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RESEARCH ARTICLE
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Characterization of virulent *Listeria monocytogenes* isolates recovered from ready-to-eat meat products and consumers in Cairo, Egypt

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Abstract

Aim: This study aimed to investigate the occurrence of some virulence genes distributed in *Listeria monocytogenes* isolated from ready-to-eat (RTE) meat products and consumers in Cairo province, Egypt.

Materials and Methods: A total of 120 beef luncheon, chicken luncheon and frankfurter beef (40 samples, each) were collected from 10 different local shops situated in Al-salam city, Cairo province, Egypt. Stool samples were collected from 40 people who had the habit of consuming RTE meat. The suspected *L. monocytogenes* isolates were subjected to a multiplex polymerase chain reaction (PCR) for rapid speciation and virulence determination using primers specific for *inIA*, *inIC*, and *inIJ* genes.

Results: Culture examination of all samples on Oxford media revealed presence of colonies characteristic to *L. monocytogenes* in 6 beef luncheon (15%), 4 chicken luncheon (10%), 1 frankfurter beef (2.5%) and 1 human stool (2.5%) samples. Species identity of *L. monocytogenes* was verified through the amplification of a 800 bp fragment with *inIA* primers in 2 out of 6 culture isolates from beef luncheon (5%), and 1 out 4 culture isolates from chicken luncheon (2.5%) samples. Statistical analysis revealed no significant difference between the occurrence of *L. monocytogenes* in different food samples examined ($p>0.05$). The virulence of these strains was ascertained by the presence of 517 bp and 238 bp fragments of *inIC* and *inIJ* genes, respectively in the isolates that contained the 800 bp fragment. The culture isolates obtained from one frankfurter beef sample, and one human stool sample were found negative by multiplex PCR for the presence of *L. monocytogenes* and its virulence specific genes.

Conclusion: It could be concluded that *L. monocytogenes* are circulating in beef and chicken luncheon sold in Cairo, Egypt. Multiplex PCR is reliable for confirmation of *L. monocytogenes*. This study suggests the implementation of hygienic measures at all levels from production to consumption in order to improve food safety. Furthermore, authors recommended consumption of beef frankfurter or any RTE meat sold in their original intact packing due to low level of contamination.

Keywords: *Listeria monocytogenes*,, consumers, ready-to-eat meat, speciation and virulence determination.

Introduction

Listeriosis has now been recognized as an emerging food borne zoonoses [1]. *Listeria monocytogenes* has been recognized as an important opportunistic human pathogen since 1929 and as food borne pathogen since 1981 [2]. Ready-to-eat (RTE) meat products are, usually, cooked during manufacture and consumed without further heating. Hence, they present high risk to the consumers due to possible cross contamination with food borne pathogens and further growth of such pathogens [3]. The extended distribution throughout the food processing environment and asymptomatic human carriers [4] and the psychrotrophic character of *Listeria* species appear to be the main causes of the prevalence in different kinds of refrigerated RTE meat products and contamination could occur either pre- or post-processing [5].

Of the 20 RTE food categories evaluated by the Food and Drug Administration and the Food Safety and Inspection Service, deli meats were classified in the very high risk category to be the principal potential source of *L. monocytogenes* [6]. In general, consumption of food contaminated with *L. monocytogenes* may cause listeriosis which may result in serious human illness with symptoms of septicemia, meningitis, encephalitis and gastroenteritis particularly in children, the elderly and immunosuppressed individuals. It may also cause miscarriage in pregnant women [7]. *L. monocytogenes* had the second highest fatality rate (20%) and the highest hospitalization rate (90%) in virulence [8]. Multistate outbreaks of food borne listeriosis were recorded [9].

Given the close morphological and biochemical resemblances of *L. monocytogenes* to other *Listeria* species, and the non-specific clinical manifestations of listeriosis [10], the availability of rapid, specific and sensitive diagnostic tests capable of distinguishing *L. monocytogenes* from other *Listeria* species is essential for the effective control of the disease.

