Detection and identification of Salmonella species in minced beef and chicken meats by using Multiplex PCR in Assiut city

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Abstract

The present study was undertaken to determine the incidence and distribution of Salmonella species in selected meat and chicken products purchased from retail supermarkets in Assiut, Egypt. A total of 75 samples including 25 samples each of minced frozen beef, frozen chicken legs and frozen chicken fillets were collected over a 7-month period between January and July 2009 and examined for the presence of Salmonella species. In addition, 28 children stool cultures were collected from hospitalized children resident in Pediatric University Hospital with diarrhea or fever. Out of the total 75 meat samples examined, Salmonella was detected in 5 (20%) of minced frozen beef, 9 (36%) of frozen chicken leg and 13 (52%) of frozen chicken fillet samples analyzed. Regarding the examined 28 children stool cultures, 3 (10.71 %) were found Salmonella positive. Of the total 30 Salmonella positive samples from all examined samples, five selected Salmonella isolates were further identified using multiplex PCR (m-PCR). Two serovars were the dominant serovar identified was *Salmonella entrica subsp. entrica serovar Enteritidis* (2 chicken leg isolates and 2 chicken breast fillets) followed by *Salmonella entrica subsp. entrica serovar Kentucky* (one minced beef isolate). The public health hazards of Salmonella were discussed and the suggestive measures to protect the consumers and improve the quality of meat and chicken products were given.

Keywords: Salmonellosis, Minced beef, Chicken legs, Chicken breast fillet, Incidence, m-PCR, Food borne illness, Zoonosis.

Introduction

Food-borne illness is a major international health problem (1,2). Each year, millions of persons become ill from food-borne diseases, though many cases are not reported (3).

Salmonella is one of the most important pathogenic genera implicated in food-borne bacterial outbreaks and diseases (1,4). Salmonella infections are worldwide and constitute an important public health problem in many parts of the world (5,6). There are several transmission routes for salmonellosis, but the majority of human infections are derived from the consumption of contaminated foods especially those of animal origin (7). A variety of food products, especially poultry and other types of meat products including minced meats, are the most important sources of human Salmonella infection (8,9,10,11). International outbreaks caused by a range of foodstuffs contaminated with different Salmonella serotypes have been reported (12,13,14). Most serotypes of *S. enterica* cause self-limiting gastroenteritis characterized by diarrhoea, abdominal cramps and sometimes vomiting and fever. Two serotypes, *S. typhi* and *S. paratyphi*, causes systemic fevers that can be fatal without therapy. Typhoid and paratyphoid fevers are associated with poor sanitation and are primarily diseases of developing countries (15).

In humans, Salmonella are the cause of two diseases called salmonellosis: enteric fever (typhoid), resulting from bacterial invasion of the bloodstream, and acute gastroenteritis, resulting from a food-borne infection/intoxication (16).

Outbreaks have been associated with a wide variety of foods. Dominant serotypes from clinical cases vary with geographical region: for example, S. enteritidis is the most common in Europe, while S. typhimurium is the most common in Oceania (17).

In many countries, poultry meat products continue to be a major cause of human enteritis (18,19) and outbreaks are reported regularly involving Salmonella species (20,21). Moreover, Salmonella is the most common food poisoning bacteria associated with refrigerated poultry (22).

The detection and identification of Salmonella spp. is time consuming to the food industry (23). To detect Salmonellae more rapidly, an alternative method to the conventional culture method was evaluated using polymerase chain reaction (PCR). PCR has been

Table-1: Primers used for identification of Salmonellae by multiplex PCR.

