

Molecular Diagnosis of *Salmonellae* in Frozen Meat and Some Meat Products

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Abstract

A total of one hundred and twenty random samples of luncheon, fresh raw sausage, frozen packed minced meat and frozen meat (30 samples of each) were collected from different supermarkets at Kalyobia Governorate. The collected samples were transferred directly to the laboratory in an ice box under complete aseptic conditions. The samples were immediately examined bacteriologically for the detection of *Salmonellae*. The *Salmonellae* isolates were confirmed by PCR.

Salmonellae failed to be detected in luncheon beef samples but the percentage of *Salmonellae* in fresh sausage was 10% and the isolated serovars were *S. typhi* (3.3%), *S. typhimurium* (3.3%) and *S. enteritidis* (3.3%). In frozen packed minced meat, the percentage of *Salmonellae* was 6.7% and the isolated serovars were *S. typhi* (3.3%) and *S. typhimurium* (3.3%) and in frozen meat, the percentage of *Salmonellae* was 13.3% and the isolated serovars were *S. papuana* (6.7%), *S. paratyphi A* (3.3%) and *S. vircho* (3.3%). The virulence of the isolated strains was confirmed through molecular technique by polymerase chain reaction (PCR) of the isolated *Salmonellae* strains for detection of the virulence factor (*invA* gene).

Keywords: Luncheon; Sausage; Minced meat; Frozen meat; *Salmonellae*; Serovars; PCR

Introduction

Food borne illnesses are considered by the World Health Organization (WHO) as diseases either infectious or toxic made by causative agents in ingested food. The reports in 2005 recorded 1.8 million people died from diseases causing diarrhea and high proportion of which was attributed to contamination of food and drinking water [1]. *Salmonellae* food poisoning is one of the most common and widely distributed diseases in the world [2], estimated to cause 1.3 billion cases of gastroenteritis and 3 million deaths worldwide [3].

Recently, some food borne diseases are considered as emerging diseases. Various food borne pathogens have been identified for food borne illness. *Campylobacter*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonellae* are found to be responsible for most of food origin outbreaks [4].

Meat products such as sausage, luncheon and minced meat are more popular because they are considered as quick easily prepared meat meals and solve the problem of fresh meat shortage which is not within the reach of many peoples.

Microorganisms may contaminate meat products during a long chain of processing from the time of preparation, handling,

processing, distribution and storage as well as marketing. Such contamination may render the products of inferior quality or even unfit for human consumption and at times may constitute a public health hazard. The possibility of contamination of meat products with food poisoning bacteria especially *Salmonellae* organisms has been extensively reported [5].

Traditional and conventional methods for identification of microbial pathogens depend on specific bacteriological and biochemical identification [6], which are time consuming, laborious and less accurate [7] and slow because *Salmonellae* needs 5-7 days to confirm its presence in meat products [8]. Several Polymerase Chain Reaction (PCR) assays have been developed by targeting various *Salmonellae* genes, such as *invA* [9,10]. The *invA* gene, is widely used as a target in PCR assays for *Salmonella* detection [11-13].

Materials and Methods

Isolation and identification of *Salmonellae*

The techniques adopted were carried out according to ICMSF (1978) [14]. Samples were cultivated for the isolation of *Salmonellae* species on MacConkey agar. After 24 h of incubation at 37°C, suspected colonies with typical characteristics of *Salmonellae* were sub-cultured on XLD (Xylose Lysine Deoxycholate) agar for 24 h at

37°C and *Salmonella shigella* (S.S) agar plates then incubated at 37°C for 24 h.

Molecular identification of *Salmonellae* isolates by PCR

Extraction of genomic DNA according to QIAamp DNA mini kit instructions: Genomic DNA was extracted from cell suspensions of bacteria grown overnight on XLD broth at 37°C using QIAamp® DNA Mini kit from Qiagen according to Lee SH, et al. [15]. A single colony from *Salmonellae* was resuspended in 3 ml XLD liquid media and grown overnight at 37°C. The bacterial culture was precipitated by centrifugation in a microcentrifuge at 5000 xg for 10 min. The bacterial pellets were resuspended in 180 µl AL buffer (supplied in the QIAamp DNA Mini Kit) for complete lysis. The samples were immediately cooled on ice for 5 min, and the cell lysates were digested with 20 µl of proteinase K (final concentration 800 µg/ml) at 56°C for 3 h. 200 µl ethanol (96 to 100%) was added to each tube and vortexed for 15 s. The mixtures were loaded onto the QIAamp Mini spin column and centrifuged at 6000 xg for 1 min. The filtrate was discarded and the spin column was placed in a clean collection tube (2 ml). A 500 µl of AW1 wash buffer was added to the QIAamp spin column and centrifuged at 6000 xg for 1 min. The washing step was repeated twice using 500 µl of washing buffer AW2. To elute bacterial DNA, 100 µl elution buffer (AE) was added to the center of the column, and the column was incubated at 37°C for 5 min then centrifuged at 20,000 xg for 1 min. DNA purity and quantity was determined using spectrophotometer (GeneSys 10UV, Thermo Scientific, USA).

