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Microbiological and Molecular Characterization of *Salmonella* Species in Frozen Meat and Organs Imported Into Egypt: A Public Health Importance

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Abstract **Original Research Article**

The aims of this study are to isolate and microbiologically characterize *Salmonella* spp. in frozen meat and organs imported into Egypt and to evaluate the potential virulence of the isolates by detecting the presence of the enterotoxin (*stn*) gene. A total of 1363 frozen imported meat and organ samples (962 meat, 281 liver, 69 heart, 51 kidney) were collected from original packets while lots are in their primary destination before market distribution. Isolation and microbiological identification of salmonellae were performed according to ISO 6579/2002; in addition, different *Salmonella* isolates were tested for harboring enterotoxin gene (*stn*) using PCR. Results revealed existence of the pathogen in all kinds of examined samples (meat 1.7%, liver 0.36%, heart 1.5% and kidney 3.9%); moreover, enterotoxin (*stn*) gene was detected in 85% of the isolates. The occurrence of these pathogens in relation to the country of origin was presented. Statistical analysis of the results demonstrated significant difference in prevalence rate of *Salmonella* species among the potential meat and organ exporters to Egypt; where India showed the highest rate (3.39%) followed by Brazil (1.20%), USA (0.83%) and Australia (0%).

Keywords: Imported frozen meat and organs, *Salmonella* spp., Enterotoxin (*stn*) gene, PCR.

INTRODUCTION

Non-typhoidal *Salmonella* (NTS) constitutes one of the major worldwide zoonotic food-borne pathogens. The disease caused by NTS is mainly manifested by gastroenteritis; however bacteraemia and localization in different organs with chronic sequelae may also occurs [1, 2]. Many pathogenic mechanisms are involved in NTS-induced diarrhea including production of enterotoxin which is encoded by *stn* gene [3, 4]. The most encountered serotypes in majority of food-borne human salmonellosis are *S.* Typhimurium, *S.* Enteritidis, *S.* Heidelberg, and *S.* Newport [5-8].

Globally, the annual health burden of gastroenteritis attributed to NTS has been estimated to be 94 million cases with 155, 000 deaths [1]. Moreover, there has been a substantial economic impact related to direct and indirect costs of health care and lower productivity [9, 10]. The disease also has a negative effect on food-industry/business as a result of recalls and loss of market share [11].

Poultry, eggs and pork are the most identified global sources of NTS for humans; however, during the last two decades, beef was incriminated in several large outbreaks in the USA and in many European countries [12, 13]. Contamination of animal meat and organs usually results from infected animals used in food production or through cross-contamination during slaughtering operations [14-18].

Egypt, like most of developing countries, depends mainly on importation of frozen meat and organs to face the continuous increase of human population and shortage of local animal protein. The aims of this study are to isolate and microbiologically characterize *Salmonella* spp. in frozen meat and organs imported into Egypt and to evaluate the potential virulence of the isolates by detecting the presence of the *stn* enterotoxin gene using PCR.

MATERIAL AND METHODS

Sample collection

A total of 1363 imported frozen meat and organ samples (962 meat, 281 liver, 69 heart, 51 kidney) were collected from original packets while lots are in their primary destination (Cairo Airport, Port Said Port, Alexandria Port) before market distribution. All samples were transferred refrigerated under aseptic conditions to Food Microbiology Laboratory at Central

Public Health Laboratories (CPHL) for further processing and testing.

Isolation and identification of *Salmonella* spp

Isolation and identification of *Salmonella* spp. were executed according to ISO 6579/2002 [19]. Briefly, the pre-enrichment step in non-selective medium was executed by inoculation of homogenized 25g of each sample in 225 ml buffered peptone water and incubation at 37 °C for 24 h. Enrichment was carried out by inoculation of 0.1 ml aliquots of pre-enrichment broth in the selective Rappaport-Vassiliadis with soya (RVS broth) medium and incubation at 42 °C for 24 h. A loopful from RVS broth was streaked on Xylose lysine deoxycholate agar (XLD agar) then the plates were incubated at 37°C for 24 h. Suspected colonies were biochemically confirmed by triple sugar iron (TSI), lysine decarboxylation iron agar (LIA), urea, indole, and citrate utilization tests. Pure cultures were also serologically tested using slide agglutination test according to White Kauffman scheme [20].

Molecular identification of enterotoxin (*stn*) gene

DNA extraction

Salmonella pure colonies on XLD agar were grown overnight in 5 ml buffer peptone water at 37°C. One ml of culture medium was centrifuged and the pellet was washed twice and then resuspended in 200 µl TE buffer. For cell lysis, the suspension was boiled at 100°C for 10 minutes and then placed in refrigerator for 5 minutes. Finally the mixture was centrifuged at 13,000 xg for 5 minutes and 5 µl of supernatant was used as DNA template in PCR reaction.

