



Prevalence and phylogenetic characterization of *Listeria monocytogenes* isolated from processed meat marketed in Egypt

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Received 1 April 2016; revised 22 May 2016; accepted 5 June 2016

Available online 18 June 2016

KEYWORDS

L. monocytogenes;
Processed meat;
Egypt;
Phylogenetic analysis

Abstract Because of its high case fatality rate, listeriosis locates among the most frequent causes of death due to food-borne illness. In this study, a total of 150 processed meat samples were collected from Giza Governorate, Egypt. Phenotypic and genotypic identification of *Listeria monocytogenes* was performed using PCR incorporating listeriolysin O virulence gene *hlyA* followed by DNA sequence analysis. *L. monocytogenes* was confirmed in 4% of each of beef burger, minced meat, and luncheon samples. Phylogenetic analysis showed that all the six Egyptian isolates have high homology with Colombian isolate (EF030606), except one Egyptian isolate which showed high homology with Indian isolate (EU840690). The public health significance of these pathogens as well as recommended sanitary measures were discussed.

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1. Introduction

The genus *Listeria* includes only two pathogenic strains, *Listeria monocytogenes* is a well-known cause of abortion, encephalitis and septicemia in man and animals. *Listeria ivanovii* is of major veterinary importance as a cause of

abortions, still births and neonatal septicemias in sheep and cattle, and a rare human infection [24].

L. monocytogenes has become remarkably important as a food-borne pathogen. The ability to persist in diverse conditions such as low temperature and pH, high salt concentrations and multiplication under refrigeration temperatures makes it a serious threat to public health [7,12]. Because of its high case fatality rate, listeriosis ranks among the most frequent causes of death due to food-borne illness with the highest hospitalization rates (91%) and mortality rate up to 30% [13].

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Peer review under responsibility of National Research Center, Egypt.

<http://dx.doi.org/10.1016/j.jgeb.2016.06.001>

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The detection of this pathogen in food by routine culture methods is difficult by the sporadic or low levels of contamination, by the presence of a high level of background microflora [17]. Moreover, these methods are laborious and time consuming [1], while immediate action should be taken in case of contamination especially in the case of foods having short shelf-lives, such as meat or dairy products. In general, DNA-based typing approaches are recognized as simple and cost-effective methods that have better discriminatory power than phenotypic approaches and being better for investigating *L. monocytogenes* outbreaks [5]. The classification based on the nucleic acid sequence has become more common. The gene *hly* encoding listeriolysin O is one such target gene for specific detection of *L. monocytogenes* [21].

Molecular fingerprinting is by far considered the most precise method for studying the epidemiology of foodborne diseases and allows prediction of the relationships between *Listeria* isolates, regardless of the origin or the geographical location [9].

Usually, *L. monocytogenes* is susceptible to a wide range of antibiotics, but resistance to multiple antibiotics was recorded [18]. The presence of multiple key virulence factors (Virulence markers) such as listeriolysin O (LLO encoded by *hlyA*) significantly regulates the virulence and pathogenicity of *L. monocytogenes* [21]. Moreover, food and host environments may present variable expression of virulence genes, resulting in varied infectivity [4]. Therefore, it is essential to study the epidemiological significance and distribution of virulence determinants within these isolates [23]. Thus the aim of this study was to determine the prevalence and phylogenetic characterization of *L. monocytogenes* isolated from processed meat collected from markets in Egypt.

2. Materials and methods

2.1. Samples

A total of 150 meat samples were collected from Giza Governorate over the period of October 2013 to September 2014. These samples included 25 minced meats, 25 luncheons, 50 sausages, 50 beef burger.

2.2. Isolation and identification of *L. monocytogenes*

L. monocytogenes was isolated from the examined food samples according to the International Organization for Standardization procedure [11]. Suspected colonies were transferred to tryptic soya agar plates with 0.6% yeast extract (TSA-YE) for further biochemical identification using *Listeria* Microbact 12L (Oxoid, UK). For DNA extraction, colonies were suspended in 500 µl of PBS, pH 7.2, washed 3 times in PBS. The cell suspension was centrifuged for 10 min at 800g, then the supernatant was discarded carefully and the pellet was dried and stored at -20 °C till use.

2.3. Polymerase chain reaction (PCR) analysis and DNA sequencing

2.3.1. DNA extraction

For extraction of DNA, bacterial pellets were re-suspended with 200 µl PBS. DNA was extracted from *L. monocytogenes*

isolates using the DNA extraction Kits (GF-1, Vivantis Co., Malaysia) according to manufacturer's instructions.

