Occurrence of *Listeria species* in meat, chicken products and human stools in Assiut city, Egypt with PCR use for rapid identification of *Listeria monocytogenes*

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Abstract

The present research was conducted to check the presence of Listeria spp. in some meat and chicken products purchased from retail supermarkets in Assiut (Egypt). A total of 100 samples including 25 samples each of minced frozen beef, luncheon, frozen chicken legs and frozen chicken breast fillets were collected over a 7-month period between January and July 2009 and analyzed for the presence of *Listeria spp*. In addition, 28 stool cultures examined for *Listeria spp.* from hospitalized children resident in Assiut Pediatric University Hospital with diarrhea or fever. Out of the total 100 meat samples examined, Listeria spp. were detected in 8 (32%) of minced frozen beef, 8 (32%) of luncheon, 13 (52%) of frozen chicken leg and 14 (56%) of frozen chicken fillet samples analyzed, respectively. Regarding the examined 28 stool cultures from hospitalized children with underlying disease in Assiut Univ. hospital, 2 (7.14%) were found positive for Listeria spp. For identification of *L. monocytogenes* using polymerase chain reaction (PCR), two primers were selected to detect 217-pb fragment ofthe prfA (transcriptional activator of the virulence factor) gene for *L. monocytogenes*. 13 selected Listeria isolates displayed beta-haemolysis on sheep blood agar and positive CAMP test were further identified using PCR. PCR results showed that *L. monocytogenes* were confirmed in one of minced imported frozen meat examined, two of luncheon samples and two of frozen chicken legs with the total incidence of 5 isolates (5%) from the total 100 examined food samples. This suggests the presence of a significant public health hazard linked to the consumption of these meat and chicken products sold in Assiut city contaminated with *L. monocytogenes*. The public health significance of these pathogens as well as recommended sanitary measures was discussed.

Keywords: Listeria spp., Minced beef, Luncheon, Chicken meat, Human stool, PCR, Laboratory Technique.

Introduction

The marked increased of contamination in food industry especially meat and chicken products by pathogenic bacteria has raised a great concern of the public. Listeria spp. especially *L. monocytogenes* has been associated with a wide variety of food sources particularly meat and chicken (Endang et al., 2003). Listeria spp. is ubiquitous bacteria widely distributed in the natural environment. The ubiquitous character of the bacteria inevitably results in contamination of numerous food products (Farber and Peterkin, 1991).

The genus Listeria includes 6 different species (*L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seegligeri and L. grayi*). Both *L. ivanovii* and *L. monocytogenes* are pathogenic in mice, but only *L. monocytogenes* is consistently associated with human illness (Seafood Network Information Center, 2007).

Listeria spp. has been isolated from poultry, red meat and meat products in many countries around the world such as Yugoslavia (Buncic, 1991), Belgium (Uyttendaele et al., 1999), New Zealand (Hudson et al., 1992), Australia (Ibrahim and Mac Rae, 1991), and Japan (Ryu et al., 1992), although these foods have not been associated with documented outbreaks of human listeriosis. The detection of Listeria spp. in meat is of particular concern in terms of consumer safety, as these organisms are capable of growing on both raw

Table-1: Oligonucleotide sequences used for identification of Listeria monocytogenes by PCR

Target gene	Primer sequence (5'-3')	Amplified fragment length	Reference
prfA gene	LIS-F: TCA TCG ACG GCA ACC TCG G LIS-R: TGA GCA ACG TAT CCT CCA GAG T	217 bp	Germini et al. (2009)

and cooked meat at refrigeration temperatures (Walker et al., 1990).

In the past 25 years, *L. monocytogenes* has become increasingly important as a food-associated pathogen. Because of its high case fatality rate, listeriosis ranks among the most frequent causes of death due to food-borne illness. *L. monocytogenes* infections are responsible for the highest hospitalisation rates (91%) amongst known food-borne pathogens and have been linked to sporadic episodes and large outbreaks of human illness worldwide. The ability to persist in food-processing environments and multiply under refrigeration temperatures makes *L. monocytogenes* a significant threat to public health (Jemmi and Stephan 2006).

Moreover, *L. monocytogenes* is an important food-borne pathogen that can cause septicemia, meningitis (or meningoencephalitis), encephalitis and gastroenteritis, particularly in children, the elderly and immunosuppressed individuals; it also causes miscarriage in pregnant women. The mortality rate can be as high as 30% (Robinson et al., 2000 and Churchill et al., 2006).