Oligonucleotide primers for *Salmonellae* spp. (*invA* gene): Primer sets for the pathotypes and virulence genes for the isolated *Salmonellae* spp. according to Oliveira, et al. [16] (Table 1).

DNA amplification: The samples were done in BioRad Thermal cycler (T100-England) with the following thermal profile: initial denaturation at 95°C for 5 min then denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension for 30 sec at 72°C for 35 cycles and after the last cycle, the mixture was incubated for final extension at 72°C for 10 min.

Detection of PCR products according to Sambrook J, et al. [17]: Amplified products were detected in 1.5% agarose gel electrophoresis pre-stained with ethidium bromide, at 80 V for 1 hour. Specific amplicons were observed under ultraviolet transillumination, compared with the marker, the gel was transferred to UV cabinet, and the gel was photographed by a gel documentation system and the data was analyzed through computer software.

Results and Discussion

In this work, a total of one hundred and twenty random samples of meat products, (30 samples of luncheon beef, 30 samples of fresh sausage, 30 samples of frozen minced meat and 30 samples of frozen meat) were examined for *Salmonellae*. The virulence of the isolated serotypes was confirmed by detection of the *invA* gene which is responsible for the pathogenicity of *Salmonella* serotypes.

Incidence of *Salmonellae*

Table 1: Primers used for the detection of *Salmonellae* sp.

Primer	Sequence	Amplified product
<i>invA</i>	GTGAAATTATCGCCACGTTCCGGGCAA	284 bp
	TCATCGCACCGTCAAAGGAACC	

Salmonellae were detected in 7.5 % of the examined meat product samples. The percentage of *Salmonellae* in luncheon meat, fresh sausage, frozen minced meat and frozen meat was 0%, 10% and 6.7% and 13.3% respectively (Table 2).

The results present in table 2 revealed that *Salmonellae* failed to be detected in the examined luncheon samples.

These results agree with that reported by Gouda HI, Saleh AA, Edris A, Nashed-Heba F, Moussa MM, et al, Fathi S, et al, Aiedia HA, Abd-El-Aziz A, S, et al., Ouf-Jehan M, Eleiwa-Nesreen ZH, Edris AM, et al, Abd El-Kader-Hanaa A, et al. and Shaltout FA, et al. [18-30]. The result disagrees with that reported by Mohamed (1988) [31] who recorded that *Salmonellae* could be detected out of 100 examined luncheon samples and Mohamed K [32] who recorded that *Salmonellae* could be detected out of 10 examined luncheon samples (10%).

The results presented in table 2 revealed that *Salmonellae* were present in 10% of the examined samples of fresh sausage. Similar results were recorded by Edris AM, et al. [28]. Nearly similar results were obtained by Ouf-Jehan M, Eleiwa-Nesreen ZH and Mohamed FA [26,27,33] with an incidence of (8%) for each of them, Cabedo L, et al. (11.1) and El-Kader HA, et al. (13.3) [34,29]. While higher results were reported by Banks JG, et al. [35] (55 %) and Shaltout FA, et al. (16%) [30]. Lower results obtained by Zaki EMS (5%) and Ahmed AAH, et al. (5 %) [36,37]. Some investigators failed to detect *Salmonellae* in meat products as Vazgecer B, et al. and Ismail AH [38,39].

It's clear from the results reported in table 2 that 6.7% of the examined samples of frozen packed minced meat were positive for *Salmonellae*. This result agrees with that of Edris AM, et al. and El-Kader HA, et al. [28,29].

Nearly similar results were obtained by Abd El-Aziz AS, et al. (5%), and Shaltout FA (6%) [25,40]. While lower results were recorded by Roberts TA, et al. and Abd El-Atty NS, et al. [41,42] as (2%) for each of them. On the other hands, higher results were reported by Molla B, et al. (32.7%), Ejeta G, et al. (14.4%), Mohamed K (40%), AL-Jobori KM, et al. (36%), and Shaltout FA, et al. (40%) [43,44,32,7,30].

Salmonellae were present in 13.3 % of the examined samples of frozen meat (Table 2). Nearly similar results were recorded by El-Kader HA, et al. [29] (10%), but higher results reported by AL-Jobori KM, et al. [7] (40%) and Mahmood NR, et al. [45] who isolated *Salmonellae* spp. at a rate of 24.76% and Elsayed MS, et al. (18%) [46].

From the results recorded in table 2 it's clear that frozen meat had the higher incidence of *Salmonellae* contamination followed by fresh sausage and frozen packed minced meat while *Salmonellae* could not be detected in luncheon.

