WFL Publisher
Science and Technology
Meri-Rastilantie 3 B, FI-00980
Helsinki, Finland
e-mail: info@world-food.net

Journal of Food, Agriculture & Environment Vol.11 (2): 197-201. 2013 *www.world-food.net*

Molecular characterization of Salmonella virulence genes isolated from different sources relevant to human health

Ihab Mohamed Moussa [1], Yosra Samy Aleslamboly [2], Abdullah Abdulaziz Al-Arfaj [1], Ashgan Mohamed Hessain [3]*, Azza Said Gouda [4] and Refai Mohamed Kamal [2]

[1] Department of Botany and Microbiology, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia.[2] Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, P. O. Box 11441, Giza 12452, Egypt. [3] Department of Health Science, College of Applied Studies and Community Service, King Saud University, P. O. Box 22459 Riyadh11495, Saudi Arabia. [4] Department of Microbiology, Desert Research Center, P. O. Box 12451, Cairo 14521,Egypt.
**e-mail:ahessan@ksu.edu.sa ashgan319@yahoo.com*

Received 10 January 2013, accepted 26 April 2013.

Abstract

Between 14 January 2010 to 30 April 2012, bacteriological examination of 1075 samples (290 rectal swabs from cattle, 335 rectal swabs from sheep, 450 cloacal swabs from poultry) from diarrheic animals revealed the isolation of 68 Salmonella belonging to 13 different Salmonella serovars. This work was done at Faculty of Veterinary Medicine, Cairo University as well as Center of Excellence in Biotechnology Research, King Saud University. The most common serovars were *Salmonella* Typhimurium (16 isolates), *Salmonella* Enteritidis (13 isolates), *Salmonella* Kentucky (8 isolates) and *Salmonella* Arizona (7 isolates). Other serovars typed were *Salmonella* Heidelberg (4), *Salmonella* Cerro (4), *Salmonella* Gallinarum (3), *Salmonella* Virginia (3), *Salmonella* Paratyphi-A (3), *Salmonella* Dublin (2), *Salmonella* Agona (2), *Salmonella* Hadar (2) and *Salmonella* Bardo (1). All isolates of *Salmonella* Enteritidis and *Salmonella* Typhimurium were examined for the virulence genes *fim*A, *vag, and invA* and *spv*Cgenes by Multiplex PCR. It was evident that *fim*A was the most common in both serovars, followed by *inv*A, while *vag* and *spv*C had almost equal prevalence *Salmonella* Enteritidis and *Salmonella* Typhimurium isolates from cattle, sheep, poultry and human.

Key words: *Salmonella* Typhimurium, *Salmonella* Enteritidis, virulence genes *fim*A, *vag, and invA* and *spv*Cgenes, m-PCR.

Introduction

Salmonellosis is one of the major food-borne diseases. Salmonella infection has been associated with the consumption of raw and undercooked poultry and other meat products; however, many outbreaks have also been associated with contaminated fresh fruits and vegetables [1]. *Salmonella* Typhimurium and *Salmonella* Enteritidis are the most frequently isolated serovars from foodborne outbreaks throughout the world [2].

S. Enteritidis is a major cause of food-borne diseases [3], and during the last two decades it has been isolated worldwide in increasing numbers [2].

S. Typhimurium is a facultative intracellular pathogen that causes a self-limiting gastroenteritis in humans and, less commonly, a non-typhoidal bacteremia [4]. *S.* Typhimurium pathogenesis depends upon a large number of virulence proteins. Pathogenesis of salmonellosis depends up on a large number of factors controlled by an array of genes responsible for the actual virulence of Salmonella [5].

Several rapid and sensitive methods have been developed for identification of Salmonella serovars from clinical samples [6]. These methods, however, still lack the necessary sensitivity and specificity. In vitro amplification of DNA by the polymerase chain reaction (PCR) method is a powerful tool in microbiological diagnostics [7]. Multiplex PCR provides us with a specific method and superior ability to detect *S. enterica* and the serovar *S.* Enteritidis and/or *S.* Typhimurium in the presence of other bacteria simultaneously [8]. In this method several genes are used to detect *Salmonella* genus or serovars including: virulent chromosomal genes such as *invA* [6,7] and plasmid genes such as *spv.* [10]. Therefore, the purpose of this study was to examine Salmonella sero-types (*S.* Enteritidis and *S.*Typhimurium) and virulence genes profiles from cattle, sheep, and poultry as well as from human clinical cases that can pose public health serious.