Primer	Length(nucleotides)	Primer sequence	Amplicon product (bp)	References
Rfbj-F	24	5'-CCAGCACCAGTTCCAACTTGATAC	663	(28)
Rfbj-R	24	5'-GGCTTCCGGCTTTATTGGTAAGCA		
Flic-F	24	5'-ATAGCCATCTTTACCAGTTCCCCC	183	(28)
Flic-R	24	5'-GCTGCAACTGTTACAGGATATGCC		
Fljb-F	24	5'-ACGATTGGTACGGCTTCTGTAACC	526	(28)
Fljb-R	24	5'-TACCGTCGATAGTAACGACTTCGG		
Sdf-I-R	23	5'-TGTGTTTTATCTGATGCAAGAGG-3'	293	(29)
Sdf-I-F	23	5'-CGTTCTTCTGGTACTTACGATGAC-3'		

demonstrated to be a very specific and sensitive method for the detection of Salmonellae (24). In the last decade, there has been a wide interest in the use of the multiplex PCR (mPCR) technique. mPCR approaches have been largely applied to detect different species of several microbial niches, to differentiate closely related species and to recognize single species (25).

Therefore, the goal of this study was to determine the incidence of Salmonella spp. in minced beef, chicken meats and human in Assiut (Egypt) and to use multiplex PCR (m-PCR) for their identification.

Material and Methods

Collection of samples: A total of 75 random samples of meat and chicken products (25 samples each of minced frozen beef, frozen chicken legs, and frozen chicken breast fillets without skin) were collected from different retail supermarkets and groceries in Assiut city. The minced frozen beef samples were collected in presterilized plastic bags while, frozen chicken meat samples obtained in their casing as sold to the consumers. The collected samples were transferred directly to the laboratory in an ice box with a minimum of delay, where they were prepared for bacteriological examination.

Preparation of samples: At the laboratory, frozen samples were thawed by overnight refrigeration. Each sample was aseptically and carefully freed from its casings and mixed thoroughly in sterile mortar.

Human samples: To identify the occurrence of Salmonella infections in hospitalized children in Assiut, the cases of study was conducted. The cases were defined as the children, resident in Pediatric Uni. Hospital; Assiut Uni., with diarrhea or fever between January and July 2009. A stool culture examined for Salmonella. The parents of the cases were interviewed, by a single investigator using a standardized questionnaire addressing the family's consumption of, and purchasing and preparation conditions for, various foods such as poultry and beef, and their contacts with people having presented with an episode of diarrhea.

Isolation of Salmonella spp. (26)

Pre-enrichment: Twenty five grams of the examined samples were weighed aseptically into sterile blender container and thoroughly homogenized with 225 ml of sterile lactose broth. The homogenate was incubated at 37°C for 24 h.

Selective Enrichment: 0.1 ml and 1 ml of the incubated pre-enrichment homogenate were transferred to 10 ml Rappaport-Vassiliadis broth (RV) (Oxoid) and Selenite cystine (SC) broth (Difco) as selective enrichment, respectively. RV broth incubated at 42°C for 24 h and SC broth incubated at 37°C for 24 h.

Selective Plating: At the end of the incubation period, a loopful from each of the selective enrichment broths was streaked onto Salmonella-Shigella (SS) agar (BBL), and incubated at 37°C for 24 h. The plates were examined for the presence of typical colonies of Salmonella (transparent colonies with black centers on SS agar).

Confirmation: Smears of suspected colonies were stained with Gram's stain and examined morphologically for staining characters. Presumptive Salmonella colonies were then subjected to initial screening tests using triple sugar iron agar (TSI) slant, lysine iron agar (LIA) slant (Merck), urea broth (Merck) and lysine decarboxylase broth (Oxoid). TSA incubated at 37°C for 24 h and LIA incubated at 37°C for 24-48 h. All biochemical tests were performed at 37°C for 18–24 hrs including citrate utilization, indol production, methyl red, motility, urease and Voges-Proskauer (27).

DNA extraction for multiplex PCR assay

DNA was extracted from selected pure cultures of bacteria that had been enriched at 37°C for 18 h in brain heart infusion (BHI) broth. One ml of the BHI broth was collected in an eppendorf tube, centrifuged at 15000 rpm for 5 min and the supernatant discarded. The cell pellets were resuspended in 1 ml phosphate buffered saline (PBS, Oxoid), centrifuged at 15000 rpm for 5 min and the supernatant discarded. DNA extractions were performed on the cell pellet using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

For the multiplex PCR, four primer pairs were used. All of the primer sequences are shown in Table 1. Multiplex PCR reactions were performed with Techne Cyclogene Thermocycler using a total volume of 25 µl, which was contained within a 0.5 ml thin-walled PCR tube. The optimal amplification reaction mixture contained 12.5 µl 2 × QIAGEN Multiplex PCR Kit

Table-2: Incidence of Salmonella in minced beef, chicken meat and human samples.