PCR and agarose gel electrophoresis

All PCR amplifications were performed in 25-µl reaction mixtures containing 5 µl of each DNA template, 5 µl of 5X PCR Master Mix and 10 pmoles of each *Stn* P1-Forward (5- TTG TGT CGC TAT CAC TGG CAA CC -3) and *Stn* M13-Reverse (5- ATT CGT AAC CCG CTC TCG TCC -3) primer supplied by Metabion, Germany. This oligonucleotide set targets the enterotoxin gene (*stn*) of *Salmonella* organisms [21]. The following thermocycle profile was used: an initial 3-min denaturation at 94°C, 25 cycles (each consisting of a 1-min denaturation at 94°C, a 1-min annealing at 59°C, and a 1-min extension at 72°C) and a 10-min final extension at 72°C. A positive and negative (No DNA) controls were included in each run. Aliquots of amplified PCR products were analyzed on 1.5% agarose gel by electrophoresis and seen under UV with ethidium bromide. Products of 617 bp indicate positive results.

Statistical Analysis

The analysis of the results was conducted using the computer software SPSS (SPSS Inc., Chicago, IL, USA; version 16.0). The significance difference of *Salmonella* prevalence among potential countries, from which meat and organs were imported, was evaluated by chi-square test. Post hoc test for determination of source of difference was performed [22]. The *p*-value < 0.05 was considered statistically significant.

RESULTS

Results of this study are presented in tables 1 to 3 and Figure 1. The general chi-square test showed a significant difference between the prevalence of isolated *Salmonella* species among the potential meat and organ exporters to Egypt (Brazil, India, Australia and USA). The post hoc test with the adjusted *p*-value of 0.00625 (level of significance), due to multiple testing, showed that India had the highest significant prevalence of *Salmonella* (3.39%) followed by Brazil (1.20%), USA (0.83%) and Australia (0%).

Table-1: Occurrence of *Salmonella* spp. in frozen meat and organ samples imported into Egypt

Country of Origin	Sample								Total tested	Total +ve (%)
	Meat		Liver		Heart		Kidney			
	No tested	+ve (%)	No tested	+ve (%)	No tested	+ve (%)	No tested	+ve (%)		
Brazil	552	6 (1.1)	3	0 (0)	15	0 (0)	15	1 (6.7)	585	7 (1.2)
India	293	10 (3.4)	NA	-	2	0 (0)	NA	-	295	10 (3.4)
Australia	62	0 (0)	12	0 (0)	NA	-	NA	-	74	0 (0)
Canada	21	0 (0)	5	0 (0)	NA	-	NA	-	26	0 (0)
America	18	0 (0)	258	1 (0.39)	51	1 (2)	36	1 (2.8)	363	3 (0.8)
South Africa	9	0 (0)	NA	-	NA	-	NA	-	9	0 (0)
New Zealand	4	0 (0)	3	0 (0)	1	0 (0)	NA	-	8	0 (0)
Argentina	2	0 (0)	NA	-	NA	-	NA	-	2	0 (0)
Netherlands	1	0 (0)	NA	-	NA	-	NA	-	1	0 (0)
Total	962	16 (1.7)	281	1 (0.36)	69	1 (1.5)	51	2 (3.9)	1363	20 (1.5)

NA = Not Available

Table-2: Recovered *Salmonella* serovars from imported frozen meat and organ samples in relation to country of origin

Country of Origin	Sample											
	Meat			Liver			Heart			Kidney		
	No tested	No +ve	Serovar (Isolate no.)	No tested	No +ve	Serovar (Isolate no.)	No tested	No +ve	Serovar (Isolate no.)	No tested	No +ve	Serovar (Isolate no.)
Brazil	552	6	*S.* Typhimurium (3) *S.* Newport (3)	3	0	-	15	0	-	15	1	*S.* Typhimurium (1)
India	293	10	*S.* Typhimurium (5) *S.* Newport (1) *S.* Hadar (1) *S.* Infantis (1) *S.* Muenchen (1) *S.* Winston (1)	NA	-	-	2	0	-	NA	-	-
Australia	62	0	-	12	0	-	NA	-	-	NA	-	-
Canada	21	0	-	5	0	-	NA	-	-	NA	-	-
America	18	0	-	258	1	*S.* Typhimurium (1)	51	1	*S.* Typhimurium (1)	36	1	*S.* Duisburg (1)
South Africa	9	0	-	NA	-	-	NA	-	-	NA	-	-
New Zealand	4	0	-	3	0	-	1	0	-	NA	-	-
Argentina	2	0	-	NA	-	-	NA	-	-	NA	-	-
Netherlands	1	0	-	NA	-	-	NA	-	-	NA	-	-

NA = Not Available

Table-3: Occurrence of *stn* gene in recovered *Salmonella* isolates from food samples

Recorded serotypes	Total no. of isolates	No. positive for *stn* gene (%)
S. Typhimurium	11	10 (90.9)
S. Newport	4	3 (75)
S. Hadar	1	1 (100)
S. Infantis	1	1 (100)
S. Muenchen	1	1 (100)
S. Winston	1	0 (0)
S. Duisburg	1	1 (100)
Total	20	17 (85)