Materials and Methods

Samples: A total of 1075 samples were collected from diarrheic cattle, sheep and poultry to be subjected for isolation of Salmonella (290 rectal swabs from cattle, 335 rectal swabs from sheep and 450 cloacal swabs from poultry). Samples were obtained from El-Basateen-slaughter house and private farms.

Salmonella strains: The human strains of *Salmonella* Enteritidis and *Salmonella* Typhimurium were obtained from the hospital of the Faculty of Medicine in Beni Swif University.

Isolation and identification of Salmonella isolates: Samples were cultured in selenite F broth and incubated at 37°C for 18 hour. A loopful from inoculated broth was streaked onto the surface of S.S. and XLD agar plates, and then incubated at 37°C for 24-48

hours. Suspected colonies were purified and subcultured into nutrient agar slopes and incubated at 37°C for 24 hours. The purified cultures were subjected to further investigation. Films from the prepared pure cultures were stained with Gram stain and examined microscopically. Salmonella are Gram negative, medium size bacilli. Motility was tested on 0.4% soft agar. The pure cultures of the isolates were identified according to Quinn *et al.* [10].

Serological identification: Typical Salmonella isolates were serotyped by using Denka-Seiken (Tokyo, Japan) antisera following Kauffman-White serotyping scheme [11].

Extraction of DNA: The DNA of *S.* Enteritidis and *S.* Typhimurium isolates were extracted using boiling method according to Croci *et al.* [12].

PCR design and amplification: Amplification of Salmonella strains DNA[13, 14] using oligonucleotide primers synthesized by Metabion Company, Germany. Four sets of primers were used; the first pair was designed with reference to sequence published by Cohen *et al.* [15], *fimA* F- ´CCTTTCTCCATCGTCCTGAA´3 and *fimA* R-5´´TGGTGTTATCTGCCTGAC-CA´3. The second pair was designed with reference to sequence published by Malkawi [8], *vag* F- 5´TTGTAGCTGCTTATGGGGCGGG´3 and *vag* R 5´TGGAGAA ACGACGCACTG TACT GC´3. The third pair was designed with reference to sequence published by Chiu and Ou [15], *inv*A F- 5´ACA GTG CTC GTT TAC GAC CTG AAT´3 and *inv*A R 5´AGA CGA CTG GTA CTG ATC GAT AAT´3. The third pair was designed with reference to sequence published Swamy *et al.* [16] *spv*A F- 5´GAT GCC GGT ATC CCA CTT TA´3 and *spv*A R 5´CGT GAG GAA CCG TTT TAT CG´3. All reactions were carried out in a final volume of 50 µl in micro-amplification tube (PCR tubes) The reaction mixtures consisted of 1µl (200 ng) of the extracted DNA template for the bacterial cultures, 5 µl 10X PCR buffer (75 mM Tris –HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl , 20 mM $(NH_4)_2SO_4$) , 1µl dNTPs (40 M), 1 µl (1U Ampli Taq DNA polymerase), 1µl (50 pmol) from the forward and reverse primers. Each primer pair was used separately and the volume of the reaction mixture was completed to 50 µl using DDW. 40 µl paraffin oil was added and the thermal cycler was adjusted as follows: Initial denaturation at 94°C for 5 min. Followed by 35 cycles of denaturation at 94°C for 1 min. Annealing at 55°C for 1 s. Extension 72°C for 21 s. Final extension at72°C for 7 min .The PCR products were stored in the thermal cycler at 4°C until they were collected. The PCR products were visualized by agarose gel electrophoresis according to Sambrook *et al.* [13].

Results

Bacteriological examination of 1075 samples (290 rectal swabs from cattle, 335 rectal swabs from sheep, 450 cloacal swabs from poultry) from diarrheic animals were examined for Salmonella. 68 samples 6.3% harbored various *Salmonella* species. The highest rate of isolation 8% was obtained from cloacal swabs in poultry, followed by rectal swabs in sheep 5.6% and finally the rectal swabs in cattle 4.4%. The *Salmonella* isolates recovered from the examined samples could be typed into13 different Salmonella serovars, *S.* Typhimurium was more frequent 23.5% than *S.* Enteritidis 20.6% among the total Salmonella isolates. On the other hand, the rate of *S.* Enteritidis was higher 38.5% than that of *S.* Typhimurium 30.8% in cattle, while *S.* Typhimurium was more common than *S.* Enteritidis in sheep and poultry (26.3% and19.4%, versus 21.1% and 13.8% , respectively). Other serovars were detected as shown in Tables 1 and 2.