Unlike most other enteric pathogens, *L. monocytogenes* is notable for its ability to grow at refrigeration temperatures. This has considerable significance for food safety, as it means that chilling to 4º C cannot be relied upon to prevent the growth of the organism to dangerous levels (Pal et al., 2008).

In addition, because of its ability to survive and proliferate at refrigeration temperature, *L. monocytogenes* may cause disease through frozen foods (Schillinger et al., 1991). Due to its ubiquitous character, *L. monocytogenes* easily enters the human food chain and may multiply rapidly (Farber and Peterkin, 1991).

The standard microbiological methods for identification of Listeria spp. Are laborious and time consuming requiring a minimum of five days to recognize Listeria spp. And about 10 days to identify *L. monocytogenes* by confirmation tests (Amagliani et al., 2007) while rapid response should be carried out in case of confirmation since it is of principal importance to ensure the safety of foods. In the few past years, progressing in biotechnology has resulted in the development of rapid methods that reduce the analysis time and offer great sensitivity and specificity in the detection of pathogens. Among these, PCR has been increasingly used for the rapid, sensitive and specific detection of food borne pathogens (Norton, 2000).

Therefore, the goal of this study was to determine the incidence of Listeria spp. and *L. monocytogenes* in minced frozen beef, luncheon, and frozen chicken meats as well as in human stools from hospitalized children resident in Pediatric University Hospital in Assiut (Egypt).

Materials and Methods

Collection of samples: One hundred random samples of meat and chicken products [25 samples each of minced frozen beef, luncheon, frozen chicken legs, and frozen chicken breast fillets (frozen raw fillets without skin)] were collected from different retail supermarkets and groceries in Assiut city. The collected samples were transferred directly to the laboratory in an ice box for recovery of Listeria spp.

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 Listeria infections in hospitalized children in Assiut, the cases-control study was conducted. The cases were defined as the children, resident in Pediatric Univ. Hospital; Assiut Univ., with diarrhea or fever between January and July 2009. A stool culture examined for Listeria spp. 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 Listeria spp. (FAO, 1992):

Enrichment procedures: The initial procedure of isolation involves the use of Listeria selective enrichment broth (LSEB) to enhance the growth of Listeria spp. LSEB base consists of trypticase soy broth with 0.6% yeast extract supplemented with Listeria selective supplement (Hi Media laboratories) which contains acriflavin-HCL (15 mg/L), nalidixic acid (40 mg/L) and cycloheximide (50 mg/L). Ten grams of samples as well as swabs from human stools were aseptically added to 90 ml LSEB and mixed thoroughly. All the primary enrichment broths were incubated at 37°C and 30 °C for 24-48 h.

Selective plating: Following the enrichment procedure, a loopful of homogenate was streaked onto Listeria selective agar base (Hi Media laboratories)

Table-2: Isolation rate of Listeria spp. in different food and human samples

Type of samples	No. of analyzed samples	Positive samples	
		No.	%
Minced frozen meats	25	8	32
Luncheon	25	8	32
Frozen chicken fillets	25	11	44
Chicken frozen legs	25	14	56
Human stools	28	2	7.14
Total	128	43	33.59

and Oxford - Listeria Selective Agar and the plates were incubated at 35 °C for 24-48 h.

Confirmation: Colonies suspected to be Listeria were characterized using Gram stain; catalase reaction; umbrella-shaped motility pattern by using motility test medium; haemolysis on sheep blood agar; fermentation of mannitol, rhamnose and xylose and CAMP test performed according to Bergey's Manual of Systematic Bacteriology (Seeliger and Jones, 1987).

Genomic DNA Extraction : For each Listeria strain, a 10-ml culture was grown to mid-log phase in Tryptose Soya (TSY) broth, and 1 ml of cells was pelleted by centrifugation (13.000 ×g for 5 min). The cell pellets were resuspended in 1ml of sterile water. The resuspended cells were re-centrifuged at 12.500 ×g for 15 min. The pelleted cells were then used for DNA extraction.

Genomic DNA from suspected Listeria strains was extracted using the Wizard genomic DNA purification kit (Promega, USA) as recommended by the manufactures. Protocol for Gram positive bacteria, cellular lyses was carried out by enzymatic fragment with lysozyme. DNA samples were stored at -20 °C until use.