2.3.2. PCR

This PCR amplifies a 234 bp region of the *hlyA* gene (*q*-Hemolysin, listeriolysin O) encoding listeriolysin O. PCR was performed according to Furrer et al. [8]. 50 µl volume containing 2 µl of each primer (10 µM), 25 µl of 2X Taq Master Mix (Cat. No. PLMM01, Vivantis Co., Malaysia), Primers, LMA: CGGAGG TTCC GCAAAGATG and LMB: CCTCCAGAGTGATCGATGTT. Polymerase chain reaction (PCR) amplification conditions were: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 45 s at 55 °C, 45 s at 72 °C and a final extension of five min at 72 °C. The PCR products were analyzed using 1% agarose gel electrophoresis.

The positive PCR products were then sequenced in MACROGEN Company (Korea) on 3730 × L sequencers (Applied Biosystem, USA). The accuracy of data was confirmed by two-directional sequencing with the forward and reverse primers used in PCR.

The nucleotide sequences obtained in this study were analyzed using the BioEdit 7.0.4.1 and ClustalW2 (<http://www.clustal.org/>) programs. The resulting sequences were aligned with *hlyA* gene of reference sequences of *Listeria* spp. using a neighbor-joining analysis of the aligned sequences implemented in the program CLC Sequence Viewer 6.

3. Results and discussion

Contamination of the meat with *L. monocytogenes* generally occur after the slaughter and may come from the skin of the animals, the hands of the workers, the equipment and the tools used [16]. 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 favor the persistence of *L. monocytogenes* [2].

In the present study, PCR results showed that *L. monocytogenes* were confirmed in 8 out of 25 of beef burgers examined (4%), one out of 25 of minced meat (4%), and one out of 25 of Luncheon (4%) from the total 100 examined food samples. The sausage samples were totally negative.

This suggests the presence of a significant public health hazard linked to the consumption of this meat sold in Giza Governorate contaminated with *L. monocytogenes*.

3.1. Nucleotide sequence accession numbers

Six sequences PCR samples (Giza 1–6) used in this study have been deposited in the Gene Bank database under accession no: KR812472, KR812473, KR81274, KR534875, KR812475 and KR812476 respectively. Phylogenetic analysis showed that all the six Egyptian isolates have high homology with Colombian isolate (EF030606), except one Egyptian isolate which showed high homology with Indian isolate (EU840690). This may be due to the importation of animals and raw meat from the Latin America and India. This suggests the presence of a significant public health hazard linked to the consumption of this meat sold in Egypt contaminated with *L. monocytogenes*.

From Fig. 1, the differences in the sequences of the six Egyptian isolates (Giza 1–6) which we examined in comparison with the other Gene Bank isolates from different isolation