All isolates of *Salmonella* Enteritidis and *Salmonella* Typhimurium in diarrheic cattle, sheep, poultry, in addition to that of human were examined for the virulence genes *fim*A, *vag, inv*A and *spv*Cgenes. It was evident that *fim*A was the most common in both serovars, followed by *inv*A, while *vag* and *spv*C had almost equal prevalence. *fim*A gene was detected in 89.4% of *S.* Enteritidis and 80% of *S.* Typhimurium isolates. *Inv*A gene was found in 47.3% and 50% in *S.* Enteritidis and *S.* Typhimurium, respectively. The incidence of *vag* and *spv*C genes was between 26.3% and 31.5 in *S.* Enteritidis and 30% in *S.* Typhimurium as shown in Table 2.

The combination of *fim*A, *vag, inv*A *and spv*C gene in *S.* Enteritidis strains isolated from different sources, revealed the, the 4 genes were present in 3 isolates (2 human and 1 poultry), 4 isolates contained 3 genes (2 contained fimA, *vag and spv*C and 2 *fim*A, *inv*A *and spv*C). Two genes were found in 4 isolates, all of them had *fim*A combined with *inv*A, while 6 isolates contained one virulence gene, namely the gene *fim*A and 2 isolates contained

Table 1. Salmonella serovars isolated from different animal species.

Animal species	Samples			Salmonella isolates		
	No.tested	No.positive	%	Serovars	No.	%
Cattle	290	13	4.4	Typhimurium	4	30.7
				Enteritidis	4	30.7
				Agona	2	15.3
				Dublin	2	15.3
				Paratyphi A	1	07.6
Sheep	335	19	5.6	Typhimurium	5	26.3
				Enteritidis	4	21.0
				Heidelberg	4	21.0
				Kentucky	3	15.7
				Paratyphi A	2	10.5
				Bardo	1	5.2
Poultry	450	36	8%	Typhimurium	7	19.4
				Arizona	7	19.4
				Enteritidis	5	13.8
				Kentucky	5	13.8
				S. Cerro	4	11.1
				Verginia	3	08.3
				Gallinarum	3	08.3
				Hadar	2	05.5
Total	1075	68	6.3	-	-	-

Table 2. Distribution of some virulence genes in *S.* Enteritidis and *S.* Typhimurium.

Salmonella Enteritidis (19)										
Genes	Cattle (4)		Sheep (4)		Poultry (5)		Human (6)		Total	
	(+)	(%)	(+)	(%)	(+)	(%)	(+)	(%)	No	%
*fim*A	4	100%	3	75%	4	80%	6	100%	17	89.4%
Vag	1	25%	1	25%	1	20%	2	33%	5	26.3%
*inv*A	2	50%	2	50%	2	40%	3	50%	9	47.3%
*spv*C	1	25%	1	25%	2	40%	2	33%	6	31.5%
Salmonella Typhmuruim (20)										
Genes	Cattle (4)		Sheep (5)		Poultry (7)		Human (4)		Total	
	(+)	(%)	(+)	(%)	(+)	(%)	(+)	(%)	No	%
*fim*A	3	75%	4	80%	5	71.4%	4	100%	16	80%
Vag	1	25%	1	20%	2	28%	2	50%	6	30%
*inv*A	2	50%	2	40%	3	42.8%	3	27%	10	50%
*spv*C	1	25%	1	20%	2	28%	2	50%	6	30%

none of the 4 genes. From the table it is evident that, *fim*A was the most common, as it was detected in 17 out of 19 isolates, followed by *inv*A (9), *spv*C (7) and *vag* (5) genes as shown in Table 3.

In *S.* Typhimurium strains, 4 genes were present in 3 isolates (one each of cattle, poultry and human isolates), 2 isolates contained 3 genes (one from sheep and from human); 6 isolates contained 2 genes and 8 isolates contained one gene, while one isolate contained none of the 4 genes. From the Table it is evident that, *fim*A was the most common, as it was detected in 16 out of 20 isolates, followed by *inv*A (10), *spv*C,*vag* (each5 genes) as shown in Table 4.

