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Protein Expression Diversity amongst Different Serovars of *Salmonella enterica* Using Quantitative Real Time PCR

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Abstract: The present endeavor was initiated to focus the study on closely related *Salmonella* serovars Gallinarum, Shubra, Typhimurium, Newport, Agona, Saintpaul and Kentucky, which are associated with the majority of infections of mammalian and avian hosts in Egypt. The objective was to consider the level of variation in the protein expression patterns of the five resolved over-regulated proteins [*Salmonella* pathogenicity island 1 effector protein (SPI 1 effector protein), response regulator protein (RRP), T cell inhibitor protein (STI), rfbS and heat shock protein 90 (HSP 90)]. These selected 5 genes showed over-expression on the level of protein in *S.* Gallinarum versus *S.* Enteritidis. Primers were designed using gene-specific sequences deposited in Gene Bank after aligning each gene with the same gene in different *Salmonella* serovars to choose the highly conserved regions. Different expression levels for the five proteins, HSP90, SPI1, TCI, rfbS and RRP, were examined among seven *Salmonella* serovars using QRT-PCR. The gene expression data were normalized to the 16sRNA gene as reference house keeping gene. There was an over expression in the level of the mRNA in case of the 5 examined proteins in *S.* Gallinarum over the other *Salmonella* serovars. Hypothetically, this emphasize the involvement of such proteins in host specificity or virulence in a specific host.

Key words: HSP90 · SPI1 · TCI · Rfbs · RRP · *Salmonella* Serovars

INTRODUCTION

Salmonella is a group of organisms comprised of two species: *Salmonella bongori* and *Salmonella enterica*, which is divided into 6 subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica* [1]. Most isolates from humans and warm-blooded animals belong to *S. enterica* subspecies Enterica which is associated with disease in warm-blooded animals differing in their prevalence and the diseases that they cause in different hosts [2]. Other *S. enterica* subspecies and *S. bongori* occur more commonly in cold-blooded animals and the environment and are of lower pathogenicity [3]. S. *enterica* serovars are defined by antigenic variation at lipopolysaccharide moieties (O antigen), flagellar antigens (H antigen) and capsular polysaccharides (Vi antigen). Using the Kauffman-White scheme there are over 2500 *Salmonella* serovars that have been defined on that basis within subspecies 1 [1].

Since the mid 80s, there has been a marked increase in the incidence of human salmonellosis in many countries. This has been associated with *Salmonella* contamination of human food products particularly, poultry meat and eggs. *Salmonella* is a zoonotic agent and salmonellosis is a transmissible animal disease of a socio-economic importance with public health consequences [3]. Some of the 2,500 different serovars of *Salmonella* are host adapted, like *S.* Cholerasuis in pigs or *S.* Pullorum/Gallinarum in poultry while some others affect all species, for example, the *S.* Typhimurium, the most common pathogen for humans [3]. The results of a Swedish study that looked at the comparative burden of

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salmonellosis [4], showed a high, linear correlation between *Salmonella* prevalence in laying hens and human illness. Of basic importance for the control of *Salmonella* in food-producing animals is that meat products contaminated by any serotype of *Salmonella* are by law forbidden to be sold (Food Act. SFS 1971:511) and declared unfit for human consumption [6].

S. enterica serovar Gallinarum is non-motile host-adapted *Salmonella* that causes fowl typhoid, a severe systemic disease responsible for heavy economic losses to the commercial poultry industry [7]. Fowl typhoid remains endemic in many countries of Africa, the Middle East, Central and South America and Asia [8]. Several investigations involving comparative studies between *S.* Gallinarum and other *Salmonella* serotypes have disclosed subtle differences in the cellular mechanisms that may be responsible for specificity of *S.* Gallinarum to an avian host [9-13]. In contrast to other *Salmonella* serotypes, very little is known about the genetic basis of *S.* Gallinarum virulence and the molecular mechanisms involved in systemic infection and development of FT [14]. Reserve transcription combined with Real time PCR technology has been adapted to perform quantitative RT-PCR which is a powerful method to quantify gene expression [15, 16]. Relative quantification describes the change in expression of the target gene relative to some reference group. Analysis of difference of the relative gene expression data was done using real-Time quantitative PCR and the $2^{-\Delta\Delta CT}$ method among *S.* Gallinarum and other tested serovars [17].