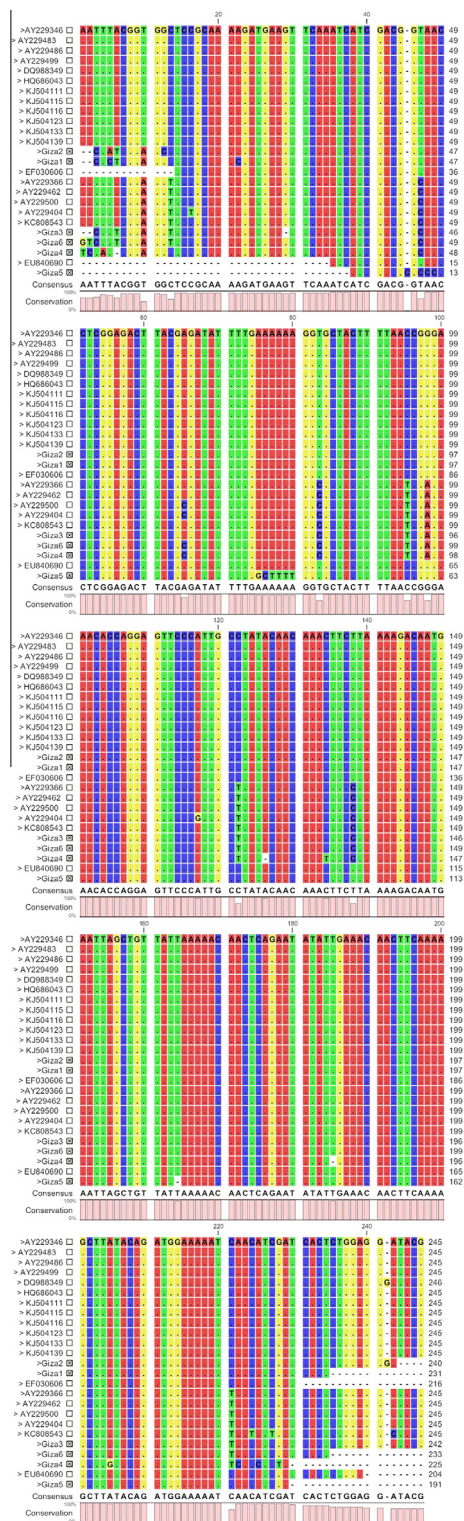


Figure 1 Sequence alignment of *Listeria monocytogenes* (present study) isolated from processed meat (Giza 1–6) with the closely related isolates from different sources. Only variable sites are shown with different color. Dashes in the middle indicate gaps.

sources depending on the analysis of the *hlyA* gene may reflect sequencing artifacts or may be true differences. Sallen et al. [20] reported the common presence of sequence polymorphism between different isolates of the same *Listeria* species.

DNA sequencing of representative samples confirmed the identification of the *Listeria* species. Blasting the obtained sequences with those in database and the deduced phylogenetic analysis (Figs. 2 and 3) were induced. Clear sequences of *hlyA* gene were obtained from the 6 isolates. Homology results (98–99%) proved that the six isolates were *L. monocytogenes*. The neighbor-Joining (NJ) phylogenetic analysis based on the *hlyA* gene (Fig. 2) showed that all the six Egyptian isolates were clustered with relevant sequences of *L. monocytogenes* EU840690 and EF030606 isolates in the same group (Lineage 1) as shown in Fig. 2.

Concerning the geographical distribution (Fig. 2), *hlyA* gene analysis of our isolates in comparison with other *L. monocytogenes* isolates from India, Colombia, Brazil and USA showed that all the Egyptian isolates have high homology with Colombian isolate (EF030606), Except Egypt 5 isolate which showed high homology with Indian isolate (EU840690). This may be due to the importation of animals and raw meat from the Latin America and India.

This was confirmed by Phylogenetic analysis based on the *hly* gene of *L. monocytogenes* applied by Headley et al. [10] which revealed the identification of *L. monocytogenes* isolates from cattle and small ruminants in Brazil with encephalitic listeriosis. This finding is of significant importance to understand the epidemiology of *L. monocytogenes* in Egypt.

The phylogenetic tree based on *hlyA* gene sequence clearly differentiates between the *L. monocytogenes*, *Listeria innocua*, *L. ivanovii* and *Listeria seeligeri*. It was noticed from Fig. 2 that our isolates were in a different cluster (lineage I) from that of the other *Listeria* spp. as shown in Lineage III.

This was different when using the 16S rRNA gene for phylogenetic analysis as reported by Soni and Dubey [21] and Soni et al. [22], that noticed that due to the close relationship (> 99%) between the members of *Listeria* species, other *Listeria* sp. (*L. innocua*, *L. ivanovii*, *L. seeligeri* and *Listeria welshimeri*) are also clustered in same *L. monocytogenes* group because of the highly conserved nature of the 16S rRNA gene among its species.

In order to ascertain the presence of pathogens in their respective environment, the detection of one of the major virulence factors is a better option. Among the various virulence factors, LLO (a 58 kDa hemolysin protein encoded by *hly* gene) is the main virulence factor and pathogenic marker for the detection of *Listeria* sp. [3].

In the present study, we identified 6 isolates of *L. monocytogenes* originating from processed meat based on *hlyA* gene sequence. On the basis of sequence similarity, all the Egyptian Isolates (except Egypt 5) were found to be closely related to *L. monocytogenes* isolate EF030606, isolated from human clinical samples and from food processing plants and food (Hams and chicken) in Colombia while Egyptian isolate5 was closely related to isolate EU840690 separated from raw and packaged meat and meat products in India as shown in the lineage (4) of phylogenetic tree (Fig. 3). The details of isolate, strain isolation source, country and accession numbers are shown in Table 1.

