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Prevalence, virulence and antibiotic susceptibility of *Listeria monocytogenes* isolated from sheep

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**ABSTRACT**

**Objective:** This study was undertaken to determine the prevalence, virulence, and antibiotics susceptibility of *Listeria monocytogenes* isolated from hindbrain, spinal cord, milk, and intestinal content collected from sheep in the Dakahila Governorate, Egypt.

**Design:** Observational study.

**Samples:** We analyzed 472 samples, including 72 hindbrain/spinal cord samples from emergency slaughtered sheep, 300 raw-milk samples from apparently healthy sheep, and 100 intestinal content sample from slaughtered sheep at three abattoirs.

**Procedures:** Isolation and identification of *L. monocytogenes* were performed using conventional technique. The biochemically identified isolates were confirmed by 16S rRNA gene sequencing and examined for virulence-associated genes (*hlyA* and *iap*) as well as for antimicrobial susceptibility.

**Results:** In total, 16 (3.39%) out of 472 sheep samples (5.56% (4/72) in hindbrain/spinal cord, 4% (12/300) in milk, and 0% (0/100) in intestinal content samples) were found to be positive for *L. monocytogenes*. All the confirmed isolates were positive for the *hlyA* gene (100%); meanwhile, none of them exhibited the *iap* gene. Antibiotic susceptibility testing showed high resistance rates to amoxicillin, cefotaxime, erythromycin (50% each), and vancomycin (37.5%). Sulfamethoxazole–trimethoprim and tetracycline resistance rates were 25% and 12.5%, respectively. On the contrary, all isolates were susceptible to amikacin, ciprofloxacin, and norfloxacin. Interestingly, 37.5% (6/16) of *L. monocytogenes* isolates exhibited multidrug resistance (MDR). The multiple antibiotic resistances (MAR) index of isolates ranged from 0.1 to 0.6.

**Conclusion and clinical relevance:** Our data highlights the importance of awareness of virulent strains or MDR *L. monocytogenes* of sheep samples and potentially samples from other domestic animals in Egypt.

**Keywords:** *Listeria monocytogenes*; *hlyA* gene; *iap* gene; virulence; antibiotic susceptibility; sheep.

1. INTRODUCTION

*Listeria monocytogenes* is a facultative intracellular bacterium, that can cause severe food-borne infection in humans and invasive diseases in different animal species, especially in ruminants [1]. *L. monocytogenes* is widely disseminated in environmental locations including moist soil, the gastrointestinal tract of healthy animals, and in feedstuff such as silage [2, 3]. Lightly acidified silage affords an excellent survival and even multiplication substrate [4]. It has the ability to survive at low temperatures and pH, excessive salt or bile concentrations, oxidative stress, carbon starvation, and in other hostile environments [5].

In sheep, *L. monocytogenes* cause various infections, including meningoencephalitis, abortion, neonatal mortality, stillbirth, septicaemia, and enteritis [6, 7]; rarely, listeric myelitis is seen [8]. Somewhere else, encephalitis, frequently related to silage feeding, is more common [9]. *Listeria* might be excreted in milk during lactation and the presence of *Listeria* in milk samples is considered to indicate a high risk for contamination of dairy products [10]. Sheep milk has traditionally been recognized as a likely source of listeriosis [11]. Intermittent shedding of *L. monocytogenes* has also been reported in asymptomatic small ruminants [12].

Several virulence factors in *Listeria* permit invasion, survival, multiplication, and bacterial mobility in the intracellular environment [13].

Listeriolyisin O (LLO), a cholesterol-dependent cytolysin and a pore-forming exotoxin with hemolytic activity [17, 18], is encoded by the *hly* gene. LLO mediates lysis of bacterium-containing phagocytic vacuoles, causing release of *L. monocytogenes* from the phagosome into the cytosol following phagosome acidification. The intracellular mobility and cell-to-cell spread are facilitated by the surface protein actA (ActA). ActA is responsible for intracellular movement through actin polymerization and also has a role in cell adhesion and invasion [14, 15]. Moreover, internalins are surface proteins used in adhesion and entrance to target cells, while phospholipase C and lecinthase are important in mediating membrane lysis [16]. Ivanolysin is another cholesterol-dependent cytolysin. Apart from the *hly* gene, relevant markers for the identification of *L. monocytogenes* is the *iap* gene—another important virulence-associated gene in *L. monocytogenes*, which encodes a surface protein with murein hydrolase activity.