The focus of the study was on *Salmonella* serovars Gallinarum, Shubra, Typhimurium, Newport, Agona, Saintpaul and Kentucky, which are associated with the majority of infections of mammalian and avian hosts [3-5]. The objective was to consider the level of variation in the protein expression patterns of the five resolved over-regulated proteins (HSP90, SPI1, TCI, rfbS and RRP) on mRNA level of these closely related *Salmonella* serovars and with very different pathogenic potentials, in order to search for protein factors with levels of expression or post-translational modifications characteristic for each serovar.

MATERIALS AND METHODS

Bacterial Strains, Media and Chemicals: Bacterial strains used in this study included triplicate of seven *Salmonella* serovars; *S.* Gallinarum, *S.* Shubra, *S.* Typhimurium, *S.* Newport, *S.* Agona, *S.* Saintpaul and *S.* Kentucky isolated from local and imported poultry flocks in the National lab for Veterinary Quality Control on Poultry Production, Egypt and were typed in the Central lab of the Ministry of Health [18]. *S.* Gallinarum JOL394 and *S.* Typhimurium Wtx3339 reference strains were kindly provided by the Division of Biological Sciences, Pusan National University, Busan 609-735, South Korea. *Salmonella* strains used in RNA extraction for Quantitative Real time PCR were grown in 5ml of LB-broth (Becton Dickinson, France) for 16 h with shaking at 37°C, cultures were started from lyophilized aliquots. Three plates of LB-Agar were used for culturing each *Salmonella* strain.

RNA Extraction and RT-PCR: *Salmonella* colonies of the 3 LB-agar plates were collected in an eppendorf tube, centrifuged at 14,000 × g for 5 min and the supernatant discarded. The pellets were resuspended in 1 ml phosphate buffered saline (PBS, Oxoid), centrifuged at 14,000 × g for 5 min and the supernatant discarded. Total RNA was extracted from different *Salmonella* serovar using RNA Ambion RiboPure total RNA isolation kit according to the manufacturer's instructions. The absorbencies of RNA samples were checked at 260 and 280 nm for determination of sample concentration and purity using Nano drop. The ratio of A260 to A280 values is a measure of RNA purity [18]. The RNA concentration was adjusted to 100ng/µl. Total RNA was reverse-transcribed to cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Total RNA (1-2 µg) were mixed with 2µl Oligo(dT) and the total volume was made to 12 µl by nuclease-free water. Tubes were spun briefly and heated for 3 min at 85°C, chilled, spun briefly and placed on ice. The following components were added: 2 µl 10X RT Buffer, 4 µl dNTP mix, 1 µl RNase Inhibitor and1 µl SuperScript II reverse transcriptase, mixed gently, spun and incubated at 44°C for one hour, followed by incubation at 92°C for 10 min to inactivate the reverse transcriptase [18]. The reactions were stored at-20°C until use.

Primers used in Quantitative Real-Time PCR Assay: The design of the primers was based on the multiple alignments of the 5 selected genes among different *Salmonella* serovars. These selected 5 genes showed over-expression on the level of protein in *S.* Gallinarum versus *S.* Enteritidis.

Table 1: Primer sequences for Real time PCR assay

Gene			Primer Sequence	Length	Start seq.	Product size	Seq. Size	Acc. No.
Pathogenicity island 1 effector protein	SPI1	F	CGTGACCACCTTTCCATCTT	20	802	199	2058	gi|16761659
		R	CCATTCGACTAACAGCAGCA	20	1000			
Heat shock protein 90	HSP90	F	TACGTTGACCATTGCCGATA	20	246	161	1899	gi|16418995
		R	AGAAGCCTACGCCAAACTGA	20	406			
Rfbs effector protein	Rfbs	F	TGGCTTAGCAAGGAAGAGGA	20	42	247	721	gi|17227066
		R	TGGCAGTGATGTTCCACAAT	20	288			
Response regulator	RR	F	TATGACTTTCCCGCAGTTCC	20	108	249	675	gi|16764586
		R	AAGAGGTAATGGCGCGTATG	20	356			
T cell inhibitor	TCI	F	TGCGGTAAGAACCGTAGGAC	20	399	177	2298	gi|16765496
		R	ATCGCTTTCACCATGGTTTC	20	575			
Reference Gene	16S rRNA	F	CCTCAGCACATTGACGTTAC	20	468	248	1541	gi:1899235
		R	TTCCTCCAGATCTCTACGCA	20	715			