Table 3. Combination of *fimA, vag, invA and spvC* gene in *S.* Enteritidis strains isolated from different sources.

Animal species	S. Enteritidis strains	*fimA*	*vag*	*invA*	*spvC*
Cattle	Strain 1	+	+	-	+
	Strain 2	+	-	+	-
	Strain 3	+	-	+	-
	Strain 4	+	-	-	-
Sheep	Strain 1	+	+	-	+
	Strain 2	+	-	+	-
	Strain 3	+	-	+	-
	Strain 4	-	-	-	-
Poultry	Strain1	+	+	+	+
	Strain 2	+	-	+	+
	Strain 3	+	-	-	-
	Strain 4	+	-	-	-
	Strain 5	-	-	-	-
Human	Strain 1	+	+	+	+
	Strain 2	+	+	+	+
	Strain 3	+	-	+	+
	Strain 4	+	-	-	-
	Strain 5	+	-	-	-
	Strain 6	+	-	-	-

Table 4. Combination of *fim*A,*vag*,*inv*A *and spv*C genes in *S.* Typhimurium strains isolated from different sources.

Animal species	S. Typhimurium strains	*fim*A	*vag*	*inv*A	*spv*C
Cattle	Strain 1	+	+	+	+
	Strain 2	+	-	+	-
	Strain 3	+	-	-	-
	Strain 4	-	-	-	-
Sheep	Strain 1	+	+	-	+
	Strain 2	+	-	+	-
	Strain 3	+	-	-	-
	Strain 4	+	-	-	-
	Strain 5	-	-	+	-
Poultry	Strain 1	+	+	-	-
	Strain 2	+	+	+	+
	Strain 3	+	-	+	-
	Strain 4	-	-	-	+
	Strain 5	+	-	-	-
	Strain 6	+	-	-	-
	Strain 7	-	-	+	-
Human	Strain 1	+	+	+	+
	Strain 2	+	+	-	+
	Strain 3	+	-	+	-
	Strain 4	+	-	+	-

Discussion

In the present work, bacteriological examination of 1075 samples from diarrheic animals showed that 68 samples harbored various *Salmonella* species. The highest rate of isolation 8% was obtained from cloacal swabs in poultry, followed by rectal swabs in sheep 5.6% and finally the rectal swabs in cattle 4.4%.Similar results have been reported in Egypt [18-20] and other countries of the world [21-23]. The obtained percentage was lower, when compared with that recorded by Cortez *et al.* [24], who reported that the percent of positive Salmonella samples was 10%. On the other hand, Antunes [25] recorded 44% for *S.* Enteritidis.

Differences in prevalence may be the result of using different sample types, or different methods for detection of salmonellae. The incidence of various *Salmonella* species seems to vary with geographic location and the types of food consumed [26]. However, it was not the aim of the present work to study the incidence of Salmonella in animals poultry and man, rather than to obtain fresh cultures of *S.* Typhimurium and *S.* Enteritidis for molecular characterization of Egyptian isolates. These two serovars were chosen, because they are the most common Salmonella serovars in man, animals and poultry [4, 23, 27].

Serotyping was done in the present work, indicated the isolation of 13 different Salmonella serovars. The results revealed that *S.* Typhimurium (23.5%) and *S.* Enteritidis (20.6%) were most frequent among the total Salmonella isolates. This finding substantiates the report of the CDC [28], which indicated that the two leading *Salmonella enterica* serovars that cause salmonellosis in the United States are *S.* Typhimurium and *S.* Enteritidis (22% and 17% of all Salmonella infections) and Smith-Palmer *et al.* [27], who concluded that the two serovars of Salmonella, which were currently of particular importance in both human and animal infection in Scotland were *S.* Enteritidis and *S.* Typhimurium.