The existence of the isolate KC808543 in separate group (cluster1) may be due to that it was isolated from swine not bovine slaughter house, while cluster 2 was isolated from human clinical samples and poultry meat. Finally, the cluster 3 was isolated from different sources (clinical samples, river water, poultry meat, cow's milk and vegetables). This explains

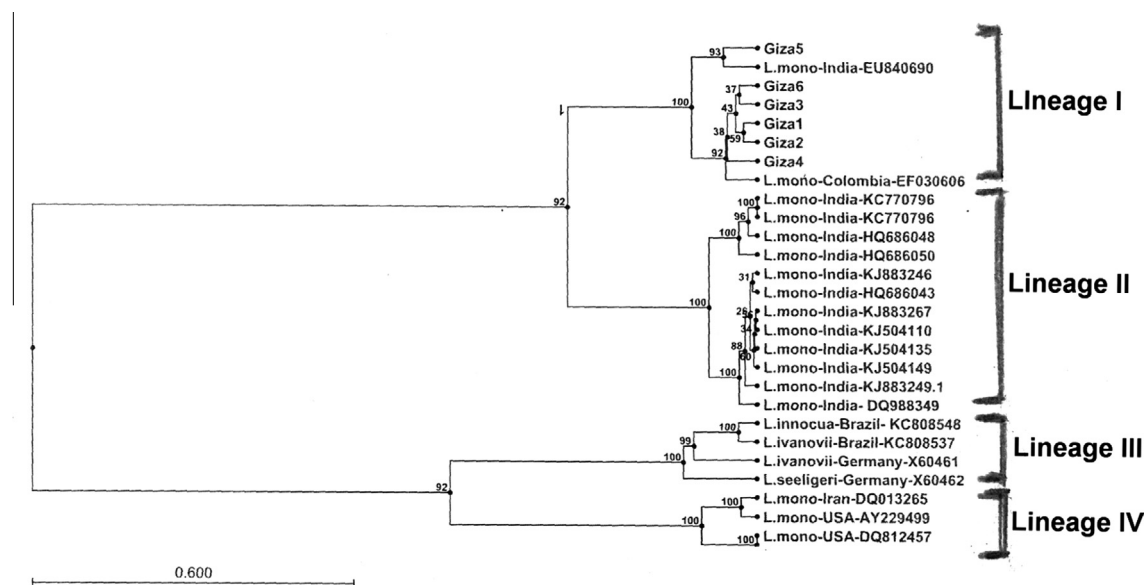


Figure 2 Phylogenetic relationship of selected strains of *Listeria* spp. from different geographical areas, representing the four distinct lineages, based on the listeriolysin gene. The Gene Bank accession numbers of the isolates used are given.

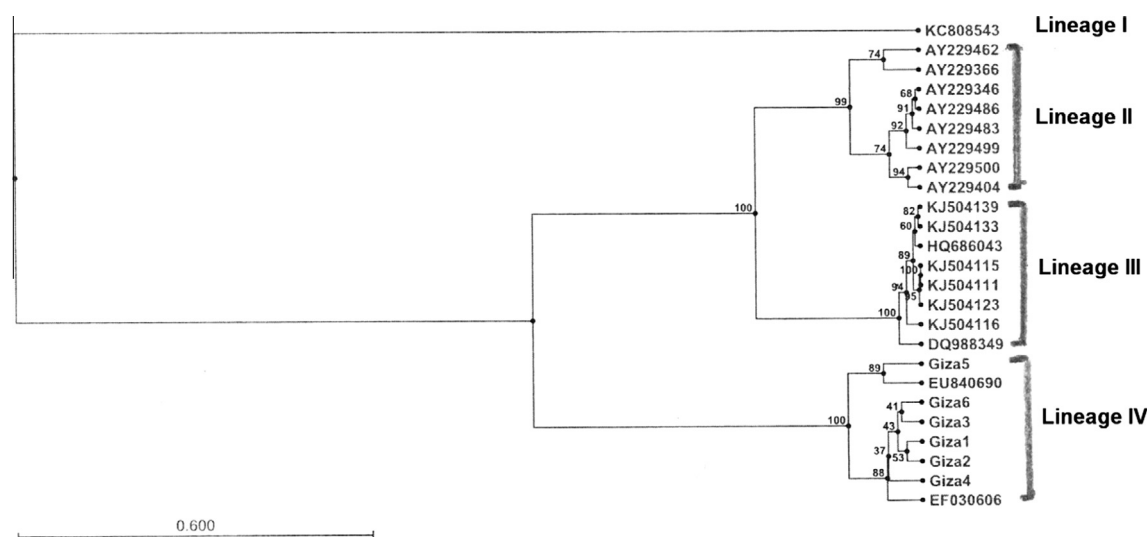


Figure 3 Phylogenetic relationship of selected strains of *Listeria* spp. from different sources, representing the four distinct lineages, based on the listeriolysin gene. The Gene Bank accession numbers of the isolates used are given.

why our isolates were grouped in the cluster 4 which were isolated from raw, packaged meat and food processing plants.

