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Research Article

Real Time and Conventional PCR for Characterization of *Salmonella sp.* from Imported Meat to Egypt

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ABSTRACT

This study was aimed to detect *Salmonella spp.* in imported frozen beef and buffalo meat cuts from India, Brazil, Australia and Newzeland. One hundred frozen meat samples were collected from supermarkets located at Alexandria province and from Alexandria port. *Salmonella* was biochemically identified in 18/100 (18%) samples. Serologically, *S. paratyphi A* and *S. typhi* were detected in 10/18 (55.56%) and 8/18 (44.44%) positive samples, respectively. Using conventional PCR, 18/18 (100%) were confirmed to be pathogenic by specific primer to *rfbS* gene of the pathogenic strains of *Salmonella* responsible for the most food poisoning while all of these pathogenic isolates 18/18 (100 %) were also confirmed by real time PCR amplifying the *invA* gene of *Salmonella spp.*

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INTRODUCTION

Microbial contamination of food is serious problem and trading between countries raises the liability for outbreaks. 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 (WHO, 2007). 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 *Salmonella* are found to be responsible for most of food origin outbreaks (Chemburu et al., 2005). Mainly, many foods are source of food borne outbreaks if these are contaminated with *Salmonella spp.* These foods are: undercooked eggs, poultry, meat, raw milk, dairy products, seafood, chocolate, salad and spices. The reported infective dose of about 15–20 microorganisms are sufficient to cause pain in stomach. Mostly diarrhea, nausea, chills, fever, and headache appears after incubation period of 12 to 24 hrs (Abadias et al., 2008). Sometimes fatal infections of adults and children can occur from typhoid and paratyphoid fever due to bacteremia and destructive inflammation of the intestine and other organs. Traditional and conventional methods for identification of microbial pathogens rely mainly upon specific bacteriological and biochemical identification. Culture and isolation have high reliability for accurate detection of foodborne pathogen. Although culture based methods are considered to be standard techniques for detection of single bacteria like *S. aureus*, *Salmonella*, *Coliforms* , *E.coli*, etc. (Ayçiçek et al., 2004), however they are labour intensive and time consuming as it take 3 days to obtain the results. Implementation of immunological methods for detection of bacterial agents, spores, toxins and viruses expressed great success (Iqbal et al., 2000). Monoclonal antibodies have been used in the diagnosis of food borne pathogens as *Salmonella enterica* (Schneid et al., 2005), *Salmonella typhi* (Kumar et al., 2003). Most of immunological tests used for detection of foodborne pathogens are agglutination test (Matar et al., 1997) western blot test (Rasooly and Rasooly, 1998), enzyme immunoassay (EIA) (Borck et al., 2002), enzyme linked immunosorbent assay (ELISA) (Bennett, 2005). The sensitivity and specificity of the polymerase chain reaction (PCR) is very high as it is able to detect even single bacterium that's because it detects a single copy of targeted sequence of DNA, amplification of the target rather than the signal makes it promising, and lowers the liability of false–positive results as well as lower time required for performing the technique (Batt, 2007). Identification and differentiation between Typhi and Paratyphi A using rfbS primers targeting the gene *prt* that encodes CDP–paratose synthase, which essentially converts CDP–4–keto–3,6–dideoxyglucose to CDP–paratose. The gene *prt* is present in both serovars (Hirose et al., 2002). *Salmonella* virulence mainly relies on chromosomal and plasmid factors. Chromosomally situated invasion gene *inv*A is thought to enable the invasion of salmonellae into cultured epithelial cells (Galan and Curtiss, 1989). The *invA* gene is present on

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Salmonella pathogenicity island 1 (SPI–1) that enables *salmonella in* invading epithelial cells. This gene is highly conserved in almost all *Salmonella* serotypes and has been used as a potential target for *Salmonella* detection (Li et al., 2012).

MATERIAL AND METHODS

Preparation of Samples (APHA, 1982)

25 grams of frozen meat sample was transferred to 225 ml of sterile peptone water (should be representative and taken from the different parts of the carcass), with application of hygienic measures.