The absence of *Salmonellae* in luncheon meat may be due to the addition of food additives such as spices and preservatives, which have

Table 2: Incidence of *Salmonellae* in the examined meat products and frozen meat samples.

Samples	No. of samples	Positive	%
Luncheon (beef)	30	0	0
Fresh sausage	30	3	10
Frozen packed minced meat	30	2	6.7
Frozen meat	30	4	13.3
Total	120	9	7.5

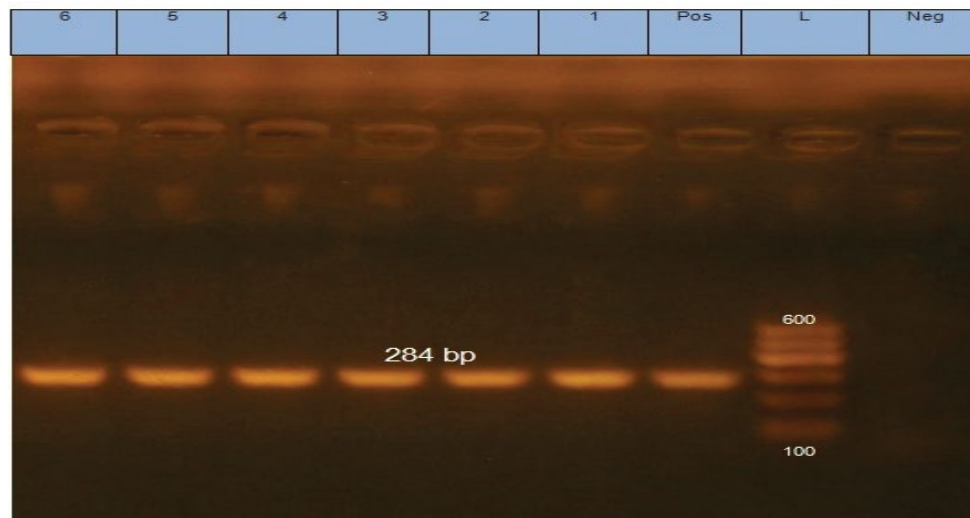


Figure 1: Agarose gel electrophoresis 2% showing PCR products of *invA* gene (284 bp) Specific for characterization of all *Salmonella* species. Lanes (1) to (6): Positive *Salmonella* species for *invA* gene.

Lane L: Molecular marker DNA ladder (100 bp) (cat. No. 239035) supplied from QIAGEN (USA)

Lane Pos: Control positive *Salmonella* strain for *invA* gene

Lane Neg: Control negative

Lane 1: *Salmonella typhi*

Lane 2: *Salmonella typhimurium*

Lane 3: *Salmonella enteritidis*

Lane 4: *Salmonella paratyphi A*

Lane 5: *Salmonella papuana*

Lane 6: *Salmonella vircho*

an antimicrobial activity and inhibit survival and multiplication of micro-organisms [47]. This also may be attributed to the exposure to high temperature during processing and cooking procedures.

High incidence of *Salmonellae* in fresh sausage may be due to faults in certain practices of slaughtering and handling processes such as the use of contaminated knives, tools, rags, saws, boards, etc., as well as unhygienic slaughtering, dressing, washing, transporting, handling and cutting in abattoirs and butcher shops that affect the incidence of *Salmonellae*. High incidence of *Salmonellae* in fresh sausage may be due to the fact that this product is made from raw meat in addition to natural casing which is often used in their manufacture and may be act as an important source for *Salmonella* [48].

The high incidence of *Salmonellae* in frozen packed minced meat may be due to cutting and contamination of meat besides the increase in its water and oxygen contents as well as contamination from grinders, air, packaging materials and hands of the workers. Temperature rise (2-4°C) during grinding could also increase the incidence of *Salmonella* organisms [49].

The incidence of *Salmonellae* in frozen meat may be originated through a different infected touch of food handlers and butchers, improper clothing, fecal hand contamination, may be through rodent and other insects or unhygienic practice in slaughter house area. There is a risk of chances for developing Salmonellosis is major due to survival of *Salmonella* at very low temperature too. They may come in different serotypes *S. enteritidis*, *S. typhi*, *S. typhimurium*, etc. [50].

Serotyping of the isolated *Salmonellae*

From the result recorded in table 3, it is clear that 3 *Salmonella* serovars were identified from fresh sausage samples, 1 (3.3 %) strains as *S. typhi*, 1 (3.3 %) strains as *S. typhimurium* and 1 (3.3%) strain as *S. enteritidis*.

Similar results were obtained by Abd EL-Kader-Hanaa, et al. [29] where they could isolate *S. typhi* and *S. typhimurium* with a percentage of 3.3 % for each strain but higher results were recorded for *S. enteritidis* with a percentage of 6.7%.