Detection of Salmonella is by standard bacteriological, biochemical and serological are generally time-consuming, tedious and costly as they require hundreds of antisera as well as well-trained technicians [29]. In our study, PCR and multiplex PCR were used to identify *S.* Enteritidis and *S.* Typhimurium virulence genes, which are specific for the genus *Salmonella*. The result denoted that *fim*A was the most common in both serovars, followed by *inv*A, while *vag* and *spv*C had almost equal prevalence. *fim*A *gene* was detected in 89.4% of *S.* Enteritidis and 80% of *S.* Typhimurium isolates. *fim*A gene was the most common in all isolates of *S.* Enteritidis recovered from different sources, as it was present in all cattle and human isolates 100%, in 80% of poultry isolates and 75% of sheep isolates. In case of *S.* Typhimurium, it was present in all human isolates 100%, in 80% of sheep isolates and 75% of cattle isolates.

This was considered by Naravaneni and Jamil [30] very useful in the diagnosis of Salmonella organisms at the genus level, as it can differentiate between *Salmonella* and non-*Salmonella* species. This strengthens the finding of the high prevalence of *fim*A in both *S.* Enteritidis and *S.*Typhimurium in the present study, as it indicates the usefulness of this gene in the rapid detection these salmonellae by PCR. It should however be taken in consideration that in this case about 10-20% of the isolates could be missed due to the absence of this gene. This may be corrected by application of multiplex PCR using more virulence genes, as done in the present work.

This finding was consistent with many previous reports [31, 32] that established the presence of *inv*A gene in nearly all Salmonella irrespective of serovar or source. Also Murugkar *et al.* [33] reported that all salmonellae possessed the genetic information of invasion (*inv*A).

In the present work, the *inv* gene was found in only 47.3% and 50% in *S.* Enteritidis and *S.* Typhimurium, respectively. It was

found in 50% of *S.* Enteritidis of cattle, sheep and human origin and 40% of poultry isolates. *inv*A gene was detected in only 27% of human isolates, 40% of sheep isolates, 42% of poultry isolates and 50% of cattle isolates of *S.* Typhimurium.

The *inv*A is essential for full virulence in Salmonella and is thought to trigger the internalization required for invasion of deeper tissue. This gene is important in the invasion of phagocytic epithelial cells and entry into the intestinal mucosa. This was confirmed by Khan *et al*. [34] who showed that *S.* Typhimurium strains carrying mutation in *inv*A genes are unable to selectively invade in the follicle-associated epithelium of murine Peyer' patches. The *inv*A gene of Salmonella contains sequences unique to this genus and has been proved to be a suitable PCR target with potential diagnostic application [31]. This gene is recognized as an international standard for detection of *Salmonella* genus [7].

Based on the results obtained in our study, the use of *fim*A detected only 80% of *S.* Typhimurium isolates, the combination of *fim*A and *inv*A, raised the rate of positivity to 90%. A finding which confirms the use of more than one gene for detection of more Salmonella by PCR.

With regard to *spv*C gene, Amini *et al.* [32] reported that the use of this gene yielded different and unexpected results. The *spv* genes were not detected in 5.9% of isolates of the *S.* Enteritidis as compared with 70% of the isolates reported by Wood *et al.* [35].

In the present study, the incidence of *spv*C genes was 31.5% in *S.* Enteritidis and 30% in *S.* Typhimurium. The *spv*C gene was detected in 25% of cattle and sheep isolates, 33% of human isolates and 40% of poultry isolates of *S.* Enteritidis. In case of *S.*Typhimurium, *spv*C gene was highest 50% in human isolates, followed by 28% in poultry isolates, 25% in cattle isolates and 20% in sheep isolates. Wood *et al.* [36] found that this gene is only present in 30% of S. Enteritidis strains isolated from poultry. In the study of Amini *et al*. [32], the presence of *spv*C gene in *S.* Enteritidis from human source was 90%, while this gene was present in 100% of the bovine source isolates. In the case of *S.* Enteritidis in poultry source, presence of *spv* C was 88.6%. Another study has reported lack of *spv*C gene of the samples from human, pig, and poultry sources [36].

Detection of these *spv* genes allows us to decide whether the pathogenesis of the isolates from positive clinical samples is attributable to chromosome or plasmid born virulence factor [37].

Based on the results obtained in our study, the use of *fim*A detected only 80% of *S.* Typhimurium isolates, the combination of *fim*A and *inv*A, raised the rate of positivity to 90%, while the combination of *fim*A and *inv*A and *spv* genes, raised the positivity to 95%.