PCR identification of Listeria monocytogenes : For *L. monocytogenes* PCR identification, two primers were selected based on the prfA (transcriptional activator of the virulence factor) gene for *L. monocytogenes* as mentioned by (Germini et al., 2009). Primer sequences used in the PCR are listed in Table 1.

All PCR reactions were performed in a final volume of 25µl using 2µl of extracted DNA as template. Each reaction mixture contained 12.5 µl GoTaq® Green Master Mix (Promega, M7122) 1µl of 500 ?M forward primer (LIS-F);1µl of 500 pM reverse primer (LIS-R) and 8 µl of Ultra-Pure DNase/RNase-Free Distilled Water (Gibco, Grand Island, NY, USA).

The amplification profile was as follows: pre incubation at 95 °C for 5 min; 40 cycles consisting of dsDNA denaturation at 95 °C for 30 s, primer annealing at 54°C for 30 s, primer extension at 72 °C for 30 s; final elongation at 72 °C for 5 min. Reactions were thermally cycled in a Techne Cyclogene.

Gel Electrophoresis: All amplification products were resolved in 1% agarose gel, stained with ethidium bromide, detected under a short-wavelength UV light source, and photographed with EDVOTEK Gel documentation system. The 1-KB plus DNA Ladder (Invitrogen) was used as molecular size marker.

Results and discussion

Meat and chicken products have been frequently contaminated with *L. monocytogenes* and may serve as vehicle of other pathogenic organisms. The frequent occurrence of *L. monocytogenes* in meat and chicken may pose a potential risk for consumers (Mahmood et al., 2003). Human infections primarily result from eating contaminated food and may lead to serious and potentially life-threatening listeriosis (Posfay-Barbe and Wald, 2004).

Of the total of 100 meat and chicken product samples examined, 41 (41%) isolates of Listeria spp. were recovered. Of these 13 isolates displayed beta-haemolysis on sheep blood agar and positive CAMP test. These 13 selected Listeria isolates were further identified using PCR.

The wide application of nucleic acid amplification techniques and the increasing industrial interest toward rapid methods has led to the development and application of PCR based methods for the detection of microbial pathogens in food (Germini et al., 2009).

Analyzing the PCR profiles, 5 out of 13 isolated Listeria strains showed one amplified product (217 bp), (Fig. 1) that is specific for *L. monocytogenes*. Thus, *L. monocytogenes* were confirmed in one of minced imported frozen meat examined, two of luncheon samples and two of frozen chicken legs with the total incidence of 5 isolates (5%) from the total 100 examined samples (Table 3 and Figure 1). This suggests the presence of a significant public health hazard linked to the consumption of these meat and chicken products sold in Assiut city contaminated with *L. monocytogenes.*

The percentage of culture positivity of *L. monocytogenes* in meat and chicken products in the present study is in agreement with the reported incidences in other countries such as 5.1% in Ethiopia (Molla et al., 2004), 4.9% in Belgian meat products (Uyttendaele, 1999) and 3.6% in processed meat

Table-3: Incidence of Listeria spp. in meat and chicken products as well as in human stools

Samples	*L. monocytogenes*		*L. ivanovii*		*L. innocua*		*L. welshimeri*		*L. seeligeri*		*L. grayi*		*L. murrayi*	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Minced meat	1	4	-	-	7	28	-	-	-	-	-	-	-	-
Luncheon	2	8	1	4	-	-	2	8	2	8	1	4	-	-
Frozen chicken fillet	-	-	3	12	2	8	2	8	-	-	4	16	-	-
Frozen chicken leg	2	8	3	12	3	12	3	12	-	-	2	8	1	4
Human stools	-	-	-	-	-	-	-	-	1	3.8	1	3.8	-	-
Total	5	-	7	-	12	-	7	-	3	-	8	-	1	-

products in Chile (Cordano and Rocourt 2001).

When several studies in various countries are compared, *L. monocytogenes* isolation rates seem to vary significantly. This wide variation may be explained in terms of geographic location, isolation methods and kinds of media employed (Akpolat et al., 2004).

Concerning minced imported frozen meat examined in our study, Listeria spp. were isolated from 8 (32%) of 25 examined samples. *L. monocytogenes* occurred in one (4%) and *L. innocua* in 7 (28%) of tested samples, respectively (Tables 2 & 3).

