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# Rabbit Meat as a Possible Source for Multidrug Resistant *Listeria monocytogenes*

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## Abstract

Rabbit meat is a rich source of protein with low fat and cholesterol contents making it a healthy meat source. *Listeria monocytogenes* is one of foodborne pathogens that can cause a serious disease named listeriosis in humans. This study firstly investigated the prevalence of *Listeria spp*., particularly *Listeria monocytogenes* in rabbit meat and offal. Secondly, the expression of virulence associated genes and the antibiogram of the identified *Listeria monocytogenes* were further examined. The achieved results revealed that *Listeria spp*. was isolated from rabbit thigh muscles, shoulder muscles, loin, liver and kidneys at 25%, 15%, 10%, 15% and 5%, respectively. Five *Listeria spp*. namely, *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri* and *L. seeligeri* were serologically identified. Multidrug pathogenic *L. monocytogenes* was particularly isolated from thigh muscles and loin only at 12.5% and 7.5%, respectively. *L. monocytogenes* isolates showed a complete resistance (100%) to both of kanamycin and neomycin.

**Keywords:** *Listeria monocytogenes*; Rabbit meat; Antibiogram; Virulence genes

## Introduction

Rabbit meat industry is developing in many Middle-Eastern countries including Egypt as rabbits are characterized by the short production cycle, high feed conversion ratio, high fertility rates, and require small space area [1]. Rabbit meat is also of high protein and low fat and cholesterol levels making it very healthy meat source [2]. However, rabbit meat like any kind of meat might be contaminated with potential pathogenic microorganisms during slaughtering, processing, evisceration or transportation [3]. Therefore, rabbit meat may be considered as a possible source for transmission of bacterial foodborne pathogens such as *Salmonella* spp., *Escherichia coli* and *Staphylococcus aureus* [4]. However, few reports studied the microbial status of rabbit meat, particularly in Egypt.

*Listeria* spp. is considered of major public health significance, in particular the most important pathogenic species, *L. monocytogenes* [5]. *Listeria monocytogenes* is a widespread foodborne pathogen, which is able to grow and survive over a wide range of temperatures, pH and water activities making it among the most serious threats to human health [6,7]. Almost 99% of human listeriosis has resulted from consumption of contaminated foods [8]. The symptoms of human listeriosis include fatigue, chills, headache, and gastroenteritis. If not properly treated, the disease can develop into septicemia, abortion, meningitis, encephalitis and finally death [9]. Therefore, continuous monitoring for the prevalence rates of *L. monocytogenes* in retailed meat and offal in Egypt is a matter of importance for both consumer's safety and food hygiene.

The extensive use of antibiotics in animal farms including rabbit intensive rearing systems is continuous in Egypt for the prevention and control of bacterial diseases and as animal feed additives, however, the uncontrolled irregular use of such antibiotics may result in development of drug-resistant pathogens, which make the treatment of such bacterial diseases in diseased humans and animals of high difficulty [10].

In sight of the previous facts, this study aimed at investigation of the prevalence of multidrug-resistant *Listeria spp*. in the retailed rabbit meat and offal in Egypt. The antibiogram and the expression of virulence-associated genes in the isolated and identified *L. monocytogenes* were also carried out.

## Materials and Methods

### Collection of samples

A total of 40 random rabbit carcasses were collected from rabbit butchery shops at different sanitation levels in Zagazig city, Sharkia Governorate, Egypt. From each rabbit carcass, thigh muscles, shoulder muscles, loin, liver and kidney (n=40 each) were collected. The collected samples were defined and packed in sterile plastic bags then labeled and immediately transferred under sanitary precautions in an icebox without undue delay to the Laboratory of Meat Hygiene and Technology, Faculty of Veterinary Medicine, Zagazig University, Egypt. The collected samples were examined bacteriologically for the presence of *Listeria* monocytogenes.

### Bacteriological examination

**Isolation and identification of Listeria** spp: Detection and enumeration of *Listeria* monocytogenes in the examined samples were done according to the methods described before [11].

**Enrichment procedures:** Under complete aseptic conditions, 10 grams from each sample were aseptically transferred into a sterile

blender containing 90 ml of sterile peptone water 1%. The contents were homogenized at 3000 rpm for 3 min at 25°C, then allowed to stand for 5 min. The homogenate was incubated at 37°C for 24 h for pre-enrichment of the samples. After incubation, 1 ml of the culture was transferred into a tube containing 9 ml of secondary enrichment medium (Full Fraser broth), then incubated at 37°C for 48 h.