The least detected gene in the present study was the virulence associated gene (*vag* gene), which was present in 26.3-30% of the isolates of *S.* Enteritidis and *S.* Typhimurium, respectively. In case *S.* Enteritidis, the highest rate 33% was found in human isolates and the lower 20% in poultry isolates, while 25% of cattle and sheep isolates were positive for this gene. In case *S.* Typhimurium, the highest incidence of *vag gene* 50% was recorded in human isolates, followed by poultry isolates 28%, cattle isolates 25% and sheep isolates 20% . Bakshi *et al*. [38] reported the detection of this gene in all *Salmonella* spp. in 24 *S.* Enteritidis isolated by using PCR assay.

It is clear in the present study that, there were some discrepancies about distribution of virulence plasmid of various strains of *S.* Typhimurium and *S.* Enteritidis from human and animal origins. This was reported Amini *et al.* [32] where their study reported a higher distribution of *spv*C genes in bovine sources and lower distribution in poultry sources compared with human-origin sources. Drastic genetic variations in Salmonella could be derived from transfer of this organism between human-origin and animal-origin strains. Whether this can transfer virulence plasmid from animal-origin strains to human-origin strains or vice versa remains to be investigated. Strains of Salmonella bacterium (Particularly Typhimurium and Enteritidis serovars) which carry virulence plasmid can cause systemic disease, while plasmidless strains can cause local or asymptomatic disease [39].

Presence of virulence gene in *Salmonella* species mainly enhances the induction of gastroenteritis, organs focal infection and systemic febrile disease in animal and human cases [6, 17]. Spread of Salmonella is mainly occurred through contamination of animal and human foods especially poultry and poultry product, so the initial interaction between *Salmonella* species and intestinal mucosa is mainly regulated by the expression level of *inv*A. While *spv*C is one of virulence genes which play role in control the immune response and regulate the interaction of the host cell as well as *spv*C is control the growth rate of Salmonella inside the host cell Swamy *et al*. [17] So presence of *spv*C gene and *inv*A gene are essential for full expression of virulence of *Salmonella* species in animal and human.

Conclusions

It was evident that *fim*A was the most common in both serovars (*Salmonella* Enteritidis and *Salmonella* Typhimurium), followed by *inv*A, while *vag* and *spv*C had almost equal prevalence in the isolates from cattle, sheep, poultry and human.

Acknowledgements

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for supporting the work through the research group project No.: RGP-VPP-176.

References

[1]Gallegos-Robles, M., Loredo, A., Ojeda, G., Vega, A., Chew, Y., Velarde, S. and Fratamico, P. M. 2008. Identification of Salmonella serovars isolated from cantaloupe and Chile pepper production systems in Mexico by PCR-Restriction Fragment Length Polymorphism. J. Food Protect. **71**(11):2217-2222.

[2]Brenner, F. W., Villar, R. G., Angulo, F. J., Tauxe, R. and Swaminathan, B. 2000. Salmonella nomenclature. J. Clin. Microbiol. **38**:2465-2467.

[3]Herikstad, H., Motarjemi, Y. and Tauxe, R. V. 2002. Salmonella surveillance: A global survey of public health serotyping. Epidemiol. Infect. **129**:1-8.

[4]Velge, P., Cloeckaert, A. and Barrow, P. 2005. Emergence of Salmonella epidemics: The problems related to *Salmonella* Enteritidis and multiple antibiotic resistances in other major serotypes. Vet. Res. **36**:267–288.

[5]Murugkar, H., Rahman, H. and Dulta, P. 2003. Distribution of virulence genes in Salmonella serovars isolated from man and animals. Indian J. Medical. **117**:66-70.

[6]Zahraei, T., Salehi, M., Mahzounieh, and E. Khaksar 2010. Detection of Salmonella serovars in zoo and pet reptiles, rabbits, and rodents in Iran by culture and PCR methods. Comp. Clin. Pathol. **19**:199–202.

[7]Malorny, B., Hoorfar, J., Hugas, M., Heuvelink, A., Fach, P., Ellerbroek, L., Bunge, C., Dorn, C. and Helmuth, R. 2003. Interlaboratory diagnostic accuracy of a Salmonella specific PCR-based method. Inter. J. Food Microbiol. **89**:241-249.

[8]Malkawi, H. and Gharaibeh, R. 2004. Rapid and simultaneous identification of two *Salmonella enterica* serovars, Enteritidis and Typhimurium from chicken and meat products by Multiplex PCR. Biotechnology **3**(1):44-48.