All primers (Table 1) were designed using gene-specific sequences deposited in Gene Bank after aligning each gene with the same gene in different *Salmonella* serovars using Multalin software to choose the highly homologues regions to allocate the primers [19]. Sequences were taken from Gene Bank accession numbers relative to the identified protein using MALDI TOF mass spectrometry and nucleotide entries on NCBI website (http://www.ncbi.nlm.nih.gov). Primers were designed using the Primer 3 software [20]. The specificity of the primer sequences was tested by homology searches in the nucleotide database [NCBI, nucleotide BLAST (blastn)].

Gradient PCR: Each reaction mixture included 10 PCR sample mixtures; each sample was subjected to a different annealing temperature following a gradient temperature to determine the proper annealing temperature for each primer set. The reaction mixture (200 µl) was prepared by adding 20µl 10X buffer, 16µl of 2.5mM nucleotide Mix, 8µl of forward and reverse primers, 1µl Taq DNA polymerase, 8µ cDNA template and water to a final volume of 200 µl. The tubes were mixed, divided among 10 PCR tubes, spun and incubated in Biometra T Professional gradient thermal cycler and the program was adjusted as follows: 1 cycle at 95°C for 5 minutes followed by heating to 95°C for 30 seconds, 50-70°C for 30 seconds and 72°C for 1 minute. These 3 steps were repeated for 35 cycles followed by final extension to 72°C for 1 min for 1 cycle. The best annealing temperature can be determined after visualizing the product on agarose gel.

Quantitative Real-time PCR Assay (QRT-PCR): The five proteins that showed over-expression in SG versus SE in a previous proteomic study [18] were subjected for further study to check the changes in the mRNA levels. Different expression levels for the five proteins, HSP90, SPI1, TCI, rfbS and RRP, were examined among 6 *Salmonella* serovars field isolates from poultry using QRT-PCR. The gene expression data were normalized to the 16sRNA gene as reference house keeping gene that was used as an internal standard for this study. Normalization of data and calculation of results were performed as previously described by Livak and Schmittgen [17]. Quantitative real-time PCRs [18] were performed in a final volume 20 µl of 2X iQ SYBER green Supermix (Bio-Rad Laboratories) containing cDNA synthesized as described above and the specific primer pair. Real-time PCR reactions were performed in triplicates using iCycler iQ real-time detection system (Bio-Rad, Hercules, CA, USA).

The reaction mixture consisted of 1 µl cDNA, 0.5 mM of each primer, iQ SYBR GREEN PERMIX (BIO-RAD) in a total volume of 20 µl. The Fast Start polymerase was activated and cDNA denatured by a pre incubation for 10 min at 95°C, the template was amplified for 50 cycles of denaturation programmed for 20 s at 95°C, annealing of primers at 62°C programmed for 20 s and extension at 72°C programmed for 30 s. Fluorescent data were acquired during each extension phase. After 50 cycles, a melting curve was generated by heating the samples to 95 °C followed by cooling down to 55 °C for 7 s and slowly heating the samples at 0.1°C/s to 95°C while measuring fluorescence continuously. Each assay included three serial dilutions of tested cDNAs and no-template negative control.

Calculation of Relative Expression: CT is the average of the CT data for each sample expressed as mean ± S.D. The ΔCT value is calculated by the subtraction of the average 16sRNA CT from average tested gene CT [17]. The ΔΔCT value is calculated by subtraction of each *Salmonella* serotype ΔCT from *S.* Gallinarum ΔCT. The expression relative to each *Salmonella* serovar is calculated using the equation $2^{-\Delta\Delta CT}$.

Statistical Analyses: Results are expressed as means ± SEM. Data were analyzed using paired Student's t test and the differences between the two groups were considered significant at $P<0.05$.

