



Molecular Characterization of Salmonella Enterica Isolated from Chicken Meat and its Products by Multiplex PCR

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Key words

S. typhimurium - *S. enteritidis* - *S. Kentucky* - chicken meat - Multiplex-PCR

ABSTRACT:

The present study was conducted for identification of molecular characterization of *Salmonella enterica* isolated from chicken meat by using multiplex-PCR isolated from 200 samples of fresh chicken meat and 100 samples of ready to eat chicken meat. Samples were assessed by performing the pre-enrichment and enrichment culture, biochemically characterized by the analytical profiling index (API 20E system). The primers were selected for *invA*, *stn*, *avrA*, *sefA*, *fliC* and *stm 4495* genes, specific for the detection of *Salmonella enteritidis*, *Salmonella typhimurium* and *Salmonella kentucky*. Standard bacteriological examination revealed that 7 *S. enterica* isolated from 200 chicken meats with a percentage of (3.5%), including 5 *S. typhimurium* with a percentage of (2.5%) and 1 for each of *S. enteritidis* and *S. kentucky* with a percentage of (0.5 %), while could not isolate *S. enterica* from ready to eat chicken meat. The results of multiplex PCR for the three isolated serotypes of *Salmonella enterica* (1 for each *S. typhimurium*, *S. Kentucky* and *S. enteritidis*) could detect the universal gene (*invA*) and virulence genes (*avrA* and *stn*) in all examined serotypes, while (*fliC*) gene was detected in both *S. typhimurium* and *S. kentucky* only while not detected in *S. enteritidis*, (*stm 4495*) gene was detected only in *S. typhimurium* but was not detected in *S. kentucky* and *S. enteritidis*. (*sefA*) gene was detected only in *S. enteritidis* but was not detected in *S. typhimurium* and *S. kentucky*.

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1- INTRODUCTION

Salmonella enterica serovar *typhimurium* and *Salmonella enterica* serovar *enteritidis* are the most frequently isolated serovars from food borne out breaks throughout the world (Herikstad et al., 2002). Several genes have been used to detect *Salmonella* in natural environmental samples as well as food and feces samples. Virulence chromosomal genes including; *invA* (Malorny et al., 2003b), *sefA* (Szabo and Mackey, 1999), *fliC* (Soumet et al., 1998) are target genes for PCR amplification of *Salmonella* species.

Salmonellae still a major cause of food-borne human disease in most parts of the world (Carraminana et al., 2004). The most common human non-typhoidal salmonellosis is caused by *S. enterica* serovar *S. typhimurium* and *enteritidis* (Yang et al., 2010), both of which are frequently found in contaminated foodstuffs, particularly in meat (Forshell and Wierup, 2006).

Multiplex PCR provides us with a specific method and superior ability to detect *Salmonella enterica* and the serovar *S. enteritidis* and/or *S. typhimurium* in the presence of other bacteria simultaneously (Yan and Sekaran, 2010).

Salmonellosis in humans most often results from consumption of contaminated food, in particular meat, raw vegetables, poultry and eggs (Braden and Tauxe, 2013).

In Egypt, a significant increase in the number of *Salmonella* isolates from animal and chicken meat has been observed (Ahmed and Shimamoto, 2014).

Salmonellosis is a serious zoonotic food-borne disease which causes outbreaks and sporadic cases of gastroenteritis in human worldwide as well as high medical and economical costs (Lee, 2015). The study was planned for detection of molecular characterization of *Salmonella enterica* isolated from chicken meat by using multiplex-PCR.

2. MATERIAL AND METHODS

2.1. Collection of Samples:

A total of 300 samples included of 200 fresh chicken meats were collected from chicken retail markets and 100 samples of ready to eat chicken meat from markets in Alexandria Governorate. All samples were labeled and placed in ice box to keep the samples cool, and transferred quickly as soon as possible for bacteriological examination.

2.2. Isolation and identification of *Salmonella enterica*:

The procedures for isolation of *Salmonella enteric* according to the techniques recommended by ISO 6579 (2002), morphological and biochemical identification were carried out according to Cruickshank et al. (1975) and Kauffmann (1972) and further confirmation of the presumptive *Salmonella* isolates was carried out with a commercial bacterial identification kit (Bio Merieux, Marcy, France) by the Analytical Profile Index (API) system followed by serological identification of *Salmonella* "O" and "H" antigens as well as the phase of the organism were detected by using agglutination sera test. The sera used were purchased from Welcome Research Laboratories Beckenham, England, according to the Kauffman-White scheme at *Salmonella* Reference Laboratory in Animal Health Research Institute, Dokky, Egypt.

