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Molecular Characterization of Different *Salmonella Enterica* Serotypes Isolated From Frozen Meat in Minoufiya Governorate

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ABSTRACT

Salmonella is one of the most important causative agents of food poisoning and gastroenteritis in humans. This study spot highlights on isolation, identification and molecular characterization of salmonella serovars from imported frozen meat using the convential and modern molecular tools**.** Methods: A cross-sectional study was carried out on 100 samples of frozen meat collected from different supermarkets from Minufiya governorate, Egypt. Results: The prevalence of Salmonella were 6%. Serotyping of the obtained salmonella isolated revealed that *Salmonella enteritidis*, *Salmonella typhimurium* and *Salmonella .paratyphi* were the prevalent serotypes in the examined samples. *S. typhimurium*, only 3 samples (3%) ,*Salmonella enteritidis* was isolated from only 2 sample (2%) and *S.paratyphi* only 1 samples (1%). The application of conventional PCR for the six obtained isolates of Salmonella serotypes using universal gene (*invA*) was effective tool for identification and genotypic of pathogenic *Salmonella serotypes.* Conclusions: This study concluded that Salmonella is among the most important food borne pathogens worldwide contaminating a wide range of animal products including meat products. Also indicated that the cPCR was specific and rapid method for identification and genotyping of pathogenic salmonella serotypes.

Keywords: *S. enteritidis*, *S. typhimurium*, Food poisoning, *invA* gene

INTRODUCTION

Meat is a suitable media for growth of different micro-organisms such as Salmonella , Echerechia coli and others pathogenic microorganism . Among food-borne diseases of animal origin, Salmonellosis is considered as one of the main causes of bacterial gastroenteritis in humans (Otero, Garci, & Moreno, 1998). Salmonella is a life-threatening bacterium and it is a cause of food-borne bacterial illnesses in humans. Salmonella is listed as the second predominant bacterial cause of foodborne gastroenteritis worldwide. Salmonella serotypes can grow and survive in many different foods products which transmitted through the ingestion of contaminated foods with Salmonella (Mead *et al.*, 1999).

Salmonella is a gram-negative, non-spore forming rod and facultative anaerobe of the family Enterobacteriaceae that have the ability to ferment glucose. Most salmonella strains are motile with peritrichous flagella and can reduce nitrate to nitrite (Grimont, Grimont, & Bouvet, 2000).

Different Salmonella serotypes are responsible for most cases of gastroenteritis, enteric fever, septicemia, and are capable of surviving outside their host for various periods of time (Duffy *et*

al., 2004). 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 phenotypic identification methods of salmonella species was basically depend on culturing followed by morphological and biochemical characterization (Böhme *et al.*, 2012). Recently, modern and advanced molecular techniques have been developed for detection of foodborne microbes depending on nucleic acid amplification such as cPCR which is a quick, sensitive and specific tool for detection of many organism of the genus Enterobacteriaceae (Mckillip and Drake, 2004)

MATERIALS AND METHODS

Sample collection and processing

A total of 100 frozen meat samples were randomly collected from different supermarkets in Minufiya governorate, Egypt. Samples were collected aseptically and transferred for further bacteriological examination at the bacteriology labs, Faculty of Veterinary Medicine, University of Sadat City, Minufiya, Egypt. Samples were then cultivated in peptone water (pre enrichment); one ml of pre enriched broth was transferred aseptically to 10 ml of tetrathionate broth then incubated at 37°C for 24 hours, a loopful of enriched broth was streaked onto plates of Xylose Lysine Desoxycholate agar (XLD agar). Then inoculated plates were incubated at 37^0C for 24 hours. The suspected isolates were identified biochemically according to (Quinn *et al.*, 2002; Bendanarski, 2006; Murray *et al.*, 2009 and England, 2014) and serologically according to Kauffmann white scheme. Typical Salmonella colonies were examined for their size, colour, consistency, shape and microscopic examination after Gram's staining. For the conformation of Salmonella, biochemical reactions are very important for serotyping the isolates. In the present study, all the 6 isolates were subjected to biochemical characteristics on the basis of IMViC reaction, gas production and sugar fermentation as described by (Andrews *et al.*, 1998) .

Serotyping of Salmonella isolates

The isolates that were preliminarily identified biochemically as Salmonella were subjected to serological identification and carried out according to modified Kauffman- white scheme as described by WHOCC – Salm. (2007) as follow:

Suspected isolates were cultured on T.S.I. and incubated at 37°C for 24 h. A loopful was homogenized in a drop of physiological saline on slide so as to exclude rough strain which showed auto agglutination. Only smooth strain which showed homogenous suspension are tested further by using polyvalent **"O" and "H" antisera**. Agglutination usually occurred within 30 – 60 seconds after mixing the bacteria with antiserum. Cultures which showed agglutination with corresponding polyvalent **"O" and** polyvalent **"H" antisera.** Are tested with each of the **"O"** grouping sera and then with the respective mono- specific **"O" antisera.**