RESULTS

***Salmonella* Pathogenicity Island Effector Protein Expression Profiling:** Results of real-time quantitative PCR among different *Salmonella* serovars showed that the SPI effector protein mRNA expressed significantly higher levels in *S.* Gallinarum when compared with the other *Salmonella* serovars (*S.* Shubra, *S.* Typhimurium, *S.* Newport, *S.* Agona, *S.* Saintpaul and *S.* Kentucky). The SPI mRNA level in *S.* Gallinarum was over expressed by $1.6X10^5$, $6.8X10^2$, $3.7X10^3$, $2.9X10^5$, 45.3 and $2.9X10^3$ than that found in *S.* Shubra, *S.* Typhimurium, *S.* Newport, *S.* Agona, *S.* Saintpaul and *S.* Kentucky, respectively (Table 2).

Rfbs Expression Profiling: A dramatic absence of Rfbs mRNA was observed in *S.* Shubra, *S.* Typhimurium, *S.* Newport and *S.* Agona. On the other hand, the Rfbs mRNA expression level was significantly higher in *S.* Gallinarum when compared with the *S.* Saintpaul ($4.8 X 10^3$) and *S.* Kentucky ($2.4 X 10^3$) (Table 3).

Heat Shock Protein 90 Expression Profiling: Using the Quantitative Real time PCR, the HSP90 mRNA in *S.* Gallinarum and other salmonellae revealed a higher expression on the mRNA level in *S.* Gallinarum in comparison with the other *Salmonella* serovars by 3.8, 4.5, 7, 8.6, 38.6 and 26.7 (*S.* Shubra, *S.* Typhimurium, *S.* Newport, *S.* Agona, *S.* Saintpaul and *S.* Kentucky, respectively) (Table 4).

T Cell Inhibitor Expression Profiling: By examining the T cell inhibitor mRNA in *S.* Gallinarum and the other *Salmonella* serovars using quantitative real time PCR, it was found that there was higher expression on the mRNA level in *S.* Gallinarum in comparison with *S.* Shubra, *S.* Typhimurium, *S.* Newport, *S.* Agona and *S.* Saintpaul (9.85, 19.2, 5.3, 43.1 and 2.1, respectively) respectively. Contrarily, the T cell inhibitor mRNA in *S.* Kentucky was higher than that in *S.* Gallinarum (Table 5).

Response Regulator Protein Expression Profiling: Finally, In case of response regulator protein, *S.* Gallinarum showed higher expression level on the mRNA level over other salmonellae using quantitative real time PCR. RRP mRNA was 9.6, 11, 10.8, 11.9, 14.2 and 14.5 times higher in *S.* Gallinarum than *S.* Shubra, *S.* Typhimurium, *S.* Newport, *S.* Agona, *S.* Saintpaul and *S.* Kentucky respectively (Table 6).

Table 2: Quantitative analysis of the relative changes in *Salmonella* pathogenicity island effector protein mRNA expression levels using real-time quantitative PCR among different *Salmonella* serovars

	Average CT [a]	ΔCT [b]	ΔΔCT [c]	Exp. [d]
SG16sRNA	16.17 ± 0.48	-0.6 ± 0.3	-17.3± 0.84	$1.6X10^5$
SG SPI	15.57 ± 0.3			
S. Shubra 16sRNA	11.77 ± 0.84	16.7 ± 0.84		
S. Shubra SPI	28.47 ± 0.47			
ST-16sRNA	10.37 ± 0.39	8.8 ± 0.39	-9.4 ± 0.39	$6.8X10^2$
ST-SPI	19.17 ± 0.22			
S. Newport 16sRNA	10.13 ± 0.38	11.24± 0.38	-11.84 ± 0.38	$3.7X10^3$
S. Newport SPI	21.37 ± 0.35			
S. Agona 16sRNA	8.9 ± 0	17.57 ± 0	-18.17 ± 0	$2.9X10^5$
S. Agona SPI	26.47 ± 0.26			
S. Saintpaul 16sRNA	17.03 ± 0.19	4.9 ± 0.19	-5.5 ± 0.19	45.3
S. Saintpaul SPI	21.93 ± 0.09			
S. Kentucky 16sRNA	17.67 ± 0.43	10.9± 0.43	-11.5 ± 0.43	$2.9X10^3$
S. Kentucky SPI	28.57 ± 0.07			

[a] The average of the CT data for each sample.

[b] The ΔCT value is calculated by the subtraction of the average 16sRNA CT from average HSP90 CT.

[c] The ΔΔCT value is calculated by subtraction of each *Salmonella* serotype ΔCT from SG ΔCT.

[d] The expression relative to S. Gallinarum is calculated using the equation $2^{-\Delta\Delta CT}$.