2.3. Molecular typing of *Salmonella enterica* using M-PCR:

Aliquots of the storage solutions were plated on trypticase soy agar (Becton Dickinson) with overnight incubation at 37°C. Bacterial genomic DNA was then extracted after growth

overnight in Luria-Bertani broth (Difco Laboratories, Detroit, MI, USA) by using the DNA extraction kit (Qiagen) according to the manufacturer's protocol and stored at 4°C (Lee, 2009).

The specificity of the oligonucleotide primers were carried out by testing all the recovered *Salmonella* strains in addition to the standard positive and standard negative strains with M-PCR using the primer pairs as the first three sets specific for the genus *Salmonella* amplified 284bp fragment within the *invA* gene (Oliveira et al., 2003), 617bp fragment within the *stn* gene (Murugkar et al., 2003) and 422bp fragment within the *avrA* gene (Huehn et al., 2010). The fourth set amplified a 559bp fragment specific for *S. typhimurium* (Fli15) and *S. kentucky* (Tym) within the *fliC* gene (Soumet et al., 1999). The fifth set amplified a 310bp fragment specific for *S. enteritidis* within the *sefA* gene (Rahn et al., 1992). The sixth set amplified a 915bp fragment specific for *S. typhimurium* within the *stm* gene (Liu et al., 2012). The sequence of oligonucleotide primers used in M-PCR showed in Table (1), temperature and time conditions of the two primers during PCR are shown in Table (2).

Table (1) Target genes used in M-PCR assays:

Sex sets of primers selected from different genomic sequences were used in multiplex PCR :

Strains	Primer	Target gene	Primer sequence	Length of amplified product	Reference
<i>Salmonella</i> Spp.	S139	<i>invA</i>	GTGAAATTATCGCCACGTTCTGGGCAA	284 bp	Rabie N.,2012
	S141		TCATCGCACCGTCAAAGGAACC		
Virulence primer		<i>Stn</i>	TTGTGTCGCTATCACTGGCAA CC	617 bp	Osman KM.,2014
Virulence primer		<i>AvrA</i>	CCTGTATTGTTGAGCGTCTGG	422 bp	Sallam K.I,2014
<i>S. kentucky</i>	<i>fli 15</i>	<i>fliC</i>	CGGTGTTGCCAGGTTGGTAAT	559 bp	Harris WV.,2004
	<i>tym</i>		ACTCTTGCTGGCGGTGCGACTT		
<i>S. enteritidis</i>	SEFA2	<i>sefA</i>	GCAGCGGTTACTATTGCAGC	310 bp	Islam SM.,2005
	SEFA4		TGTGACAGGGACATTTAGCG		
<i>S. Typhimurium</i>	S. Typhimurium-STM-F	STM4495	GGT GGC AAG GGA ATG AA	915 bp	Gracias KS.,2004
	S. Typhimurium-STM-R		CGC AGC GTA AAG CAA CT		
	rfbSG		TAT TCA CGA ATT GATATACTC		

Table (2) Cycling conditions of the different primers during PCR:

	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Temperature	95°C	95°C	58°C	72°C	35	72°C
Time	10 min	1 min	1 min	1 min	-	10 min

Two multiplex PCR assays were performed: The first assay was carried out to determine the positivity of each *Salmonella* serovar for the presence of the three common genes, in addition to the related discriminatory gene. The another assay was carried out to investigate whether the regions designed for the primers of the discriminatory genes are not serovar unique region and there is another positivity found in the other *Salmonella enterica*.