**Isolation procedures:** A loopeful from the Full Fraser broth culture was streaked onto Oxford media (Himedia, Mumbai, India) with *Listeria* Oxford supplement (Himedia, Mumbai, India) and incubated for 24-48 h at 35°C and then observed for the presence of typical *Listeria* colonies. Colonies presumptive for *Listeria* spp. (showing morphological characters as dew drop-like, black with brown hallow, or dark brown colonies 1-2 mm in diameter) were inoculated into Tryptone Soya broth supplemented with 0.6% yeast extract and kept at 4°C for further identification.

**Identification of Listeria isolates:** Pure presumptive isolates were identified morphologically and biochemically [12,13] and serologically using the Oxoid *Listeria* Test Kit (Oxoid, Basingstoke, Hampshire, England) according to the manufacturer's instructions.

## Molecular identification of *Listeria monocytogenes* virulent genes

Application of multiplex-PCR for molecular characterization of virulence factors of the isolated *L. monocytogenes* strains represented by invasive associated protein (iap), haemolysin (hylA) and actin polymerization protein (actA) genes was essentially performed using Primers (Pharmacia Biotech) as shown in Table 1.

| Target gene | Oligonucleotide sequence (5′ → 3′) | Product size (bp) |
|---|---|---|
| iap (F) | 5′ ACAAGCTGCACCTGTTGCAG ′3 | 131 |
| iap (R) | 5′ TGACAGCGTGTGTAGTAGCA ′3 | |
| hlyA (F) | 5′ GCAGTTGCAAGCGCTTGGAGTGAA ′3 | 456 |
| hlyA (R) | 5′ GCAACGTATCCTCCAGAGTGATCG ′3 | |
| actA (F) | 5′ CGCCGCGGAAATTAAAAAAAGA ′3 | 839 |
| actA (R) | 5′ ACGAAGGAACCGGGCTGCTAG ′3 | |

**Table 1:** Oligonucleotide primer sequences used in the present study.

## DNA extraction using QIA amp kit

The technique recommended by Shah et al. [14] was applied with some modifications. All detected *L. monocytogenes* strains were grown overnight on brain heart infusion broth at 37°C, and the suspension was then heated at 100°C for 20 min. Accurately, 50-200 µl of the culture were placed in Eppendorf tube and kept frozen at -40°C till use. The obtained lysate (5 µl) was used as DNA template in PCR reaction mixture.

## Amplification reaction of *L. monocytogenes*

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). A multiplex PCR was performed with comprising three virulence-associated genes (iap, hlyA and actA). The multiplex PCR was set up in 50 µl reaction volume. The cycling conditions for PCR included an initial denaturation of DNA at 95°C for 2 min followed by 35 cycles each of 15 sec denaturation at 95°C, 30 sec annealing at 60°C and 1 min extension at 72°C, followed by a final extension of 10 min at 72°C and held at 4°C. Amplified DNA fragments were analyzed by 1.5% of agarose gel electrophoresis (Applichem, Germany, GmbH) in 1x TBE buffer stained with ethidium bromide and captured as well as visualized on UV transilluminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

| Antimicrobial agent | Sensitivity disc content (µg) | Resistant (mm) | Intermediate (mm) | Susceptible (mm) |
|---|---|---|---|---|
| Neomycin (N) | 30 | 12 or less | 13-16 | 17 or more |
| Ampicillin (AM) | 10 | 13 or less | 14-17 | 18 or more |
| Chloramphenicol (C) | 30 | 12 or less | 13-17 | 18 or more |
| Ciprofloxacin (CP) | 5 | 15 or less | 15-19 | 20 or more |
| Erythromycin (E) | 15 | 13 or less | 14-22 | 23 or more |
| Cephalothin (CN) | 30 | 14 or less | 15-17 | 18 or more |
| Gentamicin (G) | 10 | 12 or less | 13-14 | 15 or more |
| Enrofloxacin (EN) | 5 | 11 or less | 12 | 13 or more |

| | | | | |
|---|---|---|---|---|
| Kanamycin (K) | 30 | 13 or less | 14-17 | 18 or more |
| Oxacillin (OX) | 1 | 10 or less | 11-Dec | 13 or more |
| Streptomycin (S) | 10 | 11 or less | Dec-14 | 15 or more |
| Oxytetracycline (T) | 30 | 14 or less | 15-18 | 19 or more |
| Nalidixic acid (NA) | 30 | 13 or less | 14-18 | 19 or more |
| Sulphamethoxazol (SXT) | 25 | 10 or less | Nov-15 | 16 or more |