Table 3: Quantitative analysis of the relative changes in Rfbs protein mRNA expression levels using real-time quantitative PCR among different *Salmonella* serovars

	Average CT (a	ΔCT (b	ΔΔCT (c	Exp. (d
SG16sRNA	25 ± 0.12	3.57 ± 0.29	-	-
SG Rfbs	28.57 ± 0.29			
S. Shubra 16sRNA	23.73 ± 0.32	-		
S. Shubra Rfbs	ND			
ST-16sRNA	23.53 ± 0.59	-	-	-
ST-Rfbs I	ND			
S. Newport 16sRNA	23.87 ± 0.41	-	-	-
S. Newport Rfbs	ND			
S. Agona 16sRNA	22.4 ± 0.15	-	-	-
S. Agona Rfbs	ND			
S. Saintpaul 16sRNA	22.13 ± 0.12	5.84 ± 0.12	-2.27 ± 0.12	4.8
S. Saintpaul Rfbs	27.97 ± 0.12			
S. Kentucky 16sRNA	21.2 ± 0.2	14.8 ± 0.2	-11.23 ± 0.2	2.4 X 103
S. Kentucky Rfbs	36 ± 1.05			

[a] The average of the CT data for each sample.

[b] The ΔCT value is calculated by the subtraction of the average 16sRNA CT from average HSP90 CT.

[c] The ΔΔCT value is calculated by subtraction of each *Salmonella* serotype ΔCT from SG ΔCT.

[d] The expression relative to *S.* Gallinarum is calculated using the equation $2^{\Delta\Delta\Delta CT}$.

Table 4: Quantitative analysis of the relative changes in heat shock protein 90 (HSP90) mRNA expression levels using real-time quantitative PCR among different *Salmonella* serovars.

	Average CT (a	ΔCT (b	ΔΔCT (c	Exp. (d
SG16sRNA	24.27 ± 0.22	3.53	-1.91	3.8
SG HSP 90	27.8 ± 0.36			
S. Shubra 16sRNA	26.13 ± 1.07	5.44		
S. Shubra HSP 90	31.75 ± 1.13			
ST-16sRNA	22.2 ± 0.25	5.7	-2.17	4.5
ST HSP 90	27.9 ± 0.1			
S. Newport 16sRNA	23.37± 0.2	6.33	-2.8	7
S. Newport HSP 90 P	29.7 ± 0.2			
S. Agona 16sRNA	22.87± 0.3	6.63	-3.1	8.6
S. Agona HSP 90	29.5 ± 0.4			
S. Saintpaul 16sRNA	21.2 ± 0.15	8.8	-5.27	38.6
S. Saintpaul HSP 90	30 ± 0.15			
S. Kentucky 16sRNA	20.8 ± 0.06	8.27	-4.74	26.7
S. Kentucky HSP 90	29.07 ± 0.42			

[a] The average of the CT data for each sample.

[b] The ΔCT value is calculated by the subtraction of the average 16sRNA CT from average HSP90 CT.

[c] The ΔΔCT value is calculated by subtraction of each *Salmonella* serotype ΔCT from SG ΔCT.

[d] The expression relative to *S.* Gallinarum is calculated using the equation $2^{\Delta\Delta\Delta CT}$.

Table 5: Quantitative analysis of the relative changes in T cell inhibitor (TCI) mRNA expression levels using real-time quantitative PCR among different *Salmonella* serovars

	Average CT (a	ΔCT (b	ΔΔCT (c	Exp. (d
SG16sRNA	16.17 ± 0.48	15.2 ± 0.09	-3.3 ± 0.84	9.85
SG TCI	31.37 ± 0.09			
S. Shubra 16sRNA	11.77 ± 0.84	18.5 ± 0.84		
S. Shubra TCI	30.27 ± 1.16			
ST-16sRNA	10.37 ± 0.39	19.46 ± 0.39	-4.26 ± 0.39	19.2
ST TCI	29.83 ± 0.19			
S. Newport 16sRNA	10.13 ± 0.38	17.6± 0.38	-2.4 ± 0.38	5.3
S. Newport TCI	27.73 ± 0.49			
S. Agona 16sRNA	8.9± 0	20.63	-5.43	43.1
S. Agona TCI	29.53 ± 0.24			
S. Saintpaul 16sRNA	17.03 ± 0.19	16.3 ± 0.19	-1.1 ± 0.19	2.1
S. Saintpaul TCI	33.6 ± 0.47			
S. Kentucky 16sRNA	17.67 ± 0.43	13.73 ± 0.43	1.47 ± 0.43	0.4
S. Kentucky TCI	31.4 ± 0.26			

[a] The average of the CT data for each sample.