Isolation of Salmonella Spp.

a) Pre–enrichment onto un–selective liquid buffered peptone water, incubation at 37ºC for 24hrs.
b) Enrichment onto Rappaport–Vasiliadis soya broth is incubated at 41.5ºC for 24hrs and Muller–kauffmann tetrathionate/novobiocin broth alternatively 37ºC for 24 hrs.
c) Plating out of *Salmonella* from the cultures obtained in the previous step, onto Xylose lysine Deoxycholate (XLD) agar and Hektoen enteric agar (HK) then incubated at 37ºC for 24hours,
d) Purification: all typical or suspect colonies streaked onto the surface of nutrient agar sloops then incubate at 37ºC for 24hours.

Identification of Salmonella Isolates

a) Biochemical identification using Triple Sugar Iron agar, Urea agar, L–lysine Decarboxylation medium according to (koneman et al., 1992).
b) Serological Identification of *Salmonella* isolates according to the method of Edwards and Ewing, 1972.

Molecular Identification of Salmonella Isolates

I. Extraction of genomic DNA was done according to the method of Sambrook et al., 1989:
   a) Preparation of the overnight culture: One colony from each strain grown on each media was picked up and cultured in 10 ml Lauria bertani broth and incubated at 37ºC overnight in shaker incubator.
   b) Preparations of the bacterial isolates for DNA extraction: overnight cultured cells cooled on ice for 10 minutes, centrifuged at 4°C for 5 minutes to be pelleted down, resuspended in 0.5 ml Tris EDTA buffer, allowed for 2 cycles freezing and thawing. The cells were then incubated 1 hour at 37°C with 1µl lysozymes (final concentration 100µg/ml). Proteinase–K was added 1µl/0.5 ml (final concentration 100µg/ml) and incubated for further 1 hour at 56°C with shaking.
   c) Trizol extraction: one milliliter trizol was added and after 5 minutes of incubation at room temperature, chloroform 0.4 ml added, vortexed for 15 seconds, and kept for 3 minutes at room temperature. Then it was centrifuged at 14000 rpm for10 minutes at 4°C. The upper liquid layer containing RNA was completely removed; the DNA in the interphase was precipitated with 0.6 ml absolute ethanol and kept for 3 minutes at room temperature before centrifugation at 4000 rpm for 5 minutes at 4°C. The supernatant removed and the pelleted DNA washed twice with sodium citrate 0.1 in 10% ethanol. The DNA pellet was kept in the washing solution at room temperature for 30 minutes with periodical mixing, and then centrifugation was done at 4000 rpm for 5 minutes at 4°C. Following the 2 washes, the DNA was resuspended in 2ml of 75% ethanol, kept at room temperature for 20 minutes with periodical mixing and then centrifuged. The DNA pellet was finally dried briefly for 5 minutes under vacuum and dissolved in 50µl of 8 mM NaOH. The pH was then adjusted at 8 by adding 115 µl/ml of 0.1 M HEPES. Two µl of RNAase were then added and incubated at 37°C for 1 hour.
   d) Purification of DNA: DNA was mixed with 1ml of wizard DNA clean up resin in 1.5 ml microfuge tube. Resin/DNA mix was then transferred to the minicolumn provided with the kit and the solution was drained out by adding slight pressure over the minicolumn. Resin/DNA mix was then washed with 2ml of 80% isopropanol and dried by centrifugation at 14,000 rpm/ 2 minutes at 4°C. DNA was then eluted with 50µl of hot 80°C TrisEDTA buffer.

Polymerase Chain Reaction

The primers used were as follows: forward (5'–CTT GCT ATG GAA GAC ATA ACG AAC C–3') reverse (5'–CGT CTC CAT CAA AAG CTC CAT AGA–3'). Primers GenBank accession no. M29682 synthesized by BioBasic, Canada, purified by HPLC and concentrated to 100 pg/µl. Each reaction volume was 50µl.The mixture contained 0.3µM of each primer, 200 µM of dATP, dCTP, and dGTP, 190µM dTTP, 10µM DIG–11–dUTP (Roche Diagnostics), 0.5 U of Taq polymerase, 5µl of 10xPCR buffer, and 1.5 mM of $MgCl_2$ with various concentrations (from 50ng to 0.5pg) of genomic DNA. The PCR program conducted was as following: 94ºC for 5 min, followed by 5, 8 or 30 cycles at 94, 60 and 72ºC respectively, each for 1 min and the final extension at 72ºC for 5 minutes. Then, 5µl of every product loaded into wells of 2% agarose gel with 0.5µg/ml ethidium bromide. Molecular weight marker 100bp ladder (Fermentas) was used. PCR products were electrophoresed, visualized with UV light and images stored using a gel documentation system (Gel Doc1000; BioRad, Hercules, CA) expect product size (bp) 258 bp (Hirose et al., 2002).