Quick, specific, and sensitive diagnostic methods able to differentiate *L. monocytogenes* from other *Listeria* species are essential for efficient control of the listeriosis. As *L. monocytogenes* exhibit strain variations in virulence and pathogenicity, the ability to identify potentially pathogenic strains of *L. monocytogenes* precisely and quickly is necessary...
to limit the spread of *L. monocytogenes* infection and reduce food-related diseases [19, 20]. Accordingly, polymerase chain reaction (PCR) assays have been developed for identification of virulent strains of *L. monocytogenes* isolated from the brain stem and sillage and associated with outbreaks in sheep [21, 22].

*Listeria* species are generally sensitive to numerous antibiotics; however, drug-resistant strains are emerging in food products [23]. Antimicrobial resistance in *Listeria* strains is caused by the horizontal transfer of antimicrobial resistance genes and genetic interchange between different *Listeria* species [24, 25]. *L. monocytogenes* is commonly susceptible to antimicrobial agents with an effect on Gram-positive bacteria. However, within the last decades the drug resistance has emerged in *L. monocytogenes* [24].

Although *L. monocytogenes* causes losses in Egyptian sheep herds and might be associated with food-borne diseases, *L. monocytogenes* has been studied only to a very limited extent in Egypt. Consequently, the current study was undertaken to screen the for the presence of *L. monocytogenes* in samples of hindbrain and spinal cord, milk and intestinal content collected from sheep in the Dakahlia province in Egypt and to determine the virulence and antimicrobial susceptibility in identified isolates.

### 2. MATERIALS AND METHODS

#### 2.1. Samples

A total of 472 samples, including 72 hindbrain and spinal cord samples from emergency-slaughtered sheep with or without neurological signs from three abattoirs located in Aga, Tonamel, and Mansoura, 300 raw-milk samples from asymptomatic sheep reared at different farms in Sonbokht, Aga, and Alderis, and 100 intestinal content samples from slaughtered sheep in abattoirs were collected from the Dakahlia province in Egypt over the period lasting from November 2016 to April 2017. All samples were collected in sterile packages under aseptic conditions, kept on ice during transportation, and transferred immediately to the laboratory for microbiological analysis.

#### 2.2. Isolation and identification of *L. monocytogenes*

For bacterial isolation, 25 g or mL of each sample was inoculated into 225 mL of *Listeria* enrichment broth (Oxoid, England) containing *Listeria*-selective enrichment supplement, then homogenized using a stomacher for 30 s, and incubated at 30 °C for 48 h. A loopful from the previously incubated broth was cultured onto *Listeria*-selective agar (Oxoid, England) with *Listeria*-selective supplement and incubated at 37 °C for 24-48h [26]. Five colonies showing typical morphological characters of *Listeria* were picked up and subjected to Gram staining, catalase test, cold enrichment, and laboratory testing for hemolytic activity and CAMP reaction on 5% sheep blood agar, motility testing, and a acid production from sugar [27, 28].

#### 2.3. Molecular characterization of *L. monocytogenes*

DNAs from the isolated *L. monocytogenes* strains were subtype to PCR assays targeting the 16S rRNA gene and the virulence-associated genes hlyA and iap. The DNAs were extracted by the boiled lysate method [29]. DNA amplification was performed using the primers and conditions listed in Table 1. The amplified DNA products were separated by electrophoresis on 1.5% agarose gels and photographed by a gel documentation system. DNA from the reference strain *L. monocytogenes* ATCC 35152 was included in the testing as a positive control.

#### 2.4. Determination of antibiotic susceptibility

*L. monocytogenes* isolates were tested for antibiotic susceptibility against nine different antibiotics by the standard disk diffusion method [30] using Mueller-Hinton agar plates (Difco). The following antimicrobial disks (Oxoid) were used: amoxicillin (AML, 10µg), vancomycin (VA, 30µg), amikacin (AK, 30µg), ciprofloxacin (CIP, 5µg), norfloxacin (NOR, 10µg), tetracycline (TE, 30µg), cefotaxime (CTX, 30µg), erythromycin (E, 15µg), and sulfamethoxazole–trimethoprim (SXT, 25µg). Resistance was detected by measuring the inhibition zone around the antimicrobial disk in accordance with the CLSI standards [30]. The reference strain *L. monocytogenes* ATCC 35152 was applied as a control. A MAR index was determined for each isolate by using the formula MAR = A–B, with (A) indicating the number of antibiotics to which the examined isolate depicted resistance and (B) being the total number of antibiotics to which the examined isolate was evaluated for susceptibility [31].

#### 2.5. Statistical Analysis

Statistical analysis of the results by calculation of the ratio was made using the SPSS Statistics 17.0 software program.