However, we have found that strains from lineage 4 may be more likely to survive thermal inactivation than strains from the other three lineages of *L. monocytogenes*, indicating that lineages 4 may be better adapted to the food-processing environment than the other three lineage isolates, this was agreed by De Jesus and Whiting [6], which explains that with the development of unique ecological adaptations for these isolates such as enhanced psychrotolerance and growth at refrigeration temperatures.

The close relation between our isolates and Colombian isolate (EF030606) that was isolated from some human clinical samples demonstrate the close relationship between the disease in human and animals. Finally this proof that our Egyptian

isolates are more frequently isolated in food-borne epidemics of listeriosis and in sporadic cases of human and animal listeriosis. This was agreed by Liu et al. [14] and Roberts et al. [19].

Finally, in order to minimize human listeriosis, foods should be cooked to an internal temperature of 70 °C for more than 20 min 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 as reported by Mahmoud et al. [15].

In conclusion, the hlyA gene sequence information could be the reliable option to indicate the presence of *L. monocytogenes*, tracking the source of infection and identifying the geographical distribution and divergence. More work, however, is

Table 1 Details of *L. monocytogenes* isolates used in the present study with the available ones in Gene Bank from different habitats and country.

Ser. no	Isolate	Strain	Serotype	Source of Isolation	Country	Access number
1	Giza 1	<i>L. monocytogenes</i>	–	processed meat	Egypt	KR812472
2	Giza 2	<i>L. monocytogenes</i>	–	processed meat	Egypt	KR812473
3	Giza 3	<i>L. monocytogenes</i>	–	processed meat	Egypt	KR812474
4	Giza 4	<i>L. monocytogenes</i>	–	processed meat	Egypt	KR534875
5	Giza 5	<i>L. monocytogenes</i>	–	processed meat	Egypt	KR812475
6	Giza 6	<i>L. monocytogenes</i>	–	processed meat	Egypt	KR812476
7	72	<i>L. monocytogenes</i>	4d	Clinical and environmental (poultry meat)	USA	AY229346
8	57	<i>L. monocytogenes</i>	3b	Clinical and environmental (poultry meat)	USA	AY229366
9	193	<i>L. monocytogenes</i>	3a	Clinical and environmental (poultry meat)	USA	AY229404
10	126	<i>L. monocytogenes</i>	1/2b	Clinical and environmental (poultry meat)	USA	AY229462
11	101	<i>L. monocytogenes</i>	1/2c	Clinical and environmental (poultry meat)	USA	AY229483
12	88	<i>L. monocytogenes</i>	4a	Clinical and environmental (poultry meat)	USA	AY229486
13	168	<i>L. monocytogenes</i>	4b	Clinical and environmental (poultry meat)	USA	AY229499
14	172	<i>L. monocytogenes</i>	1/2a	Clinical and environmental (poultry meat)	USA	AY229500
15	–	<i>L. monocytogenes</i>	–	Poultry meat	India	DQ988349
16	LMO6	<i>L. monocytogenes</i>	–	Human clinical samples and from food processing plants and food (Hams and chicken)	Colombia	EF030606
17	62TP_BRSL3	<i>L. monocytogenes</i>	–	Raw and packaged meat and meat products	India	EU840690
18	W1	<i>L. monocytogenes</i>	–	River gangs water	India	HQ686043
19	Lm4	<i>L. monocytogenes</i>	–	Swine slaughter house & meat market	Brazil	KC808543
20	Pb1	<i>L. monocytogenes</i>	–	Human placental bit	India	KJ504111
21	VS1	<i>L. monocytogenes</i>	–	Human vaginal swab	India	KJ504115
22	M2	<i>L. monocytogenes</i>	–	Cow milk	India	KJ504116
23	VB1	<i>L. monocytogenes</i>	–	Vegetables	India	KJ504123
24	VT1	<i>L. monocytogenes</i>	–	Vegetables	India	KJ504133
25	VC1	<i>L. monocytogenes</i>	–	Vegetables	India	KJ504139

required on *L. monocytogenes* in order to ascertain its presence in clinical and environmental samples in Egypt as there is limited information available on such aspects for tropical countries including Egypt. The molecular technologies such as the next generation sequencing are likely to be helpful in rapid acquisition of sequence data to facilitate detection and characterization of other pathogenic strains of *L. monocytogenes*.

Acknowledgements

The authors wish to express their gratitude to the National Research Centre, Cairo, for providing and financial support of this study.

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