**Table 2:** Antimicrobial discs, concentration and interpretation of the reactions on the isolated pathogens.

### Antibiogram of the isolated *Listeria monocytogenes*

Antimicrobial susceptibility was tested by the single diffusion method. Sensitivity discs with variable concentrations were used to determine the susceptibility of the isolated bacterial strains (Oxoid Limited, Basingstoke, Hampshire, UK) (Table 2). Agar plate method was applied by using of nutrient agar as a substrate for growth of the tested bacterium for its antibiotic sensitivity. The antimicrobial susceptibility testing was applied according to the guidelines stipulated by National Committee for Clinical Laboratory Standards (NCCLS) [15]. The tested strains were evaluated as susceptible, intermediate and resistant. Multiple Antibiotic Resistance (MAR) index for each strain was determined according to the formula stipulated by Singh et al. [16] as follow:

MAR index=Number of resistance (Isolates classified as intermediate were considered sensitive for MAR index)/Total Number of tested antibiotics.

## Results and Discussion

The achieved results in the present study revealed that *Listeria* spp. was isolated from rabbit thigh muscles, shoulder muscles, loin, liver and kidneys at 25%, 15%, 10%, 15% and 5%, respectively (Table 3). The overall isolation percentage of *Listeria* spp. from rabbit meat in Egypt in the present study was 14%. This level is corresponding to the isolation rate (11%) of *Listeria spp*. from rabbit meat products and carcasses retailed in Italy [17]. Serological identification of the isolated *Listeria* spp. revealed the incidence of five *Listeria* spp. namely, *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri* and *L. seeligeri*. The total incidence of these species in the examined rabbit samples was 28.57%, 21.43%, 35.72%, 7.14% and 7.14% respectively (Table 4). *L. monocytogenes* was particularly isolated from thigh muscles and loin only at 12.5% and 7.5%, respectively. In agreement with the recorded results, *L. monocytogenes* was previously isolated from minced rabbit meat in Egypt [1] and rabbit carcasses in Italy [17]. *Listeria* spp. is an opportunistic intracellular pathogen that is able to survive under extreme pH, osmolarity and temperature, it has been detected in a variety of meat products and rabbit meat processing plants [18,19]. Contamination of rabbit meat with foodborne pathogens gives an indication about the unsatisfactory hygienic measures adopted during processing of such an important meat source [3].

| **Samples** | **Number** | **Positive *Listeria* spp.** | |
|---|---|---|---|
| | | **Number** | **%** |
| Thigh muscles | 40 | 10 | 25% |
| Shoulder muscles | 40 | 6 | 15% |
| Loin | 40 | 4 | 10% |
| Liver | 40 | 6 | 15% |
| Kidneys | 40 | 2 | 5% |
| total | 200 | 28 | 14% |

**Table 3:** Prevalence rates of *Listeria* spp. isolated from the examined rabbit samples.

| **Samples** | **Listeria monocytogenes** | **Listeria ivanovii** | **Listeria innocua** | **Listeria welshimeri** | **Listeria seeligeri** |
|---|---|---|---|---|---|
| Thigh muscles | 5 (12.5%) | 0% | 3 (7.5%) | 1 (2.5%) | 1 (2.5%) |
| Shoulder muscles | 0% | 2 (5%) | 0% | 1 (2.5%) | 0% |

| | | | | | |
|---|---|---|---|---|---|
| Loin | 3 (7.5%) | 0% | 2 (5%) | 0% | 1 (2.5%) |
| Liver | 0% | 4 (10%) | 2 (5%) | 0% | 0% |
| Kidneys | 0% | 0% | 2 (5%) | 0% | 0% |
| Total | 8 (28.57%) | 6(21.43%) | 10(35.72%) | 2 (7.14) | 2 (7.14%) |