Type of samples	No of examined samples	Positive samples	
		No	%
Frozen chicken breast fillets	25	13	52.00%
Frozen chicken legs	25	9	36.00%
Minced frozen meats	25	5	20.00%
Human samples	28	3	10.71 %
Total	103	30	29.13 %

Table-3: Multiplex PCR results of selected Salmonella spp. in minced beef, chicken meat and human samples.

Type of samples	Positive examined samples	mPCR result
Frozen chicken breast fillets	2	S. enteritidis
Frozen chicken legs	2	S. enteritidis
Minced frozen meats	1	S. kentucky
Total	5	

(Qiagen) containing HotStar Taq polymerase, multiplex PCR buffer, dNTP mix and 3 mM MgCl2, a final concentration of 0.4 µM for each primer, 4.5 µl sterile RNase-free water and 2 µl of test or control DNA.

The PCR protocol consisted of the following steps: (i) an initial denaturation step of 2 min at 95°C; (ii) 30 cycles, with 1 cycle consisting of 1 min at 95°C, 1 min at 57°C, and 1 min at 72°C; and (iii) a final elongation step of 10 min at 72°C. The PCR products were electrophoresed in 1% (wt/vol) D-1 agarose (Pronadisa, Madrid, Spain), stained with 2 µg of ethidium bromide (Sigma-Aldrich, Madrid, Spain) per ml, and photographed under UV light. In each PCR run, a nontemplate control was included to detect possible external DNA contamination.

Results and Discussion

The obtained results of this study are summarized in Tables 2 & 3 and Figure 1.

Screening of minced frozen meats for Salmonella

Out of 25 minced frozen meat samples examined, five samples (20.0%) were found to be contaminated with Salmonella (Table 2). Previous studies conducted in Assiut, Egypt, indicated the occurrence of Salmonella in different food animals, meat and meat products (30,31,32). Other investigators could recover the organisms from minced meat samples in variable percentages such as (30) (5%), (33) (12%), (34) (5%), (35) (1.5%), (36) (11.4%), (37) (6.3%), (38) (12.1%) and (32) (6%). Summarized data from several European countries showed that Salmonella prevalence in minced beef ranged from 0.0% to 3.6%, with a mean of 1.1% (39).

Contradictory report had been published on the absence of Salmonella in the examined 30 minced meat samples collected from different localities in Assiut city (31). Likewise, many researchers such as (40,41,42,43), could not detect Salmonella species from samples of minced beef. They concluded that this negative result not indicates the absence of the bacteria, but this result may be due to low sensitivity and specificity of the method used in isolation.

In this study, the identical strain was identified using m-PCR as Salmonella enterica subsp. enterica serovar Kentucky (S. Kentucky).

Salmonella enteritidis has become the predominant strain that causing food poisoning. Investigation of Salmonella enteritidis outbreaks indicate that its emergence is largely related to consumption of poultry or eggs (44).

Food-borne salmonellosis often follows consumption of contaminated animal products, which usually results from infected animals used in food production or from contamination of the carcasses or edible organs (45,46).