Real time qRT–PCR utilizing primers and probe targeting invA gene were as follows: forward primer 5'–GCG TTC TGA ACC TTT GGT AAT AA–3' reverse primer 5'–CGT TCG GGC AAT TCG TTA–3' Sal–TM (probe) 5'–FAM–TGTTG CGGTG GGTTT GTTGTCTT– TAMRA–3' Synthesized by BioBasic, Canada, purified by HPLC and concentrated to 100pg/ul. The primers (GenBank accession no. NC_006511.1) and probe (GenBank accession no. NC 015761.1) amplify the *invA* gene of *Salmonella* spp. The quantitation was done by Q–PCR using probe based method. The PCR mix was performed as follow : 2X reagent Mix 2.5 µl, 1.0 µl of magnesium chloride sol 25mM, 0.01 µl forward primer, 0.01 µl reverse primer, Probe 0.01µl, DNA 0.5 µl, RNAase– DNase free water To 25 µl. The reaction was done using agilant MX3005 probe real time PCR. The program was adjusted as follow: thermo start activation step 95°C for 10 min 1 cycle, and 30 cycles consisted of denaturation 95°C for15 sec, annealing 57°C for 30 sec and extension 60°C for 30 sec .The immersion light created by the hydrolysis of the Taq man probe was read after the end of each extension step. Interpretation of results was made

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with the help of the real–time PCR system user guide instructions. Briefly, in every PCR assay Ct value >16.9 for the *invA* probe considered positive and zero result of Ct values (non–amplified, NA) considered negative, avoidance of PCR inhibitors made by repeating PCR assay with tenfold diluted DNA template suspension (Novinscak et al., 2007).

Data Analysis

The prevalence to every test calculated after dividing number of positive samples by number of all samples under test within the specified period.

Table 1: Conventional and real time PCR of *Salmonella* serotypes isolated from different meat cuts imported from Brazil, India, Australia and Newzeland.

Country	Species	No. of isolates	Isolated serotypes		Conventional PCR of using primer for *rfbS*	Real time PCR using primer and probe for *invA* gene
Brazil	Beef	13	*S.typhi**	1	1/1 (100%)	1/1 (100%)
			*S.paratyphiA***	2	2/2 (100%)	2/2(100%)
	Buffalo	11	*S.typhi*	1	1/1 (100%)	1/1(100%)
			S.paratyphi A	2	2/2 (100%)	2/2 (100%)
India	Beef	6	*S.typhi*	1	1/1 (100%)	1/1(100%)
			S.paratyphi A	1	1/1 (100%)	1/1 (100%)
	Buffalo	12	*S.Typhi*	1	1/1 (100%)	1/1(100%)
			S.paratyphi A	1	1/1 (100%)	1/1 (100%)
Australia	Beef	5	*S.typhi*	1	1/1 (100%)	1/1(100%)
			S.paratyphi A	2	2/2 (100%)	2/2(100%)
	Buffalo	7	*S.typhi*	1	1/1 (100%)	1/1 (100%)
			S.paratyphi A	–	–	–
New zealand	Beef	6	*S.typhi*	1	1/1 (100%)	1/1 (100%)
			S.paratyphi A	1	1/1 (100%)	1/1 (100%)
	Buffalo	5	*S.typhi*	1	1/1 (100%)	1/1 (100%)
			S.paratyphi A	1	1/1 (100%)	1/1 (100%)
Total	100	65		18 (18)	18/18 (100%)	18/18 (100%)

From 100 imported meat cuts from Brazil, India, Australia and Newzeland, 65 isolates were obtained on specific media for isolation of Salmonella, identified and serotyped isolates were *S.typhi* and *S.paratyphi A* and represented 18/65 (18%) { *S.Typhi* * 8/18 (44.44%) and *S. paratyphi A*** 10/18 (55.6%)}. Result of conventional PCR confirmation using *rfbS* was 9/18 (50%) while real time PCR using primers and probe specific to invA was 6/9 (66.7%).