The same procedure is applied to **"H" (**phase 1 and phase 2) **antisera.** Both phases (H1 and H2) were determined. In all agglutination tests only strong rapid agglutinin are considered as positive. The final decision of typing is made with the help of **Kauffman- white scheme.**

Molecular characterization of Salmonella isolates

Extraction and purification of DNA

One milliliter of freshly enriched *Salmonella* culture was transferred to a micro-centrifuge tube with a capacity of 1.5 mL. The cell suspension was centrifuged for 10 minutes at 14,000 × g. The pellet was resuspended in 300 µL of DNase-RNase-free distilled water and centrifuged at 14,000 × g for 5 minutes. The supernatant was carefully discarded and the pellet was resuspended in 200 µL of DNase-RNase-free distilled water, incubated for 15 minutes at 100°C and immediately chilled on ice, then centrifuged for 5 minutes at 14,000 × g at 4°C. An aliquot of 5 µL of the supernatant was used as the template DNA in the PCR.

Conventional PCR procedure

The isolated Salmonella strains were detected by conventional PCR for the presence of *invA* gene.

Targeted gene and its primer sequences used in the amplification studies are summarized in (Table 1)

Table 1 : Oligonucleotide primer sequence and amplified PCR product for *Salmonella* virulence genes used in PCR.

Target gene	Sequence	Amplified product	Reference
invA	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	284 bp	**Oliveira *et al*., 2003**

PCR ampilification cycling of the gene was applied with the temperature and time conditions of primer during cPCR that are shown in (Table).

Table 2 : Cycling conditions of the primer during cPCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
invA	94˚C / 5 min	94˚C / 30 sec.	55˚C / 40 sec.	72˚C / 30 sec	35	72˚C / 7 min.

The amplification was carried out in 50 µL reaction PCR tubes containing 5 µL master mix (10 ×, Fermentas, Leon-Rot, Germany), 5 µL of 20 Mm dTNPs mix, 0.15 µL of Taq polymerase (5 U/L µL, Fermentas, Leon-Rot, Germany), 1 µL of 0.1 mM forward and reverse primers, and 1 µL of DNA template. PCR products obtained were subjected to horizontal gel electrophoresis in 1.5% agarose, and the size of the amplicon was determined by comparing it with the DNA marker.

RESULT

Prevalence of Salmonella species from frozen meat samples in Minufiya governorate

The results in table3, revealed that the prevalence of salmonella species in frozen meat in three different area in Minufiya governorate ; Tala, Shibin and Sadat City were 7.5%, 3.33% and 6.66% respectively. While the overall prevalence rate from all collected samples (100) was 6%.

Table 3 : Overall prevalence rate of Salmonella in examined frozen meat samples.

City	No samples	No of positive samples	%*
Tala	40	3	7.5
Shibin	30	1	3.33
Sadat	30	2	6.66
Total	100	6	6

* percentages were calculated according to examined samples of each city.

Phenotypic and Biochemical identification of Salmonella species obtained from frozen meat samples

Suspected colonies were identified by Gram staining which appeared as Gram-negative short rods, non-capsulated and non- spore forming, also biochemical test oxidase reaction was done . Both Gram-negative and oxidase-negative isolates were subculture onto XLD, SS agar medium, at which Salmonella colonies were pink with a black center with a lightly transparent zone and colorless with black centers respectively**.** Regarding to the biochemical identification of Salmonella All tested isolates were confirmed using different biochemical tests as in **(**Table

Table 4: Results of biochemical tests used for identification of Salmonella isolates.

Biochemical test	Results
Oxidase	pale colour (-ve)
Citrate	Blue colour (+ve)
Urease	yellow colour (-ve)
Reaction on TSI medium	K/A/G/ H2S

Serotyping of Salmonella isolates from frozen meat samples:

The data presented in table (5) showed that, the serotyping of Salmonella spp. from the examined frozen meat samples were mainly *S. enteritidis* and *S. typhimurium* and *S. paratyphi*. *S. enteritidis* was isolated from only 2 sample (2%), while in case of *S. Typhimurium*, only 3samples (3%) and *S.para typhi* in only 1samples (1%).

Table 5: Incidence and serologically identification of Salmonella spp.

Salmonella	No	%
S.typhimurium	3	50
S.enterditis	2	33.33
S.para typhi	1	16.66

Molecular detection of salmonella serovars using *invA* gene

The results revealed that *invA* gene was detected in 3 isolates (50 %) of tested salmonella isolates by PCR reaction.