The true incidence of salmonellosis both in humans and animals is difficult to evaluate because of lack of an epidemiological surveillance system in place, which is particularly true in developing countries. However, in countries with a reporting system, the number of outbreaks particularly in humans has increased considerably in recent years (47,48). Carrier states of humans are of concern to the food manufacturing and food service industries because of the perceived risk of contamination of food by infected food handlers and the risk of food-borne disease outbreaks (49). Meat processing and packaging at the wholesale or retail levels are likely to contribute to the higher levels of contamination in minced beef compared to beef carcasses. The presence of even small numbers of Salmonella in carcass meat and edible offals may lead to heavy contamination of minced meat and sausage. When

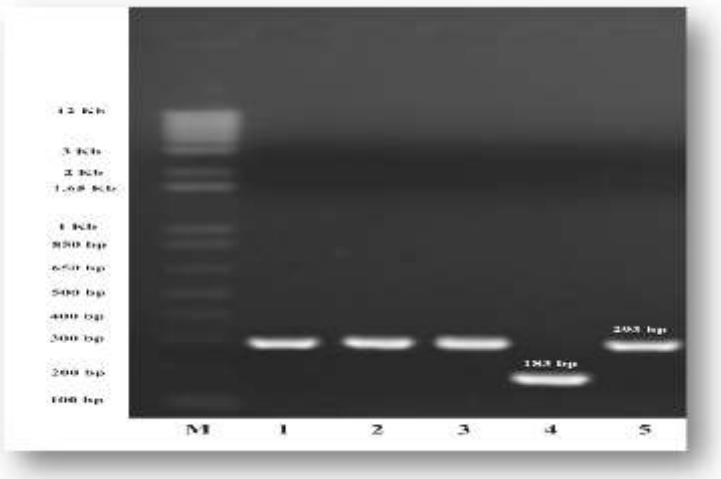

Figure. 1 Agarose gel analysis of multiplex PCR assay results for the isolated Salmonella serotypes indicated above each lane. The sizes of DNA ladder (M) are indicated in base pairs to the left of the gel image.

Lane 1. Chicken fillet (F 2): *Salmonella entrica subsp. entrica serovar Enteritidis.*
Lane 2. Chicken fillet (F 5): *Salmonella entrica subsp. entrica serovar Enteritidis.*
Lane 3. Chicken leg (L 15): *Salmonella entrica subsp. entrica serovar Enteritidis.*
Lane 4. Minced meat (M 1): *Salmonella entrica subsp. entrica serovar Kentucky.*
Lane 5. Chicken leg (L 10): *Salmonella entrica subsp. entrica serovar Enteritidis.*

meat is cut into pieces, more microorganisms are added to the surfaces of exposed tissue. Raw meats, particularly minced meats have very high total counts of microorganisms and Salmonellae are likely to be present in large numbers (30).

During conventional slaughter procedures and further processing to prepare poultry and meats for consumption, microorganisms are introduced into and onto carcasses (50). Prevention of contamination during slaughtering and subsequent processing has therefore been identified as by far the most important factor in safeguarding the microbiological quality of poultry (51). Several gastroenteritis outbreaks have been reported in Jordan in the pervious years, many of which were owing to *S. enterica* as a causative agent as a result of consumption of contaminated food-products (1).

Screening of chicken meats for Salmonella

The findings outlined in Table (2) showed that nine samples (36.00%) of frozen chicken legs and thirteen samples (52.00%) of frozen chicken breast fillets were contaminated with Salmonella.

Chicken products are widely acknowledged to be a significant reservoir for Salmonella. They have frequently been incriminated as a source of Salmonella contamination and consequently thought to be major sources of the pathogen in humans (52,9). Furthermore, one of the commonest causes of Salmonella infection reported in humans has been through the handling of raw poultry carcasses and products, together with the consumption of undercooked poultry meat (53).

Varying incidence rates of Salmonella in chicken and/or chicken parts were reported. (54) found Salmonella in 26.3% of fresh whole chickens, 26.7% of breasts, 14.3% of legs, 0% of drumsticks, 0% of thighs, and 40% of wings. (55) reported Salmonella contamination around 40% for each of wings, legs and giblets. Moreover, Straver et al. (56) pointed out that nineteen fillets (8.6%) were contaminated with Salmonella on chicken breast fillet (chilled raw fillets without skin) collected from five local retail outlets in The Netherlands.

Also, poultry meat was extensively contaminated with Salmonella (40%) in Sao Paulo, Brazil (57). Contradictory report has been published on the absence of Salmonella from the examined 30 chicken quarter samples collected from different localities in Assiut city (31).