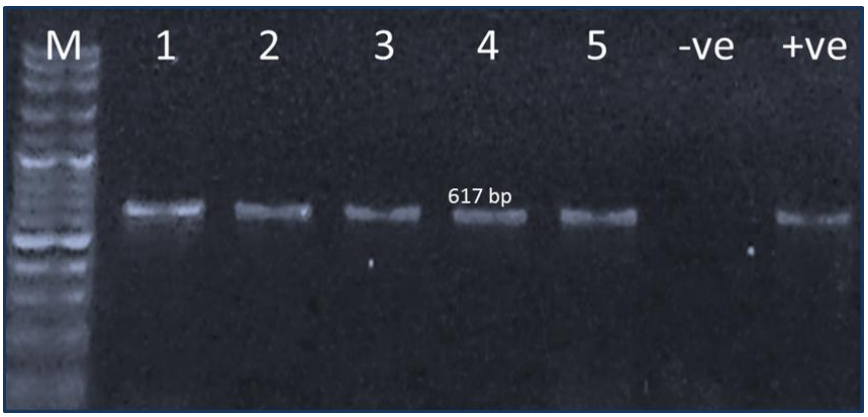

Fig-1: Agarose gel electrophoresis of PCR products obtained by amplification of *stn* gene in *Salmonella* isolates from imported frozen meat and organs. Lane M, molecular size marker (100bp DNA Ladder). Lanes 1 to 5, positive samples. Lanes –ve and +ve represent negative and positive control respectively

DISCUSSION

In spite of advances achieved in food science and technology, food poisoning remains a major global health problem [23, 24]. Non-typhoidal salmonellosis is one of the most prevalent foodborne illnesses, which occurs mainly due to consumption of contaminated animal products including imported frozen cattle and buffalo meat and organs [1, 2, 12-18]. Contamination of such food arises from infected animals used in food production or through cross-contamination of carcasses during slaughtering operations. Hides, feces, water, intestinal contents, lymph nodes, processing equipment, and humans are probable sources of contamination, which occur during skin removal and evisceration [25-27]. Indeed, the type of *Salmonella* and extent of contamination of meat and organs reflect the hygienic and safety measures undertaken from the early beginning of food chain in the farm till storage of the final product.

To the best of our knowledge, no work was executed to investigate the occurrence and prevalence of *Salmonella* spp. in frozen meat and organs imported into Egypt from their original packets while lots are in their primary destination before market distribution. Therefore, the results of this study reflect the actual status of contamination in the country of origin (exporting country) and this will in turn help the importation authorities to take correct decisions.

The prevalence of *Salmonella* spp. in imported meat from different countries is expected to be variable. This is attributable to the differences in number, type, and amount of sample as well as method of detection and the principals of result interpretation.

Tables 1 & 2 show that *Salmonella* spp. were recovered from all kinds of samples (meat, liver, heart, and kidney) and only samples imported from India, Brazil, and America showed evidence of contamination. The statistical analysis of our results showed a significant difference between the prevalence of isolated *Salmonella* species among the potential meat and organ exporters to Egypt (Brazil, India, Australia and USA). It is showed that India had the highest significant prevalence of *Salmonella* (3.39%) followed by Brazil (1.20%), USA (0.83%) and Australia (0%). This result is supported by a previous study performed in India [28] which concluded that most of slaughter houses in India are laden with the pathogen and contamination of frozen buffalo meat cuts in slaughter houses occur during slaughtering, carcass washing, chilling, deboning, packing and freezing. This study also revealed that *Salmonella* can survive freezing conditions. Therefore, importation of frozen buffalo meat from India may introduce a foodborne pathogen into the country.

Table 2 displays different isolated *Salmonella* serovars. It is shown that *S.* Typhimurium is the predominant detected strain. This result is in agreement with a previous study conducted in Egypt which recorded that the most prevalent serotype detected in frozen meat, liver and heart was *S.* Typhimurium. This serotype is the most common serotype associated with human salmonellosis [29].

The mechanisms involved in *Salmonella* pathogenicity are still not well understood. It has been shown that *Salmonella*-induced diarrhea is mediated in part by enterotoxins which are encoded by *stn* gene [3, 4]. *Salmonella* serotypes which are associated with gastroenteritis and diarrhea in man and animals have been shown to produce enterotoxin [30]. Figure 1 and Table 3 show the results of PCR detection and prevalence of *stn* gene among the bacterial isolates. Seventeen out of 20 (85%) of isolates were documented to harbor *stn* gene. Noteworthy, different serotypes from different counties were demonstrated to carry this virulence gene. This result suggests that *stn* gene is widely distributed among *Salmonella*e irrespective of their serovars and source of isolation; therefore, this gene could be considered a suitable target for the detection of *Salmonella* spp.

In conclusion, this study revealed that imported frozen meat and organs may constitute a foodborne illness hazard due to contamination with enterotoxin producing *Salmonella* spp. In addition, our results demonstrated that India has the highest significant prevalence of *Salmonella* followed by Brazil, USA and Australia. This is a flashing warning signal for the concerned authorities on the hygienic measures employed in slaughter houses in countries from which frozen meat and organs are imported.

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