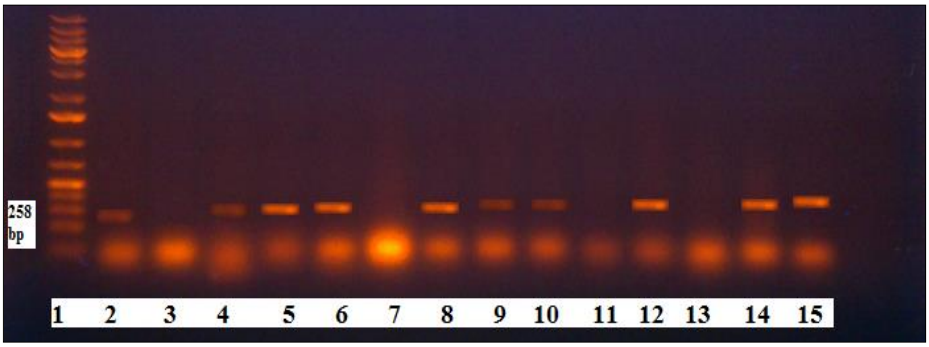
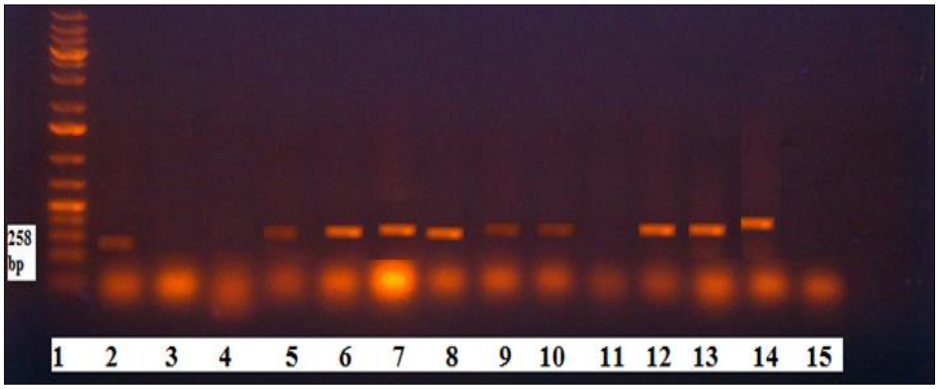


Figure 1.: Conventional PCR result using *rfbS* primer lane (1) 1000 bp ladder, lane (2) control positive, lane (3) control negative while, lanes (4, 5, 6, 8, 9, 10, 12, 14 and 15) are positive and lanes (7, 11 and 13) were negative

Figure 2 : Conventional PCR result using *rfbS* primer lane (1) 1000bp ladder, lane (2) control positive, lane (3) control negative while, lanes (5, 6, 7, 8, 9,10, 12, 13, and14) are positive and lanes (4, 11 and 15) were negative



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RESULTS

One hundred frozen meat samples were collected from supermarkets located at Alexandria province and from Alexandria port. *Salmonella* was biochemically identified in 18/100 (18%) samples. Serologically *S. paratyphi A* and *S. typhi* were detected in 10/18 (55.56%) and 8/18 (44.44%) of the positive samples, respectively. Using Conventional PCR, 18/18 (100%) were confirmed to be pathogenic by specific primer to *rfbS* gene of the pathogenic strains of *Salmonella* responsible for the most food poisoning while, all of these pathogenic isolates 18/18 (100 %) were confirmed by real time PCR using primers and probe that amplify the *invA* gene of *Salmonella* spp Table 1; Figure 1, Figure2).

DISCUSSION

Historically meat, poultry and eggs are considered as major sources of high quality animal protein. Potentially they may harbor, or become environmentally contaminated with certain pathogenic microorganisms during preharvest production or processing throughout the food chain (Forsythe, 1996).Therefore; these food types have been considered vehicles for salmonellosis transmission to human beings. (Dallal et al., 2009).