[9]Soumet, C., Ermel, G., Rose, N., Rose, V., Drouin, P., Salvat, G. and Colin, P. 1999. Evaluation of a multiplex PCR assay for simultaneous identification of *Salmonella* sp., *Salmonella* Enteritidis and *Salmonella* Typhimurium from environmental swabs of poultry houses. Lett. Appl. Microbiol. **28**:113-117.

[10]Quinn, P. J.,Carter, M. E., Markey, B. K., Donnelly, W. J. and Leonard, F. C. 2002. Veterinary Microbiology and Microbial Disease. S. Livingstone Limited, Edinburgh and New York, pp. 47-67.

[11]Popoff, M. Y. 2001. Antigenic formulas of the *Salmonella* serovars. 8th edn..WHO Collaborating Centre for Reference and Research on Salmonella, Pasteur Institute, Paris, France, pp. 63-75.

[12]Croci, L., Dlibato, E., Volpe, G., Medici, D. D. and Palleschi, G. 2004. Comparison of PCR, Electrochemical Enzyme linked Immunosorbent Assays, and the standard culture method for detection Salmonella in meat products. Appl. Env. Mic. **70**:1393-1396.

[13]Sambrook, J., Friscgh, E. F. and Meniates, T. 1989. Molecular Cloning: A Laboratory Manual.Vol.1. Cold Spring Harbor Laboratory Press, New York, pp. 54-60.

[14]Oliveira, S. D., Rodenbusch, C. R., Ce, M. C., Rocha, S. L. S. and Canal, C. W. L. 2003. Evaluation of selective and non-selective enrichment PCR. Lett. Appl. Microbiol. **36**(4):217-221.

[15]Cohen, H. J., Mechanda, S. M. and Lin, W. 1996. PCR amplification of the fimA gene sequence of *Salmonella* Typhimurium, a specific method for detection of *Salmonella* spp. Appl. Environ. Microbiol. **62**(12):4303–4308.

[16]Chiu, C. H. and Ou, J. T. 1996. Rapid identification of Salmonella serovars in feces by specific detection of virulence genes, invA and spvC, by an enrichment broth culture-multiplex PCR combination assay. J. Clin. Microbiol. **34**:2619–2622.

[17]Swamy, S. C., Barnhart, H. M., Lee, M. D. and Dreesen, D. W. 1996. Virulence determinants invA and spvC in Salmonellae isolated from poultry products, wastewater, and human sources. Appl. Environ. Microbiol. **62**(10):3768–3771.

[18]Stevens, A., Kerouanton, A., Marault, M., Millemann, Y., Brisabois, A., Cavin, J.F. and Dufour, B. 2008. Epidemiological analysis of *Salmonella enterica* from beef sampled in the slaughter house and retailers in Dakar (Senegal) using pulsed-field gel electrophoresis and antibiotic susceptibility testing. J. Food Microbiol. **123**:191-197.

[19]Kang, M., Besser, T. E. Hancock, D., Porwollik, S., McClelland, M., and Call, D. 2006. Identification of specific gene sequences conserved in contemporary epidemic strains of *Salmonella enterica*. Appl. Environ. Microbiol. **72**(11):6938–6947.

[20]Gürakan, G. C., Aksoy, C., Ögel, Z. B. and Ören, N. G. 2008. Differentiation of *Salmonella* Typhimurium from *Salmonella* Enteritidis and other Salmonella serovars using Random Amplified Polymorphic DNA Analysis. Poult. Sci. **87**:1068-1074.

[21]Moussa, I. M., Ashgan, M. H., Mohamed, M. S., Mohamed, K. H. F. and Al-Doss, A. A. 2010. Rapid detection of *Salmonella* species in new borne calves by polymerase chain reaction. Int. J. Genetics. Mol. Biol. **2**(4):062-066.

[22]Khalil, A. M., Mofarlane, R. G., Shand, N. A., Leslie, S. E. and Christensen, N. H. 2001. Experimental infection of pregnant sheep with attenuated *Salmonella* Typhimurium. Proceedings of the New Zealand. Soc. Animal Prod. **61**(4):74-77.

[23]Genic, O. 2002. The isolation, identification and serotyping of Salmonella isolated from domestic animals in Kars District. Kafkas-Universitesi Veteriner. Fakultesi Dergisi **8**(1):23-30.