Nearly similar results were obtained by Rao NM, et al. [51] where they could isolate *S. typhimurium* and *S. enteritidis* with a percentage of 2.5 % for each strain, Edris AM, et al. [28] where they could isolate *S. typhi*, *S. typhimurium* and *S. enteritidis* with a percentage of 4%, 4% and 2% respectively and Shaltout FA, et al. [30] where they could detect *S. enteritidis* (4%).

From the result recorded in table 4 it is clear that 2 *Salmonellae* serovars were identified in the examined frozen packed minced meat samples and identified as 1 (3.3%) strain as *S. typhimurium* and 1 (3.3%) strain as *S. typhi*.

This result agrees with that obtained by El-Kader HA, et al. [29] where they recorded that the incidence of *S. typhimurium* in the examined frozen packed minced meat samples was 3.3 %.

Nearly similar results were obtained by Edris AM, et al. [28] where they could isolate *S. typhi* (2%) and *S. typhimurium* (4%) and Shaltout FA, et al. [30] where they could detect *S. typhi* (4%), but higher results were recorded for *S. typhimurium* (8%).

From the result recorded in table 5 it's clear that 3 *Salmonella* serovars were identified in the examined frozen meat samples and identified as 2 (6.7%) strains as *S. papuana*, 1 (3.3%) strain as *S. paratyphi A* and 1 (3.3%) strain as *S. vircho*. Higher results were reported by Elsayed MS, et al. [46] where they stated that *S. paratyphi A* was detected in 8/18 (44.44%) positive samples.

Identification of *Salmonella* by PCR

The specificity of the oligonucleotide primers were carried out by testing of all *Salmonella* strains with PCR using the primer pairs targeting the *invA* gene (specific for all members of *Salmonella* species).

All the 6 *Salmonella* isolates detected by bacteriological examination were tested by PCR using the same primer pair after selective enrichment media on XLD agar and all *Salmonella* serovars were positive for amplification of 284 bp fragments of *invA* gene as shown in figure 1.

The present study supports the ability of this specific primer set to confirm the isolates as *Salmonella*. Isolates were subjected to *Salmonella* specific gene (*invA*) and were confirmed as *Salmonella* positive by the predicted product of 284 bp DNA fragments. The results obtained in the present study were in accordance with Nagappa K, et al., Abd El-Kader-Hanaa A, et al., and Moustafa NY, et al. [52,29,53] where they could amplify 284 bp of *invA* gene which is specific for demonstration and characterization of the isolated *Salmonella* species by using PCR.

The ability of *Salmonella* specific primers to detect *Salmonella* species rapidly and accurately in the present study is primarily due to the primer sequences that are selected from the gene *invA* of *S.*

Table 3: Serotyping of *Salmonellae* isolated from the examined fresh sausage samples (n=30).

<i>Salmonella</i> serovars	No.	%*	%**
<i>S. typhi</i>	1	33.3	3.3
<i>S. typhimurium</i>	1	33.3	3.3
<i>S. enteritidis</i>	1	33.3	3.3

%*=percentage collected from only positive *Salmonella* samples
%**=percentage collected from total examined samples

Table 4: Serotyping of *Salmonellae* isolated from the examined frozen packed minced meat samples (no=30).

<i>Salmonella</i> serovars	No.	%*	%**
<i>S. typhi</i>	1	50	3.3
<i>S. typhimurium</i>	1	50	3.3

%*=percentage collected from only positive *Salmonella* samples
%**=percentage collected from total examined samples

Table 5: Serotyping of *Salmonellae* isolated from the examined frozen meat samples (n=30).

<i>Salmonella</i> serovars	No.	%*	%**
<i>S. papuana</i>	2	50	6.7
<i>S. paratyphi A</i>	1	25	3.3
<i>S. vircho</i>	1	25	3.3

%*=percentage collected from only positive *Salmonella* samples
%**=percentage collected from total examined samples

typhimurium as reported by Darwin KH, et al., Nucera DM, et al., and Craciunas C, et al. [54-56].

Culture techniques are universally recognized as the standard methods for the detection of bacterial pathogens, such as *Salmonella* in food stuffs [57]. These techniques generally take longer time [58] and are less sensitive compared to PCR based methods [55,56]. The use of *invA* gene specific PCR method in most diagnostic and research laboratories is possible and through the molecular basis of *Salmonella* identification techniques, this method is the simplest and less expensive.

Conclusion

In conclusion, PCR is a rapid and specific method for the detection of the virulent strains of *Salmonellae* in meat products and frozen meat samples. It gives the ability to detect *Salmonella* cells and identify the virulence of the isolated strains within a little time and PCR was demonstrated to be accurate methods for *S. enterica* identification.

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