[b] The ΔCT value is calculated by the subtraction of the average 16sRNA CT from average TCI CT.

[c] The ΔΔCT value is calculated by subtraction of each *Salmonella* serotype ΔCT from SG ΔCT.

[d] The expression relative to *S.* Gallinarum is calculated using the equation $2^{\Delta\Delta\Delta CT}$.

Table 6: Quantitative analysis of the relative changes in response regulator (RRP) mRNA expression levels using real-time quantitative PCR among different *Salmonella* serovars

	Average CT [a]	ΔCT [b]	ΔΔCT [c]	Expression [d]
SG16sRNA	25.3 ± 0.17	5.57 ± 0.09	-3.26 ± 0.35	9.6
SG RRP	30.87 ± 0.09			
S. Shubra 16sRNA	24.1 ± 0.35	8.83 ± 0.35		
S. Shubra RRP	32.93 ± 0.17			
ST-16sRNA	23.8 ± 0.57	9.03 ± 0.57	-3.46 ± 0.57	11
ST RRP	32.83 ± 0.41			
S. Newport 16sRNA	24.2 ± 0.42	9 ± 0.42	-3.43 ± 0.42	10.8
S. Newport RRP	33.2 ± 0.5			
S. Agona 16sRNA	22.73 ± 0.12	9.14 ± 0.12	-3.57 ± 0.12	11.9
S. Agona RRP	31.87 ± 0.45			
S. Saintpaul 16sRNA	22.43 ± 0.12	9.4 ± 0.12	-3.83 ± 0.12	14.2
S. Saintpaul RRP	31.83 ± 0.03			
S. Kentucky 16sRNA	21.57 ± 0.17	9.43 ± 0.17	-3.86 ± 0.17	14.5
S. Kentucky RRP	31.0 ± 0.85			

[a] The average of the CT data for each sample.

[b] The ΔCT value is calculated by the subtraction of the average 16sRNA CT from average RRP CT.

[c] The ΔΔCT value is calculated by subtraction of each *Salmonella* serotype ΔCT from SG ΔCT.

[d] The expression relative to *S.* Gallinarum is calculated using the equation $2^{\Delta\Delta\Delta CT}$.

DISCUSSION

Gene-expression analysis is increasingly important in many fields of biological research. Understanding patterns of expressed genes is expected to provide insight into complex regulatory networks and will most probably lead to the identification of genes relevant to new biological processes, or implicated in disease. RT-PCR provides the simultaneous measurement of gene expression in many different samples for a limited number of genes and is especially suitable when only a small number of cells are available [21-23]. At the same time, mRNA expression profiling quantification requires normalization and several variables need to be controlled for in gene-expression analysis [24].

In the present study we used QRT-PCR to examine differential expression profiles of some of NCBI identified proteins that are found to correlate virulence among *S.* Gallinarum and *S.* Enteritidis serovars in comparison to 16 sRNA as reference gene. QRT-PCR results obtained from measuring the differences in expression level among *Salmonella* pathogenicity island effector protein SPI 1 showed that SPI 1 mRNA level in *S.* Gallinarum was higher than SPI 1 mRNA in *S.* Shubra, *S.* Typhimurium, *S.* Newport, *S.* Agona, *S.* Saintpaul and *S.* Kentucky.