[24]Threlfall, E. J., Daya, M., de Pinna, E., Charlett, A. and Goodyear, K. L. 2006. Assessment of factors contributing to changes in the incidence of antimicrobial drug resistance in *Salmonella* Enteritidis and Typhimurium from humans in England and Wales in 2000, 2002 and 2004. Int. J. Antimicrob. Agents **28**:389–395.

[25]Cortez, A. L. L., Carvalho, A. C. F.B., Ikuno, A. A., Bürger, K. P. and Vidal-Martins, A. M. C. 2006. Identification of *Salmonella* spp. isolates from chicken abattoirs by multiplex-PCR. Res. Vet. Sci. **81**:340–344.

[26]Antunes, P., Reu, C., Sousa, J. C., Peixe, L. and Pestana, N. 2003. Incidence of Salmonella from poultry products and their susceptibility to antimicrobial agents. Int. J. Food Microbiol. **82**:97– 103.

[27]Padungtod, P. and Kaneene, J. B. 2006. Salmonella in food animals and humans in northern Thailand. Int. J. Food Microbiol. **108**:346-354.

[28]Smith-Palmer, A., Stewaret, W. C., Mather, H., Greig, A., Cowden, J. M. and Reilly, W. J. 2003. Epidemiology of *Salmonella* Enteritidis and Typhimurium in animal and people in Scotland between 90-2001.Vet. Rec. **153**(17):517-520.

[29]Centers of Disease Control and Prevention "CDC"2003. Salmonella—Annual Summary 2002. Centers for Disease Control and Prevention, Atlanta, pp. 106-115.

[30]Echeita, M. A., Herrera, S., Garaizar, J. and Usera, M. A. 2002. Multiplex PCR based detection an identification of the most common Salmonella second-phase flagellar antigen. Res. Microbiol. **153**:107-113.

[31]Naravaneni, R. and Jamil, K. 2005. Rapid detection of food-borne pathogens by using molecular techniques. J. Med. Microbiol. **54**:51–54.

[32]Murugkar, H., Rahman, H. and Dulta, P. 2003. Distribution of virulence genes in Salmonella serovars isolated from man and animals. Indian J. Medical. **117**:66-70.

[33]Jamshidi, A., Bassamim, M. R. and Afshari-Nic, S. 2008. Identification of Salmonella serovars Typhimurium by a multiplex PCR-Based assay from poultry carcasses in Mashhad-Iran. Int. J. Vet. Res. **3**(1):43-48.

[34]Amini, K., Zahraei, T., Nikbakht, G., Ranjbar, R., Javid Amini, J. and Ashrafganjooe, S. 2010. Molecular detection of *inv*A and *spv* virulence genes in *Salmonella* Enteritidis isolated from human and animals in Iran. Afr. J. Microbiol. Res. **4**(21):2202 - 2210.

[35]Khan, A. A., Nawaz, M. S., Khan, S. and Sernigelia, C. E. 1999. Detection of multidrug resistant *Salmonella* Typhimurium DT104 by multiplex polymerase chain reaction. FEEMS Microbiol. Lett. **182**:355-360.

[36]Wood, M. W., Mahon, J. and Lax, A. J. 1994. Development of a probe and PCR primers specific to virulence plasmid of *Salmonella* Enteritidis. Mol. Cell Probes **8**:473-479.

[37]Castilla, K. S., Ferreira, C. S. A., Moreno, A. M., Nunes, I. A. and Ferreira, A. J. P. 2006. Distribution of virulence genes *sef*C, *pef*A and *spv*C in *Salmonella* Enteritidis phage type 4 strains isolated in Brazil. Br. J. Microbiol. **37**(2):1-7.

[38]Trafny, E. A., Kozlowska. K. and Szpakowska, M. 2006. A novel Multiplex PCR assay for the detection of *Salmonella enterica* serovar Enteritidis in human faeces. Lett. Appl. Microbiol. **43**:673-679.

[39]Bakshi, C. S., Singh, V. P., Malik, M., Singh, R. K. and Sharma, B. 2003. 55kb plasmid and virulence associated genes are positively correlated with *Salmonella* Enteritidis pathogenicity in mice and chickens. Vet. Res. Comm. **27**(6):425-432.