Internalin genes (*InlA, InlJ and InlC)* are a panel of virulence-specific genes that involved directly in *L. monocytogenes* passage through the intestinal barriers as well as involvement in the subsequent stages of infection [11]. *InlA* was intended for species- specific recognition while *InlJ and InlC* were designed for virulence determination of *L. monocytogenes*. Polymerase chain reaction (PCR) is deemed to be more reliable than conventional methods of identification [12].

The increase of RTE food consumption due to changes in the lifestyle and the ability of *L. monocytogenes* to attach to different surfaces forming biofilms and consequently its persistence in food environment necessitate periodically repeated surveys for determining the prevalence and the distribution of some virulence genes in *L. monocytogenes* isolated from RTE food and consumers.

Materials and Methods

Ethical approval

Human samples were obtained after having an informed consent.

Sampling

The samples used in this study were:

RTE meats that did not require any further cooking prior to consumption. The categories of RTE meats purchased were beef luncheon, chicken luncheon and frankfurter beef (40 samples, each). Beef luncheon, chicken luncheon were repacked at the markets and local shops. However, the frankfurter beef samples were purchased in their original intact packing. Immediately after purchase, the samples were transferred on ice to the laboratory and stored at 4°C until analysis.

Stool samples: After contacting of about 100 people, a total of 40 persons of different age groups and have the habit of eating RTE meat of the same categories examined, were investigated for the presence of *L. monocytogenes* in their stool. The forty persons were apparently healthy at the time of stool collection; however, eight of them had got a transient diarrhea within 1-2 months before being investigated. Stool cups were distributed 1 day before collection. Stool samples were transported to the laboratory on ice and stored at 4°C until analysis.

Isolation and identification of *L. monocytogenes*

RTE meat: The packages containing RTE meats were disinfected at the incision site by swabbing with 70% ethanol [8]. Analytical portion (25 g) was aseptically weighed using a sterile spatula and blended for 1 min with 225 ml of buffered peptone water (BPW, Oxoid, CM509). The homogenate was transferred to a sterile bottle jar and was incubated at 30°C for 24 h. 1 ml of the incubated homogenate was added to 9 ml of *Listeria* selective broth (Himedia, 569-500G) and further incubated for 48 h at 30°C. Thereafter, the homogenate was streaked onto the Oxford agar plates (Himedia, MV1145-500G with *Listeria* Oxford supplement Himedia, FD071), incubated for 48 h at 37°C. Grey colonies surrounded by black zone (suspected *L. monocytogenes*) were picked up and purified on nutrient agar plates.

Stool samples: A swab taken from each stool sample using a sterile swab was immersed in sterile BPW tubes under aseptic condition. The tubes were labeled with respect to name, age, sex, and date of collection. The remaining steps were carried out as those applied for RTE meat samples.

Multiplex PCR for speciation and virulence determination of *L. monocytogenes* culture isolates

The *L. monocytogenes* culture isolates obtained from RTE and human stool samples were subjected to a multiplex PCR specific for *L. monocytogenes* speciation and virulence determination.

Genomic DNA extraction: For each suspected *L. monocytogenes* isolate, a 10 ml culture was grown in Tryptose soya broth, and 1 ml of cells was pelleted by centrifugation at 10,000 rpm for 30 s. The cell pellets were re-suspended in 200 µl physiological saline. The re-suspended cells were re-centrifuged at 10,000 rpm for 30 s. The cell pellets were then used for DNA extraction using bacterial DNA extraction kit (spin-column) (BioTeke Corporation, DP2001) as recommended by the manufacturers. Protocol for Gram-positive bacteria, cellular lyses, was carried out by enzymatic fragment with lysozyme. DNA samples were stored at −20°C until used.

Primers used: The oligonucleotide primers targeting *L. monocytogenes* internalin genes *inIA* (intended for species-specific recognition) and the *inIC* and *inIJ* (intended for virulence determination of *L. monocytogenes*) as mentioned by Liu *et al.,* [13] were used in this study. Primer sequences used are listed in Table-1.