Compared to the results of these studies, incidence that we have obtained appears to be quite high. The Salmonella strains detected in our survey were isolated from chicken samples purchased from different supermarkets. Some earlier reports (58,55) indicated lower percentage of Salmonella in chickens bought from butchers' and poultries' shops compared to supermarkets. It is possible that the chicken parts may be contaminated by Salmonella at multiple steps such as production, processing and distribution until retail marketing.

Chicken packaging may be a potential vehicle for introduction of pathogens in retail and in particular for the cross-contamination of Salmonella (58).

In the current study, the identical strains were identified using m-PCR as *Salmonella enterica subsp. enterica serovar entertidis* (*S. entertidis*). The isolation of S. entertidis from chicken carcasses and/or chicken parts has been reported by some other researchers (59,60,61). Worldwide, *S. entertidis* is a clinically prevalent Salmonella serovar, which is associated with the consumption of foods containing eggs or poultry meat from systemically infected chickens (60). In Finland, *S. entertidis* has been the most common serovar in poultry and humans (59).

The identification of *S. enteritidis* and/or *S. kentucky* in meats was applied on DNA isolated from the selected pure cultures of bacteria; because Salmonella is not uniformly distributed throughout contaminated samples (62). Consequently, it is essential to increase the processed quantity of the sample to enhance the probability of detecting such bacterium and to avoid sampling errors.

This finding recommends that PCR could be used to gain a quick and a reliable idea about the status of the sample and the microbiological techniques could be used to further confirm the presence of certain microorganism and/or isolating it.

The m-PCR procedure described here may also be extended for the routine detection and/or identification of *S. enterica*, *S. enterica serotype S. enteritidis* and/or *S. enterica serotype S. typhimurium* from environmental and clinical samples.

Screening of human samples for Salmonella

Results given in Table 2 revealed that Salmonella could be isolated from 3 (10.71%) out of 28 samples of human sources. *Salmonella typhimurium* typically accounts for 20% of human cases (63). Bennet et al. (64) performed faecal cultures from 71 Addis Ababa infants. They reported that Salmonella spp. were found in 12 of 61 hospitalised infants. Also, 1,025 faecal samples from hospitalized diarrhoeal children in and around Calcutta, India were screened for enteropathogens. Four *S. enterica* serotypes were identified in 157 (15.3%) cases as a single pathogen. *S. enterica serotype Typhimurium* was detected in 110 (70%) cases. *S. Seftenberg*, *S. Infantis*, and *S. Virchow* were detected in 28 (17.8%), 14 (8.9%), and 5 (3.2%) cases respectively. *S. Typhimurium* was isolated from 11 (3.2%) non-diarrhoeal control children. All of these children had acute watery diarrhoea, and 5% of them had severe dehydration, 40% had some dehydration, and 55% had no dehydration. Vomiting, fever, and diffused pain in abdomen were the associated presentations of these children (65).

PCR results

For identification of selected Salmonella species using multiplex PCR, four primer sets were used in the same reaction mixture (Table 1). Analyzing the PCR profiles, 4 out of Salmonella isolated strains showed one amplified product (293-bp), (Fig. 1) that is specific for serovar Enteritidis and one strain showed amplification product at (183-bp) that is specific for serovar Kentucky. In the current study, the identical strains were identified using m-PCR as *Salmonella enterica subsp. enterica serovar Entertidis (S. entertidis)* and *Salmonella enterica subsp. enterica serovar Kentucky (S. kentucky).*

From the results of the present study, it could be concluded that Salmonella is widespread in minced frozen beef, frozen chicken legs and fillets samples obtained from retail supermarkets in Assiut, Egypt. It could be a potential vehicle for food-borne infections and implementation of preventive measures and consumer food safety education efforts are needed. Proper cooking of meat and chicken products before consumption and improving personal and meat hygiene in the line of meat production from farm to fork should be adopted to ensure the safety of meat and meat products for human consumption.

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