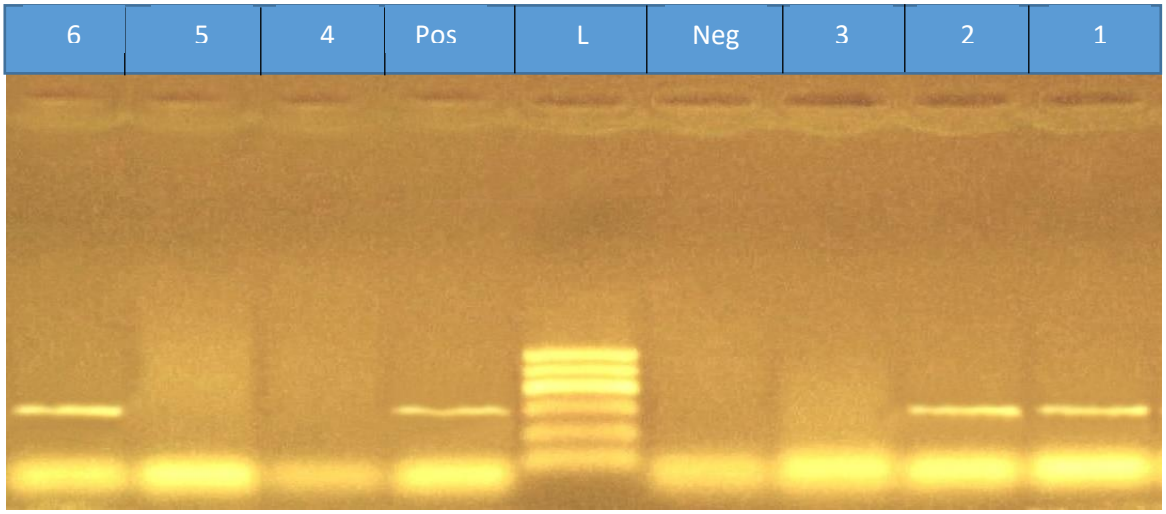

Fig. 1 : 1.5% Agarose gel electrophoresis of PCR product of *inv*A gene at 248pb of *Salmonella*. P : for positive control, "Neg"; Negative control ; Lane L (100-600bp marker); Lanes 1,2, 6 (3 Positive) at 284bp , Lanes 3,4,5 (3 Negative) at 284bp.

DISCUSSION

Salmonella is considered one of the frequently pathogenic bacterium incriminated in many food poisoning outbreaks (Gouws *et al.*, 1998). Its prevalence was worldwide distributed and constitute potential public health hazard (Erdem *et al.*, 2005).

Meat is considered the main reservoir of Salmonellae as well as improper processing, evisceration, backing, insufficient cooking, all are implicated in increased level of bacterial contamination of meat products in particular poultry meat products (Zhang *et al.*, 2001).

In the present study, out of 100 frozen meat samples examined, six samples (6 %) were found to be contaminated with *Salmonella*. These was in consonant with other studies such as (White *et al.*, 2001;Abd-Allah, 2003; (Ghafir *et al.*, 2005) Anon *et al.*, (2006) Anonymous *et al.*, (2008) reported (2%), (7.5%), (4.1%), (3.6%) and (1%) respectively. While, higher prevalence rates were obtained by (Tolba, 1994); Abd EL-Aziz *et al.*, 1996; Mohamed *et al.*, 1998) (20%), (Ejeta *et al.*, 2004)(25%),

(Zaidi *et al.,* 2006)(54%), moffat(McCuddin *et al.,* 2006)(31%). Concerning to the serotyping results, *S. enteritidis* , *S. typhimurium* and *S.para typhi* were the prevalent serotypes. Moreover, *S. enteritidis* was isolated from 2 sample (2%), while *S. Typhimurium*, 3samples (3%) and *S.paratyphi* only 1samples (1%) . This was supported by Zhao *et al*., (2001) who recovered S.*Typhimurium* in 5 samples out of 14 isolates as well as (Ramya *et al.,* 2012) who reported that S.*Typhimurium* was the most predominant in chicken and beef. Furthermore, Margarita *et al*., (2017) demonstrated that S. Typhimurium was found in minced meat. This is in contact with (Foley and Lynne, 2008) mentioned that *S. typhimurium* and *S. enteritidis* were the predominant serotypes of *Salmonella* ssociated with human salmonellosis.

In this study six salmonella isolates were subjected for genotypic identification of *S. enterica* using designed primers of *invA* gene by cPCR and the results revealed that *invA* gene wae detected in 3 (50 %). These findings were similar to (Siala *et al.,* 2017)who reported that *invA* DNA was detected in *Salmonella* isolates from food samples by qPCR . In addition to, (Hassanein *et al.,* 2011) identified two serotypes of salmonella (Salmonella entrica subsp. entrica serovar Enteritidis and Salmonella entrica subsp. entrica serovar Kentucky) with multiplex PCR from chicken leg and minced meat.

CONCLUSSION

The prevalence of Salmonella from frozen meat showed the importance of maintaining good biosecurity in production, proper processing and handling of meat. The role of meat in the persistence and transmission of Salmonella infection and the reduction of meat contamination should be studied in detail. Additionally, the high occurrence of *S. paratyphi* among salmonellae serotypes from frozem meat assumed its potential public health for human infection with Typhoid fever. Further studies are needed to provide an accurate knowledge about prevalence of salmonellae among meat & meat products and hygienic measures to prevent the dissemination of infection.

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