Therefore, our study focused on isolation and identification of *Salmonella* spp. from meat cuts imported from (Brazil, India, Australia and Newzeland) to Egypt. Salmonellosis is serious zoonotic disease of great public health concern because of its endemic nature, higher morbidity levels and being associated with a wide range of foods. Results obtained in Table 1 which mainly represent the prevalence of isolated and biochemically identified *Salmonella* was 18/100 (18%) (Table 1) found in our study are in agreement with the result found by (D'Aoust et al., 2000), who reported that *salmonella* prevalence in post–slaughter beef ranges from 0.6 to 20.3% in about 23045 samples tested from Denmark, Germany, Nigeria, Portugal and USA. Also prevalence of *Salmonellae* in meats at retail level of raw beef ranges from 1.3 to 21.5% in about 3743 samples tested in Denmark, Japan, Mexico, Thailand and Netherlands. Also, similar prevalence of *Salmonella spp.* was reported by Dallal et al., 2010 from beef in Iran with prevalence of 38/189 (20.1%) and (Yang et al., 2010) who reported prevalence of *Salmonella* from beef in China was 13/78 (16.7%).

The studies on the serotyping identified *S.typhi** with percentage of 8/18 (44.44%) and *S.paratyphi A*** with percentage of 10/18 (55.56%) These data nearly comply with that obtained by Jegadeeshkumar et al., 2010 who reported *Salmonella* prevalence from meat was 20% and the serotype isolated after examining three food types (fish, fruits and meat) were S.typhi, *S.paratyphi A* ,*S.paratyphi B* and *S.typhimurium* 55.5%, 48.1%, 25.9% and 22.2% respectively in positive samples.

Salmonella typhi has well adapted and is responsible for causing deadly invasive typhoid fever in human beings leading to high morbidity and mortality. *Salmonella Paratyphi A* dT+ since the end of the 1990s has become increasingly prominent. Many outbreaks associated with this variant in France (Desenclos et al., 1996) in Canada (Gaulin et al., 2002) in Australia and several European countries (Denny et al., 2007) have been recorded. High prevalence of isolation of the multi–drug resistant *salmonella paratyphi A* dT+clone has been recorded from poultry and poultry products in Germany and Netherlands (Miko et al., 2002). Contamination of meat with bacterial pathogens has been reported in many countries (Kinsella et al., 2008). Others recognized open air bacterial spoilage of meat by presence of gram–negative organisms (Eribo and Jay, 1985). Unhygienic abattoirs environment and practices during post–process handling has serious impact on health (Abdullahi et al., 2006). Not only environment, but also the animals will be slaughtered can constitute contamination source (Sofos et al., 2000). Live animals harboring the pathogens and contaminated environment constitute sources for contamination during slaughtering and meat products during processing, storage and handling. In addition, role of animals in contaminating the carcass was confirmed by (Arthure et al., 2008) who found that lymph nodes of culled cattle meat contain high prevalence of *Salmonella* than did those from fed cattle carcasses consequently liability of increasing number of *Salmonella* in carcass is present. Additionally, butcher hands, dress and slaughtering equipments have also been considered as other contamination sources (Aftab et al., 2012). Serotyped isolates were confirmed to be *Salmonella* with conventional PCR using *rfbS* Primer and real time PCR using primer and probe for *invA* gene with percentage of 18/ 18(100%). The sensitivity of PCR in detection of *Salmonella* was confirmed by Mccarthy et al., 2009 who optimized multiplex PCR assay and quantitative real–time PCR assay for detection and differentiation of *S. enterica* Typhimurium and Heidelberg in foods. The multiplex PCR assay detected *S. Enterica* isolates at concentrations as low as 1 CFU/g of inoculated Cheddar cheese, raw turkey, and cooked turkey. They concluded that PCR has significantly saved time needed to identify *S.enterica* Typhimurium and Heidelberg, making this rapid, sensitive, specific and selective diagnostic tool. Also higher PCR sensitivity was confirmed by Robles et al., 2009 who detected 4 *Salmonella* containing meat samples out of 50 using PCR which were more than culture technique that detected only 3 out of 50, therby confirming higher sensitivity than cultivation.

CONCLUSION

Periodical testing of imported meat is of great importance so as to prevent foodborne outbreaks. Furthermore traditional methods should be substituted by advanced molecular methods for detection of serious bacterial agents because they are fast, sensitive and labor saving.

AUTHOR CONTRIBUTIONS

Dr Mohamed Elsayed shared in PCR identification, data analysis and writing this paper, Dr Eman Abdeen shared in isolation, Dr Akiela and Dr Rasha Zahran shared in paper revision while Dr Thaer Farouk helped in finishing PCR.

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