Multiplex PCR steps for amplification of the target genes

Multiplex PCR was conducted in primus thermocycler (MWG-Biotech) in a volume of 25 µl containing 0.8 U Taq DNA polymerase (Fisher Scientific, Houston, TX), ×1 PCR buffer, 200 µM dNTPs and 10 ng each Listeria DNA, together with 40 pmol each *inIA*, 30 pmol each *inIC* and 20 pmol each *inIJ* primers. Reaction mixture with no DNA template and known *L. monocytogenes* isolate were incorporated as negative and positive control, respectively in each run. The cycling program consisted of ×1 94°C for 2 min; ×30 94°C for 20 s, 55°C for 20 s, and 72°C for 50 s; and ×1 72°C for 2 min [13].

Gel electrophoresis: All amplification products were resolved in 1.25% agarose gel, stained with ethidium bromide, detected under Ultraviolet Transilluminator (Spectroline). The 100 bp ladder was used as a molecular size marker.

Results

Table-2 shows that out of 120 RTE meat samples examined by culture, 11 samples (9.16%) were

Table-1: Oligonucleotide sequences of the primers used in multiplex PCR assay for *L. monocytogenes*.

Target gene	Coding sequences	Primer sequences (5-3)	Nucleotide positions	Expected PCR product (bp)
inIA	94534-96936	ACGAGTAACGGGACAAATGC	94612-94631	800
		CCCGACAGTGGTGCTAGTTT	95411-95392	
inIC	107200-108090	AATTCCCACAGGACACAACC	107306-107325	517
		CGGGAATGCAATTTTTCACTA	107822-107802	
inIJ	188153-190708	TGTAACCCCGCTTACACAGTT	188989-189009	238
		AGCGGCTTGGCAGTCTAATA	189226-189207	

PCR=Polymerase chain reaction, *L. monocytogenes=Listeria monocytogenes*

Table-2: Suspected *L. monocytogenes* isolates detected in RTE meat products and human stool samples.

Source	Number of analyzed samples	Number of positive	% of positive
Beef luncheon	40	6	15
Chicken luncheon	40	4	10
Frankfurter beef	40	1	2.5
Total	120	11	9.16
Human stool	40	1	2.5

RTE=Ready-to-eat, *L. monocytogenes=Listeria monocytogenes*

positive for the presence of suspected *L. monocytogenes* isolates. These 11 isolates were; 6 from beef luncheon (15%), 4 from chicken luncheon (10%), and 1 from frankfurter beef (2.5%). Suspected *L. monocytogenes* isolate was detected in only one (2.5%) stool sample out of the 40 human stool samples. Statistical analysis revealed no significant difference between the occurrence of *L. monocytogenes* in different food samples examined ($p>0.05$).

Table-3 and Figure-1 show the results of multiplex PCR for speciation and virulence determination of the suspected *L. monocytogenes* isolates obtained from the 11 RTE meat samples and one human stool sample. Species specific gene (*inIA*, 800 bp) of *L. monocytogenes* and the virulence genes (*inIC* and *inIJ* that have molecular weight of 517 and 238, respectively) were detected in two suspected *L. monocytogenes* isolates out of six from beef luncheon (5%) and one isolate out of four from chicken luncheon (2.5%). On the other hand, suspected *L. monocytogenes* isolates from one frankfurter beef, and one human stool were negative by multiplex PCR (0%). All samples positive for *inIA* (Species identity gene) were also positive for the presence of *inIC* and *inIJ* (virulence specific genes).