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**Table 1. Oligonucleotide primer sequences used in this study.**

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Amplified product</th>
<th>Primary denaturatio n</th>
<th>Amplification (35 cycles )</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>GGAAGGGGCTTATACCTGAGGAC</td>
<td>1200 bp</td>
<td>94°C/5min</td>
<td>94°C/30 sec</td>
<td>60°C/1 min</td>
</tr>
<tr>
<td>hlyA</td>
<td>CTGCTGAGGGTCATTATCCTCCTAC</td>
<td>174 bp</td>
<td>50°C/30 sec</td>
<td>72°C/30 sec</td>
<td>72°C/7 min</td>
</tr>
<tr>
<td>iap</td>
<td>GTAATCGAGGAGGCTTACCTCC</td>
<td>131 bp</td>
<td>60°C/30 sec</td>
<td>72°C/30 sec</td>
<td>72°C/7 min</td>
</tr>
</tbody>
</table>

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3. RESULTS

3.1. Prevalence of L. monocytogenes in sheep and virulence-associated genes

In total, 16 (3.39%) of 472 sheep samples were found positive for L. monocytogenes. All the 16 biochemically identified strains of L. monocytogenes were confirmed by PCR (Figure 1). Specifically, the proportion of samples positive for L. monocytogenes was 5.56% (4/72), 4% (12/300), and 0% (0/100) in hindbrain/spinal cord, milk, and intestinal content samples, respectively.

Moreover, all 16 isolates were examined for the two virulence-associated genes hlyA and iap. The hlyA gene was present in 100% (16/16) of the isolates (Figure 2), while the iap gene was absent (Figure 3).

### Table 2. Antibiotic susceptibility of Listeria monocytogenes strains (n=16) from sheep samples.

<table>
<thead>
<tr>
<th>Antibiotic disk</th>
<th>Susceptible (%)</th>
<th>Intermediate (%)</th>
<th>Resistant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>8 (50%)</td>
<td>0</td>
<td>8 (50%)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>4 (25%)</td>
<td>6 (37.5%)</td>
<td>6 (37.5%)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>16 (100%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>16 (100%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>16 (100%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>2 (12.5%)</td>
<td>12 (75%)</td>
<td>2 (12.5%)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>8 (50%)</td>
<td>0</td>
<td>8 (50%)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>8 (50%)</td>
<td>0</td>
<td>8 (50%)</td>
</tr>
<tr>
<td>Sulfamethoxazole-trimethoprim</td>
<td>12 (75%)</td>
<td>0</td>
<td>4 (25%)</td>
</tr>
</tbody>
</table>

### Table 3. Antibiotic resistance pattern and multiple antibiotic resistance index among Listeria monocytogenes isolates (n=16) from sheep.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Antibiotic resistance pattern</th>
<th>MAR index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AML,E,SXT</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>AML,VA,CTX,E</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>CXT</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>TE</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>AML</td>
<td>0.1</td>
</tr>
<tr>
<td>6</td>
<td>VA,E</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>AML,VA,CTX,E,SXT</td>
<td>0.6</td>
</tr>
<tr>
<td>8</td>
<td>CTX</td>
<td>0.1</td>
</tr>
<tr>
<td>9</td>
<td>AML,VA,CTX,E</td>
<td>0.4</td>
</tr>
<tr>
<td>10</td>
<td>CTX</td>
<td>0.1</td>
</tr>
<tr>
<td>11</td>
<td>AML</td>
<td>0.1</td>
</tr>
<tr>
<td>12</td>
<td>TE</td>
<td>0.1</td>
</tr>
<tr>
<td>13</td>
<td>VA,E</td>
<td>0.2</td>
</tr>
<tr>
<td>14</td>
<td>CTX</td>
<td>0.1</td>
</tr>
<tr>
<td>15</td>
<td>AML,VA,CTX,E,SXT</td>
<td>0.6</td>
</tr>
<tr>
<td>16</td>
<td>AML,E,SXT</td>
<td>0.3</td>
</tr>
</tbody>
</table>


### 3.2. Antimicrobial susceptibility results

Results from antimicrobial susceptibility testing are shown in Table 2. High resistance rates to amoxicillin, cefotaxime, erythromycin (50% each), and vancomycin (37.5%) were observed. Resistance to sulfamethoxazole–trimethoprim and tetracycline was present in 25% and 12.5% of the isolates, respectively. On the contrary, all strains were susceptible to amikacin, ciprofloxacin, and norfloxacin. Interestingly, 37.5% (6/16) of the L. monocytogenes isolates were MDR, displaying resistance to more than two classes of antibiotics, displaying seven resistance patterns. The MAR index of the strains ranged from 0.1 to 0.6 (Table 3).
4. DISCUSSION