The same results were encountered with HSP 90, response regulator protein, RfbS protein and T cell inhibitor protein. This coincides with the results previously obtained by Osman *et al*. [18] that showed over expression of these proteins in *S.* Gallinarum versus *S.* Enteritidis using 2 dimensional electrophoresis and MALDI TOF Mass spectrometry and also on the level of the mRNA when tested with QRT-PCR. This result gives a strong evidence of the specificity of these proteins to *S.* Gallinarum which could increase the speculation of its role in host adaptation and/or virulence among the poultry species. This coincides with the previous results of Dieye *et al*. [25] who reported that, SPI1 contributes to the colonization of both the cecum and spleen of the chicken while in the absence of SPI1, cecal colonization was inhibited. The *Salmonella* (SPI) 1 and 2 are two major virulence determinants of *S. enterica* as they encode type III secretion systems (TIIISS) that form syringe-like organelles on the surface of Gram-negative bacteria and enable the injection of effector proteins directly into the cytosol of eukaryotic cells [26, 27]. SPI1 primarily promotes the invasion of non-phagocytic intestinal epithelial cells and the initiation of the inflammatory responses in the intestines [23, 28]. It is also involved in the survival and persistence of *Salmonella* in the systemic compartment of the host [23, 28]. A functional study carried out by Wigley *et al.* [12] determined the SPI1 role in cell invasion *in vitro* and in virulence *in vivo*. The SPI1 mutant showed decreased invasiveness for chicken cells but was capable of causing disease in orally infected day-old chicks, although it showed some reduction in virulence. Hypothetically, we could indicate the important role of SPI1 in invasion of *S.* Gallinarum and adherence to the epithelial cells which highlights the role of such protein in host adaptation and virulence. In contrast, Jones *et al*. [29] confirmed that SPI1 is not essential for cell invasion and the production of systemic fowl typhoid suggesting that, it may play a role in the early stages of infection.

T cell inhibitor function was previously studied by Matsui [30] who observed that the purified substance of *Salmonella* Typhimurium-derived inhibitor of T-cell proliferation (STI) had an immunosuppressive effect accompanied by augmentation of interferon-gamma (IFN-gamma) secretion and inhibition of interleukin-2 (IL-2) secretion. Furthermore, STI acts directly on T-cells in the absence of accessory cells, such as macrophages and down-regulates IL-2 receptor (IL-2R) function. In the light of the above findings, we can consider STI to be one of the virulence factors in *S*. Gallinarum infection in poultry.

Response regulator protein (phoP) activates transcription of genes whose products play a role in bacterial resistance to anti-microbial peptides by chemically modifying lipopolysaccharide [31]. phoP has been shown to play a role in bacterial resistance to bile [32]. In addition, phoP represses *Salmonella* pathogenicity island 1 genes that are important for bacterial entry into epithelial cells [33]. This reflects the important role of response regulator protein as a means in *S.* Gallinarum virulence which we found to be over-expressed in *S.* Gallinarum versus other tested serovars.

Although the exact role of HSPs in *S.* Gallinarum pathogenesis remains unknown and not yet studied, our data coincide with many previous publications that proved the importance of these proteins in *Salmonella* pathogenesis. Chaperone Hsp90, or stress proteins are synthesized by all cells in response to various types of environmental stress and significant HSPs, of microbial pathogens, appear to be involved in pathogenesis and host immune responses [34]. There is strong evidence that they act as immunodominant antigens of many pathogenic microorganisms and are able to activate a major portion of the immune repertoire of an infected host. Clearly, HSPs could be advantageous in ensuring the organism's survival under such a wide range of potentially hostile growth conditions [35]. Also, HSPs have close involvement in virulence and pathogenesis as it is responsible for the binding of the bacterium to intestinal mucus and could thus be viewed as a virulence factor [36]. Also, it is thought that they contribute to bacterial survival within macrophages by stabilizing bacterial macromolecular complexes after exposure to the toxic and degradative products found within the macrophages [37]. The over expression of HSP90 in *S.* Gallinarum versus other *Salmonella* serovars, revealed in the present study, together with previously reported data about HSP90 its hypothetical importance in *Salmonella* pathogenicity which requires further study.

rfbS protein gene cluster, encodes glycosyl synthase and transferase enzymes for the biosynthesis of oligosaccharide-repeating units. The rfb genes are required for the biosynthesis of bacterial polysaccharide O antigens [38, 39]. Putatively, the regions encoding the 3,6-dideoxyhexosyl synthases determine the antigenic specificities of *Salmonella* serogroups and specifically, the presence of rfbS gene was found in *Salmonella* serovars serogroup D [40].

In conclusion, we could suggest that, there is a major role played by either one or combination of these identified over-expressed proteins in *S.* Gallinarum adaptation or virulence in poultry species. This in turn

makes the functional analysis of these proteins a must in order to find out their exact role in *S.* Gallinarum pathogenicity among poultry species.

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