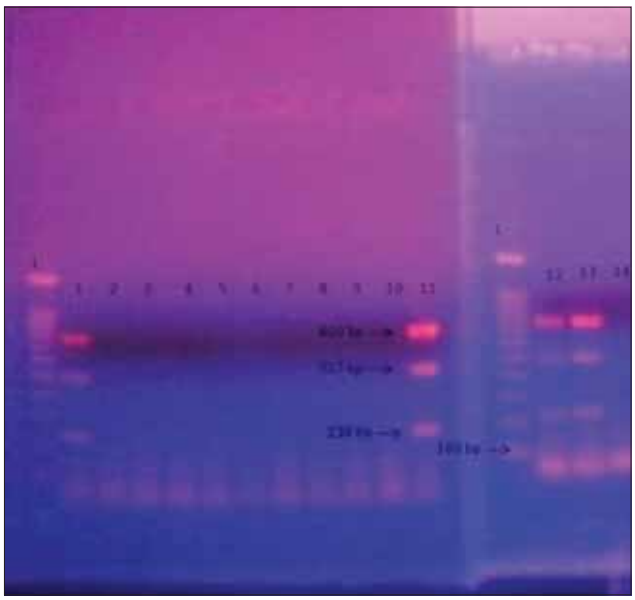

Figure-1: Agarose gel electrophoresis of multiplex polymerase chain reaction products obtained from representative suspected *L. monocytogenes* culture isolates. Lanes (source): L (100 bp ladder); 1 (Positive control); 2 (HS); 3, 9, 11, 4 (CL); 5, 10, 7, 8, 12, 13 (BL); 10 (CL); 6 (FB); 14 (Negative control). HS: Human stool, CL: Chicken luncheon, BL: Beef luncheon, FB: Frankfurter beef.

Discussion

The primary vehicle for *Listeria* infection is food. Normally, meat and meat products, vegetables, fish, dairy products, minimal processed food and RTE foods are potential sources of transmission [14]. The overall prevalence of *L. monocytogenes* in all RTE meat products examined (9.16%) in the current study agreed with previous studies showing that prevalence of *L. monocytogenes* in RTE meat products may vary from 0 to 72%, with different level of CFU per gram up on expiry of the shelf life [15]. Lower prevalence of 3.4, 1.2 and 1.5% in meat product samples were previously reported in Czech Republic [16], Sweden [17], and Jordon [18], respectively.

L. monocytogenes was previously detected in 12 and 17% of beef luncheon and chicken luncheon examined in Egypt [19]. They attributed the higher prevalence of *L. monocytogenes* to the nature of samples they examined. Their samples were collected from street vendors lacking refrigeration facilities, sources of running water, personal hygiene and public toilets. A lower prevalence of *L. monocytogenes* in beef luncheon was previously reported by Gombas *et al*., [20]. A higher prevalence (11%) of *L. monocytogenes* was previously recorded in frankfurter beef samples in Sweden [21].

In the current study, the prevalence of suspected *L. monocytogenes* was higher in RTE meat samples repacked at local shops (beef and chicken luncheon) than those purchased in their original intact packing (frankfurter beef). These results agreed with Gelbicova and Karpiskova, [16] who found 83% of the positive samples originated from sliced products. They stated that the undesirable bacterial contamination of meat

Table-3: Multiplex PCR for speciation and virulence determination of 12 suspected *L. monocytogenes* isolates using internalin genes primers (*inIA, inIC, and inIJ*).

Source	Number of isolates	Multiplex PCR positive isolates(%)		
		Isolates containing *inIA* (800 bp)	Isolates containing *inIC* (517 bp)	Isolates containing *inIJ* (238 bp)
Beef luncheon	6	2 (5)[a]	2 (5)	2 (5)
Chicken luncheon	4	1 (2.5)	1 (2.5)	1 (2.5)
Frankfurter beef	1	0 (0)	0 (0)	0 (0)
Total	11	3 (2.5)	3 (2.5)	3 (2.5)
Human stool	1	0 (0)	0 (0)	0 (0)

[a]The percentage of samples positive by PCR to total examined. PCR=Polymerase chain reaction, *L. monocytogenes=Listeria monocytogenes*

products could occur either directly in the manufacturing process or as a result of subsequent handling, storage or distribution. Moreover, Garrido *et al.*, [22] in Spain reported higher incidence in in-store-packaged products (8.5%) as compared to products packaged by manufacturer (2.7%). They suggested that the slicing equipment could be a source for the pathogen dissemination.