4. Antimicrobial susceptibility testing:

3. RESULTS

3.1. Bacteriological examination:

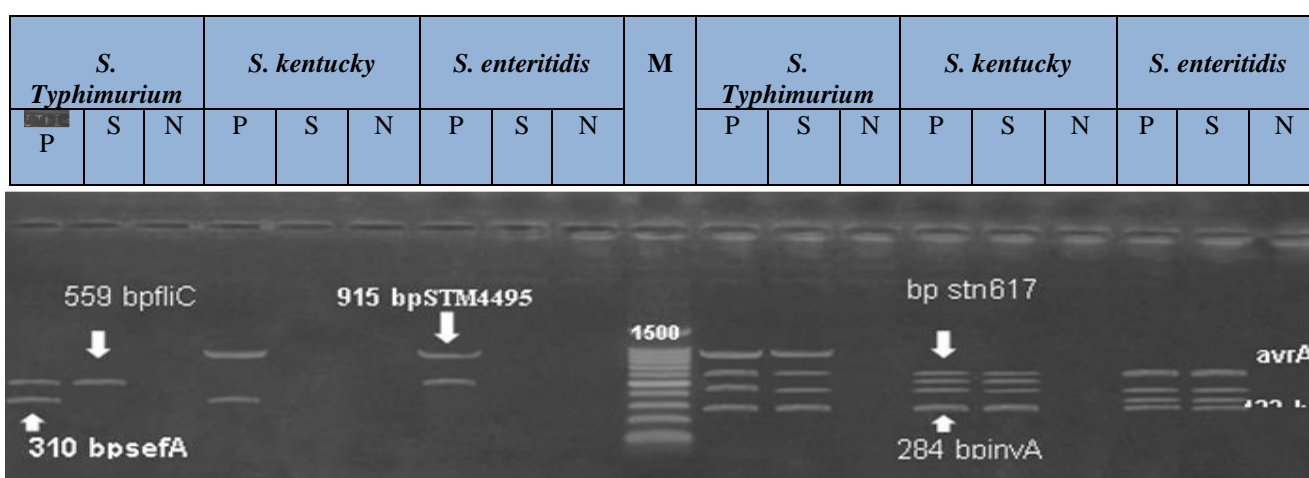
Data showed in Table (3) explored that out of 200 fresh chicken meat samples, 5 (2.5%), 1 (0.5%) and 1 (0.5%) were positive for *S. typhimurium*, *S. kentucky* and *S. enteritidis*, respectively. So total no. of *Salmonella enteric* are 7 (3.5%) could be recovered from ready to eat chicken products.

3.2. M-PCR:

Sex Primer sets chosen from different genes were assessed in order to detect the genus *Salmonella* and their serotypes. Multiplex PCR applied in the present study gave positive results by all tested serovars yielding three fragments of 284 bp (*invA*), 422 bp (*avrA*) and 617 bp (*stn*) corresponding to genus *Salmonella*, and additional three distinct amplified fragments of 559 bp (*fliC*), 915 bp (*STM*) and 310 bp (*sefA*) which allowed identification of *S. typhimurium*, *S. kentucky* and *S. enteritidis* (Fig. 1). The Tym primer was used in combination with the Fli15 primer for both *S. typhimurium* and *S. kentucky*. However, the STM primer set was used to differentiate between the two species. Under the conditions used, both *S. typhimurium* and *S. Kentucky* gave a 559 bp amplified fragment with the Fli15-Tym primer set. However, only *S. typhimurium* gave a 915 bp amplified fragment with the STM primer set (Fig. 1).

Table (3). Incidence of *Salmonella* Enteritidis and *Salmonella* Typhimurium. *Salmonella* Kentucky isolated from 300 chicken meat samples

Type of Samples	No. of Samples	S. enteric isolates		Serotyping of S. enteric isolates					
		No.	%	Salmonella Typhimurium		Salmonella Enteritidis		Salmonella Kentucky	
				No.	%	No.	%	No.	%
Fresh chicken meat	200	7	3.5%	5	2.5%	1	0.5%	1	0.5%
Ready to eat chicken meat	100	0	0	0	0	0	0	0	0

**Fig. (1)** Ethidium bromide stained agarose gel electrophoresis containing the PCR products along with 100bp DNA ladder (lane M). P: positive control; N: negative control which is (Nuclease free water) and S: tested sample.

Duplex PCR for:

***S. Typhimurium*:** PCR was carried out using 2 primers specific for *FliC* (559bp) and *SefA* (310bp) genes: the tested sample give positive with *FliC* gene only.

***S. Kentucky*:** PCR was carried out using 2 primers specific for *STM* (915bp) and *SefA* (310bp) genes: the tested sample give negative.

***S. enteritidis*:** PCR was carried out using 2 primers specific for *STM* (915bp) and *FliC* (559bp) genes: the tested sample give negative.

Multiplex PCR for:

***S. Typhimurium*:** PCR was carried out using 4 primers specific for *InvA* (284bp), *SefA* (310bp), *Stn* (617bp) and *STM* (915bp) genes; the tested sample give positive with the four primers like the positive control.

***S. kentucky*:** PCR was carried out using 4 primers specific for *InvA* (284bp), *SefA* (310bp), *AvrA* (422bp) and *Stn* (617bp) genes; the tested sample give positive with the four primers like the positive control.

