some serovars along the production chain.

CONCLUSION

The widespread dissemination of the investigated virulence genes underscores the pathogenic potential of the studied *Salmonella* strains which have been causing infection in humans and contaminating meat in Algeria. These findings are a great concern regarding salmonellosis infection and transmission. A comprehensive study involving all of the major serovars should be carried out to establish a definitive status in regards to the existence of these genes among various *Salmonella* serotypes and the role of the products phenotypically expressed by these virulence factors in the pathogenesis of salmonellosis in humans and animals.

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