In healthy adults, consumption of *L. monocytogenes* contaminated food usually causes only a short period of shedding without any illness; however, in the high risk groups (pregnant, newborns, the elderly, and adults with weakened immune system) oral exposure can lead to listeriosis. Asymptomatic fecal carriage occurs in approximately 3% of healthy human, with point prevalence increasing to 26% in high-risk groups [23]. *L. monocytogenes* was found to be positive in 1.15% (10 out of 868) and 2% (1/50) of stool samples in Austria [24] and Egypt [25]. Grif *et al.*, [24] attributed the lower isolation rate of *L. monocytogenes* from human stool samples to the secretion of gastric acid that provides an important protective factor against the passage of potentially pathogenic organisms. A drastically increased prevalence of *L. monocytogenes* in the stool of patients receiving long-term H2 blockers was shown compared to the prevalence in patients with normal gastric secretion [26].

The standard microbiological methods for identification of *Listeria* spp. are laborious, and time-consuming, requiring a minimum of 5 days to recognize *Listeria* spp and about 10 days to identify *L. monocytogenes* by confirmation test [27]. The wide application of nucleic acid amplification techniques and the increasing industrial interest toward rapid and sensitive method has led to the development and application of PCR based methods for the detection of microbial pathogen in food [28]. Typing of *L. monocytogenes* is necessary when performing epidemiological investigations [29].

Species specific gene (*inIA*, 800 bp) of *L. monocytogenes* and the virulence genes (*inIC* and *inIJ* that have molecular weight of 517 and 238, respectively) were detected in two suspected *L. monocytogenes* isolates out of six from beef luncheon and one isolate out of four from chicken luncheon obtained during this study. On the other hand, suspected *L. monocytogenes* isolates obtained from one frankfurter beef, and one human stool were found to be negative by multiplex PCR. As it was illustrated in Tables-2 and 3, there were a difference between the prevalence of *L. monocytogenes* in RTE meat products and human stool depending on the technique used for detection. Difference in the prevalence rate of *L. monocytogenes* in the examined RTE meat using bacteriological examination compared to PCR technique were previously recorded in USA [11] and Egypt [30]. Similar difference was recorded in the examined human stool samples. Abdel-Malek *et al.*, [30] detected suspected *L. monocytogenes* isolates in two human stool samples out of 28 examined by culture. Upon confirmation using PCR technique using *Prf A* (transcriptional activator of the virulence factor gene of), the two isolates were not *L. monocytogenes* but were found to be *L. seeligeri* and *L. murray* (1 isolate, each). *L. monocytogenes* isolated from raw meat samples were positive to some virulence genes such as *act*A, *fla*A, *hly*A, *iap*, *inl*A and *inl*B [31]. The difference in the results between bacteriological examination and PCR technique may be due to false positive results with bacteriological examination. Some bacteria have cultural morphology similar to that of *L. monocytogenes*. *Staphylococcus aureus*, a *Listeria* like organism, and *Enterobacter faecium* can cause false positive results in conventional test, and occasionally showed slight discoloration of the selective broth and similar colonies on Oxford agar [11].

The results of this study reveal a potential public health hazard linked to the consumption of RTE meat products and highlights the need for more effective and efficient processing and storage to keep such products safe for consumption. The multiplex PCR used in this study could be a sensitive and rapid procedure for the specific detection of the virulent strain of *L. monocytogenes.* To our knowledge this is the first attempt in Egypt using multiplex PCR to confirm the prevalence of a virulent strain of *L. monocytogenes* in RTE foods. This assay could be of great importance in the epidemiological investigations in the case of food borne illnesses and trace back for the source of contamination. The data suggest a need to improve consumer education regarding food processing and storage practices in Egypt. The importance of storing

RTE meat at low temperatures (≤4°C) and taking into account the maximum storage time before consumption should be emphasized in consumer education.

Conclusion

It could be concluded that *L. monocytogenes* are circulating in beef and chicken luncheon sold in Cairo, Egypt. Multiplex PCR is reliable for confirmation of *L. monocytogenes*. This study suggests the implementation of hygienic measures at all levels from production to consumption in order to improve food safety. Furthermore, authors recommended consumption of beef frankfurter or any RTE meat sold in their original intact packing due to low level of contamination.

Authors' Contribution

AMAI: Designed the study and collected RTE meat product samples & human stools, shared in the practical part in the laboratory. SIIA shared in the practical part in the laboratory and collection of papers. AMAI drafted and revised the manuscript. Both authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing of interests.

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