***S. enteritidis*:** PCR was carried out using 4 primers specific for *InvA* (284bp), *SefA* (310bp), *AvrA* (422bp) and *Stn* (617bp) genes; the tested sample give positive with the four primers like the positive control.

4. DISCUSSION

Salmonella, a genus within *Enterobacteriaceae*, remains as an important human pathogen and it has been reported to be the most common food-borne bacterial disease in the world (Coburn et al., 2007). Poultry is one of the most important reservoirs of *Salmonellae* that can be transmitted to humans through the dealing with chicken meat and/or consumption of uncooked meat and eggs (Wales and Davies, 2011; Nawar and Khedr 2014). In present study, incidence of *Salmonella enterica* showed in Table (3) revealed isolation of 7 *Salmonella enterica* a with percentage of (3.5%); including 5 (2.5%) *S. typhimurium* and 1(2.5%) for each of *S. enteritidis* and *Salmonella kentucky*, while could not isolate *Salmonella enterica* from ready to eat chicken meat samples this result nearly similar to Prebaf (2008) who detected *Salmonella* sp. by both M-PCR and conventional microbiological methods from chicken with a percentage of (2.58%) and Nagwa et al. (2012) who isolated *Salmonella* sp. with a percentage of (4%) from raw frozen chicken meat while lower than Jamshidi et al. (2009) who isolated *Salmonella* sp (8.3%) and *Salmonella typhimurium* (1.6%) from poultry carcasses, and (Zhao et al., 2001) who

reported that the prevalence of *Salmonella* in poultry carcasses, with contamination percentages ranged from 3% to 66%. This result disagreed with Fuzihara et al. (2000) who reported that the serotype *S. enteritidis* was the most prevalent in carcasses of poultry meat, using microbiological methods.

On contrast, this study showed that no *Salmonella enterica* recovered from 100 samples of ready-to-eat chicken meat, this result agreed with Bohaychuk et al. (2006) who found that no *Salmonella* in chicken wieners, however, disagreed with Arumugaswamy (1995) who recorded *Salmonella* contamination in several ready-to-eat chicken meat products with a percentage of 14% and Osaili et al. (2014) who examined 478 ready-to-eat chicken products and published that the prevalence of *Salmonella* serovars was 0.8%, this may be due to difference in hygienic measures followed, sampling protocol and sources as well as sanitation of chicken farms.

Molecular detection and differentiation of the isolated *Salmonella* serovars that contaminated the retail chicken meat in Alexandria, Egypt by using multiple virulence genes-based M-PCR gave positive results by all tested serovars yielding three fragments of 284bp (*invA*), 422bp (*avrA*) and 617bp (*stn*) corresponding to genus *Salmonella*, and additional three distinct amplified fragments of 559bp (*fliC*), 915bp (*stm*) and 310bp (*sefA*) which allowed identification of *S. typhimurium*, *S. kentucky* and *S. enteritidis* (Fig. 1), similar studies performed by Streckel et al. (2004) who said that effector gene (*avrA*) found in 80% and Diarra (2014) who found that the invasin gene (*invA*) present in 97.9% of *Salmonella enterica* serovars and the heat-labile *Salmonella* enterotoxin (*stn*) serve as effector proteins, which are involved in the pathogenesis of salmonellosis, so the primer sets designed in the present study for *invA*, *avrA* and *stn* genes allow simultaneous identification of all pathogenic *Salmonella* within the genus level. So this assay suggested being useful especially for rapid screening of large numbers of samples contaminated with different *Salmonella* species. Although, several studies have been focused on the molecular identification of *S. typhimurium*, and *S. enteritidis* isolated from retail chicken meat (Cortez, 2006 and Abd-Elghany, 2014), few studies have been carried out on *S. kentucky* (Diarra, 2014). In the present study, the Tym primer was used in combination with the *Fli15* primer for both *S. typhimurium* and *S. kentucky*. However, the *STM* primer set was used to differentiate between the two species. Under the conditions used, both *S.*

typhimurium and *S. kentucky* gave a 559bp amplified fragment with the Fli15-Tym primer set. However, only *S. typhimurium* gave a 915bp amplified fragment with the STM primer set (Fig. 1). These findings suggested that M-multiplex PCR assays described here is reliable and easy to perform, and has a high ability for species identification. This method can be an alternative or complementary method to traditional conventional identification of *Salmonella* species.

5. REFERENCES

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