Similarly, the isolation rate of *L. monocytogenes* in minced beef samples was 5% in a study in Turkey conducted by Akpolat et al (2004). Nearly, similar results obtained by other researchers such as Abd El-Aziz (2004) (6%) and Marinsek and Grebenc (2002) who isolated *L. monocytogenes* from 3 of the minced meat samples (6.81 %).

On the other hand, higher records were reported by several investigators as Hassan et al (2001) who found Listeria spp. in 17 (73.9%) of 23 samples of imported frozen beef in Malaysia and Donald et al (1991) who reported that imported frozen beef examined harbored seven Listeria spp.: 15 *L. monocytogenes*, 18 *L. ivanovii,* 32 *L. innocua*, 2 *L. seeligeri*, 11 *L. grayi*, 7 *L. murrayi*, and 13 *L. welshimeri* isolates, respectively in Canada. Also, (Inoue et al., 2000) isolated *L. monocytogenes* in 12.2% of minced meat samples in Japan and Buncic (1991) detected *L. monocytogenes* in 69% of minced meat samples in Yugoslavia.

It is interesting to note that *L. innocua* was isolated predominantly among Listeria spp. in minced frozen meat in this study (Table 3). This finding is in agreement with other studies where *L. innocua* was the most common species in raw and cooked meats, while other Listeria spp. were less frequently (Choi et al., 2001 and De Simon et al., 1992). As similar, *L. innocua* was the most predominantly isolated species in a variety of meat samples. It was detected in 83.3% of the raw minced meat, 57.6% of the raw chicken meat, 63.1% of the raw beef, 9.6% of the cooked red meat and 10.7% of the cooked chicken samples (Yucel et al., 2005).

Furthermore, detection of *L. monocytogenes* in foods can be difficult as these bacteria are normally found in very low numbers in the presence of a heterogenous microflora. The most frequent Listeria isolates from food are *L. monocytogenes* and *L. innocua*. Several studies have demonstrated that *L. innocua* is found in food more frequently than *L. monocytogenes* (Walsh et al., 1998). The reasons for the higher frequency of recovery of *L. innocua* remain unclear yet. However, this may result from either a naturally higher prevalence or from preferential selection of *L. innocua* during laboratory detection procedures (Gnanou Besse et al., 2005).

Contamination of the meat with *L. monocytogenes* generally occurs after the slaughter and may come from the skin of the animals, the hands of the workers, the equipment and the tools used (Marinsek and Grebenc, 2002).

Regarding luncheon samples as shown in Table (3), Listeria spp. isolated were *L. monocytogenes* comprising (8 %0) of the samples, followed by *L. welshimeri* (8%), *L. seeligeri* (8%), *L. ivanovii* (4%) and *L. grayi* (4%).

In this research, *L. monocytogenes* was determined in 2 (8%) samples of luncheon meat. Lower incidence was obtained by Gombas et al (2003) who found *L. monocytogenes* in 0.89% of luncheon meat. In contrast, *L. monocytogenes* could not be isolated from luncheon samples (Elgazzar and Sallam, 1997; Mohamed and Ali, 1999 and Saad et al., 2001).

Cross-contamination, which can occur within the environment of food-processing equipment, is considered to be a possible source of Listeria contamination in processed meat such as luncheon. *L. monocytogenes* is able to attach to and survive on various working contact surfaces (Borucki, 2003). One reason may be its ability to form biofilms (Wong, 1998). Furthermore, during further transformation processes of raw meat into meat products *L. monocytogenes* can be introduced, where the amount depends on the extent of cross-contamination, personal and general hygienic measures and the process parameters (Glass and Doyle, 1989).

In addition, minced/chopped meat products as

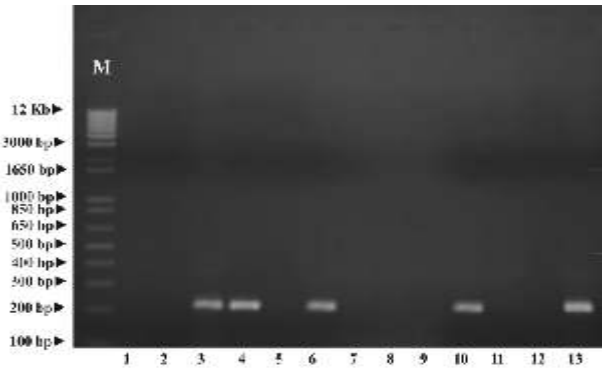

FIG. 1: Agarose gel electrophoresis of PCR products from Listeria monocytogenes isolates from examined samples: Lan M, DNA size marker (1-KB Plus DNA Ladder); lane 1, (L 1), lane 2, (L 4); lane 3, (L 3), lane 4, (L 18); lane 5, (F 9); lane 6, (Lu 9); Lan7, (Lu 7); Lan 8, (Lu 13); Lan 9 (F 14); Lan 10, (M 16), Lan 11, (L 11); Lan 12, (H 26); Lan 13, (Lu 18).
L: chicken leg isolate, F: chicken fillet isolate, Lu: luncheon isolate, M: minced meat, H: Human stool isolate.