*L. monocytogenes* is the causal agent of listeriosis, affecting a wide range of mammalian species, including monogastric animals, ruminants, and humans. Among ruminants, sheep are commonly affected by *L. monocytogenes* infection, which may be associated with various clinical presentations, including meningocerebritis and mastitis [32-34]. In the present study, we applied 16S rRNA gene PCR as a confirmatory tool for identification of *L. monocytogenes*. A low detection rate of *L. monocytogenes* (3.39%, 16/472) was observed in this panel of sheep samples. Four hindbrain/spinal cord samples (5.56%) were positive. Similarly, Aldabbagh [35] identified two out of 50 (4%) isolates in sheep brain samples. In the present work, 12/300 (4.0%) of the milk samples were found positive. This result was consistent with previous studies where *L. monocytogenes* was detected in 2.5% and 3.9% from raw milk and sheep milk in Europe and Egypt, respectively [36,37]. The results in the current investigation indicate a low likelihood of contamination related to the occurrence of *L. monocytogenes* in raw sheep milk. Moreover, the absence of *L. monocytogenes* in sheep feces in this study is compatible with a previous study, which reported a very low prevalence (0.58%) of *L. monocytogenes* in sheep feces [38]. In contrast, Wesley [39] noted a higher fecal shedding of *L. monocytogenes* in sheep (8%). Fecal shedding of *Listeria* usually lasts for 10 days with *Listeria* diffusing over the entire volume of the fore-stomach within 4 h, and throughout the whole gastrointestinal tract within 2 h [40].

With regard to virulence genes, corresponding results were obtained in an earlier investigation, where the hylA gene was shown to exist in nearly all of the tested *L. monocytogenes* strains; however, no iap gene was identified [43]. On the contrary, Osman et al. [37] found both of the tested virulence-associated genes (hylA and iap) in *L. monocytogenes* isolated from sheep milk. Surveillance of such virulence-associated genes in *L. monocytogenes* strains is critical to identify and discriminate hyper-virulent from less virulent strains.

With regard to antimicrobial susceptibility tests, the resistance of *L. monocytogenes* isolates to cefotaxime, sulfamethoxazole–trimethoprim, and tetracycline was previously reported in Egypt [37,43], indicating the necessity of the continuous monitoring of antimicrobial susceptibility of *L. monocytogenes* from different sources. Meanwhile, full susceptibility of all isolates to amikacin, ciprofloxacin, and norfloxacin was observed in a previous study [37]. In the present study, 37.5% of *L. monocytogenes* isolates exhibited MDR. These results could indicate that the evolution of multiresistant strains of *L. monocytogenes* is associated with extensive and perhaps inexpedient usage of antibiotics as a growth promoter in for example farm animals. MDR in *Listeria* is not unprecedented [24]. The existence of virulence-associated gene (hylA gene) in drug-resistant *L. monocytogenes* could increase the difficulty in treating animals suffering from *L. monocytogenes* infection.

Pathogens with a MAR index value below 0.2 would typically come from a lower-hazard source, while those with an MAR index value higher than 0.2 indicated would typically come from a higher-risk source and reflect inappropriate use of these antibiotics in the surrounding area.

Conclusion

From this study, we investigated the prevalence of virulent and multidrug-resistant *L. monocytogenes* isolated from hindbrain/spinal cord, milk, and fecal samples collected from sheep in the Dakahlia province in Egypt. The awareness of drug-resistant pathogens should be increased in Egypt, where intensive and inexpedient utilization of antimicrobial agents may be a critical issue. It is necessary for humans to sustain appropriate hygienic measures and pasteurization of milk of sheep to minimize the risk of infection with *L. monocytogenes*.

Acknowledgment

Conflict of interest statement

No conflict of interest.

Research Ethics Committee Permission

The current research work is permitted to be executed according to standards of Research Ethics committee, Faculty of Veterinary Medicine, Mansoura University.

Authors’ contribution

Gamal A. Younis designed the experiment and revised the manuscript. Gamal A. Younis, Mohamed Elhadidy, Rasha M. Elkenany supervised in carrying out the practical part. Rasha M. Elkenany shared in writing the paper and took the responsibility of correspondence to the journal. Shimaa W.A. Elbar collected samples and carried out the practical part. All authors approved the final version of the manuscript for publication.

5. REFERENCES