**Table 4:** Incidence of the serologically identified *Listeria spp*. isolated in the examined rabbit samples.

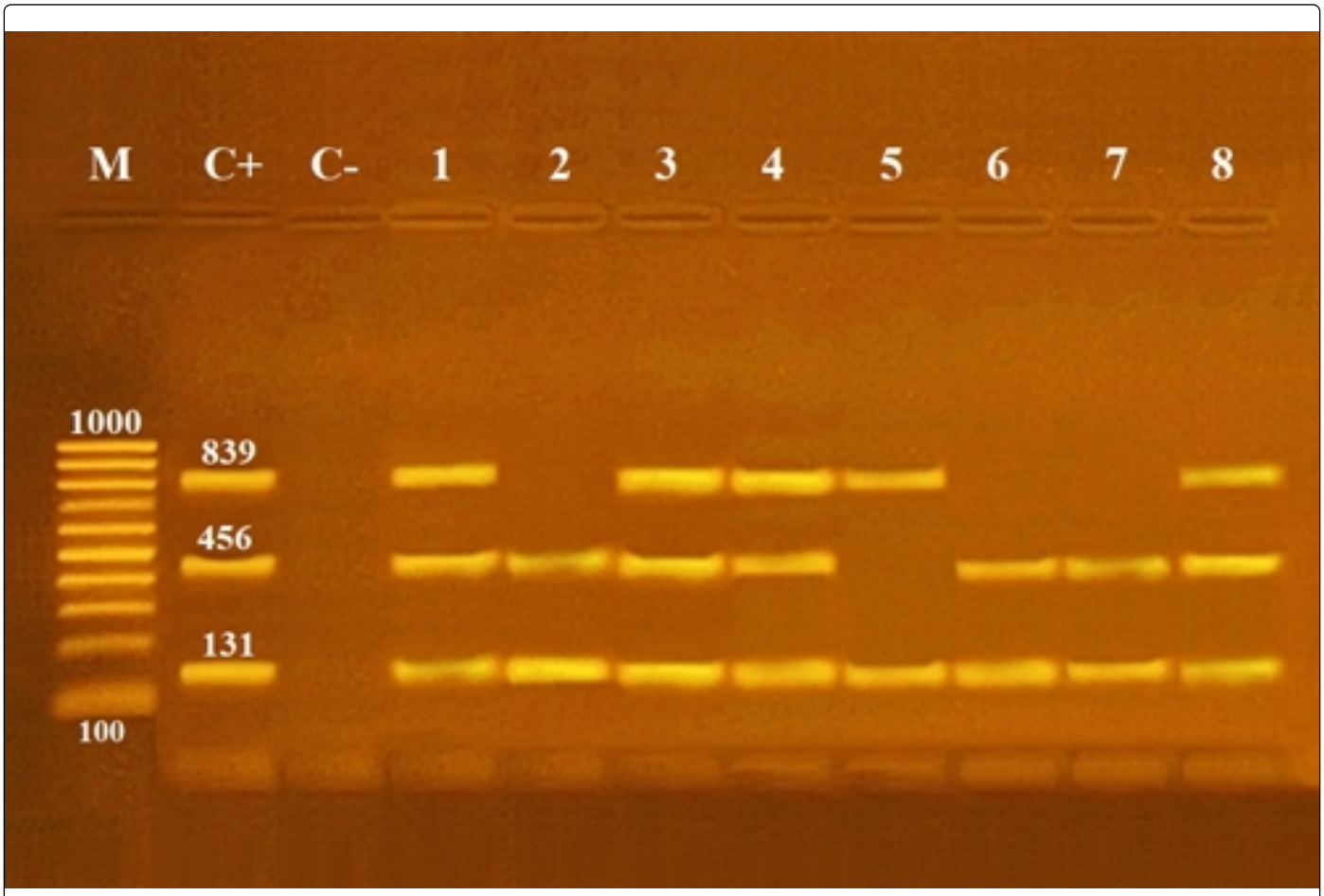


**Figure 1:** Virulence-associated gene expressions among identified *L. monocytogenes* isolates An agarose gel electrophoresis of multiplex PCR of iap (131 bp), hylA (456 bp) and actA (839 bp) virulence genes for characterization of *L. monocytogenes*. Lane M: 100 bp ladder as molecular size DNA marker. Lane C+: Control positive *L. monocytogenes* for iap, hylA and actA genes. Lane C-: Control negative. Lanes 1, 3, 4 and 8: Positive *L. monocytogenes* for iap, hylA and actA genes. Lanes 2, 6 and 7: Positive *L. monocytogenes* strains for iap and hylA genes. Lane 5: Positive *L. monocytogenes* strain for iap and actA genes.