luncheon, by their nature, undergo extensive processing and handling during their production. This leads to greater opportunities for *L. monocytogenes* contamination (Tompkin et al., 1992 and Uyttendaele, 1997).

The ability of *L. monocytogenes* to multiply at refrigeration temperatures could be considered of a significance in food intended for consumption without further cooking as luncheon meat and foods which have received cooking presumed sufficient to eliminate Listeria, but nevertheless intended be received further cooking prior to consumption where the potential competitive microflora has been largely eliminated and thus even low numbers could pose a potential hazard if proper storage conditions are not adhered to (Schuchat et al., 1992).

Frozen chicken breast fillet examined harbored four Listeria spp.: 3 (12%) *L. ivanovii*, 2 (8%) *L. innocua,* 4 (16%) *L. grayi* and 2 (8%) *L. welshimeri* isolates, respectively (Table 3).

In this study, *L. monocytogenes* were not isolated from any of the 25 samples of frozen chicken breast fillet examined. On the other hand, *L. monocytogenes* was determined in 9 samples of mechanically-deboned chicken meat (15.78 %) (Marinsek and Grebenc, 2002). Also, Hindy (2006) isolated listeria spp. from 28% and 20% of chicken fillet and meat samples, from which 8% and 4% were *L. monocytogenes*. Of the 25 Frozen chicken legs samples examined, 14 (56 %) were found to be contaminated with Listeria spp. (Table 2).

L. monocytogenes was determined in 2 (8%) samples of chicken leg analyzed in the present study. This obtained result was in agreement with other reports as in the study by Arslan et al (1999), who mentioned that *L. monocytogenes* was found at 15%, 10% and 5% in wing, leg and washing water, respectively, of 20 chickens. In the contrary, several studies showed that the rates of *L. monocytogenes* varied between 23% and 60% (Pini and Gilbert, 1988; Skovgaard and Morgen, 1988). Poultry can harbour *L. monocytogenes* in their intestinal tract and as such are a potential source of contamination (Capita et al., 2002).

Therefore, higher incidence of Listeria in chicken meat products could be attributed to contamination caused by chopping board, mincing machine, knives, cleaning cloth, other working surfaces and more human contact (Lowry and Tiong, 1985).

Moreover, *L. monocytogenes* has been strongly implicated particularly in the contamination of foods stored at low temperatures. Storage of such products under such low temperature conditions may allow the growth of significant numbers of these organisms leading to food-borne illnesses among consumers (Beumer et al., 1996; Walker et al., 1990).

Concerning the examined 28 stool cultures from hospitalized children with underlying disease in Assiut Univ. hospital, 2 (7.14%) were found positive for Listeria spp. from which one *L. seeligeri* and one *L. grayi.*

It is also important to comment that the presence of any Listeria spp. may be indicative of poor hygiene and cross-contamination scenarios which could favour the persistence of *L. monocytogenes* (Azevedo et al., 2005).

In order to minimize human listeriosis, foods should be cooked to an internal temperature of 70 °C for more than 20 minutes to ensure destruction of *L. monocytogenes*. Reheat cooked food thoroughly (70 °C), immediate aseptic packaging of the finished product to avoid post processing environmental contamination. Proper cold storage of meat and meat products (freezing-18 °C) and proper personal hygiene

of food handlers is advisable (Mahmood et al., 2003).

It was significantly important for public health to detect Listeria spp., and particularly *L. monocytogenes*, in meat products sold in Assiut, since consumers are frequently exposed to these products. Therefore, meat and meat products must be thoroughly cooked or grilled before consumption so *L. monocytogenes* is likely to be eliminated.

In conclusion, this study has demonstrated the presence and distribution of *L. monocytogenes* and other Listeria spp. in a variety of meat and chicken products in Assiut city. The study also suggests the need for improved food safety through the implementation of hygienic measures at all levels from production to consumption.

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