Listeriosis is a disease caused by *L. monocytogenes* infections in human, particularly in the elderly, infants and immunocompromised patients and lead to several symptoms including meningitis, encephalitis, abortion and even death [20]. The occurrence of such symptoms is mainly related to the expression of virulence-associated genes in Listeria isolates. The expression profile of three virulence determinants, iap, hylA and actA, in the current investigation is shown in Figure 1. It was obvious that a clear polymorphism occurred among the obtained Listeria isolates. All obtained Listeria isolates harbored iap (100%), while 7 isolates (87.5%) expressed hylA and only 5 (62.5%) isolates expressed actA. The presence of such virulent genes in the Listeria isolates is necessary for the pathogenicity and indicate the possibility of disease occurrence among consumers if such contaminated meat is ingested without proper handling. Polymorphism among Listeria isolates is reported in several studies in USA and Brazil [18,21].

| Antimicrobial agent | S | | I | | R | |
|---|---|---|---|---|---|---|
| | n | % | n | % | n | % |
| Kanamycin (K) | - | - | - | - | 8 | 100 |
| Neomycin (N) | - | - | - | - | 8 | 100 |
| Nalidixic acid (NA) | - | - | 1 | 12.5 | 7 | 87.5 |
| Penicillin G (P) | - | - | 2 | 25 | 6 | 75 |
| Cephalothin (CN) | 1 | 12.5 | 1 | 12.5 | 6 | 75 |
| Erythromycin (E) | - | - | 3 | 37.5 | 5 | 62.5 |
| Sulphamethoxazol (SXT) | 2 | 25 | 2 | 25 | 4 | 50 |
| Cefotaxim (CF) | 3 | 37.5 | 1 | 12.5 | 4 | 50 |

**Table 5:** Percentages of antimicrobial susceptibility of *L. monocytogenes* (n=8). Where n refers to number of isolates; S is sensitive; I is intermediate; R is resistant.

| No. | Listeria strains | Antimicrobial resistance profile | |
|---|---|---|---|
| 1 | *L. monocytogenes* | K, N, NA, P, CN, E, SXT, CF, T, CP, AM, DO, G, AK | 1 |
| 2 | *L. monocytogenes* | K, N, NA, P, CN, E, SXT, CF, T, CP, AM, DO | 0.857 |
| 3 | *L. monocytogenes* | K, N, NA, P, CN, E, SXT, CF, T, CP, AM | 0.786 |
| 4 | *L. monocytogenes* | K, N, NA, P, CN, E, SXT, CF | 0.571 |
| 5 | *L. monocytogenes* | K, N, NA, P, CN, E | 0.428 |
| 6 | *L. monocytogenes* | K, N, NA, P, CN | 0.357 |
| 7 | *L. monocytogenes* | K, N, NA | 0.214 |
| 8 | *L. monocytogenes* | K, N | 0.143 |
| Average 0.535 | | | |

**Table 6:** Antimicrobial resistance profile of *L. monocytogenes* strains. K: Kanamycin N: Neomycin, NA: Nalidixic acid, P: Penicillin G, CN: Cephalothin, E: Erythromycin, SXT: Sulphamethoxazol, CF: Cefotaxim, T: Oxytetracycline, CP: Ciprofloxacin, AM: Ampicillin, DO: Doxycycline, G: Gentamicin, AK: Amikacin, (number of *L. monocytogenes* isolates=8).

The extensive and abuse of antibiotics in animal farms led to development of multidrug resistant bacterial strains. In the present study, *L. monocytogenes* isolates showed a complete resistance (100%) to both of kanamycin and neomycin. The percentage of the resistance among the tested antimicrobials were as following nalidixic acid (87.5%), penicillin G (75%), cephalothin (75%), erythromycin (62.5%), sulphamethoxazol (50%) and cefotaxime (50%). However, the obtained isolates of *L. monocytogenes* showed marked sensitivity to ampicillin (62.5%), doxycycline (62.5%), gentamicin (75%) and amikacin (87.5%) (Table 5). The average MAR index for the identified *L. monocytogenes* isolates was 0.535 (Table 6). These results go in agreement with Yucel et al. [22] who reported that all *L. monocytogenes* isolates from raw or cooked meat product in Turkey were resistant to cephalothin and nalidixic acid and 66% of isolates were resistant to sulfamethoxazole, ampicillin, and trimethoprim. Similarly, in China, 73% of 167 *L. monocytogenes* isolated from retail food products were resistant to sulfonamide, 8.4% were resistant to tetracycline and 1.8% were resistant to ciprofloxacin [23]. In conclusion, the current study revealed the isolation of multidrug pathogenic *L. monocytogenes* from rabbit meat and offal retailed in Egypt. Therefore, strict hygienic measures should be followed during preparation and processing of rabbit meat before serving to humans.

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## Conflict of Interest

None.

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